Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing Klebsiella pneumoniae isolate.

Claire Hennequin, Claire Aumeran, Frédéric Robin, Ousmane Traore, Christiane Forestier

J Antimicrob Chemother 2012; **67**: 2123–2130
doi:10.1093/jac/dks169 Advance Access publication 10 May 2012

Journal of Antimicrobial Chemotherapy

Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing *Klebsiella pneumoniae* isolate

Claire Hennequin[1,2]*, Claire Aumeran[1,3], Frédéric Robin[2,4–7], Ousmane Traore[1,3] and Christiane Forestier[1]

[1]*Clermont Université, UMR CNRS 6023 'Laboratoire Microorganismes: Génome Environnement (LMGE)', Université d'Auvergne, F-63000 Clermont-Ferrand, France;* [2]*CHU Clermont-Ferrand, Service de bactériologie, F-63003 Clermont-Ferrand, France;* [3]*CHU Clermont-Ferrand, Pôle REUNNHIR, Service d'Hygiène Hospitalière, F-63003 Clermont-Ferrand, France;* [4]*Centre National de la Résistance aux Antibiotiques, laboratoire associé Entérobactéries, Clermont-Ferrand, France;* [5]*Clermont Université, Université d'Auvergne, M2iSH, BP10448, F-63000 Clermont-Ferrand, France;* [6]*Inserm, UMR 1071, F-63000 Clermont-Ferrand, France;* [7]*INRA, USC2018, Clermont-Ferrand, France*

*Corresponding author. Tel: +33-47-317-7998; Fax: +33-47-317-8370; E-mail: claire.hennequin@u-clermont1.fr

Received 16 January 2012; returned 14 February 2012; revised 3 April 2012; accepted 11 April 2012

Objectives: To characterize a CTX-M-15-producing *Klebsiella pneumoniae* isolate that was identified during an outbreak involving 16 patients who had undergone endoscopic retrograde cholangiopancreatography between December 2008 and August 2009. The strain was also detected in one endoscope used for these examinations.

Methods: Disc diffusion assays, MICs and isoelectric focusing were used to characterize the plasmidic CTX-M-15 β-lactamase. PCRs were used to check for the presence of genes associated with virulence or antibiotic resistance. Antibiotic tolerance tests and plasmid transfer were carried out in both planktonic and biofilm conditions.

Results: The strain belonged to sequence type 14 and to the virulent capsular serotype K2, but produced little glucuronic acid. It contained a 62.5 kb conjugative plasmid carrying the $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$ and *aac(6′)-Ib-cr* genes and harboured few virulence genes (*uge*, *wabG*, *kfu* and *mrkD*). The strain was highly resistant to cefotaxime (MIC 516 mg/L) and the presence of this antibiotic at sub-MIC concentrations enhanced biofilm formation. The isolate was susceptible to ofloxacin (MIC 2 mg/L), but the bactericidal effect of this antibiotic was greater in planktonic cultures and 6 h old biofilm than in 24 or 48 h old biofilms. The *K. pneumoniae* strain was notable for its ability to transfer its plasmid, especially in biofilm conditions, in which the rate of plasmid transfer was about 0.5/donor.

Conclusions: These findings demonstrate the ability of this strain to survive in a hospital environment and to transfer its extended-spectrum β-lactamase-encoding plasmid.

Keywords: Enterobacteriaceae, extended-spectrum β-lactamases, ST14

Introduction

In their natural environment, bacteria not only exist as isolated cells but also grow and survive in organized communities termed biofilms, where they are kept together by a self-produced biopolymer matrix. Biofilm growth occurs on natural surfaces, such as the teeth, heart valves and lungs, but also on indwelling medical devices, such as prosthetic joints, endoscopes and intravenous catheters.[1] Medical devices are typically contaminated by organisms that comprise the natural flora surrounding the site of material insertion. Microorganisms growing in biofilms exhibit phenotypic characteristics that are distinct from those of planktonic organisms, including increased resistance to host immune defences and to antimicrobial compounds.[2] The molecular nature of this resistance has not been fully elucidated. The resistance could be due to the slowly growing state of the cells in the deeper layers of thick biofilms, which have less access to antibiotics and nutrients, and to the impaired diffusion of antimicrobial molecules within the biofilms.[3] There is increased horizontal gene transmission in biofilms, with high plasmid transfer rates,[1,4] which aggravates the problem of resistance. The close proximity of bacterial cells in biofilms creates an environment conducive to the exchange of genetic material, especially via conjugative pili.[5]

Klebsiella pneumoniae is a Gram-negative pathogen involved in hospital outbreaks of nosocomial infections, causing bloodstream, biliary tract and other infections.[6,7] *K. pneumoniae* isolates are frequently resistant to broad-spectrum cephalosporins, due to the production of extended-spectrum β-lactamases (ESBLs),[8] evidence of the role of *K. pneumoniae* as a reservoir

for plasmids carrying antibiotic resistances genes. Over the last few decades the prevalence of ESBL-producing Enterobacteriaceae has increased worldwide, mainly due to the dissemination of *Escherichia coli* and *K. pneumoniae* producing CTX-M-type ESBLs. CTX-M-15 is the most widespread CTX-M type, and the predominant type in various countries.[9] In addition, *K. pneumoniae* produces a number of virulence factors that contribute to pathogenesis, including a thick polysaccharide capsule (which is considered to be the dominant virulence property), fimbrial and non-fimbrial adhesins and siderophores.[10–13] Epidemiological studies have shown that the gastrointestinal tract is colonized before infection and therefore constitutes a reservoir for *K. pneumoniae*.[14] The infections are often linked to the use of invasive medical devices.

Between December 2008 and August 2009, 16 patients at the teaching hospital of Clermont-Ferrand, France, were identified as having an ESBL-type CTX-M-15-producing *K. pneumoniae*; there were eight bloodstream infections, four biliary tract infections and four faecal carriages. All of the patients had previously undergone endoscopic retrograde cholangiopancreatography (ERCP). The strain was also detected in the inner channels of one endoscope used for ERCP despite its being cleaned and dried after use.[15] The endoscope had probably been contaminated by one patient's intestinal tract flora and thereafter caused the strain to spread. The clonal relatedness of all the isolates was shown by PFGE.[15]

In this study, we investigated several virulence factors of this particular isolate of *K. pneumoniae*, assessed its ability to form biofilm in the presence of subinhibitory concentrations of two major antibiotics (cefotaxime and ofloxacin) and measured the conjugative rates of its CTX-M-15-encoding plasmid in biofilm versus planktonic cultures.

Materials and methods

Bacterial strains, growth conditions and determination of hypermucoviscosity phenotype

Clinical isolate *K. pneumoniae* LM21 was used as control in biofilm and bile challenge assays and in measurements of growth and adhesion to epithelial cells.[16] All bacterial strains were stored at −20°C and −80°C in lysogeny broth (LB) medium containing 20% glycerol. Strains were grown in LB or Mueller–Hinton (MH) broth or agar at 37°C for 18–24 h. When needed, media were supplemented with relevant antibiotics: ofloxacin (0.1–8 mg/L), cefotaxime (1–1024 mg/L), streptomycin (50 mg/L) and ceftazidime (2 mg/L). Bacterial growth was monitored by measuring the optical density (OD) at 620 nm, and the numbers of cfu were quantified by plating serial dilutions of the suspension on LB agar plates. To examine bacterial growth *in vitro*, bacterial strains were grown in LB, diluted 1:100 into fresh broth and shaken for 7 h. Values for the OD of each sample were recorded at regular intervals. The isolates were classified phenotypically as mucoid or non-mucoid by the colony loop lift test. Mucoid phenotype was defined as being present when a string-like growth was observed to attach to the loop as it was lifted from the plate.[17]

Characterization of the β-lactamases: susceptibility to β-lactams, isoelectric focusing and MICs

Antibiotic-containing discs (ampicillin, amoxicillin/clavulanate, ticarcillin, ticarcillin/clavulanate, piperacillin, piperacillin/tazobactam, cefalotin, cefuroxime, cefoxitin, ceftazidime, cefotaxime, ceftriaxone, aztreonam, cefepime, imipenem, ertapenem, meropenem, nalidixic acid, norfloxacin, ciprofloxacin, tobramycin, gentamicin, netilmicin, amikacin, tetracycline, co-trimoxazole, chloramphenicol, colistin and tigecycline) were used for antibiotic susceptibility testing by the disc diffusion assay (MAST Diagnostic, Amiens, France). Results of susceptibility testing were analysed according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM).[18]

Isoelectric focusing of β-lactamases was performed with polyacrylamide gels containing ampholines with a pH range of 3.5–10.0, as previously described,[19] with TEM-39 (pI 5.2), TEM-1 (pI 5.4), TEM-2 (pI 5.6), SHV-1 (pI 7.7), CTX-M-15 (pI 8.6) and CMY-4 (pI 9) as standards.

MICs of ofloxacin and cefotaxime were determined by a microdilution method and interpreted according to guidelines of CA-SFM.[18]

PCRs

PCRs were performed to check for the presence of genes (*magA*, *allS*, *rmpA*, *kfu*, *cf29a*, *fimH*, *uge*, *wabG*[20] and *mrkD*[21]) associated with virulence in *K. pneumoniae*. Genomic DNA from strains NTUH-K2044,[17] KP52145[22] and MGH 78578 were used as controls. Primers ORF 1907-1908.1 and ORF 1907-1908.2 were used to check for the presence of colibactin.[23] Primers CTX-M-1A and CTX-M-1B, and OXA-1A and OXA-1B were used to amplify and sequence $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ genes, respectively, as previously described.[24] Primers AAC6′-A and AAC6′-B[25] were used to amplify and sequence the *aac(6′)* gene. Multilocus sequence typing (MLST) was performed as described by Diancourt *et al*.[26] (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Preparation of plasmid DNA

Plasmid extractions of clinical isolates and transconjugants were performed by the method of Kado and Liu.[27] The size of the plasmid was estimated by comparing their electrophoretic mobilities with those of known reference plasmids.

Extraction, quantification and genotyping of capsular polysaccharides (cps)

Bacterial strains were grown in LB and cps were extracted with Zwittergent 3–14 detergent. The amount of glucuronic acid was then measured according to the method described by Domenico *et al*.[28] In parallel, serial dilutions of the bacterial culture were plated to determine the number of cfu, and the concentration of cps was expressed according to the amount of glucuronic acid (μg) for 10^9 cfu per mL of sample. Each experiment was performed in triplicate.

cps genotyping was performed by PCR detection of K serotype-specific alleles *wzy* and *wzx* loci as previously described to identify K1 and K2 alleles.[17] Strains NTUH-K2044 and KP52145 were used as positive controls.

Biofilm assays

We used a slightly modified version of the microtitre plate assay developed by O'Toole and Kolter.[29] Briefly, 4 μL of overnight culture was inoculated into 100 μL of M63B1 in a 96-well culture-treated polystyrene microtitre plate (Nunc) with or without antibiotics. Wells filled with growth medium alone were included as negative controls. After 3 h of incubation at 37°C, surface-adherent biofilm formation was measured by staining bound cells for 15 min with a 0.5% (w/v) aqueous solution of crystal violet. After rinsing with distilled water, the bound dye was released from the stained cells using 95% ethanol, and OD at 590 nm was determined.

Cell cultures and adhesion assays

Intestine-407 (Int-407) cells derived from human embryonic jejunum and ileum were cultivated as described elsewhere and adhesion to the different cell lines was assayed as described previously.[30] These experiments were performed in triplicate.

Biological assay for siderophore production

Lawn of the bacterial strain LG1522[31] was grown on appropriate M9 minimal agar containing α,α′ dipyridyl. Strains to be tested were inoculated onto the lawn and incubated at 37°C overnight. Aerobactin production was detected as a halo of growth of the indicator strain around an inoculum.

Bile challenge assay

For the growth assay, 5 mL of LB broth with or without the addition of 10% of crude ox bile extract (Sigma) was inoculated with cells from an overnight culture to reach 4×10^7 cfu/mL. The cultures were incubated aerobically with shaking for 5 h and the numbers of viable bacteria were determined. The results are expressed as the ratio of the number of cfu obtained from LB cultures containing 10% of bile to the number of cfu obtained from control cultures (LB alone).

Antibiotic tolerance testing

The ability of bacteria to resist antibiotics in biofilm cultures was studied with ofloxacin according to a slightly modified method of Zuroff *et al.*[32] Briefly, the colony biofilm systems consisted of MH agar plates, sterile 0.22 μm pore nitrocellulose membranes (Millipore) and the bacteria. The membrane was inoculated with 100 μL of an exponentially growing culture (diluted to $OD_{620} = 0.1$). The culture was grown for 6, 24 or 48 h and the inoculated membrane was then transferred either to a plate containing the tested antibiotic at different concentrations (ofloxacin at 0.1, 1, 2 and 4 mg/L) or to a control plate for an additional 24 h. After incubation, some of the colony biofilms were aseptically transferred to tubes pre-filled with 5 mL of sterile PBS. The colony biofilm was vortexed vigorously for 1 min to separate the cells from the membrane and homogenized for 5 min using an ultrasonic bath. The culture was then serially diluted and the numbers of cfu per membrane were determined. Each experiment was performed in triplicate. The other biofilms were coloured with Live/Dead® Fixable Dead Cell Stain kits (L-7012, Invitrogen), according to the manufacturer's instructions, and analysed by confocal microscopy. The biofilm sample was observed with an LSM510 Meta microscope (Carl Zeiss MicroImaging, Inc., le Pecq, France) with a 40XNA 1.3 Plan-Neofluar lens. All imaging was performed at room temperature. Figures were processed using Fiji Image Browser software (http://pacific.mpi-cbg.de/wiki/index.php/Main_Page).

In parallel, the ability of bacteria to resist antibiotics in planktonic cultures was studied according to the method of Zuroff *et al.*[32] with the same antibiotic at similar concentrations and the same range of bacterial inocula. Briefly, 50 mL cultures were grown exponentially for 6 h with shaking at 37°C in MH broth. The cells were collected by centrifugation and resuspended in 50 mL of fresh medium containing different concentrations of ofloxacin or in control medium for another 24 h at 37°C with shaking. The viable cell counts were determined using serial dilutions and enumerations. Each experiment was performed in triplicate. We calculated the logarithmic reductions ($\log_{10}$ cfu) between the bacterial numerations of the control culture and the bacterial numerations after the action of ofloxacin. The mean logarithmic reductions were calculated for each experiment and compared by the Mann–Whitney test. The significance level was set at 0.05. Analyses were performed with SAS (SAS Institute Inc.).

The effect of cefotaxime and ofloxacin on the formation of biofilm was studied in microtitre plates as described above using concentrations of cefotaxime or ofloxacin ranging from 1 to 64 mg/L or 0.5 to 8 mg/L, respectively.

Transfer of plasmids

In planktonic conditions, conjugation experiments were carried out as previously described.[33] Briefly, 10^8 cfu of overnight culture of donor (CTX-M-15-producing *K. pneumoniae*) and recipient (a streptomycin-resistant mutant of *E. coli* MG1555) strains were inoculated into 1000 μL of LB broth for 3 h. In biofilm conditions, conjugation experiments were carried out on LB agar and sterile 0.22 μm pore nitrocellulose filters (Millipore) with the same inoculum.[34] After 3 h of incubation, the colony biofilms were harvested and bacteria resuspended in saline by vortexing and sonication. Transconjugants and donor strains were selected and quantified on LB agar containing streptomycin (50 mg/L) and ceftazidime (2 mg/L) and on LB agar containing ceftazidime (2 mg/L), respectively. Each experiment was performed in triplicate.

Results and discussion

CTX-M-15-producing *K. pneumoniae*

Susceptibility results

The strain was resistant to all penicillins alone or in combination with β-lactamase inhibitors (clavulanate and tazobactam), to cefalotin, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, aztreonam and cefepime. It was susceptible to cefoxitin, imipenem, ertapenem and meropenem. The double disc diffusion test confirmed the production of an ESBL.[15] The strain was also intermediate or resistant to nalidixic acid and norfloxacin, all aminoglycosides except gentamicin, and to tetracycline and cotrimoxazole. It was susceptible to ciprofloxacin, chloramphenicol, colistin and tigecycline. Isoelectric focusing showed that the strain produced two β-lactamases of pI 7.5 and 8.6. PCR and sequencing experiments identified these two enzymes as the penicillinases OXA-1 and CTX-M-15, respectively. A gene encoding AAC(6′)-Ib-cr was also detected in the strain's genome. Plasmid content analysis revealed the presence of one plasmid, with an approximate size of 61.5 kb (data not shown).

Virulence and strain typing

This CTX-M-15-producing strain was further characterized in order to determine its background specificities, including the presence of virulence-associated genes and its genotype. It harboured the genes *wabG* and *uge*, which encode proteins that play a role in colonization and virulence through their involvement in lipopolysaccharide synthesis.[35] Gene *mrkD* was also detected, which encodes type 3 fimbrial adhesin,[36] known to play a role in biofilm formation. Gene *fimH*, which encodes type 1 fimbrial adhesin, and gene *kfu*, which encodes a *Klebsiella* ferric iron uptake system that leads to higher virulence in a mouse model, were also present in the genome of this isolate.[22] These virulence genes are widespread among *K. pneumoniae* strains.[20] Specific *cps* PCRs indicated that the strain belonged to serotype K2, which is associated with virulence. MLST resulted in sequence type 14, which has already been linked to CTX-M-15-producing *K. pneumoniae*,[37,38] and seems to be well adapted to survival in the hospital environment and

able to gather resistance genes. However, extraction and quantification of cps showed that the isolate was a low glucuronic acid producer (<5 μg/0.5 mL of overnight culture) and no hypermucoviscosity phenotype was observed. No other virulence gene markers were detected. Under iron-deprived conditions of growth, no cross-feeding was observed with strain LG1522,[31] demonstrating the absence of expression of the aerobactin iron uptake system. We then compared the isolate with the laboratory non-ESBL-producing *K. pneumoniae* LM21. Their rates of growth in LB broth were similar, suggesting the absence of fitness cost due to the ESBL. Using a microtitre plate experimental model, we showed that both strains formed biofilm with a similar biomass (data not shown). No significant difference was observed between the two strains in the bile resistance assay. However, the CTX-M-15-producing *K. pneumoniae* strain adhered 1.8-fold (±0.09) more to Int-407 cells than strain LM21 ($P = 0.001$).

These results are consistent with the clinical observations of Aumeran *et al*.,[15] who found the clinical isolate of *K. pneumoniae* in 16 patients contaminated by the endoscopes. It is therefore likely that the strain's ability to form biofilm contributed to contamination of the endoscopes and that its ability to adhere to epithelial cells favoured colonization of the patients' gastrointestinal tract. However, although belonging to the virulent serotype K2, the strain was not considered clinically virulent since no death was reported and some patients did not need to be treated despite having bacteraemia. Indeed, the link between antibiotic resistance and virulence is complex. In most cases, CTX-M production has been observed in *E. coli* with few virulence factors or causing minor infections,[39–41] but in some reports the strains have been associated with high virulence expression.[42,43] Although the strain isolated in our study was highly resistant to antibiotics and exhibited colonization properties, its low virulence level probably prevented an outbreak of more severe infections.

Properties of the CTX-M-15-producing K. pneumoniae strain in biofilm versus planktonic cultures

The primary source of the epidemic strain was most likely the gut of the patients, with cross-contamination to patients who subsequently underwent endoscopy.[15] The strain's ability to initially form biofilm on such devices and the detachment of cell clumps from biofilm aggregates during the clinical investigation was probably responsible for contamination. Since most patients were receiving antibiotics, we assessed, in addition to the determination of MICs with planktonic bacteria, the behaviour of biofilm forms of this isolate in the presence of antibiotics.

Antibiotic killing rate

The bactericidal effects of cefotaxime and ofloxacin, two antibiotics frequently used in the treatment of infectious diseases, were determined on sessile bacteria using 6, 24 and 48 h old biofilms. The percentage of surviving cells showed that ofloxacin at concentrations of 1, 2 and 4 mg/L (MIC/2, MIC and 2× MIC, respectively) was far less effective against 24 and 48 h old biofilms than 6 h old biofilm or planktonic cultures (Figure 1). Confocal scanning microscopy observation of 24 h old biofilms indicated that a majority of bacteria in contact with a sub-MIC antibiotic (1 mg/L, MIC/2) were dead (red) and mostly at the bottom of the biofilm, i.e. at the closest point between bacteria and the antibiotic contained in the agar medium (Figure 2a). In contrast, without ofloxacin almost all the bacteria were alive (green) (Figure 2b).

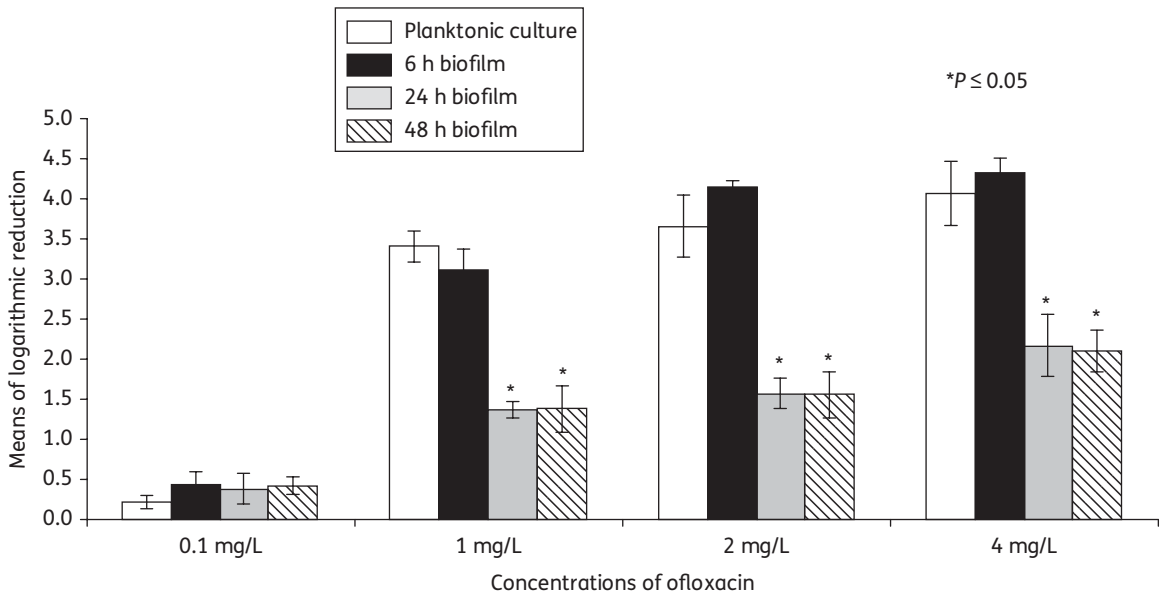

Figure 1. Bacterial logarithmic reductions of biomass (cfu) after action of ofloxacin (0.1, 1, 2 or 4 mg/L; MIC of ofloxacin for this strain, 2 mg/L) on planktonic cells and on 6, 24 or 48 h old biofilms. *$P \leq 0.05$: comparison by a Mann–Whitney test of mean logarithmic reductions between planktonic and 24 or 48 h biofilm cultures.

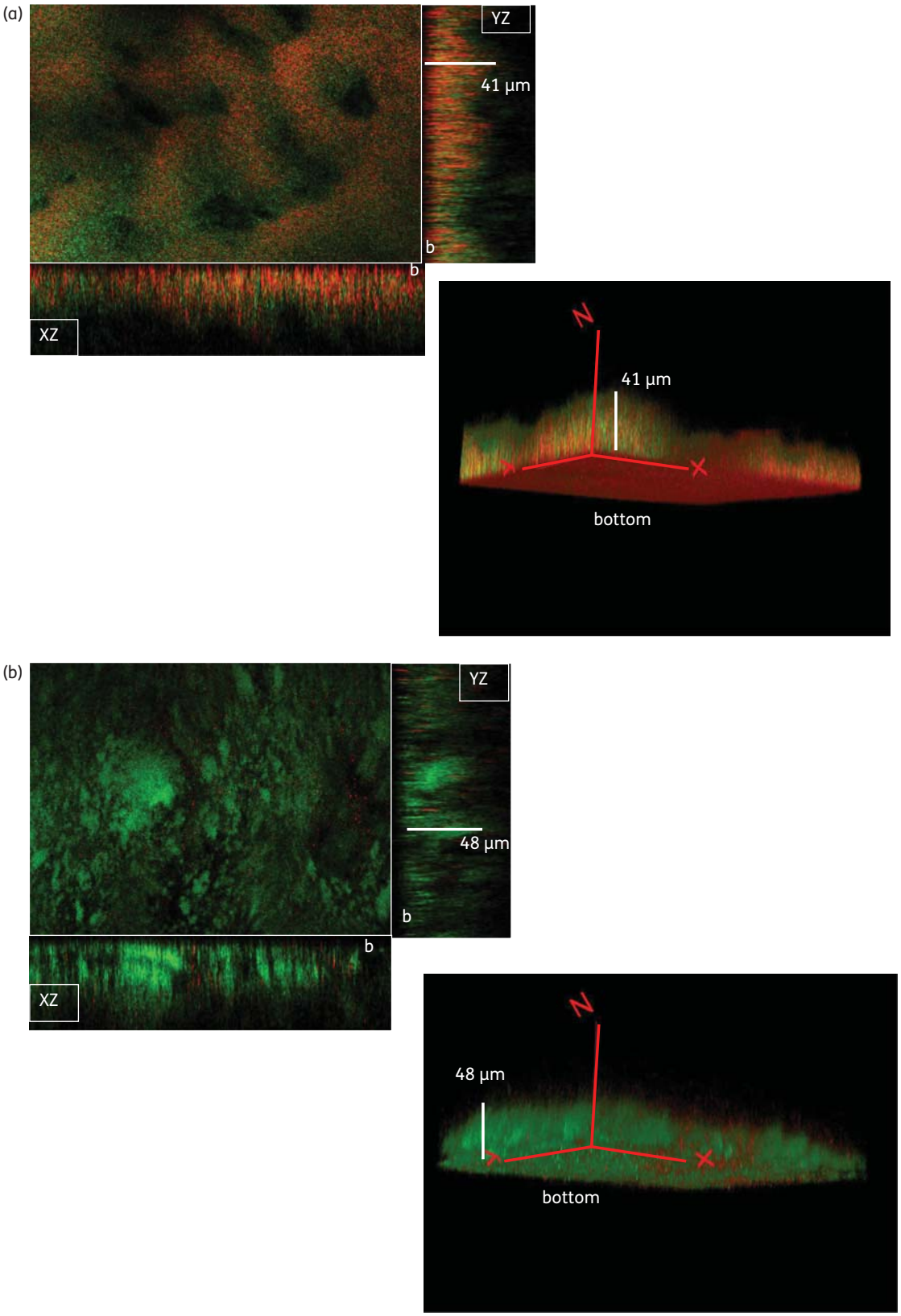

Figure 2. Confocal laser scanning microscopy images: Live/Dead® coloration of 24 h old biofilm formed in the presence (a) or in the absence (b) of ofloxacin at a concentration of 1 mg/L (sub-MIC) with the Live/Dead® kit. The bottom ('b') portion is in contact with the nitrocellulose membrane on the MH agar.

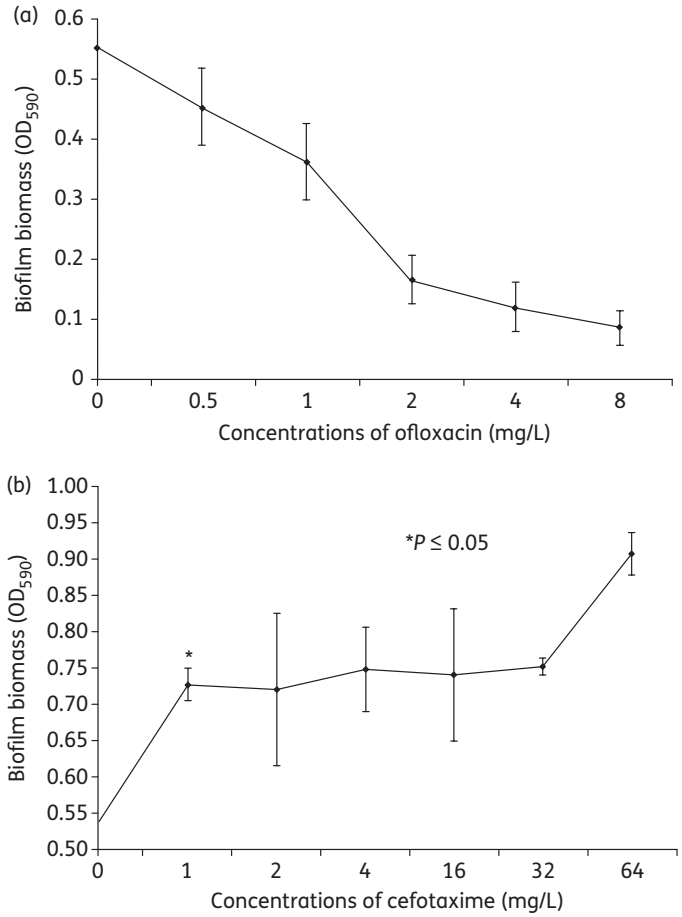

Figure 3. Quantification of the biofilm biomass by crystal violet staining (OD_{590}) in the presence of increased concentrations of ofloxacin (a) and cefotaxime (b) in microtitre plates after 3 h of incubation. *Significant differences ($P \leq 0.05$).

Sessile bacteria have increased levels of resistance to antibiotics, with the MICs of antimicrobial agents 100- to 1000-fold higher than for bacteria in planktonic form.[11] This increased resistance occurs mainly in mature biofilms and is attributed to the formation of antibiotic-resistant or -tolerant subpopulations in the deeper layers of mature biofilms in combination with impaired molecule diffusion.[3] Mature biofilm of CTX-M-15-producing *K. pneumoniae* was established after 24 h in this experimental setting, and no significant difference in antibiotic resistance was observed between 24 and 48 h old biofilms, suggesting that the impaired killing mechanism occurred after 24 h of biofilm maturation.

We then studied the effect of cefotaxime and ofloxacin on the formation of biofilm. In the presence of sub-MICs of ofloxacin, the biomass decreased and was inversely related to the antibiotic concentrations (Figure 3a). In contrast, when the cefotaxime concentration of the antibiotic increased, so did the biofilm biomass (Figure 3b). Since all concentrations tested were at least 4-fold lower than the MIC, we can infer that subinhibitory concentrations of this antibiotic enhance biofilm formation. The induction of biofilm formation by β-lactams, such as imipenem and ceftazidime, has already been observed with *Pseudomonas aeruginosa* in association with an increase in alginate production.[44] Balasubramanian *et al*.[45] have shown the existence of a co-regulatory network between β-lactam resistance, alginate production, quorum-sensing and virulence factor production, with AmpR playing a central role. *ampR* is not present in the genome of *K. pneumoniae* strains, but a previous study from our laboratory showed that type 2 quorum sensing plays a role in biofilm formation[46] and could therefore be involved in this process through another regulator.

Transfer of plasmids

K. pneumoniae is known to contain many plasmid-mediated resistance determinants and to favour the spread of these antibiotic resistances in the hospital environment and hence we decided to study the plasmid transfer ability of this particular isolate in planktonic and biofilm conditions.

In planktonic cultures, the transfer rate of the plasmid harbouring the CTX-M-15 gene was about 10^{-3}/donor. Sandegren *et al*.[47] were able to transfer the CTX-M-15-producing plasmid pUUH239.2 via conjugation to laboratory strains of *E. coli*, but with lower frequencies (5×10^{-6}–3×10^{-8}/donor). In our study, plasmid transfer occurred at a higher frequency in biofilm cultures, reaching the value of 0.5/donor. Gene transfer by plasmid conjugation and transformation has been found to occur frequently and effectively in many bacterial biofilms, both in natural environments and in more artificial settings.[4] One explanation could be that the conjugative pili act as cell adhesins, which connect the cells and stabilize biofilm structure. It is noteworthy that the presence of the plasmid in transconjugant *E. coli* did not increase its ability to form biofilm (data not shown), suggesting that the plasmid does not carry genes involved in biofilm formation.

Conclusions

In conclusion, our study provides new information about the *K. pneumoniae* strains involved in clinical infections, in particular their ability and that of their conjugative plasmids to spread. It is likely that the CTX-M-15 plasmid ended up in a *K. pneumoniae* strain that is particularly able to disseminate, but relatively non-virulent. The antibiotic resistance genes harboured by this plasmid may have given the strain a selective advantage for successful survival in a hospital environment.

Funding

The study was carried out as part of our routine work.

Transparency declarations

None to declare.

References

1 Hoiby N, Bjarnsholt T, Givskov M *et al*. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 2010; **35**: 322–32.

2 Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* 2002; **292**: 107–13.

3 Ito A, Taniuchi A, May T *et al*. Increased antibiotic resistance of *Escherichia coli* in mature biofilms. *Appl Environ Microbiol* 2009; **75**: 4093–100.

4 Molin S, Tolker-Nielsen T. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotechnol* 2003; **14**: 255–61.

5 Ong CL, Beatson SA, McEwan AG *et al*. Conjugative plasmid transfer and adhesion dynamics in an *Escherichia coli* biofilm. *Appl Environ Microbiol* 2009; **75**: 6783–91.

6 Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 1998; **11**: 589–603.

7 Sahly H, Podschun R. Clinical, bacteriological, and serological aspects of *Klebsiella* infections and their spondylarthropathic sequelae. *Clin Diagn Lab Immunol* 1997; **4**: 393–9.

8 Sirot D, Chanal C, Labia R *et al*. Comparative study of five plasmid-mediated ceftazidimases isolated in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 1989; **24**: 509–21.

9 Carrer A, Nordmann P. [CTX-M-15-producing *Klebsiella pneumoniae*: a change in the epidemiology of ESBL]. *Pathol Biol (Paris)* 2011; **59**: e133–5.

10 Chou HC, Lee CZ, Ma LC *et al*. Isolation of a chromosomal region of *Klebsiella pneumoniae* associated with allantoin metabolism and liver infection. *Infect Immun* 2004; **72**: 3783–92.

11 Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001; **9**: 34–9.

12 Regue M, Climent N, Abitiu N *et al*. Genetic characterization of the *Klebsiella pneumoniae waa* gene cluster, involved in core lipopolysaccharide biosynthesis. *J Bacteriol* 2001; **183**: 3564–73.

13 Williams P, Ciurana B, Camprubi S *et al*. Influence of lipopolysaccharide chemotype on the interaction between *Klebsiella pneumoniae* and human polymorphonuclear leucocytes. *FEMS Microbiol Lett* 1990; **57**: 305–9.

14 Markowitz SM, Veazey JM Jr, Macrina FL *et al*. Sequential outbreaks of infection due to *Klebsiella pneumoniae* in a neonatal intensive care unit: implication of a conjugative R plasmid. *J Infect Dis* 1980; **142**: 106–12.

15 Aumeran C, Poincloux L, Souweine B *et al*. Multidrug-resistant *Klebsiella pneumoniae* outbreak after endoscopic retrograde cholangiopancreatography. *Endoscopy* 2010; **42**: 895–9.

16 Favre-Bonte S, Darfeuille-Michaud A, Forestier C. Aggregative adherence of *Klebsiella pneumoniae* to human intestine-407 cells. *Infect Immun* 1995; **63**: 1318–28.

17 Fang CT, Lai SY, Yi WC *et al*. *Klebsiella pneumoniae* genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. *Clin Infect Dis* 2007; **45**: 284–93.

18 Comité de l'Antibiogramme de la Société Française de Microbiologie. *Recommandations 2011*. http://www.sfm-microbiologie.org/UserFiles/file/CASFM/casfm_2011.pdf (1 December 2011, date last accessed).

19 Bonnet R, Sampaio JL, Labia R *et al*. A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae isolated in Brazil. *Antimicrob Agents Chemother* 2000; **44**: 1936–42.

20 Brisse S, Fevre C, Passet V *et al*. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS One* 2009; **4**: e4982.

21 Hennequin C, Forestier C. *oxyR*, a LysR-type regulator involved in *Klebsiella pneumoniae* mucosal and abiotic colonization. *Infect Immun* 2009; **77**: 5449–57.

22 Nassif X, Fournier JM, Arondel J *et al*. Mucoid phenotype of *Klebsiella pneumoniae* is a plasmid-encoded virulence factor. *Infect Immun* 1989; **57**: 546–52.

23 Putze J, Hennequin C, Nougayrede JP *et al*. Genetic structure and distribution of the colibactin genomic island among members of the family Enterobacteriaceae. *Infect Immun* 2009; **77**: 4696–703.

24 Dutour C, Bonnet R, Marchandin H *et al*. CTX-M-1, CTX-M-3, and CTX-M-14 β-lactamases from Enterobacteriaceae isolated in France. *Antimicrob Agents Chemother* 2002; **46**: 534–7.

25 Park CH, Robicsek A, Jacoby GA *et al*. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* 2006; **50**: 3953–5.

26 Diancourt L, Passet V, Verhoef J *et al*. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 2005; **43**: 4178–82.

27 Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* 1981; **145**: 1365–73.

28 Domenico P, Schwartz S, Cunha BA. Reduction of capsular polysaccharide production in *Klebsiella pneumoniae* by sodium salicylate. *Infect Immun* 1989; **57**: 3778–82.

29 O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 1998; **30**: 295–304.

30 Favre-Bonte S, Joly B, Forestier C. Consequences of reduction of *Klebsiella pneumoniae* capsule expression on interactions of this bacterium with epithelial cells. *Infect Immun* 1999; **67**: 554–61.

31 Carbonetti NH, Williams PH. A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. *Infect Immun* 1984; **46**: 7–12.

32 Zuroff TR, Bernstein H, Lloyd-Randolfi J *et al*. Robustness analysis of culturing perturbations on *Escherichia coli* colony biofilm β-lactam and aminoglycoside antibiotic tolerance. *BMC Microbiol* 2010; **10**: 185.

33 Lesage DD, Gerbaud GR, Chabbert YA. [Genetic map and structure in "*Escherichia coli*" K12 of a resistance plasmid isolated from "*Salmonella ordonez*" (author's transl)]. *Ann Microbiol (Paris)* 1975; **126A**: 435–48.

34 Roux A, Beloin C, Ghigo JM. Combined inactivation and expression strategy to study gene function under physiological conditions: application to identification of new *Escherichia coli* adhesins. *J Bacteriol* 2005; **187**: 1001–13.

35 Izquierdo L, Coderch N, Pique N *et al*. The *Klebsiella pneumoniae wabG* gene: role in biosynthesis of the core lipopolysaccharide and virulence. *J Bacteriol* 2003; **185**: 7213–21.

36 Jagnow J, Clegg S. *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiology* 2003; **149**: 2397–405.

37 Oteo J, Cuevas O, Lopez-Rodriguez I *et al*. Emergence of CTX-M-15-producing *Klebsiella pneumoniae* of multilocus sequence types 1, 11, 14, 17, 20, 35 and 36 as pathogens and colonizers in newborns and adults. *J Antimicrob Chemother* 2009; **64**: 524–8.

38 Poirel L, Al Maskari Z, Al Rashdi F *et al*. NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. *J Antimicrob Chemother* 2011; **66**: 304–6.

39 Branger C, Zamfir O, Geoffroy S *et al*. Genetic background of *Escherichia coli* and extended-spectrum β-lactamase type. *Emerg Infect Dis* 2005; **11**: 54–61.

40 Lavigne JP, Blanc-Potard AB, Bourg G *et al*. Virulence genotype and nematode-killing properties of extra-intestinal *Escherichia coli* producing CTX-M β-lactamases. *Clin Microbiol Infect* 2006; **12**: 1199–206.

41 Pitout JD, Laupland KB. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis* 2008; **8**: 159–66.

42 Clermont O, Lavollay M, Vimont S *et al*. The CTX-M-15-producing *Escherichia coli* diffusing clone belongs to a highly virulent B2 phylogenetic subgroup. *J Antimicrob Chemother* 2008; **61**: 1024–8.

43 Dubois D, Prasadarao NV, Mittal R *et al*. CTX-M β-lactamase production and virulence of *Escherichia coli* K1. *Emerg Infect Dis* 2009; **15**: 1988–90.

44 Bagge N, Schuster M, Hentzer M *et al*. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and β-lactamase and alginate production. *Antimicrob Agents Chemother* 2004; **48**: 1175–87.

45 Balasubramanian D, Kong KF, Jayawardena SR *et al*. Co-regulation of β-lactam resistance, alginate production and quorum sensing in *Pseudomonas aeruginosa*. *J Med Microbiol* 2010; **60**: 147–56.

46 Balestrino D, Haagensen JA, Rich C *et al*. Characterization of type 2 quorum sensing in *Klebsiella pneumoniae* and relationship with biofilm formation. *J Bacteriol* 2005; **187**: 2870–80.

47 Sandegren L, Linkevicius M, Lytsy B *et al*. Transfer of an *Escherichia coli* ST131 multiresistance cassette has created a *Klebsiella pneumoniae*-specific plasmid associated with a major nosocomial outbreak. *J Antimicrob Chemother* 2012; **67**: 74–83.