TERESA MARIA PINTO GONÇALVES RIBEIRO

EXTENDING THE KNOWLEDGE ON DRIVERS OF INTRINSIC AND ACQUIRED RESISTANCE TO CEPHALOSPORINS AND QUINOLONES IN *ENTEROBACTERIACEAE*

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na especialidade de Microbiologia

Trabalho realizado sob orientação de:

Professora Doutora Luísa Vieira Peixe (Professora Associada com agregação da Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; chefe de grupo BacT_Drugs Lab do UCIBIO@REQUIMTE, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal)

Professora Doutora Elisabete Machado (Professora Auxiliar da Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; Investigadora UCIBIO@REQUIMTE, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal)

Doutora Ângela Novais (Investigadora UCIBIO@REQUIMTE, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal).

July 2016

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Teresa Maria Pinto Gonçalves Ribeiro

This work was performed at the Microbiology Group of the Faculty of Pharmacy, University of Porto (FFUP, Porto, Portugal) and Faculty of Healthy Sciences, University Fernando Pessoa (UFP, Porto, Portugal). The author received financial support through an individual pre-doctoral fellowship funded by the Foundation for Science and Technology (FCT) of Portugal with the reference SFRH/BD/75752/2011. The experimental research performed in this work was supported by grants from the European Union (FEDER funds) through Programa Operacional Fatores de Competitividade – COMPETE), and from national funds [FCT and Quadro de Referência Estratégica Nacional (QREN)] through projects numbers Pest-C/EQB/LA0006/2013, PTDC/AAC-AMB/103386/2008, UID/Multi/04378/2013, UID/Multi/04546/2013 and NORTE-07-0124-FEDER-000066.

Governo da República Portuguesa

UNIÃO EUROPEIA
Fundo Social Europeu

Ao Nuno, à Maria e aos meus pais,

Por tudo o que são para mim.

AGRADECIMENTOS (ACKNOWLEDGEMENTS)

Apesar de serem poucas as palavras, deixo um sentido e profundo agradecimento a todos os que colaboraram, direta ou indiretamente, na realização deste trabalho.

À Professora Doutora Luísa Peixe, Professora Doutora Elisabete Machado e Doutora Ângela Novais, as minhas orientadoras, agradeço os conhecimentos transmitidos, a disponibilidade, constante motivação, as críticas e sugestões relevantes, o rigor científico e, sobretudo, a oportunidade de realizar este trabalho. Cada uma foi essencial e contribuiu de forma única para orientar este percurso e desenvolver o meu conhecimento científico e, implicitamente, o pessoal. Muito obrigada.

À unidade de investigação à qual pertenço, UCIBIO@REQUIMTE, agradeço a disponibilização das condições necessárias para a realização e desenvolvimento deste trabalho e à Professora Doutora São José Nascimento por me ter permitido desenvolver e realizar este trabalho no Laboratório de Microbiologia da Faculdade de Farmácia da Universidade do Porto.

À Fundação para a Ciência e Tecnologia, agradeço o apoio financeiro concedido ao longo destes quatro anos.

Agradeço à Cristina Pinto da Costa e ao Nuno Oliveira do Laboratório de Microbiologia da Faculdade de Farmácia da Universidade do Porto, pela amizade, colaboração, disponibilidade e apoio demonstrados ao longo deste período.

À Doutora Filipa Grosso, Doutora Ana Freitas, Professora Doutora Carla Novais e Professora Doutora Patrícia Antunes o meu muito obrigado pela vossa ajuda, partilha de conhecimento, dedicação e palavras de ânimo, que tanto me incentivaram nos momentos mais difíceis.

À Joana Campos, Liliana Silva, Joana Mourão e João Botelho expresso a minha gratidão pelo apoio nesta fase crucial da minha vida, não podendo deixar de salientar a vossa alegria contagiante, risos cúmplices, carinho e predisposição.

À Carla Rodrigues, vizinha de bancada do laboratório, amiga e companheira deste percurso académico, que comigo partilhou gargalhadas e angústias, ilusões e certezas, que sempre me incentivou e aconselhou, que sempre esteve disponível para me ouvir a qualquer hora e em qualquer momento, o meu muito obrigado.

À Professora Doutora Eduarda Silveira, antiga colega de laboratório, pela amizade indubitável desde os tempos de Licenciatura ao qual nem a distância pôs termo, pela partilha de conhecimento e palavras de incentivo.

À Doutora Raquel Branquinho, amiga e antiga colega de laboratório, pela disponibilidade ao longo deste percurso, pela partilha de conhecimento e palavras de incentivo.

A todos os amigos, aos quais privei da minha companhia, provisoriamente, por força das circunstâncias e que compreenderam o meu silêncio.

Ao meu irmão e à minha cunhada por todo o apoio prestado aquando da colheita de amostras em Angola.

Aos meus pais por todo o amor, preocupação, palavras de ânimo e apoio incondicional.

Por fim, ao Nuno e à Maria.
Ao Nuno, o meu porto de abrigo, por toda a compreensão, pelo abraço apertado, por todo o teu amor. O último ano não foi fácil, mas nunca deixaste de acreditar que eu era capaz de superar todas as adversidades. À Maria, o meu bem mais precioso e o meu escape nestes últimos tempos, por toda a tua alegria, brincadeiras e amor. Por teres feito a mãe perceber que embora este seja um dos momentos cruciais do meu percurso académico há sempre tempo para os momentos em família.

ABSTRACT

Cephalosporins and fluoroquinolones are critical antibiotics for the treatment of human infections caused by *Enterobacteriaceae* isolates, although the expansion of acquired β-lactamases and plasmid-mediated fluoroquinolone resistance mechanisms have seriously compromised their success.

In Portugal, *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) are endemic in different niches, while carbapenemases arise recently in the clinical settings. Regarding the production of acquired AmpC β-lactamases (qAmpCs), trends have been scarcely investigated. Although the contribution of world globalization in the dispersion of antibiotic resistant (AbR) bacteria is well recognized, data on acquired mechanisms conferring resistance to cephalosporins in *Enterobacteriaceae* from underdeveloped countries, particularly from African country are scarce. Indeed, molecular epidemiological data of bacteria producing acquired β-lactamases from Angola, a country with an emerging economy and close commercial or travel relationships with Portugal, have not yet been reported.

The *qnrB* genes, encoding proteins responsible for decreased susceptibility to fluoroquinolones, constitute the most prevalent and diverse *qnr* family, with plasmid located qnrB genes thought to be originated from *Citrobacter* sp. Indeed, *qnrB* genes are known to be intrinsic of *Citrobacter* spp., contributing for a natural phenotype of reduced susceptibility to fluoroquinolones. Nevertheless, the absence of correlation of these genes with particular *Citrobacter* species within this genus, together with the lack of detailed characterization of their genetic platforms, hindered a clear establishment of their origin.

The main goals of this work were to extend the knowledge on dispersion drivers (species, clones and plasmids) of genes encoding acquired resistance to cephalosporins in *Enterobacteriaceae* from different ecological niches in two countries with close commercial and travel relationships (Portugal and Angola), and to elucidate *qnrB* origins throughout a phylogenetic analysis of housekeeping (e.g. *leuS* and *recN*) and intrinsic genes (*qnrB*) responsible for decreased susceptibility to fluoroquinolones in *Citrobacter* spp.

A total of 2107 *Enterobacteriaceae* isolates non-susceptible to extended-spectrum cephalosporins obtained from different Portuguese clinical institutions, 581 samples recovered from several non-clinical sources (healthy humans, animals and environment) of Portugal and Angola (n=518 and n=63, respectively), *Citrobacter* spp. isolates and genomes from diverse origins (n=21 and n=40, respectively) and *Enterobacteriaceae* plasmid sequences (n=16) were analyzed. Bacterial identification was performed by conventional methods and/or genotypic markers. Antibiotic susceptibility testing and conjugation assays were performed by standard methods. Genes encoding qAmpCs, ESBLs, carbapenemases or decreased susceptibility to fluoroquinolones, as well as their corresponding genetic environments were characterized by polymerase chain reaction (PCR) and sequencing. Affiliation of *qnrB* was performed by phylogenetic analysis, clonal relatedness was established by *Xba*I-pulsed-field gel electrophoresis (PFGE) and/or *mulilocus* sequencing typing

(MLST) and *Escherichia coli* phylogenetic groups were determined by PCR. The location of genes was assessed by hybridization of *Xba*I- or S1-/I-*CeuI*-digested genomic DNA, and plasmid characterization included identification of incompatibility groups (Inc) by PCR, sequencing, hybridization and plasmid MLST.

A low (5%, 99/2107) and stable occurrence of qAmpCs in different Portuguese clinical institutions was observed throughout time, corresponding to DHA-1 (82%), CMY-2 (17%) or DHA-6 (1%). DHA-1 persisted throughout time (2006-2013) in different institutions, species and clones (predominance of *Klebsiella pneumoniae* ST11 and ST1380), and particular plasmids (IncR or IncHI2), while the increase of CMY-2 in the last years (2009-2013) was linked to multiple *Escherichia coli* clones and to IncI1 plasmids. On the other hand, CMY-2 was the only qAmpC-type detected in *E. coli* from healthy humans (n=3) and uncooked chicken carcass (n=1) from Portugal. It was observed an increase in the proportion of healthy humans colonized with qAmpC-producing *Enterobacteriaceae* from 2001-2004 (0%) to 2013-2014 (2.1%) linked to plasmid dissemination, although an indistinguishable PFGE pattern was found from two-family related individuals. Regarding Angola, qAmpC or carbapenemases were not detected among recent *Enterobacteriaceae* isolates (2013) collected from different non-clinical niches. However, ESBL genes, mostly $bla_{\text{CTX-M-15}}$, were identified in different species (mainly *E. coli*) and almost all samples. Despite the high clonal diversity observed among *E. coli* isolates (n=34/23 PFGE-types) and the absence of the pandemic B2-ST131 clone, some widespread clonal complexes (CCs) from A (CC10 and CC168), B1 (CC156) or D (CC38) phylogroups were detected. $bla_{\text{CTX-M-15}}$ was variably identified on typeable (29%; IncFII, IncFIIK6, IncHI2 and IncY) or non-typeable (16%) plasmids or on the chromosome (14%), while for 41% of the isolates its specific location was not determined. Finally, identification of *Citrobacter* species by different genotypic markers revealed that *recN* gene presented the most discriminatory power, allowing the identification of putative novel species, including the identification of *Citrobacter europaea* novel species. Phylogenetic analysis of all *qnrB* genes revealed seven main clusters and two branches. Specific genetic platforms were associated with each one of these *qnrB* clusters which evolved in different *Citrobacter* species. Furthermore, a high identity was observed between some of the platforms identified in the chromosome of *Citrobacter* spp. and in different plasmids of *Enterobacteriaceae*.

The overall results of this Thesis indicate that qAmpCs are disseminated in clinical and non-clinical settings in Portugal linked to the amplification of specific qAmpC-types with different genetic backgrounds in particular *Enterobacteriaceae* species. Furthermore, CTX-M-15-producing *Enterobacteriaceae* are widespread in diverse non-clinical niches in Angola, an atypical epidemiology highlighting the need for antimicrobial resistance surveillances in different regions of Africa. Moreover, and beyond the contribution to accurately discriminate *Citrobacter* species, the present work also suggests divergent evolution of closely related *qnrB* genes/platforms in particular *Citrobacter* spp, with plasmidic *qnrB* originated from particular species.

Keywords: *qnrB*, acquired β-lactamases, phylogenetic analysis, *Citrobacter*, Africa

RESUMO

As cefalosporinas e as fluoroquinolonas são antibióticos críticos para o tratamento das infeções humanas por isolados de *Enterobacteriaceae*, embora a expansão de β-lactamases adquiridas e de mecanismos de resistência às fluoroquinolonas tenha gravemente comprometido o seu sucesso.

Em Portugal, isolados de *Enterobacteriaceae* produtores β-lactamases de espetro alargado (ESBLs) são endémicos em diferentes nichos, enquanto as carbapenemases surgiram mais recentemente em nichos clínicos. Relativamente à evolução da taxa de *Enterobacteriaceae* produtoras de β-lactamases adquiridas do tipo AmpC (qAmpC) esta tem sido ainda fracamente explorada. Embora a contribuição da globalização mundial na dispersão de bactérias resistentes aos antibióticos seja reconhecida, dados sobre mecanismos adquirida de resistência a cefalosporinas em *Enterobacteriaceae* de países em desenvolvimento, particularmente de países Africanos são escassos. De facto, dados de epidemiologia molecular de bactérias produtoras de β-lactamases adquiridas de Angola, um país com uma economia emergente e com estreitas relações comerciais e circulação de pessoas com Portugal, ainda não foram relatados.

Os genes *qnrB*, a mais prevalente e diversa família de *qnr,* codificam proteínas responsáveis pela susceptibilidade reduzida a fluoroquinolonas, sendo atribuído ao *Citrobacter* sp. a origem dos *qnrB* de localização plasmídica. De facto, os genes *qnrB* são conhecidos por serem intrínsecos em *Citrobacter* spp., contribuindo para um fenótipo natural de suscetibilidade reduzida a fluoroquinolonas. No entanto, a ausência de correlação destes genes com determinadas espécies de *Citrobacte*r, em conjunto com a falta de caraterização detalhada das suas plataformas genéticas, impede um estabelecimento claro da sua origem.

Os principais objetivos deste trabalho são o aumento do conhecimento sobre os veículos de dispersão (espécies, clones e plasmídeos) de genes adquiridos que conferem resistência a antibióticos β-lactâmicos em *Enterobacteriaceae* de diferentes nichos ecológicos de dois países com estreitas relações comerciais e circulação de pessoas (Portugal e Angola) e descrever as trajectórias evolutivas de genes intrínsecos (*qnrB*) responsáveis pela diminuição da suscetibilidade às fluoroquinolonas em *Citrobacter*.

Dois mil cento e sete isolados de *Enterobacteriaceae* não suscetíveis às cefalosporinas de espectro alargado obtidos de diferentes instituições clínicas portuguesas, 581 amostras provenientes de diversas origens não-clínicas (humanos saudáveis, animais e meio ambiente) de Portugal e Angola (n=518 e n=63, respetivamente), isolados e genomas de *Citrobacter* spp. (n=21 e n=40, respetivamente) e sequências plasmídicas de *Enterobacteriaceae* (n=16) foram analisados. A identificação dos isolados foi realizada por métodos clássicos e/ou por marcadores genotípicos. Os estudos de suscetibilidade a antibióticos e os ensaios de conjugação foram realizados por métodos clássicos. Os genes que codificam para qAmpCs, ESBLs ou diminuição da suscetibilidade às fluoroquinolonas e o seu ambiente genético foram amplificados por PCR e sequenciação. A afiliação do gene *qrnB* foi realizada por análise filogenética, a relação clonal entre isolados foi estabelecida por *Xba*I-eletroforese em campo pulsado (PFGE) e/ou tipagem por sequenciação de múltiplos *loci* (MLST) e os grupos filogenéticos de isolados de *Escherichia coli* foram determinados por PCR. A localização dos genes e a análise de plasmídeos incluiu *Xba*I-PFGE ou S1-/I-*Ceu*I-PFGE com posterior hibridação com sondas, identificação de grupos de incompatibilidade (Inc) por PCR, sequenciação e *pMLST*.

A ocorrência de qAmpCs em instituições clínicas Portuguesas foi baixa (5%, 99/2107) e estável ao longo do tempo, correspondentes à identificação de qAmpCs do tipo DHA-1 (82%), CMY-2 (17%) ou DHA-6 (1%). DHA-1 persistiu ao longo do tempo (2006-2013) em diferentes instituições, espécies e clones (predominância dos clones *Klebsiella pneumoniae* ST11 e ST1380) e plasmídeos particulares (IncR ou IncHI2), enquanto o aumento de CMY-2 nos últimos anos (2009-2013) foi associado a vários clones de *E. coli* e plasmídeos do tipo IncI1. Em contrapartida, apenas a qAmpC do tipo CMY-2 foi detetada em isolados de *E. coli* provenientes de humanos saudáveis (n=3) e de carne de frango crua (n=1) de Portugal. Neste estudo, a proporção de humanos saudáveis colonizados com *Enterobacteriaceae* produtoras de qAmpCs aumentou significativamente entre 2001-2004 (0%) e 2013-2014 (2.1%), associado com a disseminação de plasmídeos, embora tenham sido detectados 2 perfis de PFGE iguais entre dois familiares. Em Angola, não foram detectadas a presença de qAmpCs ou carbapenemases em isolados de *Enterobacteriaceae* obtidos recentemente (2013) de nichos não clínicos. Contudo, genes que codificam para ESBLs, predominantemente $bla_{\text{CTX-M-15}}$, foram identificados em isolados de diferentes espécies (maioritariamente *E. coli*) obtidos de quase todas as amostras. Apesar de ter sido observada uma elevada diversidade clonal entre os isolados de *E. coli* (n=34/23 pulsotipos) e do clone pandémico B2-ST131 não ter sido identificado, alguns complexos clonais (CCs), do grupo filogenético A (CC10 e CC168), B1 (CC156) ou D (CC38) foram detetados. $bla_{\text{CTX-M-15}}$ foi variavelmente identificada em plasmídeos tipáveis (29%; IncFII, IncFIIK6, IncHI2 and IncY) ou não-tipáveis (16%) ou no cromossoma (14%), enquanto em 41% dos isolados a sua localização específica não foi determinada. Por fim, a análise filogenética de diferentes marcadores genotípicos para identificação das diferentes espécies de *Citrobacter*, revelou que o gene *recN* apresenta o maior poder discriminatório, permitindo a identificação de supostas novas espécies, incluindo a identificação da nova espécie *Citrobacter europaeus*. A análise filogenética de todos os genes *qnrB* revelou sete principais grupos e dois ramos. Plataformas genéticas específicas foram associadas a cada um dos grupos de *qnrB* que evoluíram em diferentes espécies de *Citrobacter*. Para além disso, uma elevada identidade foi observada entre algumas plataformas identificadas no cromossoma de *Citrobacter* spp. e em diferentes plasmídeos de *Enterobacteriaceae*.

Como conclusões gerais deste trabalho salientamos a disseminação de qAmpCs em nichos clínicos ou não-clínicos em Portugal, associados à amplificação de tipos específicos de qAmpCs veiculados por diferentes clones e plasmídeos em determinadas espécies de *Enterobacteriaceae*. Adicionalmente, isolados de *Enterobacteriaceae* produtores de CTX-M-15 estão disseminados em diversos nichos não clínicos em Angola, destacando-se diferenças em relação ao tipo de ESBLs, ocorrência e origens genéticas responsáveis pela sua disseminação em países desenvolvidos. Este trabalho também sugere o envolvimento de determinadas espécies de *Citrobacter* em evoluções divergentes de genes *qnrB* próximos/plataformas e na origem de *qnrB* localizados em plasmídeos.

Palavras-chave: *qnrB*, β-lactamases adquiridas, análises filogenéticas, *Citrobacter*, África.

CONTENTS

Chapter 1

Introduction

"A ciência realmente só tem alcançado tornar mais intensa e forte uma certeza: a velha certeza socrática da nossa irreparável ignorância. De cada vez sabemos mais...que não sabemos nada."

Eça de Queirós

1.1. REVIEW ARTICLE

The global epidemiology of acquired AmpC β-lactamases in clinical *Enterobacteriaceae*

The global epidemiology of acquired AmpC β-lactamases among clinical *Enterobacteriaceae*

T. G. Ribeiro[1], Â. Novais[1], E. Machado[1,2], L. Peixe[1*]

[1]UCIBIO@REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

***Corresponding author:** Luísa Vieira Peixe, UCIBIO@REQUIMTE, Laboratory of Microbiology, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal; Tel: (+351) 220 428 580; E-mail: lpeixe@ff.up.pt

Running title: Global epidemiology of qAmpCs in clinical *Enterobacteriaceae*.

Abstract

Acquired AmpC β-lactamases (qAmpCs) inactivate important b-lactams antibiotics, constituting a serious public health threat. There are a growing number of reviews of qAmpC-producing *Enterobacteriaceae* found either in bacteria from human or non-human sources. However, an update and comprehensive review of the literature on qAmpCs in *Enterobacteriaceae* recovered from different clinical settings across continents is not available. This mechanism of resistance to extended-spectrum β-lactams has been found around the world and appears to be increasing in occurrence. CMY-2 is the most globally widespread and dominant qAmpC, reported mostly in *E. coli* and *Salmonella* spp. recovered from different clinical settings worldwide, whereas DHA-1 has been confined mostly to nosocomial *K. pneumoniae* of particular countries. Clonal expansion and/or mobile genetic elements (mainly plasmids) have been the main reasons for the widespread *Enterobacteriaceae* producing qAmpCs. Furthermore, irrespective of the particular qAmpC, most isolates are multidrug-resistant. This article reviews the occurrence, distribution and diversity of qAmpCs in clinical *Enterobacteriaceae* isolates and highlights the role of particular clones and plasmids in the dissemination of qAmpCs worldwide.

Keywords: plasmid, clone, Ambler class C β-lactamase, DHA-1, CMY-2

Introduction

The emergence and expansion of *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs), acquired AmpC β-lactamases (qAmpCs) and/or carbapenemases in different settings represent are major concerns in many countries (1–7), as these acquired β-lactamases inactivate several β-lactam antibiotics relevant in the treatment of human infections. This problem has been lead to serious implications for both public health and infection control practices (8).

The molecular epidemiology of ESBL- or carbapenemase-producing *Enterobacteriaceae* has been worldwide described in detail (1,9–14), but there is no recent data summarizing epidemiological features of *Enterobacteriaceae* producing qAmpCs across continents in different clinical settings (hospital, community).

In this review, we sought to (i) describe the occurrence, distribution and diversity of qAmpCs in clinical *Enterobacteriaceae* isolates recovered worldwide, (ii) highlight the contribution of the clonal expansion of certain high-risk clones to the global epidemiology, and (iii) describe the role of epidemic plasmids, other mobile genetic elements (MGEs) and integrons in the current qAmpC spread. All surveys performed worldwide and covering isolates recovered after 2000s were included, whereas studies reporting only AmpC phenotype without genotype confirmation and characterization were excluded.

Origin, classification and resistance phenotypes of AmpCs

Genes encoding AmpC β-lactamases were originally described at the chromosome of diverse *Enterobacteriaceae* species (e.g. *Escherichia coli*, *Shigella* spp., *Citrobacter* spp., *Serratia* spp., *Hafnia alvei*, *Morganella* spp., *Enterobacter* spp.), *Acinetobacter* spp., *Pseudomonas* spp., and amongst other genus (Figure 1) (15,16). Six to fourteen years later, AmpC β-lactamases located at plasmids were also recognized in different bacteria, including in species naturally lacking the chromosomal bla_{AmpC} genes, such as *Klebsiella* spp., *Proteus mirabilis* and *Salmonella* spp. (15–17).

Over 240 allelic variants of AmpC β-lactamases have been described worldwide, being recognized to date eight AmpC-types (ACC, ACT, CFE, DHA, FOX, LAT, MIR, MOX) (ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Lahey.tab). Affiliations of chromosomal- and plasmid-encoded AmpC amino acid sequences (Figure 1) demonstrates the high diversity of chromosomal AmpCs and the close relationship of qAmpCs to chromosomal enzymes of particular species. Therefore, qAmpCs can be divided in five sublineages or groups, according with affiliations with the most closely chromosomal-encoded AmpC described in particular species or bacterial families: *Citrobacter freundii* group (LAT and most of the CMY types; 94% amino acid identity), *Enterobacter* group (ACT-1 and MIR-1; 85% to 98% amino acid identity), *Morganella morganii* group (DHA-1

and DHA-2; 100% amino acid identity) and *H. alvei* group (ACC-1; 100% amino acid identity), which might be their origin (Figure 1). The phylogeny of CMY-1, CMY-8, CMY-9, MOX, and FOX qAmpCs is unclear, despite their low sequence similarities (≤77% amino acid identity) with chromosomal AmpC β-lactamases from *Aeromonadaceae* group (Figure 1) (16–18). The novel qAmpC CDA-1 recently described in a *Cedecea davisae* clinical isolate is phylogenetically and biochemically close to the chromosome-encoded β-lactamases from *Enterobacter* (19).

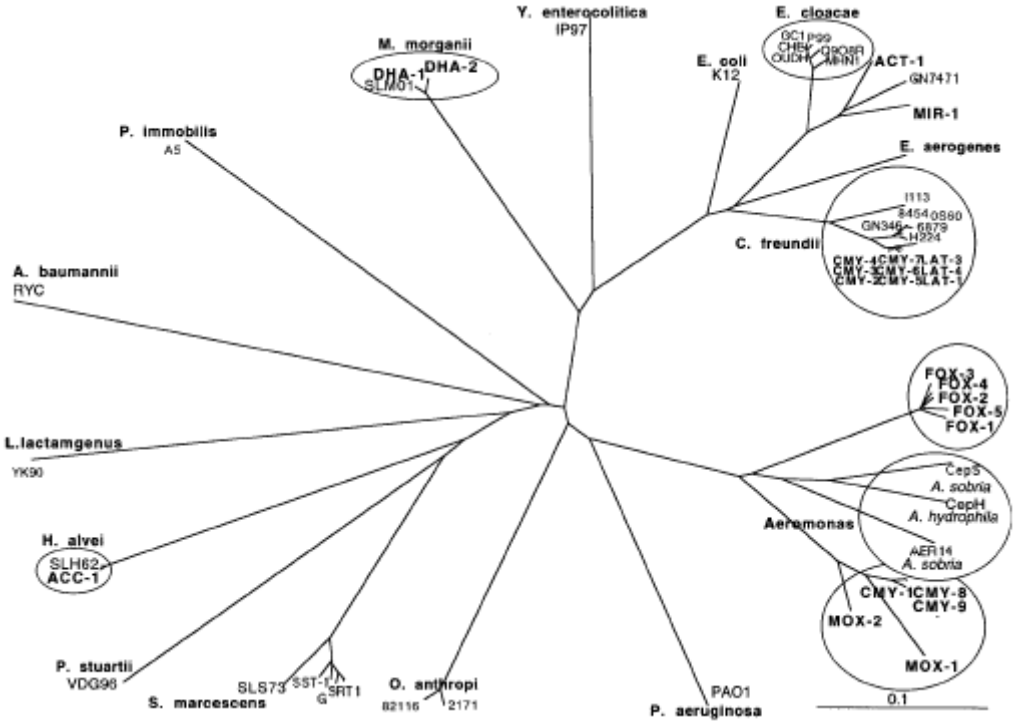


FIGURE 1. Affiliations of chromosomal- and plasmid-encoded AmpC amino acid sequences [reprinted with permission from reference (16)].

According to their amino acid sequence, these enzymes with serine in the active site were positioned in the class C of the Ambler structural classification of β-lactamases (20).

AmpC enzymes are resistant to β-lactamase inhibitors [clavulanate (CLA)] and hydrolyze aminopenicillins, first generation cephalosporins (C1G), and depending on the enzyme, second generation cephalosporins (C2G) including cephamycins [e.g. cefoxitin (FOX), cefotetan (CTT)]. These enzymes also hydrolyze carboxipenicillins, ureidopenicillins and third generation cephalosporins (C3G) to a lesser extent (21). ACC-1 is exceptional in not conferring resistance to cephamycins and is actually cefoxitin inhibited (15). These hydrolytic and inhibition profiles justify the inclusion of the AmpC β-lactamases in the group 1 of Bush-Jacoby-Medeiros classification scheme (22,23). According with resistance

phenotypes conferred by AmpC production, *Enterobacteriaceae* are divided in different groups: *Enterobacteriaceae* lacking AmpC (group 0) or producing intrinsic ("wild-type") (group 1), low- (group 2) or high-level cephalosporinase (group 3), and acquired AmpC β-lactamases (group 4), as shown in Table 1, and detailed below.

Salmonella spp. and *P. mirabilis* lack natural AmpC β-lactamase genes, being intrinsically susceptible to aminopenicillins, carboxipenicillins, ureidopenicillins, aztreonam, cephalosporins and carbapenems (group 0). This susceptibility phenotype is also naturally exhibited by *E. coli* and *Shigella* spp. (group 1) (21), despite they produce very low levels of a non-inducible (lacks an *ampR* gene) chromosomal AmpC-type cephalosporinase (15,21). In many *Enterobacteriaceae* species (e.g. *Enterobacter cloacae*, *Enterobacter aerogenes*, *C. freundii*, *Providencia* spp., *M. morganii*, *H. alvei*, *Serratia* spp., *Pantoeae agglomerans*), the chromosomal AmpC expression is inducible by β-lactams (strong inducers include FOX, imipenem, and CLA) (group 2) (15,21). The low-level cephalosporinase comprises resistance to aminopenicillins, aminopenicillins/β-lactamase inhibitor combinations, and C1G (group 2). They can be divided in sub-groups according with their behavior towards cefuroxime (CXM) and FOX: (i) species usually susceptible to CXM and FOX (*H. alvei*, *Providencia rettgeri*, *Providencia stuartii*, and *Providencia agglomerans*); (ii) species more resistant to FOX than CXM (*E. cloacae*, *E. aerogenes*, and *C. freundii*); and (iii) species more resistant to CXM than FOX (*Serratia marcescens* and *M. morganii*) (21).

Hyperproduction of chromosomal AmpC β-lactamases ("high-level cephalosporinase") is characterized by more or less marked resistance to penicillins (except to amdinocillin or temocillin), C1G, C2G, at least one C3G and aztreonam. C4G are usually more active (group 3). The synergy test (usually performed as a phenotypic test to detect ESBL expression) is negative between clavulanate and C3G, C4G or aztreonam. Cephamycins are inactive except in the species *H. alvei* due to production of ACC-1. Resistance conferred by AmpCs to different β-lactams can be partially or totally restored in the presence of cloxacillin (an AmpC β-lactamase inhibitor) (21). The hyperproduction of AmpC in species with inducible expression of this enzyme (e.g. *Enterobacter* spp., *C. freundii*, *Providencia* spp., and *S. marcescens*) is thought to be derived from the action of the LysR-type transcriptional regulator (LTTR) AmpR, whereas in *E. coli* the high-level cephalosporinase is mainly due to mutations in the promoter or attenuator region and/or gene duplication (16,21,24). Finally, the high-level cephalosporinase phenotype can result from acquisition of a plasmid encoded bla_{AmpC} gene (qAmpC).

Worldwide epidemiology of qAmpCs among *Enterobacteriaceae* from clinical settings

qAmpCs have spread across countries and continents, however their prevalence, distribution and types differ according to geographical location, bacterial species, settings, and time periods analysed (15,25,26). Furthermore, comparing qAmpCs prevalence between countries and even within the same country is a difficult task, if not impossible, since the rates of occurrence of qAmpCs vary according to the sample selected for analysis (isolates resistant to cephalosporins, cefoxitin, ampicillin, fluoroquinolones, or cotrimoxazole; ESBL or carbapenemase producers; presumptive AmpC producers).

The qAmpCs are not linked exclusively to *Enterobacteriaceae* isolated from human origin. The primary reservoirs, however, of such organisms are still contentious and might be multiple (27). In fact, qAmpC-producing *E. coli* and *Salmonella* have been isolated from food-producing (particularly poultry and cattle), wild, stray and domestic animals, from aquatic environments, and also from food (2,27–36). All these reports raise important questions about the possible role of different ecological reservoirs, being suggested an important role of livestock in the selection or enrichment of qAmpC-producing bacteria, particularly of CMY-2 like producers (2,27,29,37,38). Moreover, the global mobility of people (international travel, human migration, patients transfer), and food and livestock trade seem to contribute to the spread or shifts in the occurrence and diversity of qAmpCs in different locations (39,40).

CMY-2 is clearly the most globally widespread and dominant qAmpC, reported mostly in *E. coli* isolates, but also noticeable among *Salmonella* spp., being recovered from different clinical settings and also from non-human hosts as mentioned above (2,6,12,41–48) (Table 2). DHA-1, the second most prevalent qAmpC, has been particularly detected among *Klebsiella pneumoniae* recovered from hospitals (Table 2). Other qAmpC-types have been only sporadically reported (Table 2). Most of qAmpC producers other than *Salmonella* have been associated with urinary tract infections (Table 2).

The relevance of clonal expansion, plasmid dissemination or both mechanisms in the spread of qAmpCs varies according with the bacterial species and/or the qAmpC-type analysed. Overall, the spread of $bla_{\text{CMY-2}}$ among clinical *E. coli* has been largely due to dissemination of some specific plasmid families [please see section "*The impact of MGEs in the qAmpC epidemiology*"], with clonal outbreaks of $bla_{\text{CMY-2}}$ carrying *Salmonella* described in different geographic locations (45,46,49,50). Moreover, outbreaks of particular DHA-1-producing *K. pneumoniae* clones, which have been observed in some countries, have emerged subsequently to plasmid spread.

The following section of this manuscript is dedicated to present an overview of the distribution and population structure of qAmpC-producing *Enterobacteriaceae* recovered

from different clinical settings worldwide. However, few surveys analysed the clonal contribution to the spread of qAmpCs, with most of them being performed in Europe (Table 2), as will be further detailed.

The Americas

In *North America*, CMY-2 is the most commonly detected qAmpC in *E. coli* and *Salmonella* spp. both in hospital and community clinical settings (Table 2) (12,43,48,51,52). Other qAmpC-types have been sporadically reported among different *Enterobacteriaceae* species (Table 2). However, the selected isolates might not be representative of qAmpC-producing *Enterobacteriaceae*, as most of the studies only analysed specific *Enterobacteriaceae* species (e.g. *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *P. mirabilis* or *Salmonella* spp.) (Table 2).

Remarkably, one USA study comparing CMY- or ESBL-producing *E. coli* recovered from different clinical sources in the same period of time (2007), showed that infections caused by CMY-producing *E. coli* were almost as frequent as those involving ESBL-producing *E. coli* (53). Recently (2009-2011), 14% and 7.4% of *E. coli* producing CMY-2 were obtained from community-dwelling human patients and from hospital patients with bloodstream infections, respectively (43,54). In this country, the dissemination of CMY-2 among *Salmonella* multi-drug resistant (MDR) involved in human infections first emerged in *Salmonella* enterica serotype Typhymurium (hence forward *S.* Typhymurium) and latter in *S.* Newport due, in part, to the emergence of "Newport-MDRAmpC" (55–58). The acronym "MDRAmpC" refers to strains that are resistant to 9 antimicrobial agents (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, amoxicillin/clavulanic acid, cephalothin, cefoxitin, and ceftiofur), exhibit either decreased susceptibility or resistance to extended-spectrum cephalosporins (e.g. ceftriaxone), and are sometimes resistant to trimethoprim-sulfamethoxazole (56). These strains are of particular concern, as ceftriaxone is the drug of choice for treating complicated salmonellosis in children (50,57). Subsequent epidemiological studies showed that the CMY-producing *Salmonella* strains were widely spread among livestock animals in various parts of the country being also possible for the majority of these cases to identify an epidemiological link to the handling or consumption of contaminated food products (e.g. beef, egg, chicken, milk) (56–59). Interestingly, during 2000-2002, Moland *et al.* (2006) sampled 63 USA sites (hospitals, clinical laboratories, health care systems) in 34 states and observed that FOX-*like* was the most predominant qAmpC-type, being identified among *K. pneumoniae* and *K. oxytoca* isolates, while in 2010, among *Enterobacteriaceae* bloodstream isolates collected from 26 hospitals located in 20 USA states, FOX-5 was the second most prevalent qAmpC identified mostly in *K. pneumoniae* (43,60). However, in both studies the role of

particular clones in the spread of these enzymes was not evaluated. Recently, phylogenomic analysis of FOX-5-producing *E. coli* and *Klebsiella* isolates from a cohort study investigating the colonization by qAmpC producers from patients in ICUs, demonstrated a considerable genomic diversity (61). Four isolates were identified as likely to have been acquired or selected during the stay at the ICU, since the patients were negative for qAmpC producers upon admission to the ICU. The authors showed that the FOX gene is mobile and that the genomic backbone of *E. coli* or *Klebsiella* is not a determining factor in the acquisition of this feature (61).

In Canada, one study involving patients in Intensive Care Units (ICUs) from 19 tertiary-care hospitals, in a close time period (2005-2006), showed that qAmpC-producing *E. coli* (5.5%; mainly CMY-2) were even more prevalent than ESBL-producing *E. coli* (3.7%; mostly CTX-M-15), which was attributed to the implementation of improved infection control measures to minimize the spread of ESBLs producers, whereas AmpC-producers, which generally are unidentified were not subjected to such infection control measures (62). More recently, a shift in the distribution of acquired β-lactamases was noticed by the CANWARD study (2007-2011), with an increase of ESBL-producing *E. coli* (5.3%; mostly CTX-M-15) over qAmpC-producing *E. coli* (1.3%; mostly CMY-2) (8). In these studies, bacterial typing by Pulsed-Field Gel Electrophoresis (PFGE) revealed that qAmpC-producing *E. coli* were genetically unrelated (8,62,63). The first reported case of CMY-2-producing *Salmonella*, isolated from human patients in Canada, occurred in 2002 during a small outbreak of *S.* Newport (64). Other studies showed that CMY-2 was also frequent among *Salmonella* Heidelberg, *S.* Typhimurium, amongst other serotypes (31,51). Characterization of CMY-2-producing *S.* Heidelberg population recovered from different Canadian sources (livestock, abattoir, retail meat, and human patients) revealed limited genetic diversity amongst isolates of human and non-human origin, suggesting livestock and food of animal origin as an important source of *Salmonella* carrying $bla_{\text{CMY-2}}$ (51).

Regarding *Latin America*, CMY-2 was the most commonly identified qAmpC-type, although available studies analysing qAmpC occurrence are few (Table 2). Nevertheless, the occurrence of CMY-2 varied according to time period, clinical setting, bacterial species and sample selected for analysis.

In Brazil, the presence of qAmpC-producing *Enterobacteriaceae* has been sporadically reported throughout time. In 2001, qAmpCs were not detected among *K. pneumoniae*, *P. mirabilis* or *E. coli* isolates recovered at a large university hospital (65). Latter studies (2002-2003, 2006) reported a single FOX producer in *E. coli* and a single CMY-2-producing *K. pneumoniae* (43,66). On the other hand, $bla_{\text{CMY-2-}\textit{like}}$ was detected in two clonally related *E. coli* strains isolated from the body of a single patient in 2007 (67). More recently (2012), clonally unrelated CMY-producing *E. coli* isolates (n=12;

11 CMY-2, 1 CMY-4) were identified in outpatients (0.46%) and inpatients (1.8%) with suspected urinary tract infections (68).

In Argentina, CMY-2 (or CMY-2-*like*) was the dominant qAmpC-type reported in 2006 and 2009, being identified among clonal related *Shigella flexneri* recovered from paediatric patients and among clonally unrelated *E. coli* recovered from hospital or community settings, respectively (69,70). Nevertheless, CIT group qAmpCs [including LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1 (71)] were identified among different *Enterobacteriaceae* species from six hospitals in Argentina (2009) (72) and have also been detected in a collection of *P. mirabilis* in Chile, of which 59% were recovered from community-acquired infections (73). The high genetic variability observed among *P. mirabilis* isolates suggests that the increase of CIT qAmpCs from 2006 (0.17%) to 2009 (4.5%) might be mainly due to plasmid dissemination (73).

Reports of *bla*$_{CMY-2}$-producing *Salmonella* are infrequent in countries outside North America (55). Possible explanations for this low frequency could be the lack of systematic screening for *Salmonella* isolates carrying such qAmpCs. From 2000 to 2005, an integrated surveillance system of the food chain in Mexico, that included samples from humans with systemic infections and humans with symptomatic/asymptomatic enteric infections, retail meat and food-animal intestines, revealed the emergence and rapid dissemination of MDR *bla*$_{CMY-2}$-producing *S.* Typhimurium in food animals and humans (50,55). PFGE analysis of the MDR *bla*$_{CMY-2}$-producing *S.* Typhimurium isolates revealed high clonal diversity, yet in some of the clusters human isolates were found to be closely related with those from swine intestine or retail meat, supporting the hypothesis of *Salmonella* transmission through the food chain (50,55). In Uruguay, only two faecal isolates of *S.* Typhimurium, obtained from children with diarrhea, were reported as producing CMY-2 (74), while in Honduras, a *S.* Infantis isolate producing CMY-2, CTX-M-15 and SHV-5 was originated from a pediatric patient (75). In Argentina, CMY-2 was described in one *S.* Heidelberg isolate recovered from a diarrheal stool sample of an HIV adult (76).

Europe

The overall data confirm the dispersion of qAmpCs in most European countries (Table 2). Studies involving multiple clinical settings or performed during large periods of time, revealed CMY-2 as the dominant qAmpC, with exception of one Portuguese study reporting the dominance of DHA-1 among *Enterobacteriaceae* isolates (see text below) (26)(Table 2). In Norway, two multicenter studies (12 to 13 laboratories; 2003-2005; 2003-2007) revealed CMY-2 as the dominant qAmpC among a collection of clinical *E. coli* isolates (77,78). In Spain, a multicenter study covering 35 hospitals during a 6-month period and a longitudinal study (1999-2007) performed at one hospital showed that CMY-2 was also the most widely

spread qAmpC among different *Enterobacteriaceae* species (*E. coli*, *P. mirabilis*, *Klebsiella* spp., *Salmonella* spp., *Citrobacter koseri*, *Proteus penneri*), although other qAmpCs (individually or associated) were also identified (Table 2) (25,41). In Poland, a multicenter study involving three hospitals and analyzing *Enterobacteriaceae* isolates during a 3-month period, identified CMY-2-*like* variants as the dominant qAmpC-type, although it was only found in *P. mirabilis* (79). CMY-2 was also the most qAmpC reported in Belgium, Bosnia and Herzegovina, Denmark, France and Switzerland, being mostly identified among *E. coli* recovered from hospitals or community settings (Table 2). In studies only analyzing *Salmonella enterica* isolates, CMY-2 was reported as the dominant qAmpC (France, Germany, Portugal, Romania, Spain) (Table 2) (6,45,47,49,80). Remarkably, in Portugal, a longitudinal study performed during 2002-2013 revealed a distribution of qAmpCs different from that found in other European countries. DHA-1 was detected in different clinical institutions and mostly in *K. pneumoniae* isolates, representing 96% of the qAmpCs identified in 2002-2008 *versus* 55% in 2009-2013, while CMY-2 was mostly recovered from *E. coli* isolates, representing 4% of the qAmpCs identified in 2002-2008 *versus* 45% in 2009-2013, the latter linked to isolates recovered from community (53%) or hospitalized (47%) patients (26). In Bulgaria, the first (2008) and the only reported qAmpC to date, to the best of our knowledge, was identified as ACC-1 in *K. pneumoniae* isolate collected from an hospitalized patient (81).

The characterization of CMY-2 producing *E. coli* population revealed a high clonal diversity and the absence of any predominant clone (Table 2), which could reflect multiple acquisitions possibly from different reservoirs. Nevertheless, some widespread lineages of *E. coli* belonging to phylogenetic group B2 [sequence type (ST) 95, ST131], D (ST69, ST393, ST405, ST648), and A e B1 clonal complexes (ST10, ST155, ST168, ST448) have been detected (Table 2). Sporadic cases and outbreaks in humans in France during 2000-2005 were associated with MDR *S. enterica* serotype Newport (80,82). The authors hypothesized that during 2000–2005 some isolates likely entered France from North America through imported food or they could have come to France and North America from some other country (80). In fact, French and USA MDR-AmpC *S.* Newport shared some characteristics: ST45 and IncH1 plasmid carrying $bla_{\text{CMY-2}}$. In Romania, *Salmonella* isolates (12 *S.* Typhimurium and 1 *S.* Heidelberg) recovered from children with gastroenteritis produced CMY-2. The similarity of the PFGE patterns observed among *S.* Typhimurium isolates suggested an outbreak (49).

Unlike the predominance of CMY-2 in *E. coli* and *Salmonella* spp. isolates recovered from different clinical settings, most of reports showed DHA-1 as the predominant qAmpC among *K. pneumoniae* isolates recovered from hospital settings, with dissemination of particular clones in some countries (Table 2). The international ST11-*K. pneumoniae* clone

(belonging to clonal group CG258) was associated with the spread of DHA-1 in Czech Republic and Portugal (Table 2), despite it had been more commonly linked to the worldwide spread of different carbapenemases (KPC-2/-3; VIM-1/-4; NDM-1; OXA-48/-245) or ESBLs (SHV-5/-12; CTX-M-3/-15; SFO-1) (4,83–88). In Portugal, 48% of DHA-producing *K. pneumoniae* isolates belonged to ST11, obtained from several clinical institutions (hospital and community) at different regions (26). In Czech Republic, all 38 DHA-producing *K. pneumoniae* (of which only 9 were identified as DHA-1) belonged to ST11 (89). In France, the emergence of DHA-1-producing *K. pneumoniae* was associated with ST37, ST48 and ST1263 clones (90,91). The high-risk ST37-*K. pneumoniae* clone, which has a worldwide distribution and is relatively common in healthcare environments (92), was indicated as the responsible for the DHA-1 increase observed from 2008 (0.2%) to 2010 (3.1%) (90), while ST48 and ST1263 clones were linked to the emergence of DHA-1-producing *K. pneumoniae* in surgical ICU at one French hospital (2006-2010) (91). Clonal analysis of DHA-1-producing *K. pneumoniae* isolates associated with an outbreak in the burns ICU at one Spanish hospital, showed the involvement of ST37 (92). The characterization of 26 DHA-1-producing *K. pneumoniae* from other Spanish hospital, revealed that 54% belonged to ST17 (clonal group CG17) (93), which has been frequently associated with the worldwide spread of CTX-M-15 among clinical or commensal isolates (4), and more recently with dissemination of carbapenemases (NDM-1, VIM, KPC, OXA-181) in Egypt, Greece, China, and Singapore (94–99).

Outbreaks of *Enterobacteriaceae* producing other qAmpC-types than CMY-2 or DHA-1 have also been described, but in most cases they seemed to be only of local significance. Clonal outbreaks associated with ACC-1-producing *K. pneumoniae* have been reported in French and Irish hospitals (100,101), while FOX-7-producing *K. pneumoniae* belonging to ST26 or ST14 were involved in a large clonal outbreak in a neonatal ICU in Italy (102). Another large outbreak involving a single *P. mirabilis* clone expressing CMY-16 was also observed in different hospitals and long-term care and rehabilitation facilities in Northern Italy (103–105).

Shifts in the occurrence and diversity of qAmpCs among *Enterobacteriaceae* isolates in some European countries might be associated with patients traveling outside Europe or transferred from one country to other (40,106). In Belgium, for example, one patient who had been previously hospitalized in Montenegro was colonized with *E. coli* producing CMY-16 and NDM-1 carbapenemase (107). In one Serbian patient admitted at a Swiss hospital, and in a Swiss patient transferred from the ICU of a hospital located in Serbia, it was observed infection or intestinal colonization with CMY-16-producing *K. pneumoniae*, respectively (108,109). These isolates also co-produce NDM-1 and/or OXA-48 carbapenemases, and/or CTX-M-15 ESBL.

Asia

CMY-2 and DHA-1 were the most reported qAmpC-types in Asian countries (China, Japan, Korea, and Malaysia) (110–116) (Table 2). Nevertheless, it should be highlighted that most of the study designs were based in biased samples, as the occurrence of qAmpCs has been determined mostly among *E. coli* and/or *K. pneumoniae* isolates. Few studies analysed the occurrence and diversity of qAmpCs among diverse *Enterobacteriaceae* species and most of them did not characterize the population structure of qAmpC producers (Table 2).

CMY-2 was the most common qAmpC in studies only analyzing *E. coli*, recovered either from hospital (China, Iran, Japan, Russia, Thailand) or community settings (China, Japan) (46,97,117–119), while DHA-1 was the most predominant qAmpC among *K. pneumoniae* mainly recovered from nosocomial settings (China, Korea, Japan, Taiwan) (110–114,119–123), and only sporadically associated with outpatients (Japan) (119) (Table 2). Interestingly, two Chinese studies revealed that CMY-2 was as frequent as DHA-1 among *E. coli* (110,111), whereas another study showed DHA-*like* as the predominant qAmpC-type among *E. coli* (124). One study performed in Korea during 2007-2010 revealed that DHA-1-producing *K. pneumoniae* were as frequent as DHA-1-producing *E. coli* (125).

In India, the predominant qAmpC-type varied according with the clinical setting analysed. DHA group qAmpCs (including DHA-1, DHA-2) or CIT and EBC (including MIR-1 and ACT-1) and CIT group qAmpCs were reported as the predominant among *E. coli* and *K. pneumoniae* isolates from different hospitals during the same time period (2009-2010), respectively (126–128), while another Indian study covering five tertiary care centres showed that co-production of CIT and FOX qAmpC-types was the most frequent situation among *E. coli* and *K. pneumoniae* isolates (2007-2008) (129). Furthermore, in a collection of one hundred nosocomial *K. pneumoniae* isolates, CMY-2 was the most detected (2008-2009) (130).

Sporadic descriptions or clonal outbreaks of CMY-2 producing *Salmonella* have been described in Asia. In Thailand, CMY-2 was identified in *S.* Stanley, which is a pig-associated serotype common in Southeast Asia and the second most common serotype implicated in human salmonellosis between 2002 and 2008 (40). Interestingly, authors also showed that at least some of the *Salmonella* Stanley isolates collected from patients in Europe were acquired during travel to Southeast Asia (40). Moreover, an outbreak of CMY-2 producing *Shigella sonnei* involving infected children in two elementary Taiwan schools was described in 2001 (131).

Clonal outbreaks involving MDR *K. pneumoniae* producing DHA-1 have been disclosed in Korea, Japan and China, yet the population structure has not been addressed (114,132,133), whereas TSAR (Taiwan Surveillance of Antibiotic Resistance) surveillance

programme showed that ST11-*K. pneumoniae* producing DHA-1 was the dominant clone (123) (Table 2), although the ST15-*K. pneumoniae* clone has also been identified mainly when isolates were involved in bloodstream infections (122). Dissemination of DHA-1-producing *K. pneumoniae* in a Korean tertiary care hospital was linked both to horizontal and clonal spread (112). A complex epidemiology of qAmpCs involved also clonal and plasmid spread was also observed for DHA-1 and CMY-2 among *E. coli* from a university hospital in China during 2003 to 2005 (110).

Africa

Few African studies evaluated the occurrence of qAmpC-producing *Enterobacteriaceae*, with most of them being conducted in North Africa (Algeria, Egypt, and Morocco) (Table 2), and overall the most predominant qAmpC-type vary between countries. A shift in the CMY-type has been reported in Algeria, which might be related with a temporal evolution by a step-by-step mutation. CMY-2 was the most commonly identified qAmpC in clinical *Enterobacteriaceae* collected between 2003 and 2007 from three Algiers hospitals (134), while CMY-4 (differing from CMY-2 in W661R) was the most prevalent CMY-type identified in different *Enterobacteriaceae* species, mostly (60%) associated with community-acquired infections, reported by a multicenter and longitudinal study involving 5 hospitals and 4 private laboratories in Algeria carried out during 2005-2010 (135). In Egypt, CMY-2 or CMY-2-*like* were the most commonly identified qAmpCs in different *Enterobacteriaceae* species (mostly *E. coli*) (2008, 2011-2012), whereas in Morocco (2010), ACT (ACT-2 and ACT-7) was the dominant qAmpC-type, although sporadically reported among producing *K. pneumoniae* isolates recovered from patients with community-acquired UTIs (136).I In Kenya, *E. coli* co-producing CMY-2-*like* and CTX-M-15 ESBL were responsible for community-acquired UTIs (137). In Nigeria and South Africa, qAmpCs were sporadically reported (Table 2). In Tunisia (2000, 2002-2003), a large clonal outbreak involving ACC-1-producing *S.* Livingstone was reported affecting pediatric wards from three different hospitals (138). Latter (2005-2006), other outbreak was described involving *P. stuartii* producing CMY-2-*like* (4 identified as CMY-16) recovered from patients at a burn ward of one hospital (139). Interestingly, the authors observed that the use of the same aspirator containing CMY-2-producing *P. stuartii* strains was concomitant with the beginning of the outbreak.

Oceania

There are only limited data on the occurrence of qAmpC-producing *Enterobacteriaceae* in this continent, with two studies being performed in Australia and one in New Zealand (Table 2). The only qAmpC identified in *E. coli* was CMY-2 reported

between 2008 and 2010 from three Australian hospitals, with the majority of CMY-2-producing *E. coli* belonging to phylogenetic groups D (60%) and B2 (24%) (12). Also in Australia, *S.* Typhimurium co-producing CMY-7 and SHV-9 was isolated from a stool sample of one hospitalized patient after a trip from Australia through Pakistan, Turkey and Saudi Arabia (140). In New Zealand (2006), the only qAmpC identified in unrelated *E. coli* was also CMY-2, with all the isolates categorized as community-acquired and they were all from the Auckland region (141).

The impact of plasmids and other MGEs in the qAmpC epidemiology among clinical *Enterobacteriaceae*

Although qAmpCs are normally encoded by genes located in plasmids, the capture and dissemination of bla_{qAmpC} between the same or different *Enterobacteriaceae* species are also associated with the horizontal transmission of other MGEs. MGEs can be generally divided in two types: (i) elements that can move from one bacteria to another, as conjugative plasmids, transposons and integrative and conjugative elements (ICEs); and (ii) elements that can move from one genetic location to another in the same cell (transposons, integrons/*gene cassettes*) (142).

Several epidemiological studies showed a high diversity of plasmid backbones (different size, self-transfer ability, and antibiotic resistance patterns) carrying bla_{qAmpC} genes (16,26,51,78,143,144). Molecular epidemiological data available indicate that a diversity of plasmids with the dominance of IncI1 and IncA/C have been associated with spread of $bla_{\text{CMY-2}}$ among members of the family *Enterobacteriaceae* (mostly clonally unrelated *E. coli* or *Salmonella* spp.) recovered from clinical (Table 3) or non-clinical settings (51,54,145–149). Several other bla_{CMY} gene variants were also linked to the IncI1 and IncA/C plasmid families (Table 3). More recently, IncA/C plasmids carrying $bla_{\text{CMY-4}}$ or $bla_{\text{CMY-16}}$ have acquired other antibiotic resistance genes, such as genes coding for carbapenemases ($bla_{\text{NDM-1}}$, $bla_{\text{VIM-4}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-204}}$) and/or quinolone resistance (QNR) proteins (*qnrA6*), ESBLs ($bla_{\text{CTX-M-15}}$, $bla_{\text{PER-1}}$), 16S rRNA methylases (*armA*), and fosfomycin resistance (*fosA3*) (108,109,150–154). The IncK was the third most common plasmid replicon-type associated with $bla_{\text{CMY-2}}$ (Table 3). Diverse plasmid families were associated with $bla_{\text{DHA-1}}$ identified in *K. pneumoniae*, although the most commonly reported include the IncL/M, IncHI2, IncR and IncFIA groups. These plasmids carrying $bla_{\text{DHA-1}}$ have also been reported among other *Enterobacteriaceae* species (Table 3). Regarding FOX-5, the majority of the $bla_{\text{FOX-5}}$–carrying plasmids among *E. coli* and *Klebsiella* spp. in the USA were categorized in the IncA/C group (Table 3).

Plasmids carrying qAmpCs genes ($bla_{\text{CMY-2-}like}$, $bla_{\text{DHA-1-}like}$, $bla_{\text{FOX-}like}$, $bla_{\text{ACT-1}}$, $bla_{\text{ACT-2}}$, $bla_{\text{ACT-7}}$, $bla_{\text{ACT-16}}$) often carry multiple other β-lactam resistance genes, including genes

coding for ESBLs (TEM-type, SHV-type, CTX-M-type, PER-1, OXA-1) and/or carbapenemases (OXA-48/-162/-204, VIM-1/-4, IMP-1/-4, NDM-1, KPC-2, CARB-2/PSE-1) (Table 2) (61,109,133,150,152,153,155–160), as well as resistance determinants to non-β-lactam antibiotics, such as aminoglycosides (e.g. *armA*, *rmtB*, *rmtC*), fluoroquinolones [e.g. *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*], and sulfonamides (e.g. *sul1*), amongst others (26,86,88,109,155,161–164). These resistance genes can be found in identical or different plasmid backbone (109,164). This situation is of public health concern because complicate the management of infections due to *Enterobacteriaceae* being resistant to several antimicrobial agents, especially last resort drugs (e.g. carbapenems).

The insertion sequence (IS) *Ecp1* can co-mobilize flanking DNA fragments via a one-ended transposition mechanism, and it also can supply an efficient promoter for the high-level expression of neighbouring genes (15). This transposable element (IS*Ecp1*/*Δ*IS*Ecp1*) has been responsible for the mobilization of genes encoding CMY-2-*like* [CMY-2, CMY-4, CMY-5, CMY-7, CMY-12, CMY-14 to CMY-16, CMY-21, CMY-23, CMY-25, CMY-27, CMY-31, CMY-36, CMY-40, CMY-42, CMY-48, CMY-57, CMY-58, CMY-59, CMY-60], ACC-1 and ACC-4 from the chromosome of *Enterobacteriaceae* species to plasmids (15,29,143,144,165). Other IS, namely IS*26* has been associated with the mobilization of DHA-1 (144). A composite transposon comprising a *C. freundii*-derived sequence of 4,252 bp which including the *bla*$_{\text{CMY-13}}$-*ampR* region, was flanked by two directly repeated IS*26* elements in *E. coli*, indicating that this gene might have spread from the *Citrobacter* chromosome to a plasmid through an IS*26*-dependent mobilization, similarly as described for *bla*$_{\text{SHV}}$ genes in *S.* Typhimurium and *E. coli* (166).

Although plasmids have proven to be one of the main mechanisms involved in the rapid dissemination of *bla*$_{\text{qAmpC}}$ among *Enterobacteriaceae*, other MGEs such as SXT/R391-*like* ICEs have been associated with the mobilization of *bla*$_{\text{CMY-2}}$ in *P. mirabilis* (26,142,167). ICEs are self-mobile elements found in the genome of some bacteria (168). They are able to integrate into bacterial chromosomes, to excise from the chromosome, and to transfer themselves from one bacteria to another by conjugation, despite they are not able to replicate by themselves (142,168). Integration of the ICE is usually site-specific and frequently occurs at the 3′ end of a tRNA gene (168).

Genes encoding other qAmpCs (CMY-1, -8, -9, -10, -11, and -19; DHA-1, -6, -7; MOX-1; ACC-1) have been found adjacent to an IS*CR1* involved in gene mobilization into complex class 1 integrons (15,101,144). Class 1 integrons are derivatives of Tn*402* transposons, but they are defective for self-transposition and their dissemination occurs through association with transposons (mostly Tn*3*/Tn*21* family) and/or plasmids (169). The *bla*$_{\text{FOX-3}}$, *bla*$_{\text{FOX-8}}$, *bla*$_{\text{FOX-10}}$ (GenBank accession number NZ_AKYX01000140) and *bla*$_{\text{ACT-10}}$ were located in a class 1 integron (144,170), whereas *bla*$_{\text{FOX-7}}$ was associated with a transposon (Tn*6240*)

(171). In summary, the horizontal spread of *bla*$_{qAmpC}$ genes among strains belonging to the same or different *Enterobacteriaceae* species seems to be associated with a complex dissemination process involving different MGEs.

Conclusions

Epidemiological data regarding qAmpCs among *Enterobacteriaceae* can be characterized at the best as incomplete. Therefore, only estimations of their worldwide prevalence can be made, a situation that reflects the lack or insufficiency of surveillance systems in many countries (mostly African, South American and Oceanian countries). Furthermore, it is difficult to compare the occurrence of qAmpCs among clinical *Enterobacteriaceae* from different countries of the world, as there is no uniformity regarding the bacterial species, time period and type clinical setting(s) analysed in the different studies. Notwithstanding these limitations, some general epidemiological features are highlighted. First, it is clear that CMY-2 is the predominant qAmpC described in different clinical settings mostly among *E. coli* or *Salmonell*a spp. isolates, whereas DHA-1-producing *K. pneumoniae* isolates are more related to hospital setting. Second, the mechanisms of qAmpC spread, amplification and persistence might be complex, involving clonal spread of bacteria (usually with only local impact) and also plasmid dissemination, which has proven to have an important role in the rapid dissemination of *bla*$_{qAmpC}$ among *Enterobacteriaceae*, although other MGEs may also be relevant for the epidemiology of qAmpCs. In fact, particular plasmids have been associated with the global spread of *bla*$_{CMY-2}$ among *Enterobacteriaceae*, while the participation of clones and/or plasmids contributed to the amplification of DHA-1 in hospital settings. Third, in the last years, increasing qAmpC-producing *Enterobacteriaceae* resistant also to other antibiotics, including to last resort drugs (e.g. carbapenems), by carrying other mechanisms of resistance, constitute a complex multifactorial problem of high public health significance that deserves a deep analysis and the implementation of specific interventions at different levels.

Funding

This work received financial support from the European Union (FEDER funds) through Programa Operacional Fatores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through projects UID/Multi/04378/2013 and UID/Multi/04546/2013. T. G. R. and Â. N. were supported by fellowships from FCT (SFRH/BD/75752/2011 and SFRH/BPD/104927/2014, respectively) through Programa Operacional Capital Humano (POPH).

Transparency declarations

None to declare.

Table 1. AmpC resistance phenotypes conferred by AmpC β-lactamases intrinsic in some *Enterobacteriaceae* species.

Antibiotic(s)	Species lacking β-lactamases (group 0)	Species intrinsically producing AmpC (group 1)	Species producing low-level intrinsic AmpC (group 2)	Species producing intrinsic/acquired high-level AmpC (hyperproduction) (group 3)			qAmpC (group 4)
	Salmonella spp., *P. mirabilis*	*E. coli*, *Shigella* spp.	*E. cloacae*, *E. aerogenes*, *S. marcescens*, *C. freundii*, *M. morganii*, *H. alvei*, *P. stuartii*, *P. rettgeri*, *P. agglomerans*	*Salmonella* spp., *P. mirabilis*, *E. coli*, *Sighella* spp.	*K. pneumoniae*, *K. oxytoca*, *C. koseri*, *C. amalonaticus*, *E. hermanii*	*E. cloacae*, *E. aerogenes*, *S. marcescens*, *C. freundii*, *M. morganii*, *H. alvei*, *P. stuartii*, *P. rettgeri*, *P. agglomerans*, *P. vulgaris*, *P. penneri*	
Aminopenicillins	S	S/I	R	R	R	R	R
Aminopenicillins/ CLA	S	S/I	R	R	R	R	R
Carboxypenicillins	S	S	S	R (I)	R (I)	R (I)	R
Carboxypenicillins/CLA	S	S	S	R (I)	R (I)	R (I)	R
Ureidopenicillins	S	S	S	R (I)	R (I)	R (I)	R
C1G	S	S/I	R	R	R	R	R
C2G	S	S	R/I/S[a]	R/I/(S→I)	R/I/(S→I)	R/I/(S→I)	R
FOX	S	S	R/I/S[a]	R/I	R/I	R/I (S[g])	R (S[g])
C3G	S	S	S	R[c]/I/(S→I)	R[c]/I/(S→I)	R[c]/I/(S→I)	R
C4G	S	S	S	S (I/R[d])	S	S (I/R[h])	S (R)
Carbapenems	S	S	S	S[e]	S[f]	S[i]	S

CLA, clavulanic acid; C1G, first-generation cephalosporins; C2G, second-generation cephalosporins; C3G, third-generation cephalosporins; C4G, fourth-generation cephalosporins; FOX, cefoxitin; I, intermediate; R, resistance; S, susceptible.

[a] Result depends on species and isolate.

[b] →, proposed interpretation; (), rarely observed.

[c] At least one third-generation cephalosporin is not susceptible.

Table 1. *Continued*

[d] A very small number of *E. coli* produce AmpC mutants capable of hydrolyzing fourth-generation cephalosporins.

[e] I or R if there is also resistance due to impermeability (alteration of porins). The combined resistance phenotype has been mainly observed in *E. coli*.

[f] I or R if there is also resistance due to impermeability (alteration of porins). The combined resistance phenotype has been mainly observed in *K. pneumoniae*.

[g] Intrinsic or acquired cephalosporinase hyperproduction in *H. alvei*.

[h] A very small number of *E. cloacae*, *C. freundii* and *S. marcescens* produce AmpC mutants with increased activity towards fourth-generation cephalosporins.

[I] I or R if there is also resistance due to impermeability (alteration of porins). This resistance phenotype has been mainly observed in association with AmpC hyperproduction in *E. cloacae*, *E. aerogenes*, and *C. freundii*.

Table 2. Worldwide epidemiology of qAmpCs among *Enterobacteriaceae* from clinical settings.

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
America									
Argentina	CMY-2 (18)	*E. coli* (17), *S.* Heidelberg* (1)				urine (12), abdominal fluid (4), blood (4), skin (1), perianal abscess (1), faeces (1), NI (19)	2006, 2009, 2012	C, H	(69,70,72,76)
	CIT group genes (15)	*P. mirabilis* (8), *E. coli* (4), *K. pneumoniae* (1), *Salmonella* spp. (1), *S. flexneri* (1)							
	CMY-2 like (7)	*E. coli** (3), *S. flexneri** (3), *P. mirabilis** (1)							
	DHA-1 (2)	*P. mirabilis* (1), *K. pneumoniae* (1)							
Brazil	CMY-2 (11)	*E. coli**				urine (13), blood (2) , abdominal fluid (1)	2000-2003, 2006, 2007, 2012	C, H	(43,65–68)
	CMY-2-like (3)	*K. pneumoniae*, *E. coli**							
	CMY-4 (1)	*E. coli**							
	FOX-5-like (1)	*E. coli**							
Canada	CMY-2 (463)	*S. enterica** (290), *E. coli** (173)	D (16), A (8), B2 (2), ST131 (1), NP (116)			urine (23), blood (11), stool (6), wound (1), respiratory tract (1), NI (524)	2000-2012	C, H	(8,51,62,63,172–175)
	CIT group genes (110)	*E. coli**							
	ACT-1 (1)	*E. coli**	A (1)						
	FOX-1 (1)	*E. coli**							
	FOX-5 (1)	*E. coli**							
Canada, USA[b]	CMY-2 (12)	*S. enterica** (12)				NI	2005-2007	H	(48)

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Chile	CIT group genes (34)	*P. mirabilis**				urine (17), ulcers and wounds (14), respiratory tract (1), catheter (1), blood (1)	2006-2009	C, H	(73)
Honduras	CMY-2	*S.* Infantis*			SHV-5, CTX-M-15	NI	2002	H	(75)
Mexico	CMY-2-like (80)	*S.* Typhimurium*				blood (1), NI (79)	2000-2005, 2011	H	(50,55,176)
Uruguay	CMY-2-like (2)	*S.* Typhimurium*				faeces	2009, 2010	H	(74)
USA	CMY-2 (111)	*E. coli* (50+46*), *P. mirabilis* (13), *K. pneumoniae* (1+1*)			CTX-M-14 (2), TEM-1-*like* (2), TEM-2-*like* (1)	urine (35), blood (4), NI (109)	2000-2002, 2004, 2005-2008, 2009-2011	C, H	(43,52–54,60,177,178)
	FOX-like (30)	*K. pneumoniae** (26), *K. oxytoca** (4)			SHV-ESBL (18)				
	CMY-like (6)	*P. mirabilis** (4), *Salmonella* spp.* (2)							
	FOX-5 (9)	*K. pneumoniae* (3), *E. coli** (3), *P. mirabilis*(1+1*), *E. cloacae* (1)							
	CMY-2-like (3)	*E. coli**							
	CMY-108 (2)	*E. coli**							
	DHA-like (2)	*K. pneumoniae**, *K. oxytoca**							
	DHA-1 (2)	*E. coli**, *P. mirabilis*							
	CMY-18 (1)	*E. coli**							
	CMY-32 (2)	*E. coli**	D (2)						
	CMY-33 (1)	*E. coli**	A (1)						

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
	FOX-like (1)	*E. coli**							
	ACT-1-like (1)	*K. pneumoniae**							
Europe									
Belgium	CMY-2 (10)	*E. coli**	D (6), B2 (2), B1 (1), A (1)			NI	2004-2006	H	(106)
	ACC-1 (1)		BI						
Bosnia and H erzegovina	CMY-2 (4)	*E. coli* (2), *K. oxytoca* (1), *P. mirabilis* (1)			CTX-M	urine	2009-2010	C, H	(179)
	DHA-1 (3)	*K. pneumoniae* (1), *K. oxytoca* (1), *P. mirabilis (1)*							
Bulgaria	ACC-1 (1)	*K. pneumoniae** (1)				blood	2008	H	(81)
Czech Republic	DHA group genes (27)	*K. pneumoniae**		ST11 /CG258		urine (13),wound (6), throat (4), stomach (3), sputum (3), BAL (2), blood (3), abscess (1), tracheostomy tube (1)	2006	H	(89)
	DHA-1 (9)								
Denmark	CMY-2 (8)	*E. coli** (4), *S.* Stanley* (4)	ST46/CC46, ST48/CC10, ST448/CC448, ST652			blood (2), NI (8)	2006, 2008, 2009	H	(40,180,181)
	CMY-22 (2)	*E. coli**	B2						

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
France	CMY-2 (76)	*S.* Newport* (47), *E. coli** (29)	B1 (7), B1-ST156/CC156 (8), B1-ST155/CC155, A-ST46/CC46 (4), A-ST167/CC10, A-ST540, D-ST354/CC354 (2), D-ST68, D-ST117, D-ST420, B2-ST12/CC12, B2-ST978			stool (54), blood (2), catheter (2), urine (2), blood (1), broncho-alveolar lavage (1), bronchoscopy with protected brush catheter (1), NI (50)	2000-2009	H	(80,82,90,91,182,183)
	DHA-1 (23)	*K. pneumoniae**		ST48 (9), ST37 (5), ST1263 (4), ST359 (1), NP (4)	SHV-12 (4)				
	CMY-like (12)	*S.* Newport*							
	CMY-2-like (1)	*E. coli**	A-ST46/CC46						
	ACC-1 (1)	*E. coli**	BI						
Germany	CMY-2 (8) ACC-1 (6)	*S. enterica**				NI	2005-2011	NI	(45)
Italy	CMY-16 (71)	*P. mirabilis**			TEM-92 (3)	urine (62), skin and soft-tissue (4), lower respiratory tract (3), blood (1), cutaneous ulcer (1)	2003-2006	C, H	(103–105)

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Norway	CMY-2 (44)	*E. coli**	D-ST38/CC38, D-ST69/CC69, D-ST393/CC31, D-ST405/CC405, D-ST648/CC648, D-ST117, D-ST420, D-ST778(4), D-ST963, D-ST981, A-ST10/CC10 (2), A-ST448/448 (2), A-ST88/CC23, A-ST155/CC155, A-ST93/CC168, A-ST359, A-ST453, A-ST976, B2-ST131/CC131 (4), B2-ST979/CC95, B2-ST127, B2-ST636, B1-ST448/448 (3), B1-ST88/CC23, B1-ST155/CC155, NP(9)		TEM (7)	blood (38), NI (10)	2003-2007	H, NI	(77,78)
	CMY-7 (2)		A-ST167/CC10, NP						
	DHA-1 (2)		A-ST23/CC23, B1-ST977						

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Poland	CMY-2-like (47) CMY-15 (19) CMY-12 (4) CMY-38 (1)	*P. mirabilis*			CTX-M (8)	urine (50), NI (21)	2003-2004	H	(79)
Portugal	DHA-1 (81)	*K. pneumoniae* (50), *Enterobacter* spp. (17), *K. oxytoca* (6), *E. coli* (5), *K. variicola* (3)	B1 (2), A1 (2), B2 (1)	ST11/GC258 (24), ST1380 (14), ST440 (2), ST1224 (2), ST17 (1),ST323 (1), ST416 (1), ST443 (1), ST1871 (1), ST1872 (1), ST1873 (1), ST1874 (1)	SHV-12 (28), SHV-90 (6), SHV-5 (5), SHV-145 (1), CTX-M-32 (1)	urine (43), sputum (20), blood (5), exudate (5), catheter (2), peritoneal fluid (1), faeces (1), NI (23)	2002-2013	H, C	(26,184)
	CMY-2 (18)	*E. coli* (16), *P. mirabilis* (1), *S. enterica** (1)	B1 (7), A (4), B2-ST131 (2), B2-ST95/CC95 (1), D-ST57/350 (1), D-ST117 (1)		CTX-M-15 (2), CTX-M-1 (1)				
	DHA-6 (1)	*K. pneumoniae*		ST11/GC258					
Romania	CMY-2 (13)	*S.* Typhimurium* (12), *S.* Heidelberg* (1)				stool	2000-2001	C, H	(49)

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Spain	CMY-2 (937)	*E. coli* (529+286*), *P. mirabilis* (82+5*), *K. pneumoniae* (24), *S. enterica* (5*+2), *K. oxytoca* (2), *C. koseri* (1), *P. penneri* (1)	B2 (52), B1 (54), A (42), D (25), F(20), B2 (19), C (10), B2-ST131 (6), D-ST354/CC354 (4), D-ST57/CC350 (4), D-ST115 (3), D-ST393/CC31 (3), D-ST420 (3), D-ST38/CC38 (2), D-ST405/CC405 (1), D-ST648 (1), NP (529), NI (37)		CTX-M-1 (2), CTX-M-14 (2), CTX-M-9 (2), TEM-4 (2), CTX-M-1 (1), CTX-M-2 (1), CTX-M-14 (1), CTX-M-15 (1), SHV-12 (1)VIM-1 (1)	urine (225), skin and soft-tissue (30), blood (29), fluids and tissue (17), respiratory tract (7), wound (2), catheter (2), anal exudate (1), abdominal exudate (1), ascitic liquid (1), bronchoalveolar fluid (1), tracheal fluid (1), peritoneal fluid (1), NI (936)	1999-2012	C, H	(25,39,41,42,47,92,93,143,144,185–189)
	DHA-1 (270)	*E. coli* (82+63*), *K. pneumoniae* (82+26*), *K. oxytoca* (9), *P. mirabilis* (4), *C. koseri* (2), *S. enterica* (1*+1)	B2 (21), F (8), B1 (6), A (6), C (5), D (5), E (1), NP (56) , NI (11)	ST37 (27), ST17 (14), ST326 (3), ST427 (3), ST416 (2), ST13 (1), ST440 (1), ST428 (1), NP (83)	CTX-M-15 (7), CTX-M-14 (4), SHV-12 (2), SHV-2 (1), VIM-1 (1)				
	ACC-1 (12)	*E. coli* (5), *K. pneumoniae* (4), *P. mirabilis* (3)							
	CMY-4 (7)	*E. coli* (5+2*)	B1-ST19 (1), D (1), NP (5)						
	CMY-27 (4)	*E. coli*							
	CMY-2-like (3)	*E. coli*							
	FOX-8 (2)	*E. coli*							

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
	DHA-7 (2)	*E. cloacae*			VIM-1 (2)				
	CMY-7 (2)	*E. coli*							
	CMY-42 (2)	*E. coli**	B1-ST58/CC155, NI						
	CMY-25 (1)	*K. pneumoniae*							
	CMY-30 (1)	*E. coli**							
	CMY-40 (1)	*E. coli*							
	CMY-43 (1)	*E. coli*							
	CMY-48 (1)	*E. coli*							
	CMY-54 (1)	*E. coli*							
	CMY-55 (1)	*E. coli*							
	CMY-56 (1)	*K. pneumoniae*							
	CMY-57 (1)	*E. coli*							
	CMY-96 (1)	*K. pneumoniae*							
	DHA-6 (1)	*E. coli*							
	FOX-3 (1)	*E. coli*							
	CMY-2+DHA-1 (1)	*K. pneumoniae*							
	ACC-1+FOX-3 (1)	*E. coli*							
Switzerland	CMY-2 (14)	*E. coli* (12), *P. mirabilis* (1), *K. pneumoniae* (1)				urine (1), NI (17)	2006-2007, 2009-2010	H	(108,190)
	DHA-1 (2)	*E. coli*							
	CMY-31 (1)	*K. pneumoniae*							
	CMY-16 (1)	*K. pneumoniae*		ST25	NDM-1, CTX-M-15				

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
The Netherlands	CMY-2 group (27)	*E. coli* (22), *P. mirabilis* (4), *K. pneumoniae* (1)			CTX-M-15 (2), CTX-M-1 (1)	NI	2009	NI	(191)
	MIR-1/-2/-3 (2)	*E. coli*, *K. pneumoniae*			CTX-M-15 (1), SHV-4 group (1)				
	ACT-5 (1)	*E. coli*							
	ACT-like (1)	*E. coli*							
	DHA-1 (1)	*K. pneumoniae*			CTX-M-1 group+SHV-4 group (1)				
UK[a]	CIT group genes (60)	*E. coli** (59), *K. pneumoniae** (1)			CTX-M-1 group (20)	NI	2004-2007	H, NI	(101,192)
UK (Ireland)	ACC group genes (14)	*K. pneumoniae** (10), *E. coli** (4)							
	ACC-1 (13)	*K. pneumoniae**							
	DHA-1 (2)	*K. pneumoniae**							
UK (Wales)	FOX group genes (11)	*K. pneumoniae** (8), *E. coli** (3)							
UK (Ireland, Scotland)	DHA group genes (3)	*K. pneumoniae** (2), *E. coli** (1)							
Turkey	CMY-2 (13)	*E. coli**				blood (5), NI (34)	2007-2012	C, H	(193–195)
	CIT group genes (9)								
	MOX group genes (8)								
	EBC group genes (3)								
	ACT-1 (2)	*K. pneumoniae**							
	CIT+FOX group genes (1)	*E. coli**							

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
	MOX group (3)	*K. pneumoniae**							
Asia									
	CMY-2 like (4)	*E. coli*			CTX-M-55 (1)	blood	2007-2010	H	(196)
Cambodia	DHA-1 (2)	*K. pneumoniae*			CTX-M-15+CTX-M-14 (1)				
China	DHA-1 (129)	*K. pneumoniae** (107), *E. coli** (21), *K. oxytoca** (1)			CTX-M-*like*+SHV-*like* (20), SHV-*like* (17), KPC-2 (2), CTX-M-14 (2), CTX-M-3 (1)	NI	2003-2006, 2010-2011, 2012	H, C	(110,111,124,165,197)
	CMY-2 (28)	*E. coli** (27), *K. pneumoniae** (1)			CTX-M-*like* (9), CTX-M-15 (2), CTX-M-24 (1)				
	DHA-like (11)	*E. coli**							
	CMY-42 (1)	*E. coli**	A-ST3835		NDM-1, CTX-M-15, SHV-12				
	ACC-like (1)	*E. coli**							
India	CIT group genes (54)	*E. coli**(34+NI), *K. pneumoniae** (17+NI)			CTX-M-15 (NI), CTX-M-9-*like* (NI)	urine (65), skin and soft tissue (17), blood (18), other body fluids (31), NI (87)	2007-2010	C, H	(126–130)
	DHA group genes (47)	*E. coli** (24+NI), *K. pneumoniae** (22+NI)							
	CIT+EBC group genes (31)	*E. coli** (28), *K. pneumoniae** (4)			CTX-M (NI)				
	CIT+ FOX group genes (25)	*E. coli** (NI), *K. pneumoniae** (NI)							
	EBC group genes (21)	*E. coli** (9+NI), *K. pneumoniae** (2+NI)			CTX-M (NI)				

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
	CMY-2 (12)	*K. pneumoniae**							
	MOX group genes (13)	*E. coli**(11), *K. pneumoniae** (2)							
	DHA-1 (8)	*K. pneumoniae**							
	EBC+DHA group genes (5)	*E. coli** (2+NI), *K. pneumoniae** (2+NI)							
	FOX group genes (4)	*E. coli** (NI), *K. pneumoniae** (NI)							
	EBC+ACC group genes (2)	*E. coli** (NI), *K. pneumoniae** (NI)							
	ACC group genes (2)	*K. pneumoniae**, *E. coli**							
	FOX+DHA group genes (1)	*E. coli** (NI), *K. pneumoniae** (NI)							
	FOX+EBC+DHA group genes (1)	*E. coli** (NI), *K. pneumoniae** (NI)							
Iran	CMY-2 (14)	*E. coli**				urine, wound	2011-2012	H	(117,118)
Japan	DHA-1 (47)	*K. pneumoniae** (41), *K. pneumoniae* (6)			SHV-12 (13)	urine (19), pus (3), sputum (36), PTCD (2), artificial graft (2), pus (2), faeces (2), CVC (1), blood (1), bile (1), drain (1), joint liquid (1), throat (1)	2000-2008	C, H	(119,121)
	CMY-2 (22)	*E. coli* (17), *K. oxytoca* (2), *K. pneumoniae** (2), *P. mirabilis* (1)			CTX-M-*like* (1), SHV-12+IMP-1 (1)				
	CMY-8 (2)	*K. pneumoniae*							
	MOX-1 (1)	*K. oxytoca*							

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Korea	DHA-1 (176)	*K. pneumoniae* (93*+37), *E. coli** (39), *K. oxytoca* (5), *Salmonella* spp. (1), *P. mirabilis** (1)				urine (93), sputum (83), wound (20), blood (11), body fluid (10), NI (261)	2002-2004, 2007-2010	H	(112–115,125)
	DHA-1 group (110)	*K. pneumoniae** (93), *E. coli** (10)							
	DHA-1 like* (72)	*K. pneumoniae** (55), *E. coli** (8), *K. oxytoca** (8), *P. mirabilis** (1)							
	CMY-2 group (39): CMY-2(3), CMY-18 (1), ND (35)	*E. coli** (38), *K. pneumoniae** (1)							
	CMY-2 (65)	*E. coli** (45), *K. pneumoniae* (10*+1), *P. mirabilis** (9)							
	CMY-1 group (15): CMY-10 (6), ND (8)	*E. coli** (14), *K. pneumoniae** (2)							
	CMY-1 (4)	*E. coli** (1), *K. pneumoniae* (1*+1), *P. mirabilis** (1)							
	CMY-10 (2)	*P. mirabilis**			PER-1+TEM-52 (1)				
	ACT-1 (1)	*K. pneumoniae* (1)							
Malaysia	DHA-1-like	*Enterobacter* spp.*				urine	2012-2014	NI	(116)
Pakistan	CIT group genes (23) MOX group genes (16) EBC group genes (4) FOX group genes (2)	*E. coli**			CTX-M-1 group (1)	urine (25), pus (12), cerebrospinal fluid (4), blood (1), NI (3)	2008-2009	C, H	(198)
Russia	CMY-2 (1)	*S. enterica**				NI	2002-2005	H	(46)

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Singapore	CIT group genes (41)	*E. coli** (34), *P. mirabilis** (4), *K. pneumoniae** (3)				urine (46), blood (3), NI (3)	NI	C, H	(199)
	DHA group genes (11)	*K. pneumoniae** (10), *E. coli** (1)							
Tawain	CMY-2 like (137)	*E. coli** (127), *K. pneumoniae** (10)			SHV-5-*like* (5)	blood (20), stool (8), NI (46)	2001, 2004, 2002-2009, 2011, 2002-2012	C, H	(122,123,131,200,201)
	CMY-like (48)	*P. mirabilis**			ESBL (7)				
	DHA-1 like (31)	*K. pneumoniae**			SHV-5-*like* (5)				
	DHA-1 (31)	*K. pneumoniae**		ST15 (6), ST11 (7), ST6 (1), ST48 (1), ST76 (1), ST101 (1) ST147 (3), ST152 (1), ST219 (1), ST322 (1), ST367 (1), ST685 (1), ST1180 (1), ST1380 (1), ST1926 (1)	SHV (6), CTX-M (2), CTX-M+SHV (1), CTX-M-3 (7), SHV-12+CTX-M-3 (3), SHV-12+CTX-M-15 (1)				
	CMY-2 (9)	*Shigella sonnei** (8), *K. pneumoniae** (1)		ST48	SHV-5 (8), CTX-M-14 (1)				
	DHA-like (6)	*P. mirabilis**			ESBL (7)				
Thailand	CMY-2	*S.* Stanley* (19)				urine (19), pus (13), sputum (9), body fluid (9), blood (2), NI (19)	2005-2006, 2008	H	(40,202)
	CMY-2 like (46)	*E. coli** (40), *K. pneumoniae** (6)			ESBL (15)				
	CMY-1 like (4)	*E. coli** (3), *K. pneumoniae** (1)							
	DHA-1 (2)	*E. coli** (1), *K. pneumoniae** (1)							

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
Africa									
Algeria	CMY-4 (14)	*E. coli* (9), *K. pneumoniae* (4), *P. mirabilis* (1)	D (3), B1 (3), B2 (2), F (1)	ST1618 (2), ST17, ST1617	CTX-M-15 (2)	urine (17), faeces (5), blood (2), pus (2)	2003-2010	C, H	(134,135)
	CMY-2 (8)	*P. stuartii (3), E. coli* (2), *K. pneumoniae (1), P. mirabilis(1), S. marcescens (1)*			CTX-M-15 (3)				
	DHA-1 (4)	*K. pneumoniae* (1), *E. coli* (1), *E. cloacae* (2)		ST834	SHV-12 (2), CTX-M-15 (1), CTX-M-3+SHV-12				
Egypt	CMY-2 (18)	*E. coli* (11), K. *pneumoniae* (4), *K. oxytoca* (3)				urine (22), NI (34)	2008, 2011-2012	C, H	(44,203)
	CMY-2 variant (14)	*E. coli* (11), *Klebsiella* spp. (2), *P. mirabilis* (1)							
	CMY (13)	NI			CTX-M-15 (10)				
	DHA (4)	NI							
	DHA-1 (6)	*K. pneumoniae* (3), *E. coli* (2), *P. mirabilis* (1)							
	CMY-4 (1)	*K. pneumoniae*							
	CMY-4 variant (1)	*E. coli* (1)							
Kenya	CMY-2-like (12)	*E. coli**			CTX-M-15 (12)	urine	2004-2005	H, C	(137)
Morocco	ACT-7 (2)	*K. pneumoniae**			CTX-M-15 (1), CTX-M-15+TEM-3 (1)	urine	2010	C	(204)
	ACT-2 (1)				SHV-12				
	DHA-1 (1)				CTX-M-15				
Nigeria	DHA-1 (3)	*K. pneumoniae*, *P. mirabilis*, *M. morganii*			CTX-M-15 (4)	ear (1), aspirate (1), wound (1),	2005-2007	H	(205,206)

Table 2. *Continued*

Continent Country	qAmpC enzyme(s) (no.)c	Species* (no.*)	*E. coli* phylogenetic group-ST (no.)	*K. pneumoniae* ST (no.)	Co-production of other ESBL or carbapenemases (no.)	Sample type (no.)	Year(s) of isolation	Clinical settings[d]	References
	CMY-2 (1) ACT-1 (1)	*C. freundii* *K. pneumoniae*				blood (1), catheter tip (1)			
South Africa	CMY-2 (3)	*S .enterica**			CTX-M-37+TEM-63 (1), TEM-63 (1), SHV-2 (1)	stool	2001	H	(207)
Tunisia	ACC-1 (111) CMY-2-like (59) CMY-16 (4) DHA-1 (2)	*S.* Livingstone* *P. stuartii** *P. stuartii** *Providencia* spp.*			SHV-2a (2) TEM-24 (2)	catheter (1), blood (1), NI (169)	2002-2003, 2005-2006, 2008	H	(138,139,208)
Oceania									
Australia	CMY-2 (67) CMY-7 (1)	*E. coli** *S.* Typhimurium*	D (40), B2-non ST31 (10), B2-ST131 (6), A (8), B1 (3)		CTX-M-15 (7) SHV-9 (1)	urine (54), rectal swabs (4) respiratory tract (3), intra-abdominal (2), wound (2), blood (1), faeces (1), unspecified sterile site (1) faeces	2000, 2008-2010		(12,140)
New Zealand	CMY-2 (5) CMY-29 (1)	*E. coli**				urine	2006	C	(141)

Table 2. *Continued*

BAL, bronchoalveolar lavage; CVC, central venous catheter; NP, not performed; NI, not indicated; PTCD, extract from percutaneous transhepatic cholangial drainage; ST, Sequence type.

[a]UK, United Kingdom (includes England, Scotland, Wales, and Northern Ireland).

[b]USA, United States of America.

[c]MOX group qAmpCs (including MOX-1, MOX-2, CMY-1, and CMY-8 to CMY-11), CIT group qAmpCs (including LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1), FOX group qAmpCs (including FOX-1 to FOX-5b), EBC group qAmpCs (including MIR-1 and ACT-1), DHA group qAmpCs (including DHA-1, DHA-2) according to Pérez-Pérez *et al.*, 2002 (71).

[d]C, Community (Long-Term Care and Rehabilitation Facilities; private laboratories; nursing homes); H, Hospital.

Table 3. Overview of plasmid families associated with qAmpC β-lactamases in *Enterobacteriaceae* recovered from different clinical settings.

Enzyme	Replicon(s)[a]/ Relaxases[b]	Species	Total no. of plasmids	Country(ies)	Reference(s)
CMY-2	I1	*E. coli* (115), *S. enterica* (80), *K. pneumoniae* (2), *P. mirabilis* (1), *C. koseri* (1)	199	Argentina, Australia, Canada, Italy, Norway, Portugal, Spain, Thailand, UK, Uruguay, USA	(12,26,30,40,47,51,54,69,74,78,143–145,173,174,184,188,209,210)
	A/C	*S. enterica* (82), *E. coli* (29), *P. mirabilis* (9), *K. pneumoniae* (4), *P. stuartii* (2)	126	Algeria, Canada, France, Greece, Honduras, Iraq, Mexico, Norway, Portugal, Spain, Thailand, USA	(26,30,40,46,47,50,51,78,134,143,144,173,174,210)
	K	*E. coli* (47), *P. mirabilis* (1), *K. pneumoniae* (1)	49	Argentina, Australia, Canada, Portugal, Spain	(12,26,30,31,69,143,144,188)
	FII	*E. coli* (13)	13	Argentina, Norway, Spain, USA	(54,69,78,143,188)
	FIA	*E. coli* (2), *S. enterica* (1)	3	Mexico, Spain	(176,188)
	ColE	*E. coli* (2)	2	Spain	(188)
	L/M	*E. coli* (2)	2	USA	(54)
	FIB	*E. coli* (1)	1	Spain	(188)
	Y	*E. coli* (2)	2	Argentina	(69)
	N	*E. coli* (1), *S. enterica* (1)	2	Argentina	(69,76)
	K+FIA	*E. coli* (1)	1	Spain	(188)
	I1+F	*E. coli* (6)	6	Norway, Spain	(78,143)
	I1+A/C	*S. enterica* (2)	2	Canada, Spain	(51,143)

Table 3. *Continued*

Enzyme	Replicon(s)[a]/ Relaxases[b]	Species	Total no. of plasmids	Country(ies)	Reference(s)
	I1+K	*E. coli* (4)	4	Canada, Spain	(143,173)
	I1+FIB	*E. coli* (1)	1	Spain	(144)
	A/C+FIB+F	*E. coli* (2)	2	Spain	(143)
	A/C+FIA+FIB	*E. coli* (1)	1	Spain	(143)
	A/C+FII	*E. coli* (4)	4	Norway	(78)
	A/C+Y	*S. enterica* (2)	2	Thailand	(40)
	I1+A/C+F	*E. coli* (1)	1	Norway	(78)
	NT	*E. coli* (14)	14	Australia, Canada, Portugal, Spain	(12,26,173,188)
CMY-4	A/C	*P. stuartii* (8), *E. coli* (4), *K. pneumoniae* (3), *K. oxytoca* (1), *P. mirabilis* (1), *C. koseri* (1), *Salmonella* spp. (2), *E. cloacae* complex (1)	21	Algeria, India, Italy, Tunisia, United Arab Emirates, UK	(135,150–153,210,211)
	I1	*E. coli* (3), *K. pneumoniae* (2)	5	Spain	(144,188)
	F	*E. coli* (1)	1	Spain	(143)
	Q	*E. coli* (1)	1	Greece	(212)
	K+FIB	*E. coli* (1)	1	Spain	(144)
	NT	*E. coli* (1)	1	Spain	(143)
CMY-5	ColE	*K. oxytoca* (1)	1	Sweden	(213)
CMY-7	I1	*E. coli* (5)	5	Norway, Pakistan, Spain, UK	(78,144,210)
CMY-13	N	*E. coli* (1)	1	Greece	(166)
CMY-16	A/C	*P. stuartii* (4), *K. pneumoniae* (1)	5	Switzerland, Tunisia	(108,139)

Table 3. *Continued*

Enzyme	Replicon(s)[a]/ Relaxases[b]	Species	Total no. of plasmids	Country(ies)	Reference(s)
	Q1	*P. mirabilis* (1)	1	Italy	(214)
CMY-25	NT	*K. pneumoniae* (1)	1	Spain	(143)
CMY-27	I1	*E. coli* (2)	1	Spain	(144)
	F	*E. coli* (1)	1	Spain	(143)
	MOBP12	*E. coli* (1)	1	Spain	(143)
CMY-31	ColE	*Salmonella* spp. (1)	1	Greece	(215)
CMY-38	ColE	*K. pneumoniae* (1)	1	Greece	(215)
CMY-42	I1	*E. coli* (3)	2	China, Egypt, Spain	(165,188,216)
CMY-54	K+FIB	*E. coli* (1)	1	Spain	(144)
CMY-55	A/C	*E. coli* (1)	1	Spain	(144)
CMY-56	A/C	*K. pneumoniae* (1)	1	Spain	(144)
CMY-57	I1	*E. coli* (1)	1	Spain	(144)
CMY-2+DHA-1	cr+FIIA	*K. pneumoniae* (1)	1	Spain	(144)
DHA-1	L/M	*K. pneumoniae* (58), *K. oxytoca* (3), *E. coli* (8)	69	France, Spain	(91,93,143)
	HI2	*K. pneumoniae* (22), *E. cloacae* complex (17), *K. oxytoca* (3), *K. variicola* (3), *E. coli* (2)	47	France, Portugal	(26,91)
	R	*K. pneumoniae* (26), *K. oxytoca* (3), *E. coli* (2)	31	Portugal	(26)
	FIA	*K. pneumoniae* (15), *E. coli* (7)	22	Spain, Taiwan	(122,188)
	F	*E. coli* (9)	9	Spain	(188)
	I1	*E. coli* (5)	5	Spain	(144,188)

Table 3. *Continued*

Enzyme	Replicon(s)[a]/ Relaxases[b]	Species	Total no. of plasmids	Country(ies)	Reference(s)
	FIB	*E. coli* (4)	4	Spain	(188)
	A/C	*E. cloacae* complex (2), *P. stuartii* (2), *K. pneumoniae* (1), *E. coli* (1)	6	Algeria, Spain, Tunisia	(134,144)
	ColE	*E. coli* (3)	3	Spain	(188)
	K	*E. coli* (2)	2	Spain	(188)
	L/M+FIA	*E. coli* (2)	2	Spain	(143)
	L/M+FIC	*K. pneumoniae* (1), *K. oxytoca* (1)	2	Spain	(143)
	FIIK	*K. pneumoniae* (1)	1	France	(26,91)
	L/M+N	*P. mirabilis* (1)	1	Spain	(143)
	N	*E. coli* (1)	1	Spain	(143)
	LVPK	*K. pneumoniae* (1)	1	Algeria	(135)
	NT	*E. coli* (8), *K. pneumoniae* (6), *K. oxytoca* (3), *C. koseri* (1), *Salmonella* spp. (1), *P. mirabilis* (1)	20	Argentina, Norway, Portugal, Spain,	(26,69,78,93,143,144,188)
DHA-6	R	*K. pneumoniae* (1)	1	Portugal	(26)
	I1	*E. coli* (1)	1	Spain	(144)
ACT-1	L/M	*C. freundii* (1)	1	China	(217)
ACT-16	FII	*E. cloacae* complex (1)	1	India	(155)
ACC-1	MOBF11	*E. coli* (1), *K. pneumoniae* (1)	2	Spain	(143)
	N	*E. coli* (1), *K. pneumoniae* (1)	2	Spain	(144)
	I1	*E. coli* (1)	1	Spain	(143)

Table 3. *Continued*

Enzyme	Replicon(s)[a]/ Relaxases[b]	Species	Total no. of plasmids	Country(ies)	Reference(s)
	NT	*K. pneumoniae* (2), *P. mirabilis* (1)	3	Spain	(144)
ACC-1+FOX-3	NT+U	*E. coli* (1)	1	Spain	(144)
FOX-3	U	*E. coli* (1)	1	Spain	(144)
FOX-5	A/C	*E. coli* (14), *K. pneumoniae* (14), *K. oxytoca* (1)	29	USA	(61)
FOX-7	L/M	*K. pneumoniae* (2), *K. oxytoca* (1), *Pantoea agglomerans* (1)	4	Italy	(171)
FOX-10	A/C	*Klebsiella* spp. (1)	1	USA	(61)

[a]NT, plasmids that were untypeable.

[b]F includes all the IncF plasmids amplified by F-simplex PCR using the PBRT method; MOBP11 belongs to the MOBP relaxase family (includes IncP-1 plasmids); MOBP12 belongs to the MOBP relaxase family (includes IncI, K and B/O); MOBF11 belongs to the MOBF relaxase family (includes IncN and IncW).

References

1. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, *et al.* Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infect Dis. 2013;13(9):785–96.
2. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012;18(7):646–55.
3. Rodrigues C, Machado E, Pires J, Ramos H, Novais A, Peixe L. Increase of widespread A, B1 and D *Escherichia coli* clones producing a high diversity of CTX-M-types in a Portuguese hospital. Futur Microbiol. 2015;10(7):1125–31.
4. Rodrigues C, Machado E, Ramos H, Peixe L, Novais A. Expansion of ESBL-producing *Klebsiella pneumoniae* in hospitalized patients: a successful story of international clones (ST15, ST147, ST336) and epidemic plasmids (IncR, IncFIIK). Int J Med Microbiol. 2014;304(8):1100–8.
5. Freitas F, Machado E, Ribeiro TG, Novais Â, Peixe L. Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings. Eur J Clin Microbiol Infect Dis. 2014;33(4):551–8.
6. Antunes P, Mourao J, Campos J, Peixe L. Salmonellosis: the role of poultry meat. Clin Microbiol Infect. 2016;22(2):110–21.
7. Rodrigues C, Bavlovič J, Machado E, Amorim J, Peixe L, Novais Â. KPC-3-Producing *Klebsiella pneumoniae* in Portugal Linked to Previously Circulating Non-CG258 Lineages and Uncommon Genetic Platforms (Tn4401d-IncFIA and Tn4401d-IncN). Front Microbiol. 2016;7:1000.
8. Denisuik AJ, Lagace-Wiens PR, Pitout JD, Mulvey MR, Simner PJ, Tailor F, *et al.* Molecular epidemiology of extended-spectrum beta-lactamase-, AmpC beta-lactamase- and carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from Canadian hospitals over a 5 year period: CANWARD 2007-11. J Antimicrob Chemother. 2013;68 Suppl 1:i57–65.
9. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. Euro Surveill. 2008;13(47).
10. Canton R, Akova M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, *et al.* Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin Microbiol Infect. 2012;18(5):413–31.
11. Bonelli RR, Moreira BM, Picao RC. Antimicrobial resistance among *Enterobacteriaceae* in South America: history, current dissemination status and

associated socioeconomic factors. Drug Resist Updat. 2014;17(1-2):24–36.

12. Sidjabat HE, Seah KY, Coleman L, Sartor A, Derrington P, Heney C, *et al*. Expansive spread of IncI1 plasmids carrying *bla*$_{CMY-2}$ amongst *Escherichia coli*. Int J Antimicrob Agents. 2014;44(3):203–8.
13. Ribeiro TG, Novais A, Peixe L, Machado E. Atypical epidemiology of CTX-M-15 among *Enterobacteriaceae* from a high diversity of non-clinical niches in Angola. J Antimicrob Chemother. 2016;71(5):1169–73.
14. Manenzhe RI, Zar HJ, Nicol MP, Kaba M. The spread of carbapenemase-producing bacteria in Africa: a systematic review. J Antimicrob Chemother. 2015;70(1):23–40.
15. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009;22(1):161–82.
16. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type beta-lactamases. Antimicrob Agents Chemother. 2002;46(1):1–11.
17. Barlow M, Hall BG. Origin and evolution of the AmpC beta-lactamases of *Citrobacter freundii*. Antimicrob Agents Chemother. 2002;46(5):1190–8.
18. Raskine L, Borrel I, Barnaud G, Boyer S, Hanau-Bercot B, Gravisse J, *et al*. Novel plasmid-encoded class C beta-lactamase (MOX-2) in *Klebsiella pneumoniae* from Greece. Antimicrob Agents Chemother. 2002;46(7):2262–5.
19. Ammenouche N, Dupont H, Mammeri H. Characterization of a novel AmpC β-lactamase produced by a carbapenem-resistant *Cedecea davisae* clinical isolate. Antimicrob Agents Chemother. 2014;58(11):6942–5.
20. Ambler RP. The structure of beta-lactamases. Philos Trans R Soc L B Biol Sci. 1980;289(1036):321–31.
21. Bonnet, R, Bonomo RA. Beta-lactams and *Enterobacteriaceae*. In: Courvalin P, Leclercq R, Rice L, editors. Antibiogram. 1st ed. Portland: ASM Press; 2009. p. 135–55.
22. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 1995;39(6):1211–33.
23. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrob Agents Chemother. 2010;54(3):969–76.
24. Mark BL, Vocadlo DJ, Oliver A. Providing β-lactams a helping hand: targeting the AmpC β-lactamase induction pathway. Future Microbiol. 2011;6(12):1415–27.
25. Mata C, Miro E, Rivera A, Mirelis B, Coll P, Navarro F. Prevalence of acquired AmpC beta-lactamases in *Enterobacteriaceae* lacking inducible chromosomal ampC genes at a Spanish hospital from 1999 to 2007. Clin Microbiol Infect. 2010;16(5):472–6.
26. Ribeiro TG, Novais Â, Rodrigues C, Nascimento R, Freitas F, Machado E, *et al*. Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases

over 12 years: unusual DHA dominance in an European country. J Antimicrob Chemother. 2016 (*Under Editorial Revision*)

27. Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos A-P, Mevius D, *et a*l. Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin Infect Dis. 2013 Apr [cited 2016 Jul 1];56(7):1030–7.
28. Dimude JU, Amyes SGB. Molecular characterisation and diversity in *Enterobacter cloacae* from Edinburgh and Egypt carrying $bla_{\text{CTX-M-14}}$ and $bla_{\text{VIM-4}}$ beta-lactamase genes. Int J Antimicrob Agents. 2013;41(6):574–7.
29. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, *et al.* Broad-spectrum beta-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. FEMS Microbiol Rev. 2010;34(3):295–316.
30. Mataseje LF, Baudry PJ, Zhanel GG, Morck DW, Read RR, Louie M, *et al.* Comparison of CMY-2 plasmids isolated from human, animal, and environmental *Escherichia coli* and *Salmonella* spp. from Canada. Diagn Microbiol Infect Dis. 2010;67(4):387–91.
31. Mataseje LF, Neumann N, Crago B, Baudry P, Zhanel GG, Louie M, *et al.* Characterization of cefoxitin-resistant *Escherichia coli* isolates from recreational beaches and private drinking water in Canada between 2004 and 2006. Antimicrob Agents Chemother. 2009;53(7):3126–30.
32. Ben Said L, Jouini A, Alonso CA, Klibi N, Dziri R, Boudabous A, *et al.* Characteristics of extended-spectrum beta-lactamase (ESBL)- and pAmpC beta-lactamase-producing *Enterobacteriaceae* of water samples in Tunisia. Sci Total Env. 2016;550:1103–9.
33. Taguchi M, Seto K, Yamazaki W, Tsukamoto T, Izumiya H, Watanabe H. CMY-2 beta-lactamase-producing *Salmonella enterica* serovar Infantis isolated from poultry in Japan. Jpn J Infect Dis. 2006;59(2):144–6.
34. Bajaj P, Singh NS, Kanaujia PK, Virdi JS. Distribution and molecular characterization of genes encoding CTX-M and AmpC beta-lactamases in *Escherichia coli* isolated from an Indian urban aquatic environment. Sci Total Env. 2015;505:350–6.
35. Titilawo Y, Obi L, Okoh A. Antimicrobial resistance determinants of *Escherichia coli* isolates recovered from some rivers in Osun State, South-Western Nigeria: Implications for public health. Sci Total Env. 2015;523:82–94.
36. M'Ikanatha N M, Sandt CH, Localio AR, Tewari D, Rankin SC, Whichard JM, *et al.* Multidrug-resistant *Salmonella* isolates from retail chicken meat compared with

human clinical isolates. Foodborne Pathog Dis. 2010;7(8):929–34.

37. Dierikx CM, van der Goot JA, Smith HE, Kant A, Mevius DJ. Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. PLoS One. 2013;8(11):e79005.
38. Laube H, Friese A, von Salviati C, Guerra B, Käsbohrer A, Kreienbrock L, *et al.* Longitudinal monitoring of extended-spectrum-beta-lactamase/AmpC-producing *Escherichia coli* at German broiler chicken fattening farms. Appl Environ Microbiol. 2013;79(16):4815–20.
39. Gude MJ, Seral C, Saenz Y, Gonzalez-Dominguez M, Torres C, Castillo FJ. Evaluation of four phenotypic methods to detect plasmid-mediated AmpC beta-lactamases in clinical isolates. Eur J Clin Microbiol Infect Dis. 2012;31(8):2037–43.
40. Hendriksen RS, Le Hello S, Bortolaia V, Pulsrikarn C, Nielsen EM, Pornruangmong S, *et al.* Characterization of isolates of *Salmonella enterica* serovar Stanley, a serovar endemic to Asia and associated with travel. J Clin Microbiol. 2012;50(3):709–20.
41. Miro E, Aguero J, Larrosa MN, Fernandez A, Conejo MC, Bou G, *et al.* Prevalence and molecular epidemiology of acquired AmpC beta-lactamases and carbapenemases in *Enterobacteriaceae* isolates from 35 hospitals in Spain. Eur J Clin Microbiol Infect Dis. 2013;32(2):253–9.
42. Gude MJ, Seral C, Saenz Y, Cebollada R, Gonzalez-Dominguez M, Torres C, *et al.* Molecular epidemiology, resistance profiles and clinical features in clinical plasmid-mediated AmpC-producing *Enterobacteriaceae*. Int J Med Microbiol. 2013;303(8):553–7.
43. Castanheira M, Pereira AS, Nicoletti AG, Pignatari ACC, Barth AL, Gales AC. First report of plasmid-mediated *qnrA1* in a ciprofloxacin-resistant *Escherichia coli* strain in Latin America. Antimicrob Agents Chemother. 2007;51(4):1527–9.
44. Helmy MM, Wasfi R. Phenotypic and molecular characterization of plasmid mediated AmpC beta-lactamases among *Escherichia coli, Klebsiella* spp., and *Proteus mirabilis* isolated from urinary tract infections in Egyptian hospitals. Biomed Res Int. 2014;2014:171548.
45. Eller C, Simon S, Miller T, Frick JS, Prager R, Rabsch W, *et al.* Presence of beta-lactamases in extended-spectrum-cephalosporin-resistant *Salmonella enterica* of 30 different serovars in Germany 2005-11. J Antimicrob Chemother. 2013;68(9):1978–81.
46. Egorova S, Kaftyreva L, Grimont PA, Weill FX. Prevalence and characterization of extended-spectrum cephalosporin-resistant nontyphoidal *Salmonella* isolates in adults in Saint Petersburg, Russia (2002-2005). Microb Drug Resist. 2007;13(2):102–7.

47. Gonzalez-Sanz R, Herrera-Leon S, de la Fuente M, Arroyo M, Echeita MA. Emergence of extended-spectrum beta-lactamases and AmpC-type beta-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. J Antimicrob Chemother. 2009;64(6):1181–6.
48. Glenn LM, Lindsey RL, Frank JF, Meinersmann RJ, Englen MD, Fedorka-Cray PJ, *et al.* Analysis of antimicrobial resistance genes detected in multidrug-resistant *Salmonella enterica* serovar Typhimurium isolated from food animals. Microb Drug Resist. 2011;17(3):407–18.
49. Miriagou V, Filip R, Coman G, Tzouvelekis LS. Expanded-spectrum cephalosporin-resistant *Salmonella* strains in Romania. J Clin Microbiol. 2002;40(11):4334–6.
50. Wiesner M, Zaidi MB, Calva E, Fernández-Mora M, Calva JJ, Silva C. Association of virulence plasmid and antibiotic resistance determinants with chromosomal multilocus genotypes in Mexican *Salmonella enterica* serovar Typhimurium strains. BMC Microbiol. 2009;9:131.
51. Andrysiak AK, Olson AB, Tracz DM, Dore K, Irwin R, Ng LK, *et al.* Genetic characterization of clinical and agri-food isolates of multi drug resistant *Salmonella enterica* serovar Heidelberg from Canada. BMC Microbiol. 2008;8:89.
52. Hanson ND, Moland ES, Hong SG, Propst K, Novak DJ, Cavalieri SJ. Surveillance of community-based reservoirs reveals the presence of CTX-M, imported AmpC, and OXA-30 beta-lactamases in urine isolates of *Klebsiella pneumoniae* and *Escherichia coli* in a U.S. community. Antimicrob Agents Chemother. 2008;52(10):3814–6.
53. Sidjabat HE, Paterson DL, Qureshi ZA, Adams-Haduch JM, O'Keefe A, Pascual A, *et al.* Clinical features and molecular epidemiology of CMY-type beta-lactamase-producing *Escherichia coli*. Clin Infect Dis. 2009;48(6):739–44.
54. Bortolaia V, Hansen KH, Nielsen CA, Fritsche TR, Guardabassi L. High diversity of plasmids harbouring $bla_{\text{CMY-2}}$ among clinical *Escherichia coli* isolates from humans and companion animals in the upper Midwestern USA. J Antimicrob Chemother. 2014;69(6):1492–6.
55. Zaidi MB, Leon V, Canche C, Perez C, Zhao S, Hubert SK, *et al.* Rapid and widespread dissemination of multidrug-resistant $bla_{\text{CMY-2}}$ *Salmonella* Typhimurium in Mexico. J Antimicrob Chemother. 2007;60(2):398–401.
56. Gupta A, Fontana J, Crowe C, Bolstorff B, Stout A, Van Duyne S, *et al.* Emergence of multidrug-resistant *Salmonella enterica* serotype Newport infections resistant to expanded-spectrum cephalosporins in the United States. J Infect Dis. 2003;188(11):1707–16.
57. Harbottle H, White DG, McDermott PF, Walker RD, Zhao S. Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, and antimicrobial

susceptibility typing for characterization of *Salmonella enterica* serotype Newport isolates. J Clin Microbiol. 2006;44(7):2449–57.

58. Varma JK, Marcus R, Stenzel SA, Hanna SS, Gettner S, Anderson BJ, *et al.* Highly resistant *Salmonella* Newport-MDRAmpC transmitted through the domestic US food supply: a FoodNet case-control study of sporadic *Salmonella* Newport infections, 2002-2003. J Infect Dis. 2006;194(2):222–30.
59. Miriagou V, Tassios P, Legakis N, Tzouvelekis LS. Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. Int J Antimicrob Agents. 2004;23(6):547–55.
60. Moland ES, Hanson ND, Black JA, Hossain A, Song W, Thomson KS. Prevalence of newer beta-lactamases in gram-negative clinical isolates collected in the United States from 2001 to 2002. J Clin Microbiol. 2006;44(9):3318–24.
61. Hazen TH, Zhao L, Boutin MA, Stancil A, Robinson G, Harris AD, *et al.* Comparative genomics of an IncA/C multidrug resistance plasmid from *Escherichia coli* and *Klebsiella* isolates from intensive care unit patients and the utility of whole-genome sequencing in health care settings. Antimicrob Agents Chemother. 2014;58(8):4814–25.
62. Baudry PJ, Nichol K, DeCorby M, Mataseje L, Mulvey MR, Hoban DJ, *et al.* Comparison of antimicrobial resistance profiles among extended-spectrum-beta-lactamase-producing and acquired AmpC beta-lactamase-producing *Escherichia coli* isolates from Canadian intensive care units. Antimicrob Agents Chemother. 2008;52(5):1846–9.
63. Simner PJ, Zhanel GG, Pitout J, Tailor F, McCracken M, Mulvey MR, *et al.* Prevalence and characterization of extended-spectrum beta-lactamase- and AmpC beta-lactamase-producing *Escherichia coli*: results of the CANWARD 2007-2009 study. Diagn Microbiol Infect Dis. 2011;69(3):326–34.
64. Pitout JDD, Reisbig MD, Mulvey M, Chui L, Louie M, Crowe L, *et al.* Association between handling of pet treats and infection with *Salmonella enterica* serotype Newport expressing the AmpC beta-lactamase, CMY-2. J Clin Microbiol. 2003;41(10):4578–82.
65. da Silva Dias RC, Borges-Neto AA, D'Almeida Ferraiuoli GI, de-Oliveira MP, Riley LW, Moreira BM. Prevalence of AmpC and other beta-lactamases in enterobacteria at a large urban university hospital in Brazil. Diagn Microbiol Infect Dis. 2008;60(1):79–87.
66. Campana EH, Barbosa PP, Fehlberg LC, Gales AC. Frequency of plasmid-mediated AmpC in Enterobacteriaceae isolated in a Brazilian Teaching Hospital. Braz J Microbiol. 2013;44(2):477–80.
67. Pavez M, Neves P, Dropa M, Matté MH, Grinbaum RS, Elmor de Araújo MR, *et al.*

Emergence of carbapenem-resistant *Escherichia coli* producing CMY-2-type AmpC beta-lactamase in Brazil. J Med Microbiol. 2008;57(Pt 12):1590–2.

68. Rocha DA, Campos JC, Passadore LF, Sampaio SC, Nicodemo AC, Sampaio JL. Frequency of Plasmid-Mediated AmpC beta-Lactamases in *Escherichia coli* Isolates from Urine Samples in Sao Paulo, Brazil. Microb Drug Resist. 2015
69. Cejas D, Fernandez Canigia L, Quinteros M, Giovanakis M, Vay C, Lascialandare S, *et al.* Plasmid-Encoded AmpC (pAmpC) in *Enterobacteriaceae*: epidemiology of microorganisms and resistance markers. Rev Argent Microbiol. 2012;44(3):182–6.
70. Rapoport M, Monzani V, Pasteran F, Morvay L, Faccone D, Petroni A, *et al.* CMY-2-type plasmid-mediated AmpC beta-lactamase finally emerging in Argentina. Int J Antimicrob Agents. 2008;31(4):385–7.
71. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002;40(6):2153–62.
72. Jure MA, Presti C, Cudmani NM, Grellet LM, Lopez C, Musa EH, *et al.* CMY-2-type plasmid-mediated AmpC ss-lactamases emerging in Tucuman, Argentina. Rev Argent Microbiol. 2011;43(1):24–7.
73. Trevino M, Navarro D, Barbeito G, Areses P, Garcia-Riestra C, Regueiro BJ. Plasmid-mediated AMPc producing *Proteus mirabilis* in the Health Care Area of Santiago de Compostela: molecular and epidemiological analysis by rep-PCR and MALDI-TOF. Rev Esp Quim. 2012;25(2):122–8.
74. Cordeiro NF, Yim L, Betancor L, Cejas D, García-Fulgueiras V, Mota MI, *et al.* Identification of the first *bla*$_{\text{CMY-2}}$ gene in *Salmonella enterica* serovar Typhimurium isolates obtained from cases of paediatric diarrhoea illness detected in South America. J Glob Antimicrob Resist. 2013;1(3):143–8.
75. Liebana E, Batchelor M, Torres C, Briñas L, Lagos LA, Abdalhamid B, *et al.* Pediatric infection due to multiresistant *Salmonella enterica* serotype Infantis in Honduras. J Clin Microbiol.2004;42(10):4885–8.
76. Cejas D, Vignoli R, Quinteros M, Marino R, Callejo R, Betancor L, *et al.* First detection of CMY-2 plasmid mediated β-lactamase in *Salmonella* Heidelberg in South America. Rev Argent Microbiol. 2014;46(1):30–3.
77. Haldorsen B, Aasnaes B, Dahl KH, Hanssen AM, Simonsen GS, Walsh TR, *et al.* The AmpC phenotype in Norwegian clinical isolates of *Escherichia coli* is associated with an acquired ISEcp1-like ampC element or hyperproduction of the endogenous AmpC. J Antimicrob Chemother. 2008;62(4):694–702.
78. Naseer U, Haldorsen B, Simonsen GS, Sundsfjord A. Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and

ST131 in Norway. Clin Microbiol Infect. 2010;16(2):171–8.

79. Empel J, Baraniak A, Literacka E, Mrowka A, Fiett J, Sadowy E, *et al.* Molecular survey of beta-lactamases conferring resistance to newer beta-lactams in *Enterobacteriaceae* isolates from Polish hospitals. Antimicrob Agents Chemother. 2008;52(7):2449–54.
80. Egorova S, Timinouni M, Demartin M, Granier SA, Whichard JM, Sangal V, *et a*l. Ceftriaxone-resistant *Salmonella enterica* serotype Newport, France. Emerg Infect Dis. 2008;14(6):954–7.
81. Markovska R, Schneider I, Marteva-Proevsk Y, Mitov I, Bauernfeind A, Markova B. First detection of the AmpC beta-lactamase ACC-1 in a *Klebsiella pneumoniae* isolate in Bulgaria. J Chemother. 2012;24(5):307–8.
82. Espié E, De Valk H, Vaillant V, Quelquejeu N, Le Querrec F, Weill FX. An outbreak of multidrug-resistant *Salmonella enterica* serotype Newport infections linked to the consumption of imported horse meat in France. Epidemiol Infect. 2005;133(2):373–6.
83. Chudackova E, Bergerova T, Fajfrlik K, Cervena D, Urbaskova P, Empel J, *et al.* Carbapenem-nonsusceptible strains of *Klebsiella pneumoniae* producing SHV-5 and/or DHA-1 beta-lactamases in a Czech hospital. FEMS Microbiol Lett. 2010;309(1):62–70.
84. Mathers AJ, Peirano G, Pitout JD. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant *Enterobacteriaceae*. Clin Microbiol Rev. 2015;28(3):565–91.
85. Ruiz-Garbajosa P, Curiao T, Tato M, Gijon D, Pintado V, Valverde A, *et al.* Multiclonal dispersal of KPC genes following the emergence of non-ST258 KPC-producing *Klebsiella pneumoniae* clones in Madrid, Spain. J Antimicrob Chemother. 2013;68(11):2487–92.
86. Hidalgo L, Gutierrez B, Ovejero CM, Carrilero L, Matrat S, Saba CK, *et al. Klebsiella pneumoniae* sequence type 11 from companion animals bearing ArmA methyltransferase, DHA-1 beta-lactamase, and QnrB4. Antimicrob Agents Chemother. 2013;57(9):4532–4.
87. Baraniak A, Grabowska A, Izdebski R, Fiett J, Herda M, Bojarska K, *et al.* Molecular characteristics of KPC-producing *Enterobacteriaceae* at the early stage of their dissemination in Poland, 2008-2009. Antimicrob Agents Chemother. 2011;55(12):5493–9.
88. Guo Q, Wang P, Ma Y, Yang Y, Ye X, Wang M. Co-production of SFO-1 and DHA-1 beta-lactamases and 16S rRNA methylase ArmA in clinical isolates of *Klebsiella pneumoniae*. J Antimicrob Chemother. 2012;67(10):2361–6.

89. Empel J, Hrabak J, Kozinska A, Bergerova T, Urbaskova P, Kern-Zdanowicz I, *et al.* DHA-1-producing *Klebsiella pneumoniae* in a teaching hospital in the Czech Republic. Microb Drug Resist. 2010;16(4):291–5.
90. Illiaquer M, Caroff N, Bemer P, Aubin GG, Juvin ME, Lepelletier D, *et al.* Occurrence and molecular characterization of *Klebsiella pneumoniae* ST37 clinical isolates producing plasmid-mediated AmpC recovered over a 3-year period. Diagn Microbiol Infect Dis. 2012;74(1):95–7.
91. Compain F, Decre D, Fulgencio JP, Berraho S, Arlet G, Verdet C. Molecular characterization of DHA-1-producing *Klebsiella pneumoniae* isolates collected during a 4-year period in an intensive care unit. Diagn Microbiol Infect Dis.2014;80(2):159–61.
92. Lopez-Camacho E, Gomez-Gil R, Tobes R, Manrique M, Lorenzo M, Galvan B, *et al.* Genomic analysis of the emergence and evolution of multidrug resistance during a *Klebsiella pneumoniae* outbreak including carbapenem and colistin resistance. J Antimicrob Chemother. 2014;69(3):632–6.
93. Diestra K, Miro E, Marti C, Navarro D, Cuquet J, Coll P, *et al.* Multiclonal epidemic of *Klebsiella pneumoniae* isolates producing DHA-1 in a Spanish hospital. Clin Microbiol Infect. 2011;17(7):1032–6.
94. Gamal D, Fernandez-Martinez M, Salem D, El-Defrawy I, Montes LA, Ocampo-Sosa AA, *et al.* Carbapenem-resistant *Klebsiella pneumoniae* isolates from Egypt containing $bla_{\text{NDM-1}}$ on IncR plasmids and its association with rmtF. Int J Infect Dis. 2016;43:17–20.
95. Giakkoupi P, Papagiannitsis CC, Miriagou V, Pappa O, Polemis M, Tryfinopoulou K, *et al.* An update of the evolving epidemic of $bla_{\text{KPC-2}}$-carrying *Klebsiella pneumoniae* in Greece (2009-10). J Antimicrob Chemother. 2011;66(7):1510–3.
96. Pantelidou IM, Galani I, Georgitsi M, Daikos GL, Giamarellos-Bourboulis EJ. Interactions of *Klebsiella pneumoniae* with the innate immune system vary in relation to clone and resistance phenotype. Antimicrob Agents Chemother. 2015;59(11):7036–43.
97. Zhang X, Li X, Wang M, Yue H, Li P, Liu Y, *et al.* Outbreak of NDM-1-producing *Klebsiella pneumoniae* causing neonatal infection in a teaching hospital in mainland China. Antimicrob Agents Chemother. 2015;59(7):4349–51.
98. Jin Y, Shao C, Li J, Fan H, Bai Y, Wang Y. Outbreak of multidrug resistant NDM-1-producing *Klebsiella pneumoniae* from a neonatal unit in Shandong Province, China. PLoS One. 2015;10(3):e0119571.
99. Balm MN, Ngan G, Jureen R, Lin RT, Teo JW. OXA-181-producing *Klebsiella pneumoniae* establishing in Singapore. BMC Infect Dis. 2013;13:58.

100. Ohana S, Leflon V, Ronco E, Rottman M, Guillemot D, Lortat-Jacob S, *et al.* Spread of a *Klebsiella pneumoniae* strain producing a plasmid-mediated ACC-1 AmpC beta-lactamase in a teaching hospital admitting disabled patients. Antimicrob Agents Chemother. 2005;49(5):2095–7.
101. Roche C, Boo TW, Walsh F, Crowley B. Detection and molecular characterisation of plasmidic AmpC beta-lactamases in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Dublin, Ireland. Clin Microbiol Infect. 2008;14(6):616–8.
102. Arena F, Giani T, Becucci E, Conte V, Zanelli G, D'Andrea MM, *et al.* Large oligoclonal outbreak due to *Klebsiella pneumoniae* ST14 and ST26 producing the FOX-7 AmpC beta-lactamase in a neonatal intensive care unit. J Clin Microbiol. 2013;51(12):4067–72.
103. Luzzaro F, Brigante G, D'Andrea MM, Pini B, Giani T, Mantengoli E, *et al.* Spread of multidrug-resistant *Proteus mirabilis* isolates producing an AmpC-type beta-lactamase: epidemiology and clinical management. Int J Antimicrob Agents. 2009;33(4):328–33.
104. Migliavacca R, Nucleo E, D'Andrea MM, Spalla M, Giani T, Pagani L. Acquired AmpC type beta-lactamases: an emerging problem in Italian long-term care and rehabilitation facilities. New Microbiol. 2007;30(3):295–8.
105. D'Andrea MM, Nucleo E, Luzzaro F, Giani T, Migliavacca R, Vailati F, *et al.* CMY-16, a novel acquired AmpC-type beta-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from northern Italy. Antimicrob Agents Chemother. 2006;50(2):618–24.
106. Bogaerts P, Rodriguez-Villalobos H, Bauraing C, Deplano A, Laurent C, Berhin C, *et al.* Molecular characterization of AmpC-producing *Escherichia coli* clinical isolates recovered at two Belgian hospitals. Pathol Biol. 2010;58(1):78–83.
107. Bogaerts P, Bouchahrouf W, de Castro RR, Deplano A, Berhin C, Pierard D, *et al.* Emergence of NDM-1-producing *Enterobacteriaceae* in Belgium. Antimicrob Agents Chemother. 2011;55(6):3036–8.
108. Poirel L, Schrenzel J, Cherkaoui A, Bernabeu S, Renzi G, Nordmann P. Molecular analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland. J Antimicrob Chemother. 2011;66(8):1730–3.
109. Seiffert SN, Marschall J, Perreten V, Carattoli A, Furrer H, Endimiani A. Emergence of Kl*ebsiella pneumoniae* co-producing NDM-1, OXA-48, CTX-M-15, CMY-16, QnrA and ArmA in Switzerland. Int J Antimicrob Agents. 2014;44(3):260–2.
110. Li Y, Li Q, Du Y, Jiang X, Tang J, Wang J, *et al.* Prevalence of plasmid-mediated AmpC beta-lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-Type AmpC beta-lactamase resistance in China. J Clin Microbiol.

2008;46(4):1317–21.

111. Ding H, Yang Y, Lu Q, Wang Y, Chen Y, Deng L, *et al.* The prevalence of plasmid-mediated AmpC beta-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from five children's hospitals in China. Eur J Clin Microbiol Infect Dis. 2008;27(10):915–21.
112. Yong D, Lim Y, Song W, Choi YS, Park DY, Lee H, *et al.* Plasmid-mediated, inducible AmpC beta-lactamase (DHA-1)-producing Enterobacteriaceae at a Korean hospital: wide dissemination in *Klebsiella pneumoniae* and *Klebsiella oxytoca* and emergence in Proteus mirabilis. Diagn Microbiol Infect Dis. 2005;53(1):65–70.
113. Lee K, Lee M, Shin JH, Lee MH, Kang SH, Park AJ, *et al.* Prevalence of plasmid-mediated AmpC beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Korea. Microb Drug Resist. 2006;12(1):44–9.
114. Song W, Lee KM, Kim HS, Kim JS, Kim J, Jeong SH, *et al.* Clonal spread of both oxyimino-cephalosporin- and cefoxitin-resistant *Klebsiella pneumoniae* isolates co-producing SHV-2a and DHA-1 beta-lactamase at a burns intensive care unit. Int J Antimicrob Agents. 2006;28(6):520–4.
115. Park YJ, Lee S, Kim YR, Oh EJ, Woo GJ, Lee K. Occurrence of extended-spectrum (beta)-lactamases and plasmid-mediated AmpC (beta)-lactamases among Korean isolates of *Proteus mirabilis*. J Antimicrob Chemother. 2006;57(1):156–8.
116. Mohd Khari FI, Karunakaran R, Rosli R, Tee Tay S. Genotypic and Phenotypic Detection of AmpC beta-lactamases in *Enterobacter* spp. Isolated from a Teaching Hospital in Malaysia. PLoS One. 2016;11(3):e0150643.
117. Shayan S, Bokaeian M. Detection of ESBL- and AmpC-producing *E. coli* isolates from urinary tract infections. Adv Biomed Res. 2015;4:220.
118. Shayan S, Bokaeian M, Shahraki S. Prevalence and molecular characterization of AmpC-producing clinical isolates of *Escherichia coli* from southeastern Iran. Microb Drug Resist. 2014;20(2):104–7.
119. Yamasaki K, Komatsu M, Abe N, Fukuda S, Miyamoto Y, Higuchi T, *et al.* Laboratory surveillance for prospective plasmid-mediated AmpC beta-lactamases in the Kinki region of Japan. J Clin Microbiol. 2010;48(9):3267–73.
120. Liu X-Q, Liu Y-R. Detection and genotype analysis of AmpC β-lactamase in *Klebsiella pneumoniae* from tertiary hospitals. Exp Ther Med. 2016;12(1):480–4.
121. Muratani T, Kobayashi T, Matsumoto T. Emergence and prevalence of beta-lactamase-producing *Klebsiella pneumoniae* resistant to cephems in Japan. Int J Antimicrob Agents. 2006;27(6):491–9.
122. Yan JJ, Zheng PX, Wang MC, Tsai SH, Wang LR, Wu JJ. Allocation of *Klebsiella pneumoniae* Bloodstream Isolates into Four Distinct Groups by ompK36 Typing in a

Taiwanese University Hospital. J Clin Microbiol. 2015;53(10):3256–63.

123. Ma L, Lu PL, Siu LK, Hsieh MH. Molecular typing and resistance mechanisms of imipenem-non-susceptible *Klebsiella pneumoniae* in Taiwan: results from the Taiwan surveillance of antibiotic resistance (TSAR) study, 2002-2009. J Med Microbiol. 2013;62(Pt 1):101–7.

124. Liu X, Liu Y. Detection of plasmid-mediated AmpC β-lactamase in *Escherichia coli*. Biomed reports. 2016;4(6):687–90.

125. Park MJ, Kim TK, Song W, Kim JS, Kim HS, Lee J. An Increase in the clinical isolation of acquired AmpC beta-lactamase-producing *Klebsiella pneumoniae* in Korea from 2007 to 2010. Ann Lab Med. 2013;33(5):353–5.

126. Mohamudha PR, Harish BN, Parija SC. Molecular description of plasmid-mediated AmpC beta-lactamases among nosocomial isolates of Escherichia coli & *Klebsiella pneumoniae* from six different hospitals in India. Indian J Med Res. 2012;135:114–9.

127. Shahid M, Sobia F, Singh A, Khan HM. Concurrent occurrence of bla_{ampC} families and $bla_{\text{CTX-M}}$ genogroups and association with mobile genetic elements ISEcp1, IS26, ISCR1, and sul1-type class 1 integrons in *Escherichia coli* and *Klebsiella pneumoniae* isolates originating from India. J Clin Microbiol. 2012;50(5):1779–82.

128. Shanthi M, Sekar U, Arunagiri K, Sekar B. Detection of Amp C genes encoding for beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. Indian J Med Microbiol. 2012;30(3):290–5.

129. Manoharan A, Sugumar M, Kumar A, Jose H, Mathai D, Khilnani GC, *et al*. Phenotypic & molecular characterization of AmpC beta-lactamases among *Escherichia coli*, *Klebsiella* spp. & *Enterobacter* spp. from five Indian Medical Centers. Indian J Med Res. 2012;135:359–64.

130. Gupta V, Kumarasamy K, Gulati N, Garg R, Krishnan P, Chander J. AmpC beta-lactamases in nosocomial isolates of *Klebsiella pneumoniae* from India. Indian J Med Res. 2012;136(2):237–41.

131. Huang IF, Chiu CH, Wang MH, Wu CY, Hsieh KS, Chiou CC. Outbreak of dysentery associated with ceftriaxone-resistant *Shigella sonnei*: First report of plasmid-mediated CMY-2-type AmpC beta-lactamase resistance in *S. sonnei*. J Clin Microbiol.2005;43(6):2608–12.

132. Roh KH, Uh Y, Kim JS, Kim HS, Shin DH, Song W. First outbreak of multidrug-resistant *Klebsiella pneumoniae* producing both SHV-12-type extended-spectrum beta-lactamase and DHA-1-type AmpC beta-lactamase at a Korean hospital. Yonsei Med J. 2008;49(1):53–7.

133. Yu F, Ying Q, Chen C, Li T, Ding B, Liu Y, *et al*. Outbreak of pulmonary infection

caused by *Klebsiella pneumoniae* isolates harbouring *bla*$_{IMP-4}$ and *bla*$_{DHA-1}$ in a neonatal intensive care unit in China. J Med Microbiol. 2012;61(Pt 7):984–9.

134. Iabadene H, Messai Y, Ammari H, Alouache S, Verdet C, Bakour R, *et al.* Prevalence of plasmid-mediated AmpC beta-lactamases among *Enterobacteriaceae* in Algiers hospitals. Int J Antimicrob Agents. 2009;34(4):340–2.
135. Gharout-Sait A, Touati A, Guillard T, Brasme L, de Champs C. Molecular characterization and epidemiology of cefoxitin resistance among *Enterobacteriaceae* lacking inducible chromosomal ampC genes from hospitalized and non-hospitalized patients in Algeria: description of new sequence type in *Klebsiella pneumoniae* isolate. Braz J Infect Dis. 2015;19(2):187–95.
136. Barguigua A, El Otmani F, Talmi M, Reguig A, Jamali L, Zerouali K, *et al.* Prevalence and genotypic analysis of plasmid-mediated beta-lactamases among urinary *Klebsiella pneumoniae* isolates in Moroccan community. J Antibio. 2013;66(1):11–6.
137. Kariuki S, Revathi G, Corkill J, Kiiru J, Mwituria J, Mirza N, *et al. Escherichia coli* from community-acquired urinary tract infections resistant to fluoroquinolones and extended-spectrum beta-lactams. J Infect Dev Ctries. 2007;1(3):257–62.
138. Ktari S, Arlet G, Verdet C, Jaoua S, Kachrid A, Ben Redjeb S, *et al.* Molecular epidemiology and genetic environment of acquired bla ACC-1 in *Salmonella enterica* serotype Livingstone causing a large nosocomial outbreak in Tunisia. Microb Drug Resist. 2009;15(4):279–86.
139. Arpin C, Thabet L, Yassine H, Messadi AA, Boukadida J, Dubois V, *et al.* Evolution of an incompatibility group IncA/C plasmid harboring *bla*$_{CMY-16}$ and *qnrA6* genes and its transfer through three clones of *Providencia stuartii* during a two-year outbreak in a Tunisian burn unit. Antimicrob Agents Chemother. 2012;56(3):1342–9.
140. Hanson ND, Moland ES, Hossain A, Neville SA, Gosbell IB, Thomson KS. Unusual *Salmonella enterica* serotype Typhimurium isolate producing CMY-7, SHV-9 and OXA-30 beta-lactamases. J Antimicrob Chemother. 2002;49(6):1011–4.
141. Heffernan C, Carter, PHP. Identification of extended-spectrum β-lactamase types, plasmid-mediated ampC β-lactamases and strains among urinary *Escherichia coli* and *Klebsiella* in New Zeland in 2006. 2007.
142. Mata C, Navarro F, Miro E, Walsh TR, Mirelis B, Toleman M. Prevalence of SXT/R391-like integrative and conjugative elements carrying *bla*$_{CMY-2}$ in *Proteus mirabilis*. J Antimicrob Chemother. 2011;66(10):2266–70.
143. Mata C, Miro E, Alvarado A, Garcillan-Barcia MP, Toleman M, Walsh TR, *et al.* Plasmid typing and genetic context of AmpC beta-lactamases in *Enterobacteriaceae* lacking inducible chromosomal ampC genes: findings from a Spanish hospital 1999-2007. J Antimicrob Chemother. 2012;67(1):115–22.

144. Zamorano L, Miro E, Juan C, Gomez L, Bou G, Gonzalez-Lopez JJ, *et al.* Mobile genetic elements related to the diffusion of plasmid-mediated AmpC beta-lactamases or carbapenemases from *Enterobacteriaceae*: findings from a multicenter study in Spain. Antimicrob Agents Chemother. 2015;59(9):5260–6.

145. Accogli M, Fortini D, Giufre M, Graziani C, Dolejska M, Carattoli A, *et al.* IncI1 plasmids associated with the spread of CMY-2, CTX-M-1 and SHV-12 in *Escherichia coli* of animal and human origin. Clin Microbiol Infect. 2013;19(5):E238–40.

146. Endimiani A, Hilty M, Perreten V. CMY-2-producing *Escherichia coli* in the nose of pigs. Antimicrob Agents Chemother. 2012;56(8):4556–7.

147. Ben Sallem R, Ben Slama K, Rojo-Bezares B, Porres-Osante N, Jouini A, Klibi N, *et al.* IncI1 plasmids carrying *bla*$_{\text{CTX-M-1}}$ or *bla*$_{\text{CMY-2}}$ genes in *Escherichia coli* from healthy humans and animals in Tunisia. Microb Drug Resist. 2014;20(5):495–500.

148. Haenni M, Saras E, Metayer V, Medaille C, Madec JY. High prevalence of *bla*$_{\text{CTX-M-1}}$/IncI1/ST3 and *bla*CMY-2/IncI1/ST2 plasmids in healthy urban dogs in France. Antimicrob Agents Chemother. 2014;58(9):5358–62.

149. Doublet B, Praud K, Nguyen-Ho-Bao T, Argudin MA, Bertrand S, Butaye P, *et al.* Extended-spectrum beta-lactamase- and AmpC beta-lactamase-producing D-tartrate-positive S*almonella enterica* serovar Paratyphi B from broilers and human patients in Belgium, 2008-10. J Antimicrob Chemother. 2014;69(5):1257–64.

150. Caltagirone M, Bitar I, Piazza A, Spalla M, Nucleo E, Navarra A*, et al.* Detection of an IncA/C plasmid encoding VIM-4 and CMY-4 beta-lactamases in *Klebsiella oxytoca* and *Citrobacter koseri* from an inpatient in a cardiac rehabilitation unit. New Microbiol. 2015;38(3):387–92.

151. Sarkar A, Pazhani GP, Chowdhury G, Ghosh A, Ramamurthy T. Attributes of carbapenemase encoding conjugative plasmid pNDM-SAL from an extensively drug-resistant *Salmonella enterica* Serovar Senftenberg. Front Microbiol. 2015;6:969. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26441902

152. Potron A, Nordmann P, Poirel L. Characterization of OXA-204, a carbapenem-hydrolyzing class D beta-lactamase from *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2013;57(1):633–6.

153. Mnif B, Ktari S, Chaari A, Medhioub F, Rhimi F, Bouaziz M, *et al.* Nosocomial dissemination of *Providencia stuarti*i isolates carrying *bla*$_{\text{OXA-48}}$, *bla*$_{\text{PER-1}}$, *bla*$_{\text{CMY-4}}$ and *qnrA6* in a Tunisian hospital. J Antimicrob Chemother. 2013;68(2):329–32.

154. Villa L, Guerra B, Schmoger S, Fischer J, Helmuth R, Zong Z, *et al.* IncA/C Plasmid Carrying *bla*$_{\text{NDM-1}}$, *bla*$_{\text{CMY-16}}$, and *fosA3* in a *Salmonella enterica* Serovar Corvallis Strain Isolated from a Migratory Wild Bird in Germany. Antimicrob Agents Chemother. 2015;59(10):6597–600.

155. Datta S, Mitra S, Viswanathan R, Saha A, Basu S. Characterization of novel plasmid-mediated beta-lactamases (SHV-167 and ACT-16) associated with New Delhi metallo-beta-lactamase-1 harbouring isolates from neonates in India. J Med Microbiol. 2014;63(Pt 3):480–2.
156. Voulgari E, Poulou A, Dimitroulia E, Politi L, Ranellou K, Gennimata V, *et al.* Emergence of OXA-162 Carbapenemase- and DHA-1 AmpC Cephalosporinase-Producing Sequence Type 11 *Klebsiella pneumoniae* Causing Community-Onset Infection in Greece. Antimicrob Agents Chemother, 2015;60(3):1862–4.
157. Martinez-Martinez L, Gonzalez-Lopez JJ. Carbapenemases in *Enterobacteriaceae*: types and molecular epidemiology. Enferm Infecc Microbiol Clin. 2014;32 Suppl 4:4–9.
158. Pitout JD, Nordmann P, Poirel L. Carbapenemase-Producing *Klebsiella pneumoniae,* a Key Pathogen Set for Global Nosocomial Dominance. Antimicrob Agents Chemother. 2015;59(10):5873–84.
159. Lascols C, Hackel M, Marshall SH, Hujer AM, Bouchillon S, Badal R, *et al.* Increasing prevalence and dissemination of NDM-1 metallo-beta-lactamase in India: data from the SMART study (2009). J Antimicrob Chemother. 2011;66(9):1992–7.
160. Kuai S, Shao H, Huang L, Pei H, Lu Z, Wang W, *et al.* KPC-2 carbapenemase and DHA-1 AmpC determinants carried on the same plasmid in *Enterobacter aerogenes*. J Med Microbiol. 2014;63(Pt 3):367–70.
161. Rasheed JK, Kitchel B, Zhu W, Anderson KF, Clark NC, Ferraro MJ, *et al.* New Delhi metallo-beta-lactamase-producing *Enterobacteriaceae*, United States. Emerg Infect Dis. 2013;19(6):870–8.
162. Roy S, Singh AK, Viswanathan R, Nandy RK, Basu S. Transmission of imipenem resistance determinants during the course of an outbreak of NDM-1 *Escherichia coli i*n a sick newborn care unit. J Antimicrob Chemother. 2011;66(12):2773–80.
163. Yang J, Ye L, Wang W, Luo Y, Zhang Y, Han L. Diverse prevalence of 16S rRNA methylase genes *armA* and *rmtB* amongst clinical multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates. Int J Antimicrob Agents. 2011;38(4):348–51.
164. Rahman M, Shukla SK, Prasad KN, Ovejero CM, Pati BK, Tripathi A, *et al.* Prevalence and molecular characterisation of New Delhi metallo-beta-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant *Enterobacteriaceae* from India. Int J Antimicrob Agents. 2014;44(1):30–7.
165. Feng Y, Yang P, Xie Y, Wang X, McNally A, Zong Z. *Escherichia coli* of sequence type 3835 carrying $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-42}}$ and $bla_{\text{SHV-12}}$. Sci Rep. 2015;5:12275.
166. Miriagou V, Tzouvelekis LS, Villa L, Lebessi E, Vatopoulos AC, Carattoli A, *et al.*

CMY-13, a novel inducible cephalosporinase encoded by an *Escherichia coli* plasmid. Antimicrob Agents Chemother. 2004;48(8):3172–4.

167. Mac Aogain M, Rogers TR, Crowley B. Identification of emergent *bla*$_{CMY-2}$ -carrying *Proteus mirabilis* lineages by whole-genome sequencing. New Microbes New Infect. 2016;9:58–62.
168. Gyles C, Boerlin P. Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. Vet Pathol. 2014;51(2):328–40.
169. Partridge SR, Tsafnat G, Coiera E, Iredell JR. Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiol Rev. 2009;33(4):757–84.
170. Ma XJ, Yang HF, Liu YY, Mei Q, Ye Y, Li HR, *et al.* ACT-10, a novel plasmid-encoded class C beta-lactamase in a *Serratia marcescens* isolate from China. Diagn Microbiol Infect Dis. 2014;80(4):343–4.
171. Di Pilato V, Arena F, Giani T, Conte V, Cresti S, Rossolini GM. Characterization of pFOX-7a, a conjugative IncL/M plasmid encoding the FOX-7 AmpC-type beta-lactamase, involved in a large outbreak in a neonatal intensive care unit. J Antimicrob Chemother. 2014;69(10):2620–4.
172. Pitout JD, Gregson DB, Church DL, Laupland KB. Population-based laboratory surveillance for AmpC beta-lactamase-producing *Escherichia coli*, Calgary. Emerg Infect Dis. 2007;13(3):443–8.
173. Baudry PJ, Mataseje L, Zhanel GG, Hoban DJ, Mulvey MR. Characterization of plasmids encoding CMY-2 AmpC beta-lactamases from *Escherichia coli* in Canadian intensive care units. Diagn Microbiol Infect Dis. 2009;65(4):379–83.
174. Mataseje LF, Xiao J, Kost S, Ng L-K, Doré K, Mulvey MR, *et al.* Characterization of Canadian cefoxitin-resistant non-typhoidal *Salmonella* isolates, 2005-06. J Antimicrob Chemother. 2009;64(4):723–30.
175. Karlowsky JA, Adam HJ, Baxter MR, Lagacé-Wiens PRS, Walkty AJ, Hoban DJ, *et al.* In vitro activity of ceftaroline-avibactam against gram-negative and gram-positive pathogens isolated from patients in Canadian hospitals from 2010 to 2012: results from the CANWARD surveillance study. Antimicrob Agents Chemother. 2013;57(11):5600–11.
176. Wiesner M, Calva JJ, Bustamante VH, Pérez-Morales D, Fernández-Mora M, Calva E, *et al.* A multi-drug resistant *Salmonella* Typhimurium ST213 human-invasive strain (33676) containing the *bla*$_{CMY-2}$ gene on an IncF plasmid is attenuated for virulence in BALB/c mice. BMC Microbiol. 2016;16:18.
177. Deshpande LM, Jones RN, Fritsche TR, Sader HS. Occurrence of plasmidic AmpC type beta-lactamase-mediated resistance in *Escherichia coli*: report from the SENTRY Antimicrobial Surveillance Program (North America, 2004). Int J

Antimicrob Agents. 2006;28(6):578–81.

178. Waltner-Toews RI, Paterson DL, Qureshi ZA, Sidjabat HE, Adams-Haduch JM, Shutt KA, *et al.* Clinical characteristics of bloodstream infections due to ampicillin-sulbactam-resistant, non-extended- spectrum-beta-lactamase-producing *Escherichia coli* and the role of TEM-1 hyperproduction. Antimicrob Agents Chemother. 2011;55(2):495–501.
179. Ibrahimagic A, Bedenic B, Kamberovic F, Uzunovic S. High prevalence of CTX-M-15 and first report of CTX-M-3, CTX-M-22, CTX-M-28 and plasmid-mediated AmpC beta-lactamase producing *Enterobacteriaceae* causing urinary tract infections in Bosnia and Herzegovina in hospital and community settings. J Infect Chemother. 2015;21(5):363–9.
180. Jorgensen RL, Nielsen JB, Friis-Moller A, Fjeldsoe-Nielsen H, Schonning K. Prevalence and molecular characterization of clinical isolates of *Escherichia coli* expressing an AmpC phenotype. J Antimicrob Chemother. 2010;65(3):460–4.
181. Hansen F, Olsen SS, Heltberg O, Justesen US, Fuglsang-Damgaard D, Knudsen JD, *et al.* Characterization of third-generation cephalosporin-resistant *Escherichia coli* from bloodstream infections in Denmark. Microb Drug Resist. 2014;20(4):316–24.
182. Mammeri H, Eb F, Berkani A, Nordmann P. Molecular characterization of AmpC-producing *Escherichia coli* clinical isolates recovered in a French hospital. J Antimicrob Chemother. 2008;61(3):498–503.
183. Corvec S, Cremet L, Leprince C, Dauvergne S, Reynaud A, Lepelletier D, *et al.* Epidemiology of *Escherichia coli* clinical isolates producing AmpC plasmidic beta-lactamase during a 5-year period in a French teaching Hospital. Diagn Microbiol Infect Dis. 2010;67(3):277–81.
184. Antunes P, Coque TM, Peixe L. Emergence of an IncIγ plasmid encoding CMY-2 ß-lactamase associated with the international ST19 OXA-30-producing ß-lactamase *Salmonella* Typhimurium multidrug-resistant clone. J Antimicrob Chemother. 2010;65(10):2097–100.
185. Aragon LM, Mirelis B, Miro E, Mata C, Gomez L, Rivera A, *et al.* Increase in beta-lactam-resistant *Proteus mirabilis* strains due to CTX-M- and CMY-type as well as new VEB- and inhibitor-resistant TEM-type beta-lactamases. J Antimicrob Chemother. 2008;61(5):1029–32.
186. Oteo J, Cercenado E, Cuevas O, Bautista V, Delgado-Iribarren A, Orden B, *et al.* AmpC beta-lactamases in *Escherichia coli*: emergence of CMY-2-producing virulent phylogroup D isolates belonging mainly to STs 57, 115, 354, 393, and 420, and phylogroup B2 isolates belonging to the international clone O25b-ST131. Diagn Microbiol Infect Dis. 2010;67(3):270–6.

187. Ortega A, Oteo J, Aranzamendi-Zaldumbide M, Bartolome RM, Bou G, Cercenado E, *et al*. Spanish multicenter study of the epidemiology and mechanisms of amoxicillin-clavulanate resistance in *Escherichia coli*. Antimicrob Agents Chemother.2012;56(7):3576–81.

188. Alonso N, Miro E, Pascual V, Rivera A, Simo M, Garcia MC, *et al*. Molecular characterisation of acquired and overproduced chromosomal bla_{AmpC} in *Escherichia coli* clinical isolates. Int J Antimicrob Agents. 2016;47(1):62–8.

189. Galan-Sanchez F, Aznar-Marin P, Marin-Casanova P, Rodriguez-Iglesias M. Diversity of *bla* genes and low incidence of CTX-M in plasmid-mediated AmpC-producing *Escherichia coli* clinical isolates. APMIS. 2014;122(9):796–9.

190. Adler H, Fenner L, Walter P, Hohler D, Schultheiss E, Oezcan S, *et al*. Plasmid-mediated AmpC beta-lactamases in *Enterobacteriaceae* lacking inducible chromosomal ampC genes: prevalence at a Swiss university hospital and occurrence of the different molecular types in Switzerland. J Antimicrob Chemother. 2008;61(2):457–8.

191. Voets GM, Platteel TN, Fluit AC, Scharringa J, Schapendonk CM, Stuart JC, *et al*. Population distribution of Beta-lactamase conferring resistance to third-generation cephalosporins in human clinical *Enterobacteriaceae* in the Netherlands. PLoS One. 2012;7(12):e52102.

192. Woodford N, Reddy S, Fagan EJ, Hill RL, Hopkins KL, Kaufmann ME, *et al*. Wide geographic spread of diverse acquired AmpC beta-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. J Antimicrob Chemother. 2007;59(1):102–5.

193. Yilmaz NO, Agus N, Bozcal E, Oner O, Uzel A. Detection of plasmid-mediated AmpC beta-lactamase in *Escherichia coli* and *Klebsiella pneumoniae*. Indian J Med Microbiol. 2013;31(1):53–9.

194. Balikci H, Acikgoz ZC, Guvenman S, Celikbilek N, Ozdem B. Detection of plasmid-mediated AmpC beta-lactamase production in *Escherichia coli* and *Klebsiella* spp. isolates]. Mikrobiyol Bul. 2014;48(1):82–93.

195. Sari AN, Bicmen M, Gulay Z. Investigation of plasmid mediated AmpC beta-lactamases among *Escherichia coli* and *Klebsiella pneumoniae* isolated from blood cultures. Mikrobiyol Bul. 2013;47(4):582–91.

196. Vlieghe ER, Huang TD, Phe T, Bogaerts P, Berhin C, De Smet B, *et al*. Prevalence and distribution of beta-lactamase coding genes in third-generation cephalosporin-resistant *Enterobacteriaceae* from bloodstream infections in Cambodia. Eur J Clin Microbiol Infect Dis. 2015;34(6):1223–9.

197. Zhang J, Zheng B, Zhao L, Wei Z, Ji J, Li L, *et al*. Nationwide high prevalence of CTX-

M and an increase of CTX-M-55 in *Escherichia coli* isolated from patients with community-onset infections in Chinese county hospitals. BMC Infect Dis. 2014;14:659.

198. Hussain M, Hasan F, Shah AA, Hameed A, Jung M, Rayamajhi N, *et al.* Prevalence of class A and AmpC beta-lactamases in clinical *Escherichia coli* isolates from Pakistan Institute of Medical Science, Islamabad, Pakistan. Jpn J Infect Dis. 2011;64(3):249–52.
199. Tan TY, Ng SY, Teo L, Koh Y, Teok CH. Detection of plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. J Clin Pathol. 2008;61(5):642–4.
200. Yan JJ, Hong CY, Ko WC, Chen YJ, Tsai SH, Chuang CL, *et al.* Dissemination of *bla*$_{\text{CMY-2}}$ among *Escherichia coli* isolates from food animals, retail ground meats, and humans in southern Taiwan. Antimicrob Agents Chemother. 2004;48(4):1353–6.
201. Wang JT, Chen PC, Chang SC, Shiau YR, Wang HY, Lai JF, *et al.* Antimicrobial susceptibilities of *Proteus mirabilis*: a longitudinal nationwide study from the Taiwan surveillance of antimicrobial resistance (TSAR) program. BMC Infect Dis.2014;14:486.
202. Singtohin S, Chanawong A, Lulitanond A, Sribenjalux P, Auncharoen A, Kaewkes W, *et al.* CMY-2, CMY-8b, and DHA-1 plasmid-mediated AmpC beta-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from a university hospital, Thailand. Diagn Microbiol Infect Dis. 2010;68(3):271–7.
203. Fam D, El Said M, El Defrawy I, El Dadei E, El Attar S, Sorur A, Ahmed S, Klena JNG. Prevalence of plasmid-mediated ampC genes in clinical isolates of *Enterobacteriaceae* from Cairo, Egypt. Br Microbiol Res J. 2013;3(4):525–37.
204. Barguigua A, El Otmani F, Talmi M, Zerouali K, Timinouni M. Prevalence and types of extended spectrum beta-lactamases among urinary *Escherichia coli* isolates in Moroccan community. Microb Pathog. 2013;61-62:16–22.
205. Ogbolu DO, Daini OA, Ogunledun A, Alli AO, Webber MA. High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria. Int J Antimicrob Agents. 2011;37(1):62–6.
206. Ogbolu DO, Daini OA, Ogunledun A, Terry Alli OA, Webber MA. Dissemination of IncF plasmids carrying beta-lactamase genes in Gram-negative bacteria from Nigerian hospitals. J Infect Dev Ctries. 2013;7(5):382–90.
207. Usha G, Chunderika M, Prashini M, Willem SA, Yusuf ES. Characterization of extended-spectrum beta-lactamases in *Salmonella* spp. at a tertiary hospital in Durban, South Africa. Diagn Microbiol Infect Dis. 2008;62(1):86–91.
208. Mahrouki S, Chihi H, Bourouis A, Ayari K, Ferjani M, Moussa MB, *et al.* Nosocomial

dissemination of plasmids carrying *bla*$_{TEM-24}$, *bla*$_{DHA-1}$, *aac(6')-Ib-cr*, and *qnrA6* in Providencia spp. strains isolated from a Tunisian hospital. Diagn Microbiol Infect Dis. 2015;81(1):50–2.

209. de Toro M, Garcia P, Rodriguez I, Rojo-Bezares B, Helmuth R, Saenz Y, *et al.* Characterisation of plasmids implicated in the mobilisation of extended-spectrum and AmpC beta-lactamase genes in clinical *Salmonella enterica* isolates and temporal stability of the resistance genotype. Int J Antimicrob Agents.2013;42(2):167–72.
210. Hopkins KL, Liebana E, Villa L, Batchelor M, Threlfall EJ, Carattoli A. Replicon typing of plasmids carrying CTX-M or CMY beta-lactamases circulating among *Salmonella* and *Escherichia coli* isolates. Antimicrob Agents Chemother. American Society for Microbiology (ASM); 2006;50(9):3203–6.
211. Sonnevend A, Ghazawi A, Yahfoufi N, Al-Baloushi A, Hashmey R, Mathew M, *et al.* VIM-4 carbapenemase-producing *Enterobacter cloacae* in the United Arab Emirates. Clin Microbiol Infect. 2012;18(12):E494–6.
212. Kotsakis SD, Tzouvelekis LS, Lebessi E, Doudoulakakis A, Bouli T, Tzelepi E, *et al.* Characterization of a mobilizable IncQ plasmid encoding cephalosporinase CMY-4 in *Escherichia coli*. Antimicrob Agents Chemother. 2015;59(5):2964–6.
213. Wu SW, Dornbusch K, Kronvall G, Norgren M. Characterization and nucleotide sequence of a *Klebsiella oxytoca* cryptic plasmid encoding a CMY-type beta-lactamase: confirmation that the plasmid-mediated cephamycinase originated from the *Citrobacter freundii* AmpC beta-lactamase. Antimicrob Agents Chemother.1999;43(6):1350–7.
214. D'Andrea MM, Giani T, Henrici De Angelis L, Ciacci N, Gniadkowski M, Miriagou V, *et al.* Draft Genome Sequence of *Proteus mirabilis* NO-051/03, Representative of a Multidrug-Resistant Clone Spreading in Europe and Expressing the CMY-16 AmpC-Type beta-Lactamase. Genome Announc.2016;4(1).
215. Zioga A, Whichard JM, Kotsakis SD, Tzouvelekis LS, Tzelepi E, Miriagou V. CMY-31 and CMY-36 cephalosporinases encoded by ColE1-like plasmids. Antimicrob Agents Chemother. 2009;53(3):1256–9.
216. Soliman AM, Khalifa HO, Ahmed AM, Shimamoto T, Shimamoto T. Emergence of an NDM-5-producing clinical *Escherichia coli* isolate in Egypt. Int J Infect Dis. 2016;48:46–8.
217. Wei Y, Wang J. Identification of ACT-1 plasmid-mediated AmpC beta-lactamase producing *Citrobacter freundii* from a Chinese patient. Ann Lab Med. 2013;33(1):86–8.

1.2. Review Article

Epidemiology of acquired β-lactamases involved in resistance to extended-spectrum β-lactams in *Enterobacteriaceae* in Africa: a global overview and recent trends

Epidemiology of acquired β-lactamases involved in resistance to extended-spectrum β-lactams in *Enterobacteriaceae* in Africa: a global overview and recent trends

T. G. Ribeiro[1], Â. Novais[1], E. Machado[1,2], L. Peixe[1*]

[1]UCIBIO@REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

***Corresponding author:** Luísa Vieira Peixe, UCIBIO@REQUIMTE, Laboratory of Microbiology, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal; Tel: (+351) 220 428 580; E-mail: lpeixe@ff.up.pt

Running title: Epidemiology of acquired β-lactamases among *Enterobacteriaceae* in Africa.

Abstract

Available epidemiological data suggests that the dissemination of *Enterobacteriaceae* resistant to extended-spectrum β-lactams constitutes an important problem in Africa with a probable impact in the global epidemiology. However, detailed molecular data from African countries, and especially on acquired β-lactamases are fragmented and limited to certain bacterial species, clinical settings and/or countries hindering to establish a global scenario. In this review, we describe the current status regarding the occurrence, diversity and distribution of acquired β-lactamases (EAAC) [extended-spectrum β-lactamases (ESBLs), acquired AmpC β-lactamases (qAmpC) and carbapenemases] among *Enterobacteriaceae* isolates recovered from clinical and non-clinical settings in Africa. The contribution of particular *Enterobacteriaceae* clones and/or plasmids for the dissemination of antibiotic resistance determinants to extended-spectrum β-lactams in Africa is also reviewed.

Keywords: carbapenemases, ESBL, AmpC, environment, clinical

Introduction

Africa is the second largest and most populous continent, after Asia. It includes 54 nations that altogether comprise approximately 1.2 billion people, representing nearly 15% of the world population (http://www.unicef.org/publications/files/UNICEF_Africa_Generation_2030_en_11Aug.pdf). Most of the African countries reflect low human development indexes (HDI), according to the last HDI rank from 2014 (http://hdr.undp.org/en/composite/HDI). Relatively to Public Health, the lack of financial and political resources, nutrition deprivations, poor sanitation, overcrowding living conditions, inadequate hygiene and infection control measures in healthcare settings affect large areas of Africa and might constitute favorable conditions for the dissemination of antimicrobial resistant bacteria (AMR) (1,2). In a recent report launched by the World Health Organization (WHO), it is recognized that "*…information concerning the true extent of antimicrobial resistance in the African Region is limited because surveillance of drug resistance is carried out in only a few countries. There is a scarcity of accurate and reliable data on antibiotic resistance for many common and serious infectious conditions that are important for public health in the region*" (http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf).

Arguably, the most important concern regarding antibiotic resistance during the last decades has been the emergence and spread of acquired β-lactamases (EAAC) [extended-spectrum β-lactamases (ESBLs), acquired AmpC β-lactamases (qAmpC) and carbapenemases] in bacteria from different ecological niches (3–7). Although the compilation of some African studies suggest that EAAC-producing *Enterobacteriaceae* are already established in Africa (8–12), most data are fragmented and biased towards particular acquired β-lactamases, clinical settings, infections (e.g. bacteremia) and countries. In addition, there is no comprehensive review of clones and/or plasmids linked to the spread of these β-lactamases in Africa, preventing to understand reservoirs and transmission routes and ultimately the contextualization with data from topic. We will provide an overview on the current status regarding the occurrence of genes encoding EAAC among *Enterobacteriaceae* isolates recovered from clinical and non-clinical niches in Africa, as well as clones and plasmids associated with their dissemination.

Acquired β-lactamases conferring resistance to extended-spectrum β-lactams in clinical *Enterobacteriaceae*

Multidrug-resistant (MDR) *Enterobacteriaceae* isolates are frequently reported in many African countries especially linked to the production of ESBLs, qAmpCs and/or carbapenemases both in hospital and community settings, although their prevalence has

been shown to vary between countries (8–10). They have been identified in all regions of Africa, though most existing reports and especially detailed molecular data are from North African countries, particularly Central Maghreb (Tunisia, Algeria, Morocco) (8,9,13–15). Below, we present a brief overview on the current epidemiology of *Enterobacteriaceae* producing main acquired β-lactamases in Africa.

(i) Extended-spectrum β-lactamases (ESBLs)

ESBLs constitute the most frequent mechanism of resistance to extended-spectrum β-lactams (16). They have the ability to confer bacterial resistance to all β-lactams except carbapenems and cephamycins, and are inhibited by β-lactamase inhibitors such as clavulanic acid (17). The occurrence of ESBL-producing *Enterobacteriaceae* seems to be low in most Central, Eastern and Southern African countries, compared to countries from North and Western Africa (Figure 1). However, the low occurrence rates reported for those regions could result from the absence of routine culture and antibiotic susceptibility testing in hospital and community settings (18). The diversity of particular ESBLs also varies between countries, although CTX-M-15 is the most frequently reported among *Enterobacteriaceae* causing human infections. Other ESBLs are prevalent in specific geographic regions such as CTX-M-3 in Algeria, CTX-M-14 in Kenya, SHV-12 in Tunisia and SHV-28 in Senegal (Figure 1), sometimes associated with focal outbreaks. Furthermore, a shift in the distribution of ESBL-types, with a recent increase of CTX-M-15 over SHV or TEM was also reported in some countries.

North Africa

In Algeria, CTX-M-15 and in a lesser extent CTX-M-3 are widely distributed among diverse *Enterobacteriaceae* species in different hospitals (15,19–31). This situation is comparable to that reported in Eastern European countries and China (3,5). Interestingly, the rate of ESBL-producing *Enterobacter cloacae* (CTX-M-15, CTX-M-3) has increased throughout time: 2.3% in 2004 (19), 17.7% in 2007 (22), 47% in 2009 (29), and 67% in 2013 (28). Clonal outbreaks caused by CTX-M-3-producing *Salmonella enterica* serotype Senftenberg and CTX-M-15-producing *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *S. enterica*) were described in the same or different wards at Algerian hospitals (15,24,25,27,28). In 1998, *S.* Senftenberg producing CTX-M-3 and ArmA 16S RNA methylase (conferring resistance to aminoglycosides) was described for the first time in one Algerian hospital (26). Latter (2011-2013), in the same hospital, an outbreak or a suspected outbreak associated with CTX-M-15 and ArmA was described in a different *Salmonella* serovar (Infantis) and in *S. marcescens* (27,31). The authors

hypothesize that CTX-M-3 gene might probably had evolved into a CTX-M-15 variant (27), however *de novo* acquisition of CTX-M-15 could not be discarded.

In Egypt, different ESBL-types have been identified as the most dominant, depending on the species. Overall, CTX-M-15 was the most frequently reported ESBL among *E. coli* recovered from hospital and community infections or *K. pneumoniae* from nosocomial infections (32–35). However, other ESBL were also frequent in particular species (SHV-2a, SHV-5 or SHV-12 among *K. pneumoniae* and CTX-M-1, CTX-M-9 or both among *E. coli*) (34,36,37).

The prevalence of ESBL producers in Morocco was variable according to the population analyzed, with a predominance of CTX-M-15-producing *E. coli* or *K. pneumoniae* causing community-acquired urinary tract infections (UTIs) (38–40). Two studies performed during 2004-2007 and 2004-2009, showed a prevalence of 1.3% of ESBL-producing *E. coli* (38,40). This prevalence is similar to that reported in France in 2006 (1.1%), a country with close commercial and cultural relations with Morocco (41,42). However, in 2010 the prevalence of ESBL-producing *K. pneumoniae* in Morocco rose to 7.5% (39). Interestingly, CTX-M-28 and SHV-12 were the most common ESBL detected among a collection of *qnr* positive *Enterobacteriaceae* (43,44).

Regarding Tunisia, the current population of ESBL-producing *Enterobacteriaceae* causing hospital or community-acquired infections produces in most cases CTX-M-15 (45–50). In fact, a temporal shift in the distribution of ESBL-types among *K. pneumoniae* was observed in different Tunisian hospitals, with a dramatic increase of CTX-M-15 enzyme over SHV-12 after 2003 (45,48,51), a scenario similar to that reported in Europe (5). Nosocomial outbreaks involving SHV-2-producing *S. enterica* serotype Wien (Sfax Hospital in 1998), TEM-4-producing *S. enterica* serotype Mbandaka (University Hospital in Tunis from 1995 to 1999), CTX-M-27-producing *S. enterica* serotype Livingstone (Farhat Hached Hospital in 2002) have also been reported (52–54).

West Africa

Available epidemiological data from West Africa are mainly from Ghana, Nigeria and Senegal. In Ghana, ESBL-producing *E. coli* isolates were more identified among hospitalized patients (69%) than in outpatients (31%) (55). CTX-M-15 was the dominant ESBL, mainly identified in *E. coli* from hospitalized patients and *K. pneumoniae* from community-acquired infections and nosocomial origin (18,56).

In Nigeria, most of the studies did not characterize the ESBL-type, but similar ESBL occurrences were reported in different hospital institutions (23.6%-30% among *E. coli* and *K. pneumoniae* and 20% among *E. cloacae*) (57–59). CTX-M-15 was the most prevalent

ESBL detected among Gram-negative enteric isolates (mostly *E. coli*) recovered from different hospitals (60–63).

In Senegal, a longitudinal study (1999-2009) analyzing the prevalence and diversity of ESBLs among clinical *S. enterica* isolates reported an ESBL prevalence of 0.6% (12). SHV-ESBLs (SHV-2 and SHV-12) were detected in different *S. enterica* (Miami and Agona and Keurmassar serotypes, respectively) in 1990 and early 2000s, while CTX-M-15 was reported since mid-2000s among different *S. enterica* serotypes (Kentucky, Havana, Telelkebir, Grumpensis, Typhimurium, Carmel) (12). In 2011, a nosocomial outbreak caused by CTX-M-109-producing *S. enterica* serovar Grumpensis was reported in Dakar, however the source of the infection was never established (64). Diverse *Enterobacteriaceae* species other than *Salmonella* spp. (*E. coli*, *K. pneumoniae*, *Enterobacter* spp., *Morganella morganii*) producing CTX-M-15 or co-producing CTX-M-15 and SHV-28 have been identified causing hospital-acquired infections since 2008 (65–67).

Central Africa

Few studies have analyzed the occurrence and diversity of ESBLs in isolates recovered from clinical institutions in Central Africa. SHV (mostly SHV-12) was the dominant ESBL among hospitalized patients from Cameroon (at least until 2000s) (68), with CTX-M-15 being reported afterwards (2005) among *E. coli* and *K. pneumoniae* isolates recovered from patients with UTI acquired during hospitalization (68). In Central African Republic, a significant increase in the proportion of ESBL-producers in UTIs was noteworthy: 3.7% in 2004, 8.9% in 2005 and 19.3% in 2006 (69).

East Africa

In Kenya, during a 18-year period (1992-2010), CTX-M-15 and CTX-M-14 were the dominant ESBLs among *E. coli* isolates recovered from inpatients and outpatients, while other CTX-M and ESBL-types (SHV and TEM) were detected less frequently (Figure 1) (70,71).

In Tanzania, CTX-M-15 is widely distributed (72–76), and epidemic strains of *K. pneumoniae, E. coli* and *Enterobacter* spp. have emerged (74–76).

In Madagascar, 9.7% of *Enterobacteriaceae* involved in community-acquired UTIs between 2004 and 2006 were ESBL producers (77). A further study encompassing clinical *Enterobacteriaceae* isolates from patients at surgery and intensive care units (2006-2008) reported a rate of ESBL producers of 21.3% (78). More recently (2012-2013), 44% of *Enterobacteriaceae* isolates recovered from neonatal units from two hospitals were ESBL producers (CTX-M-15-producing *E. cloacae* or *K. pneumoniae*) (79).

In Malawi, only data from *Enterobacteriaceae* involved in bacteremia is available: <1% of ESBL producers, with CTX-M-15, TEM-63, SHV-12 and SHV-27 being identified (80).

South Africa

A shift in the diversity of ESBLs has occurred in South Africa. Until 2003, SHV-*like* and TEM-*like* were the most commonly reported ESBLs, mainly in *Salmonella* spp. and *K. pneumoniae* (81–83). After 2003, CTX-M enzymes have been identified (Figure 1), mainly CTX-M-15 and CTX-M-14 in *E. coli* and *Shigella* spp. recovered from different hospitals (84–86).

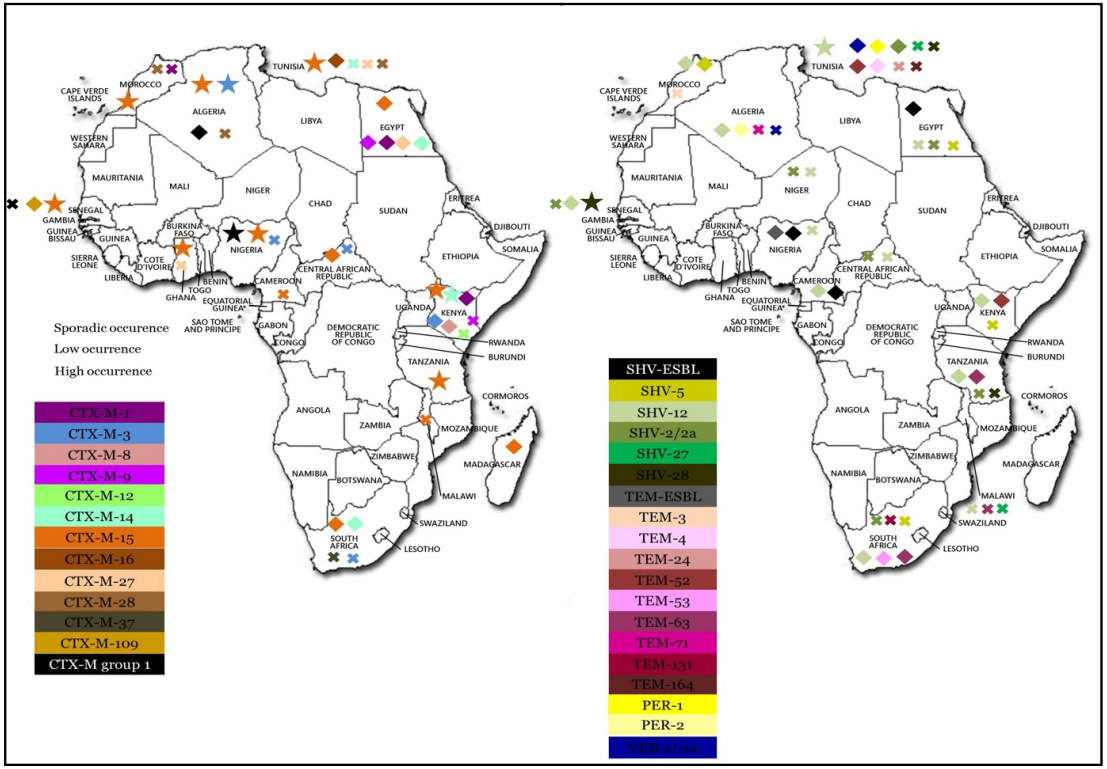


Figure 1. Geographical distribution of different ESBL-types among *Enterobacteriaceae* from Africa (8,12,15,18,21,31,33,34,48,49,51–53,56,63–68,71–76,79,80,82,87–93).

It is important to highlight that co-production of different ESBLs is increasingly reported in African countries. In fact, *Enterobacteriaceae* isolates expressing ESBLs, namely different CTX-M enzymes (CTX-M-15 and CTX-M-14; CTX-M-1 and CTX-M-9), CTX-M and SHV (CTX-M-15 and SHV-2a, SHV-12 or SHV-28), CTX-M and TEM (CTX-M-15 and TEM-3, TEM-24 or TEM-63; CTX-M-37 and TEM-63), TEM and SHV (TEM-4 and SHV-2a) or ESBL and carbapenemases (CTX-M-14 and VIM-4; CTX-M-15 and NDM-1 and SHV-5; CTX-M-15 and OXA-48, IMP-1 or NDM-1; SHV-28 and OXA-48; SHV-12 and IMP-1) have been linked to community and hospital-acquired infections (33–35,39,44,48,53,66,67,81,93–99).

(ii) Acquired AmpC β-lactamases (qAmpCs)

Acquired AmpC β-lactamases constitute a large group of Ambler class C enzymes, within which seven classes have been recognized (CMY, DHA, ACC, FOX, MIR, ACT and MOX). With the exception of ACC-1, they can hydrolyze efficiently penicillins and oxyimino-cephalosporins (including cephamycins) and are not inhibited by clavulanic acid (100). The qAmpC enzymes have been identified throughout the world, but their occurrence is thought to be much lower than that reported for ESBLs, although it might be underestimated due to difficulties in their detection (e.g. differentiation regarding ESBL production) and/or because they are not systematically sought (100,101). CMY-2 is the most frequently encountered variant with the broadest geographic distribution, being an important cause of β-lactam resistance especially among *Salmonella* (particularly in North America) and *E. coli* (3,100,102,103). DHA-1 is the second most prevalent qAmpC reported, more frequently in *K. pneumoniae* in Asia but also increasingly implicated in outbreaks in different European countries (104–107). Other qAmpCs were occasionally reported in nosocomial outbreaks (e.g. ACC-1 in France and Ireland; FOX-7 or CMY-16 in Italy) (108–112).

The first qAmpC reported in Africa was CMY-4, described in a Tunisian clinical isolate of P. mirabilis (1996), eight years later from the first qAmpC report described in the USA (MIR-1 in a *K. pneumoniae* clinical isolate in 1998) (114,115). Taking into account the few studies conducted in Africa (mostly from North Africa), these acquired β-lactamases were mostly reported since the beginning of 2000s. Moreover, the overall occurrence of qAmpCs seems to be low, although their occurrence might also be underestimated as previously mentioned. Figure 2 illustrates the distribution of different qAmpC-types in African countries. CMY-2 and DHA-1, the most common variants detected in different African countries (Figure 2), have been identified in several *Enterobacteriaceae* species (*E. coli*, *K. pneumoniae*, *E. cloacae*, *M. morganii*, *Providencia stuartii*, *P. mirabilis*, *S. marcescens*, *Salmonella* spp., *Shigella* spp., *Citrobacter freundii*) from hospitals and community clinical settings (39,43,81,85,92,93,115–117), ACC-1 and CMY-4 were linked to nosocomial

outbreaks (as detailed further below), while ACT was sporadically reported and FOX, MIR and MOX enzymes were not detected (Figure 2).

North Africa

Analyzing the epidemiological situation in more detail in different regions of Africa, it is noticed that in *North Africa* the dominant qAmpC-type varies according with the country, the source of isolation, the sample studied (e.g. isolates resistant to extended-spectrum β-lactams and fluoroquinolones or ESBL producers) and/or the bacterial species.

Overall, CMY-2 (Algeria) or other CMY variants (Algeria, Egypt, Tunisia), DHA-1 (Morocco, Tunisia), and ACC (Tunisia) are the qAmpC-types most frequently identified (Figure 2). CMY-2 was the most prevalent qAmpC reported among *Enterobacteriaceae* between 2003 and 2007 in different Algerian hospitals (115,116). Additionally, CMY-4 was reported as the dominant qAmpC among *K. pneumoniae* and *E. coli* recovered from different Algerian clinical settings between 2005 and 2006 (118), being also associated with *K. pneumoniae* and *P. stuartii* clonal outbreaks (2006, 2011) (119,120), whereas in Tunisian hospitals it was only sporadically reported (114,121,122).

One of the first reports of ACC-1 in Europe (France in 1998) was associated with a *K. pneumoniae* outbreak following the admission of a patient transferred from Tunisia (123). Further investigations showed that ACC-1 is probably widespread in Tunisian hospitals, being identified in different *Enterobacteriaceae* species (*K. pneumoniae*, *P. mirabilis*, and *S. enterica* serotype Livingstone and serotype Mbandaka) (53,124), and linked to a large nosocomial outbreak caused by *S.* Livingstone between 1999 and 2003 (125).

In Egypt, CMY-2-*like* were the most common qAmpC types in *Enterobacteriaceae* from hospitalized patients (September 2011 to October 2012) (126), while in Morocco DHA-1 was commonly detected among SHV-12- or SHV-28-producing *E. cloacae*, *E. coli* or *K. pneumoniae* identified among hospitalized patients (43) or ACT-2 sporadically detected among community acquired infections (39).

West Africa

Studies performed in Niger, Nigeria and Senegal, reported variable qAmpCs among different *Enterobacteriaceae* species associated with hospital or community-acquired infections: CMY-2 (*E. coli*, *C. freundii*), CMY-30 (*E. coli*), DHA-1 (*P. mirabilis*, *K. pneumoniae*, *M. morganii*), and ACT-1 (*K. pneumoniae* and *E. coli*) (60,66,98,127) (Figure 2).

East and South Africa

In Kenya (East Africa) and South Africa (South Africa), CMY-2 or CMY-2-*like* were the dominant qAmpCs identified in *E. coli* from community-acquired UTIs (2004-2005) (71), different *S. enterica* serotypes (Typhimurium, Schwarzengrund, Kissi, Isangi) in hospitalized patients (2001-2003) (81,83), or ESBL-producing *Shigella* spp. from 2003-2009 (85). In addition, CMY-20 was identified for the first time in South Africa among clinical *E. coli* isolates (128).

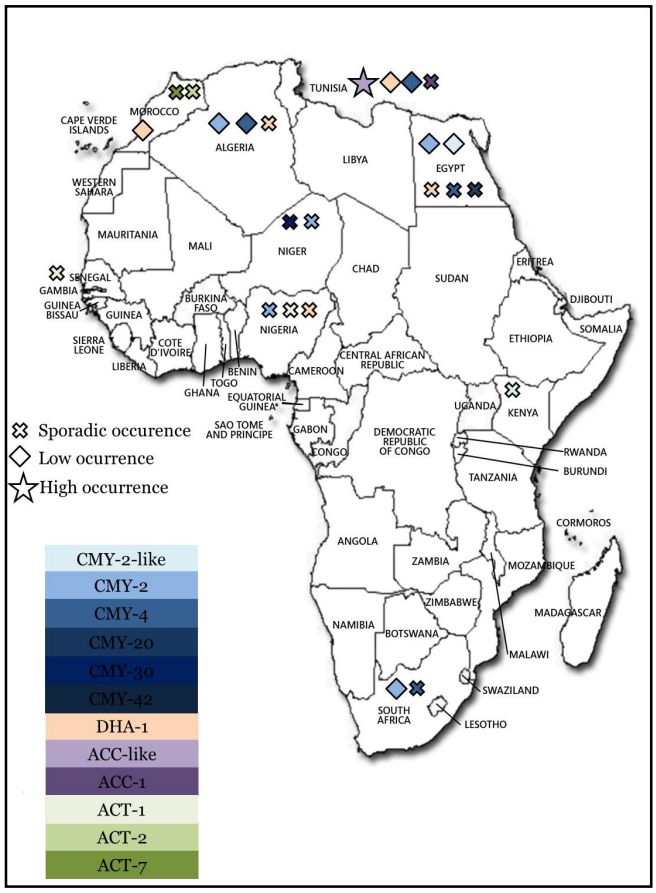


Figure 2. Geographical distribution of different qAmpC-types among *Enterobacteriaceae* from Africa (8,53,66,71,83,92,93,114,116,118,120–122,125–130)

(iii) Carbapenemases

The most widespread carbapenemases among *Enterobacteriaceae* are the KPC enzymes (class A), the metallo-β-lactamases (class B)] represented mostly by VIM, IMP and NDM types, and finally OXA-48-like enzymes (class D) (131,132). Carbapenemases enzymes represent the most versatile family of β-lactamases, with a variable hydrolysis spectrum that most often affects almost all extended-spectrum β-lactams (4). They have quickly spread worldwide after first description and are variably endemic in different geographic areas or countries: KPC in northeastern United States, Latin American

(Argentina, Brazil, Puerto Rico, Colombia), European (Greece, Italy), and Asian countries (Israel, China); VIM in Greece; IMP in Japan and Taiwan; NDM-1 in the Indian subcontinent, Balkans and the Gulf region; and OXA-48 type in some Mediterranean (Morocco, Tunisia) European (Spain, Belgium) and Asian countries (Turkey, India) (1,4,11,132–135).

Overall, the occurrence of acquired carbapenemases among *Enterobacteriaceae* is still low in Africa (with the exception of North Africa), with most studies reporting these β-lactamases after 2010s, as observed in European countries (136). However, there is a lack of information concerning detailed molecular data of carbapenemase-producing bacteria, since antibiotic susceptibility testing for diagnostic purposes and active surveillance in most African countries are absent or deficient (11). Nevertheless, from the current available reports, OXA-48-producing *Enterobacteriaceae* are endemic in North African countries, namely in Morocco and Tunisia (Figure 3), being identified mostly from *K. pneumoniae* and *E. coli* isolates collected from both hospital and community settings. NDM (mostly identified in *K. pneumoniae*) or VIM variants were reported in all but one African region (Central Africa), while KPC has been sporadically reported and other carbapenemases were confined to particular countries (Figure 3). Figure 3 illustrates the distribution of the different carbapenemases all over Africa. Interestingly, several European studies reported carbapenemase-producing *Enterobacteriaceae* from patients transferred from African countries as further detailed, underscoring the importance of an early warning system and active surveillance including colonization screening upon admission of patients transferred across borders and/or healthcare systems (137).

North Africa

In Algeria, carbapenemases have been sporadically reported in different *Enterobacteriaceae* associated with hospital infections: KPC-3 in *K. pneumoniae* (2013); VIM-19 in *E. coli*, *K. pneumoniae* and *P. stuartii* (2008), and OXA-48 in *E. coli* and *K. pneumoniae* (2012, 2014) (138–141). Interestingly, NDM-5-producing *E. coli* were isolated from three Algerian patients in 2014, three years later from the first description of this NDM variant (142).

KPC-producing *K. pneumoniae* isolated from hospitalized patients were first reported in Egypt in 2011 (143). VIM-1 was identified in different *Enterobacteriaceae* species (*E. coli*, *K. pneumoniae* and *P. stuartii*), whereas VIM-4 or VIM-29 were detected in *E. cloacae* or *E. coli* (respectively) recovered from hospitalized patients (35,94,144). NDM-1 was first identified in 2013 in one *K. pneumoniae* isolate, and subsequently in other *Enterobacteriaceae* (145), whereas NDM-5 was identified in *E. coli* from two different hospitals, with one case showing a patient with no history of travel beyond the Egyptian

border (145,146), which might suggest a local emergence of this NDM-5. OXA-48, OXA-161 and OXA-163 have been sporadically detected in *K. pneumoniae* or *E. coli* (10,147,148).

In Libya, two studies reported OXA-48 as the unique carbapenemase described so far in *K. pneumoniae* isolates (10,149). Interestingly, many studies reported OXA-48-producing *K. pneumoniae* from patients transferred from Libya to European countries (e.g. Denmark, Italy, Slovenia) (137,150,151).

In Morocco, VIM-1 was described in a *K. pneumoniae* which additionally co-produced two other carbapenemases (NDM-1 and OXA-48) (152). IMP-1 was reported in a *K. pneumoniae* isolate co-producing CTX-M-15 and *E. cloacae* co-producing additionally CTX-M-15 and CMY-2 or SHV-12 and DHA-1, associated with a community-acquired infection (153). NDM-1 was reported in *K. pneumoniae* isolates producing additionally CTX-M-15 and SHV-5 (95). OXA-48-producing *Enterobacteriaceae* are endemic in Morocco and Tunisia, being described in hospital and community settings (4,10,134). In France, $bla_{\text{OXA-48}}$-positive *E. cloacae* isolates were recovered from patients who had been previously hospitalized in Morocco (154). OXA-204 (a point mutant analog of OXA-48) and NDM-1 were sporadically reported in Tunisian hospitals (155–157) and VIM-4 was linked to a hospital clonal outbreak by *K. pneumoniae* co-producing CTX-M-15 and CMY-4 (120).

West and Central Africa

In 2011, several carbapenemases (NDM, GES, VIM) were identified mostly in *K. pneumoniae* from four tertiary hospitals in Nigeria (158), while in Senegal, OXA-48 was the unique carbapenemase reported in different *Enterobacteriaceae* species (mostly *K. pneumoniae*) causing community- or hospital-acquired infections (66). In Sierra Leone (2010-2011), DIM-1 was detected among *Enterobacter* spp. and *E. coli*, and VIM in different *Enterobacteriaceae* species (159). Surprisingly, the draft genome sequence of one *E. cloacae* isolate revealed that this strain harbored a complete $bla_{\text{OXA-58}}$ open reading frame surrounded by flanking sequences that were 100% identical to IS*Aba3*, found in a number of *Acinetobacter* plasmids (159). To date, this enzyme was exclusively found in *Acinetobacter* species (160).

Regarding Central Africa, there are no studies analyzing the occurrence and diversity of carbapenemases. Nevertheless, from a rectal swab specimen collected from a patient transferred from Cameroon to France, two *E. coli* isolates were found to co-produce NDM-4 and CTX-M-15 or NDM-1 (161), which might indicate that NDM carbapenemases in Africa are probably underscored.

East Africa

The prevalence of *Enterobacteriaceae* producing carbapenemases reported at tertiary hospital in Tanzania between 2007 and 2012 was 42%, with several types of carbapenemases being detected (162). IMP enzymes were predominant (19%; mostly in *E. coli*), followed by VIM (11%; mainly in *K. pneumoniae*), OXA-48 (5%), KPC (4%) and NDM (3%). Interestingly, a recent study conducted at one hospital in Uganda (2013-2014), also revealed a high prevalence (28.6%) and diversity of carbapenemase-producing *Enterobacteriaceae* (mostly *K. pneumoniae* and *E. coli*) (163). However, in this study the most prevalent gene was bla_{VIM} (10.7%), followed by $bla_{\text{OXA-48}}$ (9.7%), bla_{IMP} (6.1%), bla_{KPC} (5.1%) and $bla_{\text{NDM-1}}$ (2.6%). In Ethiopia, carbapenemase producers were identified (*E. coli*, *K. pneumoniae*, *E. aerogenes*) in hospitalized patients, however determination of the allelic variant was not performed (164). NDM-1 was reported among clonally related *K. pneumoniae* collected between 2007 and 2009 at one hospital in Kenya, prior to the first known identification of an NDM-1 producer (2008) from a Swedish patient previously hospitalized in India (165,166). Interestingly, similar pulsed-field gel electrophoresis (PFGE) patterns were observed among NDM-1-producing *K. pneumoniae* reported from Kenya, and the first cases of NDM-1-producing *K. pneumoniae* isolates identified in Sweden (Europe) and Sultanate of Oman (Asia), for which a connection with India was established, which might indicate that the introduction of NDM-1-producing *K. pneumoniae* in the Kenyan population could be linked to the Indian subcontinent (166).

South Africa

In 2011, KPC-2-producing *K. pneumoniae* or *E. cloacae* were identified in two different hospitals (167), and VIM-1-producing *K. pneumoniae* was isolated from a patient without recent travel outside South Africa (168). The first report of NDM-1 in South Africa occurred in 2011 identified in one nosocomial *E. cloacae* isolate. One year later, a direct epidemiological link between NDM endemic areas was established, NDM-1-producing *E. cloacae* was reported from a patient who had been previously hospitalized in India (169,170). OXA-48 or OXA-181 (the second most common OXA-48 derivative) have been identified in nosocomial *Enterobacteriaceae* isolates (*K. pneumoniae*, *S. marcescens* or *K. pneumoniae*, respectively) (171).

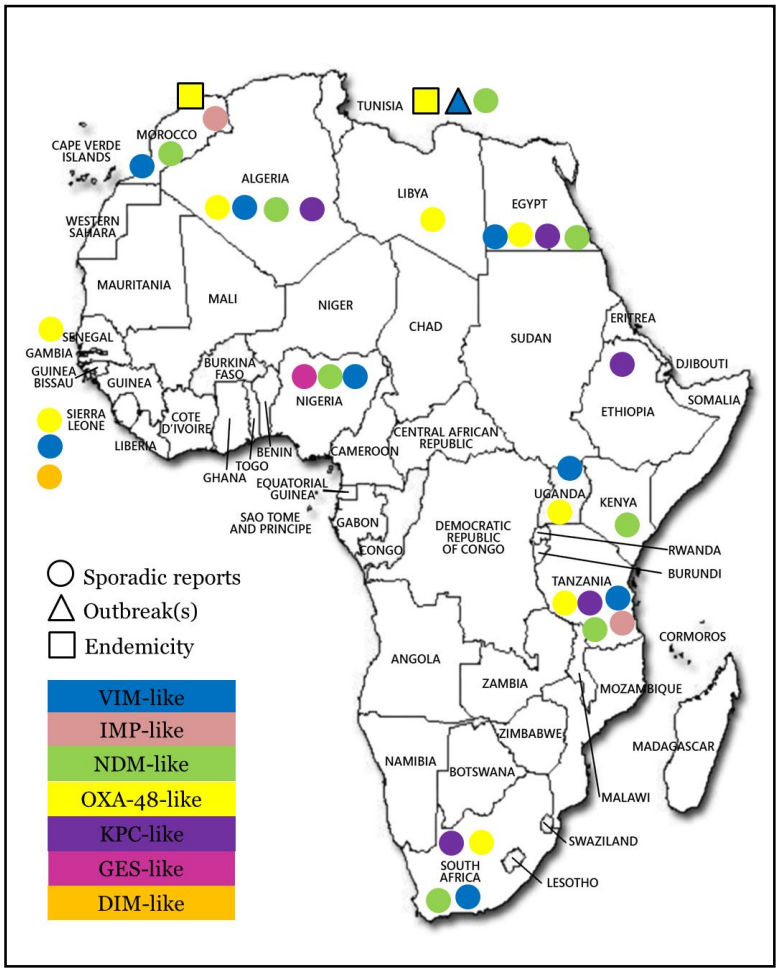


Figure 3. Geographical distribution of different carbapenemase-types among *Enterobacteriaceae* from Africa (4,10,134,138,140–142,158,163,164,166,172–174).

Mechanisms of dissemination of EAAC genes among clinical *Enterobacteriaceae*

Detailed molecular epidemiology studies in the last decades have demonstrated that the successful dissemination (locally or in wide geographic areas) of the most widespread EAAC genes in *Enterobacteriaceae* is a result of the combination of the expansion of particular clones mainly from *E. coli* and *K. pneumoniae* and/or plasmid transfer between isolates from the same or different species (5,175,176). Outbreaks other than *E. coli* or *K. pneumoniae* isolates have also been described, but in most cases they seem to be of local significance (5). In addition, the identification of identical clones and/or plasmids in human infections and food-producing animals or healthy humans suggests a food-borne origin for particular antibiotic resistant genes and the potential role of direct or indirect transmission through the food chain, although there is no clear evidence (7,177,178). Though the majority of African studies did not address these points, a brief overview of the bacterial clonal and

plasmid structure involved in the dissemination of acquired β-lactamase genes in this geographic region is presented below.

Clonal spread

The dissemination of CTX-M-15 in different African countries seems to have mainly been driven by the pandemic B2-ST131 *E. coli* clone (21,32,46,56,62,75,98,179), as occurred in developed countries. In fact, this clone is currently spread worldwide among humans and also increasingly recovered from animals and the environment (5,7,180) linked mostly to the CTX-M-15 pandemic, but also with other ESBLs, qAmpCs (mostly CMY) or carbapenemases (5,181–186). CTX-M-15 has also been sporadically linked to ST405 *E. coli* clone from phylogenetic group D in Egypt, Tunisia, Tanzania, and Cameroon (35,47,75,161). This clone is also relatively frequent in other continents associated with the spread of CTX-M enzymes, qAmpCs, carbapenemases (NDM, OXA-48) and/or methylases (ArmA, RmtB) enzymes among different hosts (187–192).

Other *E. coli* clones identified among isolates producing carbapenemases, ESBLs and/or qAmpCs in Africa were ST23 (VIM-1 and SHV-12), ST38 (OXA-48 and CTX-M-14 and SHV-12), ST101 (VIM-1, OXA-48 and VIM-29 and CMY-2), ST617 (OXA-48), ST648 (OXA-48), ST2659 (NDM-5 and CTX-M-15), and ST5018 (NDM-5 and CMY-42) (35,142,144,146,156,161).

Regarding *K. pneumoniae* isolates, the ST101 clone was one of the most predominant in different geographic contexts and linked to diverse acquired β-lactamases (CTX-M-15, OXA-48, OXA-181, OXA-204, NDM-1, KPC-2) in hospital or clinical community settings (45,48,99,155,156,193–196), highlighting its potential for the acquisition of different platforms. Moreover, this clone has been involved in cases of importation of OXA-48 and/or CTX-M-15 to Poland or Libya after patient hospitalization in Tunisia (149–151,197). ST101 with $bla_{\text{VIM-1}}$ and $bla_{\text{CTX-M-15}}$ has also been identified in Egypt (144).

The worldwide spread ST11-*K. pneumoniae* seems also to be dispersed in Africa, where it has been linked to the production of OXA-48, co-production of NDM-1 and CTX-M-15 or NDM-1 and OXA-48 in Egypt and Tunisia (144,147,157). This clone is also extensively distributed in different continents linked to the production of diverse ESBLs (mainly CTX-M) or carbapenemases (KPC, OXA-48, NDM, VIM) (4,187,198–200). Other widespread or emergent *K. pneumoniae* clones linked to the production of acquired β-lactamases in Africa were: ST14 (OXA-48, NDM-1 and CTX-M-15) in Kenya and Egypt (144,166), ST15 (NDM-1 and/or CTX-M-15) in Algeria and Morocco (21,95) and ST147 (OXA-48 and CTX-M-15, OXA-204 and CMY-4) in Libya and Tunisia (134,149,150,155). All of them are known to be frequent *K. pneumoniae* lineages increasingly involved in nosocomial infections in many other geographic regions (198,200). This, the globalization

of humans, trade and food might have played an essential role in the emergence of carbapenemase-producers in Africa, but these genetic backgrounds seem to be crucial to provide stable platforms for the maintenance and dissemination of these antibiotic resistance genes (176,201,202).

In Tunisia, *K. pneumoniae* isolates co-producing VIM-4, CTX-M-15, and CMY-4 were genetically identical but were different from VIM-1 producers responsible for the Greece outbreak. Furthermore, *bla*$_{VIM-1}$ has been identified among *K. pneumoniae* ST17, ST101, and ST569 strains from Egypt and South Africa (144,168).

The majority of African studies reporting *Enterobacteriaceae* producing qAmpCs or class A, DIM or IMP carbapenemases did not provide data on the bacterial population structure (143,153,158,162,167).

Horizontal gene transfer

It is known the involvement of particular plasmid types assigned to IncFII, IncI1, HI2, IncP, IncN, IncL, IncM and IncA/C plasmid incompatibility groups in the spread of specific ESBLs, qAmpC or carbapenemases among different *Enterobacteriaceae* species and/or niches worldwide (5,187,203–205), although the situation in developing countries is underestimated.

Besides clonal spread, plasmid transfer seems to have also influenced the dissemination of carbapenemases in Africa. In Africa, the predominant plasmid carrying *bla*$_{CTX-M-15}$ in *E. coli* belongs to the IncF group (mostly in *E. coli*) (Ghana, Kenya, Morocco, Niger, Tanzania, Tunisia) (45,48,56,75,98,166,179,206), also the most reported in clinical settings worldwide (5). However, other plasmids have also been linked to the dissemination of *bla*$_{CTX-M-15}$, such as IncFIIK in *K. pneumoniae* (Ghana, Tunisia) (48,56), and IncFIIAs (mostly *K. pneumoniae* and *E. coli*) (Tunisia) (179), and more rarely IncL/M (probably the currently designated IncL group) (Algeria, Tunisia), IncFIC (Tunisia), IncK (Tunisia), IncHI1 (Tunisia) and untypeable plasmids (Tunisia, Tanzania) (45,48,76,115,179).

One study performed in Tunisia reported *K. pneumoniae* carrying *bla*$_{SHV-12}$ in 60–180 kb untypeable plasmids, and more rarely in IncHI2, while SHV-2a was carried by different Inc groups (IncFII, IncL/M) (45). Plasmids assigned to the IncL/M group carrying *bla*$_{CTX-M-3/-15}$ and *armA* genes were reported in *S. enterica* serotypes Infantis or Senftenberg or *S. marcescens* in Algeria (26,27,31), whereas *bla*$_{CTX-M15/-27}$ and *bla*$_{SHV-12}$ were located in 65 kb IncL/M, 80 kb IncFII, and untypeable plasmids in *K. pneumoniae* isolates recovered from the same Tunisian clinical institution (45). IncA/C plasmids carrying *bla*$_{CTX-M-15}$ and *bla*$_{TEM-52}$ were reported in *E. coli* isolates recovered from Tunisia (207).

Plasmids carrying qAmpC genes were only characterized in North African countries (Algeria, Tunisia). The majority of the *bla*$_{CMY-2/CMY-4}$–carrying plasmids identified in diverse

Enterobacteriaceae species in Algeria belonged mostly to the IncA/C2 group, followed by IncI1. Plasmids carrying bla_{DHA-1} or both bla_{DHA-1} and bla_{TEM-24} genes were identified as IncA/C among *E. cloacae* (Algeria) (115,117,118). The clonal outbreak of ACC-1-producing *S. enterica* serotype Livingstone reported in Tunisia might also involve plasmid dissemination, since bla_{ACC-1} was located on 100-kb IncFI plasmid in three representative isolates (125).

The bla_{NDM-1} identified only in *K. pneumoniae* isolates from different African countries (Egypt, Kenya, Morocco, Nigeria, Tunisia) was associated with several Inc groups (A/C, N, L/M, H, R) and untypeable plasmids (96,157,158,166,174). Some of these plasmids also harbored $bla_{CTX-M-15}$, *rmtC* or *rmtF* methylase genes (96,158,166,174). Other NDM variants where reported in *E. coli* from Cameroon and Egypt, linked to IncFIA (120 kb; bla_{NDM-4} and $bla_{CTX-M-15}$) or IncI1 (> 93 kb; bla_{NDM-5}, $bla_{CTX-M-15}$ and bla_{CMY-42}), respectively (146,161). In contrast, plasmids carrying the bla_{OXA-48} gene were mostly characterized among clinical *K. pneumoniae* from North Africa (Egypt, Libya, Tunisia), and belonged to the IncL/M (currently recognized as IncL), as observed by others (4,134,135,144,149,151,157,204). IncL/M plasmids identified among *K. pneumoniae* from Libya (2011) contain bla_{OXA-48} and $bla_{CTX-M-15}$ (149), an association that was also described in European countries (France, Spain) (208–210). The bla_{VIM-19} gene was identified in 160-180 kb plasmids, and located inside a class 1 integron in diverse *Enterobacteriaceae* species collected from two Algerian hospitals (139,211), while bla_{VIM-1} was identified within an IncFII plasmid in one *K. pneumoniae* from South Africa (168). bla_{VIM-4} and $bla_{CTX-M-14}$ were carried on the same 300 kb plasmid in two *E. cloacae* complex isolates from Egypt (94).

EAAC from non-clinical sources (humans, animals, environment)

There are a growing number of studies reporting acquired β-lactamases among non-clinical settings in developed countries (7,178,212–215). However, they are not yet systematically sought in *Enterobacteriaceae* from non-clinical sources, and especially from African countries.

Recently (2015), Ribeiro and colleagues investigated the distribution and molecular epidemiology of EAAC in *Enterobacteriaceae* from non-clinical niches in Angola, an under researched sub-Saharan country (190). An unexpected high occurrence of CTX-M-15 in diverse non-clinical niches (healthy humans, animals and aquatic environments) and *Enterobacteriaceae* species (mostly *E. coli*) was reported, whereas qAmpCs or carbapenemases were not detected. The variability observed in $bla_{CTX-M-15}$ genetic environments, genetic locations and clones suggests an extraordinary ability for acquisition and mobilization of this gene by multiple genetic backgrounds (190). Interestingly, CTX-M-15 was the most common ESBL in diverse *Enterobacteriaceae* species (mostly *E. coli*) from

non-clinical sources (healthy humans and animals, environment) recovered from Sub-Saharan Africa (comprises all African regions with exception of North Africa), and was also the most prevalent among environmental samples from Tunisia and Algeria (190). This is surprising, since in most developed countries CTX-M-15-producing *E. coli* only recently has been reported among non-clinical sources (mostly aquatic environments and healthy humans) (216–218). In Tunisia, $bla_{\text{CTX-M-1}}$ is widely disseminated among clonally unrelated *E. coli* recovered from healthy humans, livestock and pet animals and food, being also the major ESBL-type in food-producing animals in Europe (pigs, poultry and cattle) and also among companion animals (7,193). Although most of the African studies did not characterize the population structure of CTX-M-15 producers from non-clinical settings, we observed that the same clones associated with CTX-M-15 in African hospitals were detected in non-clinical sources (e.g. *E. coli* ST131, ST405, ST617; *K. pneumoniae* ST15) (21,46,47,179,190,219,220), suggesting the transmission of zoonotic pathogens to humans trough the food chain.

Regarding qAmpCs, it is known that CMY-2 is the most prevalent mainly among *E. coli* and *Salmonella* spp. from non-clinical sources in Europe, Asia and North America, associated with IncI1 or IncA/C plasmids (7,203,213,214,221,222). Data from Africa are very scarce, with CMY-2 being identified among *E. coli* isolates recovered from chickens, companion animals (dog), and wastewaters in Tunisia and, similarly, associated with IncI1 (220,223–225). However, in Angola (2013) no qAmpC was identified among *Enterobacteriaceae* isolates recovered from different ecological niches (healthy humans, animals and aquatic environments) (190).

Reports of carbapenemase-producing *Enterobacteriaceae* from non-clinical niches in Africa were rare and came from the North African region. *K. pneumoniae* producing IMP and VIM were described in Tunisian rivers (226), while *E. coli* producing NDM-5 were identified among companion animals in Algeria and retail chicken meat in Egypt (33,227). OXA-48-producing *E. coli* have been described in companion animals in Algeria, and OXA-48-producing *S. marcescens* in water puddles from Morocco (227,228).

The wide distribution of ESBLs, qAmpCs and carbapenemases in different compartments in Africa highlights the existence of possible reservoirs in different ecological niches in this continent, and eventually a dynamic genetic exchange between them through the food chain that might also play a role in the global epidemiology of antibiotic resistance.

Conclusions

Enterobacteriaceae resistant to extended-spectrum β-lactams have been reported all over Africa, with a great variability in the occurrence, diversity and geographical distribution the different acquired beta-lactamases. Overall, most of African data are biased

towards North African countries, which probably for this reason show the highest occurrence and diversity of acquired β-lactamases both in clinical and non-clinical settings. Furthermore, it is remarkable the description of such a diversity of acquired β-lactamases both in clinical and non-clinical settings in Africa, where sociocultural conditions might exacerbate this situation and demand an urgent need of quick and accessible methodologies to detect these antibiotic resistance mechanisms. Also, the description of clones and/or MDR plasmids encoding acquired β-lactamases in Africa similar to those responsible for the dissemination of these enzymes worldwide highlights the role of international travel, tourism and trade in the emergence and spread of these antibiotic resistant determinants. Indeed, infection or colonization by *Enterobacteriaceae* producing acquired β-lactamases in countries outside Africa have been associated with patients transfer or previously hospitalization on African epidemic areas.

Funding

This work received financial support from the European Union (FEDER funds) through Programa Operacional Fatores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through projects UID/Multi/04378/2013 and UID/Multi/04546/2013. T. G. R. and Â. N. were supported by fellowships from FCT (SFRH/BD/75752/2011 and SFRH/BPD/104927/2014, respectively) through Programa Operacional Capital Humano (POPH).

Transparency declarations

None to declare.

References

1. Bonelli RR, Moreira BM, Picao RC. Antimicrobial resistance among *Enterobacteriaceae* in South America: history, current dissemination status and associated socioeconomic factors. Drug Resist Updat. 2014;17(1-2):24–36.
2. Leopold SJ, van Leth F, Tarekegn H, Schultsz C. Antimicrobial drug resistance among clinically relevant bacterial isolates in sub-Saharan Africa: a systematic review. J Antimicrob Chemother. 2014;69(9):2337–53.
3. Hawkey PM, Jones AM. The changing epidemiology of resistance. J Antimicrob Chemother. 2009;64 Suppl 1:i3–10.
4. Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis. 2011;17(10):1791–8.
5. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing

Enterobacteriaceae in Europe. Euro Surveill. 2008;13(47).

6. Sidjabat HE, Paterson DL. Multidrug-resistant *Escherichia coli* in Asia: epidemiology and management. Expert Rev Anti Infect Ther. 2015;13(5):575–91.
7. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012;18(7):646–55.
8. Storberg V. ESBL-producing *Enterobacteriaceae* in Africa - a non-systematic literature review of research published 2008-2012. Infect Ecol Epidemiol. 2014;4.
9. Tansarli GS, Poulikakos P, Kapaskelis A, Falagas ME. Proportion of extended-spectrum beta-lactamase (ESBL)-producing isolates among *Enterobacteriaceae* in Africa: evaluation of the evidence--systematic review. J Antimicrob Chemother. 2014;69(5):1177–84.
10. Manenzhe RI, Zar HJ, Nicol MP, Kaba M. The spread of carbapenemase-producing bacteria in Africa: a systematic review. J Antimicrob Chemother. 2015;70(1):23–40.
11. Sekyere JO, Govinden U, Essack S. The Molecular Epidemiology and Genetic Environment of Carbapenemases Detected in Africa. Microb Drug Resist. 2016;22(1):59–68.
12. Harrois D, Breurec S, Seck A, Delauné A, Hello S Le, Gándara MP de la, *et al.* Prevalence and characterization of extended-spectrum β-lactamase-producing clinical *Salmonella enterica* isolates in Dakar, Senegal, from 1999 to 2009. Clin Microbiol Infect. 2014;20(2):O109–16.
13. Muvunyi CM, Masaisa F, Bayingana C, Mutesa L, Musemakweri A, Muhirwa G, *et al.* Decreased susceptibility to commonly used antimicrobial agents in bacterial pathogens isolated from urinary tract infections in Rwanda: need for new antimicrobial guidelines. Am J Trop Med Hyg. 2011;84(6):923–8.
14. Beyene G, Nair S, Asrat D, Mengistu Y, Engers H, Wain J. Multidrug resistant *Salmonella* Concord is a major cause of salmonellosis in children in Ethiopia. J Infect Dev Ctries. 2011;5(1):23–33.
15. Baba Ahmed-Kazi Tani Z, Decré D, Genel N, Boucherit-Otmani Z, Arlet G, Drissi M. Molecular and epidemiological characterization of enterobacterial multidrug-resistant strains in Tlemcen Hospital (Algeria) (2008-2010). Microb Drug Resis. 2013;19(3):185–90.
16. Tal Jasper R, Coyle JR, Katz DE, Marchaim D. The complex epidemiology of extended-spectrum β-lactamase-producing *Enterobacteriaceae*. Future Microbiol. 2015;10(5):819–39.
17. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrob

Agents Chemother. 2010;54(3):969–76.

18. Eibach D, Campos CB, Krumkamp R, Al-Emran HM, Dekker D, Boahen KG, *et al.* Extended spectrum beta-lactamase producing *Enterobacteriaceae* causing bloodstream infections in rural Ghana, 2007–2012. Int J Med Microbiol. 2016;2007–12.
19. Touati A, Benallaoua S, Forte D, Madoux J, Brasme L, de Champs C, *et al.* First report of CTX-M-15 and CTX-M-3 beta-lactamases among clinical isolates of *Enterobacteriaceae* in Béjaia, Algeria. Int J Antimicrob Agents. 2006;27(5):397–402.
20. Touati A, Brasme L, Benallaoua S, Madoux J, Gharout A, de Champs C. *Enterobacter cloacae* and *Klebsiella pneumoniae* isolates producing CTX-M-15 recovered from hospital environmental surfaces from Algeria. J Hosp Infect. 2008;68(2):183–5.
21. Ahmed ZB, Ayad A, Mesli E, Messai Y, Bakour R, Drissi M. CTX-M-15 extended-spectrum beta-lactamases in *Enterobacteriaceae* in the intensive care unit of Tlemcen Hospital, Algeria. East Mediterr Heal J. 2012;18(4):382–6.
22. Iabadene H, Messai Y, Ammari H, Ramdani-Bouguessa N, Lounes S, Bakour R, *et al.* Dissemination of ESBL and Qnr determinants in *Enterobacter cloacae* in Algeria. J Antimicrob Chemother. 2008;62(1):133–6.
23. Messai Y, Iabadene H, Benhassine T, Alouache S, Tazir M, Gautier V, *et al.* Prevalence and characterization of extended-spectrum beta-lactamases in *Klebsiella pneumoniae* in Algiers hospitals (Algeria). Pathol Biol. 2008;56(5):319–25.
24. Ramdani-Bouguessa N, Mendonça N, Leitão J, Ferreira E, Tazir M, Caniça M. CTX-M-3 and CTX-M-15 extended-spectrum beta-lactamases in isolates of *Escherichia coli* from a hospital in Algiers, Algeria. J Clin Microbiol. 2006;44(12):4584–6.
25. Naas T, Lezzar A, Bentchouala C, Smati F, Scheftel J-M, Monteil H, *et al.* Multidrug-resistant *Salmonella enterica* serotype Senftenberg isolates producing CTX-M beta-lactamases from Constantine, Algeria. J Antimicrob Chemother. 2005;56(2):439–40.
26. Naas T, Bentchouala C, Lima S, Lezzar A, Smati F, Scheftel J-M, *et al.* Plasmid-mediated 16S rRNA methylases among extended-spectrum-beta-lactamase-producing *Salmonella enterica* Senftenberg isolates from Algeria. J Antimicrob Chemother. 2009;64(4):866–8.
27. Naas T, Bentchouala C, Cuzon G, Yaou S, Lezzar A, Smati F, *et al.* Outbreak of *Salmonella enterica* serotype Infantis producing ArmA 16S RNA methylase and CTX-M-15 extended-spectrum β-lactamase in a neonatology ward in Constantine, Algeria. Int J Antimicrob Agents. 2011;38(2):135–9.
28. Khennouchi NC el H, Loucif L, Boutefnouchet N, Allag H, Rolain J-M. MALDI-TOF

MS as a Tool To Detect a Nosocomial Outbreak of Extended-Spectrum-β-Lactamase- and ArmA Methyltransferase-Producing *Enterobacter cloacae* Clinical Isolates in Algeria. Antimicrob Agents Chemother. 2015;59(10):6477–83.

29. Nedjai S, Barguigua A, Djahmi N, Jamali L, Zerouali K, Dekhil M, *et al.* Prevalence and characterization of extended spectrum beta-lactamase-producing *Enterobacter cloacae* strains in Algeria. J Infect Dev Ctries. 2013;7(11):804–11.
30. Nedjai S, Barguigua A, Djahmi N, Jamali L, Zerouali K, Dekhil M, *et al.* Prevalence and characterization of extended spectrum β-lactamases in *Klebsiella-Enterobacter-Serratia* group bacteria, in Algeria. Médecine Mal Infect. 2012;42(1):20–9.
31. Batah R, Loucif L, Olaitan AO, Boutefnouchet N, Allag H, Rolain JM. Outbreak of *Serratia marcescens* Coproducing ArmA and CTX-M-15 Mediated High Levels of Resistance to Aminoglycoside and Extended-Spectrum Beta-Lactamases, Algeria. Microb Drug Resist. 2015;21(4):470–6.
32. Fam N, Leflon-Guibout V, Fouad S, Aboul-Fadl L, Marcon E, Desouky D, *et al.* CTX-M-15-producing *Escherichia coli* clinical isolates in Cairo (Egypt), including isolates of clonal complex ST10 and clones ST131, ST73, and ST405 in both community and hospital settings. Microb Drug Resist. 2011;17(1):67–73.
33. Abdallah HM, Reuland EA, Wintermans BB, Al Naiemi N, Koek A, Abdelwahab AM, *et al.* Extended-Spectrum beta-Lactamases and/or Carbapenemases-Producing *Enterobacteriaceae* Isolated from Retail Chicken Meat in Zagazig, Egypt. PLoS One. 2015;10(8):e0136052.
34. Newire EA, Ahmed SF, House B, Valiente E, Pimentel G. Detection of new SHV-12, SHV-5 and SHV-2a variants of extended spectrum beta-lactamase in *Klebsiella pneumoniae* in Egypt. Ann Clin Microbiol Antimicrob. 2013;12:16.
35. Abdelaziz MO, Bonura C, Aleo A, Fasciana T, Calà C, Mammina C. Cephalosporin resistant *Escherichia coli* from cancer patients in Cairo, Egypt. Microbiol Immunol. 2013;57(5):391-5.
36. Hassan WM, Hashim A, Domany R. Plasmid mediated quinolone resistance determinants *qnr*, *aac(6')-Ib-cr*, and *qep* in ESBL-producing *Escherichia coli* clinical isolates from Egypt. Indian J Med Microbiol. 2012;30(4):442–7.
37. Khalaf NG, Eletreby MM, Hanson ND. Characterization of CTX-M ESBLs in *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates from Cairo, Egypt. BMC Infect Dis. 2009;9:84.
38. Bourjilat F, Bouchrif B, Dersi N, Claude JDPG, Amarouch H, Timinouni M. Emergence of extended-spectrum beta-lactamases-producing *Escherichia coli* in community-acquired urinary infections in Casablanca, Morocco. J Infect Dev Ctries. 2011;5(12):850–5.

39. Barguigua A, El Otmani F, Talmi M, Reguig A, Jamali L, Zerouali K, *et al.* Prevalence and genotypic analysis of plasmid-mediated beta-lactamases among urinary *Klebsiella pneumoniae* isolates in Moroccan community. J Antibiot. 2013;66(1):11–6.

40. Barguigua A, El Otmani F, Talmi M, Bourjilat F, Haouzane F, Zerouali K, *et al.* Characterization of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from the community in Morocco. J Med Microbiol. 2011;60(9):1344–52.

41. Arpin C, Quentin C, Grobost F, Cambau E, Robert J, Dubois V, *et al.* Nationwide survey of extended-spectrum {beta}-lactamase-producing *Enterobacteriaceae* in the French community setting. J Antimicrob Chemother. 2009;63(6):1205–14.

42. Thomas L. Encyclopedia of the Developing World. 1st ed. New York: Routledge Taylor & Francis Group; 2006.

43. Bouchakour M, Zerouali K, Gros Claude JD, Amarouch H, El Mdaghri N, Courvalin P, *et a*l. Plasmid-mediated quinolone resistance in expanded-spectrum beta-lactamase producing *Enterobacteriaceae* in Morocco. J Infect Dev Ctries. 2010;4(12):779–803.

44. Barguigua A, Zerouali K, Katfy K, El Otmani F, Timinouni M, Elmdaghri N. Occurrence of OXA-48 and NDM-1 carbapenemase-producing *Klebsiella pneumoniae* in a Moroccan university hospital in Casablanca, Morocco. Infect Genet Evol. 2015;31:142–8.

45. Elhani D, Bakir L, Aouni M, Passet V, Arlet G, Brisse S, *et al.* Molecular epidemiology of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* strains in a university hospital in Tunis, Tunisia, 1999-2005. Clin Microbiol Infect. 2010;16(2):157–64.

46. Dahmen S, Bettaieb D, Mansour W, Boujaafar N, Bouallègue O, Arlet G. Characterization and molecular epidemiology of extended-spectrum beta-lactamases in clinical isolates of *Enterobacteriaceae* in a Tunisian University Hospital.in a Tunisian University Hospital. Microb Drug Resist. 2010;16(2):163-70.

47. Ben Slama K, Ben Sallem R, Jouini A, Rachid S, Moussa L, Sáenz Y, *et al.* Diversity of genetic lineages among CTX-M-15 and CTX-M-14 producing *Escherichia coli* strains in a Tunisian hospital. Curr Microbiol. 2011;62(6):1794–801.

48. Mansour W, Grami R, Ben Haj Khalifa A, Dahmen S, Châtre P, Haenni M, *et al.* Dissemination of multidrug-resistant $bla_{\text{CTX-M-15}}$/IncFIIk plasmids in *Klebsiella pneumoniae* isolates from hospital- and community-acquired human infections in Tunisia. Diagn Microbiol Infect Dis. 2015;83(3):298–304.

49. Alibi S, Ferjani A, Boukadida J. Molecular characterization of extended spectrum

beta-lactamases produced by *Klebsiella pneumoniae* clinical strains from a Tunisian Hospital. Médecine Mal Infect. 2015;45(4):139–43.

50. Abbassi MS, Torres C, Achour W, Vinué L, Sáenz Y, Costa D, *et al.* Genetic characterisation of CTX-M-15-producing *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from stem cell transplant patients in Tunisia. Int J Antimicrob Agents. 2008;32(4):308–14.
51. Mamlouk K, Boutiba-Ben Boubaker I, Gautier V, Vimont S, Picard B, Ben Redjeb S, *et al.* Emergence and outbreaks of CTX-M beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* strains in a Tunisian hospital. J Clin Microbiol. 2006;44(11):4049–56.
52. Hammami A, Arlet G, Ben Redjeb S, Grimont F, Ben Hassen A, Rekik A, *et al.* Nosocomial outbreak of acute gastroenteritis in a neonatal intensive care unit in Tunisia caused by multiply drug resistant *Salmonella* Wien producing SHV-2 beta-lactamase. Eur J Clin Microbiol Infect Dis. 1991;10(8):641–6.
53. Makanera A, Arlet G, Gautier V, Manai M. Molecular epidemiology and characterization of plasmid-encoded beta-lactamases produced by Tunisian clinical isolates of *Salmonella enterica* serotype Mbandaka resistant to broad-spectrum cephalosporins. J Clin Microbiol. 2003;41(7):2940–5.
54. Bouallègue-Godet O, Ben Salem Y, Fabre L, Demartin M, Grimont PAD, Mzoughi R, *et al.* Nosocomial outbreak caused by *Salmonella enterica* serotype Livingstone producing CTX-M-27 extended-spectrum beta-lactamase in a neonatal unit in Sousse, Tunisia. J Clin Microbiol. 2005;43(3):1037–44.
55. Feglo P, Adu-Sarkodie Y, Ayisi, Lord, Jain R, Spurbeck RR, Springman AC, *et al.* Emergence of a novel extended-spectrum-β-lactamase (ESBL)-producing, fluoroquinolone-resistant clone of extraintestinal pathogenic *Escherichia coli* in Kumasi, Ghana. J Clin Microbiol. 2013;51(2):728–30.
56. Agyekum A, Fajardo-Lubian A, Ansong D, Partridge SR, Agbenyega T, Iredell JR. $bla_{\text{CTX-M-15}}$ carried by IncF-type plasmids is the dominant ESBL gene in *Escherichia coli* and *Klebsiella pneumoniae* at a hospital in Ghana. Diagn Microbiol Infect Dis. 2016;84(4):328–33.
57. Olonitola O, Olayinka A, Inabo H, Shaibu A. Production of extended spectrum beta-lactamases of urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. Int J Biol Chem Sci. 2007;1(2):181–5.
58. Aibinu IE, Ohaegbulam VC, Adenipekun EA, Ogunsola FT, Odugbemi TO, Mee BJ. Extended-spectrum beta-lactamase enzymes in clinical isolates of *Enterobacter* species from Lagos, Nigeria. J Clin Microbiol. 2003;41(5):2197–200.

59. Mohammed Y, Gadzama GB, Zailani SB, Aboderin AO. Characterization of Extended-Spectrum Beta-lactamase from *Escherichia coli* and *Klebsiella* Species from North Eastern Nigeria. J Clin Diagn Res. 2016;10(2):DC07–10.
60. Ogbolu DO, Daini OA, Ogunledun A, Alli AO, Webber MA. High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria. Int J Antimicrob Agents. 2011;37(1):62–6.
61. Olowe OA, Grobbel M, Büchter B, Lübke-Becker A, Fruth A, Wieler LH. Detection of $bla_{\text{CTX-M-15}}$ extended-spectrum β-lactamase genes in *Escherichia coli* from hospital patients in Nigeria. Int J Antimicrob Agents. 2010;35(2):206–7.
62. Aibinu I, Odugbemi T, Koenig W, Ghebremedhin B. Sequence Type ST131 and ST10 Complex (ST617) predominant among CTX-M-15-producing *Escherichia coli* isolates from Nigeria. Clin Microbiol Infect. 2012;18(3):E49–51.
63. Raji MA, Jamal W, Ojemeh O, Rotimi VO. Sequence analysis of genes mediating extended-spectrum beta-lactamase (ESBL) production in isolates of *Enterobacteriaceae* in a Lagos Teaching Hospital, Nigeria. BMC Infect Dis. 2015;15:259.
64. Diop A, Sambe-Ba B, Seck A, Dia ML, Timbiné LG, Niang AA, *et al*. First Description of the Extended Spectrum-Beta-Lactamase Gene $bla_{\text{CTX-M-109}}$ in *Salmonella* Grumpensis Strains Isolated from Neonatal Nosocomial Infections in Dakar, Senegal. PLoS One. 2016;11(6):e0157683.
65. Dia ML, Ngom B, Diagne R, Ka R, Lo S, Cisse MF, *et al*. Molecular detection of CTX-M-15-type β-lactamases in *Escherichia coli* strains from Senegal. New microbes New Infect. 2016;9:45–6.
66. Moquet O, Bouchiat C, Kinana A, Seck A, Arouna O, Bercion R, *et al*. Class D OXA-48 carbapenemase in multidrug-resistant enterobacteria, Senegal. Emerg Infect Dis. 2011;17(1):143–4.
67. Diene SM, Fenollar F, Fall B, Sow K, Niang B, Samba Ba P, *et al*. CTX-M-15-producing *Morganella morganii* from Hôpital Principal de Dakar, Senegal. New microbes New Infect. 2014;2(2):46–9.
68. Gangoué-Piéboji J, Bedenic B, Koulla-Shiro S, Randegger C, Adiogo D, Ngassam P, *et al*. Extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* in Yaounde, Cameroon. J Clin Microbiol. 2005;43(7):3273–7.
69. Bercion R, Mossoro-Kpinde D, Manirakiza A, Le Faou A. Increasing prevalence of antimicrobial resistance among *Enterobacteriaceae* uropathogens in Bangui, Central African Republic. J Infect Dev Ctries. 2009;3(3):187–90.
70. Kiiru J, Kariuki S, Goddeeris BM, Butaye P. Analysis of β-lactamase phenotypes and carriage of selected β-lactamase genes among *Escherichia coli* strains obtained from

Kenyan patients during an 18-year period. BMC Microbiol. 2012;12:155.

71. Kariuki S, Revathi G, Corkill J, Kiiru J, Mwituria J, Mirza N, *et al. Escherichia coli* from community-acquired urinary tract infections resistant to fluoroquinolones and extended-spectrum beta-lactams. J Infect Dev Ctries. 2007;1(3):257–62.
72. Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DS, Urassa WK, *et al.* High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. J Clin Microbiol. 2005;43(2):745–9.
73. Ndugulile F, Jureen R, Harthug S, Urassa W, Langeland N. Extended-spectrum beta-lactamases among Gram-negative bacteria of nosocomial origin from an intensive care unit of a tertiary health facility in Tanzania. BMC Infect Dis. 2005;5:86.
74. Mshana SE, Hain T, Domann E, Lyamuya EF, Chakraborty T, Imirzalioglu C. Predominance of *Klebsiella pneumoniae* ST14 carrying CTX-M-15 causing neonatal sepsis in Tanzania. BMC Infect Dis. 2013;13:466.
75. Mshana SE, Imirzalioglu C, Hain T, Domann E, Lyamuya EF, Chakraborty T. Multiple ST clonal complexes, with a predominance of ST131, of *Escherichia coli* harbouring $bla_{\text{CTX-M-15}}$ in a tertiary hospital in Tanzania. Clin Microbiol Infect. 2011;17(8):1279–82.
76. Mshana SE, Gerwing L, Minde M, Hain T, Domann E, Lyamuya E, *et al.* Outbreak of a novel *Enterobacter* spp. carrying $bla_{\text{CTX-M-15}}$ in a neonatal unit of a tertiary care hospital in Tanzania. Int J Antimicrob Agents. 2011;38(3):265-9.
77. Randrianirina F, Soares J-L, Carod J-F, Ratsima E, Thonnier V, Combe P, *et al.* Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in Antananarivo, Madagascar. J Antimicrob Chemother. 2007;59(2):309–12.
78. Randrianirina F, Vaillant L, Ramarokoto CE, Rakotoarijaona A, Andriamanarivo ML, Razafimahandry HC, *et al.* Antimicrobial resistance in pathogens causing nosocomial infections in surgery and intensive care wards in Antananarivo, Madagascar. J Infect Dev Ctries. 2010;4(2):074–82.
79. Naas T, Cuzon G, Robinson AL, Andrianirina Z, Imbert P, Ratsima E, *et al.* Neonatal infections with multidrug-resistant ESBL-producing *E. cloacae* and *K. pneumoniae* in Neonatal Units of two different Hospitals in Antananarivo, Madagascar. BMC Infect Dis. 2016;16(1):275.
80. Gray KJ, Wilson LK, Phiri A, Corkill JE, French N, Hart CA. Identification and characterization of ceftriaxone resistance and extended-spectrum beta-lactamases in Malawian bacteraemic *Enterobacteriaceae*. J Antimicrob Chemother. 2006;57(4):661–5.

81. Usha G, Chunderika M, Prashini M, Willem SA, Yusuf ES. Characterization of extended-spectrum beta-lactamases in *Salmonella* spp. at a tertiary hospital in Durban, South Africa. Diagn Microbiol Infect Dis. 2008;62(1):86–91.
82. Essack SY, Hall LM, Pillay DG, McFadyen ML, Livermore DM. Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum beta-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. Antimicrob Agents Chemother. 2001;45(1):88–95.
83. Kruger T, Szabo D, Keddy KH, Deeley K, Marsh JW, Hujer AM, *et al.* Infections with nontyphoidal *Salmonella* species producing TEM-63 or a novel TEM enzyme, TEM-131, in South Africa. Antimicrob Agents Chemother. 2004;48(11):4263–70.
84. Peirano G, van Greune CHJ, Pitout JDD. Characteristics of infections caused by extended-spectrum beta-lactamase-producing *Escherichia coli* from community hospitals in South Africa. Diagn Microbiol Infect Dis. 2011;69(4):449–53.
85. Tau NP, Smith AM, Sooka A, Keddy KH. Molecular characterization of extended-spectrum beta-lactamase producing *Shigella* isolates from humans in South Africa, 2003-2009. J Med Microbiol. 2012;61(1):162–4.
86. Ehlers MM, Veldsman C, Makgotlho EP, Dove MG, Hoosen AA, Kock MM, *et al.* Detection of bla_{SHV}, bla_{TEM} and bla_{CTX-M} antibiotic resistance genes in randomly selected bacterial pathogens from the Steve Biko Academic Hospital. FEMS Immunol Med Microbiol. 2009;56(3):191–6.
87. Sangare SA, Maiga AI, Guindo I, Maiga A, Camara N, Savadogo S, *et al.* Prevalence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* isolated from blood cultures in Africa. Med Mal Infect. 2015;45(9):374–82.
88. Mohamed Al-Agamy MH, El-Din Ashour MS, Wiegand I. First description of CTX-M beta-lactamase-producing clinical *Escherichia coli* isolates from Egypt. Int J Antimicrob Agents. 2006;27(6):545–8.
89. Rakotonirina HC, Garin B, Randrianirina F, Richard V, Talarmin A, Arlet G. Molecular characterization of multidrug-resistant extended-spectrum beta-lactamase-producing *Enterobacteriaceae* isolated in Antananarivo, Madagascar. BMC Microbiol. 2013;13:85.
90. Weill F-X, Perrier-Gros-Claude J-D, Demartin M, Coignard S, Grimont PAD. Characterization of extended-spectrum-beta-lactamase (CTX-M-15)-producing strains of *Salmonella enterica* isolated in France and Senegal. FEMS Microbiol Lett. 2004;238(2):353–8.
91. Gangoue-Pieboji J, Miriagou V, Vourli S, Tzelepi E, Ngassam P, Tzouvelekis LS. Emergence of CTX-M-15-producing enterobacteria in Cameroon and characterization of a $bla_{CTX-M-15}$-carrying element. Antimicrob Agents Chemother.

2005;49(1):441–3.

92. Mahrouki S, Chihi H, Bourouis A, Ayari K, Ferjani M, Moussa MB, *et al.* Nosocomial dissemination of plasmids carrying *bla*$_{TEM-24}$, *bla*$_{DHA-1}$, *aac(6')-Ib-cr*, and *qnrA6* in *Providencia* spp. strains isolated from a Tunisian hospital. Diagn Microbiol Infect Dis. 2015;81(1):50–2.
93. Mahrouki S, Perilli M, Bourouis A, Chihi H, Ferjani M, Ben Moussa M, *et al.* Prevalence of quinolone resistance determinant *qnrA6* among broad- and extended-spectrum beta-lactam-resistant *Proteus mirabilis* and *Morganella morganii* clinical isolates with *sul1*-type class 1 integron association in a Tunisian Hospital. Scand J Infect Dis. 2013;45(8):600–5.
94. Dimude JU, Amyes SGB. Molecular characterisation and diversity in *Enterobacter cloacae* from Edinburgh and Egypt carrying *bla*$_{CTX-M-14}$ and *bla*$_{VIM-4}$ beta-lactamase genes. Int J Antimicrob Agents. 2013;41(6):574–7.
95. Poirel L, Benouda A, Hays C, Nordmann P. Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco. J Antimicrob Chemother. 2011;66(12):2781–3.
96. Villa L, Poirel L, Nordmann P, Carta C, Carattoli A. Complete sequencing of an IncH plasmid carrying the *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$ and *qnrB1* genes. J Antimicrob Chemother. 2012;67(7):1645–50.
97. Réjiba S, Mercuri PS, Power P, Kechrid A. Emergence and dominance of CTX-M-15 extended spectrum beta-lactamase among *Escherichia coli* isolates from children. Microb Drug Resist. 2011;17(2):135–40.
98. Woerther PL, Angebault C, Jacquier H, Hugede HC, Janssens AC, Sayadi S, *et al.* Massive increase, spread, and exchange of extended spectrum β-lactamase-encoding genes among intestinal *Enterobacteriaceae* in hospitalized children with severe acute malnutrition in Niger. Clin Infect Dis. 2011;53(7):677–85.
99. Lahlaoui H, Poirel L, Barguellil F, Moussa MB, Nordmann P. Carbapenem-hydrolyzing class D β-lactamase OXA-48 in *Klebsiella pneumoniae* isolates from Tunisia. Eur J Clin Microbiol Infect Dis. 2012;31(6):937–9.
100. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009;22(1):161–82,
101. Sidjabat HE, Seah KY, Coleman L, Sartor A, Derrington P, Heney C, *et al.* Expansive spread of IncI1 plasmids carrying *bla*$_{CMY-2}$ amongst *Escherichia coli*. Int J Antimicrob Agents. 2014;44(3):203–8.
102. Sidjabat HE, Paterson DL, Qureshi ZA, Adams-Haduch JM, O'Keefe A, Pascual A, *et al.* Clinical features and molecular epidemiology of CMY-type beta-lactamase-producing *Escherichia coli*. Clin Infect Dis. 2009;48(6):739–44.
103. Miriagou V, Tassios P, Legakis N, Tzouvelekis LS. Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. Int J Antimicrob Agents. 2004;23(6):547–55.

104. Ribeiro TG, Novais Â, Rodrigues C, Nascimento R, Freitas F, Machado E, *et al*. Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country. J Antimicrob Chemother. 2016 (under *Editorial Revision*).

105. Empel J, Hrabak J, Kozinska A, Bergerova T, Urbaskova P, Kern-Zdanowicz I, *et al*. DHA-1-producing *Klebsiella pneumoniae* in a teaching hospital in the Czech Republic. Microb Drug Resist. 2010;16(4):291–5.

106. Illiaquer M, Caroff N, Bemer P, Aubin GG, Juvin ME, Lepelletier D, *et al*. Occurrence and molecular characterization of *Klebsiella pneumoniae* ST37 clinical isolates producing plasmid-mediated AmpC recovered over a 3-year period. Diagn Microbiol Infect Dis. 2012;74(1):95–7.

107. Diestra K, Miro E, Marti C, Navarro D, Cuquet J, Coll P, *et al*. Multiclonal epidemic of *Klebsiella pneumoniae* isolates producing DHA-1 in a Spanish hospital. Clin Microbiol Infect. 2011;17(7):1032–6.

108. Ohana S, Leflon V, Ronco E, Rottman M, Guillemot D, Lortat-Jacob S, *et al*. Spread of a *Klebsiella pneumoniae* strain producing a plasmid-mediated ACC-1 AmpC beta-lactamase in a teaching hospital admitting disabled patients. Antimicrob Agents Chemother. 2005;49(5):2095–7.

109. Roche C, Boo TW, Walsh F, Crowley B, Alvarez M, Tran J, *et al*. Detection and molecular characterisation of plasmidic AmpC beta-lactamases in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Dublin, Ireland. Clin Microbiol Infect. 2008;14(6):616–8.

110. Arena F, Giani T, Becucci E, Conte V, Zanelli G, D'Andrea MM, *et al*. Large oligoclonal outbreak due to *Klebsiella pneumoniae* ST14 and ST26 producing the FOX-7 AmpC β-lactamase in a neonatal intensive care unit. J Clin Microbiol. 2013;51(12):4067–72.

111. Migliavacca R, Nucleo E, D'Andrea MM, Spalla M, Giani T, Pagani L. Acquired AmpC type beta-lactamases: an emerging problem in Italian long-term care and rehabilitation facilities. New Microbiol. 2007;30(3):295–8.

112. D'Andrea MM, Nucleo E, Luzzaro F, Giani T, Migliavacca R, Vailati F, *et al*. CMY-16, a novel acquired AmpC-type beta-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from northern Italy. Antimicrob Agents Chemother. 2006;50(2):618–24.

113. Papanicolaou GA, Medeiros AA, Jacoby GA. Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 1990;34(11):2200–9.

114. Verdet C, Arlet G, Ben Redjeb S, Ben Hassen A, Lagrange PH, Philippon A. Characterisation of CMY-4, an AmpC-type plasmid-mediated beta-lactamase in a Tunisian clinical isolate of *Proteus mirabilis*. FEMS Microbiol Lett. 1998;169(2):235–40.

115. Iabadene H, Messai Y, Ammari H, Alouache S, Verdet C, Bakour R, *et al.* Prevalence of plasmid-mediated AmpC beta-lactamases among *Enterobacteriaceae* in Algiers hospitals. Int J Antimicrob Agents. 2009;34(4):340–2.

116. Fam D, El Said M, El Defrawy I, El Dadei E, El Attar S, Sorur A, *et al.* Prevalence of plasmid-mediated ampC genes in clinical isolates of *Enterobacteriaceae* from Cairo, Egypt. Br Microbiol Res J. 2013;3(4):525–37.

117. Mahrouki S, Bourouis A, Chihi H, Ouertani R, Ferjani M, Moussa MB, *et al.* First characterisation of plasmid-mediated quinolone resistance-*qnrS1* co-expressed *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{DHA-1}}$ genes in clinical strain of *Morganella morganii* recovered from a Tunisian Intensive Care Unit. Indian J Med Microbiol. 2012;30(4):437–41.

118. Gharout-Sait A, Touati A, Guillard T, Brasme L, de Champs C. Molecular characterization and epidemiology of cefoxitin resistance among *Enterobacteriaceae* lacking inducible chromosomal ampC genes from hospitalized and non-hospitalized patients in Algeria: description of new sequence types in *Klebsiella pneumoniae* isolates. Braz J Infect Dis . 2015;19(2):187–95.

119. Mnif B, Ktari S, Chaari A, Medhioub F, Rhimi F, Bouaziz M, *et al.* Nosocomial dissemination of *Providencia stuartii* isolates carrying *bla*$_{\text{OXA-48}}$, *bla*$_{\text{PER-1}}$, *bla*$_{\text{CMY-4}}$ and *qnrA6* in a Tunisian hospital. J Antimicrob Chemother. 2013;68(2):329–32.

120. Ktari S, Arlet G, Mnif B, Gautier V, Mahjoubi F, Ben Jmeaa M, *et al.* Emergence of multidrug-resistant *Klebsiella pneumoniae* isolates producing VIM-4 metallo-beta-lactamase, CTX-M-15 extended-spectrum beta-lactamase, and CMY-4 AmpC beta-lactamase in a Tunisian university hospital. Antimicrob Agents Chemother. 2006;50(12):4198–201.

121. Dahmen S, Mansour W, Charfi K, Boujaafar N, Arlet G, Bouallegue O. Imipenem resistance in *Klebsiella pneumoniae* is associated to the combination of plasmid-mediated CMY-4 AmpC beta-lactamase and loss of an outer membrane protein. Microb Drug Resist. 2012;18(5):479–83.

122. Armand-Lefevre L, Leflon-Guibout V, Bredin J, Barguellil F, Amor A, Pages JM, *et al.* Imipenem resistance in *Salmonella enterica* serovar Wien related to porin loss and CMY-4 beta-lactamase production. Antimicrob Agents Chemother. 2003;47(3):1165–8.

123. Nadjar D, Rouveau M, Verdet C, Donay L, Herrmann J, Lagrange PH, *et al.* Outbreak of *Klebsiella pneumoniae* producing transferable AmpC-type beta-lactamase (ACC-

1) originating from *Hafnia alvei*. FEMS Microbiol Lett. 2000;187(1):35–40.

124. Rhimi-Mahjoubi F, Bernier M, Arlet G, Jemaa Z Ben, Jouve P, Hammami A, *et al*. Identification of plasmid-encoded cephalosporinase ACC-1 among various enterobacteria (*Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella*) isolated from a Tunisian hospital (Sfax 997-2000). Pathol Biol. 2002;50(1):7–11.

125. Ktari S, Arlet G, Verdet C, Jaoua S, Kachrid A, Ben Redjeb S, *et al*. Molecular epidemiology and genetic environment of acquired bla_{ACC-1} in *Salmonella enterica* serotype Livingstone causing a large nosocomial outbreak in Tunisia. Microb Drug Resist. 2009;15(4):279–86.

126. Helmy MM, Wasfi R. Phenotypic and molecular characterization of plasmid mediated AmpC beta-lactamases among *Escherichia coli*, *Klebsiella* spp., and *Proteus mirabilis* isolated from urinary tract infections in Egyptian hospitals. Biomed Res Int. 2014;2014:171548.

127. Ogbolu DO, Daini OA, Ogunledun A, Terry Alli OA, Webber MA. Dissemination of IncF plasmids carrying beta-lactamase genes in Gram-negative bacteria from Nigerian hospitals. J Infect Dev Ctries. 2013;7(5):382–90.

128. Mocktar C, Govinden U, Sturm AW, Essack SY. CMY-20, a novel AmpC-type beta-lactamase from South African clinical *Escherichia coli* isolates. Diagn Microbiol Infect Dis. 2008;60(4):405–8.

119. Fam N, El Said M, Aboul-Fadl L, El Dabei E, El Attar S, Sorur A, Fouad S, Klena J, Gamal D. Detection of plasmid-mediated AmpC beta-lactamases in clinically significant bacterial isolates in a research institute hospital in Egypt. Life Sci J. 2013;10(2):2294–304.

130. Souna D, Amir AS, Bekhoucha SN, Berrazeg M, Drissi M. Molecular typing and characterization of TEM, SHV, CTX-M, and CMY-2 beta-lactamases in *Enterobacter cloacae* strains isolated in patients and their hospital environment in the west of Algeria. Med Mal Infect. 2014;44(4):146–52.

131. Miriagou V, Cornaglia G, Edelstein M, Galani I, Giske CG, Gniadkowski M, *et al*. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect. 2010;16:112–22.

132. Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. Clin Microbiol Rev. 2012;25(4):682–707.

133. Campos AC, Albiero J, Ecker AB, Kuroda CM, Meirelles LE, Polato A, *et al*. Outbreak of *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: A systematic review. Am J Infect Control. 2016;pii: S0196-6553(16)00280-7.

134. Pitout JD, Nordmann P, Poirel L. Carbapenemase-Producing *Klebsiella*

pneumoniae, a Key Pathogen Set for Global Nosocomial Dominance. Antimicrob Agents Chemother. 2015;59(10):5873–84.

135. Dortet L, Poirel L, Nordmann P. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. Biomed Res Int. 2014;2014:249856.
136. Cantón R, Akova M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, *et al.* Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin Microbiol Infect. 2012;18(5):413–31.
137. Pirš M, Andlovic A, Cerar T, Žohar-Čretnik T, Kobola L, Kolman J, *et al.* A case of OXA-48 carbapenemase-producing *Klebsiella pneumoniae* in a patient transferred to Slovenia from Libya, November 2011. Euro Surveill. 2011;16(50):20042.
138. Bakour S, Sahli F, Touati A, Rolain J-M. Emergence of KPC-producing *Klebsiella pneumoniae* ST512 isolated from cerebrospinal fluid of a child in Algeria. New microbes New Infect. 2015;3:34–6.
139. Robin F, Aggoune-Khinache N, Delmas J, Naim M, Bonnet R. Novel VIM metallo-beta-lactamase variant from clinical isolates of *Enterobacteriaceae* from Algeria. Antimicrob Agents Chemother. 2010;54(1):466–70.
140. Cuzon G, Bentchouala C, Vogel A, Héry M, Lezzar A, Smati F, *et al.* First outbreak of OXA-48-positive carbapenem-resistant *Klebsiella pneumoniae* isolates in Constantine, Algeria. Int J Antimicrob Agents. 2015;46(6):725–7.
141. Agabou A, Pantel A, Ouchenane Z, Lezzar N, Khemissi S, Satta D, *et al.* First description of OXA-48-producing *Escherichia coli* and the pandemic clone ST131 from patients hospitalised at a military hospital in Algeria. Eur J Clin Microbiol Infect Dis. 2014;33(9):1641–6.
142. Sassi A, Loucif L, Gupta SK, Dekhil M, Chettibi H, Rolain JM. NDM-5 carbapenemase-encoding gene in multidrug-resistant clinical isolates of *Escherichia coli* from Algeria. Antimicrob Agents Chemother. 2014;58(9):5606–8.
143. Metwally L, Gomaa N, Attallah M, Kamel N. High prevalence of *Klebsiella pneumoniae* carbapenemase-mediated resistance in *K. pneumoniae* isolates from Egypt. East Mediterr Heal J. 2013;19(11):947–52.
144. Poirel L, Abdelaziz MO, Bernabeu S, Nordmann P. Occurrence of OXA-48 and VIM-1 carbapenemase-producing *Enterobacteriaceae* in Egypt. Int J Antimicrob Agents. 2013;41(1):90–1.
145. Gamal D, Fernández-Martínez M, El-Defrawy I, Ocampo-Sosa AA, Martínez-Martínez L. First identification of NDM-5 associated with OXA-181 in *Escherichia coli* from Egypt. Emerg Microbes Infect. 2016;5:e30.
146. Soliman AM, Khalifa HO, Ahmed AM, Shimamoto T, Shimamoto T. Emergence of an NDM-5-producing clinical *Escherichia coli* isolate in Egypt. Int J Infect Dis.

2016;48:46–8.

147. Abdelaziz MO, Bonura C, Aleo A, Fasciana T, Mammina C. NDM-1- and OXA-163-producing *Klebsiella pneumoniae* isolates in Cairo, Egypt, 2012. J Glob Antimicrob Resist. 2013;1(4):213–5.
148. Abdelaziz MO, Bonura C, Aleo A, El-Domany RA, Fasciana T, Mammina C. OXA-163-producing *Klebsiella pneumoniae* in Cairo, Egypt, in 2009 and 2010. J Clin Microbiol. 2012;50(7):2489–91.
149. Lafeuille E, Decré D, Mahjoub-Messai F, Bidet P, Arlet G, Bingen E. OXA-48 carbapenemase-producing *Klebsiella pneumoniae* isolated from Libyan patients. Microb Drug Resist. 2013;19(6):491–7.
150. Hammerum A, Larsen A, Hansen F, Justesen U, Friis-Møller A, Lemming L, *et al*. Patients transferred from Libya to Denmark carried OXA-48-producing *Klebsiella pneumoniae*, NDM-1-producing *Acinetobacter baumannii* and meticillin-resistant *Staphylococcus aureus*. Int J Antimicrob Agents. 2012;40(2):191–2.
151. Kocsis E, Savio C, Piccoli M, Cornaglia G, Mazzariol A. *Klebsiella pneumoniae* harbouring OXA-48 carbapenemase in a Libyan refugee in Italy. Clin Microbiol Infect. 2013;19(9):E409–11.
152. Barguigua A, El Otmani F, Lakbakbi El Yaagoubi F, Talmi M, Zerouali K, Timinouni M. First report of a *Klebsiella pneumoniae* strain coproducing NDM-1, VIM-1 and OXA-48 carbapenemases isolated in Morocco. APMIS. 2013;121(7):675–7.
153. Barguigua A, Otmani F El, Talmi M, Zerouali K, Timinouni M, Barguigua A, *et al*. Emergence of carbapenem-resistant *Enterobacteriaceae* isolates in the Moroccan community. Diagn Microbiol Infect Dis. 2011;73(3):290–1.
154. Poirel L, Ros A, Carrër A, Fortineau N, Carricajo A, Berthelot P, *et al*. Cross-border transmission of OXA-48-producing *Enterobacter cloacae* from Morocco to France. J Antimicrob Chemother. 2011;66(5):1181–2.
155. Grami R, Mansour W, Ben Haj Khalifa A, Dahmen S, Chatre P, Haenni M, *et al*. Emergence of ST147 *Klebsiella pneumoniae* Producing OXA-204 Carbapenemase in a University Hospital, Tunisia. Microb Drug Resist. 2016;22(2):137–40.
156. Charfi K, Mansour W, Khalifa A, Mastouri M, Aouni M, Mammeri H. Emergence of OXA-204 β-lactamase in Tunisia. Diagn Microbiol Inect Dis. 2015;82(4):314–7.
157. Ben Nasr A, Decré D, Compain F, Genel N, Barguellil F, Arlet G. Emergence of NDM-1 in association with OXA-48 in *Klebsiella pneumoniae* from Tunisia. Antimicrob Agents Chemother. 2013;57(8):4089–90.
158. Ogbolu DO, Webber MA. High-level and novel mechanisms of carbapenem resistance in Gram-negative bacteria from tertiary hospitals in Nigeria. Int J Antimicrob Agents. 2014;43(5):412–7.

159. Leski TA, Bangura U, Jimmy DH, Ansumana R, Lizewski SE, Li RW, *et al.* Identification of *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{DIM-1}}$, and *bla*$_{\text{VIM}}$ carbapenemase genes in hospital *Enterobacteriaceae* isolates from Sierra Leone. J Clin Microbiol. 2013;51(7):2435–8.
160. Patel G, Bonomo RA. Status report on carbapenemases: challenges and prospects. Expert Rev Anti Infect Ther. 2011;9(5):555–70.
161. Dortet L, Poirel L, Anguel N, Nordmann P. New Delhi metallo-β-lactamase 4-producing *Escherichia coli* in Cameroon. Emerg Infect Dis. 2012;18(9):1540–2.
162. Mushi MF, Mshana SE, Imirzalioglu C, Bwanga F. Carbapenemase genes among multidrug resistant gram negative clinical isolates from a tertiary hospital in Mwanza, Tanzania. Biomed Res Int. 2014;2014:303104.
163. Okoche D, Asiimwe BB, Katabazi FA, Kato L, Najjuka CF. Prevalence and Characterization of Carbapenem-Resistant *Enterobacteriaceae* Isolated from Mulago National Referral Hospital, Uganda. PLoS One. 2015;10(8):e0135745.
164. Eshetie S, Unakal C, Gelaw A, Ayelign B, Endris M, Moges F. Multidrug resistant and carbapenemase producing *Enterobacteriaceae* among patients with urinary tract infection at referral Hospital, Northwest Ethiopia. Antimicrob Resist Infect Control. 2015;4:12.
165. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, *et al.* Characterization of a new metallo-beta-lactamase gene, *bla*$_{\text{(NDM-1)}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother. 2009;53(12):5046–54.
166. Poirel L, Revathi G, Bernabeu S, Nordmann P. Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. Antimicrob Agents Chemother. 2011;55(2):934–6.
167. Brink AJ, Coetzee J, Clay CG, Sithole S, Richards GA, Poirel L, *et al.* Emergence of New Delhi metallo-beta-lactamase (NDM-1) and *Klebsiella pneumoniae* carbapenemase (KPC-2) in South Africa. J Clin Microbiol. 2012;50(2):525–7.
168. Peirano G, Moolman J, Pitondo-Silva A, Pitout JD. The characteristics of VIM-1-producing *Klebsiella pneumoniae* from South Africa. Scand J Infect Dis. 2012;44(1):74–8.
169. Lowman W, Sriruttan C, Nana T, Bosman N, Duse A, Venturas J, *et al.* NDM-1 has arrived: first report of a carbapenem resistance mechanism in South Africa. S Afr Med J. 2011;101(12):873–5.
170. Govind CN, Moodley K, Peer AK, Pillay N, Maske C, Wallis C, *et al.* NDM-1 imported from India-first reported case in South Africa. S Afr Med J. 2013;103(7):476–8.
171. Brink AJ, Coetzee J, Corcoran C, Clay CG, Hari-Makkan D, Jacobson RK, *et al.* Emergence of OXA-48 and OXA-181 carbapenemases among *Enterobacteriaceae* in

South Africa and evidence of in vivo selection of colistin resistance as a consequence of selective decontamination of the gastrointestinal tract. J Clin Microbiol. 2013;51(1):369–72.

172. Ampaire LM, Katawera V, Nyehangane D, Boum Y, Bazira J. Epidemiology of Carbapenem Resistance among Multidrug Resistant *Enterobacteriaceae* in Uganda. Br Microbiol Res J. 2015;8(2):418–23.
173. Mellouk FZ, Bakour S, Meradji S, Al-Bayssari C, Bentakouk MC, Zouyed F, *et al.* First Detection of VIM-4-Producing *Pseudomonas aeruginosa* and OXA-48-Producing *Klebsiella pneumoniae* in Northeastern (Annaba, Skikda) Algeria. Microb Drug Resist. 2016 [Epub ahead of print].
174. Gamal D, Fernández-Martínez M, Salem D, El-Defrawy I, Lvarez Montes L, Ocampo-Sosa AA, *et al.* Carbapenem-resistant *Klebsiella pneumoniae* isolates from Egypt containing $bla_{\text{NDM-1}}$ on IncR plasmids and its association with rmtF. Int J Infect Dis. 2016;43:17–20.
175. Brolund A, Sandegren L. Characterization of ESBL disseminating plasmids. Infect Dis. 2016;48(1):18–25.
176. Mathers AJ, Peirano G, Pitout JD. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant *Enterobacteriaceae*. Clin Microbiol Rev. 2015;28(3):565–91.
177. Huijbers PM, Graat EA, Haenen AP, van Santen MG, van Essen-Zandbergen A, Mevius DJ, *et al.* Extended-spectrum and AmpC beta-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors and molecular characteristics. J Antimicrob Chemother. 2014;69(10):2669–75.
178. Woodford N, Wareham DW, Guerra B, Teale C. Carbapenemase-producing *Enterobacteriaceae* and non-*Enterobacteriaceae* from animals and the environment: an emerging public health risk of our own making? J Antimicrob Chemother. 2014;69(2):287–91.
179. Lahlaoui H, De Luca F, Maradel S, Ben-Haj-Khalifa A, Ben Hamouda H, Kheder M, *et al.* Occurrence of conjugative IncF-type plasmids harboring the $bla_{\text{CTX-M-15}}$ gene in *Enterobacteriaceae* isolates from newborns in Tunisia. Pediatr Res. 2015;77(1-1):107–10.
180. Novais Â, Pires J, Ferreira H, Costa L, Montenegro C, Vuotto C, *et al.* Characterization of globally spread *Escherichia coli* ST131 isolates (1991 to 2010). Antimicrob Agents Chemother. 2012;56(7):3973–6.
181. Oteo J, Cercenado E, Fernández-Romero S, Saéz D, Padilla B, Zamora E, *et al.* Extended-spectrum-β-lactamase-producing *Escherichia coli* as a cause of pediatric

infections: report of a neonatal intensive care unit outbreak due to a CTX-M-14-producing strain. Antimicrob Agents Chemother. 2012;56(1):54–8.

182. Harada K, Nakai Y, Kataoka Y. Mechanisms of resistance to cephalosporin and emergence of O25b-ST131 clone harboring CTX-M-27 beta-lactamase in extraintestinal pathogenic *Escherichia coli* from dogs and cats in Japan. Microbiol Immunol. 2012;56(7):480–5. A
183. Pires J, Taracila M, Bethel CR, Doi Y, Kasraian S, Tinguely R, *et al.* In Vivo Evolution of CMY-2 to CMY-33 beta-Lactamase in *Escherichia coli* Sequence Type 131: Characterization of an Acquired Extended-Spectrum AmpC Conferring Resistance to Cefepime. Antimicrob Agents Chemother. 2015;59(12):7483–8.
184. Naseer U, Haldorsen B, Simonsen GS, Sundsfjord A. Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. Clin Microbiol Infect. 2010;16(2):171–8.
185. Piazza A, Caltagirone M, Bitar I, Nucleo E, Spalla M, Fogato E, *et al.* Emergence of *Escherichia coli* Sequence Type 131 (ST131) and ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a Long-Term Care and Rehabilitation Facility (LTCRF) in Northern Italy. Adv Exp Med Biol. 2016;901:77-89.
186. Wang LH, Liu PP, Wei DD, Liu Y, Wan LG, Xiang TX, *et al.* Clinical isolates of uropathogenic *Escherichia coli* ST131 producing NDM-7 metallo-beta-lactamase in China. Int J Antimicrob Agents. 2016;48(1):41-5.
187. Cantón R, Gonzalez-Alba JM, Galán JC. CTX-M Enzymes: Origin and Diffusion. Front Microbiol. 2012;3:110.
188. Muller A, Stephan R, Nuesch-Inderbinen M. Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. *Sci Total Env*. 2016;541:667–72.
189. Alghoribi MF, Gibreel TM, Farnham G, Al Johani SM, Balkhy HH, Upton M. Antibiotic-resistant ST38, ST131 and ST405 strains are the leading uropathogenic *Escherichia coli* clones in Riyadh, Saudi Arabia. J Antimicrob Chemother. 2015;70(10):2757–62.
190. Ribeiro TG, Novais Â, Peixe L, Machado E. Atypical epidemiology of CTX-M-15 among *Enterobacteriaceae* from a high diversity of non-clinical niches in Angola. J Antimicrob Chemother. 2016;71(5):1169–73.
191. Tian GB, Rivera JI, Park YS, Johnson LE, Hingwe A, Adams-Haduch JM, *et al.* Sequence type ST405 *Escherichia coli* isolate producing QepA1, CTX-M-15, and RmtB from Detroit, Michigan. Antimicrob Agents Chemother. 2011;55(8):3966–7.
192. Mo SS, Slettemeas JS, Berg ES, Norstrom M, Sunde M. Plasmid and Host Strain Characteristics of *Escherichia coli* Resistant to Extended-Spectrum Cephalosporins

in the Norwegian Broiler Production. PLoS One. 2016;11(4):e0154019.

193. Czobor I, Novais Â, Rodrigues C, Chifiriuc M, Mihăescu G, Lazăr V, *et al*. Efficient transmission of IncFIIY and IncL plasmids and *Klebsiella pneumoniae* ST101 clone producing OXA-48, NDM-1 or OXA-181 in Bucharest hospitals. Int J Antimicrob Agents. 2016;pii: S0924-8579(16)30125-X.
194. Del Franco M, Paone L, Novati R, Giacomazzi CG, Bagattini M, Galotto C, *et al*. Molecular epidemiology of carbapenem resistant Enterobacteriaceae in Valle d'Aosta region, Italy, shows the emergence of KPC-2 producing *Klebsiella pneumoniae* clonal complex 101 (ST101 and ST1789). BMC Microbiol. 2015;15(1):260.
195. Melegh S, Schneider G, Horváth M, Jakab F, Emődy L, Tigyi Z. Identification and characterization of CTX-M-15 producing *Klebsiella pneumoniae* clone ST101 in a Hungarian university teaching hospital. Acta Microbiol Immunol Hung. 2015;62(3):233–45.
196. Nematzadeh S, Shahcheraghi F, Iversen A, Giske C. Successful international clones of $bla_{\text{CTX-M-15}}$-producing *Klebsiella pneumoniae* with coexpression of plasmid-mediated quinolone resistance (PMQR) determinants in Tehran hospitals. Diagn Microbiol Infect Dis. 2015;83(4):371–4.
197. Izdebski R, Bojarska K, Baraniak A, Literacka E, Herda M, Żabicka D, *et al*. NDM-1- or OXA-48-producing *Enterobacteriaceae* colonising Polish tourists following a terrorist attack in Tunis, March 2015. Euro Surveill. 2015;20(23). pii: 21150.
198. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev. 2011;35(5):736–55.
199. Sennati S, Santella G, Di Conza J, Pallecchi L, Pino M, Ghiglione B, *et al*. Changing epidemiology of extended-spectrum β-lactamases in Argentina: emergence of CTX-M-15. Antimicrob Agents Chemother. 2012;56(11):6003–5.
200. Bowers JR, Kitchel B, Driebe EM, MacCannell DR, Roe C, Lemmer D, *et al*. Genomic Analysis of the Emergence and Rapid Global Dissemination of the Clonal Group 258 *Klebsiella pneumoniae* Pandemic. PLoS One. 2015;10(7):e0133727.
201. Bruchmann S, Muthukumarasamy U, Pohl S, Preusse M, Bielecka A, Nicolai T, *et al*. Deep transcriptome profiling of clinical *Klebsiella pneumoniae* isolates reveals strain and sequence type-specific adaptation. Environ Microbiol. 2015;17(11):4690–710.
202. Hawkey PM, Gill IS, Raiser M, Bowen J, Rodrigue JP, Wellington EM, *et al*. Multidrug-resistant Gram-negative bacteria: a product of globalization. J Hosp Infect. 2015;89(4):241–7.
203. Carattoli A. Resistance plasmid families in *Enterobacteriaceae*. Antimicrob Agents Chemother. 2009;53(6):2227–38.

204. Carattoli A, Seiffert SN, Schwendener S, Perreten V, Endimiani A. Differentiation of IncL and IncM Plasmids Associated with the Spread of Clinically Relevant Antimicrobial Resistance. PLoS One. 2015;10(5):e0123063.
205. Hopkins KL, Liebana E, Villa L, Batchelor M, Threlfall EJ, Carattoli A. Replicon typing of plasmids carrying CTX-M or CMY beta-lactamases circulating among *Salmonella* and *Escherichia coli* isolates. Antimicrob Agents Chemother. 2006;50(9):3203–6.
206. Ballén V, Sáez E, Benmessaoud R, Houssain T, Alami H, Barkat A, *et al.* First report of a *Klebsiella pneumoniae* ST466 strain causing neonatal sepsis harbouring the $bla_{\text{CTX-M-15}}$ gene in Rabat, Morocco. FEMS Microbiol Lett. 2015;362(1):1–4.
207. Chouchani C, El Salabi A, Marrakchi R, Ferchichi L, Walsh TR. Characterization of IncA/C conjugative plasmid harboring $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-15}}$ extended-spectrum β-lactamases in clinical isolates of *Escherichia coli* in Tunisia. Eur J Clin Microbiol Infect Dis. 2012;31(6):1081–7.
208. Potron A, Nordmann P, Rondinaud E, Jaureguy F, Poirel L. A mosaic transposon encoding OXA-48 and CTX-M-15: towards pan-resistance. J Antimicrob Chemother. 2013;68(2):476–7.
209. Cuzon G, Ouanich J, Gondret R, Naas T, Nordmann P. Outbreak of OXA-48-Positive Carbapenem-Resistant *Klebsiella pneumoniae* Isolates in France. Antimicrob Agents Chemother. 2011;55(5):2420–3.
210. Pitart C, Sole M, Roca I, Fabrega A, Vila J, Marco F. First Outbreak of a Plasmid-Mediated Carbapenem-Hydrolyzing OXA-48 -Lactamase in *Klebsiella pneumoniae* in Spain. Antimicrob Agents Chemother. 2011;55(9):4398–401.
211. Rodriguez-Martinez J-M, Nordmann P, Fortineau N, Poirel L. VIM-19, a metallo-beta-lactamase with increased carbapenemase activity from *Escherichia coli* and *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2010;54(1):471–6.
212. Guerra B, Fischer J, Helmuth R. An emerging public health problem: acquired carbapenemase-producing microorganisms are present in food-producing animals, their environment, companion animals and wild birds. Vet Microbiol. 2014;171(3-4):290–7.
213. Antunes P, Coque TM, Peixe L. Emergence of an IncIγ plasmid encoding CMY-2 β-lactamase associated with the international ST19 OXA-30-producing ss-lactamase *Salmonella* Typhimurium multidrug-resistant clone. J Antimicrob Chemother. 2010;65(10):2097–100.
214. Carattoli A, Garcia-Fernandez A, Varesi P, Fortini D, Gerardi S, Penni A, *et al.* Molecular epidemiology of *Escherichia coli* producing extended-spectrum beta-lactamases isolated in Rome, Italy. J Clin Microbiol. 2008;46(1):103–8.

215. Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos AP, Mevius D, *et al.* Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin Infect Dis. 2013;56(7):1030–7.
216. Li S, Zhu ZC, Wang L, Zhou YF, Tang YJ, Miao ZM. Prevalence and characterization of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in spring waters. Lett Appl Microbiol. 2015;61(6):544–8.
217. Zurfluh K, Nuesch-Inderbinen M, Morach M, Zihler Berner A, Hachler H, Stephan R. Extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* isolated from vegetables imported from the Dominican Republic, India, Thailand, and Vietnam. Appl Env Microbiol. 2015;81(9):3115–20.
218. Woerther PL, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev. 2013;26(4):744–58.
219. Maamar E, Hammami S, Alonso CA, Dakhli N, Abbassi MS, Ferjani S, *et al.* High prevalence of extended-spectrum and plasmidic AmpC beta-lactamase-producing *Escherichia coli* from poultry in Tunisia. Int J Food Microbiol. 2016;231:69–75.
220. Ben Said L, Jouini A, Alonso CA, Klibi N, Dziri R, Boudabous A, *et al.* Characteristics of extended-spectrum beta-lactamase (ESBL)- and pAmpC beta-lactamase-producing *Enterobacteriaceae* of water samples in Tunisia. Sci Total Env. 2016;550:1103–9.
221. Seiffert SN, Hilty M, Perreten V, Endimiani A. Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: an emerging problem for human health? Drug Resist Updat. 2013;16(1-2):22–45.
222. Antunes P, Mourão J, Campos J, Peixe L. Salmonellosis: the role of poultry meat. Clin Microbiol Infect. 2016;22(2):110–21.
223. Ben Sallem R, Ben Slama K, Saenz Y, Rojo-Bezares B, Estepa V, Jouini A, *et al.* Prevalence and characterization of extended-spectrum beta-lactamase (ESBL)- and CMY-2-producing *Escherichia coli* isolates from healthy food-producing animals in Tunisia. Foodborne Pathog Dis. 2012;9(12):1137–42.
224. Ben Sallem R, Ben Slama K, Rojo-Bezares B, Porres-Osante N, Jouini A, Klibi N, *et al.* IncI1 plasmids carrying $bla_{(CTX\text{-}M\text{-}1)}$ or $bla_{(CMY\text{-}2)}$ genes in *Escherichia coli* from healthy humans and animals in Tunisia. Microb Drug Resist. 2014;20(5):495–500.
225. Ben Sallem R, Gharsa H, Slama K Ben, Rojo-Bezares B, Estepa V, Porres-Osante N, *et al.* First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. Vector Borne Zoonotic Dis.

2013;13(2):98–102.

226. Chouchani C, Marrakchi R, Henriques I, Correia A. Occurrence of IMP-8, IMP-10, and IMP-13 metallo-β-lactamases located on class 1 integrons and other extended-spectrum β-lactamases in bacterial isolates from Tunisian rivers. Scand J Infect Dis. 2013;45(2):95–103.
227. Yousfi M, Touati A, Mairi A, Brasme L, Gharout-Sait A, Guillard T, *et al*. Emergence of Carbapenemase-Producing *Escherichia coli* Isolated from Companion Animals in Algeria. Microb Drug Resist. 2016; 22(4):342-6.
228. Potron A, Poirel L, Bussy F, Nordmann P. Occurrence of the carbapenem-hydrolyzing beta-lactamase gene $bla_{\text{OXA-48}}$ in the environment in Morocco. Antimicrob Agents Chemother. 2011;55(11):5413–4.

1.3. Mini Review Article

Improvements on *Citrobacter* species differentiation, a key factor for understanding their clinical significance and the diversification of intrinsic antibiotic resistance genes

Improvements on *Citrobacter* species differentiation, a key factor for understanding their clinical significance and the diversification of intrinsic antibiotic resistance genes

T. G. Ribeiro[1], Â. Novais[1], E. Machado[1,2], L. Peixe[1*]

[1]UCIBIO@REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

***Corresponding author:** Luísa Vieira Peixe, UCIBIO@REQUIMTE, Laboratory of Microbiology, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal; Tel: (+351) 220 428 580; E-mail: lpeixe@ff.up.pt

Running title: Differentiation and intrinsic antibiotic resistance genes within *Citrobacter*.

Abstract

The aim of this review is to provide a comprehensive update on the classification and identification of *Citrobacter* species. *Citrobacter* spp. include Gram-negative rod-shaped bacteria that are oxidase-negative, non-spore-forming, and facultative anaerobic. They have been isolated from a wide range of habitats, including clinical settings associated with several human infections. However, *Citrobacter* strains are often misidentified, mainly due to difficulties on the characterization and classification of *Citrobacter* species, and hence their prevalence and clinical significance might be underestimated. Several methods have been investigated for differentiation of *Citrobacter* spp. species, but just recently partial DNA sequencing of a single gene (*recN*) and analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry have been proposed as simple methods for reliable discrimination within *Citrobacter* species. Accurate identification of etiological agents of human infections is essential for prevention and control of infectious diseases, as well as the knowledge of features of some intrinsic antibiotic resistance, which have been mobilized to non-natural hosts and are currently compromising the therapy of infectious diseases.

Keywords: Identification, phylogeny, clinical significance, *qnrB*, ampC

Introduction

Members of the genus *Citrobacter* (Kingdom *Bacteria*; Phylum *Proteobacteria*; Class *Gammaproteobacteria*; Order *Enterobacteriales*; Family *Enterobacteriaceae)* include rod-shaped Gram-negative, oxidase-negative, facultative anaerobic and non-spore-forming bacteria, which are phenotypically and genotypically heterogeneous (1,2). This genus was discovered by Werkmann and Gillen in 1932 (1), and currently comprises twelve species recognized by the International Committee on Systematic Bacteriology: *Citrobacter freundii, Citrobacter amalonaticus, Citrobacter braakii, Citrobacter farmeri, Citrobacter gillenii, Citrobacter koseri* (formerly *Citrobacter diversus*), *Citrobacter murliniae, Citrobacter pasteurii, Citrobacter rodentium, Citrobacter sedlakii, Citrobacter youngae*, and *Citrobacter werkmanii* (2–4).

Citrobacter spp. include ubiquitous bacteria that could be found in different environments. They are considered inhabitants of human and other animal guts and can be found in sewage, water, soil, and food, although they also constitute important agents of opportunistic infections in humans (2,4,5). In fact, *Citrobacter* spp. commonly take advantage of immunocompromised hosts, colonizing and causing a variety of opportunistic infections, including urinary tract infections (mostly), wound infections, pneumonia, septicemia, meningitis, endocarditis, skin and soft tissue infections, intra-abdominal infections and surgical site infections (4,6–11). The *Citrobacter* species most commonly associated with human infections are *C. koseri* and *C. freundii*, although *C. youngae*, *C. braakii* and *C. amalonaticus* were also reported (9,12–15). No statistically significant association between the site of infection and particular *Citrobacter* species was observed, however recent studies showed that neonatal central nervous system infection (including meningitis and brain abscesses) was attributed to *C. koseri* (11,16).

Accurate identification of etiological agents of human infections is essential for pathogenicity features and reservoirs assignments and to depict transmission routes of particular species, essential keys for infection diseases prevention and control measures. The clinical relevance of *Citrobacter* spp. imposes an accurate identification at species level. However, *Citrobacter* species are still difficult to identify and discriminate by conventional methods, and for this reason its prevalence and clinical significance for humans might be even higher than it is currently known (15,17). Moreover, intrinsic antibiotic resistance features are commonly species specific in different genus (e.g. *Acinetobacter* spp.) (18) and their knowledge would be important to guide therapy and to develop new antibiotics targeting relevant species.

In this review, it will be provide an updated overview on the main features and recent methodologies used for *Citrobacter* species discrimination, as well as their impact on reclassification and recognition of new species within this genus. Moreover, intrinsic genes

conferring resistance to extended-spectrum cephalosporins or reduced susceptibility to fluoroquinolones within *Citrobacter* spp. will be also addressed.

Methodological approaches to discriminate *Citrobacter* spp.

Several methodological approaches, since the conventional phenotypic tests (based on morphological, physiological and biochemical characteristics) to the recently and more sophisticated phenotyping systems [e.g. the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS)] have been used along the years for microbial species definition, including within *Citrobacter* spp. (3,19). Nevertheless, huge achievements in the identification and discrimination of microorganisms occurred after the introduction of genotypic approaches in the 1960's, including the DNA-DNA hybridization (DDH), phylogenetic analysis based on single or multilocus sequence analysis (MLSA) of housekeeping genes (16S rRNA gene mandatory), and data analysis [e.g. average nucleotide identity (ANI)] of whole genome sequence (WGS) (20,21). Currently, species are defined by both phenotypic and genotypic characteristics, and are represented by type strains deposited in two international culture collections (22). Relevant phenotypic or genotypic characteristics that can be used to discriminate *Citrobacter* species are further detailed.

1. Biochemical characteristics

In 1993, Brenner *et al.* (3) observed that *Citrobacter* species could be differentiated on the basis of a combination of several conventional biochemical and carbon source utilization tests. Nevertheless, problems in the identification based on the biochemical tests routinely used were early recognized, even with a small set of strains tested (23). The main differential biochemical characteristics of *Citrobacter* species are briefly summarized below (3,4).

C. freundii: positive for citrate and negative for indole, arginine dihydrolase and ornithine decarboxylase; acid production from sucrose, melibiose and inositol, but not from dulcitol and salicin; ability to utilize *m*-coumarate, *myo*-inositol, lactulose, 1-O-CH_3-α-galactoside, sucrose, but not dulcitol as sole carbon sources.

C. koseri: inability to grow in culture medium containing potassium cyanide; indole and citrate positive; ability to utilize malonate; acid production from D-adonitol, inositol, D-arabitol, α-methyl-D-glucoside.

C. amalonaticus: indole, citrate and ornithine decarboxylase positive and positive or delayed positive for arginine dihydrolase; acid production from 3-O-CH_3-D-glucose and protocatechuate but not from *m*-coumarate and 1-O-CH_3-α-galactoside.

C. farmeri: indole and citrate positive; produces arginine dihydrolase and ornithine decarboxylase; acid production from α-CH_3-D-glucoside, melibiose, raffinose, sucrose, and

esculin; ability to utilize benzoate, 4-hydroxybenzoate, maltitol, D-melibiose, 1-O-CH_3-α-galactoside, palatinose, protocatechuate, D-raffinose, and sucrose, but not from *m*-coumarate, dulcitol, and malonate as sole carbon sources.

C. youngae: indole negative, delayed positive for citrate and arginine dihydrolase, and negative for ornithine decarboxylase; acid production from dulcitol but not from melibiose; ability to utilize dulcitol, 3-phenylpropionate, and L-sorbose but not gentisate, 3-hydroxybenzoate, malonate, D-melibiose, 1-O-CH_3-α-galactoside, 3-O-CH_3-D-glucose, and tricarballylate as sole carbon sources.

C. braakii: production of indole is variable; positive or delayed positive for citrate, arginine dihydrolase, and ornithine decarboxylase; acid production from melibiose but not from sucrose; ability to utilize *m*-coumarate, 1-O-CH_3-α-galactoside, 3-phenylpropionate, and L-tyrosine (delayed) but not malonate and L-sorbose as sole carbon sources.

C. werkmanii: negative for indole production and ornithine decarboxylase; positive for citrate, malonate and arginine dihydrolase; lack of acid production from dulcitol, melibiose, sucrose, and 5-ketogluconate; ability to utilize *m*-coumarate, D-lyxose, malonate, 3-phenylpropionate, L-sorbose, and D-tartrate but not dulcitol, 4-hydroxybenzoate, D-melibiose, and 1-O-CH_3-α-galactoside as sole carbon sources.

C. sedlakii: positive for indole production, arginine dihydrolase, and ornithine decarboxylase; delayed citrate positive; production of acid from dulcitol and melibiose but not from sucrose and L-sorbose; ability to utilize benzoate, dulcitol, 4-hydroxybenzoate, *myo*-inositol, lactulose, malonate, 1-O-CH_3-α-galactoside, and protocatechuate but not 5-ketogluconate and L-sorbose as sole carbon sources.

C. rodentium: negative for indole production, citrate, and arginine dihydrolase; ornithine decarboxylase positive; lack of motility; utilization of malonate; inability to produce acid from melibiose and sucrose; ability to utilize malonate but not *m*-coumarate, glycerol, *5* - ketogluconate, D-melibiose, 1-O-CH_3-α-galactoside, and 3-O-CH_3-D-glucose as sole carbon sources.

C. gillenii: negative for indole production and ornithine decarboxylase; positive for citrate and delayed positive for arginine dihydrolase; utilization of malonate; negative urease reaction; acid production from inositol, arbutin, salicin and meliobiose but not from L-sorbose; inability to utilize gentisate, 3-hydroxybenzoate, 3-O-CH_3-D-glucose, L-sorbose, and tricarballylate as sole carbon sources.

C. murliniae: indole and citrate positive; catalase negative; delayed positive for arginine dihydrolase; ornithine decarboxylase negative; acid production from L-sorbose, dulcitol, arbutin and salicin; ability to utilize dulcitol, D-lyxose, I-O-CH_3-α-galactoside (delayed), and L-tyrosine but not malonate and protocatechuate as sole carbon sources.

C. pasteurii: negative for indole production, catalase negative and ornithine decarboxylase negative; citrate positive; acid production from L-sorbose, esculin, starch and 5-ketogluconate.

It is important to highlight that *Citrobacter* spp. differentiation based on these biochemical features is often problematic, with frequent variability in the results for a range of these biochemical tests within each species.

2. MALDI-TOF MS

MALDI-TOF MS is an accurate, fast, robust and sensitive methodology that relies in the reproducible detection of large molecules, which can be used for microbial identification and typing. This methodology relies in the comparison of experimental mass spectra obtained with a library of known reference strains or analyzing spectra for identification of species-specific biomarkers, offering an adequate alternative to genomic-based approaches (19,24,25).

MALDI-TOF MS has already been used for the identification of *Citrobater* spp., however most of the isolates were identified as *C. freundii* complex (26,27). Concerning the identification of species-specific biomarkers in *Citrobacter* spp., and to the best of our knowledge, only one study identified biomarkers common for all *Citrobacter* species (50S ribosomal proteins) and species-specific biomarkers, yet the last ones were not indicated (19).

3. DNA–DNA hybridization (DDH)

In 1965, Britten and Kohne developed the DDH technique that would become the tool to overcome many of the problems in bacterial taxonomy (28). Basically, this technique measures the degree of genetic similarity between two different species (29). Using DDH, a phylogenetically definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness (30). This methodology, until recently recognized to be the genotypic 'gold standard' for the delineation of prokaryotic species, has been also used in most of *Citrobacter* species definition (3). However, major disadvantages are implementation difficulties of DDH experiments and the high experimental error associated, as well as the impossibility of establishing a central database (31,32).

4. Phylogenetic analysis based on single housekeeping genes

a) 16S rRNA gene

Phylogenetic analysis of nucleotide sequences of *rrs* coding for the 16S ribosomal RNA (16S rRNA) was shown to distinguish relatively well *Citrobacter* from other related genera

(19). However, it failed to decipher relationships at *Citrobacter* species level, as a result of its conserved nature (4,19). In fact, phylogenetic relationships based on 16S rRNA sequences distinguished 3 *Citrobacter* groups: group I (*C. pasteurii, C. freundii, C. youngae, C. braakii, C. werkmanii, C. gillenii* and *C. murliniae*); group II (*C. amaloniticus, C. farmeri, C. sedlakii* and *C. rodentium*); and group III (*C. koseri*) (4,33).

b) *recN* gene versus other phylogenetic markers

Recently, Ribeiro and colleagues proposed the amplification of a single phylogenetic marker to discriminate species within *Citrobacter* genus (34). Individual phylogenetic analysis of *rpoB* (β subunit of RNA polymerase gene), *pyrG* (CTP synthetase gene), *fusA* (protein synthesis elongation factor-G gene), *leuS* (leucine tRNA synthetase gene) and *recN* (DNA repair protein gene), revealed that *leuS* and *recN* gene sequences provided an accurate discrimination for the currently recognized *Citrobacter* species (34).

In fact, phylogenetic analysis of *leuS* gene allowed the delineation of 12 distinct clusters, each one supported by a type strain of each *Citrobacter* species. The clusters were defined with a cutoff value of <97.5%, supported by bootstrap values >92%. It was also recognized that *recN* provided a greater resolution than *leuS* (cutoff values of <96.1%, statistically supported by bootstrap values >94%), with *recN* tree topology being overall congruent with that obtained for *leuS* sequences. However, 3 new clusters were observed besides the 12 depicted from the *leuS* tree topology, namely, *Citrobacter* sp. I*, *Citrobacter* sp. II* and *Citrobacter* sp. III*, which might correspond to isolates from novel species, not yet confirmed (Figure 1) (34).

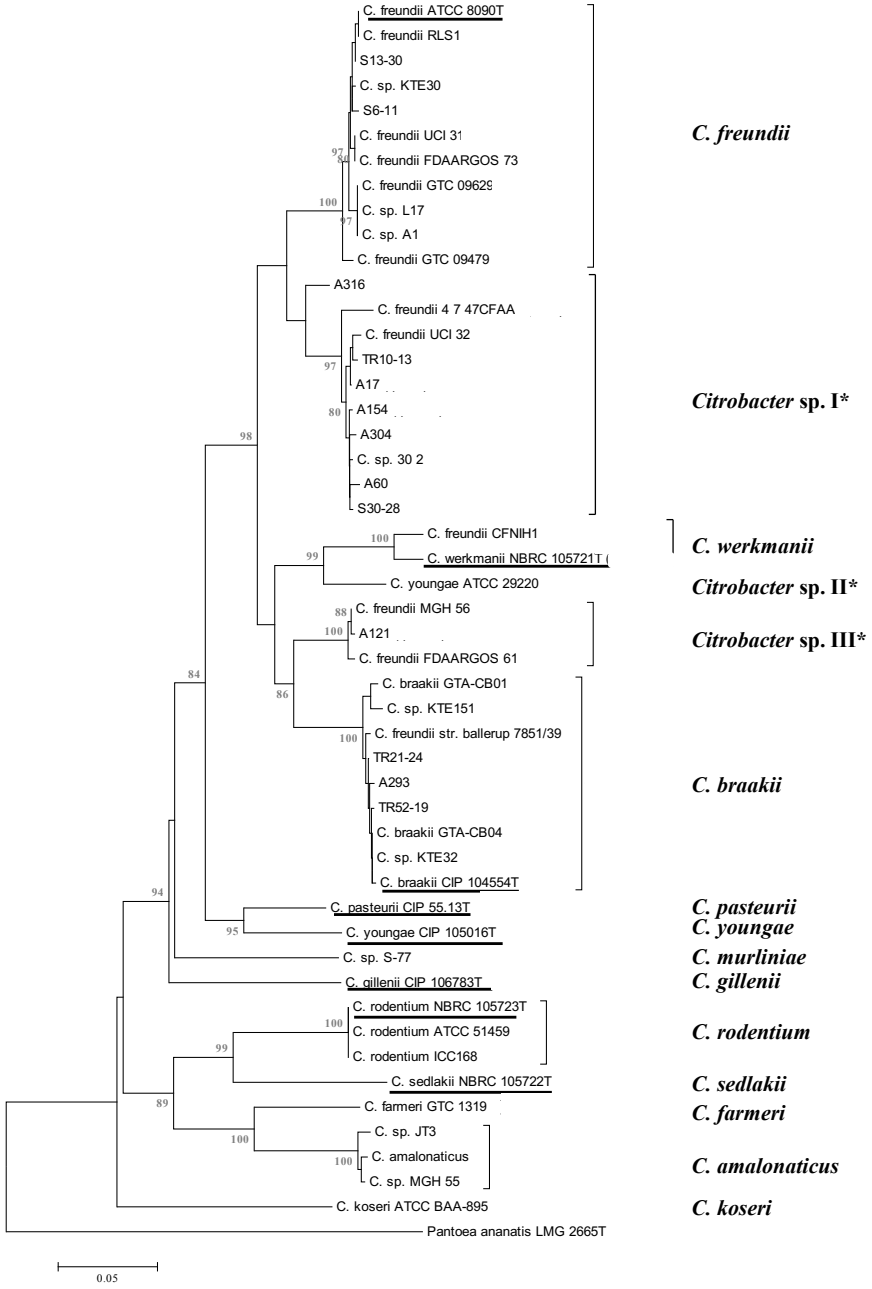


Figure 1. Neighbor-joining tree based on *recN* gene sequences of all *Citrobacter* species performed in the study carried out by Ribeiro *et al.* (2015). Genetic distances were constructed using Kimura's two-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. *Citrobacter* species type strains are underlined. *, *Citrobacter* sp. I, *Citrobacter* sp. II, and *Citrobacter* sp. III correspond to putative novel species. *Pantoea ananatis* strain LMG 2665^T was used as outgroup for tree rooting. [Adapted from reference (34)].

5. MLSA

MLSA is currently a widely used method to obtain a higher resolution on the analysis of the phylogenetic relationships among species within a genus or among genera within a family (35). In MLSA studies, partial sequences of at least four genes coding for proteins with conserved functions ('housekeeping genes') are used to generate phylogenetic trees and subsequently deduce phylogenies (35).

In 2015, Clermont and colleagues proposed the use of four concatenated housekeeping genes, namely *rpoB*, *pyrG*, *fusA*, and *leuS* to depict taxonomic affiliations among *Citrobacter* spp. isolates deposited in the Collection of l'Institut Pasteur (CIP) (Figure 2) (4). The tree obtained from concatenated genes allowed the identification of the novel *Citrobacter pasteurii* species within *Citrobacter* genus (Figure 2) (4). However, sequences of the four housekeeping genes from all *Citrobacter* species are still not available in public databases, which makes impossible for other researchers the identification of their own *Citrobacter* strains by the reproduction of the MLSA scheme described.

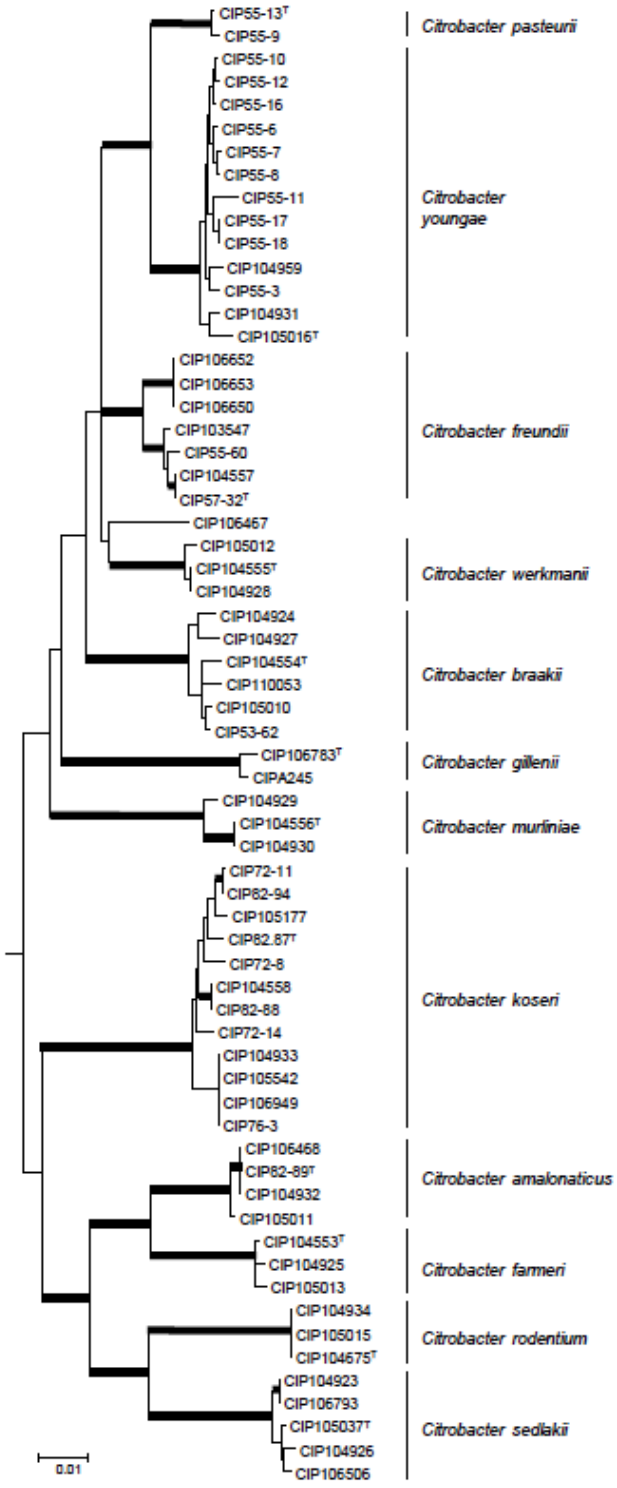


Figure 2. Phylogenetic relationships based on the concatenated alignments of nucleotide sequences of the four protein-coding genes *fusA, pyrG, leuS* and *rpoB*. The scale bar corresponds to 1% nucleotide change. *Escherichia coli* CIP 54.8T was used as outgroup for tree rooting. Branches depicted in bold were supported by bootstrap values equal or above 99% as obtained after 1,000 replicates. [Adapted from reference (4)].

6. WGS data analysis

The recent advances in sequencing technologies have given to all the microbiology laboratories (research and diagnostics) the access to whole genome sequencing (22) (https://gold.jgi.doe.gov/statistics). The continuing reduction in sequencing costs and the shortening of the 'time to result' makes this approach an attractive strategy tool to replace some older techniques previously used to identify and characterize strains at the genomic level (22). The whole genome-based average nucleotide identity (ANI) and/or digital DNA-DNA hybridization (dDDH) have largely replaced DDH on definitions of species, although dDDH has not yet been applied in *Citrobacte*r genomes (4,32,36).

The ANI is a similarity index calculated between a given pair of genomes that can be applicable to prokaryotic organisms independently of their G+C content (37). An ANI cutoff score of >95% indicates that the comparing genomes belong to the same species (37) with ANI values ≥ 95% corresponding to the traditional 70% DDH threshold (35). However, not all whole genome sequences of *Citrobacter* type strains are available in public databases (e.g. *C. amalonaticus*, *C. koseri*, *C. farmeri*, *C. murliniae*), which hinders the current identification and differentiation of all *Citrobacter* species by this approach. The recently described *C. pasteurii* species presented an ANI value of 94.71% with its closely related *C. youngae*, which was just below the generally accepted species cut-off value of 95% (4).

Intrinsic genes conferring resistance to extended-spectrum cephalosporins or reduced susceptibility to fluoroquinolones

Citrobacter spp. is an intrinsic host of several naturally occurring antibiotic resistance mechanisms and during evolution has also increasingly acquired other resistance traits (38). Antibiotic resistance genes database provide a centralized compendium of information on this topic, including those belonging to *Citrobacter* spp. (http://ardb.cbcb.umd.edu/). Resistance to extended-spectrum cephalosporins in these species may be related to: inducible chromosomal AmpC β-lactamase gene, namely CMY gene (bla_{CMY}); overexpression of chromosomal CMY gene; acquisition of plasmid-mediated extended-spectrum β-lactamases, or carbapenemase genes (38,39). Resistance or decreased susceptibility to many other antibiotic groups have been also reported in *Citrobacter* spp., including to fluoroquinolones (chromosomal *qnrB*), macrolide (*macA*, *macB*, *acrA*, *acrB*, *tolC*), trimethoprim (*dfra*), sulfonamides (*sul1*), or aminoglycosides [*aac(6')*, *aac(3)-IIa*, *aph(3')-Ia*)] (http://ardb.cbcb.umd.edu/cgi/search.cgi?db=R&term=citrobacter&field=af). The presence of chromosomal bla_{CMY} and *qnrB* genes in *Citrobacter* spp. is a matter of concern, since mobile genetic elements carrying these genes and likely originated in this genus, have shown a high potential for horizontal dissemination between isolates from the same or even

from different species (39–41). Furthermore, as some *Citrobacter* species present specific variations regarding intrinsic antibiotic resistance mechanisms, failure in the accurate species identification could lead to inappropriate antibiotic treatments, which might result in increased mortality (39,42).

Origin and evolution of chromosomal *bla*$_{CMY}$ within *Citrobacter* spp.

Citrobacter spp. produces very low levels of an inducible chromosomal AmpC-type cephalosporinase (CMY-2-*like*) which comprises resistance to aminopenicillins, aminopenicillins/β-lactamase inhibitor combinations, and first generation cephalosporins (43). The hyperproduction of AmpC in *Citrobacter* with inducible expression of this enzyme, thought to be derived from the action of the LysR-type transcriptional regulator (LTTR) AmpR (43,44), is characterized by more or less marked resistance to penicillins, aztreonam, first generation cephalosporins, second generation cephalosporins, and at least one third-generation cephalosporin is not susceptible (43).

Citrobacter freundii has been considered the ancestor of some CMY-types (CMY-2 – CMY-7, CMY-34, CMY-37, CMY-71), including the novel described CMY-122 and CMY-124 – CMY-129, mainly because of the chromosomal location of their encoding genes (5,45). Furthermore, plasmids carrying *bla*$_{CMY\text{-}2\text{-}like}$ that have been found in different *Enterobacteriaceae* species are believed to have close relationships with those from *C. freundii* because of their high identities (>94%) (46,45). Finally, the high identity observed in the region downstream *bla*$_{CMY\text{-}2\text{-}like}$ (*blc-sugE*) identified either in the chromosome of *C. freundii* and in different plasmids of *Enterobacteriaceae* (46,45,47) seems to corroborate *C. freundii bla*$_{CMY\text{-}2\text{-}like}$ as the evolutionary precursor of acquired *bla*$_{CMY}$ genes (47), rather than a homologous enzyme in the same classification group (46,45).

In a study conducted by Barlow and colleagues, they observed that the wide clinical use of β-lactams has not been a significant force directing the evolution of *bla*$_{CMY\text{-}2\text{-}like}$ alleles in *Citrobacter* spp., since the resistance levels conferred by alleles found in isolates from the pre-antibiotic era are essentially the same as those associated with alleles that have been found on plasmids from recent clinically resistant strains (46). This means that CMY-2-*like* β-lactamases are not evolving phenotypically (46), although several CMY-2-*like* variants have been described.

Although the scientific community has unequivocally accepted *C. freundii* intrinsic AmpC as the ancestor of plasmid-mediated CMY, some questions still arise. First, is *C. freundii* the unique *Citrobacter* species with a chromosomal AmpC? According with available *Citrobacter* genomes deposited in databases, we observed that CMY-48, CMY-65 and CMY-72 were the most common CMY-2 variants found among *C. freundii*, whereas other CMY-2 variants, including novel ones (not assigned), were identified among *C.*

braakii, *C. pasteurii*, *C. murliniae*, *C. youngae*, *C. werkmanii*, and the three putatively novel *Citrobacter* species described by Ribeiro *et al.* (34) (data not shown). Second, since antimicrobial agents were not considered selective and diversifying drivers of the evolutionary trajectories of *bla*$_{\text{CMY-2-}like}$ alleles in *Citrobacter* spp., it is not possible that evolution of this gene occurred within particular *Citrobacter* species? Further studies identifying *Citrobacter* isolates based on reliable methods could in future help to clarify some of these questions.

Diversification of *qnrB* within *Citrobacter* spp.

Of the six known *qnr* families (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qnrVC*) (http://www.lahey.org/qnrStudies/), *qnrB* is the most common worldwide, encoding a protein that binds to DNA gyrase and topoisomerase IV, protecting them from quinolone inhibition (48).

In 2011, Jacoby *et al.* proposed *Citrobacter* spp. as the origin of *qnrB* genes (49). However, the absence of correlation of *qnrB* genes with particular *Citrobacter* species, together with the lack of detailed characterization of *qnrB* platforms, hindered a clear establishment of the origin of *qnrB* (34). In 2015, from the phylogenetic analysis depicted from all known *qnrB* genes, Ribeiro and colleagues observed seven main clusters and two branches. They also observed that *qnrB* diversification might be favored by their association with particular host species and/or niches. In fact, specific genetic platforms were associated with each one of the *qnrB* clusters, and the reliable identification of all *Citrobacter* isolates revealed that each platform evolved in different recognizable and putatively new species. They suggest divergent evolution of closely related *qnrB* genes/platforms in particular *Citrobacter* spp. Moreover, Ribeiro *et al.* (34) also demonstrated that particular *Citrobacter* species are the origin of plasmid-encoded *qnrB* genes encountered in different *Enterobacteriaceae*.

Enterobacteriaceae with plasmid-mediated quinolone resistance (PMQR) due to *qnrB* genes have been increasingly described worldwide (34,41). Recently, it has been shown that the ciprofloxacin minimum inhibitory concentration for *E. coli* ATCC 25922 harboring any *qnr* gene was 0.03–0.125 mg/L, which is 8- to 32-fold higher than for the empty wild-type strain (41). However, since *Enterobacteriaceae* with PMQR genes and lacking other quinolone resistance mechanisms will be classified as susceptible by standard CLSI and/or EUCAST guidelines (http://shop.clsi.org/M100-S26.html, http://www.eucast.org/clinical_breakpoints/) criteria for antibiogram interpretation, their occurrence might be underestimated (50).

Conclusions

Conventional methods used on routine analysis at microbiology laboratories (as commercialized biochemical tests) do not provide sufficient discrimination for identification of many *Citrobacter* isolates at species level, which has important implications in the evaluation of their corresponding clinical significance. Currently, partial DNA sequencing of *recN*, MALDI-TOF MS and ANI analysis are reliable methods for discrimination within *Citrobacter* species. Moreover, accurate species identification is essential to elucidate the origin of antibiotic resistance genes, to understand potential routes of mobilization of intrinsic resistance genes from the host chromosome to plasmids, and to delineate further measures to contain its dissemination. Nevertheless, MALDI-TOF MS routine equipment enlarged with databases covering protein specific profiles of newly recognized species could allow a reliable identification of *Citrobacter* spp.

Funding

This work received financial support from the European Union (FEDER funds) through Programa Operacional Fatores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through projects UID/Multi/04378/2013 and UID/Multi/04546/2013. T. G. R. and Â. N. were supported by fellowships from FCT (SFRH/BD/75752/2011 and SFRH/BPD/104927/2014, respectively) through Programa Operacional Capital Humano (POPH).

Transparency declarations

None to declare.

References

1. Werkman CH, Gillen GF. Bacteria Producing Trimethylene Glycol. J Bacteriol. 1932;23(2):167–82.

2. Frederiksen W. Genus X. Citrobacter Werkman 221 and Gillen. In: D. Brenner, N. Krieg, J. Staley and GG, editor. Bergey's Manual of Systematic Bacteriology. New York: Springer; 2005. p. 651–6.

3. Brenner DJ, Grimont PAD, Steigerwalt AG, Fanning GR, Ageron E, Riddle CF. Classification of Citrobacteria by DNA Hybridization: Designation of *Citrobacter farmeri* sp. nov., *Citrobacter youngae* sp. nov., *Citrobacter braakii* sp. nov., *Citrobacter werkmanii* sp. nov., *Citrobacter sedlakii* sp. nov., and Three Unnamed *Citrobacter* genomospecies. Int J Syst Evol Microbiol. 1993;43(4):645–58.

4. Clermont D, Motreff L, Passet V, Fernandez J-C, Bizet C, Brisse S. Multilocus

sequence analysis of the genus *Citrobacter* and description of *Citrobacter pasteurii* sp. nov. Int J Syst Evol Microbiol. 2015;65(Pt 5):1486–90.

5. Liao X, Fang L, Li L, Sun J, Li X, Chen M, *et al.* Characterization of chromosomal *qnrB* and *ampC* alleles in *Citrobacter freundii* isolates from different origins. Infect Genet Evol. 2015;35:214–20.

6. Borenshtein D, Schauer D. The Genus Citrobacter. In: Workin M, Falkow S, Rosenberg E, Scheleifer KH, Stackebrandt E, editors. In the Prokaryotes. New York: Springer; 2006. p. 90–8.

7. Chao C-T, Lee S-Y, Yang W-S, Chen H-W, Fang C-C, Yen C-J, *et al. Citrobacter* peritoneal dialysis peritonitis: rare occurrence with poor outcomes. Int J Med Sci. 2013;10(9):1092–8.

8. Thapa B, Adhikari P, Mahat K, Chhetri MR, Joshi LN. Multidrug-resistant nosocomial *Citrobacter* in a hospital in Kathmandu. Nepal Med Coll J. 2009;11(3):195–9.

9. Samonis G, Karageorgopoulos DE, Kofteridis DP, Matthaiou DK, Sidiropoulou V, Maraki S, *et al.* Citrobacter infections in a general hospital: characteristics and outcomes. Eur J Clin Microbiol Infect Dis. 2009;28(1):61–8.

10. Engelkirk P. G., Duben-Engelkirk JL. Laboratory Diagnosis of Infectious Diseases: Essentials of Diagnostic Microbiology. In: Lippincott Williams & Wilkins, editor. In the Gram-negative bacilli: the family *Enterobacteriaceae*. Philadelphia; 2008. p. 308.

11. Cuadros EN, Castilla CY, Algarra CM, Perez DM, Lopez BR, Martin FJG, *et al.* Medical and neurosurgical management of *Citrobacter koseri*, a rare cause of neonatal meningitis. J Med Microbiol. 2014;63(Pt_1):144–7.

12. Arens S, Verbist L. Differentiation and susceptibility of *Citrobacter* isolates from patients in a university hospital. Clin Microbiol Infect. 1997;3(1):53–7.

13. Chen KJ, Chen TH, Sue YM. *Citrobacter youngae* and *Pantoea agglomerans* peritonitis in a peritoneal dialysis patient. Perit Dial Int 2013;33(3):336–7.

14. Manganello S, Tayara A, Perazzi B, Neira L, Famiglietti A, Pugliese L, *et al.* [Characterization and distribution of *Citrobacter* species in a university hospital]. Enfermedades Infecc y Microbiol Clínica. 2001;19(1):11–4.

15. Garcia V, Abat C, Moal V, Rolain J-M. *Citrobacter amalonaticus* human urinary tract infections, Marseille, France. New Microbes New Infect. 2016;11:1–5.

16. Deveci A, Coban AY. Optimum management of *Citrobacter koseri* infection. Expert Rev Anti Infect Ther. 2014;12(9):1137–42.

17. Lai CC, Tan CK, Lin SH, Liu WL, Liao CH, Huang YT, *et al.* Bacteraemia caused by non-*freundii*, non-*koseri* *Citrobacter* species in Taiwan. J Hosp Infect. 2010;76(4):332–5.

18. Turton JF, Woodford N, Glover J, Yarde S, Kaufmann ME, Pitt TL. Identification of *Acinetobacter baumannii* by detection of the $bla_{\text{OXA-51-like}}$ carbapenemase gene intrinsic to this species. J Clin Microbiol. 2006;44(8):2974–6.

19. Kolínská R, Spanělová P, Dřevínek M, Hrabák J, Zemličková H. Species identification of strains belonging to genus *Citrobacter* using the biochemical method and MALDI-TOF mass spectrometry. Folia Microbiol. 2015;60(1):53–9.

20. Tindall BJ, Rosselló -Móra R, Busse H, Ludwig W, Kämpfer P, Brian Tindall CJ. Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol. 2010;60(Pt 1):249-66.

21. Kämpfer P, Glaeser SP. Prokaryotic taxonomy in the sequencing era--the polyphasic approach revisited. Environ Microbiol. 2012;14(2):291–317.

22. Bertelli C, Greub G. Rapid bacterial genome sequencing: Methods and applications in clinical microbiology. Clin Microbiol Infect. 2013;19(9):803–13.

23. O'Hara CM, Roman SB, Miller JM. Ability of commercial identification systems to identify newly recognized species of *Citrobacter*. J Clin Microbiol. 1995;33(1):242–5.

24. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev. 2013;26(3):547–603.

25. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 1999;20(18):3551–67.

26. Saffert RT, Cunningham SA, Ihde SM, Jobe KEM, Mandrekar J, Patel R. Comparison of Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometer to BD Phoenix automated microbiology system for identification of gram-negative bacilli. J Clin Microbiol. 2011;49(3):887–92.

27. Ford BA, Burnham C-AD. Optimization of routine identification of clinically relevant Gram-negative bacteria by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and the Bruker Biotyper. J Clin Microbiol. 2013;51(5):1412–

20.

28. Britten RJ, Kohne DE. Nucleotide sequence repetition in DNA. Book CIWY, editor. 1965. 78-91 p.

29. O'Hara CM, Brenner FW, Miller JM. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clin Microbiol Rev. 2000;13(4):534–46.

30. Rosselló-Mora R, Amann R. The species concept for prokaryotes. FEMS Microbiol Rev. 2001;25(1):39–67.

31. Cho JC, Tiedje JM. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. Appl Environ Microbiol. 2001;67(8):3677–82.

32. Auch AF, von Jan M, Klenk H-P, Göker M. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Stand Genomic Sci. 2010;2(1):117–34.

33. Warren JR, Farmer JJ, Dewhirst FE, Birkhead K, Zembower T, Peterson LR, *et al*. Outbreak of nosocomial infections due to extended-spectrum beta-lactamase-producing strains of enteric group 137, a new member of the family *Enterobacteriaceae* closely related to *Citrobacter farmeri* and *Citrobacter amalonaticus*. J Clin Microbiol. 2000;38(11):3946–52.

34. Ribeiro TG, Novais Â, Branquinho R, Machado E, Peixe L. Phylogeny and Comparative Genomics Unveil Independent Diversification Trajectories of *qnrB* and Genetic Platforms within Particular *Citrobacter* Species. Antimicrob Agents Chemother. 2015;59(10):5951–8.

35. Glaeser SP, Kämpfer P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst Appl Microbiol. 2015;38(4):237–45.

36. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics. 2013;14:60.

37. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA. 2009;106(45):19126–31.

38. Ranjan KP, Ranjan N. *Citrobacter*: An emerging health care associated urinary pathogen. Urol Ann. 2013;5(4):313–4.

39. Caltagirone M, Bitar I, Piazza A, Spalla M, Nucleo E, Navarra A, *et al*. Detection of an

IncA/C plasmid encoding VIM-4 and CMY-4 β-lactamases in *Klebsiella oxytoca* and *Citrobacter koseri* from an inpatient in a cardiac rehabilitation unit. New Microbiol. 2015;38(3):387–92.

40. Ribeiro TG, Novais Â, Rodrigues C, Nascimento R, Freitas F, Machado E, *et al.* Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country. J Antimicrob Chemother. 2016. (under *Editorial Revision*)

41. Machuca J, Briales A, Labrador G, Díaz-de-Alba P, López-Rojas R, Docobo-Pérez F, *et al.* Interplay between plasmid-mediated and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in *Escherichia coli*. J Antimicrob Chemother. 2014;69(12):3203–15.

42. Cantón R. Lectura interpretada del antibiograma: una necesidad clínica. Enferm Infecc Microbiol Clin. 2010;28(6):375–85.

43. Bonnet R, Bonomo RA. Beta-lactams and *Enterobacteriaceae*. In: Courvalin P, Leclercq R, Rice L, editors. Antibiogram. 1st ed. Portland: ASM Press; 2009. p. 135–55.

44. Mark BL, Vocadlo DJ, Oliver A. Providing β-lactams a helping hand: targeting the AmpC β-lactamase induction pathway. Future Microbiol. 2011;6(12):1415–27.

45. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type beta-lactamases. Antimicrob Agents Chemother. 2002;46(1):1–11.

46. Barlow M, Hall BG. Origin and evolution of the AmpC beta-lactamases of *Citrobacter freundii*. Antimicrob Agents Chemother. 2002;46(5):1190–8.

47. Wu SW, Dornbusch K, Kronvall G, Norgren M. Characterization and nucleotide sequence of a *Klebsiella oxytoca* cryptic plasmid encoding a CMY-type beta-lactamase: confirmation that the plasmid-mediated cephamycinase originated from the *Citrobacter freundii* AmpC beta-lactamase. Antimicrob Agents Chemother. 1999;43(6):1350–7.

48. Jacoby GA, Strahilevitz J, Hooper DC. Plasmid-mediated quinolone resistance. Microbiol Spectr. 2014;2(5).

49. Jacoby GA, Griffin CM, Hooper DC. *Citrobacter* spp. as a source of *qnrB* alleles. Antimicrob Agents Chemother. 2011;55(11):4979–84.

50. Kanamori H, Yano H, Hirakata Y, Endo S, Arai K, Ogawa M, *et al.* High prevalence of extended-spectrum β-lactamases and *qnr* determinants in *Citrobacter* species from Japan: dissemination of CTX-M-2. J Antimicrob Chemother.

2011;66(10):2255–62.

Chapter 2

Objectives and outline of the Thesis

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein

2.1. Statement of objectives

Extended-spectrum β-lactams, as third and fourth generation cephalosporins and carbapenems, and fluoroquinolones are critical antibiotics for treatment of human infections caused by *Enterobacteriaceae* isolates. However, resistance to these important antibiotic families is increasing worldwide, compromising their use and leading to a major public health threat. The most common antibiotic resistance mechanism to extended-spectrum cephalosporins and carbapenems among *Enterobacteriaceae* is the acquisition of genes encoding the production of enzymes known as β-lactamases [e.g. extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases and carbapenemases]. Similarly, the activity of fluoroquinolones among *Enterobacteriaceae* is increasingly being compromised by the emergence of mutations in DNA gyrases (e.g. GyrA, GyrB) and topoisomerase IV (e.g. ParC, ParE), or acquisition of genes encoding acetylases [Aac(6')-Ib-cr], efflux pumps (e.g. QepA, OqxAB, AcrAB-TolC) or proteins protecting DNA gyrase and topoisomerase IV from quinolone inhibition (e.g. Qnr).

The worldwide contemporary epidemiology of *Enterobacteriaceae* resistant to extended-spectrum β-lactams seems to be largely dominated by a large expansion of ESBLs and the more recent emergence and widespread of carbapenemases. Comparatively, the available epidemiological data suggests that the incidence of acquired AmpC β-lactamases (qAmpC) seems to be lower, though it might be underestimated since they are not thoroughly sought or because qAmpC production is frequently masked by other mechanisms of resistance (hyperproduction of chromosomal AmpCs, production of other β-lactamases with similar phenotypes), hindering their detection. Indeed, most of the existing studies exploring the molecular epidemiology of *Enterobacteriaceae* producing qAmpCs are limited to species lacking inducible chromosomal AmpC genes ($_{i}$Cr-AmpCs) (e.g. *K. pneumoniae* and *E. coli*), focused on specific qAmpC-types (e.g. DHA or CMY), single institutions and short periods of time or addressing few non-human hosts (e.g. food-producing animals or food products).

In Portugal, identification of qAmpCs among clinical *K. pneumoniae* and *E. coli* isolates between 2002 and 2008 revealed the presence of bla_{qAmpC} genes in 50% of the presumptive qAmpC producers, alerting for the need of continuous surveillance. For this reason, a comprehensive analysis on the occurrence, diversity and genetic backgrounds of qAmpCs among diverse *Enterobacteriaceae* species recovered from both clinical and non-clinical niches is crucial to understand the contribution of qAmpCs for the increasing resistance rates to third-generation cephalosporins in our country.

Currently, the continuous movement of people (medical tourism, transfer of patients between countries, leisure, migrant workers, war-displaced refugees) and trade of goods (particularly food products or animals used for food) over international borders are recognized as important players in the dispersion of antibiotic resistant (AbR) bacteria, although available epidemiological data on this topic is still scarce. In fact, most of the studies analyzing the occurrence, diversity, population structure and plasmids of *Enterobacteriaceae* producing acquired β-lactamases (ESBLs, qAmpCs, carbapenemases) are from developed countries, whereas much less in known from underdeveloped countries, particularly from Africa continent. Available data is limited to specific regions (North Africa) and settings (clinical institutions) while information from sub-Saharan African countries (e.g. Angola) is scarce. Angola is a country with an emerging economy and close commercial or travel relationships with Portugal. Therefore, molecular epidemiological data of bacteria producing acquired β-lactamases from non-clinical niches in Angola constitutes an important contribution for the identification of potential reservoirs, the dynamics of spread of these AbR bacteria and a better understanding of the global epidemiology of *Enterobacteriaceae* resistant to extended-spectrum β-lactams.

Finally, phylogenetic studies designed to survey the phylogeny on intrinsic antibiotic resistance genes and their natural hosts are nowadays of utmost significance, since they might contribute to shade some light on the origin and mechanisms of diversification of genes involved in the reduced susceptibility of bacteria to clinically relevant antibiotics, besides providing tools for accurate species identification. The *qnrB* genes are known to be intrinsic of *Citrobacter* complex species contributing for a natural phenotype of reduced susceptibility to fluoroquinolones. Nevertheless, the absence of studies analyzing the correlation of these genes with particular *Citrobacter* species within this genus, together with the lack of detailed characterization of their platforms, hinders a clear establishment of their origin and furthermore to ascertain the clinical significance of the different *Citrobacter* species.

The **main goals** of this work were:

i) to extend the knowledge on dispersion drivers (bacterial species, clones and plasmids) of acquired genes conferring resistance to β-lactams in *Enterobacteriaceae* from different ecological niches from Portugal and Angola, two countries with close commercial and travel relationships;

ii) to accurately discriminate *Citrobacter* species in order to depict evolutionary trajectories of intrinsic genes (*qnrB*) responsible for decreased susceptibility to fluoroquinolones.

To accomplish these purposes, *Enterobacteriaceae* isolates non-susceptible to extended-spectrum cephalosporins from Portugal (different clinical institutions and diverse non-clinical niches throughout a large period of time) and Angola (diverse non-clinical niches) were screened for detection of acquired β-lactamases (qAmpCs, ESBLs and/or carbapenemases) genes. Furthermore, a collection of *Citrobacter* spp. isolates of diverse origins from Portugal was studied to perform a phylogenetic analysis of housekeeping and *qnrB* genes and a comparative genomics of *qnrB* surrounding genetic sequences.

The **specific aims** of this Thesis are:

1. To analyze the occurrence, diversity and genetic backgrounds of qAmpCs among *Enterobacteriaceae* species (n=2107), with and without inducible chromosomal AmpC genes (iCr-AmpCs), from diverse clinical settings in Portugal (seven hospitals and two community laboratories) over a 12-year period.

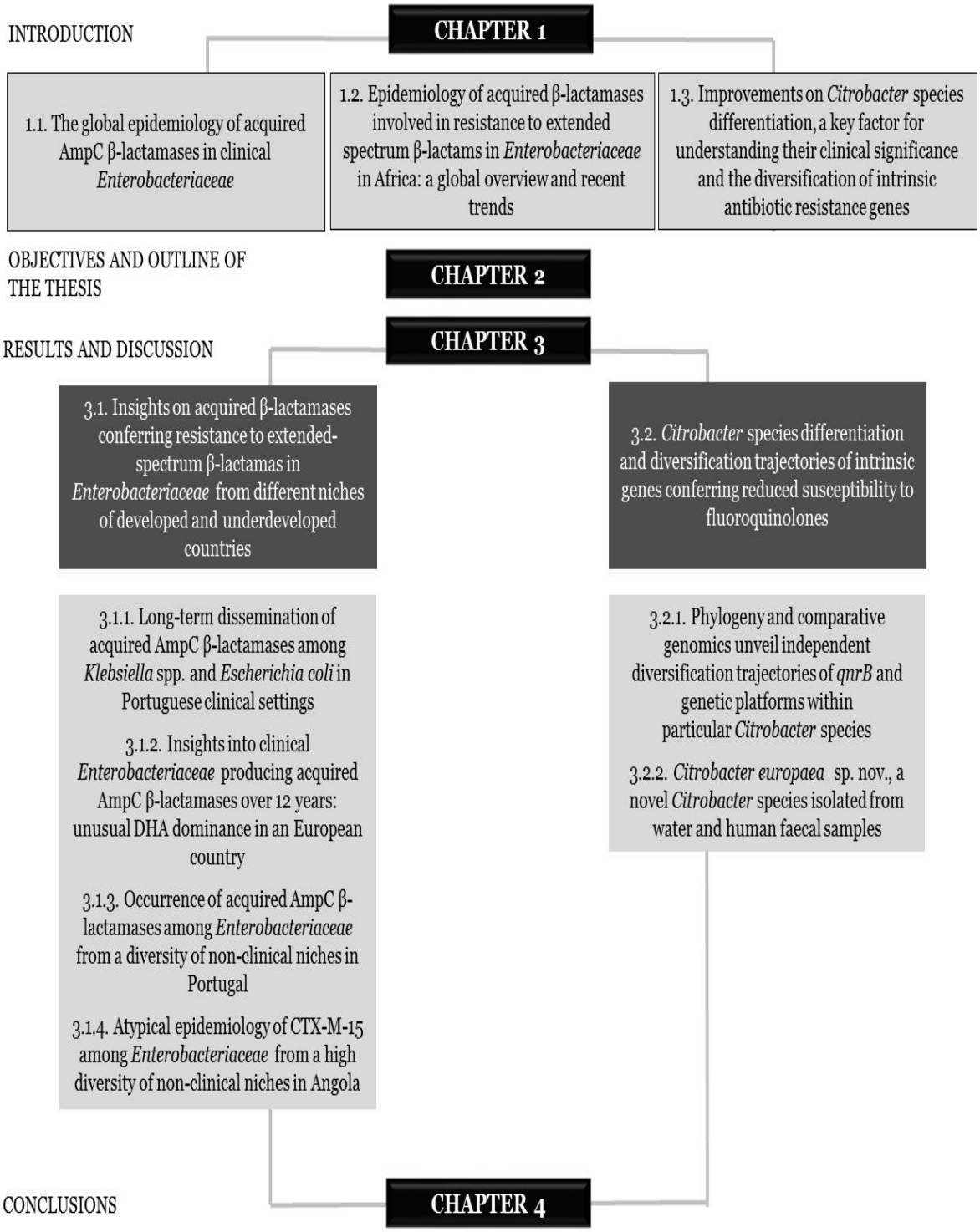
2. To assess the contribution of Portuguese non-clinical niches (n=518 samples; healthy humans, animals, and environment) as reservoirs of qAmpC-encoding plasmids and clones.

3. To investigate the distribution and molecular epidemiology of ESBLs, qAmpCs and carbapenemases in recent (2013) *Enterobacteriaceae* isolates from different non-clinical niches (n=81 samples; healthy humans, healthy animals, aquatic environments) in Angola.

4. To evaluate the potential of different housekeeping genes to accurately discriminate *Citrobacter* species.

5. To establish diversification trajectories of *qnrB* genes within *Citrobacter* species and subsequently their dispersion routes to other *Enterobacteriaceae* species.

2.2. Outline of the Thesis



This Thesis is organized in four chapters as follows:

Chapter 1 presents an overview of the state of the art on the topic of this Thesis, which is divided in three subsections. In section 1.1, the worldwide occurrence, distribution, diversity and drivers (clones, plasmids and other mobile genetic elements) of qAmpCs in clinical *Enterobacteriaceae* isolates were addressed. Section 1.2 focuses on the current status regarding the occurrence, population structure and plasmids associated with genes encoding ESBLs, qAmpCs and carbapenemases among *Enterobacteriaceae* recovered from different ecological niches in Africa. Finally, section 1.3 presents an updated overview on the main features and recent methodologies used for *Citrobacter* species discrimination. Moreover, intrinsic genes conferring resistance to β-lactams ($bla_{\text{CMY-2-}like}$) or reduced susceptibility to fluoroquinolones (*qnrB*) within *Citrobacter* spp. will be also explored.

Chapter 2 includes objectives and outline of the Thesis.

Chapter 3 presents the findings answering the specific aims of the Thesis. The results from the experimental research work performed in this study have been organized throughout scientific articles (n=6; 3 publications in peer review scientific journals, 2 manuscripts under editorial revision and 1 manuscript in preparation). This chapter is divided in two subsections:

Section 3.1. **Insights on acquired β-lactamases conferring resistance to extended-spectrum β-lactams in *Enterobacteriaceae* from different niches of developed and underdeveloped countries.**

This section includes results obtained from the molecular epidemiology of a large collection of *Enterobacteriaceae* isolates, corresponding to species with and without ¡Cr-AmpCs genes collected from several clinical institutions in the North and Centre regions of Portugal over a 12-year period (2002-2013), as well as from different non-clinical niches. Moreover, it is also presented in this section the occurrence of clinically relevant genes encoding acquired resistance to extended-spectrum cephalosporins and/or carbapenems among *Enterobacteriaceae* from non-clinical niches in an underdeveloped country (Angola). The possible contribution of clones and/or plasmids in the dissemination of $bla_{\text{CTX-M-15}}$ in Angola is also discussed. Results were organized in the following scientific articles:

- Freitas F, Machado E, **Ribeiro TG**, Novais Â, Peixe L. 2014. Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and

Escherichia coli in Portuguese clinical settings. *European Journal of Clinical Microbiology and Infectious Diseases*. 33(4):551-8.

- **Ribeiro TG**, Novais Â, Machado E, Peixe L. 2016. Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country. *Journal of Antimicrobial Chemotherapy*. (*under editorial revision*)
- **Ribeiro TG**, Novais Â, Machado E, Peixe L. 2016. Occurrence of acquired AmpC β-lactamases among *Enterobacteriaceae* from a diversity of non-clinical niches in Portugal. *Journal of Antimicrobial Chemotherapy*. (*manuscript draft*)
- **Ribeiro TG**, Novais Â, Peixe L, Machado E. 2016. Atypical epidemiology of CTX-M-15 among *Enterobacteriaceae* from a high diversity of non-clinical niches in Angola. *Journal of Antimicrobial Chemotherapy*. 71(5):1169-73.

Section 3.2. ***Citrobacter*** **species differentiation and diversification trajectories of intrinsic genes conferring reduced susceptibility to fluoroquinolones.**

The discriminatory power of different housekeeping genes in the accurate discrimination of *Citrobacter* complex species is evaluated, using the most reliable genotypic markers. Moreover, diversification trajectories of *qnrB* and genetic platforms within *Citrobacter* species are also presented. The data obtained is presented and organized in the following scientific articles:

- **Ribeiro TG**, Novais Â, Branquinho R, Machado E, Peixe L. 2015. Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species. *Antimicrobial Agents and Chemotherapy*. 59(10):5951-8.
- **Ribeiro TG**, Clermont D, Branquinho R, Machado E, Peixe L, Brisse S. 2016. *Citrobacter europaea* sp. nov., a novel *Citrobacter* species isolated from water and human faecal samples. *International Journal of Systematic and Evolutionary Microbiology*. (*manuscript submitted*)

Highlighted results from these studies are reflected in the general conclusions described in **Chapter 4**.

Additionally, the results presented in this Thesis have also been partially presented in international scientific meetings and conference proceedings, as follows:

- **Ribeiro TG***, Novais Â, Branquinho R, Machado E, Peixe L. 2015. Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic surroundings within particular *Citrobacter* species. [Oral Presentation]. Abstract available in: Abstract book of the 3rd International Symposium on the Environmental Dimension of Antibiotic Resistance (EDAR), 17-21 May, Wernigerode, Germany.
- **Ribeiro TG***, Novais Â, Nascimento R, Freitas F, Peixe L, Machado E. 2015. Persistence and spread of particular acquired AmpC β-lactamases linked to KP-ST11 international clone and/or epidemic IncR, IncHI2 or IncI1 plasmids. [ePoster Viewing - EV0113]. Abstract available in: Abstract book of the 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 25-28 April, Copenhagen, Denmark.
- **Ribeiro TG***, Novais Â, Peixe L, Machado E. 2014. Diverse non-clinical reservoirs of CTX-M-15-encoding plasmids and clones in a developing country with close relations with Europe. [Poster Presentation – P1150]. Abstract available in: Abstract book of the 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 10-13 May, Barcelona, Spain.
- **Ribeiro TG***, Novais Â, Novais C, Silva R, Sousa JC, Peixe L, Machado E. 2013. High diversity of *qnrB* genes in *Citrobacter freundii* complex isolates from an aquatic source. [Poster Presentation – P1454]. Abstract available in: Online Lecture Library of the 23rd ECCMID European Congress of Clinical Microbiology and Infectious Diseases, 27-30 April, Berlin, Germany.
- Freitas F*, Novais Â, **Gonçalves T**, Amaral S, Cantón R, Coque TM, Machado E, Peixe L. 2011. Dissemination of DHA-1 and CMY-2 beta-lactamases among *Klebsiella* spp. and *E. coli* from Portuguese clinical settings. 21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 7-10 May, Milan, Italy; [Poster Presentation – P1343]. Abstract available in: *Clinical Microbiology and Infection* 17 (Suppl 4): S373.

Chapter 3

Results and discussion

"What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on."

Jacques Yves Cousteau

3.1. Insights on acquired β-lactamases conferring resistance to extended-spectrum β-lactams in *Enterobacteriaceae* from different niches of developed and underdeveloped countries

3.1.1. Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings

3.1.2. Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country

3.1.3. Occurrence of acquired AmpC β-lactamases among *Enterobacteriaceae* from a diversity of non-clinical niches in Portugal

3.1.4. Atypical epidemiology of CTX-M-15 among *Enterobacteriaceae* from a high diversity of non-clinical niches in Angola

Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings

Francisco Freitas[1,2,3], Elisabete Machado[1,2], Teresa Gonçalves Ribeiro[1,2], Ângela Novais[1], and Luísa Peixe[1*]

[1]REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Portugal; [2]CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; [3]Laboratório de Microbiologia, Centro Hospitalar Tondela-Viseu, EPE.

European Journal of Clinical Microbiology and Infectious Diseases 2014 April; 33(4):551-8

Springer Science and Bus Media B V has authorized the reproduction of the final published PDF version of this paper in this Thesis through the License Agreement Number 3910220589380 on July 15, 2016.

Eur J Clin Microbiol Infect Dis (2014) 33:551–558
DOI 10.1007/s10096-013-1983-9

ARTICLE

Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings

F. Freitas · E. Machado · T. G. Ribeiro · Â. Novais · L. Peixe

Received: 24 July 2013 / Accepted: 10 September 2013 / Published online: 7 October 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract We investigated the occurrence, diversity and molecular epidemiology of genes coding for acquired AmpC β-lactamases (qAmpC) among clinical isolates of *Enterobacteriaceae* lacking inducible chromosomal AmpCs in Portugal. A total of 675 isolates non-susceptible to broad-spectrum cephalosporins obtained from four hospitals and three community laboratories during a 7-year period (2002–2008) were analysed. The presence of genes coding for qAmpC was investigated by phenotypic criteria, polymerase chain reaction (PCR) and sequencing. Bacterial identification, antibiotic susceptibility testing, conjugation assays and clonal analysis were performed by standard procedures. The presence of bla_{qAmpC} genes was detected in 50 % (50/100; 41 *Klebsiella pneumoniae*, 5 *Escherichia coli*, 4 *Klebsiella oxytoca*) of the presumptive qAmpC producers. DHA-1, detected in those species, was the most prevalent qAmpC (94 %, 47/50), being identified since 2003 and throughout the studied period in different institutions. Despite the high clonal diversity observed, three DHA-1-producing *Klebsiella* spp. clones were more frequently identified. CMY-2 (6 %, 3/50) was observed in B1-*E. coli* clones. Conjugative transfer was only observed in one (2 %) CMY-2-producing isolate. Most qAmpC producers (94 %, 47/50) co-expressed SHV-type and/or OXA-1 or CTX-M-32 extended-spectrum β-lactamases (ESBLs). To the authors' knowledge, this is the first description of the molecular epidemiology and the long-term dissemination of qAmpC-producing *Enterobacteriaceae* in Portuguese clinical settings, highlighting an evolution towards a more complex epidemiological situation regarding cephalosporin resistance in Portugal.

F. Freitas · E. Machado · T. G. Ribeiro · Â. Novais · L. Peixe (✉)
REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
e-mail: lpeixe@ff.up.pt

F. Freitas · E. Machado · T. G. Ribeiro
CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal

F. Freitas
Laboratório de Microbiologia, Centro Hospitalar Tondela-Viseu, EPE, Viseu, Portugal

Introduction

Acquired AmpC β-lactamases (qAmpC) are an emerging cause of resistance to broad-spectrum cephalosporins among *Enterobacteriaceae* lacking inducible chromosomal AmpC genes, such as *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis* and *Salmonella* spp. [1–4]. Genes encoding qAmpC are usually located on multidrug resistance plasmids, seriously constraining the therapeutic options against several infectious diseases [2]. qAmpC-producing *Enterobacteriaceae* have been reported worldwide, with significant geographical differences in the prevalence and epidemiology of different qAmpC types (Table 1). According to the few surveillance studies available in Europe, CMY-2-like enzymes are the most widespread variants [1, 3, 4, 17, 18] (Table 1). In the present study, we investigated the occurrence, diversity and molecular epidemiology of qAmpC β-lactamases among *Enterobacteriaceae* species lacking inducible chromosomal AmpC genes recovered from Portuguese clinical settings over a 7-year period.

Table 1 Occurrence of qAmpC-producing *Enterobacteriaceae* lacking inducible chromosomal AmpC genes in worldwide clinical settings

qAmpC enzyme(s)	Country(ies)	Epidemiological stage[a]	Species	Year(s) of isolation[b]	Reference(s)
Widespread					
CMY-2	Algiers	2	*E. coli*, *K. pneumoniae*, *P. mirabilis*	2003–2007	[5]
	Argentina	1	*E. coli*	2009	[6]
	Canada	1, 3	*E. coli*	2005–2009	[7, 8]
	China	1, 2	*E. coli*, *K. pneumoniae*	2003–2006	[9, 10]
	Denmark	1	*E. coli*	2006	[11]
	France	2	*E. coli*	2004–2008	[12]
	Japan	1	*E. coli*, *K. oxytoca*, *P. mirabilis*	2007–2010	[13, 14]
	Korea	1	*E. coli*, *K. pneumoniae*	2003–2007	[15, 16]
	Netherlands	1	*E. coli*, *K. pneumoniae*, *P. mirabilis*	2009	[17]
	Norway	2	*E. coli*	2003–2007	[18]
	Spain	2	*E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *S. enterica*	1999–2009	[3, 4, 19, 20]
	Switzerland	1	*E. coli*, *K. pneumoniae*, *P. mirabilis*	2006–2007	[21]
	Thailand	2	*E. coli*, *K. pneumoniae*	2005–2006	[22]
	USA	1	*E. coli*, *K. pneumoniae*	1994–2003	[23, 24]
DHA-1	Algiers	1	*E. coli*	2007	[5]
	Argentina	1	*K. pneumoniae*, *P. mirabilis*	2009	[6]
	Belgium	2	*K. pneumoniae*	2006	[25]
	China	1, 2	*E. coli*, *K. pneumoniae*, *K. oxytoca*	2003–2006	[9, 10]
	France	2	*K. pneumoniae*	2007–2009	[26]
	Ireland	1	*K. pneumoniae*	2005–2007	[27]
	Japan	1, 2	*E. coli*, *K. pneumoniae*	2006–2010	[13, 14]
	Korea	1	*E. coli*, *K. pneumoniae*, *K. oxytoca*, *S. enterica*	2002–2007	[15, 16]
	Netherlands	1	*K. pneumoniae*	2009	[17]
	Norway	1	*E. coli*	2006	[18]
	Spain	2	*E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *S. enterica*	2005–2009	[3, 4, 19]
	Switzerland	1	*E. coli*	2006–2007	[21]
	Thailand	1	*E. coli*, *K. pneumoniae*	2005–2006	[22]
	USA	1	*E. coli*, *K. pneumoniae*	1994–2004	[23, 24]
Limited spread					
CMY-4	Spain	1	*E. coli*	2005–2009	[3, 4, 19]
CMY-7	Norway	1	*E. coli*	2003	[18]
	Spain	1	*E. coli*	2009	[4]
CMY-25, -56	Spain	1	*K. pneumoniae*	2006–2009	[3, 4, 19]
CMY-27	Spain	1, 2	*E. coli*	2006–2009	[3, 4, 19]
CMY-31	Switzerland	1	*K. pneumoniae*	2005–2009	[21]
CMY-40, -43, -48, -54, -55, -57	Spain	1	*E. coli*	2009	[4]
CMY-4, -12, -14, -15, -16, -38, -45	France, Greece, Italy and/or Poland	4	*P. mirabilis*	1999–2008	[28]
ACC-1	Ireland	1	*K. pneumoniae*	2005–2007	[27]
	Spain	1, 2	*E. coli*, *K. pneumoniae*, *P. mirabilis*	2004–2009	[3, 4, 19]
	Tunisia	4	*S. enterica*	1999–2003	[29]
ACT-1	Canada	1	*E. coli*	2005–2006	[8]

Table 1 (continued)

qAmpC enzyme(s)	Country(ies)	Epidemiological stage[a]	Species	Year(s) of isolation[b]	Reference(s)
	Korea	1	*K. pneumoniae*	2003	[15]
	USA	3	*K. pneumoniae*	1992–2000	[24]
FOX-3	Spain	1	*E. coli*	2009	[4]
FOX-5	Canada	1	*E. coli*	2007–2009	[7]
	USA	1, 3	*E. coli*, *K. pneumoniae*	1992–2004	[23, 24]
FOX-8	Spain	2	*E. coli*	2009	[4]
CMY-1	Korea	1	*K. pneumoniae*	2003	[15]
CMY-8	Japan	1	*K. pneumoniae*	2003–2005	[14]
	Thailand	1	*E. coli*, *K. pneumoniae*	2005–2006	[22]
CMY-10, -11	Korea	1, 2	*E. coli*	1998–2007	[16, 30, 31]
MOX-1	Japan	1	*K. oxytoca*	2002	[14]

[a] Epidemiological stages as proposed by Grundmann et al. [53]: 1, sporadic occurrence; 2, single or independent hospital outbreaks; 3, regional spread; 4, interregional spread

[b] When multiple studies are considered, the time period is combined

Materials and methods

Bacterial isolates

We screened 675 isolates [*Escherichia coli* (n = 480), *Klebsiella pneumoniae* (n = 141), *Klebsiella oxytoca* (n = 27) and *Proteus mirabilis* (n = 27)] non-susceptible to broad-spectrum cephalosporins identified from the clinical samples of patients attending four hospitals (HA, HB, HC, HD; n = 572) and three community laboratories (CL1, CL2, CL3; n = 103) located in the North and Centre of Portugal between 2002 and 2008. The geographic distribution of the surveyed clinical settings is shown in Fig. 1. Bacterial identification and preliminary susceptibility testing were performed by using the automated VITEK (bioMérieux, Marcy l'Étoile, France) or Phoenix (BD Diagnostics, Sparks, MD, USA) systems.

Antimicrobial susceptibility and qAmpC characterisation

Antimicrobial susceptibility testing to β-lactam and non-β-lactam antibiotics was performed by using the standard disk diffusion method [32, http://www.eucast.org/]. Presumptive qAmpC producers were preliminarily identified using phenotypic criteria: (i) resistance to cefoxitin (FOX-R), intermediate susceptibility or resistance to amoxicillin–clavulanic acid (AMC-I/R) and to at least one oxyimino-cephalosporin (CEF-I/R); or (ii) susceptibility to cefoxitin (FOX-S), AMC-I/R, CEF-I/R and a negative double disk synergy test (DDST) (detailed below) [33]. The presence of genes coding for qAmpC enzymes (CMY, MOX, FOX, LAT, ACT, MIR, DHA, ACC) was further confirmed by polymerase chain reaction (PCR) using primers and conditions previously described [1, 34] and sequencing of both strands of each amplified *bla* gene.

Co-production of ESBLs

The production of extended-spectrum β-lactamases (ESBLs) was investigated among qAmpC-producing isolates by using the standard DDST performed with cefotaxime, ceftazidime, cefepime and aztreonam disks applied 20–30 mm apart from an amoxicillin–clavulanate disk [35] on Mueller–Hinton agar plates with and without cloxacillin (250 mg/L) (Sigma-Aldrich, St. Quentin-Fallavier, France). The characterization of ESBLs was performed by PCR using specific primers for bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$, and further sequencing [32].

Conjugation experiments

Conjugation experiments were performed as previously described using *E. coli* BM21R (nalidixic acid and

Fig. 1 Geographic locations of the hospitals [⋆] and community laboratories [●] surveyed in this study

rifampicin resistant, Lac+, plasmid free) or *E. coli* HB101 (azide and kanamycin resistant, Lac−, plasmid free) as recipients [32], transconjugants being selected on MacConkey agar supple- mented with cefoxitin (10 mg/L) (Sigma-Aldrich, St. Louis, EUA) and rifampicin (100 mg/L) (Sigma-Aldrich, St. Louis, EUA) or cefoxitin (10 mg/L) and sodium azide (200 mg/L) (Merck, Munich, Germany), respectively [24, 36].

Clonal relationships among qAmpC-producing isolates

Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE), according to the criteria proposed by Tenover et al. [37], using *Xba*I as the restriction enzyme (New England Biolabs, Ipswich, MA, USA) and the following electrophoresis conditions: 10–40 s for 21 h, 14 °C, 6 V/cm^2 [32]. *E. coli* phylogenetic groups were identified by a multiplex PCR assay described by Clermont et al. [38].

Results and discussion

One hundred (89 FOX-R, 11 FOX-S) presumptive qAmpC producers were identified, from which 50 (50 %) carried bla_{qAmpC} genes (41 *K. pneumoniae*, 5 *E. coli* and 4 *K. oxytoca*). They corresponded to 56 % (50/89) of the FOX-R isolates and were mainly recovered from urine (38 %, 19/50) and respiratory samples (26 %, 13/50) of hospitalised patients. The remaining FOX-R isolates (n =39) were ESBL producers (85 %, 33/39) which might have porin modifications and/or possible chromosomal AmpC hyperproducers (*E. coli*; 62 %, 24/39) [2, 33].

DHA-1 was the most prevalent qAmpC (94 %, 47/50), identified among *K. pneumoniae* (n =41; 12 PFGE types), *K. oxytoca* (n =4; 2 PFGE-types) and *E. coli* (n =2; 2 PFGE-types) isolates from three hospitals (HA, HB, HC) and one community laboratory (CL1) since 2003 (Table 2, Fig. 2). Despite the high clonal diversity observed, three DHA-1-producing *Klebsiella* spp. clones were more frequently identified: (i) *K. pneumoniae* KpF in HA, HB and HC (n =13, since 2006); (ii) *K. pneumoniae* KpB in HA (n =14, 2003–2004); and (iii) *K. oxytoca* KoA in HA (n =3, 2003–2004) (Table 2). Moreover, the DHA-1-producing *E. coli* isolates (HA, HB) belonged to B1 and B2 phylogroups (Table 2). Although the B2 phylogroup accounts for most human infections involving cephalosporin-resistant strains [39], DHA-1 has been previously reported in non-B2-*E. coli* clinical isolates (B1-, A-, or D-*E. coli*) [18, 40, 41]. The acquisition of virulence traits and multidrug resistance patterns (including to broad-spectrum cephalosporins) by A and B1 phylogroups, traditionally considered commensal, might contribute to their increasing identification among *E. coli* isolates causing human infections [38, 42–45; Novais and Peixe, unpublished data].

The CMY-2 enzyme was detected in 6 % (3/50) of qAmpC producers, corresponding to diverse B1-*E. coli* recovered in different hospitals in 2006 and 2007 (Table 2). Studies from France also report CMY-2 mainly in *E. coli* belonging to phylogroup B1 [12], but in Canada, USA and Norway, it has been mostly associated with D-*E. coli* isolates [8, 18, 46]. The high occurrence of DHA-1 producers observed in this study resembles that reported in Asian countries (67–93 %) [2, 9, 10, 16] and contrasts with data from Europe (3–20 %; predominance of CMY-2-like enzymes) [2–4, 17, 19, 21], a scenario probably reflecting the bias induced by the clonal dissemination of particular DHA-1-producing *K. pneumoniae* strains in Portuguese hospitals. Interestingly, the preliminary characterisation of presumptive qAmpC producers collected in 2012 indicated the maintenance of low levels of CMY-2-like enzymes and the predominance of DHA-1 in Portuguese clinical settings (Machado and Peixe, personal communication).

Isolates producing qAmpC were concomitantly resistant to kanamycin (98 %), tobramycin (96 %), sulphonamides (96 %), streptomycin (84 %), ciprofloxacin (82 %) and trimethoprim

Table 2 Characterisation of qAmpC-producing *Enterobacteriaceae* from Portuguese clinical settings (2002–2008)

qAmpC	Species (no.)	Year	Hospital[a] (no.)	PFGE-type (no.)/PhG[b]	Associated ESBLs (no.)	Non-β-lactam resistance phenotype[c,d]
CMY-2	*E. coli* (3)	2006	HB, HC	EcA/B1, EcB/B1	-	Km,(Tb),(Sm),(Gm),(Cp),Na,(Su),(Tp),(Te),(Cm)
		2007	HB	EcC/B1	-	Km,Sm,Ak,Cp
DHA-1	*E. coli* (2)	2007	HB	EcD/B1	SHV-12 + OXA-1	Km,Tb,Sm,Gm,Nt,Cp,Na,Su,Tp,Te,Cm
		2008	HA	NT[e]/B2	CTX-M-32 + OXA-1	Km,Tb,Sm,Cp,Na,Su,Tp,Te
	K. pneumoniae (41)	2003	HA (3)	KpB (3)	SHV-5 (1), SHV-90 (2)	Km,Tb,Sm,Gm,Nt,(Ak),Cp,Na,Su,Tp,Te,(Cm)
		2004	HA (12)	KpB (11), KpL (1)	SHV-5 (4), SHV-12 (4), SHV-90 (4)	Km,Tb,(Sm),(Gm),(Nt),(Ak),(Cp),(Na),Su,(Tp),(Te),(Cm),(Tg)
			CL1 (1)	KpA	OXA-1	
		2006	HA (4)	KpF (3), KpG (1)	OXA-1 (4)	Km,Tb,(Sm),(Gm),(Nt),(Ak),Cp,(Na),Su,(Tp),Te,Cm
			HB (1)	KpC	SHV-145 + OXA-1	
		2007	HA (3), HC (3)	KpF (6)	OXA-1 (6)	Km,Tb,(Sm),(Gm),(Ak),Cp,Na,Su,(Tp),(Te),(Cm)
			HB (4)	KpD (1), KpE (3)	SHV-12 + OXA-1 (4)	
		2008	HA (6)	KpF (3), KpJ (2), KpK (1)	SHV-12 (1), OXA-1 (5)	(Km),Tb,(Sm),(Gm),(Nt),(Ak),Cp,Na,Su,(Tp),(Te),(Cm),(Tg)
			HB (4)	KpF (1), KpH (1), KpI (2)	SHV-12 + OXA-1 (3), OXA-1 (1)	
	K. oxytoca (4)	2003	HA (2)	KoA (2)	SHV-12 (2)	Km,Tb,Sm,Gm,Nt,(Ak),Cp,Na,Su,Tp,(Te),Cm,(Tg)
		2004	HA (1)	KoA1	SHV-12	Km,Tb,Sm,Gm,Cp,Na,Su,Tp,Cm
		2008	HA (1)	KoB	OXA-1	Km,Tb,Sm,Cp,Na,Su,Tp,Te,Cm

[a] HA, Hospital Geral de Santo António (Porto, North); HB, Hospital de São Teotónio (Viseu, Centre); HC, Hospital Cândido de Figueiredo (Tondela, Centre); CL1, Microbiology Laboratory, Faculty of Pharmacy, University of Porto (Porto, North)

[b] PhG – *E. coli* phylogenetic group

[c] Km, kanamycin; Tb, tobramycin; Sm, streptomycin; Gm, gentamicin; Nt, netilmicin; Ak, amikacin; Cp, ciprofloxacin; Na, nalidixic acid; Su, sulphonamides; Tp, trimethoprim; Te, tetracycline; Cm, chloramphenicol; Tg, tigecycline

[d] Variable presence of a resistance phenotype is indicated by parentheses

[e] NT, non-typeable by PFGE

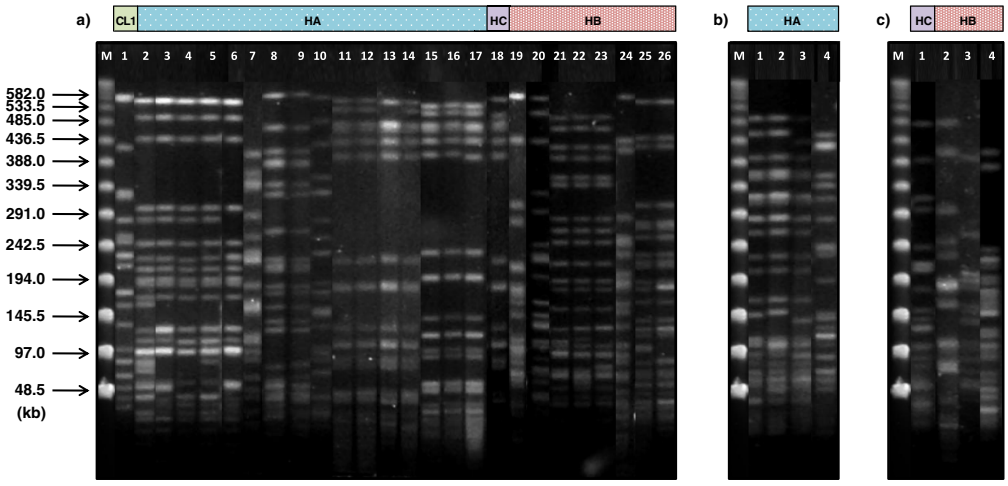


Fig. 2 PFGE patterns of *Xba*I-digested chromosomal DNA. **a)** *K. pneumoniae* harbouring bla_{DHA-1}. Lanes: M, bacteriophage lambda ladder PFGE marker (New England Biolabs, N0340S); 1, clone KpA; 2–6, clone KpB; 7, clone KpG; 8–9, clone KpJ; 10, clone KpK; 11–18, clone KpF; 19, clone KpC; 20, clone KpD; 21–23, clone KpE; 24, clone KpH; 25–26, clone KpI. **b)** *K. oxytoca* harbouring bla_{DHA-1}. Lanes: M, lambda ladder PFGE marker; 1–3, clone KoA; 4, clone KoB. **c)** *E. coli* harbouring qAmpC genes. Lanes: M, lambda ladder PFGE marker; 1, clone EcA (bla_{CMY-2}); 2, clone EcB (bla_{CMY-2}); 3, clone EcC (bla_{CMY-2}); 4, clone EcD (bla_{DHA-1})

(80 %) (Table 2), as commonly observed [2]. Most isolates (94 %, 47/50; DHA-1 producers) co-expressed ESBLs belonging mainly to SHV (SHV-5, -12, -90, -145; $n=19$) types and/or OXA-1 ($n=9/n=18$), but also CTX-M-32 ($n=1$) (Table 2). This study is the first, to the authors' knowledge, describing the co-production of DHA-1 and CTX-M-32 or highly related SHV-types. Although in Portugal an endemic situation of ESBL-encoding plasmids and clones [32, 47–50] has been implicated in the high rates of resistance to broad-spectrum cephalosporins (one of the highest in Europe) [51, http://www.ecdc.europa.eu/en/publications/surveillance_reports/arhai/Pages/arhai.aspx], the association of qAmpCs and ESBLs in different *Enterobacteriaceae* species reflects a more complex and problematic epidemiological situation regarding cephalosporin resistance in Portugal.

In contrast to most studies, the transferability of bla_{qAmpC} genes was only achieved in 2 % of the isolates (one CMY-2 producer) [2, 3], which could be justified by the loss of *tra* genes (involved in plasmid conjugative transfer) or the mobilisation of bla_{qAmpC} to the bacterial chromosome through integrative and conjugative elements (ICEs) [20], but we cannot discharge the need for other experimental conditions [52]. Although our results suggest that the dissemination of bla_{qAmpC} in Portugal occurred mainly through clonal spread, the presence of $bla_{\text{DHA-1}}$ genes in different species highlights the involvement of lateral genetic transfer elements [3, 20].

This study constitutes the first report describing the molecular epidemiology and long-term dissemination of qAmpC enzymes among *Enterobacteriaceae* (frequently co-producing ESBLs) in Portuguese clinical settings, at least since 2003. In contrast with other European countries, DHA-1 was the most frequent variant mainly associated with the spread of several *Klebsiella* spp. clones. The recent implementation of an epidemiological surveillance system of antimicrobial resistance and a stricter control on antibiotic use in Portuguese clinical institutions (http://www.dgs.pt/upload/membro.id/ficheiros/i018596.pdf) should contribute to the improvement of measures to contain the emergence and spread of these multidrug-resistant strains. Finally, deeper studies are needed in order to address the contribution of mobile genetic elements and/or particular lineages for the dissemination of acquired AmpC β-lactamases in Portugal.

Acknowledgements We thank Helena Ramos (Hospital Geral de Santo António, Porto, Portugal), Graça Ribeiro and Clementina Vital (Hospitais da Universidade de Coimbra, Coimbra, Portugal), José L. Grañeda and Conceição Faria (Centro Hospitalar Cova da Beira, Covilhã, Portugal), and Serviço de Patologia Clínica of Centro Hospitalar Tondela-Viseu (Viseu, Portugal) for kindly providing the strains.

The present work was funded by Fundação para a Ciência e Tecnologia (FCT), which belongs to the Ministry of Science, Technology and Innovation of Portugal (through grant nos. PEst-C/EQB/LA0006/2011, EXPL/DTP-EPI/0196/2012 and FCOMP-01-0124-FEDER-027745), and Fundação Ensino e Cultura Fernando Pessoa. T.G.R. was supported by a fellowship from the FCT (SFRH/BD/75752/2011) and Â.N. by a Marie Curie Intra European Fellowship within the European Commission's 7th Framework Programme (PIEF-GA-2009-255512).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. D'Andrea MM, Nucleo E, Luzzaro F, Giani T, Migliavacca R, Vailati F, Kroumova V, Pagani L, Rossolini GM (2006) CMY-16, a novel acquired AmpC-type β-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from Northern Italy. Antimicrob Agents Chemother 50(2):618–624
2. Jacoby GA (2009) AmpC β-lactamases. Clin Microbiol Rev 22(1):161–182
3. Mata C, Miró E, Alvarado A, Garcillán-Barcia MP, Toleman M, Walsh TR, de la Cruz F, Navarro F (2012) Plasmid typing and genetic context of AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *ampC* genes: findings from a Spanish hospital 1999–2007. J Antimicrob Chemother 67(1):115–122
4. Miró E, Agüero J, Larrosa MN, Fernández A, Conejo MC, Bou G, González-López JJ, Lara N, Martínez-Martínez L, Oliver A, Aracil B, Oteo J, Pascual A, Rodríguez-Baño J, Zamorano L, Navarro F (2013) Prevalence and molecular epidemiology of acquired AmpC β-lactamases and carbapenemases in *Enterobacteriaceae* isolates from 35 hospitals in Spain. Eur J Clin Microbiol Infect Dis 32(2):253–259
5. Iabadene H, Messai Y, Ammari H, Alouache S, Verdet C, Bakour R, Arlet G (2009) Prevalence of plasmid-mediated AmpC β-lactamases among *Enterobacteriaceae* in Algiers hospitals. Int J Antimicrob Agents 34(4):340–342
6. Cejas D, Fernández Canigia L, Quinteros M, Giovanakis M, Vay C, Lascialandare S, Mutti D, Pagniez G, Almuzara M, Gutkind G, Radice M (2012) Plasmid-encoded AmpC (pAmpC) in *Enterobacteriaceae*: epidemiology of microorganisms and resistance markers. Rev Argent Microbiol 44(3):182–186
7. Simner PJ, Zhanel GG, Pitout J, Tailor F, McCracken M, Mulvey MR, Lagacé-Wiens PR, Adam HJ, Hoban DJ; Canadian Antimicrobial Resistance Alliance (CARA) (2011) Prevalence and characterization of extended-spectrum β-lactamase- and AmpC β-lactamase-producing *Escherichia coli*: results of the CANWARD 2007–2009 study. Diagn Microbiol Infect Dis 69(3):326–334
8. Baudry PJ, Mataseje L, Zhanel GG, Hoban DJ, Mulvey MR (2009) Characterization of plasmids encoding CMY-2 AmpC β-lactamases from *Escherichia coli* in Canadian intensive care units. Diagn Microbiol Infect Dis 65(4):379–383
9. Li Y, Li Q, Du Y, Jiang X, Tang J, Wang J, Li G, Jiang Y (2008) Prevalence of plasmid-mediated AmpC β-lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-type AmpC β-lactamase resistance in China. J Clin Microbiol 46(4):1317–1321
10. Ding H, Yang Y, Lu Q, Wang Y, Chen Y, Deng L, Wang A, Deng Q, Zhang H, Wang C, Liu L, Xu X, Wang L, Shen X (2008) The prevalence of plasmid-mediated AmpC β-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from five children's hospitals in China. Eur J Clin Microbiol Infect Dis 27(10):915–921
11. Jørgensen RL, Nielsen JB, Friis-Møller A, Fjeldsøe-Nielsen H, Schønning K (2010) Prevalence and molecular characterization of clinical isolates of *Escherichia coli* expressing an AmpC phenotype. J Antimicrob Chemother 65(3):460–464
12. Corvec S, Crémet L, Leprince C, Dauvergne S, Reynaud A, Lepelletier D, Caroff N (2010) Epidemiology of *Escherichia coli*

clinical isolates producing AmpC plasmidic β-lactamase during a 5-year period in a French teaching Hospital. Diagn Microbiol Infect Dis 67(3):277–281
13. Matsumura Y, Yamamoto M, Higuchi T, Komori T, Tsuboi F, Hayashi A, Sugimoto Y, Hotta G, Matsushima A, Nagao M, Takakura S, Ichiyama S (2012) Prevalence of plasmid-mediated AmpC β-lactamase-producing *Escherichia coli* and spread of the ST131 clone among extended-spectrum β-lactamase-producing *E. coli* in Japan. Int J Antimicrob Agents 40(2):158–162
14. Yamasaki K, Komatsu M, Abe N, Fukuda S, Miyamoto Y, Higuchi T, Ono T, Nishio H, Sueyoshi N, Kida K, Satoh K, Toyokawa M, Nishi I, Sakamoto M, Akagi M, Nakai I, Kofuku T, Orita T, Wada Y, Jikimoto T, Kinoshita S, Miyamoto K, Hirai I, Yamamoto Y (2010) Laboratory surveillance for prospective plasmid-mediated AmpC β-lactamases in the Kinki region of Japan. J Clin Microbiol 48(9):3267–3273
15. Song W, Kim JS, Kim HS, Yong D, Jeong SH, Park MJ, Lee KM (2006) Increasing trend in the prevalence of plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking chromosomal *ampC* gene at a Korean university hospital from 2002 to 2004. Diagn Microbiol Infect Dis 55(3):219–224
16. Song W, Lee H, Lee K, Jeong SH, Bae IK, Kim JS, Kwak HS (2009) CTX-M-14 and CTX-M-15 enzymes are the dominant type of extended-spectrum β-lactamase in clinical isolates of *Escherichia coli* from Korea. J Med Microbiol 58(Pt 2):261–266
17. Voets GM, Platteel TN, Fluit AC, Scharringa J, Schapendonk CM, Stuart JC, Bonten MJ, Hall MA; National ESBL Surveillance Working Group (2012) Population distribution of beta-lactamase conferring resistance to third-generation cephalosporins in human clinical *Enterobacteriaceae* in the Netherlands. PLoS One 7(12):e52102
18. Naseer U, Haldorsen B, Simonsen GS, Sundsfjord A (2010) Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. Clin Microbiol Infect 16(2):171–178
19. Mata C, Miró E, Rivera A, Mirelis B, Coll P, Navarro F (2010) Prevalence of acquired AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *ampC* genes at a Spanish hospital from 1999 to 2007. Clin Microbiol Infect 16(5):472–476
20. Mata C, Navarro F, Miró E, Walsh TR, Mirelis B, Toleman M (2011) Prevalence of SXT/R391-like integrative and conjugative elements carrying bla_{CMY-2} in *Proteus mirabilis*. J Antimicrob Chemother 66(10): 2266–2270
21. Adler H, Fenner L, Walter P, Hohler D, Schultheiss E, Oezcan S, Frei R (2008) Plasmid-mediated AmpC β-lactamases in *Enterobacteriaceae* lacking inducible chromosomal *ampC* genes: prevalence at a Swiss university hospital and occurrence of the different molecular types in Switzerland. J Antimicrob Chemother 61(2):457–458
22. Singtohin S, Chanawong A, Lulitanond A, Sribenjalux P, Auncharoen A, Kaewkes W, Songsri J, Pienthaweechai K (2010) CMY-2, CMY-8b, and DHA-1 plasmid-mediated AmpC β-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from a university hospital, Thailand. Diagn Microbiol Infect Dis 68(3):271–277
23. Deshpande LM, Jones RN, Fritsche TR, Sader HS (2006) Occurrence of plasmidic AmpC type β-lactamase-mediated resistance in *Escherichia coli*: report from the SENTRY Antimicrobial Surveillance Program (North America, 2004). Int J Antimicrob Agents 28(6):578–581
24. Alvarez M, Tran JH, Chow N, Jacoby GA (2004) Epidemiology of conjugative plasmid-mediated AmpC β-lactamases in the United States. Antimicrob Agents Chemother 48(2):533–537
25. Vanwynsberghe T, Verhamme K, Raymaekers M, Cartuyvels R, Boel A, De Beenhouwer H (2007) Outbreak of *Klebsiella pneumoniae* strain harbouring an AmpC (DHA-1) and a bla_{SHV-11} in a Belgian hospital, August–December 2006. Euro Surveill 12(5):pii=3110
26. Illiaquer M, Caroff N, Bémer P, Aubin GG, Juvin ME, Lepelletier D, Reynaud A, Corvec S (2012) Occurrence and molecular characterization of *Klebsiella pneumoniae* ST37 clinical isolates producing plasmid-mediated AmpC recovered over a 3-year period. Diagn Microbiol Infect Dis 74(1):95–97
27. Roche C, Boo TW, Walsh F, Crowley B (2008) Detection and molecular characterisation of plasmidic AmpC beta-lactamases in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Dublin, Ireland. Clin Microbiol Infect 14(6):616–618
28. D'Andrea MM, Literacka E, Zioga A, Giani T, Baraniak A, Fiett J, Sadowy E, Tassios PT, Rossolini GM, Gniadkowski M, Miriagou V (2011) Evolution and spread of a multidrug-resistant *Proteus mirabilis* clone with chromosomal AmpC-type cephalosporinases in Europe. Antimicrob Agents Chemother 55(6):2735–2742
29. Ktari S, Arlet G, Verdet C, Jaoua S, Kachrid A, Ben Redjeb S, Mahjoubi-Rhimi F, Hammami A (2009) Molecular epidemiology and genetic environment of acquired bla_{ACC-1} in *Salmonella enterica* serotype Livingstone causing a large nosocomial outbreak in Tunisia. Microb Drug Resist 15(4):279–286
30. Lee JH, Jung HI, Jung JH, Park JS, Ahn JB, Jeong SH, Jeong BC, Lee JH, Lee SH (2004) Dissemination of transferable AmpC-type beta-lactamase (CMY-10) in a Korean hospital. Microb Drug Resist 10(3):224–230
31. Kim JY, Song JS, Bak SH, Cho YE, Kim DW, Jeong SH, Park YM, Lee KJ, Lee SH (2004) Dissemination of *Escherichia coli* producing AmpC-type beta-lactamase (CMY-11) in Korea. Int J Antimicrob Agents 24(4):320–326
32. Machado E, Coque TM, Cantón R, Novais Â, Sousa JC, Baquero F, Peixe L; Portuguese Resistance Study Group (2007) High diversity of extended-spectrum β-lactamases among clinical isolates of *Enterobacteriaceae* from Portugal. J Antimicrob Chemother 60(6): 1370–1374
33. Mirelis B, Rivera A, Miró E, Mesa RJ, Navarro F, Coll P (2006) A simple phenotypic method for differentiation between acquired and chromosomal AmpC β-lactamases in *Escherichia coli*. Enferm Infecc Microbiol Clin 24(6):370–372
34. Koeck JL, Arlet G, Philippon A, Basmaciogullari S, Thien HV, Buisson Y, Cavallo JD (1997) A plasmid-mediated CMY-2 β-lactamase from an Algerian clinical isolate of *Salmonella senftenberg*. FEMS Microbiol Lett 152(2):255–260
35. Jarlier V, Nicolas MH, Fournier G, Philippon A (1988) Extended broad-spectrum β-lactamases conferring transferable resistance to newer beta-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev Infect Dis 10(4):867–878
36. Verdet C, Benzerara Y, Gautier V, Adam O, Ould-Hocine Z, Arlet G (2006) Emergence of DHA-1-producing *Klebsiella* spp. in the Parisian region: genetic organization of the *ampC* and *ampR* genes originating from *Morganella morganii*. Antimicrob Agents Chemother 50(2):607–617
37. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33(9):2233–2239
38. Clermont O, Bonacorsi S, Bingen E (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66(10):4555–4558
39. Johnson JR, Johnston B, Clabots C, Kuskowski MA, Castanheira M (2010) *Escherichia coli* sequence type ST131 as the major cause of serious multidrug-resistant *E. coli* infections in the United States. Clin Infect Dis 51(3):286–294
40. Song S, Lee EY, Koh EM, Ha HS, Jeong HJ, Bae IK, Jeong SH (2009) Antibiotic resistance mechanisms of *Escherichia coli* isolates from urinary specimens. Korean J Lab Med 29(1):17–24
41. Arpin C, Quentin C, Grobost F, Cambau E, Robert J, Dubois V, Coulange L, André C; Scientific Committee of ONERBA (2009) Nationwide survey of extended-spectrum β-lactamase-producing

Enterobacteriaceae in the French community setting. J Antimicrob Chemother 63(6):1205–1214
42. Johnson JR, Stell AL (2000) Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis 181(1): 261–272
43. Moreno E, Prats G, Planells I, Planes AM, Pérez T, Andreu A (2006) Characterization of *Escherichia coli* isolates derived from phylogenetic groups A and B1 causing extraintestinal infection. Enferm Infecc Microbiol Clin 24(8):483–489
44. Cooke NM, Smith SG, Kelleher M, Rogers TR (2010) Major differences exist in frequencies of virulence factors and multidrug resistance between community and nosocomial *Escherichia coli* bloodstream isolates. J Clin Microbiol 48(4):1099–1104
45. Rodríguez-Baño J, Mingorance J, Fernández-Romero N, Serrano L, López-Cerero L, Pascual A; ESBL-REIPI Group (2012) Virulence profiles of bacteremic extended-spectrum β-lactamase-producing *Escherichia coli*: association with epidemiological and clinical features. PLoS One 7(9):e44238
46. Sidjabat HE, Paterson DL, Qureshi ZA, Adams-Haduch JM, O'Keefe A, Pascual A, Rodríguez-Baño J, Doi Y (2009) Clinical features and molecular epidemiology of CMY-type beta-lactamase-producing *Escherichia coli*. Clin Infect Dis 48(6): 739–744
47. Cantón R, Novais Â, Valverde A, Machado E, Peixe L, Baquero F, Coque TM (2008) Prevalence and spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Europe. Clin Microbiol Infect 14(Suppl 1):144–153
48. Giamarellou H (2005) Multidrug resistance in Gram-negative bacteria that produce extended-spectrum beta-lactamases (ESBLs). Clin Microbiol Infect 11(Suppl 4):1–16
49. Mendonça N, Leitão J, Manageiro V, Ferreira E, Caniça M (2007) Spread of extended-spectrum beta-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. Antimicrob Agents Chemother 51(6):1946–1955
50. Machado E, Coque TM, Cantón R, Baquero F, Sousa JC, Peixe L; Portuguese Resistance Study Group (2006) Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing *Enterobacteriaceae* strains containing the *aac(6')-Ib-cr* gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. Antimicrob Agents Chemother 50(9):3220–3221
51. Bouchillon SK, Johnson BM, Hoban DJ, Johnson JL, Dowzicky MJ, Wu DH, Visalli MA, Bradford PA (2004) Determining incidence of extended spectrum beta-lactamase producing *Enterobacteriaceae*, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* in 38 centres from 17 countries: the PEARLS study 2001–2002. Int J Antimicrob Agents 24(2):119–124
52. Forns N, Baños RC, Balsalobre C, Juárez A, Madrid C (2005) Temperature-dependent conjugative transfer of R27: role of chromosome- and plasmid-encoded Hha and H-NS proteins. J Bacteriol 187(12):3950–3959
53. Grundmann H, Livermore DM, Giske CG, Cantón R, Rossolini GM, Campos J, Vatopoulos A, Gniadkowski M, Toth A, Pfeifer Y, Jarlier V, Carmeli Y; CNSE Working Group (2010) Carbapenem-non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Euro Surveill 15(46):pii=19711

Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country

Teresa Gonçalves Ribeiro[1], Ângela Novais[1], Carla Rodrigues[1], Ricardo Nascimento[1], Francisco Freitas[2,3,4], Elisabete Machado[1,2], and Luísa Peixe[1*]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; [3]Centro Hospitalar Tondela, Viseu, Portugal; [4]Escola Superior de Saúde Dr. Lopes Dias, Instituto Politécnico de Castelo Branco, Portugal.

Journal of Antimicrobial Chemotherapy

Manuscript ID: JAC-2016-1045 (*under editorial revision*); June 2016

Insights into clinical Enterobacteriaceae producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country

T. G. Ribeiro[1], Â. Novais[1], C. Rodrigues[1], R. Nascimento[1], F. Freitas[2,3,4], E. Machado[1,2], L. Peixe[1*]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;

[2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal;

[3]Centro Hospitalar Tondela-Viseu, Portugal;

[4]Escola Superior de Saúde Dr. Lopes Dias, Instituto Politécnico de Castelo Branco, Portugal.

Running title: Molecular epidemiology of qAmpCs among clinical Enterobacteriaceae.

Keywords: plasmid spread, ESBL, ST11, DHA-1, CMY-2

***Corresponding author:**

Luísa Vieira Peixe, Associate Professor

UCIBIO-REQUIMTE. Lab. of Microbiology

Faculty of Pharmacy, University of Porto

Rua Jorge Viterbo Ferreira 228, 4050-313 Porto. Portugal

Direct: (+351) 220 428 580

E-mail: lpeixe@ff.up.pt, lpeixe@gmail.com

SYNOPSIS

OBJECTIVES: In order to assess the contribution of acquired AmpC (qAmpC) β-lactamases to the increasing resistance rates to third-generation cephalosporins in Portugal, we performed a longitudinal analysis of clinical Enterobacteriaceae isolates over a 12–year period.

METHODS: We analyzed 2107 Enterobacteriaceae non-susceptible to broad-spectrum cephalosporins from species with/without (n=123/n=1984) inducible chromosomal AmpC ($_{i}$Cr-AmpC) genes from nine different clinical institutions (2002-2013). Standard methods were used for bacterial identification, antibiotic susceptibility testing, identification of acquired *bla* and PMQR genes (PCR, sequencing), clonal analysis (PFGE, MLST), bla_{qAmpC} location, and plasmid characterization (S1-/I-*Ceu*I-PFGE, replicon typing, hybridization).

RESULTS: A low (5%, 99/2107) and stable occurrence of qAmpCs was observed throughout time, corresponding to DHA-1 (82%), CMY-2 (17%) or DHA-6 (1%). DHA-1 persisted throughout time (2006-2013) in different institutions, species and clones [n=81/29 PFGE-types; predominance of Kp ST11 and ST1380], and particular plasmids (IncR or IncHI2). The increase of CMY-2 in the last years (2009-2013) was linked to multiple *Escherichia coli* clones and to IncI1 plasmids. Most qAmpC producers (66%; mainly DHA-1) co-expressed ESBLs and were multidrug-resistant.

CONCLUSIONS: We observed a low rate and a stable occurrence of qAmpC throughout time, where the dominance of DHA-1 and the later emergence of CMY-2 contrast with the scenario of most European countries. Furthermore, the identification of genetic backgrounds different from those responsible for ESBL spread might have

contributed to the differential expansion of ESBL and qAmpC, which deserves further investigation.

INTRODUCTION

The worldwide contemporary epidemiology of Enterobacteriaceae resistant to broad-spectrum cephalosporins seems to be largely dominated by a greater expansion of extended-spectrum β-lactamases (ESBL) than acquired AmpC (qAmpC) β-lactamases. However, the reasons behind this differential occurrence are poorly understood. It is possible that the occurrence and clinical impact of Enterobacteriaceae producing qAmpC β-lactamases are currently underestimated, since qAmpC production is frequently masked by other mechanisms of resistance.[1,2] Moreover, most studies are limited to species lacking inducible chromosomal AmpC genes ($_{i}$Cr-AmpCs), short periods of time and/or single clinical institutions.[2,3] The most prevalent qAmpCs worldwide are CMY-2 (disseminated in different settings) and DHA-1 (mainly from nosocomial outbreaks).[2-5] In Portugal, ESBL-producing Enterobacteriaceae are endemic in different niches and have been thoroughly characterized at the molecular level (population structure, genetic platforms),[6,7] but the trends of qAmpC producers are still underexplored.[8]

We aimed to perform a comprehensive and longitudinal analysis on the occurrence, diversity and genetic backgrounds of qAmpCs among Enterobacteriaceae species recovered from both hospital and community clinical settings in Portugal over a 12-year period. This data will be of relevance to understand the contribution of qAmpC to the increasing resistance rates to third-generation cephalosporins in our country.

MATERIAL AND METHODS

Bacterial isolates

We collected 2107 Enterobacteriaceae isolates non-susceptible to broad-spectrum cephalosporins, recovered from patients attending seven hospitals (76%; HA-HG) and two community laboratories (24%; CLA and CLB) over a 12-year period in Portugal (2002-2013; North and Centre regions). They corresponded to species with [n=123; 82 *Enterobacter* spp., 21 *Serratia marcescens*, 14 *Morganella morganii*, 6 *Citrobacter* spp.] and without [n=1309; 753 *Escherichia coli* (Ec), 513 *Klebsiella pneumoniae* (Kp), 28 *Klebsiella oxytoca*, 12 *Proteus mirabilis*, 3 *Klebsiella variicola*] ¡Cr-AmpCs genes. The remaining 675 isolates [480 Ec, 141 Kp, 27 *K. oxytoca*, 27 *P. mirabilis*] from our previous publication were included for detailed epidemiological characterization and analysis of temporal shifts.[8] Bacterial identification and antibiotic susceptibility testing were performed as described.[8,9]

Characterization of qAmpC and other antibiotic resistance genes

Presumptive qAmpC producers were screened by phenotypic criteria[8] and qAmpC genes were further identified by PCR and sequencing.[8] Genes coding for ESBLs or carbapenemases were searched as described.[8,9] Plasmid-mediated quinolone resistance [PMQR; *qnr* and *aac(6')-Ib-cr*] genes were also investigated.[8-10]

Molecular characterization of isolates carrying qAmpC genes

Detection of *K. pneumoniae* and *E. coli* phylogroups, and *E. coli* O25b-ST131 were performed as described.[6,11] Clonal relatedness was established by *Xba*I-PFGE and

MLST for representative (different PFGE-patterns) *K. pneumoniae* and B2- and D-*E. coli* isolates (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html; http://bigsdb.pasteur.fr/klebsiella/primers_used.html).[9] Capsule molecular typing for ST11 *K. pneumoniae* was performed by PCR and sequencing of *wzi* gene.[12] The location of bla_{qAmpC} was assessed by hybridization of S1- or I-*Ceu*I-digested genomic DNA.[9] Plasmids carrying bla_{qAmpC} were characterized by replicon typing (PCR, sequencing and hybridization) and subtyping (http://pubmlst.org/plasmid/primers/incI1.shtml).[9,13] The bla_{qAmpC} genetic environment (ca. 20 kb) was investigated by PCR mapping and sequencing in representative isolates from different species and time periods.[10]

Statistical analysis

Statistical significance for comparison of proportions was calculated by the χ^2 test or Fisher exact test using IBM SPSS Statistics 22.0 software (*P* values of <0.05 were considered statistically significant).

RESULTS AND DISCUSSION

Persistence of DHA-1 and recent increase of CMY-2

We observed an overall occurrence of qAmpC-producing Enterobacteriaceae of 5% (99/2107), lower than that reported for ESBL-producing isolates in our country (http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-europe-2014.pdf).

The number of qAmpC producers was relatively stable throughout time, although there are variations in the number of institutions analyzed in specific years. Isolates produced mostly DHA-1 (82%), CMY-2 (17%) or DHA-6 (1%; differing from DHA-1 in A226T mutation) variably throughout time, and belonged to species with (16%) or without (84%) ¡Cr-AmpC genes from urine (44%) or sputum (20%) (Table 1).

DHA-1 represented 96% of the qAmpCs identified in 2002-2008 *versus* 55% in 2009-2013 (p<0.000001), mainly in hospital-acquired infections (93%; 75/81) and in different species (mostly Kp). CMY-2 represented 4% of the qAmpCs in 2002-2008 *versus* 45% in 2009-2013 (p<0.000001), identified in community (53%) or hospitalized (47%) patients exclusively among species without ¡Cr-AmpC genes (Table 1). DHA-6, reported here for the first time in Portugal, was only identified in one Kp (2013; CLA). The dominance and persistence of DHA-1 throughout time and in such a high diversity of species differs from the apparent predominance of CMY-2-producing *E. coli* in Europe,[2,3] and is only comparable to some Asian countries.[5,8,14]

Co-carriage of qAmpC and other antibiotic resistance genes

The majority of DHA-1 producers (96%; *Klebsiella* spp., Ec, *E. cloacae* complex) co-expressed OXA-1 with/without ESBLs (28%, mostly SHV-12/42%), and less frequently diverse SHV-types (26%) (Table 1). CTX-M-1 or CTX-M-15 were occasionally identified among CMY-2 producers (12%) (Table 1). Carbapenemase genes were not detected, despite 13 *Enterobacter* spp. (mostly *Enterobacter hormaechei*) and 9 Kp isolates producing DHA were resistant to ertapenem (25%) and/or imipenem (1% and 2%, respectively), suggesting the involvement of permeability defects.[2,5] DHA-1-producers also harboured frequently PMQR genes

[*qnrB4*, 100%; *aac-6'-Ib-cr*, 86%; *aac-6'-Ib-cr* and *qnrS1*, 8%], with the high occurrence of *qnrB4* and *aac-6'-Ib-cr* explained by their genetic vicinity to the bla_{DHA-1}.[4,15] The frequent detection of isolates co-expressing qAmpCs and ESBLs or PMQR, and exhibiting a multidrug-resistance (MDR) phenotype, highlights for limited therapeutic options in infections involving qAmpC-producing Enterobacteriaceae.

Identification of epidemic and international clones

DHA producers were clonally diverse (n=82; 30 PFGE-types), but two major MDR Kp lineages presumptively involved in nosocomial outbreaks were detected: (i) ST11 [n=25, 30%; 3 PFGE-types exhibiting *wzi*75 (n=24/25) and 1 PFGE-type with *wzi*24-K24 (n=1/25)] producing DHA-1 and OXA-1, or DHA-6, and detected in different hospitals and community clinical institutions over time; (ii) ST1380 (n=14, 17%; 1 PFGE-type), producing DHA-1 and diverse SHV-ESBLs (Table 1). The remaining DHA-1-producing Kp (n=13, 11 PFGE-types) belonged to other sporadic clones (Table 1). The epidemic lineage ST11-*wzi*75 was previously associated with the co-production of DHA-1 and CTX-M-15 in Asian countries, but capsule types other than *wzi*75 have been apparently linked to the expansion of predominant KPC enzymes within ST11 isolates.[16] (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_isolates_public&page=query). Interestingly, this lineage persisted along the 12-year period in different institutions although it was not detected among ESBL-producing *K. pneumoniae* circulating in the same hospitals and periods of time.[7]

Remarkably, three DHA-1 producing *K. variicola* isolates were identified during 2007 in different wards (paediatrics, medicine) from the same hospital, though this

167 species is more usually found in the environment or the gastrointestinal tract of animals
168 (bovines).[17] DHA-1-producing *K. oxytoca,* Ec and *E. hormaechei* isolates were clonally
169 diverse, although two clones of *E. hormaechei* were identified and linked to a
170 nosocomial outbreak in HB over a one-year period (Table 1).

171 We observed a high diversity of CMY-2-producing Ec isolates belonging to
172 phylogroups B1 (n=7; 44%), A (n=4; 25%), $B2_3$ (n=3; 19%) or D (n=2; 12%) (Table 1).
173 The higher proportion of A and B1 isolates carrying bla_{CMY-2} suggests a possible origin
174 from non-clinical reservoirs,[18,19] as observed more recently for ESBL producers.[6] The
175 detection of CMY-2 amongst the widespread B2-ST131 (n=2), B2-ST95 (n=1), D-ST57
176 (n=1) and D-ST117 (n=1) clones, frequently detected as ESBL producers in the same
177 clinical institutions, might reflect recent acquisition events.[6]

178

179 **Location of bla_{qAmpC} on specific plasmid types in different species**

180 The presence of plasmids was variable in number (1-5 plasmids) and size (20-
181 450 kb) (Table 1). The bla_{DHA-1} gene was mostly identified within ca. 175-450 kb
182 IncHI2 (n=43/81) or within ca. 40-180 kb IncR (n=31/81) plasmids, successfully
183 transferred between different *K. pneumoniae* clones and diverse Enterobacteriaceae
184 species (Table 1). The bla_{DHA-6} was located at a 95 kb IncR plasmid in ST11-Kp clone,
185 suggesting possible *in vivo* evolution of bla_{DHA} (Table 1). Thus, our study highlights the
186 role of IncHI2 or IncR plasmid types in the wide dissemination of DHA-1 among
187 different Enterobacteriaceae species and clones.

188 The bla_{CMY-2} was variably linked to different IncI1 plasmid types [60-200 kb
189 IncI1/ST12 (n=2; HD and CLA), 90 kb IncI1/ST43 (n=1; HA)], 80 kb IncK (n=2) or

150 kb IncA/C (n=1) in Ec isolates, as previously reported,[1,3] or to the chromosome of *P. mirabilis* (n=1) (Table 1).

Characterization of bla_{qAmpC} genetic environment

The genetic environment of bla_{DHA} genes was similar to that previously described in Kp plasmid pRBDHA (GenBank accession number AJ971343), with variations in the 5' region (e.g., presence of *catB3*, *arr3* and/or bla_{OXA-1} in the class 1 integron). In contrast, bla_{CMY-2} was linked to the transposon-like element IS*Ecp1* (n=12; IS*Ecp1*/ΔIS*Ecp1*-bla_{CMY-2}-*blc*-*sugE*), as described.[1,3] The *int* gene from the SXT/R391 integrative and conjugative element (ICE) family was identified in the *P. mirabilis* isolate harboring a chromosomally-encoded CMY-2, supporting the involvement of ICEs in the mobilization of bla_{CMY-2} (data not shown).[20]

CONCLUSIONS

This work constitutes one of the most comprehensive and detailed studies analysing the molecular epidemiology of clinical Enterobacteriaceae producing qAmpC during a large period of time (12 years). We confirmed a low (5%) and apparently stable occurrence of qAmpC (DHA-1 and CMY-2) throughout time, in which the predominance of DHA-1 and later emergence of CMY-2 contrasts with most European countries. Finally, we hypothesized that the identification of genetic backgrounds differing from those responsible for ESBL spread in the same institutions and time periods in our country, might have contributed to the differential expansion of ESBL and qAmpC in Portugal.

FUNDING

This work received financial support from the European Union (FEDER funds) through Programa Operacional Fatores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through projects UID/Multi/04378/2013 and UID/Multi/04546/2013. T. G. R., Â. N. and C. R. were supported by fellowships from FCT (SFRH/BD/75752/2011, SFRH/BPD/104927/2014 and SFRH/BD/84341/2012, respectively) through Programa Operacional Capital Humano (POPH).

ACKNOWLEDGMENTS

We are grateful to Helena Ramos (Centro Hospitalar do Porto – Hospital de Santo António, Porto, Portugal), Graça Ribeiro and Clementina Vital (Hospitais da Universidade de Coimbra, Coimbra, Portugal), José L. Grañeda and Conceição Faria (Centro Hospitalar Cova da Beira, Covilhã, Portugal), Sónia Magalhães (LabFafe, Fafe, Portugal), Carlos Mendes, Alexandra Gomes and Bibiana Marques (Medicina Laboratorial Dr. Carlos Torres, Unilabs, Porto, Portugal), José Amorim, Rui Campainha and Sofia Moniz (Botelho Moniz Análises Clínicas – BMAC, Santo Tirso, Portugal), and Serviço de Patologia Clínica do Centro Hospitalar Tondela-Viseu (Viseu, Portugal) for kindly providing the clinical isolates included in this study.

TRANSPARENCY DECLARATIONS

None to declare.

238 **REFERENCES**

239 1. Sidjabat HE, Seah KY, Coleman L *et al*. Expansive spread of IncI1 plasmids
240 carrying *bla*$_{CMY-2}$ amongst *Escherichia coli*. *Int J Antimicrob Agents* 2014; **44**:
241 203-8.

242 2. Jacoby GA. AmpC β-lactamases. *Clin Microbiol Rev* 2009; **22**: 161-82.

243 3. Mata C, Miró E, Alvarado A *et al*. Plasmid typing and genetic context of AmpC
244 β-lactamases in Enterobacteriaceae lacking inducible chromosomal *ampC* genes:
245 findings from a Spanish hospital 1999-2007. *J Antimicrob Chemother* 2012; **67**:
246 115-22.

247 4. Compain F, Decré D, Fulgencio JP *et al*. Molecular characterization of DHA-1-
248 producing *Klebsiella pneumoniae* isolates collected during a 4-year period in an
249 intensive care unit. *Diagn Microbiol Infect Dis* 2014; **80**: 159-61.

250 5. Matsumura Y, Tanaka M, Yamamoto M *et al*. High prevalence of carbapenem
251 resistance among plasmid-mediated AmpC β-lactamase-producing *Klebsiella*
252 *pneumoniae* during outbreaks in liver transplantation units. *Int J Antimicrob*
253 *Agents* 2015; **45**: 33-40.

254 6. Rodrigues C, Machado E, Pires J *et al*. Increase of widespread A, B1 and D
255 *Escherichia coli* clones producing a high diversity of CTX-M-types in a
256 Portuguese hospital. *Future Microbiol* 2015; **10**: 1125-31.

257 7. Rodrigues C, Machado E, Ramos H *et al*. Expansion of ESBL-producing
258 *Klebsiella pneumoniae* in hospitalized patients: a successful story of international

clones (ST15, ST147, ST336) and epidemic plasmids (IncR, IncFII$_K$). *Int J Med Microbiol* 2014; **304**: 1100-8.

8. Freitas F, Machado E, Ribeiro TG *et al*. Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 551-8.

9. Ribeiro TG, Novais Â, Peixe L *et al*. Atypical epidemiology of CTX-M-15 among Enterobacteriaceae from a high diversity of non-clinical niches in Angola. *J Antimicrob Chemother* 2016; **71**: 1169-73.

10. Quiroga MP, Andres P, Petroni A *et al*. Complex class 1 integrons with diverse variable regions, including *aac(6')-Ib-cr*, and a novel allele, *qnrB10*, associated with IS*CR1* in clinical enterobacterial isolates from Argentina. *Antimicrob Agents Chemother* 2007; **51**: 4466-70.

11. Brisse S, van Himbergen T, Kusters K *et al*. Development of a rapid identification method for *Klebsiella pneumoniae* phylogenetic groups and analysis of 420 clinical isolates. *Clin Microbiol Infect* 2004; **10**: 942-5.

12. Brisse S, Passet V, Haugaard AB *et al*. *wzi* gene sequencing, a rapid method for determination of capsular type for *Klebsiella* strains. *J Clin Microbiol* 2013; **51**: 4073-8.

13. García-Fernández A, Chiaretto G, Bertini A *et al*. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum β-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. *J Antimicrob Chemother* 2008; **61**: 1229-33.

14. Sheng WH, Badal RE, Hsueh PR; SMART Program. Distribution of extended-spectrum β-lactamases, AmpC β-lactamases, and carbapenemases among Enterobacteriaceae isolates causing intra-abdominal infections in the Asia-Pacific region: results of the Study for Monitoring Antimicrobial Resistance Trends (SMART). *Antimicrob Agents Chemother* 2013; **57**: 2981-8.

15. Alonso N, Miró E, Pascual V *et al*. Molecular characterisation of acquired and overproduced chromosomal bla_{AmpC} in *Escherichia coli* clinical isolates. *Int J Antimicrob Agents* 2016; **47**: 62-8.

16. Bowers JR, Kitchel B, Driebe EM *et al*. Genomic analysis of the emergence and rapid global dissemination of the clonal group 258 *Klebsiella pneumoniae* pandemic. *PLoS One* 2015; **10**: e0133727.

17. Holt KE, Wertheim H, Zadoks RN *et al*. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proc Natl Acad Sci U S A* 2015; **112**: E3574-81.

18. Guo YF, Zhang WH, Ren SQ *et al*. IncA/C plasmid-mediated spread of CMY-2 in multidrug-resistant *Escherichia coli* from food animals in China. *PLoS One* 2014; **9**: e96738.

19. Mataseje LF, Neumann N, Crago B *et al*. Characterization of cefoxitin-resistant *Escherichia coli* isolates from recreational beaches and private drinking water in Canada between 2004 and 2006. *Antimicrob Agents Chemother* 2009; **53**: 3126-30.

303 20. Mata C, Navarro F, Miró E *et al*. Prevalence of SXT/R391-like integrative and
304 conjugative elements carrying *bla*$_{CMY-2}$ in *Proteus mirabilis*. *J Antimicrob*
305 *Chemother* 2011; **66**: 2266-70.

306 **Table 1. Epidemiological data of qAmpC-producing Enterobacteriaceae recovered from Portuguese clinical settings (2002-2013).**

YEAR	qAmpC (no.)	Specie (no.)	PFGE-type /MLST/PhG[a] (no.)	Clinical Institutions[b]	Sample type (no.)	Plasmid content [size (kb) (Inc family)][c]	Co-production of ESBLs (no.)	Presence of PMQR genes (no.)	Non-β-lactam resistance phenotype[e]
2003	DHA-1 (7)	*K. pneumoniae* (3)	Kp1/ST1380	HA	Urine (2), Sputum (1)	290-310 (HI2), 35-190	SHV-5 (2), SHV-90 (1)	*qnrB4*, *aac-6'-Ib-cr*	KAN, (AMK), GEN, NET, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
		K. oxytoca (2)	Ko1/-	HA	Urine	295 (HI2), 136, 92	SHV-12	*qnrB4*, *aac-6'-Ib-cr*, *qnrS1*	KAN, (AMK), GEN, NET, TOB, STR, (TET), (CHL), NAL, CIP, SUL, TMP
		E. hormaechei (2)	Eh1/-	HG	Unknown	370 (HI2), 60	SHV-12	*qnrB4*, *aac-6'-Ib-cr*	KAN, (AMK), GEN, NET, TOB, STR, TET, CHL, (NAL), (CIP), SUL, TMP
2004	DHA-1 (14)	*K. pneumoniae* (13)	Kp1/ST1380 (11)	HA	Sputum (5),Catheter (2), Exudate (1), Blood (1), Unknown (2)	290-310 (HI2), 35-190	SHV-5 (4), SHV-12 (4), SHV-90 (4)	*qnrB4*, *aac-6'-Ib-cr*	KAN, (AMK), GEN, NET, TOB, STR, TET, CHL, (NAL), (CIP), SUL, TMP
			Kp2/ST1874 (1)	HA	Urine	ND	SHV-12	*qnrB4*, *aac-6'-Ib-cr*	KAN, GEN, NET, TOB, STR, TET, CHL, SUL, TMP
			Kp3/ST443 (1)	CLB	Urine	180	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, NAL, CIP, SUL
		K. oxytoca (1)	Ko1/-	HA	Urine	300	SHV-12	*qnrB4*, *qnrS1*	KAN, GEN, TOB, STR, NAL, CIP, SUL, TMP
2006	DHA-1 (13)	*E. hormaechei* (7)	Eh2/- (6)	HB	Urine (3), Sputum (2), Unknown (1)	420-450 (HI2), 60-140	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, (AMK), GEN, NET, TOB, TET, (CHL), (NAL), (CIP), SUL
			Eh3/- (1)	HC	Sputum	439 (HI2), 140, 60	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, AMK, GEN, NET, TOB, TET, CHL, SUL
		K. pneumoniae (5)	Kp4/ST11 (3)	HA	Unknown	50 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
			Kp5/ST11 (1)	HA	Unknown	50 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, STR, IPM, ERT, TET, CHL, NAL, CIP, SUL, TMP
			Kp6/ST1871 (1)	HB	Sputum	375 (HI2), 160	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, AMK, GEN, NET, TOB, TET, CHL, CIP, SUL
		E. kobei (1)	Ek1/-	HC	Sputum	420 (HI2), 150, 75, 55, 30	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, GEN, TOB, IPM, TET, CHL, CIP, SUL
	CMY-2 (2)	*E. coli* (2)	Ec1/-/B1 (1)	HC	Exudate	80 (K), 40	-	NP	KAN, NAL
			Ec2/-/B1 (1)	HB	Urine	150 (A/C), 140, 100, 95, 50	-	NP	KAN, GEN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
2007	DHA-1 (16)	*K. pneumoniae* (7)	Kp5/ST11 (6)	HA (3), HC (3)	Urine (2), Sputum (1), Unknown (3)	190, 40-50 (R)	OXA-1	*qnrB4* (6), *aac-6'-Ib-cr* (6), *qnrS1* (2)	KAN, (AMK), TOB, (STR), (TET), (CHL), NAL, CIP, SUL, TMP
			Kp7/ST1872 (1)	HB	Sputum	370 (HI2), 220	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, ANK, GEN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP

Table 1. *Continued*

YEAR	qAmpC (no.)	Specie (no.)	PFGE-type /MLST/PhG[a] (no.)	Clinical Institutions[b]	Sample type (no.)	Plasmid content [size (kb) (Inc family)][c]	Co-production of ESBLs (no.)	Presence of PMQR genes (no.)	Non-β-lactam resistance phenotype[e]
		E. hormaechei (5)	Eh3/-	HB (4), HC (1)	Urine (3), Sputum (1), Exudate (1)	420-455 (HI2), 90-420	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, AMK, GEN, NET, TOB, ERT, TET, CHL, (NAL), (CIP), SUL
		K. variicola (3)	Kv1/-	HB	Urine (2), Sputum (1)	225, 190, 180 (HI2)	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, (AMK), GEN, TOB, (STR), NAL, CIP, SUL, (TMP)
		E. coli (1)	Ec3/-/B1	HB	Blood	345 (HI2), 140, 40 (R), 35	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, GEN, NET, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
	CMY-2 (1)	*E. coli* (1)	Ec4/-/B1	HB	Exudate	100, 80 (K)	-	NP	KAN, AMK, STR, CIP
2008	DHA-1 (14)	*K. pneumoniae* (11)	Kp5/ST11 (4)	HA (3), HB (1)	Sputum (3), Urine (1)	190, 50 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, STR, (TET), CHL, NAL, CIP, SUL, TMP
			Kp8/ST1873 (1)	HB	Urine	50	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, GEN, TOB, TET, CHL, NAL, CIP, SUL
			Kp9/ST1224 (2)	HB	Blood, Peritoneal liquid	380 (HI2), 220, 40, 35	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, GEN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
			Kp10/ST440 (2)	HA	Urine	260 (ND), 240 (ND), 190, 60	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, (AMK), NET, TOB, (STR), (CHL), NAL, CIP, SUL, TMP
			Kp11/ST416 (1)	HA	Urine	ND	SHV-12	*qnrB4*, *aac-6'-Ib-cr*	GEN, TOB, STR, NAL, CIP, SUL, TMP
			Kp12/ND (1)	HB	Urine	365 (HI2), 55, 35	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, GEN, TOB, TET, CHL, NAL, CIP, SUL
		E. asburiae (1)	Ea1/-	HB	Sputum	370 (HI2), 120, 35	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, AMK, GEN, NET, TOB, STR, TET, CHL, SUL
		K. oxytoca (1)	Ko2/-	HA	Urine	80 (R)	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
		E. coli (1)	Ec5/-/B2	HA	Urine	ND	CTX-M-32, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, STR, TET, NAL, CIP, SUL, TMP
2010	DHA-1 (2)	*K. oxytoca* (2)	Ko3/-	HA	Urine	130, 85 (R)	OXA-1 (1)	*qnrB4* (2), *aac-6'-Ib-cr* (1)	KAN, (NET), TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
	CMY-2 (2)	*E. coli* (2)	Ec6/ST57/D_2 (1)	HA	Urine	145, 110, 80 (I1)	CTX-M-1	NP	STR, SUL, TMP
			Ec7/-/A_1 (1)	HA	Urine	160, 80 (ND), 20	CTX-M-15	NP	KAN, (NET), TOB, STR, TET, NAL, CIP, SUL, TMP
2011	DHA-1 (8)	*K. pneumoniae* (6)	Kp5/ST11 (5)	HA	Unknown	190, 40-50 (R)	OXA-1 (3)	*qnrB4* (5), *aac-6'-Ib-cr* (3)	KAN, (GEN), (TOB), (STR), (CHL), NAL, CIP, SUL, (TMP)
			Kp13/ST323 (1)	HA	Unknown	85 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, AMK, NET, TOB, CHL, NAL, CIP, SUL, TMP
		E. coli (2)	Ec8/-/A_1 (1)	HA	Unknown	85 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*, *qnrS1*	KAN, GEN, TOB, STR, NAL, CIP, SUL, TMP
			Ec9/-/B1 (1)	HA	Unknown	80 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, CHL, NAL, CIP, SUL
	CMY-2	*E. coli* (3)	Ec10/ST117/D_2 (1)	HA	Unknown	150, 60 (I1)	-	NP	STR

Table 1. *Continued*

YEAR	qAmpC (no.)	Specie (no.)	PFGE-type /MLST/PhG[a] (no.)	Clinical Institutions[b]	Sample type (no.)	Plasmid content [size (kb) (Inc family)][c]	Co-production of ESBLs (no.)	Presence of PMQR genes (no.)	Non-β-lactam resistance phenotype[e]
	(3)		Ec7/-/A_1 (1)	HA	Unknown	155, 145, 75 (I1), 20	-	NP	KAN, NET, TOB, STR, TET, NAL, CIP, SUL, TMP
			Ec11/-/B1 (1)	HA	Unknown	90, 80 (I1)	-	NP	NAL, CIP
2012	DHA-1 (6)	*K.pneumoniae* (5)	Kp5/ST11 (2)	CLA	Urine, Exudate	ND	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, (TET), CHL, NAL, CIP, SUL
			Kp14/ST11 (2)	HA	Urine, Blood	95 (R)	OXA-1	*qnrB4* (2), *aac-6'-Ib-cr* (2), *qnrS1* (1)	KAN, (NET), TOB, STR, (TET), (CHL), NAL, CIP, SUL, TMP
			Kp15/ST17 (1)	CLA	Urine	130, 75, 50 (R)	SHV-12, OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, TET, CIP, SUL
		E.coli (1)	Ec12/-/A_1	CLA	Sputum	175 (HI2), 80	OXA-1	*qnrB4*, *aac-6'-Ib-cr*, *qnrS1*	KAN, NET, TOB, TET, CHL, NAL, CIP, SUL, TMP
	CMY-2 (3)	*E.coli* (3)	Ec13/-/B1 (1)	HA	Urine	160, 90 (I1), 60	-	NP	TET
			Ec14/-/B1 (1)	HD	Urine	165, 85 (I1), 35	-	NP	KAN, GEN, TOB, STR, CHL, NAL, CIP, SUL, TMP
			Ec15/ST131/$B2_3$ (1)	HA	Urine	ND	-	NP	STR, NAL, CIP
2013	CMY-2 (6)	*E.coli* (5)	Ec16/-/A_0 (2)	CLA	Urine	155 (ND), 80	-	NP	KAN, GEN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
			Ec17/ST131/$B2_3$ (1)	CLA	Urine	200 (ND), 80, 45	-	NP	STR
			Ec18/ST95/$B2_3$ (1)	CLA	Urine	140 (ND), 130	-	NP	GEN, STR, TET, CHL, SUL, TMP
			Ec19/-/B1 (1)	HD	Urine	150, 100 (I1), 60	-	NP	KAN, GEN, NET, TOB, STR, TET, NAL, CIP, SUL, TMP
		P.mirabilis (1)	Pm1/-	CLA	Urine	-	-	NP	STR, TET, CHL, NAL, CIP, SUL, TMP
	DHA-1 (1)	*K.pneumoniae* (1)	Kp5/ST11	CLA	Urine	ND[d]	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, STR, TET, CHL, NAL, CIP, SUL, TMP
	DHA-6 (1)	*K.pneumoniae* (1)	Kp16/ST11	CLA	Urine	95 (R)	OXA-1	*qnrB4*, *aac-6'-Ib-cr*	KAN, TOB, CHL, NAL, CIP, SUL, TMP

AMK, amikacin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NET, netilmicin; STR, streptomycin; SUL, sulphonamides; TET, tetracyclines; TOB, tobramycin; TMP, trimethoprim; ND, not determined; NP, not performed.

[a]PhG group, *E. coli* phylogenetic group.

[b]Geographic locations of the hospitals (H) and community laboratories (CL) indicated: North (HA, CLA, CLB) and Centre (HB, HC, HD) regions of Portugal.

[c]Plasmids carrying bla_{qAmpC} genes are indicated by underlining.

[d]Plasmid content was not determined because of DNA self-degradation.

[e]Variable presence of a given resistance phenotype is indicated by parentheses.

Occurrence of acquired AmpC β-lactamases among *Enterobacteriaceae* from a diversity of non-clinical niches in Portugal

Teresa Gonçalves Ribeiro[1], Ângela Novais[1], Elisabete Machado[1,2], and Luísa Peixe[1*]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

(*manuscript draft*)

Occurrence of acquired AmpC β-lactamases among *Enterobacteriaceae* from a diversity of non-clinical niches in Portugal

T. G. Ribeiro[1], Â. Novais[1], E. Machado[1,2], L. Peixe[1*]

[1]UCIBIO@REQUIMTE. Departamento de Ciências Biológicas, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

Running title: qAmpCs in *Enterobacteriaceae* from non-clinical niches.

Keywords: CMY-2, healthy humans, animals, waters

***Corresponding author:**
Luísa Vieira Peixe
Associate Professor.
UCIBIO-REQUIMTE. Lab. of Microbiology.
Faculty of Pharmacy, University of Porto
Rua Jorge Viterbo Ferreira 228, 4050-313 Porto. Portugal
Direct: (+351) 220 428 580
E-mail: lpeixe@ff.up.pt, lpeixe@gmail.com

Sir,

Enterobacteriaceae producing acquired AmpC β-lactamases (qAmpCs), and particularly CMY-2-producing *Escherichia coli*, have been increasing reported worldwide (1–4). Nonetheless, the occurrence of qAmpCs in *Enterobacteriaceae* isolates of different non-clinical origins remains scarcely explored, with available studies focusing in specific niches (healthy humans, food, healthy/sick food-producing animals or environment) or species (*E. coli*, non-typhoidal *Salmonella*) (http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/2322.pdf) (4,5). Our goal was to investigate the occurrence, diversity and molecular epidemiology of qAmpCs genes among a large collection of *Enterobacteriaceae* isolates recovered from a diversity of non-clinical niches in Portugal.

Five hundred eighteen non-clinical samples were collected from different regions of Portugal. The samples included: (i) faeces from healthy humans [n=255; 2001-2004 (n=113) and 2013-1014 (n=142); North/Centre/South); (ii) faeces from healthy animals [78 swine: 1998 (n=21), 2004 (n=14), 2006-2007 (n=43); North/Centre/South; and 44 chickens: 2005 (n=20), 2014 (n=24); North]; (iii) uncooked chicken carcasses for human consumption (2003, 2005; n=20); and (iv) samples from aquatic environments [121: n=5 raw urban wastewaters, 2001–2002, North; n=1 river, 2003; n=2 marine waters, 2003-2004; n=66 waters used for human consumption (11 fountains, 10 boreholes, 19 water wells, 26 natural springs), 2006-2008, North/Centre; n=47 trout aquaculture samples (13 water/sediments upstream and 13 downstream trout aquacultures, 15 water/sediments from juvenile/adult fish ponds, 6 feed), 2010-2012, North]. Sample processing was performed as previously described, and included culture on MacConkey agar plates supplemented with ceftazidime (1 mg/L) or cefotaxime (1 mg/L) followed by selection of isolates representing different morphotypes and antibiotic susceptibility patterns (6–9). Bacterial identification, antibiotic susceptibility testing, detection and characterization of qAmpC and co-production of extended-spectrum β-lactamases (ESBLs) were performed as previously described (1,6). Clonal relatedness was investigated by *Xba*I-PFGE and MLST (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), and *E. coli* phylogenetic groups were identified as previously described (6). The bla_{CMY} genetic environment was also investigated (6).

We identified 4 CMY-2-producing *E. coli* isolates in 0.8% (4/518) of the samples analysed (Table 1). The CMY-2 producers were recovered from healthy humans [n=3; 1 female (aged 65) and 2 males (aged 25 and 67); 2014; North region] and uncooked chicken carcass (n=1; 2003; North). The proportion of human faecal carriage of

qAmpC-producing *Enterobacteriaceae* was 0% in 2001-2004, but increased to 2.1% (3 of 142 samples) in 2013-2014, a rate similar to those reported in few other European countries (1.3% in Netherlands, 2% in Spain, 2.4% in Denmark) and that indicates that human intestinal commensal flora might constitute an important reservoir of qAmpC genes and/or qAmpC-producing bacteria (Table S1). The detection of CMY-2 producing *E. coli* in an uncooked chicken carcass of a small set of samples (5%; 1/20) is in line with other reports worldwide, where this enzyme is frequently reported among *E. coli* and *Salmonella* spp. isolates mostly from livestock and food-products (particularly poultry and retail chicken meat), suggesting that this highly consumed food product might have contributed to the spread of such enzymes (Table S1) (10). However, in Portugal only a small number of studies have reported the presence of CMY-2 among food-production animals, being more frequently detected CTX-M-group I enzymes (10). It is also important to highlight that a recent study conducted by our research group in different Portuguese clinical settings encompassing the 2002-2013 period, revealed a recent increase of CMY-2-producing *E. coli* involved in human infections in the last years (global occurrence of 17%, corresponding to 4% of the qAmpC producers identified in 2002-2008 *versus* 44% in 2009-2013; $p<0.000001$) (1).

E. coli isolates producing CMY-2 belonged to phylogroups $B2_2$ (n=2, healthy humans), A_0 (n=1, healthy human) and A_1 (n=1, uncooked chicken carcass sample). $B2_2$-*E. coli* isolates were assigned to Sequence-Type (ST) 4953 and an indistinguishable PFGE pattern was found among these isolates that were recovered from two-family related individuals (husband and wife), suggesting a common source of CMY-faecal carriage. The other two *E. coli* isolates belonging to phylogenetic group A were assigned to ST665 (A_0) and ST48 (A_1), the last belonging to the Clonal Complex (CC) 10 which is widespread in humans (patients, healthy individuals), animals and food products, and associated with a diversity of ESBLs and less frequently with qAmpCs (Table S1) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) (11) (Table S1). All the CMY-2-producing *E. coli* isolates were multidrug resistant (MDR) and none of them produced ESBLs. The bla_{CMY-2} was associated with a similar genetic environment in all isolates, being the presence of IS*Ecp1* platform intact or truncated the only difference detected (Table 1).

In conclusion and to the best of our knowledge this is the first study analyzing the occurrence of qAmpCs producers among a large collection of *Enterobacteriaceae* isolates from diverse non-clinical niches. Our data indicate an increased rate of qAmpC in healthy humans associated with CMY-2-producing *E. coli*, which might justify the increasing trend of CMY-2 producers associated with infectious diseases in Portuguese clinical settings. Moreover, the possibility of acquisition of qAmpC-producing bacteria through the food chain, namely throughout poultry products, impose more strict food

manipulation practices and justify further surveillance in a higher diversity of food-producing animals for better understand their role in the emergence of clinical qAmpC producers, in Portugal.

Funding: This work received financial support from the European Union (FEDER funds) through Programa Operacional Fatores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through project UID/Multi/04378/2013. TGR and ÂN were supported by fellowships from FCT (SFRH/BD/75752/2011 and SFRH/BPD/104927/2014) through Programa Operacional Capital Humano (POPH). All authors declare no conflict of interest.

Transparency declarations

None to declare.

References

1. Ribeiro TG, Novais Â, Rodrigues C, Nascimento R, Freitas F, Machado E, *et al.* Insights into clinical *Enterobacteriaceae* producing acquired AmpC β-lactamases over 12 years: unusual DHA dominance in an European country. J Antimicrob Chemother. 2016 (under *Editorial Revison*).
2. Mata C, Miro E, Alvarado A, Garcillan-Barcia MP, Toleman M, Walsh TR, *et al.* Plasmid typing and genetic context of AmpC beta-lactamases in *Enterobacteriaceae* lacking inducible chromosomal ampC genes: findings from a Spanish hospital 1999-2007. J Antimicrob Chemother. 2012;67(1):115–22.
3. Sidjabat HE, Seah KY, Coleman L, Sartor A, Derrington P, Heney C, *et al.* Expansive spread of IncI1 plasmids carrying bla_{CMY-2} amongst *Escherichia coli.* Int J Antimicrob Agents. 2014;44(3):203–8.
4. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009;22(1):161–82.
5. Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos A-P, Mevius D, *et al.* Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin Infect Dis. 2013;56(7):1030–7.
6. Machado E, Coque TM, Cantón R, Sousa JC, Peixe L. Antibiotic resistance integrons and extended-spectrum beta-lactamases among *Enterobacteriaceae* isolates recovered from chickens and swine in Portugal. J Antimicrob Chemother. 2008;62(2):296–302.
7. Machado E, Coque TM, Cantón R, Sousa JC, Silva D, Ramos M, *et al.* Leakage

into Portuguese aquatic environments of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae*. J Antimicrob Chemother. 2009;63(3):616–8.

8. Rodrigues C, Machado E, Fernandes S, Peixe L, Novais Â. An update on faecal carriage of ESBL-producing *Enterobacteriaceae* by Portuguese healthy humans: detection of the H30 subclone of B2-ST131 *Escherichia coli* producing CTX-M-27. J Antimicrob Chemother. 71(4):1120–2.
9. Antunes P, Campos J, Mourao J, Pereira J, Novais C, Peixe L. Contribution of trout farms to the spread of *Enterobacteriaceae* carrying plasmid-mediated quinolone-resistance genes. In: *Twenty-third European Congress of Clinical Microbiology and Infectious Diseases*. Berlim, Germany; 2013. p. Abstract P1451.
10. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012;18(7):646-55.
11. Manges A, Johnson J. Food-Borne Origins of *Escherichia coli* Causing Extraintestinal Infections. Clin Infect Dis. 2012;55 (5): 712-719.

Table 1. Epidemiological data of CMY-2-producing *E. coli* recovered from non-clinical origins in Portugal.

Origin (sample)	PhG[a]	ST/CC/PFGE-type[b]	Genetic environment of *bla*$_{CMY-2}$	Resistance to non-β-lactams[c]
Healthy volunteer (33)	$B2_2$	ST4953/EC2	Δ*ISEcp1-bla*$_{CMY-2}$-*blc-sugE*	GEN, NET, TOB, STR, TET, CHL, NAL, CIP, SUL
Healthy volunteer (34)	$B2_2$	ST4953/EC2	Δ*ISEcp1-bla*$_{CMY-2}$-*blc-sugE*	GEN, NET, TOB, STR, TET, CHL, NAL, CIP, SUL
Healthy volunteer (97)	A_0	ST665/EC3	*ISEcp1-bla*$_{CMY-2}$-*blc-sugE*	STR, TET, NAL, SUL
Uncooked chicken carcass (6)	A_1	ST48/CC10/EC4	*ISEcp1-bla*$_{CMY-2}$-*blc-sugE*	KAN, GEN, TOB, STR, TET, CHL, SUL

[a] PhG, *E. coli* phylogenetic group; [b] ST, Sequence Type, CC, clonal complex; [c] CIP, ciprofloxacin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NET, netilmicin; STR, streptomycin; SUL, sulphonamides; TET, tetracycline; TOB, tobramycin.

Table S1. Worldwide epidemiology of qAmpCs among *Enterobacteriaceae* from non-clinical settings.

Origin										
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
Healthy Humans										
Europe	Portugal	2001-2004	113 [healthy persons]	0%	-	-	-	-	-	This study
	Portugal	2013-2014	142 [healthy persons]	2.1% (3/142)	CMY-2 (3)	*E. coli* (3)	$B2_2$ (2) A0 (1)		-	This study
	The Netherlands	2009	18 [healthy farmers]	27.8% (5/18)	CMY-2 (5)	*E. coli** (5)	NP-ST93/CC168 (1), NP-ST359 (1), NP (3)	I1/ST93 (1), K (1), ND (3)	CTX-M-1 (1)	(1)
	The Netherlands	2011	550 [healthy persons]	1.3% (7/550)	CMY-2 (7)	*E. coli* (7)	NP	NP	CTX-M-1 (1), CTX-M-15 (1)	(2)
	The Netherlands	2011	1033 [healthy persons]	0.5% (5/1033)	CMY-2 (3)	*E. coli* (3)	$B2_3$-ST131 $B2_2$-ST219 A_0-ST93/CC168	I1/ST12/CC12 I1/ST12/CC12 A/C		(3)
					DHA-1 (2)	*E. coli* (1) *C. freundii* (1)	$B2_3$-ST131 -	F1:A1:B1 NP		
	Spain	2010	1796 [outpatients]	0.39% (7/1796)	CMY-2 (7)	*E. coli* (7)	NP	NP	NP	(4)
	Spain	2010	50 [healthy persons]	2% (1/50)	CMY-2 (1)	*E. coli* (1)	D-ST405/CC405	K (78 kb)	NP	(5)
	Denmark	2008	84 [army recruits]	2.4% (2/84)	CMY-2 (2)	*E. coli* (2)	D-ST1822 B1-ST1800	-	NP	(6)
Asia	Taiwan	2001	42 [outpatients]	33.3% (14/43)	CMY-2 (14)	*E. coli** (14)	NP	NP	NP	(7)
	Japan	2003	63 [healthy persons]	1.6% (1/63)	CMY-2 (1)	*E. coli* (1)	NP	NP		(8)

Table S1. *Continued*

Origin										
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
	China	2006-2009	280 [healthy persons]	18.6% (52/280)	CMY-2 (52)	*E. coli** (52)	NP	NP	CTX-M-69 (2)	(9)
America	United States of America	2009-2011	42 [healthy persons]	19% (8/42)	CMY-2 (6)	*E. coli* (6)	NP	FII (54kb) (2), I1 (54-90 kb), L/M (54 kb)	NP	(10)
					CMY-108 (2)	*E. coli* (2)		NP		
Healthy Livestock Animals										
Europe	Spain	2000-2001	120 [chickens]	0.8% (1/120)	CMY-2 (1)	*E. coli* (1)	NP	NP	-	(11)
	Spain	2003	160 [chickens]	1.3% (2/160)	CMY-2 (2)	*E. coli* (2)	NP	NP	-	(12)
	Spain	2003	30 [pigs (10), poultry (10), rabbit (10)]	NI	CMY-2 (14)	*E. coli* (14)	D-ST115 (4)	Q+A/C (91 kb) (1)	NP	(13)
							A-NP (1)			(14)
							NP (9)	Q+A/C+N (200 kb) (1), Q+A/C (204 kb)	-	(15)
	The Netherlands	2006	153 [poultry]	2.0% (3/153)	CMY-2 (5)	*E. coli* (5)	NP	K (88 kb) (2), NP (3)	-	(16)
	The Netherlands	2009	1435 [broiler]	0.8% (12/1435)	CMY-2 (12)	*E. coli** (12)	NP-ST38/CC38 (1), NP-ST602/CC446 (1), NP-ST115 (2), NP-ST2309 (2), NP-ST70 (1), NP-ST380 (1), NP-ST789 (1), NP-ST1358 (1), NP (2)	K (3), I1/ST12/CC12 (1), Unt-p (1), NP (7)	CTX-M-1 (1)	(1)
	Italy	2009	101 [chickens (91), turkeys (10)]	4.9% (5/101)	CMY-2 (5)	*E. coli* (5)	NP	I1/ST12/CC12 (2), I1/ST86 (1), K (1), ND (1)	CTX-M-1 (1), CTX-M-2 (1)	(17)

Table S1. *Continued*

Origin										
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
	Poland	2009	880 [chickens, cattle, swines, turkey, flocks]	2.3% (20/880: 14 chickens, 4 flocks, 1 turkey, 1 swine)	CMY-2 (20)	*E. coli** (20)	NP	NP	NP	(18)
	Portugal	2009-2013	NI [breeders, broilers, layers, turkeys]	NI (4 broilers)	CMY-2 (4)	*Salmonella* spp.* (2), *E. coli** (2)	NP	NP	TEM-ESBL (2)	(19) 2015
	Belgium	2007	489 [chickens]	NI (8.8% (25/285)	CMY-2 (25)	*E. coli** (25)	NP	NP	CTX-M-1 (4)	(20)
	Belgium	2010	95 [poultry]	8.4% (8/95)	CMY-2 (8)	*Salmonella* spp* (8)	-	I1/ST12/CC12 (8)	CTX-M-2 (2)	(21)
	Switzerland	2010-2011	24 [swines]	12.5 (3/24)	CMY-2 (3)	*E. coli* (3)	A-ST2 (1), A-ST532 (1) D-ST539 (1)	I1	-	(22)
	Switzerland	2010-2011	711 [chickens [600), swines (60), cattle (51)]	1.7% (12/711: chickens)	CMY-2 (12)	*E. coli* (12)	NP-ST61/CC11 (5), NP-ST3/CC20 (1), NP-ST9/CC20 (1), NP-ST539 (2), NP-ST527 (1), NP-ST530 (1), NP-ST535 (1)	NP	NP	(23)
Asia	Japan	1999-2002	NI	NI (0.3%; 8/2747; chickens)	CMY-2 (8)	*E. coli** (8)	NP	NP	PSE-1 (1)	(24)
	Japan	2004-2006	250 [chickens (50), swines (50), cattle (100), flocks (50)]	1.6% (4/250: 3 chickens, 1 cattle)	CMY-2 (4)	*E. coli** (4)	NP	NP	-	(25)
	Taiwan	2002	62 [chickens (32), swines (30)]	12.9% (8/62: 7 swines, 1 chicken)	CMY-2 (8)	*E. coli** (8)	NP	NP	-	(7)

Table S1. *Continued*

Origin										
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
	China	2006-2009	167 [swines]	4.2% (7/167)	CMY-2 (7)	*E. coli** (7)	NP	NP	-	(9)
	China	2007-2009	896 [swines (326), chickens (316), cattle (88), ducks (58), geese (22), pigeons (61), partridges (25)]	0.2% (2/896: swines)	CMY-2 (2)	*E. coli** (2)	NP	NP	-	(26)
	China	2010-2012	520 [pigs, poultry, cattle]	NI (3.2%; 16/496; 9 swines, 5 chickens, 2 cows)	CMY-2 (15) CMY-130 (1)	*E. coli** (16)	NP	A/C (90-140 kb) (6), HI2 (291 kb) (1), Unt-p (48.5 kb) (1) HI2 (291 kb)	NP	(27)
America	Canada	1994-1999	8426 [food-producing animals, food products, animal environment]	0.1% (8/8426: 7 chickens, 1 turkey)	CMY-2 (8)	*Salmonella* spp.* (8)	-	NP	-	(28)
	Canada	1999-NI	2483 [cattle]	1.5% (37/2483)	CMY-2 (38)	*E. coli** (38)	NP	A/C (82-157 kb) (25), Unt-p (89 kb) (1), NP (12)	NP	(29)
	Canada	2001-2004	NI [chickens, swines, cattles, turkeys]	NI (chickens, swines, cattle, turkey)	CMY-2 (6)	*Salmonella* spp.* (6)	-	I1 (4), A/C and I1 (1), Unt-p (1)	NP	(30)
	Mexico	2000-2005	731 [chickens (356), swines (280), cattle (95)]	NI (6.8%; 20/295; swines)	CMY-2 (20)	*Salmonella* spp.* (8)	-	NP	-	(31)
	United States of America	2003	96 [cattle]	NI (95.1%; 116/122)	CMY-2 (116)	*E. coli** (122)	NP	NP	-	(32)

Table S1. *Continued*

Origin Continent	Country	Year(s) of isolation	Sample size [type, (no.)]	qAmpC occurrence (no. of positive samples/no. of samples analysed)	qAmpC enzyme(s) (no.)	Species (no.)	PhG-ST/CC[a]	Plasmid content [Inc family, pMLST/FAB formula, size (kb), (no.)]	Co-production of other β-lactamases	References
Africa	Tunisia	2011	80 [sheep (23), chickens (22), cattle (22), horses (6), rabbits (5), dromedaries (2)]	2.5% (2/80: chickens)	CMY-2 (2)	*E. coli* (2)	B2-ST3632 (1)	I1/ST12/CC12 (97 kb)	-	(33)
							D-ST117 (1)	I1/ST12/CC12 (97 kb)		(34)
Healthy Pet Animals										
Europe	Italy	2001-2003	NI [dogs, pets]	NI (2.0%; 1/49)	CMY-2 (1)	*E. coli** (4)	NP	NP	-	(35)
	Portugal	2009-2013	NI [dogs, cats, horses and cage birds]	NI (4; 2 dog, 1 cat, 1 cage bird)	CMY-2 (4)	*E. coli** (4)	NP	NP	-	(19)
	England	2005	183 [dogs]	NI	CMY-2 (7)	*E. coli* (7)	NP	NP	ESBL (1)	(36)
	France	2011	368 [dogs]	6.3% (23/368)	CMY-2 (23)	*E. coli* (22)	D-NP (13)	I1/ST2/CC2 (97 kb) (10), I1/ST27/CC26 (97 kb) (1), I1/ST29/CC26 (160 kb) (1), I1/- (100 kb) (1)		(37)
							B1-NP (6)	I1/ST2/CC2 (97-145 kb) (5), I1/ST133 (97 kb) (1)	CTX-M-3 (1)	
							A-NP (2)	I1/ST2/CC2 (97 kb) (2)	CTX-M-1 (1)	
							B2-NP (1)	I1/ST2/CC2 (97 kb) (1)		
						Salmonella spp. (1)	-	I1/ST2/CC2 (97 kb) (1)		

Table S1. *Continued*

Origin Continent	Country	Year(s) of isolation	Sample size [type, (no.)]	qAmpC occurrence (no. of positive samples/no. of samples analysed)	qAmpC enzyme(s) (no.)	Species (no.)	PhG-ST/CC[a]	Plasmid content [Inc family, pMLST/FAB formula, size (kb), (no.)]	Co-production of other β-lactamases	References
Asia	China	2010-2012	150 [dogs]	NI (1.6%; 2/123)	CMY-2 (2)	*E. coli** (2)	NP	-	NP	(27)
America	Canada	2002	227 [dogs (188), cats (39)]	1.3% (3/227: 3 dogs)	CMY-2 (3)	*E. coli* (3)	NP	NP	-	(38)
	Mexico	2011	53 [dogs]	11.3% (6/53)	CMY-2 (6)	*E. coli* (6)	D-ST57CC350, D-ST1465, D-NP A-ST93/CC168, A-NP B1-ST1431	NP	-	(39)
	United States of America	2009-2011	73 [dogs (65), cats (8)]	84.9% (62)	CMY-2 (62)	*E. coli* (62)	NP	I1 (80-104 kb) (29), A/C (80-104 kb) (8), FII (54-65 kb) (4), I2 (65 kb) (3), B/O (80 kb) (1), Unt-p (45 kb), ND (8)	NP	(10)
Africa	Tunisia	2010	80 [Dogs (41), Cats (39)]	1.25% (1/80: 1 dog)	CMY-2 (1)	*E. coli* (1)	B1-ST58/155 (1)	I1/ST12/CC12 (97 kb)		(34,40)
Stray Animals										
Asia	Republic of Korea	2006-2007	877 [stray dogs]	NI	CMY-2 (21)	*E. coli* (21)	D-ST648 (9) D-ST457 (1) D-ST405/CC405 (1) D-ST354/CC354 (1) B1-ST539 (2) B1-ST442 (1)	NP Unt-p (40 kb) I1 (120 kb) Unt-p (50 kb) I1 (170 kb), Unt-p (40 kb) FII (55 kb)		(41)

Table S1. *Continued*

Origin Continent	Country	Year(s) of isolation	Sample size [type, (no.)]	qAmpC occurrence (no. of positive samples/no. of samples analysed)	qAmpC enzyme(s) (no.)	Species (no.)	PhG-ST/CC[a]	Plasmid content [Inc family, pMLST/FAB formula, size (kb), (no.)]	Co-production of other β-lactamases	References
							B1-ST675 (1)	Unt-p (50 kb)		
							B1-ST1642 (1)	Unt-p (50 kb)		
							B1-ST2178 (1)	I1 (95 kb)		
							B2-ST12/CC12 (2)	A/C (50 kb) (1)		
							A-NP (1)	Unt-p (50 kb)		
					DHA-1 (1)	*E. coli* (1)	B1-ST297 (1)	NP		
Wild Animals										
Europe	Norway	2010	230 [little auks (215), glaucous gulls (15)]	0.4% (1/230: glaucous gull)	ACT-23 (1)	*E. cloacae* complex	NP	cr	-	(42)
Asia					CMY-2 (1)	*E. coli* (1)				
	Arabia Saudita	NI	75 [rock hyrax (5), Yemen Linnet (4), common kestrel (3), red foxes (3), long-tailed finches (3), caracal (2), peacock (2), rock dove (1), hamadryas baboon (1), orange-winged parrot (1), Burmese python (1), Hill Mynah (1), African gray parrot (1), common myna (1)]	2.7% (2/75; Hill mynah, Arabian red fox)	DHA-1 (1)	*E. coli* (1)	NP	NP	NI	(43)

Table S1. *Continued*

Origin										
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
Environment										
Asia	India	2011-2012	9 [river]	NI	CMY-42 (2)	*E. coli**	D-NP (2)	NP	-	(44)
America	Canada	2004-2006	15119 [private drinking water (8793), recreational water (6326)]	NI (77.5%; 110/142; 103 private drinking water, 39 recreational water)	CMY-2 (110)	*E. coli** (110)	A-NP and B1-NP (82) B2-NP and D-NP (28)	I1/ST2/CC2 (88-93 kb) (2), I1/ST12/CC12 (103-106 kb) (2) I1/ST20 (73-96 kb) (2), I1/ST18 (134 kb), I1/ST19 (122 kb),I1 (62-134 kb) (44), A/C (74-131 kb) (20), K (66-104 kb) (14), F (34-122 kb) (4), FIB and I1 (71-133 kb) (4), K/B and I1 (90-93 kb) (2), Unt-p (63-139 kb) (14)	-	(45,46)
Africa	Tunisia	2011-2012	114 [wastewater (57), surface-water samples (57)]	3.5% (4/114: 4 wastewater)	CMY-2 (4)	*E. coli* (4)	A-ST10/CC10 (2) A-ST48/CC10 (1) A-ST399/CC399 (1)	I1 (90 kb), FII (200 kb) ND (97 kb) A/C (179 kb)	-	(47)
	Nigeria	2011-2012	10 [rivers]	NI (38/172 ampicillin resistant isolates	ampC (38)	*E. coli** (38)	NP	NP	NP	(48)
Food										
Europe	The Netherlands	2010	7 [chicken]	71.4% (5/7)	CMY-2 (5)	*E. coli* (5)	B1-ST23/CC23 (2) A-ST93/CC168 (1)	K	SHV-12 (1), TEM-52 (1) SHV-12 (1)	(49)

Table S1. *Continued*

Origin									
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
							B2-ST117 (1) D-ST115 (1)			
	Sweden	2010	100 [chicken]	38% (38/100)	CMY-2 (38)	*E. coli** (38)	NP-ST38/CC38 (11), NP-ST10/CC10 (4), NP-ST69/CC69 (4), NP-ST373/CC168 (3), NP-ST648/CC648 (1), NP-ST115 (4), NP-ST2167 (4), NP-ST135 (1), NP-ST212 (1), NP-ST770 (1), NP-ST1594 (1), NP-ST2184 (1), NP-ST2207 (1), NP-ST2370 (1)	K (34), I1 (3), ND (1)	-	(50)
	Switzerland	2013	225 [chicken (75), pork (50), beef (50), veal (50)]	5.8% (13/225: 13 chicken breast)	CMY-2 (12)	*E. coli* (12)	D-ST38/CC38 (11) A-ST1564 (1)	B/O (2) B/O		(51)
					CMY-4 (1)	*E. coli* (1)	D-ST38/CC38 (1)	B/O		
	Portugal	2003-2005	20 [chicken]	5% (1/20)	CMY-2 (1)	*E. coli* (1)	A_1	A/C (150)	-	This study
Asia	Taiwan	2002	150 [chicken (100), pork (50)]	10% (15/150: 14 ground chicken, 1 ground pork)	CMY-2 (15)	*E. coli** (15)	NP	NP	-	(7)
	Japan	2004-2005	83 [chicken]	NI (6.9%; 2/29)	CMY-2 (2)	*Salmonella* spp. (2)	NP	NP	NP	(52)

Table S1. *Continued*

Origin										
Continent	**Country**	**Year(s) of isolation**	**Sample size** [type, (no.)]	**qAmpC occurrence** (no. of positive samples/no. of samples analysed)	**qAmpC enzyme(s)** (no.)	**Species** (no.)	**PhG-ST/CC**[a]	**Plasmid content** [Inc family, pMLST/FAB formula, size (kb), (no.)]	**Co-production of other β-lactamases**	**References**
	Japan	2004-2006	300 [chicken (100), pork (100), beef (100)]	0.7% (2/300: 1 chicken, 1 pork)	CMY-2 (2)	*E. coli** (2)	NP	NP	NP	(25)
America	United States of America	1998	200 [chicken (51), beef (50), turkey (50), pork (49)]	3.5% (7/200: 4 turkey, 2 chicken, 1 beef)	CMY-2 (7)	*Salmonella* spp.* (7)	NP	NP	NP	(53)
	United States of America	2006-2007	378 [chickens]	NI (21.4%; 18/84)	CMY-2 (18)	*Salmonella* spp.* (18)	-	NP	NP	(54)
	Canada	2001-2004	NI [chicken]	NI	CMY-2 (5)	*Salmonella* spp.* (4) *E. coli* (1)	- NP	I1 (5)	NP	(30)
	Mexico	2000-2005	1713 [pork (687), chicken (634), beef 392]	NI (2.9%; 39/1330; 22 pork, 14 chicken, 3 beef)	CMY-2 (39)	*Salmonella* spp.* (39)	NP	NP	-	(31)

[a] PhG, E. coli phylogenetic group; ST, Sequence Type, CC, clonal complex; * The only species analyzed in the study; NI, not identified; NP, not performed; Unt-p, untypeable plasmid; cr, chromosomal location.

References from Supplementary Table S1:

1. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius D. Extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. J Antimicrob Chemother. 2013;68(1):60–7.
2. Reuland EA, Halaby T, Hays JP, de Jongh DM, Snetselaar HD, van Keulen M, *et al.* Plasmid-mediated AmpC: prevalence in community-acquired isolates in Amsterdam, the Netherlands, and risk factors for carriage. PLoS One. 2015;10(1):e0113033.
3. van Hoek AH, Schouls L, van Santen MG, Florijn A, de Greeff SC, van Duijkeren E. Molecular characteristics of extended-spectrum cephalosporin-resistant *Enterobacteriaceae* from humans in the community. PLoS One. 2015;10(6):e0129085.
4. Garrido A, Seral C, Gude MJ, Casado C, Gonzalez-Dominguez M, Saenz Y, *et al.* Characterization of plasmid-mediated beta-lactamases in fecal colonizing patients in the hospital and community setting in Spain. Microb Drug Resist. 2014;20(4):301–4.
5. Porres-Osante N, Saenz Y, Somalo S, Torres C. Characterization of Beta-lactamases in Faecal *Enterobacteriaceae* Recovered from Healthy Humans in Spain: Focusing on AmpC Polymorphisms. Microb Ecol. 2015;70(1):132–40.
6. Hammerum AM, Lester CH, Jakobsen L, Porsbo LJ, Coque T, Baquero F, *et al.* Faecal carriage of extended-spectrum β-lactamase-producing and AmpC β-lactamase-producing bacteria among Danish army recruits. Clin Microbiol Infect. 2011;17(4):566–8.
7. Yan JJ, Hong CY, Ko WC, Chen YJ, Tsai SH, Chuang CL, *et al.* Dissemination of $bla_{\text{CMY-2}}$ among *Escherichia coli* isolates from food animals, retail ground meats, and humans in southern Taiwan. Antimicrob Agents Chemother. 2004;48(4):1353–6.
8. Kaneko K, Sato Y, Tokunaga S, Tamaki S, Okamoto R, Inoue M. AmpC beta-lactamase-mediated cefpodoxime-resistant *Escherichia coli* isolated from faecal samples of healthy volunteers. J Antimicrob Chemother. 2006;57(2):369–71.
9. Tian GB, Wang HN, Zhang AY, Zhang Y, Fan WQ, Xu CW, *et al.* Detection of clinically important beta-lactamases in commensal *Escherichia coli* of human and swine origin in western China. J Med Microbiol. 2012;61(2):233–8.
10. Bortolaia V, Hansen KH, Nielsen CA, Fritsche TR, Guardabassi L. High diversity of plasmids harbouring $bla_{\text{CMY-2}}$ among clinical *Escherichia coli* isolates from humans and companion animals in the upper Midwestern USA. J Antimicrob Chemother. 2014;69(6):1492–6.
11. Briñas L, Moreno MA, Zarazaga M, Porrero C, Sáenz Y, García M, *et al.* Detection of CMY-2, CTX-M-14, and SHV-12 beta-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. Antimicrob Agents Chemother. 2003;47(6):2056–8.
12. Briñas L, Moreno MA, Teshager T, Sáenz Y, Porrero MC, Domínguez L, *et al.* Monitoring and characterization of extended-spectrum beta-lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in 2003. Antimicrob Agents Chemother. 2005;49(3):1262–4.

13. Cortés P, Blanc V, Mora A, Dahbi G, Blanco JE, Blanco M, *et al.* Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. Appl Environ Microbiol. 2010;76(9):2799–805.
14. Blanc V, Cortés P, Mesa RJ, Miró E, Navarro F, Llagostera M, *et al.* Characterisation of plasmids encoding extended-spectrum beta-lactamase and CMY-2 in *Escherichia coli* isolated from animal farms. Int J Antimicrob Agents. 2008;31(1):76–8.
15. Mesa RJ, Blanc V, Blanch AR, Cortés P, González JJ, Lavilla S, *et al.* Extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in different environments (humans, food, animal farms and sewage). J Antimicrob Chemother. 2006;58(1):211–5.
16. Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H, Mevius D. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet Microbiol. 2010;145(3):273–8.
17. Accogli M, Fortini D, Giufre M, Graziani C, Dolejska M, Carattoli A, *et al.* IncI1 plasmids associated with the spread of CMY-2, CTX-M-1 and SHV-12 in *Escherichia coli* of animal and human origin. Clin Microbiol Infect. 2013;19(5):E238–40.
18. Wasyl D, Hasman H, Cavaco LM, Aarestrup FM. Prevalence and characterization of cephalosporin resistance in nonpathogenic *Escherichia coli* from food-producing animals slaughtered in Poland. Microb Drug Resist. 2012;18(1):79–82.
19. Clemente L, Manageiro V, Jones-Dias D, Correia I, Themudo P, Albuquerque T, *et al.* Antimicrobial susceptibility and oxymino-β-lactam resistance mechanisms in *Salmonella enterica* and *Escherichia coli* isolates from different animal sources. Res Microbiol. 2015;166(7):574–83.
20. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Catry B, *et al.* Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* Isolates in Belgian broiler farms. Antimicrob Agents Chemother. 2008;52(4):1238–43.
21. Doublet B, Praud K, Nguyen-Ho-Bao T, Argudin MA, Bertrand S, Butaye P, *et al.* Extended-spectrum beta-lactamase- and AmpC beta-lactamase-producing D-tartrate-positive *Salmonella enterica* serovar Paratyphi B from broilers and human patients in Belgium, 2008-10. J Antimicrob Chemother. 2014;69(5):1257–64.
22. Endimiani A, Hilty M, Perreten V. CMY-2-producing *Escherichia coli* in the nose of pigs. Antimicrob Agents Chemother. 2012;56(8):4556–7.
23. Endimiani A, Rossano A, Kunz D, Overesch G, Perreten V, Batchelor M, *et al.* First countrywide survey of third-generation cephalosporin-resistant *Escherichia coli* from broilers, swine, and cattle in Switzerland. Diagn Microbiol Infect Dis. 2012;73(1):31–8.
24. Kojima A, Ishii Y, Ishihara K, Esaki H, Asai T, Oda C, *et al.* Extended-spectrum-beta-lactamase-producing *Escherichia coli* strains isolated from farm animals from 1999 to 2002: report from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. Antimicrob Agents Chemother. 2005;49(8):3533–7.
25. Hiroi M, Harada T, Kawamori F, Takahashi N, Kanda T, Sugiyama K, *et al.* A survey of beta-lactamase-producing *Escherichia coli* in farm animals and raw retail meat in

Shizuoka Prefecture, Japan. Jpn J Infect Dis. 2011;64(2):153–5.

26. Zheng H, Zeng Z, Chen S, Liu Y, Yao Q, Deng Y, *et al.* Prevalence and characterisation of CTX-M β-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. Int J Antimicrob Agents. 2012;39(4):305–10.
27. Fang LX, Sun J, Li L, Deng H, Huang T, Yang QE, *et al.* Dissemination of the chromosomally encoded CMY-2 cephalosporinase gene in *Escherichia coli* isolated from animals. Int J Antimicrob Agents. 2015;46(2):209–13.
28. Allen KJ, Poppe C. Occurrence and characterization of resistance to extended-spectrum cephalosporins mediated by beta-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. Can J Vet Res. 2002;66(3):137–44.
29. Mulvey MR, Susky E, McCracken M, Morck DW, Read RR. Similar cefoxitin-resistance plasmids circulating in *Escherichia coli* from human and animal sources. Vet Microbiol. 2009;134(3):279–87.
30. Andrysiak AK, Olson AB, Tracz DM, Dore K, Irwin R, Ng LK, *et al.* Genetic characterization of clinical and agri-food isolates of multidrug resistant *Salmonella enterica* serovar Heidelberg from Canada. BMC Microbiol. 2008;8:89.
31. Zaidi MB, Leon V, Canche C, Perez C, Zhao S, Hubert SK, *et al.* Rapid and widespread dissemination of multidrug-resistant $bla_{\text{CMY-2}}$ *Salmonella* Typhimurium in Mexico. J Antimicrob Chemother. 2007;60(2):398–401.
32. Donaldson SC, Straley BA, Hegde N V, Sawant AA, DebRoy C, Jayarao BM. Molecular epidemiology of ceftiofur-resistant *Escherichia coli* isolates from dairy calves. Appl Environ Microbiol. 2006;72(6):3940–8.
33. Ben Sallem R, Ben Slama K, Sáenz Y, Rojo-Bezares B, Estepa V, Jouini A, *et al.* Prevalence and characterization of extended-spectrum beta-lactamase (ESBL)- and CMY-2-producing *Escherichia coli* isolates from healthy food-producing animals in Tunisia. Foodborne Pathog Dis. 2012;9(12):1137–42.
34. Ben Sallem R, Ben Slama K, Rojo-Bezares B, Porres-Osante N, Jouini A, Klibi N, *et al.* IncI1 plasmids carrying *bla*(CTX-M-1) or *bla*(CMY-2) genes in *Escherichia coli* from healthy humans and animals in Tunisia. Microb Drug Resist. 2014;20(5):495–500.
35. Carattoli A, Lovari S, Franco A, Cordaro G, Di Matteo P, Battisti A. Extended-spectrum beta-lactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. Antimicrob Agents Chemother. 2005;49(2):833–5.
36. Wedley AL, Maddox TW, Westgarth C, Coyne KP, Pinchbeck GL, Williams NJ, *et al.* Prevalence of antimicrobial-resistant *Escherichia coli* in dogs in a cross-sectional, community-based study. Vet Rec. 2011;168(13):354.
37. Haenni M, Saras E, Metayer V, Medaille C, Madec JY. High prevalence of *bla*CTX-M-1/IncI1/ST3 and blaCMY-2/IncI1/ST2 plasmids in healthy urban dogs in France. Antimicrob Agents Chemother. 2014;58(9):5358–62.
38. Murphy C, Reid-Smith RJ, Prescott JF, Bonnett BN, Poppe C, Boerlin P, *et al.* Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. Can Vet J.

2009;50(10):1047–53.

39. Rocha-Gracia RC, Cortés-Cortés G, Lozano-Zarain P, Bello F, Martínez-Laguna Y, Torres C. Faecal *Escherichia coli* isolates from healthy dogs harbour CTX-M-15 and CMY-2 β-lactamases. Vet J. 2015;203(3):315–9.
40. Sallem R Ben, Gharsa H, Slama K Ben, Rojo-Bezares B, Estepa V, Porres-Osante N, *et al.* First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. Vector Borne Zoonotic Dis. 2013;13(2):98–102.
41. Tamang MD, Nam H-M, Jang G-C, Kim S-R, Chae MH, Jung S-C, *et al.* Molecular characterization of extended-spectrum-β-lactamase-producing and plasmid-mediated AmpC β-lactamase-producing *Escherichia coli* isolated from stray dogs in South Korea. Antimicrob Agents Chemother. 2012;56(5):2705–12.
42. Literak I, Manga I, Wojczulanis-Jakubas K, Chroma M, Jamborova I, Dobiasova H, *et al.* *Enterobacter cloacae* with a novel variant of ACT AmpC beta-lactamase originating from glaucous gull (*Larus hyperboreus*) in Svalbard. Vet Microbiol. 2014;171(3):432–5.
43. Hassan SA, Shobrak MY. Detection of genes mediating beta-lactamase production in isolates of enterobacteria recovered from wild pets in Saudi Arabia. Vet world. 2015;8(12):1400–4.
44. Bajaj P, Singh NS, Kanaujia PK, Virdi JS. Distribution and molecular characterization of genes encoding CTX-M and AmpC beta-lactamases in *Escherichia coli* isolated from an Indian urban aquatic environment. Sci Total Env. 2015;505:350–6.
45. Mataseje LF, Neumann N, Crago B, Baudry P, Zhanel GG, Louie M, *et al.* Characterization of cefoxitin-resistant *Escherichia coli* isolates from recreational beaches and private drinking water in Canada between 2004 and 2006. Antimicrob Agents Chemother. 2009;53(7):3126–30.
46. Mataseje LF, Baudry PJ, Zhanel GG, Morck DW, Read RR, Louie M, *et al.* Comparison of CMY-2 plasmids isolated from human, animal, and environmental *Escherichia coli* and *Salmonella* spp. from Canada. Diagn Microbiol Infect Dis. 2010;67(4):387–91.
47. Ben Said L, Jouini A, Alonso CA, Klibi N, Dziri R, Boudabous A, *et al.* Characteristics of extended-spectrum beta-lactamase (ESBL)- and pAmpC beta-lactamase-producing *Enterobacteriaceae* of water samples in Tunisia. Sci Total Env. 2016;550:1103–9.
48. Titilawo Y, Obi L, Okoh A. Antimicrobial resistance determinants of *Escherichia coli* isolates recovered from some rivers in Osun State, South-Western Nigeria: Implications for public health. Sci Total Env. 2015;523:82–94.
49. de Been M, Lanza VF, de Toro M, Scharringa J, Dohmen W, Du Y, *et al.* Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. PLoS Genet. 2014;10(12):e1004776.
50. Borjesson S, Egervarn M, Lindblad M, Englund S. Frequent occurrence of extended-spectrum beta-lactamase- and transferable ampc beta-lactamase-producing *Escherichia coli* on domestic chicken meat in Sweden. Appl Env Microbiol. 2013;79(7):2463–6.
51. Vogt D, Overesch G, Endimiani A, Collaud A, Thomann A, Perreten V. Occurrence and

genetic characteristics of third-generation cephalosporin-resistant *Escherichia coli* in Swiss retail meat. Microb Drug Resist. 2014;20(5):485–94.

52. Taguchi M, Seto K, Yamazaki W, Tsukamoto T, Izumiya H, Watanabe H. CMY-2 beta-lactamase-producing *Salmonella enterica* serovar Infantis isolated from poultry in Japan. Jpn J Infect Dis . 2006;59(2):144–6.
53. White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, *et al.* The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med. 2001;345(16):1147–54.
54. M'Ikanatha N M, Sandt CH, Localio AR, Tewari D, Rankin SC, Whichard JM, *et al.* Multidrug-resistant *Salmonella* isolates from retail chicken meat compared with human clinical isolates. Foodborne Pathog Dis. 2010;7(8):929–34.

Atypical epidemiology of CTX-M-15 among *Enterobacteriaceae* from a high diversity of non-clinical niches in Angola

Teresa Gonçalves Ribeiro[1], Ângela Novais[1], Luísa Peixe[1], and Elisabete Machado[1,2*]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

Journal of Antimicrobial Chemotherapy 2016 May; 71(5):1169-73

Oxford University Press - Journal has authorized the reproduction of the final published PDF version of this paper in this Thesis through the License Agreement Number 3890930959588 on June 1, 2016.

Journal of Antimicrobial Chemotherapy Advance Access published February 16, 2016

J Antimicrob Chemother
doi:10.1093/jac/dkv489

Journal of Antimicrobial Chemotherapy

Atypical epidemiology of CTX-M-15 among Enterobacteriaceae from a high diversity of non-clinical niches in Angola

T. G. Ribeiro[1], Â. Novais[1], L. Peixe[1] and E. Machado[1,2]*

[1]*UCIBIO/REQUIMTE, Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;* [2]*FP-ENAS/CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal*

*Corresponding author. FP-ENAS/CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal. Tel: +351-225074630; Fax: +351-225074677; E-mail: emachado@ufp.edu.pt/elisabetemac@gmail.com

Received 3 September 2015; returned 26 October 2015; revised 9 December 2015; accepted 18 December 2015

Objectives: The objective of this study was to investigate the distribution and molecular epidemiology of ESBLs, acquired AmpCs and carbapenemases in Enterobacteriaceae from non-clinical niches in Angola, an under-researched sub-Saharan country.

Methods: Eighty-one samples were recovered from healthy persons ($n=18$), healthy animals ($n=33$) and their environments ($n=10$) or aquatic settings ($n=20$) in south Angola (2013). Samples were plated onto CHROMagar™ Orientation with/without antibiotics. Standard methods were used for bacterial identification, characterization of *bla* genes, antibiotic susceptibility testing and conjugation assays. Clonal analysis (XbaI-PFGE, MLST and *Escherichia coli* phylogroups), location of *bla* and plasmid characterization (S1-PFGE, I-CeuI-PFGE, replicon typing and hybridization) were also performed.

Results: ESBLs (almost exclusively CTX-M-15, 98%) were detected in 21% (45/216) of the isolates, recovered from diverse non-clinical niches and belonging to different Enterobacteriaceae species (mainly *E. coli*). Acquired AmpCs or carbapenemases were not found. The pandemic B2-ST131 *E. coli* clone was not identified, but some widespread clonal complexes (CCs) from A (CC10 and CC168), B1 (CC156) or D (CC38) phylogroups were detected. $bla_{CTX\text{-}M\text{-}15}$ was variably identified on typeable (29%; 100–335 kb; IncFII, $IncFII_{K6}$, IncHI2 and IncY) or non-typeable (16%; 70–330 kb) plasmids or on the chromosome (14%), while for 41% of the isolates its specific location was not determined.

Conclusions: This study reports, for the first time in Angola, an unexpected high occurrence of CTX-M-15 in diverse non-clinical niches and Enterobacteriaceae species, and uncovers novel plasmid replicons in under-researched geographical regions. The diffusion of $bla_{CTX\text{-}M\text{-}15}$ through such a high diversity of genetic backgrounds (clones, typeable/non-typeable plasmids and genetic environments) unveils an extraordinary ability for $bla_{CTX\text{-}M\text{-}15}$ acquisition and mobilization favoured by unrecognized ecological factors.

Downloaded from http://jac.oxfordjournals.org/ by guest on February 17, 2016

Introduction

The epidemiology of Enterobacteriaceae producing ESBLs, acquired AmpC β-lactamases (qAmpCs) and/or carbapenemases has mainly been investigated in developed countries, with studies showing their large-scale diffusion in different niches.[1–3] Currently, the role of trade globalization and human travel to endemic and/or low-income countries in the dispersion of antibiotic-resistant (AbR) bacteria is well recognized, pointing out the need to comprehensively analyse their epidemiology and ecology in these geographical regions.[4,5]

In Africa, available data on antimicrobial resistance indicate considerable resistance rates to extended-spectrum cephalosporins.[6] Nevertheless, studies analysing the occurrence and molecular epidemiology of ESBLs are limited to northern countries, certain species and/or the clinical setting, while qAmpCs or carbapenemases are sporadic.[7]

Angola is a sub-Saharan country with an emerging economy and close commercial or travel relationships with European, Asian and American countries (http://www.embangola.at/dados.php?ref=rela%E7%F5es-internacionais), for which few data on occurrence and epidemiological features of Enterobacteriaceae resistant to antibiotics are available.[8] Given that the climate, poor sanitation and uncontrolled antibiotic use might be key drivers for the emergence and dissemination of AbR bacteria, we aimed to investigate the distribution and molecular epidemiology of ESBLs, qAmpCs and carbapenemases in Enterobacteriaceae from non-clinical niches in Angola.

© *The Author 2016. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com*

Table 1. Epidemiological data of CTX-M-15-producing Enterobacteriaceae isolates recovered from different origins in Angola (2013)

Species (no.)	*E. coli* phylogenetic group (no.)	PFGE type (no.)	ST/CC	Plasmid content			Other AbR genes			Origin (sample)	Region (commune)[b]	Coresistance to non-β-lactam antibiotics[c]
				associated with $bla_{CTX-M-15}$ [size (kb) (Inc family)][a]	other							
					size (kb)	Inc family (no.)	bla_{TEM-1}	bla_{OXA-1}	*aac-6′-Ib-cr*			
E. coli ($n=34$)	A_0	I	ST4977	—	70	Y	+	–	–	healthy person (28)	Benguela	TOB, STR, KAN, CIP, SUL, TET
	A_1	II	ST5044	—	—	—	–	–	–	healthy person (28)	Benguela	TOB, STR, KAN, CIP
	A_1	III	ST181/CC168	**330**	140	—	+	+	+	healthy person (29)	Benguela	GEN, TOB, STR, KAN, NET, CIP, SUL, TMP, TET, CHL
	A_0	IV	ST2325	**330**	—	—	+	+	+	healthy person (29)	Benguela	GEN, TOB, STR, KAN, NET, CIP, SUL, TMP, TET, CHL
	A_1	V	ST5091	**200**	110	FIA	+	+	+	healthy person (31)	Baía Farta	GEN, TOB, STR, KAN, NET, CIP, NAL, SUL, TMP, TET, CHL
	A_1	VI	ST5092	—	100	—	–	–	–	floor/walls (10/Farm A)	Benguela	STR, KAN, CIP, TMP, TET
	A_0 (2)	VII (2)	ST5093	—	—	Y; P; FIB	–	+	–	floor/walls (10/Farm A)	Benguela	(GEN), TOB, STR, (CIP), (NAL), SUL, TMP, TET, CHL
	A_1 (4)	VIII (4)	ST10/CC10	—	—	N (2)	–	–	–	pigs (16, 17/Farm D)	Benguela	(GEN), (TOB), STR, (KAN), NAL, SUL, TMP, TET
	A_1	IX	ST5094	**205** (FII36:A4:B1)	—	—	–	+	+	pigs (17/Farm D)	Benguela	GEN, TOB, STR, KAN, CIP, NAL, SUL, TMP, TET, CHL
	A_1	X	ST5095	**180** (FII36:A4:B1)	—	—	–	+	+	pigs (17/Farm D)	Benguela	GEN, TOB, STR, KAN, NET, CIP, NAL, SUL, TMP, TET
	A_1	XI	ST10/CC10	**80**	—	—	+	–	+	pigs (17/Farm D)	Benguela	STR, SUL, TMP, TET
	A_0	XII	ST5096	**130**	—	FIB	+	–	–	pigs (17/Farm D)	Benguela	STR, CIP, NAL, SUL, TMP, TET
	A_0 (2)	XIII (2)	ST5154	—	—	—	–	–	–	cows (14, 15/Farm D)	Benguela	GEN, TOB, (AMK), STR, KAN, CIP, NAL, TET
	A_0 (2)	XIV (2)	ST5097	**70**	—	—	+	–	–	cows (15/Farm D)	Benguela	(TOB), STR, (NET), NAL, SUL, TMP, TET
	A_1	XV	ST617/CC10	—	130	FIA	+	–	–	wastewater (59)	Benguela	GEN, TOB, STR, KAN, CIP, NAL, SUL, TMP, TET
	A_0	XVI	ST5093	—	—	Y; P	–	+	–	wastewater (60)	Benguela	TOB, SUL, TMP, TET, CHL
	A_1	XVII	ST617/CC10	—	—	FIB; FIA	–	+	–	wastewater (60)	Benguela	GEN, TOB, STR, NET, CIP, NAL, SUL, TMP, TET
	B_1	XVIII	ST156/CC156	—	—	FII	+	–	+	healthy person (36)	Benguela	GEN, TOB, STR, KAN, CIP, NAL, SUL, TMP, CHL
	B_1	XIX	ST1727	—	—	—	–	–	–	cows (14/Farm D)	Benguela	TOB, STR, KAN
	B_1 (2)	XX (2)	ST448/CC448	**215** (FII36:A4:B1)			–	+	+	treated water for human consumption (62)	Lobito	GEN, TOB, STR, KAN, NET, CIP, NAL, SUL, TMP, TET, CHL
	B_1	XXI	ST167/CC10	**180** (FII36:A4:B1)	100	Y	+	–	+	wastewater (59)	Benguela	GEN, TOB, AMK, STR, KAN, NET, CIP, NAL, SUL, TMP, TET, CHL
	B_2	XXII	ST372	**330** (HI2)	—	—	+	+	+	healthy person (29)	Benguela	GEN, TOB, STR, KAN, NET, CIP, NAL, SUL, TMP, TET, CHL
	D_2 (5)	XXIII (5)	ST38/CC38	—[d]	100	FII	–	–	–	water for animal consumption (43, 44/Wild Animal Park)	Dombe Grande	STR, (CIP), (NAL)

Downloaded from http://jac.oxfordjournals.org/ by guest on February 17, 2016

K. pneumoniae (*n*=4)	NA	XXIV (3)	ST730	**100** (Y)	200; 377	FIIk; I1	+	–	–	chickens (8; Farm A); water for animal consumption (57/Farm A)	Benguela	STR, (TOB), (KAN), CIP, (NAL), SUL, TMP, TET, CHL
	NA	XXV	ST215	**200** ($FIIK_{kp}$)	—	—	+	+	+	pigs (17/Farm D)	Benguela	GEN, TOB, STR, KAN, NET, CIP, SUL, TMP, TET
K. oxytoca (*n*=1)	NA	XXVI	NA	**310** (HI2)	90; 170	Y	+	+	+	urban sewer line wastewater (58)	Catumbela	GEN, TOB, STR, NET, CIP, NAL, SUL, TMP, TET, CHL
E. hormaechei (*n*=2)	NA	XXVII	NA	**335** (HI2)	—	—	+	+	+	healthy person (29)	Benguela	STR, NAL, SUL, TMP, TET
	NA	XXVIII	NA	—	90	—	+	+	+	river (38)	Catumbela	GEN, TOB, STR, KAN, NET, NAL, SUL, TMP, TET, CHL
E. asburiae (*n*=1)	NA	XIX	NA	—	60	—	–	+	+	river (38)	Catumbela	GEN, TOB, KAN, TMP, TET
C. freundii (*n*=1)	NA	XXX	NA	—[d]	70	—	+	–	–	wastewater (59)	Benguela	STR, CIP, NAL, SUL, TMP, TET, CHL
C. werkmanii (*n*=1)	NA	XXXI	NA	**100** (Y)	—	—	+	+	+	wastewater (59)	Benguela	GEN, TOB, STR, KAN, NET, CIP, NAL, SUL, TMP, TET, CHL

AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NET, netilmicin; STR, streptomycin; SUL, sulphonamides; TET, tetracyclines; TMP, trimethoprim; TOB, tobramycin; NA, not applicable.

[a]Plasmids carrying *bla* genes are shown in bold and transferability of the $bla_{CTX\text{-}M\text{-}15}$ gene is indicated by underlining.

[b]Samples were collected from Benguela Province, which comprises nine municipalities (Benguela, Lobito, Bocoio, Balombo, Ganda, Cubal, Caimbambo, Baia Farta and Chongoroi), divided into >30 communes.

[c]The variable presence of a given resistance phenotype is indicated in parentheses.

[d]$bla_{CTX\text{-}M\text{-}15}$ is located on the chromosome.

Materials and methods

Sample collection

Eighty-one samples were collected from different non-clinical origins of the Benguela Province of Angola in 2013 (Figure S1, available as Supplementary data at *JAC* Online), as detailed below.

Healthy persons

Eighteen rectal swabs were taken from randomly selected healthy persons without exposure to antibiotics or hospitals in the 3 months preceding sampling. All participants provided written informed consent.

Animals and their environment

Thirty-six samples were collected from healthy food-producing animals (n=28, rectal swabs) and their environments (n=8) at Farms A and B (10–20 animals) and Farms C and D (>200 animals) [13 chickens (Farms A and C), 9 cows (Farms B and D), 6 pigs (Farm D), 3 waters for animal consumption (100 mL; Farms A–C), 3 feed samples (25 g; Farms A, C and D) and 2 swabs from floor/walls (Farms A and C)], as well as seven samples from wild animals (2 monkeys and 3 goats; faeces) and waters for animal consumption (n=2; 100 mL) at a Wild Animal Park.

Aquatic environments

Twenty samples were collected from river (n=2) or lagoon (n=1) waters (close to agricultural/residential areas, domestic animal production farms and/or septic tanks), urban sewer line wastewaters (n=3), a wastewater treatment station (n=2) and waters used for human consumption (n=12; 7 treated and 5 untreated) (100 mL).

Sample processing

Faecal samples from food-producing animals of the same type and farm were pooled in lots of three totalling 10 samples (9 pools and 1 individual). Sixty-three samples were processed as described previously[9] and cultured on CHROMagar™ Orientation with and without cefotaxime (1 mg/L), ceftazidime (1 mg/L) or imipenem (1 mg/L), followed by selection of isolates representing different colony morphotypes of presumptive Enterobacteriaceae per plate.

Detection and characterization of bla genes

ESBL, qAmpC or carbapenemase genes were identified by PCR and sequencing.[9–11] ESBL or carbapenemase production was additionally discarded by phenotypic tests (http://www.eucast.org/).[12]

Bacterial identification, antimicrobial susceptibility testing and conjugation assays

Isolates carrying acquired *bla* genes were identified by MALDI-TOF MS (Bruker Daltonik, Leipzig, Germany). *Citrobacter freundii* complex and *Enterobacter cloacae* complex were identified at species level by sequencing of genotypic markers.[13,14] Antibiotic susceptibility testing and conjugation assays (24, 30, 37 and 42°C) were performed as described previously.[10,15]

Molecular characterization of isolates carrying acquired bla genes

Escherichia coli phylogroups were identified[16] and clonal relatedness was established by XbaI-PFGE[10] and MLST for representative *E. coli* and *Klebsiella pneumoniae* isolates (different PFGE types) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html; http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers_Kpneumoniae.html).

Downloaded from http://jac.oxfordjournals.org/ by guest on February 17, 2016

The location of *bla* was investigated by hybridization of S1-, I-CeuI- or XbaI-digested (when negative with S1/I-CeuI) genomic DNA.[15] Plasmids carrying *bla* were characterized by replicon typing and subtyping (IncF plasmids; http://pubmlst.org/plasmid/primers/incF.shtml).[15,17,18] The $bla_{CTX\text{-}M\text{-}15}$ genetic environment was also investigated.[9,19]

Results and discussion

High occurrence of ESBLs, with dominance of CTX-M-15 among different species and non-clinical niches

The bla_{ESBL} genes were identified in isolates ($n=45$) of different species and almost all sample types (30.2%; 19 of 63 samples) (Table 1), while qAmpC or carbapenemase genes were not found. Thus, we report for the first time the dispersion of ESBL-producing bacteria in a wide diversity of non-clinical niches in Angola. The human faecal carriage of ESBL producers (22.2%; 4 of 18 samples) was higher than in most African (6.7%–21.7%) (Table S1) or developed (0.6%–20.3%) countries,[1] despite differences in sample size. Similarly, the occurrence of ESBLs among food-producing animals and their environments (25%–50%) or aquatic samples (30%) was higher than expected (Table 1 and Table S1). The detection of ESBL producers among healthy persons and aquatic samples reflects a worrying level of environmental contamination, which in poor living conditions poses serious risk of transmission.[5] Despite the paucity of data regarding antibiotic consumption and resistance in Angola,[6] the high rates of clinical bacteria resistant to extended-spectrum cephalosporins recently reported[20] suggests a similar scenario in clinical settings.

All but one ESBL producer (1 *K. pneumoniae*/SHV-12) encoded CTX-M-15 (98%, 44/45; Table 1). CTX-M-15 producers were identified as *E. coli* ($n=34$), *K. pneumoniae* ($n=4$), *Enterobacter hormaechei* ($n=2$), *Enterobacter asburiae* ($n=1$), *C. freundii* ($n=1$), *Citrobacter werkmanii* ($n=1$) and *Klebsiella oxytoca* ($n=1$) recovered from diverse sample types, and were frequently resistant to non-β-lactams (Table 1). The wide dispersion of CTX-M-15 in non-clinical settings has been well documented in developed countries,[1,2] but its almost exclusive detection in such a diversity of species and niches in Angola contrasts with the situation in other African countries (Table S1).[7,18] This atypical scenario could reflect a recent penetration of $bla_{CTX\text{-}M\text{-}15}$ into this geographical area and/or the local emergence of $bla_{CTX\text{-}M\text{-}15}$ driven by unrecognized factors.

CTX-M-15 producers from animals were identified on different farms, some of which import animals from Portugal (e.g. chickens/Farm A), where ESBLs other than CTX-M-15 are frequent.[9,19] To the best of our knowledge, this is the first report of CTX-M-15-producing *K. pneumoniae* among healthy food-producing animals.

Identification of international *E. coli* clones from phylogroups A, B1 and D

CTX-M-15-producing *E. coli* were clonally diverse ($n=34$; 23 PFGE types and 20 STs) and belonged to phylogroup A ($n=23$; 68%), B1 ($n=5$; 15%), D ($n=5$; 15%) or B2 ($n=1$; 3%) distributed in different niches (Table 1). The higher prevalence of CTX-M-15 among *E. coli* belonging to phylogroup A might reflect that CTX-M-15 is well established in human/animal commensal strains in sub-Saharan countries (Table S1).[8] The pandemic B2-ST131 *E. coli*[1,2] was absent (Table S1), whereas widespread clonal complexes (CCs) from A (one CC168 and seven CC10), B1 (one CC156) or D (five CC38) phylogroups were frequent (Table 1). These clones are widely represented in collections of ESBL, qAmpC and/or carbapenemase producers from non-clinical niches in developed and non-developed countries (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/GetTableInfo_html).[2,18,19] The remaining isolates ($n=20$) belonged to ST448 or STs identified here for the first time (Table 1).

CTX-M-15-producing *K. pneumoniae* belonged to ST730 ($n=3$; one PFGE type) or ST215 ($n=1$) (Table 1). The international *K. pneumoniae* clonal groups CG15 or CG258, highly represented in clinical isolates in Africa, were absent.[21] *E. hormaechei* were clonally diverse ($n=2$; two PFGE types) (Table 1).

Location of $bla_{CTX\text{-}M\text{-}15}$ in variable typeable and non-typeable plasmids or in the chromosome of different species

$bla_{CTX\text{-}M\text{-}15}$ was transferred by conjugation in 20% of the isolates (9/44; 24 and 37°C), being unequivocally identified on plasmids of variable sizes (~70–335 kb) ($n=20/44$, 45%) or on the chromosome ($n=6/44$, 14%), while in 41% ($n=18/44$) of the isolates its specific location was not determined (Table 1).

Plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ were identified as 180–215 kb IncFII (F36:A4:B1) in diverse *E. coli* ($n=5$), 310–335 kb IncHI2 in *K. oxytoca*, *E. hormaechei* and *E. coli* ($n=1$ each), 200 kb $IncFII_{K6}$ in *K. pneumoniae* ($n=1$) or 100 kb IncY in diverse *K. pneumoniae* ($n=3$) or *C. werkmanii* ($n=1$) (Table 1). For seven *E. coli*, $bla_{CTX\text{-}M\text{-}15}$ hybridized in non-typeable plasmids (~70–330 kb) (Table 1), uncovering novel replicon types in this region. The association of $bla_{CTX\text{-}M\text{-}15}$ with the F36:A4:B1 plasmid variant was previously identified in clinical isolates (Central African Republic) and pets (France).[22,23] $bla_{CTX\text{-}M\text{-}15}$ was chromosomally located in 14% (five *E. coli* and one *C. freundii*) of the isolates, a situation increasingly reported.[16] Negative results in S1/I-CeuI hybridization assays were obtained ($n=18$, 41%; *E. coli*, *E. hormaechei* and *E. asburiae*), although plasmids (60–130 kb) were detected for some isolates (data not shown). However, positive hybridization signals were observed in XbaI-digested genomic DNA (29–669 kb bands; Figure S2), as reported previously.[16] The absence of conjugative transfer and the band sizes (25% >250 kb) suggest a $bla_{CTX\text{-}M\text{-}15}$ chromosomal location, although we cannot discard non-typeable plasmids.

Diversity of $bla_{CTX\text{-}M\text{-}15}$ genetic environments

Most isolates harboured $bla_{CTX\text{-}M\text{-}15}$ flanked upstream by IS*Ecp1* or IS*26* ($n=36$ or $n=4$, respectively) and downstream by *orf477* ($n=44$), resembling common genetic platforms.[19] In two isolates, IS*3* was 116 bp after the 3′ end of IS*Ecp1*, corresponding to a novel configuration (GenBank accession number KT192055). CTX-M-15 producers harboured variably $bla_{TEM\text{-}1}$ (48%), $bla_{OXA\text{-}1}$ (41%) or *aac(6′)-Ib-cr* (39%) (Table 1). The simultaneous presence of $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$ and *aac(6′)-Ib-cr*, or $bla_{TEM\text{-}1}$ and *aac(6′)-Ib-cr* were observed mostly in isolates harbouring $bla_{CTX\text{-}M\text{-}15}$ located on plasmids (Table 1).

Conclusions

In this study, one of the few conducted in Africa embracing such a high diversity of samples, we report a high and almost exclusive occurrence of CTX-M-15 in diverse Enterobacteriaceae species

Downloaded from http://jac.oxfordjournals.org/ by guest on February 17, 2016

and non-clinical niches in Angola. The variability observed in $bla_{CTX\text{-}M\text{-}15}$ genetic environments, genetic locations and clones suggests an extraordinary ability for acquisition and mobilization of $bla_{CTX\text{-}M\text{-}15}$ by multiple genetic backgrounds, which is not comparable to that reported in developed countries. Moreover, our study unveils possible novel plasmid backgrounds involved in the spread of $bla_{CTX\text{-}M\text{-}15}$ in natural reservoirs in under-researched geographical regions.

Acknowledgements

We are very grateful to Hélder Gonçalves, Erineia Gonçalves and Walter Bonfim, for support in sample recruitment, and to all contributing farmers and healthy volunteers.

Funding

This work received financial support from the European Union (FEDER funds) through Programa Operacional Factores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through project UID/Multi/04378/2013. The work also received funds from the European Union (FEDER funds) under the framework of Quadro de Referência Estratégica Nacional (QREN) NORTE-07-0124-FEDER-000066. T. G. R. and Â. N. were supported by fellowships from FCT (SFRH/BD/75752/2011 and SFRH/BPD/104927/2014, respectively).

Transparency declarations

None to declare.

Supplementary data

Table S1, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Woerther PL, Burdet C, Chachaty E *et al*. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. *Clin Microbiol Rev* 2013; **26**: 744–58.

2 Ewers C, Bethe A, Semmler T *et al*. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect* 2012; **18**: 646–55.

3 Guerra B, Fischer J, Helmuth R. An emerging public health problem: acquired carbapenemase-producing microorganisms are present in food-producing animals, their environment, companion animals and wild birds. *Vet Microbiol* 2014; **171**: 290–7.

4 van der Bij AK, Pitout JD. The role of international travel in the worldwide spread of multiresistant Enterobacteriaceae. *J Antimicrob Chemother* 2012; **67**: 2090–100.

5 Walsh TR, Weeks J, Livermore DM *et al*. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* 2011; **11**: 355–62.

6 Leopold SJ, van Leth F, Tarekegn H *et al*. Antimicrobial drug resistance among clinically relevant bacterial isolates in sub-Saharan Africa: a systematic review. *J Antimicrob Chemother* 2014; **69**: 2337–53.

7 Storberg V. ESBL-producing Enterobacteriaceae in Africa – a non-systematic literature review of research published 2008–2012. *Infect Ecol Epidemiol* 2014; **4**: 20342.

8 Albrechtova K, Kubelova M, Mazancova J *et al*. High prevalence and variability of CTX-M-15-producing and fluoroquinolone-resistant *Escherichia coli* observed in stray dogs in rural Angola. *Microb Drug Resist* 2014; **20**: 372–5.

9 Machado E, Coque TM, Cantón R *et al*. Antibiotic resistance integrons and extended-spectrum β-lactamases among Enterobacteriaceae isolates recovered from chickens and swine in Portugal. *J Antimicrob Chemother* 2008; **62**: 296–302.

10 Freitas F, Machado E, Ribeiro TG *et al*. Long-term dissemination of acquired AmpC β-lactamases among *Klebsiella* spp. and *Escherichia coli* in Portuguese clinical settings. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 551–8.

11 Bogaerts P, Rezende de Castro R, de Mendonça R *et al*. Validation of carbapenemase and extended-spectrum β-lactamase multiplex endpoint PCR assays according to ISO 15189. *J Antimicrob Chemother* 2013; **68**: 1576–82.

12 Pires J, Novais Â, Peixe L. Blue-Carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J Clin Microbiol* 2013; **51**: 4281–3.

13 Ribeiro TG, Novais Â, Branquinho R *et al*. Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species. *Antimicrob Agents Chemother* 2015; **59**: 5951–8.

14 Andrade LN, Novais Â, Branquinho R. *et al*. Species reassignment of *Enterobacter cloacae* complex isolates by *in silico* phylogenetic analysis. In: *Abstracts of the Sixth Congress of European Microbiologists, 2015*. Abstract FEMS-2692, p. 749. Federation of European Microbiological Societies, Maastricht, The Netherlands.

15 Rodrigues C, Novais Â, Machado E *et al*. Detection of VIM-34, a novel VIM-1 variant identified in the intercontinental ST15 *Klebsiella pneumoniae* clone. *J Antimicrob Chemother* 2014; **69**: 274–5.

16 Rodríguez I, Thomas K, Van Essen A *et al*. Chromosomal location of $bla_{CTX\text{-}M}$ genes in clinical isolates of *Escherichia coli* from Germany, The Netherlands and the UK. *Int J Antimicrob Agents* 2014; **43**: 553–7.

17 Johnson TJ, Bielak EM, Fortini D *et al*. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant Enterobacteriaceae. *Plasmid* 2012; **68**: 43–50.

18 Ben Sallem R, Ben Slama K, Rojo-Bezares B *et al*. IncI1 plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ or $bla_{CMY\text{-}2}$ genes in *Escherichia coli* from healthy humans and animals in Tunisia. *Microb Drug Resist* 2014; **20**: 495–500.

19 Rodrigues C, Machado E, Peixe L *et al*. IncI1/ST3 and IncN/ST1 plasmids drive the spread of $bla_{TEM\text{-}52}$ and $bla_{CTX\text{-}M\text{-}1/\text{-}32}$ in diverse *Escherichia coli* clones from different piggeries. *J Antimicrob Chemother* 2013; **68**: 2245–8.

20 Agostinho MH. IACS – international experiences. In: *Oral Communications of the International Congress on Preventing and Fighting Healthcare-Associated Infections, Coimbra, 2014*. www.apih.pt/congresso_2014/18_iacs_angola.pdf.

21 Breurec S, Guessennd N, Timinouni M *et al*. *Klebsiella pneumoniae* resistant to third-generation cephalosporins in five African and two Vietnamese major towns: multiclonal population structure with two major international clonal groups, CG15 and CG258. *Clin Microbiol Infect* 2013; **19**: 349–55.

22 Rafaï C, Frank T, Manirakiza A *et al*. Dissemination of IncF-type plasmids in multiresistant CTX-M-15-producing Enterobacteriaceae isolates from surgical-site infections in Bangui, Central African Republic. *BMC Microbiol* 2015; **15**: 15.

23 Dahmen S, Haenni M, Châtre P *et al*. Characterization of $bla_{CTX\text{-}M}$ IncFII plasmids and clones of *Escherichia coli* from pets in France. *J Antimicrob Chemother* 2013; **68**: 2797–801.

Downloaded from http://jac.oxfordjournals.org/ by guest on February 17, 2016

Supplementary data

Table S1. Epidemiological data of ESBL-producing bacteria identified in different non-clinical niches in Africa

Origin	Region[a]	Country	Year(s) of isolation	Sample size [type, (no.)]	ESBL occurrence (no. of positive samples/no. of samples analysed)	ESBL enzyme(s) (no. of isolates)	Species (no.)	PhG-ST/CC (no.)[b]	Plasmid content [Inc family, pMLST/FAB formula, size (kb)]	References
Healthy humans	North	Tunisia	2009-2010	150 [Farmers/Veterinarians (9), Medical personnel (7), Community healthy persons (134)]	7.3% (11/150)	CTX-M-1 (10)	*E. coli*	B1-ST58/CC155 (3) B1-ST155/CC155 (1) B1-ST48/CC10 (1) A-ST10/CC10 (1) A-ST165/CC165 (1) A-ST398/CC398 (1) D-ST57/CC350 (2)	I1 (ST3) (97-100)	1, 2
						TEM-52 (1)		B2-ST219	NP	
	Sub-Saharan	Angola	2013	18 [Community healthy persons]	22.2% (4/18)	CTX-M-15 (8)	*E. coli* (7)	A-ST181/CC168 (1) A-ST2325 (1) A-ST4977 (1) A-ST5044 (1) A-ST5091 (1) B1-ST156/CC156 (1) B2-ST372 (1)	HI2 (330-335) UT (200-330)	This study
							E. hormaechei (1)			
		Cameroon	2009	150 [Students]	6,7% (10/150)	CTX-M-15 (10)	*E. coli* (9) *K. pneumoniae* (1)	NP	NP	3
		Central African Republic	2011-2012	32 [Employers (16), Villagers (14), European researchers (2)]	9.4% (3/32)	CTX-M-15+SHV-2a (1)	*K. pneumoniae*	Kp-ST1212 (1)	NR (80-330)	4
						CTX-M-15 (2)	*E. coli*	A-ST218/CC10 (1) A-ST148/CC148 (1)		
		Kenya	2009	23 [Healthy humans]	21.7% (5/23)	CTX-M-15 (5)	*E. coli*	A-NP (4), B1-NP (1)	FIB (150)	5

Table S1. *Continued*

		Madagascar	2009	484 [Patients attending for the first time health centers]	10.1% (49/484)	CTX-M-15 (46)	*E. coli* (28) *K. pneumoniae* (13) *C. freundii* (3) *E. cloacae* (1) *Pantoea* sp. (1)	A1-NP (14/31 *E. coli* isolates)	NP	6
						CTX-M-3 (2)	*E. coli*			
						CTX-M-1 (1)	*E. coli*			
						SHV-12 (2)	*E. cloacae*			
						SHV-2 (1)	*K. pneumoniae*			
						NR (1)	*Kluyvera* spp.			
		Nigeria	2006-2007	220 [Community healthy persons]	NR	NP	*K. pneumoniae* (3) *E. coli* (2) *K oxytoca* (1) *P. aeruginosa* (1)	NP	NP	7
		Senegal	NR	20 [Healthy children]	10.0% (2/20)	CTX-M-15 (5)	*E. coli*	A1-NP	FII+FIA+FIB	8
Livestock animals	North	Tunisia	2006	36 [Chickens (6), Cattle (16), Horses (14), Cats (2), Dogs (2)]	ND	ND	-	-	-	9
		Tunisia	2010	136 [Poultry]	30.1% (41/136)	CTX-M-1 (39)	*E. coli*	A-NP (20) D-NP (12) B1-NP (7)	I1	10
						CTX-M-15 (4)		A-NP (2) B1-NP (2)	NP	
		Tunisia	2011	80 [Sheep (23), Chickens (22), Cattle (22), Horses (6), Rabbits (5), Dromedaries (2)]	11.2% (9/80)	CTX-M-1 (9)	*E. coli*	B1-ST155/CC155 (2) B1-ST58/CC155 (1) B1-ST2016 (1) B1-ST88 (1) A-ST10/CC10 (1) A-ST2164 (1) D-ST57/CC350 (1) D-ST2255 (1)	I1 (ST3) (100-110)	2, 11

Table S1. *Continued*

		Tunisia	2013	65 [Chickens]	26.1% (17/65)	CTX-M-1 (16)	*E. coli*	A-NP (12) D-NP (3) $B2_3$(1)	NP	12
						ND (1)		$B2_2$(1)		
	Sub-Saharan	Nigeria	2006	200 [Chickens (100), Pigs (100)]	0.5% (1/200)	CTX-M-15 (1)	*E. coli*	NP	NP	13
		Nigeria	2010-2011	57 [Bovine]	ND	ND	*E. coli*	-	-	14
Livestock animals and their environments	Sub-Saharan	Angola	2013	10 [Chickens (5), Cows (3), Pigs (2)]	50.0% (5/10)	CTX-M-15 (16)	*E. coli* (13)	A-ST10/CC10 (5) A-ST5097 (2) A-ST5154 (2) A-ST1727 (1) A-ST5094 (1) A-ST5095 (1) A-ST5096 (1)	FII36:A4:B1 (180-205), UT (70-130), Y (100), $FIIk_6$ (200)	This study
							K. pneumoniae (3)	Kp-ST730 (2) Kp-ST1215 (1)		
				8 [Waters for animal consumption (3)	25.0% (2/8)	CTX-M-15 (1)	*K. pneumoniae*	Kp-ST730 (1)		
				Floor/walls from animal farms (2)		CTX-M-15 (3)	*E. coli*	A-ST5093 (2) A-ST5092 (1)		
				Animal feed (3)]		ND		ND		
Pet animals	North	Tunisia	2006	4 [Cats (2), Dogs (2)]	ND	ND	-	-	-	9
		Tunisia	2010	80 [Dogs (41), Cats (39)]	16.2% (13/80)	CTX-M-1 (13)	*E. coli*	B1-ST155/CC155 (1) B1-ST58/CC155 (1) B1-ST602/CC446 (1) B1-ST345 (1) B1-ST539 (1) A-ST10/CC10 (1) A-ST398/CC398 (1) A-ST1914 (1) A-ST1720 (1) A-ST1431 (1) D-ST57/CC350 (2) D-ST3562 (1)	I1 (ST3) (97-100)	2, 15

Table S1. *Continued*

		Tunisia	2011-2012	45 [Dogs (41), Cats (4)]	15.6% (7/45)	CTX-M-1 (6) CTX-M-15 (1)	*E. coli*	NP	I1 (ST3) (100-120), 1 (ST25) (115) F31:A3:B1 (160)	16
	Sub-Saharan	Kenya	2009	226 [Dogs (216), Cats (50)]	18.0% (48/266)	CTX-M-15 (48)	*E. coli*	A-NP (41) B1-NP (4) B2-ST131 (3)	FIA+FIB (150-160), FIB (110-150), I1 (90)	5
Wild animals	Sub-Saharan	Central African Republic	2011-2012	82 [Gorillas (58), Monkeys (9), Chimpanzees (7), Buffalos (4), Elephants (4), Pigs (2), Antelope (5)]	NR	CTX-M-15+SHV-62 (1) CTX-M-2 (1)	*K. pneumoniae* *K. ascorbata*	ST1211 -	NR (130-340) NP	4
Wild animals and their environments	Sub-Saharan	Angola	2013	5 [Monkeys (2) Goats (3)] 2 [Waters for animal consumption]	ND 100% (2/2)	- CTX-M-15 (5)	- *E. coli*	- D-ST38/CC38 (5)	-	This study
Stray Animals	Sub-Saharan	Angola	NR	17 [Stray dogs]	75.0% (12/16 cultivated samples)	CTX-M-15 (13)	*E. coli*	A-ST46/CC46 (5) A-ST617/CC10 (2) A-ST58/CC155 (1) A-ST3653 (1) A-ST3687 (2) A-ST3694 (1) A-ST3726 (1)	FIB, Y, N, I1, Unt-p	17
Food	North	Tunisia	2006	38 [Beef (23), Chicken (8), Turkey (2), Sheep (1), Fish (1)]	26.3% (10/38)	CTX-M-1 (5) SHV-5 (1) CTX-M-14 (2) CTX-M-8 (1) CTX-M-1 (1)	*E. coli*	B1-ST155/CC155 (3) A-ST155/CC155 (1) A-ST10/CC10 (1) B1-ST889 B1-ST2022 (2) A-ST405/CC405 (1) A-ST23/CC23 (1)	NP	9, 18
		Tunisia	2007	79 [Poultry (26), Sheep (28), Beef (14), Fish (10), Horse (1)]	12.6% (10/79) (7 poultry, 2 sheep, 1 beef)	CTX-M-1 (12) CTX-M-1+TEM-20 (1)	*E. coli*	B1-ST602/CC446 (5) B1-ST101/CC101 (3) A-ST1970 (2) A-ST522/CC522 (1) A-ST1638 (1) B1-ST224 (1)	NP	18, 19

Table S1. *Continued*

		Tunisia	2012-2013	94 [Vegetables]	8.5% (8/94)	CTX-M-15 (4)	*C. freundii* (2) *E. hormaechei* (2)	-	ND	20
						CTX-M-1 (3)	*E. coli*	B1-ST58/CC155 (2) D-ST117 (1)	I1 (97)	
						CTX-M-14 (1)	*E. coli*	A-ST10/CC10 (1)	ND	
						SHV-12 (1)	*C. freundii*	-	ND	
Environment	North	Algeria	2009	4 [Beach (3), Lake (1)]	NR	CTX-M-15 (1)	*E. coli*	NP	I1 (50)	21
		Algeria	2010	6 [Wastewater treatment plant (6: 3 raw water, 3 treated water)]	NR	CTX-M-15 (15)	*K. pneumoniae* (12)	KpI-ST147 (3) KpI-ST17 (1) KpI-ST15 (1) KpI-ST48 (1) KpI-NP (2)	FII (155), ND (14 isolates)	22
							E. coli (3)	A-ST617/CC10 (1) D-ST405/CC405 (2)		
						CTX-M-3 (6)	*E. coli* (3)	A-ST10/CC10 (2) B1-ST1431 (1)	L/M (80-98.5)	
							K. pneumoniae (3)	KpI-ST54 (1) KpI-ST17 (1) KpI-ST36 (1)		
		Tunisia	2010	2 [Rivers]	NR	SHV-12a (33)	*K. pneumoniae* (30) *P. aeruginosa* (3)	NP	NP	23
						CTX-M-15 (33)	*K. pneumoniae* (16) *E. coli* (8) *P. aeruginosa* (6) *E. aerogenes* (3)			
						TEM-15 (15)	*E. coli* (13) *P. rettgeri* (2)			
		Tunisia	2012-2013	60 [Soil (41) and Irrigation water (19) of farms]	10 (6/60)	CTX-M-15 (4)	*E. coli* (2)	B2-ST131 (1) D-ST3496	ND	20
							K. pneumoniae (1) *C. freundii* (1)			
						CTX-M-1 (2)	*E. coli* (2)	A-ST23/CC23 (1)	I1 (97)	
								B1-ST58/CC155 (1)	ND	

Table S1. *Continued*

Sub-Saharan	Angola	2013	20 [Rivers (2), Lagoon (1), Treated waters (7), Untreated water (5; 4 boreholes, 1 fountain), Urban sewer lines wastewaters (3), Wastewater treatment station (2)]	30.0% (6/20)	CTX-M-15 (11)	*E. coli* (6)	A-ST617/CC10 (2) A-ST5093 (1) B1-ST448/CC448 (2) B1-ST167/CC10 (1)	FII36:A4:B1 (180-215), HI2 (300), Y (100)	This study
						K. oxytoca (1) *E. hormaechei* (1) *E. asburiae* (1) *C. freundii* (1) *C. werkmanii* (1)			
					SHV-12 (1)	*K. pneumoniae*	NP	NP	
	Democratic Republic of the Congo	2009-2010	101 [Sachet-packaged water bags]	NR	CTX-M group I (5)	*C. freundii* (3) *K. pneumoniae* (2)	NP	NP	24
					SHV-2-*like* (1) SHV-18 (2)	*E. cloacae* *K. pneumoniae* (1) *E. coli* (1)			
	Democratic Republic of the Congo	2011	20 [Sewers (11), Rivers (9)]	40.0% (8/20: 5 sewers, 3 rivers)	CTX-M group I (10) ND (4)	*E. cloacae* (6) *K. pneumoniae* (4)	NP	NP	25

[a]Epidemiological data from Northern African countries are grey shaded. [b]PhG-ST/CC, *E. coli* phylogenetic group-sequence type/clonal complex; Kp, *Klebsiella pneumoniae*. Abbreviations: ND, not detected; NR, not reported; NP, not performed; Unt-p, untypeable plasmids.

References

1. Ben Sallem R, Ben Slama K, Estepa V *et al*. Prevalence and characterisation of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates in healthy volunteers in Tunisia. *Eur J Clin Microbiol Infect Dis* 2012; **31**: 1511-6.
2. Ben Sallem R, Ben Slama K, Rojo-Bezares B *et al*. IncI1 plasmids carrying $bla_{\text{CTX-M-1}}$ or $bla_{\text{CMY-2}}$ genes in *Escherichia coli* from healthy humans and animals in Tunisia. *Microb Drug Resist* 2014; **20**: 495-500.
3. Lonchel CM, Meex C, Gangoué-Piéboji J *et al*. Proportion of extended-spectrum ß-lactamase-producing Enterobacteriaceae in community setting in Ngaoundere, Cameroon. *BMC Infect Dis* 2012; **12**: 53.
4. Janatova M, Albrechtova K, Petrzelkova KJ *et al*. Antimicrobial-resistant Enterobacteriaceae from humans and wildlife in Dzanga-Sangha Protected Area, Central African Republic. *Vet Microbiol* 2014; **171**: 422-31.
5. Albrechtova K, Dolejska M, Cizek A *et al*. Dogs of nomadic pastoralists in northern Kenya are reservoirs of plasmid-mediated cephalosporin- and quinolone-resistant *Escherichia coli*, including pandemic clone B2-O25-ST131. *Antimicrob Agents Chemother* 2012; **56**: 4013-7.
6. Herindrainy P, Randrianirina F, Ratovoson R *et al*. Rectal carriage of extended-spectrum beta-lactamase-producing gram-negative bacilli in community settings in Madagascar. *PLoS One* 2011; **6**: e22738.
7. Afunwa RA, Odimegwu DC, Iroha RI *et al*. Antimicrobial resistance status and prevalence rates of extended spectrum beta-lactamase (ESBL) producers isolated from a mixed human population. *Bosn J Basic Med Sci* 2011; **11**: 91-6.
8. Ruppé E, Woerther PL, Diop A *et al*. Carriage of CTX-M-15-producing *Escherichia coli* isolates among children living in a remote village in Senegal. *Antimicrob Agents Chemother* 2009; **53**: 3135-7.

9. Jouini A, Vinué L, Slama KB *et al*. Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. *J Antimicrob Chemother* 2007; **60**: 1137-41.

10. Mnif B, Ktari S, Rhimi FM *et al*. Extensive dissemination of CTX-M-1- and CMY-2-producing *Escherichia coli* in poultry farms in Tunisia. *Lett Appl Microbiol* 2012; **55**: 407-13.

11. Ben Sallem R, Ben Slama K, Sáenz Y *et al*. Prevalence and characterization of extended-spectrum beta-lactamase (ESBL)- and CMY-2-producing *Escherichia coli* isolates from healthy food-producing animals in Tunisia. *Foodborne Pathog Dis* 2012; **9**: 1137-42.

12. Kilani H, Abbassi MS, Ferjani S *et al*. Occurrence of $bla_{\text{CTX-M-1}}$, *qnrB1* and virulence genes in avian ESBL-producing *Escherichia coli* isolates from Tunisia. *Front Cell Infect Microbiol* 2015; **5**: 38.

13. Fortini D, Fashae K, García-Fernández A *et al*. Plasmid-mediated quinolone resistance and β-lactamases in *Escherichia coli* from healthy animals from Nigeria. *J Antimicrob Chemother* 2011; **66**: 1269-72.

14. Inwezerua C, Mendonça N, Calhau V *et al*. Occurrence of extended-spectrum beta-lactamases in human and bovine isolates of *Escherichia coli* from Oyo state, Nigeria. *J Infect Dev Ctries* 2014; **8**: 774-9.

15. Sallem RB, Gharsa H, Slama KB *et al*. First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. *Vector Borne Zoonotic Dis* 2013; **13**: 98-102.

16. Grami R, Mansour W, Dahmen S *et al*. The $bla_{\text{CTX-M-1}}$ IncI1/ST3 plasmid is dominant in chickens and pets in Tunisia. *J Antimicrob Chemother* 2013; **68**: 2950-2.

17. Albrechtova K, Kubelova M, Mazancova J *et al*. High prevalence and variability of CTX-M-15-producing and fluoroquinolone-resistant *Escherichia coli* observed in stray dogs in rural Angola. *Microb Drug Resist* 2014; **20**: 372-5.

18. Jouini A, Slama KB, Klibi N *et al*. Lineages and virulence gene content among extended-spectrum β-lactamase-producing *Escherichia coli* strains of food origin in Tunisia. *J Food Prot* 2013; **76**: 323-7.

19. Ben Slama K, Jouini A, Ben Sallem R *et al*. Prevalence of broad-spectrum cephalosporin-resistant *Escherichia coli* isolates in food samples in Tunisia, and characterization of integrons and antimicrobial resistance mechanisms implicated. *Int J Food Microbiol* 2010; **137**: 281-6.

20. Ben Said L, Jouini A, Klibi N *et al*. Detection of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in vegetables, soil and water of the farm environment in Tunisia. *Int J Food Microbiol* 2015; **203**: 86-92.

21. Alouache S, Kada M, Messai Y *et al*. Antibiotic resistance and extended-spectrum β-lactamases in isolated bacteria from seawater of Algiers beaches (Algeria). *Microbes Environ* 2012; **27**: 80-6.

22. Alouache S, Estepa V, Messai Y *et al*. Characterization of ESBLs and associated quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from an urban wastewater treatment plant in Algeria. *Microb Drug Resist* 2014; **20**: 30-8.

23. Chouchani C, Marrakchi R, Henriques I *et al*. Occurrence of IMP-8, IMP-10, and IMP-13 metallo-β-lactamases located on class 1 integrons and other extended-spectrum β-lactamases in bacterial isolates from Tunisian rivers. *Scand J Infect Dis* 2013; **45**: 95-103.

24. De Boeck H, Miwanda B, Lunguya-Metila O *et al*. ESBL-positive Enterobacteria isolates in drinking water. *Emerg Infect Dis* 2012; **18**: 1019-20.

25. De Boeck H, Lunguya O, Muyembe JJ *et al*. Presence of extended-spectrum beta-lactamase-producing Enterobacteriaceae in waste waters, Kinshasa, the Democratic Republic of the Congo. *Eur J Clin Microbiol Infect Dis* 2012; **31**: 3085-8.

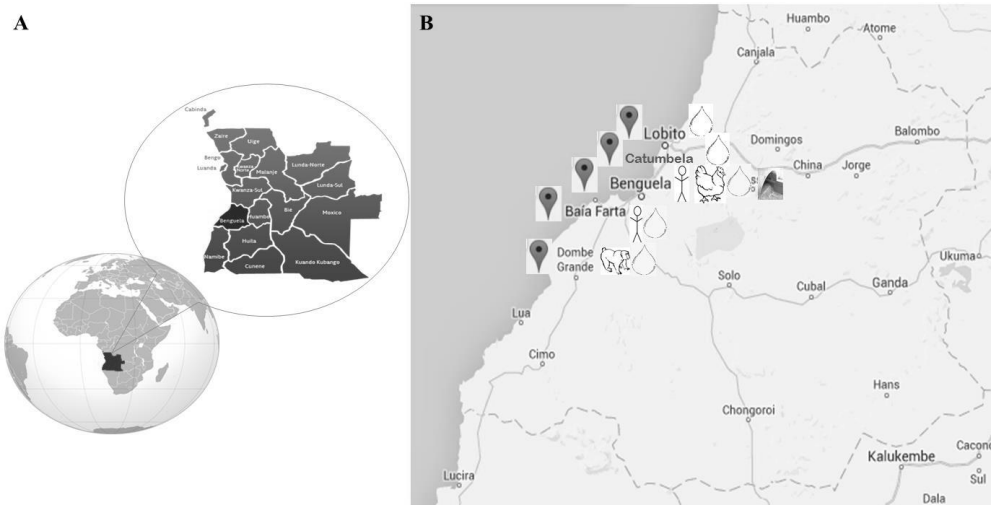


Figure S1. Schematic figure of the sampling points surveyed in this study. A- Location of Angola and its Provinces on the African continent. Benguela Province is located in the West of the Republic of Angola, on the Atlantic coast, situated about 690 km from the capital (Luanda) and covering an area of 37802 Km^2 (3.19% of the national territory). It comprises 9 Municipalities (Benguela, Lobito, Bocoio, Balombo, Ganda, Cubal, Caimbambo, Baía Farta and Chongorói), divided in more than 30 Communes, with a population estimated to be around 2 million inhabitants (http://www.welcometoangola.co.ao/?it=province_more&co=335&tp=25&LG=2). B- Sampling points of the diverse non-clinical niches surveyed in the different Communes of Benguela Province (with distances ranging from 11.5 to 89.7 miles).

Benguela Communes. healthy persons; food-producing animals; wild animals; aquatic environments (rivers, lagoon, treated and untreated waters); aquatic environments (wastewaters from urban sewer lines or treatment station).

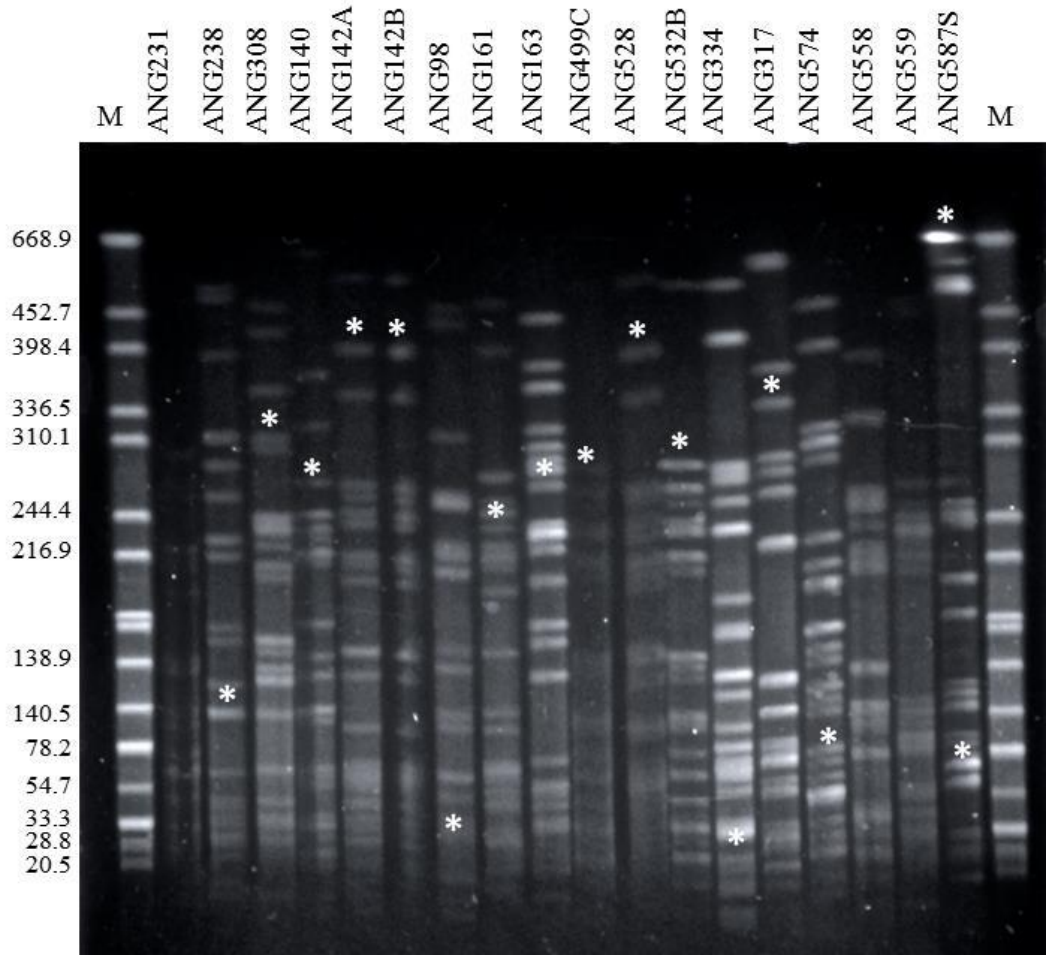


Figure S2. *Xba*I-PFGE profiles of CTX-M-15-producing isolates with negative results for hybridization of $bla_{\text{CTX-M-15}}$ with I-*Ceu*I- or S1-digested genomic DNA. Lane M, *Xba*I-digested DNA of *Salmonella enterica* serovar Braenderup H9812, used as size standard. The asterisks indicate bands that hybridized with the $bla_{\text{CTX-M-15}}$ probe.

3.2. *Citrobacter* species differentiation and diversification trajectories of intrinsic genes conferring reduced susceptibility to fluoroquinolones

Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species

Teresa Gonçalves Ribeiro[1], Ângela Novais[1], Raquel Branquinho[1], Elisabete Machado[1,2], and Luísa Peixe[1*]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; [2]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

Antimicrobial Agents and Chemotherapy 2015 October; 59(10):5951-8

American Society for Microbiology - Journals has authorized the reproduction of the final published PDF version of this paper in this Thesis through the License Agreement Number 3911910810824 on July 18, 2016.

AAC Journals.ASM.org

Phylogeny and Comparative Genomics Unveil Independent Diversification Trajectories of *qnrB* and Genetic Platforms within Particular *Citrobacter* Species

Teresa G. Ribeiro,[a] Ângela Novais,[a] Raquel Branquinho,[a] Elisabete Machado,[a,b] Luísa Peixe[a]

UCIBIO/REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal[a]; FP-ENAS/CEBIMED, Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal[b]

To gain insights into the diversification trajectories of *qnrB* genes, a phylogenetic and comparative genomics analysis of these genes and their surrounding genetic sequences was performed. For this purpose, *Citrobacter* sp. isolates ($n = 21$) and genome or plasmid sequences ($n = 56$) available in public databases harboring complete or truncated *qnrB* genes were analyzed. *Citrobacter* species identification was performed by phylogenetic analysis of different genotypic markers. The clonal relatedness among isolates, the location of *qnrB* genes, and the genetic surroundings of *qnrB* genes were investigated by pulsed-field gel electrophoresis (PFGE), S1-/I-CeuI-PFGE and hybridization, and PCR mapping and sequencing, respectively. Identification of *Citrobacter* isolates was achieved using *leuS* and *recN* gene sequences, and isolates characterized in this study were diverse and harbored chromosomal *qnrB* genes. Phylogenetic analysis of all known *qnrB* genes revealed seven main clusters and two branches, with most of them included in two clusters. Specific platforms (comprising *pspF* and *sapA* and varying in synteny and/or identity of other genes and intergenic regions) were associated with each one of these *qnrB* clusters, and the reliable identification of all *Citrobacter* isolates revealed that each platform evolved in different recognizable (*Citrobacter freundii*, *C. braakii*, *C. werkmanii*, and *C. pasteurii*) and putatively new species. A high identity was observed between some of the platforms identified in the chromosome of *Citrobacter* spp. and in different plasmids of *Enterobacteriaceae*. Our data corroborate *Citrobacter* as the origin of *qnrB* and further suggest divergent evolution of closely related *qnrB* genes/platforms in particular *Citrobacter* spp., which were delineated using particular genotypic markers.

The *qnrB* genes constitute the most prevalent and diverse (>70 allelic variants; see http://www.lahey.org/qnrStudies/) group within the *qnr* family, encoding proteins responsible for decreased susceptibility to fluoroquinolones (1–4).

Some authors have proposed *Citrobacter* spp. as the origin of *qnrB* genes, mainly based on species distribution (>60% in *Citrobacter* spp., including isolates from the preantibiotic era), location (mostly on the chromosome), and the apparent absence of mobile genetic elements in the immediate genetic environment of *qnrB* genes, mostly by characterization of clinical *Citrobacter* sp. isolates (3–5). Nevertheless, the absence of correlation of *qnrB* genes with particular *Citrobacter* species, together with the lack of detailed characterization of *qnrB* platforms, hinders a clear establishment of the origin of *qnrB*. In fact, most of the methods conventionally used to identify *Citrobacter* spp. (biochemical or phenotypic features, matrix-assisted laser desorption ionization–time of flight mass spectrometry [MALDI-TOF MS], or 16S rRNA gene sequencing) (6–8) have low discriminatory power, hindering the accurate discrimination of these species.

Recently, Clermont et al. described a multilocus sequence analysis (MLSA) based on partial sequences of *rpoB* (β subunit of RNA polymerase gene), *pyrG* (CTP synthetase gene), *fusA* (protein synthesis elongation factor-G gene), and *leuS* (leucine tRNA synthetase gene) that allowed the discrimination of the 12 recognized *Citrobacter* species, namely, *Citrobacter freundii*, *C. amalonaticus*, *C. braakii*, *C. farmeri*, *C. gillenii*, *C. koseri*, *C. murliniae*, *C. rodentium*, *C. sedlakii*, *C. werkmanii*, *C. youngae*, and *C. pasteurii* (6).

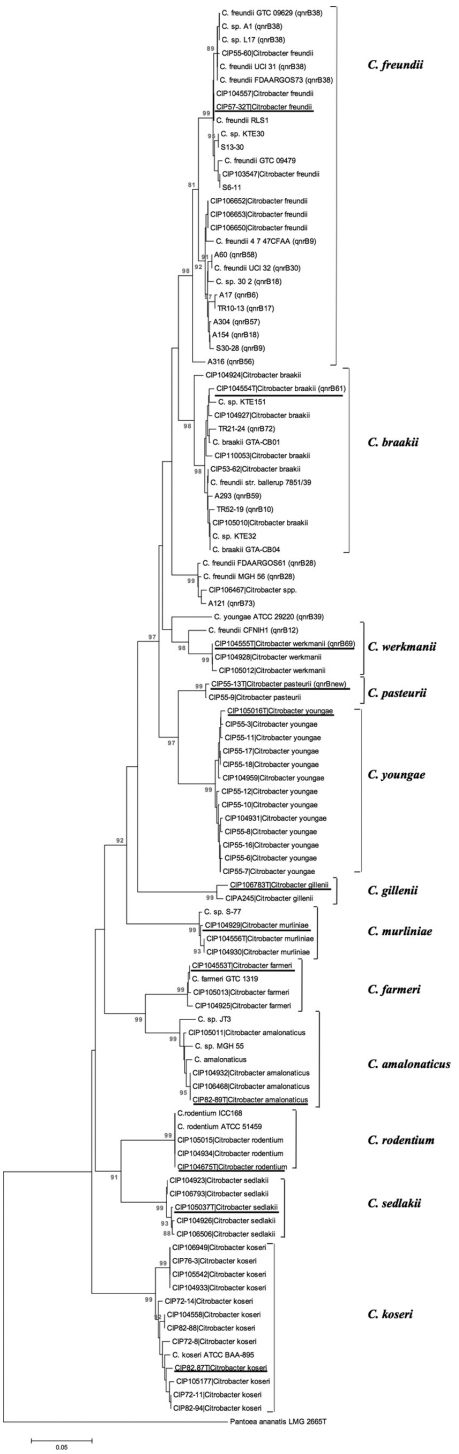
In this work, we aim to gain insights into the diversification trajectories of *qnrB* within *Citrobacter* species and to unveil *qnrB* surroundings possibly involved in the dissemination of this gene to other *Enterobacteriaceae*. For that purpose, we performed an affiliation of *Citrobacter* species and *qnrB* genes described to date and a comparative analysis of genetic sequences surrounding *qnrB* using nonclinical *Citrobacter* sp. isolates and genome and plasmid sequences deposited in public databases.

MATERIALS AND METHODS

Bacterial isolates. Twenty-one nonclinical *Citrobacter* sp. isolates harboring *qnrB* genes recovered from different nonclinical origins, including untreated waters used for human consumption ($n = 12$; 2006 to 2008), ready-to-eat salads ($n = 3$; 2010), and trout aquaculture samples (trout, feed, and sediments from a river located upstream of the trout farm) ($n = 6$; 2010 to 2012) from different geographic regions in Portugal, were included in this study (see Table S1 in the supplemental material). The isolates carried *qnrB6* ($n = 1$), *qnrB9* ($n = 1$), *qnrB10* ($n = 3$), *qnrB17* ($n = 1$), *qnrB18* ($n = 1$), *qnrB56* ($n = 3$), *qnrB57* ($n = 2$), *qnrB58* ($n = 1$), *qnrB59* ($n = 3$), *qnrB72* ($n = 2$), *qnrB73* ($n = 1$), or truncated *qnrB*

Received 7 January 2015 **Returned for modification** 13 April 2015
Accepted 6 July 2015
Accepted manuscript posted online 13 July 2015
Citation Ribeiro TG, Novais Â, Branquinho R, Machado E, Peixe L. 2015. Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species. Antimicrob Agents Chemother 59:5951–5958. doi:10.1128/AAC.00027-15.
Address correspondence to Luísa Peixe, lpeixe@ff.up.pt.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00027-15.
Copyright © 2015, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AAC.00027-15

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest



(Δ*qnrB*; $n = 2$) genes (9; P. Antunes, E. Machado, and L. Peixe, unpublished data) (see Table S1 in the supplemental material).

In addition, 40 *Citrobacter* sp. genomes and 16 *qnrB*-carrying plasmid sequences available from the Pathosystems Resource Integration Center (PATRIC) and/or the National Center for Biotechnology Information (NCBI) database were used for phylogenetic analysis and/or *qnrB* genetic surrounding comparisons.

Bacterial identification and phylogenetic analysis. Isolates included in this study were identified by biochemical methods (7), mass spectrometry (MALDI-TOF MS; Bruker Daltonik, Germany), and sequencing of 16S rRNA (8), *leuS* (leucine tRNA synthetase) (6), and *recN* (DNA repair protein) genes. PCR amplification and further sequencing of *recN* genes were performed by using primers recN-Fw (5′-ATTGCCATTGATGCTC TCGG-3′) and recN-Rv (5′-ANCGAGTCGGCCTGATCGT-3′) to amplify a 637-bp internal fragment and the following amplification conditions: one cycle of 3 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C; and 1 cycle of 1 min at 72°C.

Individual nucleotide sequences of genes included in the MLSA scheme of the *Citrobacter* genus (*rpoB*, *pyrG*, *fusA*, and *leuS*) (6) and *recN* were aligned and the average rates of similarity calculated using MEGA version 5.2.2 (http://www.megasoftware.net/) (10). The *leuS* gene sequences from Clermont et al. were included in this analysis (6). Similarity scores of the *leuS* and *recN* genes were calculated and individual phylogenetic trees were constructed in MEGA using the neighbor-joining (NJ) method (11), and genetic distances were calculated using the Kimura two-parameter model (12) in the case of nucleotide sequences and using the Jones-Taylor-Thornton (JTT) model (13) for LeuS and RecN protein sequences. The reliability of internal branches was assessed from bootstrap based on 1,000 resamplings (14). *Pantoea ananatis* strain LMG 2665^T was used as the outgroup.

Clonal relatedness. Clonal relationships among isolates belonging to the same species were established by pulsed-field gel electrophoresis (PFGE), using XbaI as a restriction enzyme and the following electrophoresis conditions: 10 to 40 s for 21 h at 14°C and 6 V/cm^2 (15). The criteria of Tenover et al. were used for comparison of band patterns obtained by PFGE, and isolates representing different PFGE-types were selected for the following studies (16).

Location, transferability, and phylogenetic analysis of *qnrB* genes. Location of *qnrB* genes was assessed by S1-/I-CeuI-PFGE and further hybridization with *qnrB* and 16S rRNA probes (17, 18). Conjugative transfer of *qnrB* was evaluated by broth and filter mating assays using *Escherichia coli* HB101 (streptomycin and azide resistant) as the recipient at a 1:2 donor-to-recipient ratio and selection plates containing ciprofloxacin (0.06 to 0.5 μg/ml) plus sodium azide (130 μm/ml) (19).

Affiliation within all *qnrB* genes described at the time of study design ($n = 74$; http://www.lahey.org/qnrStudies/) was generated as specified above for *leuS* and *recN* phylogenetic analysis.

Characterization of genetic surroundings of the *qnrB* genes. The genetic context of *qnrB* genes was characterized by PCR mapping (*pspF*, *sapA*, *intI1*, *intI2*, *intI3*, IS*Ecp1*, IS*3000*, IS*CR1*, IS*26*) and sequencing based on previously described sequences (3, 20–22). Sequences surrounding *qnrB* were further aligned and compared *in silico* with those deposited in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov.sci-hub.org/Blast.cgi) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

FIG 1 Neighbor-joining (NJ) tree based on the comparison of *leuS* gene sequences of *Citrobacter* species analyzed in this study. Genetic distances were constructed using Kimura's two-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. *Citrobacter* species type strains are underlined, and the *qnrB* alleles are shown in parentheses. *Pantoea ananatis* strain LMG 2665^T was used as the outgroup (PATRIC fig|1378093.3.peg.2577). Please refer to Table S2 in the supplemental material for accession numbers of the sequences used.

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest

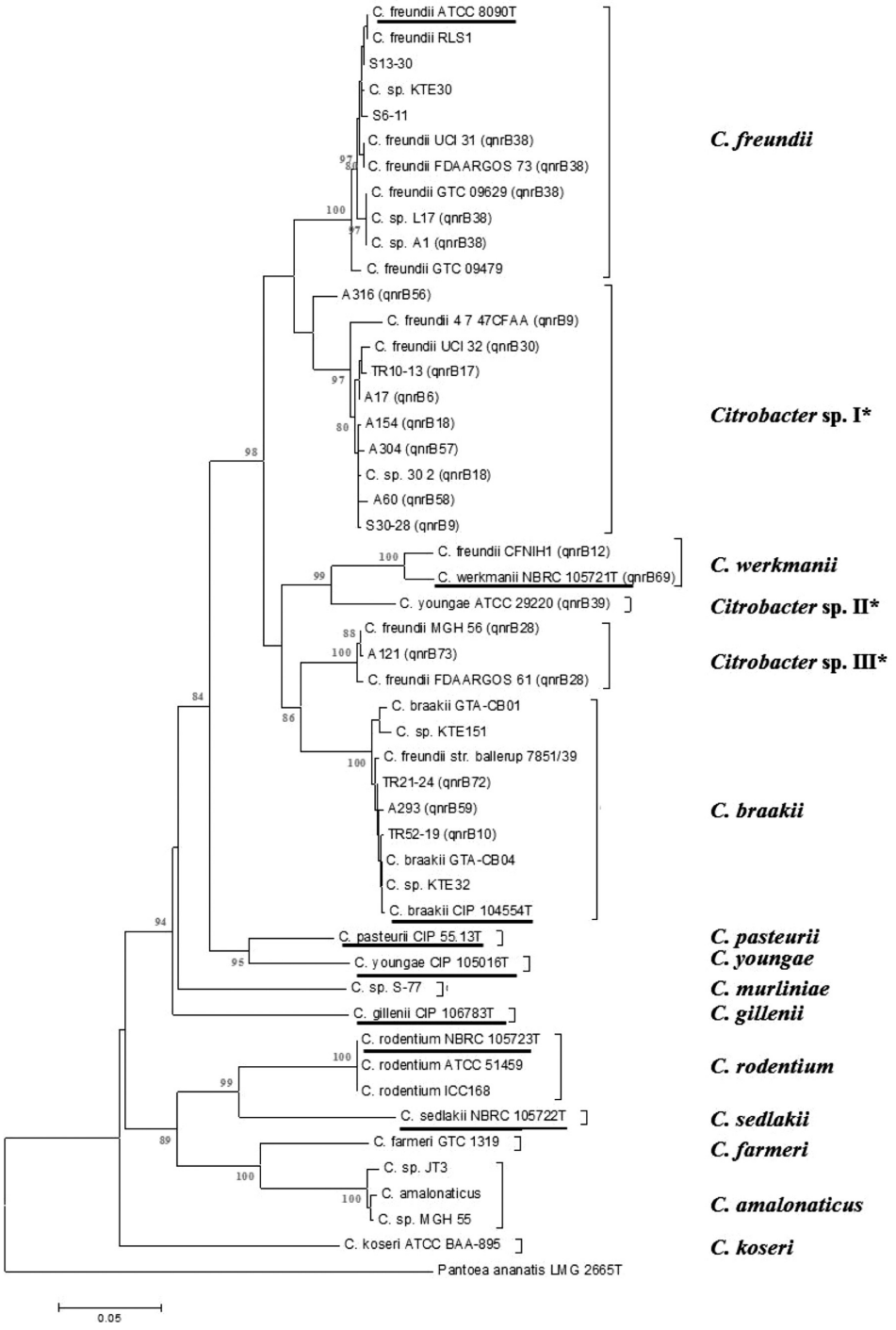
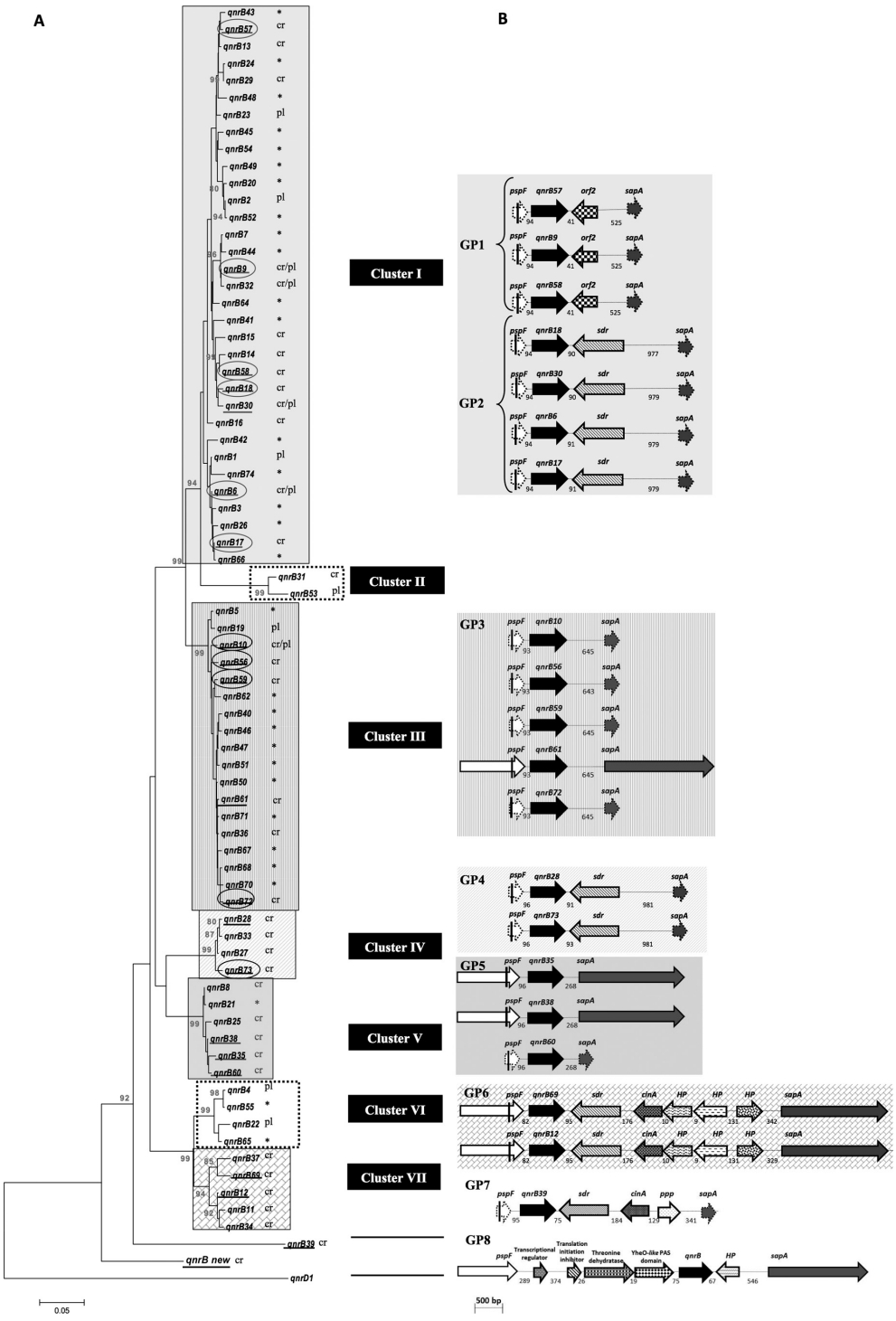


FIG 2 Neighbor-joining (NJ) tree based on the comparison of *recN* gene sequences of all *Citrobacter* species analyzed in this study. Genetic distances were constructed using Kimura's two-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. *Citrobacter* species type strains are underlined, and *qnrB* alleles are shown in parentheses. *, *Citrobacter* sp. I, *Citrobacter* sp. II, and *Citrobacter* sp. III correspond to putative novel species. *Pantoea ananatis* strain LMG 2665^T was used as the outgroup (PATRIC fig|1378093.3.peg.2577). Please refer to Table S2 in the supplemental material for accession numbers of the sequences used.

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest



Downloaded from http://aac.asm.org/ on July 19, 2016 by guest

Nucleotide sequence accession numbers. The sequences of the genetic platforms associated with the different *qnrB* alleles characterized in this study have been deposited in the GenBank database under the accession numbers KP339254 (*qnrB6*), KP339255 (*qnrB9*), KP339256 (*qnrB10*), KP339257 (*qnrB17*), KP339258 (*qnrB18*), KP339259 (*qnrB56*), KP339260 (*qnrB57*), KP339261 (*qnrB58*), KP339262 (*qnrB59*), KP339263 (*qnrB72*), and KP339264 (*qnrB73*). *recN* and *leuS* nucleotide sequence data from the different *Citrobacter* sp. isolates identified in this study are available in the GenBank database under accession numbers KR998019 (*Citrobacter* sp. I), KR998020 (*Citrobacter* sp. I), KR998021 (*Citrobacter* sp. III), KR998022 (*Citrobacter* sp. I), KR998023 (*C. braakii*), KR998024 (*Citrobacter* sp. I), KR998025 (*Citrobacter* sp. I), KR998026 (*C. freundii*), KR998027 (*C. freundii*), KR998028 (*Citrobacter* sp. I), KR998029 (*Citrobacter* sp. I), KR998030 (*C. braakii*), KR998031 (*C. braakii*), KR998032 (*Citrobacter* sp. I), KR998033 (*Citrobacter* sp. I), KR998034 (*Citrobacter* sp. III), KR998035 (*Citrobacter* sp. I), KR998036 (*C. braakii*), KR998037 (*Citrobacter* sp. I), KR998038 (*Citrobacter* sp. I), KR998039 (*C. freundii*), KR998040 (*C. freundii*), KR998041 (*Citrobacter* sp. I), KR998042 (*Citrobacter* sp. I), KR998043 (*C. braakii*), and KR998044 (*C. braakii*).

RESULTS AND DISCUSSION

***Citrobacter* species identification and clonality.** The identification at the species level of all the *Citrobacter* sp. isolates included in this study was not possible by biochemical methods, MALDI-TOF MS, or sequencing of the 16S rRNA gene (data not shown), as previously recognized (6, 23). In contrast, analysis of *leuS* and *recN* gene sequences provided an accurate discrimination of the currently recognized *Citrobacter* species, as explained below.

The *leuS* gene presented the highest discriminatory power (average rate of similarity close to 88.5%, statistically supported) of the genes included in the MLSA scheme proposed by Clermont et al. (6). Therefore, the *leuS*-based phylogenetic tree allowed the delineation of 12 distinct clusters (Fig. 1), each one supported by a type strain from each *Citrobacter* species, corroborating the topology obtained by the concatenated affiliation of the MLSA scheme (6). These clusters were defined with a cutoff value of <97.5%, supported by bootstrap values greater than 92% (Fig. 1).

The *recN* gene provided a greater resolution than *leuS*, presenting an average rate of similarity close to 85.6%. The *recN* tree topology was overall congruent with that obtained for *leuS* sequences (Fig. 2), with the presence of the same 12 clusters observed (cutoff values of <96.1% statistically supported by bootstrap values greater than 94%) supported by sequences from the available type strains. Interestingly, 3 new clusters were observed, namely, *Citrobacter* sp. I ($n = 10$), *Citrobacter* sp. II ($n = 1$), and *Citrobacter* sp. III ($n = 3$), which might correspond to isolates from novel species (Fig. 2). *Citrobacter* sp. I presented a genetic distance of 0.071 (bootstrap value of 97%) with its closest related species *C. freundii*, whereas *Citrobacter* sp. II and *Citrobacter* sp. III presented genetic distances of 0.081 (bootstrap value of 99%) and 0.073 (bootstrap value of 100%) with the closest related species *C. werkmanii* and *C. braakii*, respectively. Further studies are in progress to clearly establish the identity of the isolates included in these clusters.

Phylogenetic trees constructed based on amino acid sequences of LeuS and RecN showed that most nucleotide substitutions were synonymous, despite resulting in a less clear delineation between species due to the higher conservative character of amino acid sequences (see Fig. S1 and S2 in the supplemental material).

According to our phylogenetic analysis, *Citrobacter* sp. isolates characterized in this study were identified as *C. braakii* ($n = 83$ PFGE types), *C. freundii* ($n = 22$ PFGE types), and putatively two novel species (*Citrobacter* sp. I [$n = 107$ PFGE types] or *Citrobacter* sp. III [$n = 11$ PFGE types]) (see Table S1 in the supplemental material).

Location and affiliation of *qnrB* genes. No plasmids were detected in any of the *Citrobacter* isolates included in this study, and in all cases, *qnrB* was chromosomally located and not transferable by conjugation, further supporting the natural occurrence of this gene in the chromosome of *Citrobacter* spp. (3–5). The *qnrB* gene diversity found was in accordance with previous data (24, 25), probably driven by the interplay of different selective events (natural recombination events and/or alternative selective forces) (1, 26–28).

The phylogenetic tree constructed based on *qnrB* gene sequences (Fig. 3A) revealed seven distinct clusters (I to VII) and two branches comprising *qnrB39* and a new *qnrB* (*C. pasteurii* strain CIP 55-13^T), supported by bootstraps of ≥92% and sharing ≤92.83% identity between them. The corresponding affiliation based on amino acid sequences of QnrB showed that most nucleotide substitutions were synonymous, which resulted in a similar tree topology (see Fig. S3 in the supplemental material), with some exceptions consisting of genes showing a higher degree of nucleotide divergence (*qnrB31*, *qnrB53*, or *qnrB39*), as observed by other authors for $bla_{CTX\text{-}M}$ genes (29). Our phylogenetic analysis also showed that most of the *qnrB* genes, including those characterized in this study, belonged to cluster I ($n = 33$, including *qnrB6*, *qnrB9*, *qnrB17*, *qnrB18*, *qnrB57*, and *qnrB58*) or to cluster III ($n = 18$, including *qnrB10*, *qnrB56*, *qnrB59*, and *qnrB72*), whose diversification might be favored by their association with particular host species and/or niches (see below). Few *qnrB* genes

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest

FIG 3 Affiliation of *qnrB* genes and *qnrB* genetic platforms from *Citrobacter* spp. (A) Neighbor-joining tree based on 74 *qnrB* gene sequences (http://www.lahey.org/qnrStudies/). Genetic distances were constructed using the Kimura 2-parameter model. Numbers at branch points indicate bootstrap percentages (1,000 replications) from NJ analysis, and only values greater than 80% are shown. Horizontal bar, genetic distance of 0.05. The nucleotide sequence of *qnrD1* (GenBank accession number FJ228229) was used as the outgroup. The *qnrB* genes for which the genetic environment was first characterized in this study are surrounded by circles, whereas those available in the GenBank database are underlined. pl, plasmid-borne *qnrB*; cr, chromosomally located *qnrB*; *, *qnrB* location not assessed. (B) Schematic representation of the genetic platforms (GP) carrying chromosomally located *qnrB* genes. Numbers between ORFs indicate the size of the intergenic region in base pairs (bp). Vertical black bars represent IRR2. Genes identified in *qnrB* platforms are *pspF* (encoding a phage shock protein), *orf2* (open reading frame of a gene of unknown function), *sdr* (encoding a short-chain dehydrogenase/reductase protein), *cinA* (encoding competence/damage-inducible domain protein), *HP* (encoding a hypothetical protein), *ppp* (encoding putative periplasmic protein), and/or *sapA* (encoding a protein involved in antimicrobial peptide resistance). Genetic platforms have been deposited in the GenBank database under accession numbers KP339254 (*qnrB6*), KP339255 (*qnrB9*), ADLG01000026.1 (*qnrB9*), CP007557 (*qnrB12*), KP339256 (*qnrB10*), KP339257 (*qnrB17*), KP339258 (*qnrB18*), ACDJ02000027.1 (*qnrB18*), JMUJ01000007.1 (*qnrB28*), JTBV01000001.1 (*qnrB28*), JAPA01000008.1 (*qnrB30*), JN173057 (*qnrB35*), JN173060 (*qnrB38*), NZ_AMPE01000004.1 (*qnrB38*), NZ_AKTT01000018.1 (*qnrB38*), NZ_AOUE01000004.1 (*qnrB38*), JTBJ01000001.1 (*qnrB38*), JAPB01000002.1 (*qnrB38*), ABWL02000005.1 (*qnrB39*), KP339259 (*qnrB56*), KP339260 (*qnrB57*), KP339261 (*qnrB58*), KP339262 (*qnrB59*), AB734055 (*qnrB60*), AB734053 (*qnrB61*), BBMW01000005.1 (*qnrB69*), KP339263 (*qnrB72*), KP339264 (*qnrB73*), and CDHL01000019 (new *qnrB* from CIP 55-13^T).

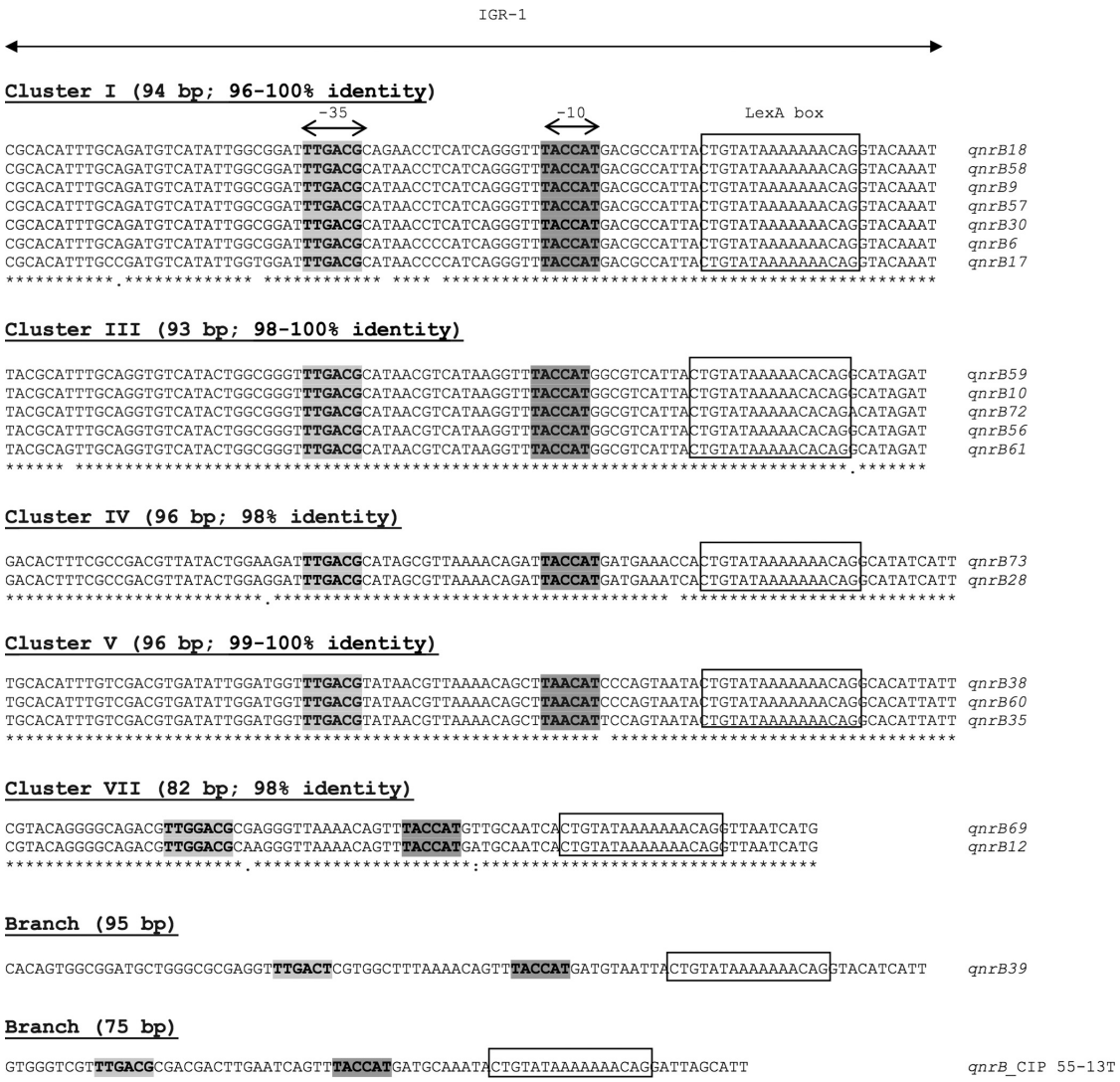


FIG 4 Nucleotide sequence alignment of intergenic regions upstream of chromosomally located *qnrB*. The −35 and −10 promoters are indicated by gray shading, and the sequence of the LexA box is boxed. Sequences were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The IGR-1 sequences represented in this figure are found in the GenBank database through the accession numbers KP339254 (*qnrB6*), KP339255 (*qnrB9*), KP339256 (*qnrB10*), CP007557 (*qnrB12*), KP339257 (*qnrB17*), KP339258 (*qnrB18*), JMUJ01000007.1 (*qnrB28*), JAPA01000008.1 (*qnrB30*), JN173057 (*qnrB35*), JN173060 (*qnrB38*), ABWL02000005.1 (*qnrB39*), KP339259 (*qnrB56*), KP339260 (*qnrB57*), KP339261 (*qnrB58*), KP339262 (*qnrB59*), AB734055 (*qnrB60*), AB734053 (*qnrB61*), BBMW01000005.1 (*qnrB69*), KP339263 (*qnrB72*), KP339264 (*qnrB73*), and CDHL01000019 (new *qnrB* from CIP 55-13^T).

were enclosed in cluster II ($n = 2$), IV ($n = 4$, including *qnrB73*), V ($n = 6$), VI ($n = 4$), or VII ($n = 5$).

Detailed characterization of *qnrB* genetic platforms. Analysis of the genetic surroundings of complete *qnrB* genes revealed eight different *qnrB* genetic platforms (GP1 to GP8) (Fig. 3B). *pspF* (encoding a phage shock protein) and *sapA* (encoding a protein involved in antimicrobial peptide resistance) genes were consistently found upstream and downstream of *qnrB* genes, respectively. A high variability was observed mostly downstream of *qnrB*, with differences in the presence of other genes (*orf2*, *cinA*, *HP*, and/or *ppp*) and in the size and identity of intergenic regions (IGRs) upstream and downstream of *qnrB* (Fig. 3B). Interestingly, we observed conserved genetic platforms (gene content and sequence identity) for closely related *qnrB* genes (i.e., those grouped in the same cluster), with an exception in cluster I, possibly explained by a recombination event (Fig. 3).

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest

As the characterization of IGRs was important to elucidate the origin and evolutionary routes of other antibiotic resistance genes (29, 30), we performed a detailed analysis of IGRs located in the *qnrB* genetic environment. In fact, the intergenic regions upstream of *qnrB* (IGR-1) were closely related (in size and in nucleotide sequence) among *qnrB* alleles that were grouped in the same cluster (identity, >96%) (Fig. 4), including those from cluster I (see above), whereas they exhibited a loss of identity between clusters (identity, 60% to 85%). This IGR-1 encompassed a LexA box consensus sequence located upstream of *qnrB* and downstream −35 and −10 promoter sequences (Fig. 4), which might directly regulate the expression of *qnrB* genes, as previously suggested (31, 32).

Interestingly, taking into consideration the similarity of the platforms carrying closely related *qnrB* genes and the identification of *Citrobacter* isolates carrying each *qnrB*, an association was found between each particular *qnrB* platform and specific *Citrobacter* species. The *qnrB* cluster I was associated with *Citrobacter* sp. I, *qnrB* cluster III with *C. braakii*, *qnrB* cluster IV with *Citrobacter* sp. III, *qnrB* cluster V with *C. freundii*, *qnrB* cluster VII with *C. werkmanii*, the branch comprising *qnrB39* with *Citrobacter* sp. II, and finally the branch comprising the new *qnrB* allele with *C. pasteurii*. One unique exception was detected (an isolate carrying *qnrB56* from cluster III belonged to *Citrobacter* sp. I instead of *C. braakii*), which may be explained by a genomic recombination event. This relationship was not established for *qnrB* alleles included in clusters II and VI due to the lack of genomic information from the corresponding strains in available databases. Thus, our findings provide additional data to support the acquisition of *qnrB* between *pspF* and *sapA* by a progenitor of at least some *Citrobacter* species prior to platform diversification. This hypothesis is further supported by the observation that 89% of isolates from particular species (*C. freundii*, *C. braakii*, *C. werkmanii*, *C. pasteurii*, *Citrobacter* sp. I, *Citrobacter* sp. II, and *Citrobacter* sp. III) carry a complete or truncated *qnrB* gene, suggesting species adaptation to variable ecological niches (see Table S2 in the supplemental material).

Analysis of the genetic environment surrounding the truncated *qnrB* genes (Δ*qnrB*) identified in this study revealed that the end of the *pspF-qnrB* intergenic region (encompassing promoter regions) and the first 360 bp of the *qnrB* gene were truncated (*pspF*-[47/49 bp]-Δ*qnrB*-[643 bp]-*sapA*). This genetic environment was identical (97% to 100%) with those described in the chromosome of other *Citrobacter* spp., including *C. freundii* strain ATCC 8090^T (GenBank accession numbers AB734052, AB734052, and AB734054), which suggests pseudogenization or deletion processes driven by insertion sequences (ISs) and eventually prophages (33, 34).

***In silico* analysis of *qnrB*-carrying plasmid platforms.** Our *in silico* analysis revealed that some of the *qnrB* genetic platforms identified in the chromosome of *Citrobacter* sp. I and *C. braakii* have already been detected in plasmids of different *Enterobacteriaceae* species (Fig. 1). This is the case for the genetic platforms containing *qnrB2*, *qnrB1*, or *qnrB6* (*qnrB* cluster I), previously identified in IncN, IncL/M, or IncFII plasmids in different *Enterobacteriaceae* species (GenBank accession numbers JX193301, JX101693, EU715254, KF193607, JX424423, JF775514, GU723682, and GU723680). Also, an identity was observed between the *qnrB10* platform detected in the chromosome of *C. braakii* and that in IncR plasmids (GenBank accession numbers EU052800, EU091084, and CP006662).

Some possibilities of mobilization of *qnrB* and/or regions surrounding *qnrB* were investigated. We did not find insertion sequences (ISs) or integrons in the *qnrB* genetic environment of the isolates characterized in this study, but an inverted repeat region (IRR; CTGAATTACTGGGT) was detected within the coding sequence of the *pspF* gene (including those associated with Δ*qnrB*). The IRR is also found in the same position in the chromosome of *Citrobacter* spp. (GenBank accession numbers AB734055, JN173060, AB734055, and AB734054) and in plasmids of different *Enterobacteriaceae* species (GenBank accession numbers EU523120, JN995611, JX101693, GU295957, JX424423, JX298080, and EU643617). This IRR is similar (0- to 5-bp mismatches) to IRR2, which was previously implicated in the mobilization of *qnrB19* after recognition by IS*Ecp1C* (35) and which might have been involved in the mobilization of other *qnrB* genes to plasmids. Nevertheless, different ISs (e.g., IS*26*, IS*CR1*, IS*Ecp1*, IS*3000*, IS*6100*) have been identified in the vicinity of diverse plasmid-mediated *qnrB* genes deposited in the GenBank database, suggesting the involvement of multiple mechanisms in the mobilization and/or assembly of the plasmid-associated *qnrB* genetic surroundings.

In conclusion, this study provides a comprehensive and extensive analysis of all *qnrB* genes and surrounding genetic platforms described to date and contributes to delineating the taxonomic positions of the different species within the *Citrobacter* genus. Our data corroborate *Citrobacter* as the origin of *qnrB* and further suggest independent diversification trajectories of specific *qnrB* genes/platforms in particular *Citrobacter* species (*C. freundii*, *C. braakii*, *C. werkmanii*, *C. pasteurii*, and in three putatively new *Citrobacter* species). Moreover, we unveil a potential route for mobilization of *qnrB* genes to plasmids, potentiating the dissemination of particular *qnrB* alleles in the clinical setting.

ACKNOWLEDGMENTS

This work received financial support from the European Union (FEDER funds through COMPETE), National Funds (Fundação para a Ciência e Tecnologia [FCT]) through projects Pest-C/EQB/LA0006/2013, PTDC/AAC-AMB/103386/2008, and UID/Multi/04378/2013, and Fundação Ensino e Cultura Fernando Pessoa. T.G.R. and R.B. were supported by Ph.D. fellowships from FCT (SFRH/BD/75752/2011 and SFRH/BD/61410/2009, respectively). A.N. was supported by funds from the European Union under the framework of QREN through Project NORTE-07-0124-FEDER-000066.

REFERENCES

1. **Baquirin MH, Barlow M.** 2008. Evolution and recombination of the plasmidic *qnr* alleles. J Mol Evol **67**:103–110. http://dx.doi.org/10.1007/s00239-008-9131-3.
2. **Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A.** 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev **22**:664–689. http://dx.doi.org/10.1128/CMR.00016-09.
3. **Jacoby GA, Griffin CM, Hooper C.** 2011. *Citrobacter* spp. as a source of *qnrB* alleles. Antimicrob Agents Chemother **55**:4979–4984. http://dx.doi.org/10.1128/AAC.05187-11.
4. **Saga T, Sabtcheva S, Mitsutake K, Ishii Y, Tateda K, Yamaguchi K, Kaku M.** 2013. Characterization of *qnrB*-like genes in *Citrobacter* species of the American Type Culture Collection. Antimicrob Agents Chemother **57**:2863–2866. http://dx.doi.org/10.1128/AAC.02396-12.
5. **Kehrenberg C, Friederichs S, de Jong A, Schwarz S.** 2008. Novel variant of the *qnrB* gene, *qnrB12*, in *Citrobacter werkmanii*. Antimicrob Agents Chemother **52**:1206–1207. http://dx.doi.org/10.1128/AAC.01042-07.

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest

6. **Clermont D, Motreff L, Passet V, Fernandez JC, Bizet C, Brisse S.** 2015. Multilocus sequence analysis of the genus *Citrobacter* and description of *Citrobacter pasteurii* sp. nov. Int J Syst Evol Microbiol **65:**1486–1490. http://dx.doi.org/10.1099/ijs.0.000122.
7. **O'Hara CM, Roman SB, Miller JM.** 1995. Ability of commercial identification systems to identify newly recognized species of *Citrobacter*. J Clin Microbiol **33:**242–245.
8. **Héritier C, Poirel L, Aubert D, Nordmann P.** 2003. Genetic and functional analysis of the chromosome-encoded carbapenem-hydrolyzing oxacillinase OXA-40 of *Acinetobacter baumannii*. Antimicrob Agents Chemother **47:**268–273. http://dx.doi.org/10.1128/AAC.47.1.268-273.2003.
9. **Campos J, Mourão J, Pestana N, Peixe L, Novais C, Antunes P.** 2013. Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. Int J Food Microbiol **166:**464–470. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.08.005.
10. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
11. **Saitou N, Nei M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406–425.
12. **Kimura M.** 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol **16:**111–120. http://dx.doi.org/10.1007/BF01731581.
13. **Jones DT, Taylor WR, Thornton JM.** 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci **8:**275–282.
14. **Felsenstein J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791. http://dx.doi.org/10.2307/2408678.
15. **Novais Â, Rodrigues C, Branquinho R, Antunes P, Grosso F, Boaventura L, Ribeiro G, Peixe L.** 2012. Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. Eur J Clin Microbiol Infect Dis **31:**3057–3063. http://dx.doi.org/10.1007/s10096-012-1665-z.
16. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol **33:**2233–2239.
17. **Barton BM, Harding GP, Zuccarelli AJ.** 1995. A general method for detecting and sizing large plasmids. Anal Biochem **226:**235–240. http://dx.doi.org/10.1006/abio.1995.1220.
18. **Liu SL, Hessel A, Sanderson KE.** 1993. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. Proc Natl Acad Sci U S A **90:**6874–6878. http://dx.doi.org/10.1073/pnas.90.14.6874.
19. **Antunes P, Mourão J, Machado J, Peixe L.** 2011. First description of qnrS1-IncN plasmid in a ST11 *Salmonella* Enteritidis clinical isolate from Portugal. Diagn Microbiol Infect Dis **69:**463–465. http://dx.doi.org/10.1016/j.diagmicrobio.2010.11.004.
20. **Machado E, Cantón R, Baquero F, Galán JC, Rollán A, Peixe L, Coque TM.** 2005. Integron content of extended-spectrum-beta-lactamase-producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. Antimicrob Agents Chemother **49:**1823–1829. http://dx.doi.org/10.1128/AAC.49.5.1823-1829.2005.
21. **Novais Â, Cantón R, Valverde A, Machado E, Galán JC, Peixe L, Carattoli A, Baquero F, Coque TM.** 2006. Dissemination and persistence of $bla_{CTX\text{-}M\text{-}9}$ are linked to class 1 integrons containing CR1 associated with defective transposon derivatives from Tn*402* located in early antibiotic resistance plasmids of IncHI2, IncP1-α, and IncFI groups. Antimicrob Agents Chemother **50:**2741–2750. http://dx.doi.org/10.1128/AAC.00274-06.
22. **Quiroga MP, Andres P, Petroni A, Soler Bistué AJ, Guerriero L, Vargas LJ, Zorreguieta A, Tokumoto M, Quiroga C, Tolmasky ME, Galas M, Centrón D.** 2007. Complex class 1 integrons with diverse variable regions, including *aac*(6′)-*Ib-cr*, and a novel allele, *qnrB10*, associated with IS*CR1* in clinical enterobacterial isolates from Argentina. Antimicrob Agents Chemother **51:**4466–4470. http://dx.doi.org/10.1128/AAC.00726-07.
23. **Brady C, Cleenwerck I, Venter S, Coutinho T, De Vos P.** 2013. Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. Syst Appl Microbiol **36:**309–319. http://dx.doi.org/10.1016/j.syapm.2013.03.005.
24. **Poirel L, Cattoir V, Nordmann P.** 2012. Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. Front Microbiol **3:**24. http://dx.doi.org/10.3389/fmicb.2012.00024.
25. **Halová D, Papousek I, Jamborova I, Masarikova M, Cizek A, Janecko N, Oravcova V, Zurek L, Clark AB, Townsend A, Ellis JC, Literak I.** 2014. Plasmid-mediated quinolone resistance genes in *Enterobacteriaceae* from American crows: high prevalence of bacteria with variable *qnrB* genes. Antimicrob Agents Chemother **58:**1257–1258. http://dx.doi.org/10.1128/AAC.01849-13.
26. **Matic I, Radman M, Taddei F, Picard B, Doit C, Bingen E, Denamur E, Elion J.** 1997. Highly variable mutation rates in commensal and pathogenic *Escherichia coli*. Science **277:**1833–1834. http://dx.doi.org/10.1126/science.277.5333.1833.
27. **Saint-Ruf C, Matic I.** 2006. Environmental tuning of mutation rates. Environ Microbiol **8:**193–199. http://dx.doi.org/10.1046/j.1462-2920.2003.00397.x-i1.
28. **Rodríguez-Martínez JM, Briales A, Velasco C, Conejo MC, Martínez-Martínez L, Pascual A.** 2009. Mutational analysis of quinolone resistance in the plasmid-encoded pentapeptide repeat proteins QnrA, QnrB and QnrS. J Antimicrob Chemother **63:**1128–1134. http://dx.doi.org/10.1093/jac/dkp111.
29. **Cantón R, González-Alba JM, Galán JC.** 2012. CTX-M enzymes: origin and diffusion. Front Microbiol **3:**110. http://dx.doi.org/10.3389/fmicb.2012.00110.
30. **Gil R, Latorre A.** 2012. Factors behind junk DNA in bacteria. Genes **3:**634–650. http://dx.doi.org/10.3390/genes3040634.
31. **Wang M, Jacoby GA, Mills DM, Hooper DC.** 2009. SOS regulation of *qnrB* expression. Antimicrob Agents Chemother **53:**821–823. http://dx.doi.org/10.1128/AAC.00132-08.
32. **Briales A, Rodriguez-Martinez JM, Velasco C, Machuca J, Díaz de Alba P, Blazquez J, Pascual A.** 2012. Exposure to diverse antimicrobials induces the expression of *qnrB1*, *qnrD* and *smaqnr* genes by SOS-dependent regulation. J Antimicrob Chemother **67:**2854–2859. http://dx.doi.org/10.1093/jac/dks326.
33. **Petty NK, Feltwell T, Pickard D, Clare S, Toribio AL, Fookes M, Roberts K, Monson R, Nair S, Kingsley RA, Bulgin R, Wiles S, Goulding D, Keane T, Corton C, Lennard N, Harris D, Willey D, Rance R, Yu L, Choudhary JS, Churcher C, Quail MA, Parkhill J, Frankel G, Dougan G, Salmond GP, Thomson NR.** 2011. *Citrobacter rodentium* is an unstable pathogen showing evidence of significant genomic flux. PLoS Pathog **7:**e1002018. http://dx.doi.org/10.1371/journal.ppat.1002018.
34. **Siguier P, Gourbeyre E, Chandler M.** 2014. Bacterial insertion sequences: their genomic impact and diversity. FEMS Microbiol Rev **38:**865–891. http://dx.doi.org/10.1111/1574-6976.12067.
35. **Cattoir V, Nordmann P, Silva-Sanchez J, Espinal P, Poirel L.** 2008. IS*Ecp1*-mediated transposition of *qnrB*-like gene in *Escherichia coli*. Antimicrob Agents Chemother **52:**2929–2932. http://dx.doi.org/10.1128/AAC.00349-08.

Downloaded from http://aac.asm.org/ on July 19, 2016 by guest

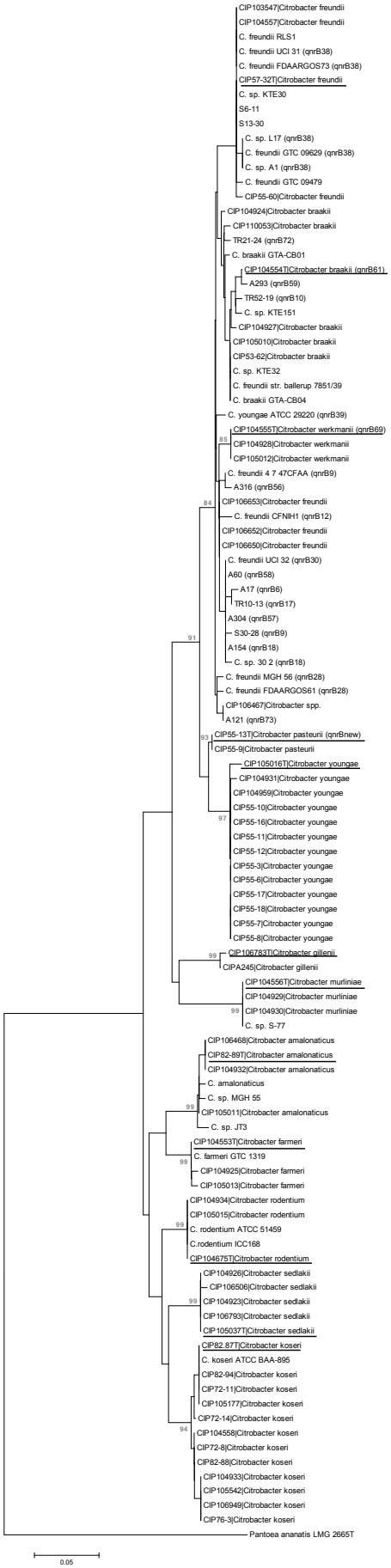

CIP103547|Citrobacter freundii
CIP104557|Citrobacter freundii
C. freundii RLS1
C. freundii UCI 31 (qnrB38)
C. freundii FDAARGOS73 (qnrB38)
CIP57-32T|Citrobacter freundii
C. sp. KTE30
S6-11
S13-30
C. sp. L17 (qnrB38)
C. freundii GTC 09629 (qnrB38)
C. sp. A1 (qnrB38)
C. freundii GTC 09479
CIP55-60|Citrobacter freundii
CIP104924|Citrobacter braakii
CIP110053|Citrobacter braakii
TR21-24 (qnrB72)
C. braakii GTA-CB01
CIP104554T|Citrobacter braakii (qnrB61)
A293 (qnrB59)
TR52-19 (qnrB10)
C. sp. KTE151
CIP104927|Citrobacter braakii
CIP105010|Citrobacter braakii
CIP53-62|Citrobacter braakii
C. sp. KTE32
C. freundii str. ballerup 7851/39
C. braakii GTA-CB04
C. youngae ATCC 29220 (qnrB39)
CIP104555T|Citrobacter werkmanii (qnrB69)
85
CIP104928|Citrobacter werkmanii
CIP105012|Citrobacter werkmanii
C. freundii 4 7 47CFAA (qnrB9)
A316 (qnrB56)
84
CIP106653|Citrobacter freundii
C. freundii CFNIH1 (qnrB12)
CIP106652|Citrobacter freundii
CIP106650|Citrobacter freundii
C. freundii UCI 32 (qnrB30)
A60 (qnrB58)
A17 (qnrB6)
TR10-13 (qnrB17)
A304 (qnrB57)
91
S30-28 (qnrB9)
A154 (qnrB18)
C. sp. 30 2 (qnrB18)
C. freundii MGH 56 (qnrB28)
C. freundii FDAARGOS61 (qnrB28)
CIP106467|Citrobacter spp.
A121 (qnrB73)
93
CIP55-13T|Citrobacter pasteurii (qnrBnew)
CIP55-9|Citrobacter pasteurii
CIP105016T|Citrobacter youngae
CIP104931|Citrobacter youngae
CIP104959|Citrobacter youngae
CIP55-10|Citrobacter youngae
97
CIP55-16|Citrobacter youngae
CIP55-11|Citrobacter youngae
CIP55-12|Citrobacter youngae
CIP55-3|Citrobacter youngae
CIP55-6|Citrobacter youngae
CIP55-17|Citrobacter youngae
CIP55-18|Citrobacter youngae
CIP55-7|Citrobacter youngae
CIP55-8|Citrobacter youngae
99
CIP106783T|Citrobacter gillenii
CIPA245|Citrobacter gillenii
CIP104556T|Citrobacter murliniae
CIP104929|Citrobacter murliniae
99
CIP104930|Citrobacter murliniae
C. sp. S-77
CIP106468|Citrobacter amalonaticus
CIP82-89T|Citrobacter amalonaticus
CIP104932|Citrobacter amalonaticus
C. amalonaticus
C. sp. MGH 55
99
CIP105011|Citrobacter amalonaticus
C. sp. JT3
CIP104553T|Citrobacter farmeri
99
C. farmeri GTC 1319
CIP104925|Citrobacter farmeri
CIP105013|Citrobacter farmeri
CIP104934|Citrobacter rodentium
CIP105015|Citrobacter rodentium
99
C. rodentium ATCC 51459
C.rodentium ICC168
CIP104675T|Citrobacter rodentium
CIP104926|Citrobacter sedlakii
CIP106506|Citrobacter sedlakii
99
CIP104923|Citrobacter sedlakii
CIP106793|Citrobacter sedlakii
CIP105037T|Citrobacter sedlakii
CIP82.87T|Citrobacter koseri
C. koseri ATCC BAA-895
CIP82-94|Citrobacter koseri
CIP72-11|Citrobacter koseri
CIP105177|Citrobacter koseri
CIP72-14|Citrobacter koseri
94
CIP104558|Citrobacter koseri
CIP72-8|Citrobacter koseri
CIP82-88|Citrobacter koseri
CIP104933|Citrobacter koseri
CIP105542|Citrobacter koseri
CIP106949|Citrobacter koseri
CIP76-3|Citrobacter koseri
Pantoea ananatis LMG 2665T
0.05

FIG S1. Affiliation of LeuS amino acid sequences. Genetic distances were constructed using Jones-Taylor-Thornton (JTT) model. Numbers at branch points indicate bootstrap percentages from Neighbor-Joining method analysis and only values greater than 80% are shown. Horizontal Bar: genetic distance of 0.05. All the different *Citrobacter* spp. type strains are underlined and *qnrB* alleles are shown in parentheses. *Pantoea ananatis* LMG 2665^{T} was used as outgroup (PATRIC ID fig|1378093.3.peg.600).

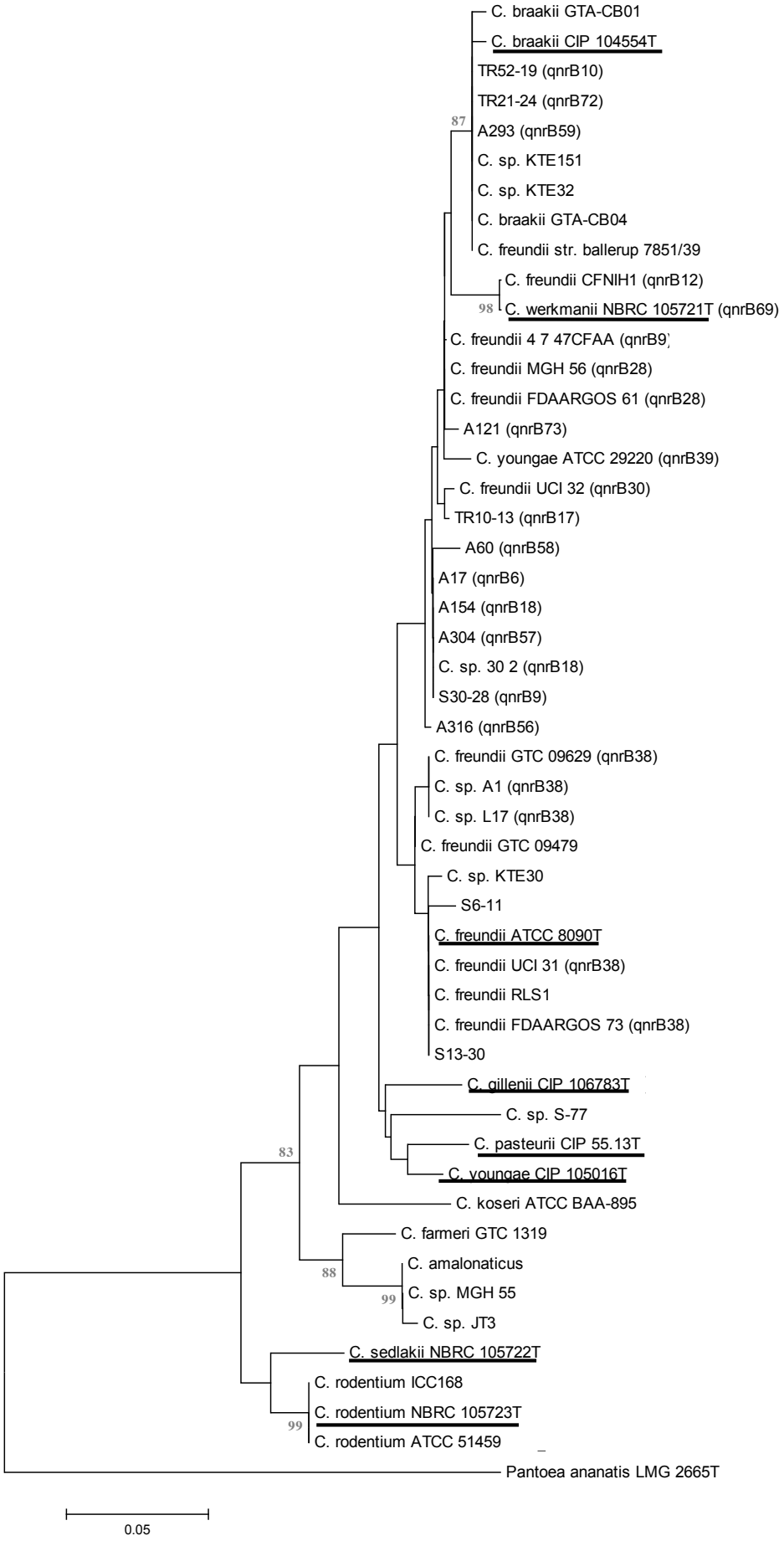

C. braakii GTA-CB01
C. braakii CIP 104554T
TR52-19 (qnrB10)
TR21-24 (qnrB72)
87
A293 (qnrB59)
C. sp. KTE151
C. sp. KTE32
C. braakii GTA-CB04
C. freundii str. ballerup 7851/39
C. freundii CFNIH1 (qnrB12)
98
C. werkmanii NBRC 105721T (qnrB69)
C. freundii 4 7 47CFAA (qnrB9)
C. freundii MGH 56 (qnrB28)
C. freundii FDAARGOS 61 (qnrB28)
A121 (qnrB73)
C. youngae ATCC 29220 (qnrB39)
C. freundii UCI 32 (qnrB30)
TR10-13 (qnrB17)
A60 (qnrB58)
A17 (qnrB6)
A154 (qnrB18)
A304 (qnrB57)
C. sp. 30 2 (qnrB18)
S30-28 (qnrB9)
A316 (qnrB56)
C. freundii GTC 09629 (qnrB38)
C. sp. A1 (qnrB38)
C. sp. L17 (qnrB38)
C. freundii GTC 09479
C. sp. KTE30
S6-11
C. freundii ATCC 8090T
C. freundii UCI 31 (qnrB38)
C. freundii RLS1
C. freundii FDAARGOS 73 (qnrB38)
S13-30
C. gillenii CIP 106783T
C. sp. S-77
83
C. pasteurii CIP 55.13T
C. youngae CIP 105016T
C. koseri ATCC BAA-895
C. farmeri GTC 1319
88
C. amalonaticus
99
C. sp. MGH 55
C. sp. JT3
C. sedlakii NBRC 105722T
C. rodentium ICC168
C. rodentium NBRC 105723T
99
C. rodentium ATCC 51459
Pantoea ananatis LMG 2665T
0.05

FIG S2. Affiliation of RecN amino acid sequences. Genetic distances were constructed using Jones-Taylor-Thornton (JTT) model. Numbers at branch points indicate bootstrap percentages from Neighbor-Joining method analysis and only values greater than 80% are shown. Horizontal Bar: genetic distance of 0.05. All the different *Citrobacter* spp. type strains are underlined and *qnrB* alleles are shown in parentheses. *Pantoea ananatis* LMG 2665^{T} was used as outgroup (PATRIC ID fig|1378093.3.peg.2577).

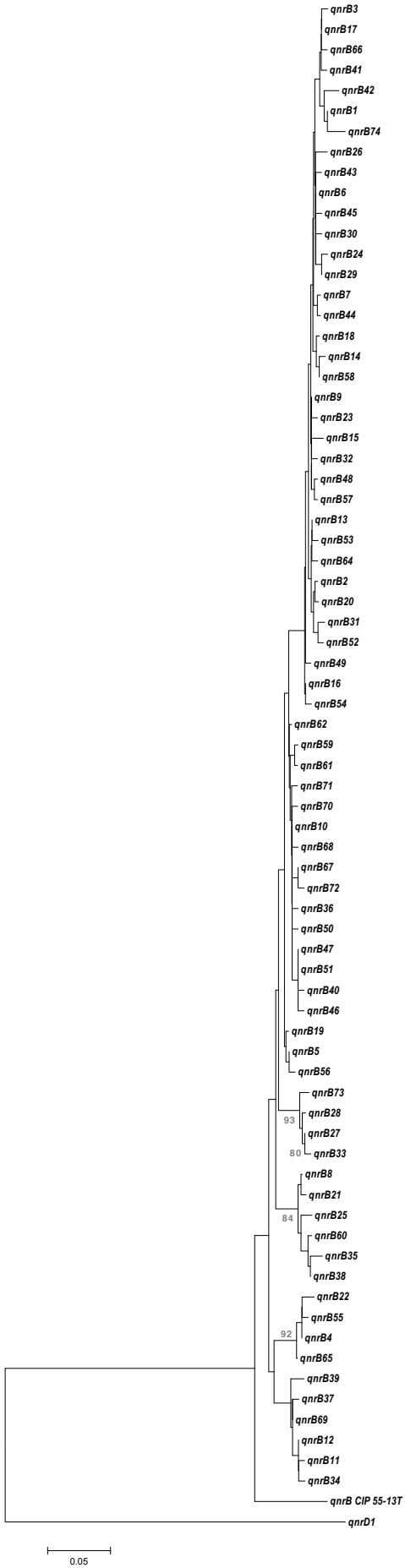

qnrB3
qnrB17
qnrB66
qnrB41
qnrB42
qnrB1
qnrB74
qnrB26
qnrB43
qnrB6
qnrB45
qnrB30
qnrB24
qnrB29
qnrB7
qnrB44
qnrB18
qnrB14
qnrB58
qnrB9
qnrB23
qnrB15
qnrB32
qnrB48
qnrB57
qnrB13
qnrB53
qnrB64
qnrB2
qnrB20
qnrB31
qnrB52
qnrB49
qnrB16
qnrB54
qnrB62
qnrB59
qnrB61
qnrB71
qnrB70
qnrB10
qnrB68
qnrB67
qnrB72
qnrB36
qnrB50
qnrB47
qnrB51
qnrB40
qnrB46
qnrB19
qnrB5
qnrB56
qnrB73
qnrB28
qnrB27
qnrB33
qnrB8
qnrB21
qnrB25
qnrB60
qnrB35
qnrB38
qnrB22
qnrB55
qnrB4
qnrB65
qnrB39
qnrB37
qnrB69
qnrB12
qnrB11
qnrB34
qnrB CIP 55-13T
qnrD1
93
80
84
92
0.05

FIG S3. Affiliation of QnrB amino acid sequences. Genetic distances were constructed using Jones-Taylor-Thornton (JTT) model. Numbers at branch points indicate bootstrap percentages from Neighbor-Joining method analysis and only values greater than 80% are shown. Horizontal Bar: genetic distance of 0.05. QnrD1 was used as outgroup (GenBank accession number ACG70184).

TABLE S1. Epidemiological data of *Citrobacter* spp. isolates harboring complete or truncated *qnrB* genes characterized in this study.

Isolate	Phylogenetic assignment[a]	Origin (sample)	PFGE-type (no.)	*qnrB* allele[b]	Antibiotic resistance pattern[c, d, e]
A17	*Citrobacter* sp. I	Water well (W1)	A (1)	*qnrB6*	IPM, NAL, SUL, (KAN), (STR)
A313	*Citrobacter* sp. I	Natural Spring (W63)	B (3)	*qnrB56*	(ATM), NAL, SUL, (TET)
A314					
A316					
A304	*Citrobacter* sp. I	Fountain (W60)	C (2)	*qnrB57*	KAN, STR, NAL, SUL, (TET)
A305					
A60	*Citrobacter* sp. I	Water well (W13)	D (1)	*qnrB58*	STR, NAL, SUL
A154	*Citrobacter* sp. I	Borehole (W33)	E (1)	*qnrB18*	STR, NAL, SUL
S30-28	*Citrobacter* sp. I	Ready-to-eat salads (S30)	F (1)	*qnrB9*	NAL
TR10-13	*Citrobacter* sp. I	Aquaculture (feed) (AQ10)	G (1)	*qnrB17*	
A121	*Citrobacter* sp. III	Water well (W23)	H (1)	*qnrB73*	NAL, SUL
A293	*C. braakii*	Borehole (W57)	I (3)	*qnrB59*	STR, NAL, SUL, (TET)
A295					
A297					
TR21-24	*C. braakii*	Aquaculture (sediment) (AQ21)	J (2)	*qnrB72*	NAL, TET
TR21-25					
TR52-19	*C. braakii*	Trout (AQ52)	K (3)	*qnrB10*	NAL, TET
TR52-21					
TR52-22					
S6-11	*C. freundii*	Ready-to-eat salads (S6)	L (1)	Δ*qnrB*	NAL, SUL, STR
S13-30	*C. freundii*	Ready-to-eat salads (S13)	M (1)	Δ*qnrB*	SUL, STR

TABLE S1. *Continued*

[a]Species identification according to the phylogenetic affiliation obtained with *leuS* and *recN* genes.
[b]All the different *qnrB* alleles were chromosomally located.
[c]Natural resistance patterns were not included.
[d]Antimicrobial agents tested [disk diffusion method according to EUCAST (http://www.eucast.org/) guidelines and breakpoints or following Clinical and Laboratory Standards Institute recommendations (http://clsi.org/) for antibiotics not considered in the EUCAST documents]: β-lactam (FEP, cefepime; ATM, aztreonam; IPM, imipenem; ERT, ertapenem) and non-β-lactam (AMK, amikacin; GEN, gentamicin; KAN, kanamycin; NET, netilmicin; STR, streptomycin; TOB, tobramycin; SUL, sulfonamides; TMP, trimethoprim; TET, tetracycline; CHL, chloramphenicol; CIP, ciprofloxacin; NAL, nalidixic acid) antibiotics.
[e]Variable presence of a given resistance phenotype is indicated in parentheses.

TABLE S2. Phylogenetic assignment of *Citrobacter* spp. isolates harboring or not *qnrB* genes.

Phylogenetic assignment[a]	Isolate	*qnrB* allele	GenBank accession number	Accession numbers (GenBank/PATRIC)	
				leuS	***recN***
C. freundii	CIP57-32^T = C. freundii ATCC 8090^T	*ΔqnrB*	AB734052	EKS58631	EKS56099
C. freundii	C. freundii GTC 09479	*ΔqnrB*	NZ_AOMS01000003	EMF20765	EMF22422
C. freundii	C. freundii RLS1	*ΔqnrB*	NZ_JATT01000060	EXF29472	EXF30442
C. freundii	C. sp. KTE30	*ΔqnrB*	ASQJ01000007	EOQ25427	EOQ21965
C. freundii	S6-11	*ΔqnrB*	NA	KR998039	KR998026
C. freundii	S13-30	*ΔqnrB*	NA	KR998040	KR998027
C. freundii	C. freundii GTC 09629	*qnrB38*	NZ_AOUE01000004	EOD61956	EOD59147
C. freundii	C. sp. A1	*qnrB38*	NZ_AKTT01000018	EJF24310	EJF20625
C. freundii	C. sp. L17	*qnrB38*	NZ_AMPE01000004	EKU36588	EKU31915
C. freundii	C. freundii UCI31	*qnrB38*	JAPB01000002	ETX72722	ETX69942
C. freundii	C. freundii FDAARGOS_73	*qnrB38*	JTBJ01000001	KGY84667	KGY86553
C. braakii	CIP 104554^T = *C. braakii* ATCC 51113^T	*qnrB61*	AB734053	KM515986	KF057886
C. braakii	C. braakii GTA-CB01	-	JRHK01000002	KHE06528	KHE04073
C. braakii	C. braakii GTA-CB04	-	JRHL01000001	KHE09771	KHE09463
C. braakii	C. freundii str. ballerup 7851/39	NA	NA	fig|670484.3.peg.401	fig|670484.3.peg.2509
C. braakii	C. sp. KTE151	*ΔqnrB*	ASQK01000005	EOQ50286	EOQ47363
C. braakii	C. sp. KTE32	-	ASQL01000009	EOQ32555	EOQ28285
C. braakii	TR52-19	*qnrB10*	KP339256	KR998044	KR998031
C. braakii	A293	*qnrB59*	KP339262	KR998036	KR998023
C. braakii	TR21-24	*qnrB72*	KP339263	KR998043	KR998030
C. youngae	CIP 105016^T	NA	NA	KM515993	KF057888

TABLE S2. *Continued*

Phylogenetic assignment[a]	Isolate	*qnrB* allele	GenBank accession number	Accession numbers (GenBank/PATRIC)	
				leuS	***recN***
C. werkmanii	CIP 104555^T = C. werkmanii NBRC 105721^T	*qnrB69*	BBMW01000005	GAL44920	GAL47081
C. werkmanii	C. freundii CFNIH1	*qnrB12*	CP007557	WP_038638761	AHY14573
C. amalonaticus	CIP 82.89^T	NA	NA	EU010040	n.a.
C. amalonaticus	C. amalonaticus	-	JMQQ01000039	KEY47803	KEY45284
C. amalonaticus	C. sp. JT3	NA	NA	fig|1451265.3.peg.2413	fig|1451265.3.peg.1330
C. amalonaticus	C. freundii MGH55	-	JMUK01000018	KDF04575	KDF07738
C. farmeri	CIP 104553^T	NA	NA	KM515987	n.a.
C. farmeri	C. farmeri GTC1319	-	NZ_BBMX01000005	GAL49434	GAL51860
C. koseri	CIP 82.87^T = C. koseri ATCC 27028^T	-	AB734056	KM515994	n.a.
C. koseri	C. koseri ATCC BAA-895	-	CP000822	YP_001454059	YP_001455447
C. rodentium	C. rodentium NBRC 105723^T	-	NZ_BBNA01000024	fig|1218085.3.peg.3980	fig|1218085.3.peg.4189
C. rodentium	C. rodentium ATCC 51459	-	JXUN01000211	KIQ50925	KIQ49016
C. rodentium	C. rodentium ICC168	-	FN543502	YP_003364286	YP_003366100
C. sedlakii	C. sedlakii NBRC 105722^T	-	NZ_BBNB01000001	fig|1218086.3.peg.1142	fig|1218086.3.peg.2278
C. pasteurii	CIP55-13^T	*qnrBnew*	CDHL01000019	CEJ66010	CEJ67563
C. murliniae	CIP 104556^T	NA	NA	KM515989	n.a.
C. murliniae	C. sp. S-77	NA	NA	fig|1080067.3.peg.1081	fig|1080067.3.peg.3390
C. gillenii	CIP 106783^T	NA	NA	KM515988	KF057887
Citrobacter sp. I	A17	*qnrB6*	KP339254	KR998032	KR998019
Citrobacter sp. I	S30-28	*qnrB9*	KP339255	KR998041	KR998028
Citrobacter sp. I	C. freundii 4_7_47CFAA	*qnrB9*	ADLG01000026	EHL82656	EHL86564

TABLE S2. *Continued*

Phylogenetic assignment[a]	Isolate	*qnrB* allele	GenBank accession number	Accession numbers (GenBank/PATRIC)	
				leuS	***recN***
Citrobacter sp. I	TR10-13	*qnrB17*	KP339257	KR998042	KR998029
Citrobacter sp. I	A154	*qnrB18*	KP339258	KR998035	KR998022
Citrobacter sp. I	C. sp. 30_2	*qnrB18*	ACDJ02000027	ZP_04561108	ZP_04560012
Citrobacter sp. I	C. freundii UCI32	*qnrB30*	JAPA01000008	ETX65167	ETX61851
Citrobacter sp. I	A304	*qnrB57*	KP339260	KR998037	KR998024
Citrobacter sp. I	A60	*qnrB58*	KP339261	KR998033	KR998020
Citrobacter sp. I	A316	*qnrB56*	KP339259	KR998038	KR998025
Citrobacter sp. II	C. youngae ATCC 29220	*qnrB39*	ABWL02000005	ZP_03835838	ZP_03838025
Citrobacter sp. III	C. freundii FDAARGOS_61	*qnrB28*	JTBV01000001	KGZ32966	KGZ31013
Citrobacter sp. III	C. freundii MGH56	*qnrB28*	JMUJ01000007	KDF21035	KDF13460
Citrobacter sp. III	A121	*qnrB73*	KP339264	KR998034	KR998021

[a]Species identification according to the phylogenetic affiliation obtained with *leuS* and *recN* genes. NA, not attainable. *Citrobacter* spp. type strains are indicated in grey shadows.

Citrobacter europaea sp. nov., a novel *Citrobacter* species isolated from water and human faecal samples

Teresa Gonçalves Ribeiro[1], Dominique Clermont[2], Raquel Branquinho[1], Elisabete Machado[1,3], Luísa Peixe[1], Sylvain Brisse[4,5]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Portugal; [2]CIP - Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; [3]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; [4]Microbial Evolutionary Genomics, Institut Pasteur, Paris, France; [5]CNRS, UMR 3525, Paris, France.

International Journal of Systematic and Evolutionary Microbiology

Manuscript ID: IJSEM-D-16-00589 (*submitted manuscript*); June 2016

International Journal of Systematic and Evolutionary Microbiology

Citrobacter europaea sp. nov., a novel Citrobacter species isolated from water and human faecal samples

--Manuscript Draft--

Manuscript Number:	
Full Title:	Citrobacter europaea sp. nov., a novel Citrobacter species isolated from water and human faecal samples
Short Title:	Citrobacter europaea sp. nov.
Article Type:	Standard
Section/Category:	New taxa - Proteobacteria
Keywords:	Enterobacteriaceae; Citrobacter; genomic sequencing; Phylogeny
Corresponding Author:	Sylvain Brisse Institut Pasteur FRANCE
First Author:	Sylvain Brisse
Order of Authors:	Sylvain Brisse
	Teresa G. Ribeiro
	Dominique Clermont
	Raquel Branquinho
	Elisabete Maria Pereira Machado, PhD degree in Microbiology
	Luisa Peixe
Manuscript Region of Origin:	FRANCE
Abstract:	Strains 97/79T and A121, recovered respectively from human faeces and well waters, were compared to currently known Citrobacter species using genotypic and phenotypic approaches. Multilocus sequence analysis based on housekeeping genes fusA, leuS, pyrG, rpoB and recN genes, showed that the two strains formed a distinct phylogenetic lineage within the Citrobacter genus. Average nucleotide identity (ANI) between strains 97/79T and A121 was 99.2%, whereas ANI values of 97/79T with the type strains of closely related Citrobacter species C. werkmanii, C. braakii, C. freundii, C. youngae and C. pasteurii were all below 93.0%. The ability to metabolize different compounds also discriminated 97/79T and A121 from other Citrobacter species. Based on these results, strains 97/79T and A121 represent a novel species of the genus Citrobacter, for which the name Citrobacter europaea sp. nov. is proposed, with strain 97/79T (=CIP 106467T= DSM 103031T) as the type strain. The G+C content of strain 97/79T is 52.0%.

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Including References (Word document)

Click here to download Manuscript Including References (Word document) Ribeiro_CitrobacterEuropaea_v4.doc

Citrobacter europaea sp. nov., a novel *Citrobacter* species isolated from water and human faecal samples

Teresa G. Ribeiro[1], Dominique Clermont[2], Raquel Branquinho[1], Elisabete Machado[1,3], Luísa Peixe[1], Sylvain Brisse[4,5]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Portugal.

[2]CIP - Collection de l'Institut Pasteur, Institut Pasteur, Paris, France.

[3]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

[4]Microbial Evolutionary Genomics, Institut Pasteur, Paris, France.

[5]CNRS, UMR 3525, Paris, France.

Corresponding author: Sylvain Brisse, Microbial Evolutionary Genomics Unit, Institut Pasteur, Paris, France. E-mail: sylvain.brisse@pasteur.fr.

Subject Category: New taxa – Proteobacteria

Running title: *Citrobacter europaea* sp. nov.

22 Footnotes:

23 The annotated genomic sequences of 97/79^T and A121 strains were deposited in the European
24 Nucleotide Archive and are available under project accession number PRJEB13613. Sequence
25 reads obtained upon genomic sequencing of strains 97/79 ^{T} and A121 are available under the
26 project accession numbers PRJEB7595 and PRJEB13613, respectively.

27 Abbreviations: *rpoB*, β-subunit of RNA polymerase gene; *pyrG*, CTP synthetase gene; *fusA*,
28 protein synthesis elongation factor-G gene; *leuS*, leucine tRNA synthetase; *recN*, DNA repair;
29 ANI, Average Nucleotide Identity.

30 Details of additional physiological and phenotypic characteristics of *C. europaea* sp. nov. 97/79^T
31 and A121 are available as supplementary material at IJSEM Online.

32

33 Abstract

34 Strains 97/79^T and A121, recovered respectively from human faeces and well waters, were
35 compared to currently known *Citrobacter* species using genotypic and phenotypic approaches.
36 Multilocus sequence analysis based on housekeeping genes *fusA*, *leuS*, *pyrG, rpoB* and *recN*
37 genes, showed that the two strains formed a distinct phylogenetic lineage within the
38 *Citrobacter* genus. Average nucleotide identity (ANI) between strains 97/79^T and A121 was
39 99.2%, whereas ANI values of 97/79^T with the type strains of closely related *Citrobacter* species
40 *C. werkmanii*, *C. braakii, C. freundii, C. youngae and C. pasteurii* were all below 93.0%. The
41 ability to metabolize different compounds also discriminated 97/79^T and A121 from other
42 *Citrobacter* species. Based on these results, strains 97/79^T and A121 represent a novel species
43 of the genus *Citrobacter*, for which the name *Citrobacter europaea* sp. nov. is proposed, with
44 strain 97/79^T (=CIP 106467^T= DSM 103031^T) as the type strain. The G+C content of strain 97/79^T
45 is 52.0%.

46

The genus *Citrobacter* includes ubiquitous bacteria that can be found in the intestinal microflora of humans and animals and in soil, water, sewage and food (Borenshtein & Schauer, 2006; Engelkirk & Duben-Engelkirk, 2008; Ribeiro *et al*., 2015; Albaser *et al*., 2016). Nevertheless, some *Citrobacter* species are considered as opportunistic human pathogens (Clermont *et al*., 2015; Engelkirk & Duben-Engelkirk, 2008; Borenshtein & Schauer, 2006). Currently, 13 species are recognized within the genus *Citrobacter*, including the two recently described species *Citrobacter bitternis* (Ko *et al.,* 2015) and *Citrobacter pasteurii* (Clermont *et al*., 2015), and the previously recognized *Citrobacter freundii*, *Citrobacter amalonaticus*, *Citrobacter braakii*, *Citrobacter farmeri*, *Citrobacter gillenii*, *Citrobacter koseri*, *Citrobacter murliniae*, *Citrobacter rodentium*, *Citrobacter sedlakii*, *Citrobacter werkmanii* and *Citrobacter youngae* species (Brenner *et al*., 1993; Frederiksen, 2005). Phylogenetic analysis of protein-coding housekeeping genes provided an accurate discrimination among *Citrobacter* species (Ribeiro *et al*., 2015; Clermont *et al*., 2015). In the latter studies, strain 97/79^T recovered from human faeces and strain A121 from well water were distinct from previously described *Citrobacter* species. The purpose of this work was to define the taxonomic position of these two strains.

Strains 97/79^T and A121 were isolated as described (Clermont *et al*., 2015; Ribeiro *et al*., 2015). *C. werkmanii* CIP 104555^T, *C. freundii* CIP 57.32^T and *C. braakii* CIP 104554^T were used for comparative phenotypic analysis. Strains were maintained on Trypticase Soy Agar (TSA; Sigma-Aldrich) for short-term storage and in Tryptic Soy Broth (TSB; Sigma-Aldrich) supplemented with 20% (v/v) glycerol at -80°C for long-term storage. Gram staining was carried out by using the bioMérieux Gram Stain kit. Oxidase activity was tested by using 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated in the presence of 3% (v/v) aqueous hydrogen peroxide solution. Growth at different NaCl concentrations [0, 3.0, 6.0, 9.0, 12.0 and 15.0% (w/v)] and temperatures (5, 10, 15, 20, 25, 30, 37, 50, 60, 65 and 70 °C) were

examined by using TSB as the basal medium. To determine the pH range for growth, basal medium was adjusted with HCl or NaOH to reach pH values of 4.0-11.0, at intervals of 1.0 pH unit. To confirm anaerobic growth, strains were inoculated into TSB tubes with paraffin on top, under optimal growth conditions. Metabolic and biochemical characterization was performed using the standardized API 20E strips (incubation 24 h at 34°C) and API 50 CH strips (incubation 48 h at 37°C) (bioMérieux, La Balme-les-Grottes, France). DNA was obtained by using the DNeasy tissue kit (Qiagen, Alameda, CA). PCR amplification of *rpoB* (β-subunit of RNA polymerase), *pyrG* (CTP synthetase), *fusA* (protein synthesis elongation factor-G), *leuS* (leucine tRNA synthetase), and *recN* (DNA repair) genes was performed using primers and PCR conditions described previously (Delétoile *et al.*, 2009; Ribeiro *et al.*, 2015). PCR products were sequenced on both strands on an ABI 3730xl DNA analyzer and chromatograms were analyzed using BioNumerics v6.6 (Applied-Maths, Sint-Martens Latem, Belgium). Further, the sequences of the five genes were subsequently confirmed from the genomic sequences. Whole genome shotgun sequencing was achieved using Illumina's MiSeq 2 x 250 nt (strain 97/79^T; Clermont *et al.*, 2015) or NextSeq-500 instrument 2 x 150 nt (strain A121). Assembly of strain 97/79^T was obtained using CLC Genomics Workbench 7.0.3 (www.clcbio.com/products/clc-genomics-workbench) and assembly of strain A121 was obtained using SPAdes v3.6.2 (Bankevich *et al.*, 2012). Average Nucleotide Identity (ANI) was calculated by using jSpecies (Richter & Rossello-Mora, 2009). Phylogenetic analyses were performed by the maximum likelihood method using PhyML (Guindon & Gascuel, 2003) with the K80 nucleotide substitution model.

The phylogenetic analysis based on the four loci *fusA*, *leuS*, *pyrG* and *rpoB*, representing a total of 2,082 nt, showed that strains 97/79^T and A121 represent a distinct branch within the group I (Warren *et al.*, 2000; Clermont *et al.*, 2015) of the *Citrobacter* genus (Figure 1). Further, these two strains were grouped together with two strains for which the genomic sequences were

publicly available, MGH 56 and FDAARGOS_61 (Figure 1). *recN* gene sequencing, previously used to discriminate *Citrobacter* species (Ribeiro *et al*., 2015), also distinguished 97/79^T and A121 from other closely related *Citrobacter* species (Figure S1).

The ANI value between the genomic sequences of strains 97/79^T and A121 was 99.2%. In contrast, the ANI values of 97/79^T compared with the type strain of *C. braakii* (93.0%), *C. freundii* (92.2%), *C. werkmanii* (90.3%), and *C. youngae* (89.2%) were clearly below the species cut-off level of 95%.

The novel isolates stained as Gram-negative. The rod shaped cells (1–2 µm in diameter and 4–5 µm in length) were motile. Growth occurred at 20ºC, 25ºC, 30 ºC, 37 ºC and 50 ºC, and in the range of 0-15% (w/v) NaCl and pH 5.0–10.0. Other phenotypic characteristics are given in supplementary Table S1. Differential biochemical characteristics of strains 97/79^T and A121 as compared to type strains of closely related *Citrobacter* species are presented in Table 1.

Overall, the genotypic and phenotypic characteristics of 97/79^T and A121 show that they represent a novel *Citrobacter,* for which the name *Citrobacter europaea* sp. nov. is proposed, with 97/79^T as the type strain.

Description of Citrobacter europaea sp. nov.

Citrobacter europaea (eu.ro.pa.'ea N.L. fem. adj. europaea of or belonging to Europe, referring to the European continent).

Gram-negative, motile, non-spore-forming short rods. Colonies are translucent, bright and 1– 2 µm in diameter and 4–5 µm in length. Facultatively anaerobic. Catalase positive and oxidase negative. Do not decompose gelatin. Voges-Proskauer test and indole production are negative.

The methyl red test is positive. Produces H_2S, reduces nitrate to nitrite and N_2 production is negative. Do not use citrate as a carbon source. L-arginine, L-lysine and L-tryptophan are not utilized. Urease activity is negative. Acid is produced from glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, N-acetylglucosamine, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-trehalose, D-raffinose, gentiobiose, L-fucose, potassium gluconate, 2-ketogluconate, 5-ketogluconate and 2-nitrophenyl-ß-D-galactopyranoside, but not from erythritol, L-xylose, D-adonitol, methyl-ß-D-xylopyranoside, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, amygdalin, esculin ferric citrate, sucrose, inulin, D-melezitose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, and L-arabitol. The DNA G+C content of the type strain is 52.0%. The type strain is 97/79^T (=CIP 106467^T= DSM 103031^T), isolated from faeces from a human with diarrhea in the USA.

Acknowledgments

This work received financial support from the European Union (FEDER funds) through Programa Operacional Factores de Competitividade – COMPETE, and from National Funds [Fundação para a Ciência e a Tecnologia (FCT)] through project UID/Multi/04378/2013, by the Institut Pasteur and the French Government Investissement d'Avenir grant ANR-10-LABX-62-IBEID. Teresa G. Ribeiro and Raquel Branquinho were supported by fellowships from FCT and UCIBIO-REQUIMTE (SFRH/BD/84341/2012 and UID/MULTI/04378/2013, respectively). We thank D. Mornico (C3BI Hub, Institut Pasteur) for assistance with genome sequence data submission.

144 References

145 **Albaser, A., Kazana, E., Bennett, M. H., Cebeci, F., Luang-In, V., Spanu, P. D. & Rossiter, J. T. (2016).**
146 Discovery of a bacterial glycoside hydrolase family 3 (GH3) β-glucosidase with myrosinase
147 activity from a *Citrobacter* strain isolated from soil. J Agric Food Chem **64**, 1520-1527.

148 **Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M.,**
149 **Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G.,**
150 **Alekseyev, M. A. & Pevzner, P. A.** (2012). SPAdes: a new genome assembly algorithm and its
151 applications to single-cell sequencing. *J Comput Biol* **19**, 455-477.

152 **Borenshtein, D. & Schauer, D. (2006).** The Genus *Citrobacter*. *In the Prokaryotes*, pp: 90-98.
153 Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Scheleifer and E. Stackebrandt: Springer,
154 New York, USA.

155 **Clermont, D., Motreff, L., Passet, V., Fernandez, J. C., Bizet, C. & Brisse, S. (2015).** Multilocus
156 sequence analysis of the genus *Citrobacter* and description of *Citrobacter pasteurii* sp. nov. *Int J*
157 *Syst Evol Microbiol* **65**, 1486-1490.

158 **Delétoile, A., Decré, D., Courant, S., Passet, V., Audo, J., Grimont, P., Arlet, G. & Brisse, S. (2009).**
159 Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by
160 multilocus gene sequencing. *J Clin Microbiol* **47**, 300-310.

161 **Engelkirk, P. G. & Duben-Engelkirk, J. L. (2008).** Laboratory Diagnosis of Infectious Diseases:
162 Essentials of Diagnostic Microbiology. *In the Gram-negative bacilli: the family*
163 *Enterobacteriaceae*. pp: 308. Lippincott Williams & Wilkins, Philadelphia, USA.

164 **Guindon, S. & Gascuel, O. (2003).** A simple, fast, and accurate algorithm to estimate large
165 phylogenies by maximum likelihood. *Syst Biol* **52**, 696-704.

166 **Ko, K. S., Choi, J. Y., Kim, J. & Park, M. K. (2015).** *Citrobacter bitternis* sp. nov. isolated from
167 bitterns. *Curr Microbiol* **70**, 894-897.

168 **Ribeiro, T. G., Novais, Â., Branquinho, R., Machado, E. & Peixe, L. (2015).** Phylogeny and
169 comparative genomics unveil independent diversification trajectories of *qnrB* and genetic
170 platforms within particular *Citrobacter* species. *Antimicrob Agents Chemother* **59**, 5951-5958.

171 **Richter, M. & Rosselló-Móra, R. (2009).** Shifting the genomic gold standard for the prokaryotic
172 species definition. *Proc Natl Acad Sci U S A* **106**, 19126-19131.

173 **Schauer D. B., Zabel B. A., Pedraza I. F., O'Hara C. M., Steigerwalt A. G. & Brenner D. J. (1995).**
174 Genetic and biochemical characterization of *Citrobacter rodentium* sp. nov. *J Clin Microbiol* **33**,
175 2064-2068.

176 **Warren, J. R., Farmer, III J. J., Dewhirst, F. E., Birkhead, K., Zembower T., Peterson L. R., Sims, L. &**
177 **Bhattacharya, M. (2000).** Outbreak of nosocomial infections due to extended-spectrum β-
178 lactamase-producing strains of enteric group 137, a new member of the family
179 *Enterobacteriaceae* closely related to *Citrobacter farmeri* and *Citrobacter amalonaticus. J Clin*
180 *Microbiol* **38**, 3946-3952.

181

Figure legend:

Figure 1. Maximum likelihood tree based on concatenated alignments of nucleotide sequences of the four protein-coding genes *fusA, pyrG, leuS* and *rpoB*. Only *Citrobacter* species that belong to group I (Warren *et al*., 2000; Clermont *et al*., 2015) were included, thus also excluding the recently described *C. bitternis* species. Previous sequence data are from Clermont *et al*., 2015. The scale bar corresponds to the proportion of changes per nucleotide. *C. koseri and C. rodentium* were used as outgroups. Branches depicted in bold were supported by bootstrap values above 95%, as obtained after 1000 replicates.

191 Table legend:

192 **Table 1.** Differential metabolic characteristics of *C. europaea* strains 97/79^T and A121 and

193 closely related *Citrobacter* type strains.

	97/79^T	A121	*C. werkmanii* CIP104555^T	*C. braakii* CIP 104554^T	*C. freundii* CIP 57.32^T
L-Arginin utilization	-	-	+	+	-
Sodium citrate utilization	-	-	+	+	+
Acid production from:					
L-Sorbose	+	+	+	-	+
Dulcitol	+	+	-	+	-
Inositol	+	+	-	-	+
Arbutin	+	+	+	-	-
Salicin	+	+	-	-	-
D-Melibiose	+	+	-	+	+
D-Raffinose	+	+	-	+	+
Starch	-	-	-	+	+
Gentiobiose	+	+	+	-	+
D-Lyxose	-	-	-	+	-
Potassium 5-Keto Gluconate	+	+	-	+	+
Sucrose	-	-	-	-	+

194

Figure 1_Phylogeny

Click here to download Figure Figure1_phylogeny.pptx

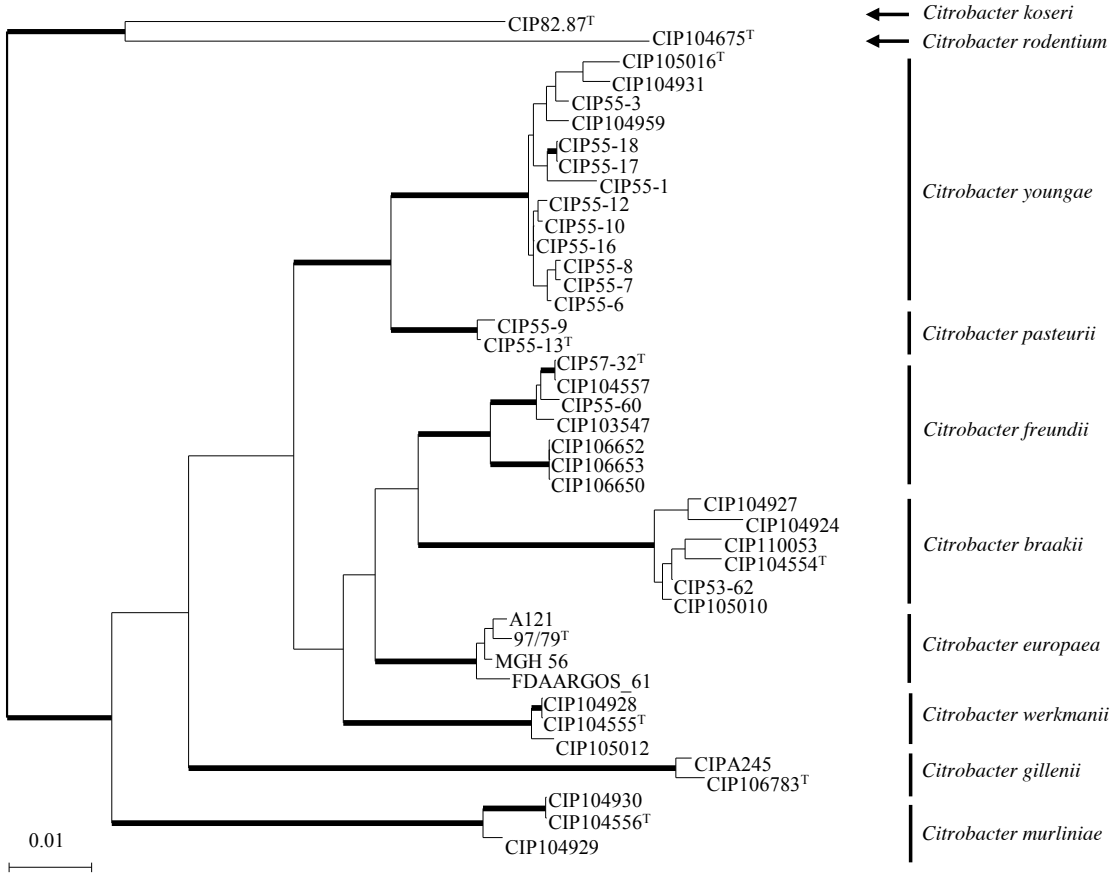
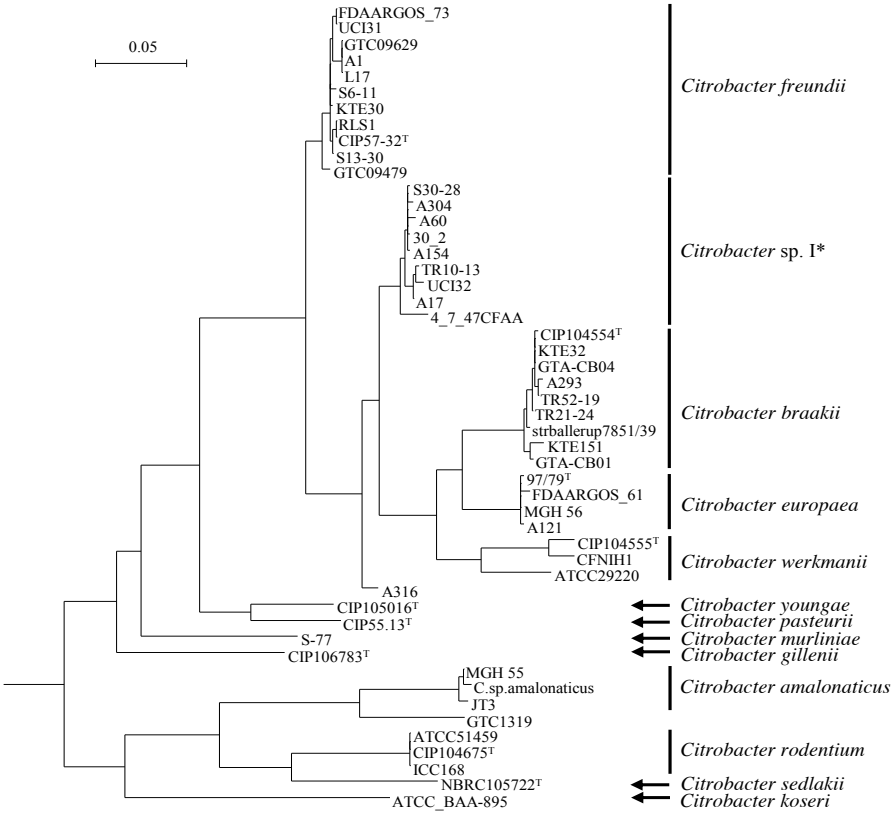


Figure S1_recN.pptx

Click here to download Figure FigureS1_recN.pptx



Manuscript Including References (Word document)

Click here to download Supplementary Material Files
Ribeiro_SupplementaryMaterial.docx

Supplementary Data

Citrobacter europaea sp. nov., a novel *Citrobacter* species isolated from water and human faecal samples

Teresa G. Ribeiro[1], Dominique Clermont[2], Raquel Branquinho[1,3], Elisabete Machado[1,4], Luísa Peixe[1], Sylvain Brisse[5,6]

[1]UCIBIO-REQUIMTE. Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Portugal; [2]CIP - Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; [3]Centro Ciência Viva de Bragança, Bragança, Portugal; [4]FP-ENAS/CEBIMED. Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; [5]Microbial Evolutionary Genomics, Institut Pasteur, Paris, France; [6]CNRS, UMR 3525, Paris, France.

Running title: *Citrobacter europaea* sp. nov.

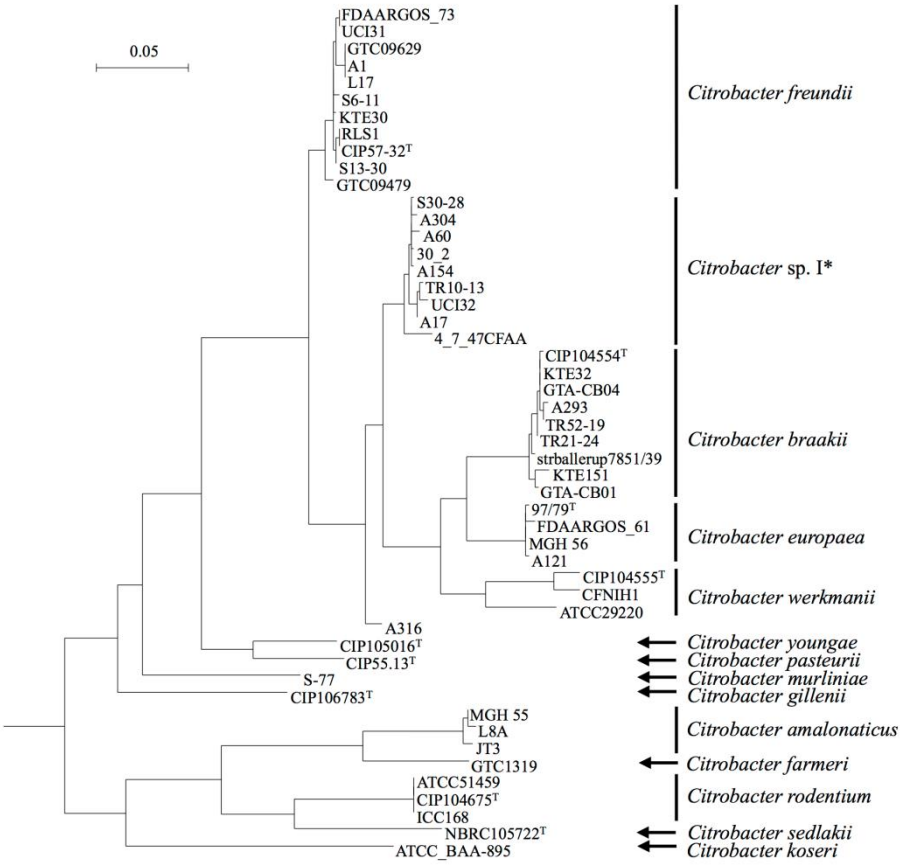
16 **Table S1.** Physiological and phenotypic characteristics of *C. europaea* sp. nov. strains.

17

Characteristics	**97/79^T**	**A121**
Catalase	**+**	**+**
Oxidase	**-**	**-**
Nitrate reduction	+	+
N_2 production	-	-
H_2S production	+	+
Indole production	-	-
Gelatin	-	-
Citrate alkalinization	+	+
Voges-Prokauer test	-	-
Methyl red test	+	+
Growth ability		
NaCl range (%, w/v)	0-15	0-15
pH range	5.0-10.0	5.0-10.0
Temperature range	20-50 °C	20-50 °C
Carbohydrates acid production profile		
Glycerol	+	+
Erythritol	-	-
D-Arabinose	+	+
L-Arabinose	+	+
D-Ribose	+	+
D-Xylose	+	+
L-Xylose	-	-
D-Adonitol	-	-
Methyl-ß-D-Xylopyranoside	-	-
D-Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
L-Sorbose	+	+
L-Rhamnose	+	+
Dulcitol	+	+
Inositol	+	+
D-Mannitol	+	+
D-Sorbitol	+	+
Methyl-α-D-Mannopyranoside	-	-
Methyl-α-D-Glucopyranoside	-	-

N-Acetylglucosamine	+	+
Amygdalin	-	-
Arbutin	+	+
Esculin Ferric Citrate	-	-
Salicin	+	+
D-Cellobiose	+	+
D-Maltose	+	+
D-Lactose (bovine origin)	+	+
D-Melibiose	+	+
Sucrose	-	-
D-Trehalose	+	+
Inulin	-	-
D-Melezitose	-	-
D-Raffinose	+	+
Starch	-	-
Glycogen	-	-
Xylitol	-	-
Gentiobiose	+	+
D-Turanose	-	-
D-Lyxose	-	-
D-Tagatose	-	-
D-Fucose	-	-
L-Fucose	+	+
D-Arabitol	-	-
L-Arabitol	-	-
Potassium Gluconate	+	+
Potassium 2-Keto Gluconate	+	+
Potassium 5-Keto Gluconate	+	+
2-Nitrophenyl-ß-D-Galactopyranoside	+	+
Amino acid utilization		
L-Arginine	-	-
L-Lysine	-	-
L-Ornithine	-	+
L-Tryptophan	-	-
Enzymatic activities		
Urease	-	-

Figure S1. Maximum likelihood tree based on the nucleotide sequence of gene *recN*. Previous sequence data are from Ribeiro *et al*., 2015. The scale bar corresponds to 1% nucleotide change. *Pantoea ananatis* LMG 2665^{T} (PATRIC ID fig|1378093.3.peg.2577) was used as outgroup for tree rooting. Sp I*: putative new species



Chapter 4

Conclusions

"Science never solves a problem without creating ten more."

George Bernard Shaw

The overall results of this Thesis reveal a low rate and a stable occurrence of qAmpC β-lactamases (CMY-2, DHA-1) throughout a large period of time in diverse clinical institutions and *Enterobacteriaceae* species in Portugal, whereas they were rarely identified in non-clinical niches. On the other hand, in Angola, CTX-M-15 was the almost exclusive acquired β-lactamase identified mostly on *E. coli* among a high diversity of non-clinical niches, an atypical epidemiology highlighting the need for antimicrobial resistance surveillance in different regions of Africa. Despite the diversity of genetic backgrounds associated with ESBLs and qAmpCs characterized in this study, some clonal lineages and plasmids were similar to those amplified in AbR populations worldwide reinforcing the role of particular genetic backgrounds in the dissemination of these β-lactamases in either developed and underdeveloped countries. Finally, this work provides tools for the accurate discrimination of *Citrobacter* species and clarifies divergent diversification trajectories of intrinsic *qnrB* genes.

Specific conclusions can be extracted from the accomplishment of the specific aims and are summarized as follows:

1. **Insights on acquired β-lactamases conferring resistance to extended-spectrum β-lactams in *Enterobacteriaceae* from different niches of developed and underdeveloped countries**

- **Epidemiology of qAmpCs from clinical and non-clinical settings in Portugal**

• We found a **low rate (5%) and a stable occurrence** of qAmpCs throughout a large period of time involving different *Enterobacteriaceae* species dispersed in diverse **clinical** institutions, in which the **predominance of DHA-1** and later emergence of CMY-2 contrasts with data from most European countries.

• Despite the high clonal diversity observed among the most frequent qAmpC-producing species (*Escherichia coli* and *Klebsiella pneumoniae*) from **clinical** origin, **particular clones** (*K. pneumoniae* ST11 and ST1380) and/or **plasmids** (IncR, IncHI2, IncI1) have been associated with the **persistence of specific qAmpC-types**.

• It is of relevance to highlight that the predominant **genetic backgrounds** identified among **qAmpC** producers (e.g. ST11 *K. pneumoniae*) were **different** from those associated with **ESBLs** spread in the same clinical institution, which might explain the **differential expansion** rates of qAmpCs and ESBLs in Portuguese clinical settings.

- We observed a **low occurrence** of qAmpCs (0.8%, only CMY-2-producing *E. coli*) in *Enterobacteriaceae* from different **non-clinical** niches in Portugal. qAmpC producers were identified among recent samples from **healthy humans** in similar rates than those reported in other European countries, including Spain. These results suggest that the **human intestinal commensal flora** might constitute actually an important **reservoir** of qAmpC genes and/or qAmpC-producing bacteria in Portugal.

- The detection of **CMY-2**-producing *E. coli* in an **uncooked chicken** carcass suggests that this highly **consumed food** product might have contributed to the **spread** of such enzymes.

➢ **Epidemiology of acquired β-lactamases from non-clinical niches in Angola**

- A remarkable **high prevalence** of **ESBL** producers and the absence of qAmpC and carbapenemases among *Enterobacteriaceae* isolates from diverse **non-clinical niches** (healthy humans, animals and their environments, and aquatic environments) is for the first time reported in the Benguela Province of Angola, reflecting a worrying level of **environmental contamination**, which in poor living conditions poses serious **risk of subsequent transmission**.

- The **high occurrence** and **wide dispersion of CTX-M-15** in such a high diversity of *Enterobacteriaceae* species and non-clinical settings in Angola could reflect a **recent penetration** of $bla_{\text{CTX-M-15}}$ into this geographical area and/or the **local emergence** of $bla_{\text{CTX-M-15}}$ driven by unrecognized factors.

- The variability observed in $bla_{\text{CTX-M-15}}$ genetic contexts (genetic environment, plasmid and clonal location) suggests an extraordinary ability for **acquisition** and **mobilization** of $\boldsymbol{bla_{\text{CTX-M-15}}}$ **by multiple genetic backgrounds**, which is **not comparable** to that reported in **developed countries**.

- Possible **novel plasmid backgrounds** involved in the **spread of** $\boldsymbol{bla_{\text{CTX-M-15}}}$ in **natural reservoirs** from an underdeveloped country were uncovered.

2. *Citrobacter* species differentiation and diversification trajectories of intrinsic genes conferring reduced susceptibility to fluoroquinolones

- Based on the **phylogenetic analysis** performed we suggest ***recN*** as the most **reliable genotypic marker** to accurately delineate the taxonomic position of the different species within the *Citrobacter* complex. The **reclassification** of most

***Citrobacter* spp.** deposited in available databases and the unveiling of putative **novel species** were also relevant findings from this study.

- A putative **new** *Citrobacter* **species** depicted by **phylogenetic analysis** and further characterized by **metabolic**, **biochemical** and **whole genome sequencing** approaches designated as ***Citrobacter europaea*** sp. nov. is proposed. Representative isolates of *C. europaea* were identified in water and human faecal samples from Portugal and the United States of America.

- **Phylogenetic and comparative genomics** analysis of all known ***qnrB*** genes and surrounding platforms confirmed the origin of *qnrB* and revealed diversification trajectories consistent with divergent evolution of different *qnrB* branches in different *Citrobacter* complex species.

Appendix

I. Permissions for inclusion of papers, figures and tables

Copyright Clearance Center

Confirmation Number: 11579115
Order Date: 07/25/2016

Customer Information

Customer: Teresa Ribeiro
Account Number: 3001032374
Organization: Teresa Ribeiro
Email: teresampg84@gmail.com
Phone: +351 916109494
Payment Method: Invoice

This is not an invoice

Order Details

Special Orders

Antimicrobial agents and chemotherapy

Billing Status: **Not Billed**

Order detail ID:	69954899
ISSN:	0066-4804
Publication Type:	Journal
Volume:	
Issue:	
Start page:	
Publisher:	AMERICAN SOCIETY FOR MICROBIOLOGY
Author/Editor:	AMERICAN SOCIETY FOR MICROBIOLOGY ; INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AN

Permission Status: **Special Order**
Special Order Update: **Response Required**

Permission type: Republish or display content
Type of use: Thesis/Dissertation
Job Ticket: 501163327

Requestor type	Academic institution
Format	Print, Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables/figures	1
Title or numeric reference of the portion(s)	Figure 1
Title of the article or chapter the portion is from	Plasmid-determined AmpC-type beta-lactamases.
Editor of portion(s)	N/A
Author of portion(s)	Philippon A, Arlet G, Jacoby GA
Volume of serial or monograph	46
Issue, if republishing an article from a serial	1
Page range of portion	6
Publication date of portion	2002
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Other territories and/or countries
Territory/Countries where you intend to distribute new product	Portugal
In the following language(s)	Original language of publication

With incidental promotional use	no
Lifetime unit quantity of new product	Up to 499
Made available in the following markets	education
The requesting person/organization	Teresa Gonçalves Ribeiro
Order reference number	
Author/Editor	Teresa Gonçalves Ribeiro
The standard identifier of New Work	https://repositorio-aberto.up.pt/browse?type=dateissued&sort_by=2&order=ASC&rpp=20&etal=-1&year=2016&month=-1&starts_with=
Title of New Work	EXTENDING THE KNOWLEDGE ON DRIVERS OF INTRINSIC AND ACQUIRED RESISTANCE TO CEPHALOSPORINS AND QUINOLONES IN ENTEROBACTERIACEAE
Publisher of New Work	Faculdade de Farmácia da Universidade do Porto
Expected publication date	Oct 2016
Estimated size (pages)	250
Customer Tax ID	PT505722232

Note: This item will be managed through CCC'S **RightsLink service.** More info **TBD**

Total order items: 1

Order Total: TBD
(Excludes TBD items)

About Us | Privacy Policy | Terms & Conditions | Pay an Invoice

Copyright 2016 Copyright Clearance Center

Copyright Clearance Center

Confirmation Number: 11576977
Order Date: 07/15/2016

Customer Information

Customer: Teresa Ribeiro
Account Number: 3001032374
Organization: Teresa Ribeiro
Email: teresampg84@gmail.com
Phone: +351 916109494
Payment Method: Invoice

This is not an invoice

Order Details

European journal of clinical microbiology & infectious diseases

Billing Status: **N/A**

Order detail ID:	69944129
ISSN:	1435-4373
Publication Type:	e-Journal
Volume:	
Issue:	
Start page:	
Publisher:	SPRINGER-VERLAG BERLIN/HEIDELBERG

Permission Status: **Granted**
Permission type: Republish or display content
Type of use: Thesis/Dissertation
Order License Id: 3910220589380

Requestor type	Academic institution
Format	Print, Electronic
Portion	chapter/article
Title or numeric reference of the portion(s)	Eur J Clin Microbiol Infect Dis. 2014 Apr;33(4):551-8.
Title of the article or chapter the portion is from	Long-term dissemination of acquired AmpC β-lactamases among Klebsiella spp. and Escherichia coli in Portuguese clinical settings.
Editor of portion(s)	N/A
Author of portion(s)	Freitas F, Machado E, Ribeiro TG, Novais Â, Peixe L
Volume of serial or monograph	33
Issue, if republishing an article from a serial	4
Page range of portion	
Publication date of portion	2014
Rights for	Main product and any product related to main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no

Lifetime unit quantity of new product	Up to 499
Made available in the following markets	education
The requesting person/organization	Teresa Gonçalves Ribeiro
Order reference number	
Author/Editor	Teresa Gonçalves Ribeiro
The standard identifier of New Work	https://repositorio-aberto.up.pt/browse?type=dateissued&sort_by=2&order=ASC&rpp=20&etal=-1&year=2016&month=-1&starts_with=
Title of New Work	EXTENDING THE KNOWLEDGE ON DRIVERS OF INTRINSIC AND ACQUIRED RESISTANCE TO CEPHALOSPORINS AND QUINOLONES IN ENTEROBACTERIACEAE
Publisher of New Work	Faculdade de Farmácia da Universidade do Porto
Expected publication date	Oct 2016
Estimated size (pages)	250
Customer Tax ID	PT505722232

Note: This item was invoiced separately through our **RightsLink service.** More info **$ 0.00**

Total order items: 1 **Order Total: $0.00**

About Us | Privacy Policy | Terms & Conditions | Pay an Invoice

Copyright 2016 Copyright Clearance Center

Copyright Clearance Center

Confirmation Number: 11566893
Order Date: 06/01/2016

Customer Information

Customer: Teresa Ribeiro
Account Number: 3001032374
Organization: Teresa Ribeiro
Email: teresampg84@gmail.com
Phone: +351 916109494
Payment Method: Invoice

This is not an invoice

Order Details

The journal of antimicrobial chemotherapy

Billing Status: **N/A**

Order detail ID: 69801209
ISSN: 0305-7453
Publication Type: Journal
Volume:
Issue:
Start page:
Publisher: OXFORD UNIVERSITY PRESS
Author/Editor: BRITISH SOCIETY FOR ANTIMICROBIAL CHEMOTHERAPY

Permission Status: **Granted**
Permission type: Republish or display content
Type of use: Thesis/Dissertation
Job Ticket: 501147899
Order License Id: 3890930959588

Requestor type	Author of requested content
Format	Print, Electronic
Portion	chapter/article
Number of pages in chapter/article	5
Title or numeric reference of the portion(s)	71(5):1169-73
Title of the article or chapter the portion is from	Atypical epidemiology of CTX-M-15 among Enterobacteriaceae from a high diversity of non-clinical niches in Angola.
Editor of portion(s)	N/A
Author of portion(s)	Ribeiro TG
Volume of serial or monograph	71
Issue, if republishing an article from a serial	5
Page range of portion	1169-1173
Publication date of portion	June, 2016
Rights for	Main product and any product related to main product
Duration of use	Current edition and up to 5 years
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Other territories and/or countries
Territory/Countries where you intend to distribute new product	Portugal

In the following language(s)	Original language of publication
With incidental promotional use	no
Lifetime unit quantity of new product	Up to 499
Made available in the following markets	education
Specified additional information	I would like to add permission to include supplementary material
The requesting person/organization	Ribeiro TG
Order reference number	
Author/Editor	Ribeiro TG
The standard identifier of New Work	SFRH/BD/75752/2011
Title of New Work	Atypical epidemiology of CTX-M-15 among Enterobacteriaceae from a high diversity of non-clinical niches in Angola.
Publisher of New Work	American Society for Microbiology
Expected publication date	Jun 2016
Estimated size (pages)	10
Customer Tax ID	PT505722232

Note: This item was invoiced through CCC'S **RightsLink service.** More info **$ 0.00**

Total order items: 1 **Order Total: $0.00**

About Us | Privacy Policy | Terms & Conditions | Pay an Invoice

Copyright 2016 Copyright Clearance Center

Copyright Clearance Center

Confirmation Number: 11576979
Order Date: 07/15/2016

Customer Information

Customer: Teresa Ribeiro
Account Number: 3001032374
Organization: Teresa Ribeiro
Email: teresampg84@gmail.com
Phone: +351 916109494
Payment Method: Invoice

This is not an invoice

Order Details

Antimicrobial agents and chemotherapy

Billing Status: **N/A**

Order detail ID:	69944159
ISSN:	0066-4804
Publication Type:	Journal
Volume:	
Issue:	
Start page:	
Publisher:	AMERICAN SOCIETY FOR MICROBIOLOGY
Author/Editor:	AMERICAN SOCIETY FOR MICROBIOLOGY ; INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AN

Permission Status: Granted

Permission type: Republish or display content
Type of use: Thesis/Dissertation
Job Ticket: 501160573
Order License Id: 3911910810824

Requestor type	Academic institution
Format	Print, Electronic
Portion	chapter/article
Title or numeric reference of the portion(s)	Antimicrob Agents Chemother. 2015 Oct;59(10):5951-8.
Title of the article or chapter the portion is from	Phylogeny and Comparative Genomics Unveil Independent Diversification Trajectories of qnrB and Genetic Platforms within Particular Citrobacter Species.
Editor of portion(s)	N/A
Author of portion(s)	Ribeiro TG, Novais Â, Branquinho R, Machado E, Peixe L
Volume of serial or monograph	59
Issue, if republishing an article from a serial	10
Page range of portion	
Publication date of portion	2015
Rights for	Main product and any product related to main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental	

promotional use	no
Lifetime unit quantity of new product	Up to 499
Made available in the following markets	education
The requesting person/organization	Teresa Gonçalves Ribeiro
Order reference number	
Author/Editor	Teresa Gonçalves Ribeiro
The standard identifier of New Work	https://repositorio-aberto.up.pt/browse?type=dateissued&sort_by=2&order=ASC&rpp=20&etal=-1&year=2016&month=-1&starts_with=
Title of New Work	EXTENDING THE KNOWLEDGE ON DRIVERS OF INTRINSIC AND ACQUIRED RESISTANCE TO CEPHALOSPORINS AND QUINOLONES IN ENTEROBACTERIACEAE
Publisher of New Work	Faculdade de Farmácia da Universidade do Porto
Expected publication date	Oct 2016
Estimated size (pages)	250
Customer Tax ID	PT505722232

Note: This item was invoiced through CCC'S **RightsLink service.** More info **$ 0.00**

Total order items: 1 **Order Total: $0.00**

About Us | Privacy Policy | Terms & Conditions | Pay an Invoice

Copyright 2016 Copyright Clearance Center