

The epidemiology and molecular evolution of the CTX-M beta-lactamases

by

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## **Abstract**

The widespread usage of extended spectrum cephalosporins in the 1980s led to the emergence of  $\beta$ -lactamases capable of destroying them. Extended spectrum  $\beta$ -lactamases (ESBL) are the most common  $\beta$ -lactamases in some parts of the world and represent a pandemic of CTX-M ESBLs. Work examining the epidemiology and distribution of specific genotypes both in the UK and across the world particularly in China and India is presented. The faecal carriage by humans and dispersal into the environment is described. The environment and release of CTX-M producing *Enterobacteriaceae* into the environment following sewage treatment is reported and that arising from fish and poultry production. The work describes the isolation and characterization of the second most important CTX-M genotype (CTX-M-14) and the development of novel methods for the characterisation of genotypes. The publications were important in the recognition of the significance of CTX-M and drew attention to the characteristic genotype distribution around the world. The importance of faecal carriage as a dispersion mechanism and the association of specific genotypes with ethnic groups was a novel finding. The work also was the first to genotype ESBLs in India identifying that CTXM-15 was the only CTX-M genotype carried by one of the world's largest populations.

## **Dedication**

To all my friends, colleagues and students in microbiology, past present and future.

Thanks to my wife Sue for all her support and love during the preparation of this thesis

## **Acknowledgements**

I acknowledge the help and inspiration of many colleagues over the years. My contribution to the papers presented in this thesis are listed below.

### **Statement of Authorship**

#### **Publication number**

1. Design of the surveillance project, recognition of the strain, data interpretation and publication writing.
2. Project idea, funding application, design, data analysis and paper writing with collaborators.
3. Project idea, design, data analysis and paper writing with collaborators.
4. Suggestion to look for CTX-M, characterisation of isolates by phenotype/PCR methods, co-wrote MSS.
5. Identified project, phenotypic characterisation of isolates, design and supervision of all molecular work. DNA sequence analysis and 1<sup>st</sup> draft MSS.
6. Partial project idea, data analysis and contribution to writing report.
7. Collection of isolates, phenotypic characterisation, data analysis and contribution to writing report.
8. Project idea, method refinement, data analysis and contribution to writing report.
9. Project idea, some experimental work, co-wrote MSS with Miss Ensor.
10. Project idea, and design, data analysis and co-wrote report.
11. Partial project idea with involvement of wider group of collaborators, data analysis and contributed to report writing.
12. Characterised strains and helped interpret data, co-wrote MSS.
13. Sole author
14. Sole author
15. Author of manuscript, A.M. Jones provided genotype data and prepared the maps.
16. Study planner, data analysis and contributed to writing report.

17. Project idea and detail design, some experimental work, 1<sup>st</sup> draft MSS and corrections.
18. Project lead (idea and funding application), data analysis and co-wrote report.
19. Project idea, data analysis, co-wrote MSS
20. Co-author with Professor Livermore.
21. Part of project design, genotyping of CTX-M with Dr Xu, contributed to data analysis and writing.
22. Project idea, some DNA sequencing and analysis, co-wrote report.
23. Project idea/design, some experimental work genotyping, co-wrote and analysed data for report.
24. Project idea, helped with data summarisation, contribution to report.
25. Involved in project design, some work on genotyping, data analysis and contributed to report writing.
26. Data analysis, provision of previously typed strains, contributed to report writing.
27. Project idea, sample collection and processing, data analysis co-wrote report with Dr Warren.
28. Wrote section on global spread; CTX-M, part of mobile resistome and sewage/manure sections.
29. Project idea, some sample processing and characterisation, data analysis co-wrote report.
30. Partial project idea and development, data analysis and writing.
31. Project idea, helped with technical development, design of evaluation and co-wrote report.
32. Project idea, helped with design of evaluation and co-wrote report.
33. Project idea, guidance and design, data analysis and report writing.

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## 1.0 Background

Extended Spectrum Beta Lactamases (ESBLs) in human medicine first came to prominence following the introduction of the extended-spectrum cephalosporins (cefotaxime, ceftazidime, ceftazidime, ceftriaxone, etc.) in the 1980's. The term was applied to mutants of the already common plasmid mediated  $\beta$ -lactamases such as SHV and TEM. One of the first detected, which originated in France, was TEM-3 ESBL which had been presumed to have been selected in the human host following the introduction and use of cefotaxime. Both of the 'parent' genotypes of these  $\beta$ -lactamase genes *bla*<sub>TEM-1</sub> *bla*<sub>TEM-2</sub> were widely disseminated amongst medically important members of *Enterobacteriaceae* and *Acinetobacter* spp. and it seemed logical that the extended-spectrum form of the  $\beta$ -lactamase would become much more widely disseminated throughout the world under the selective pressure of the use of third generation cephalosporins. This happened to a degree in that particularly some variants of TEM, such as TEM-10, TEM-24 and SHV-12, became widely distributed, particularly amongst *Klebsiella* spp. in hospital settings, but never reached high levels of prevalence (usually <20%). There also was never a significant spread of these genes into the more general environmental and commensal pool of *Enterobacteriaceae*, even in countries where ESBL phenotype rates were reported to be very high, such as in China and India. The initially named MEN-1 ESBL from an *E.coli* isolate characterised in France from a patient in Italy (1990) was found to be identical to CTX-M-1 from an *E.coli* isolated from a child in Germany (1989) (Bauernfeind et al. 1996) The same paper also reported 2 further isolates of CTX-M-1 producing *E.coli* from Germany in 1994, but they were regarded as examples of unusual  $\beta$ -lactamases because of their rarity in Europe. In the 1990s in South



America the emergence and recognition of CTX-M-2 was seen as much more serious as the enzyme was found in *Salmonella typhimurium*, *E.coli*, *Klebsiella* spp. and *Proteus mirabilis* in Argentina and Paraguay (Radice et al. 2002). We now, realise this was a turning point in the evolutionary history of  $\beta$ -lactamase in *Enterobacteriaceae*. In this particular case the gene emerged in *Enterobacteriaceae* as a result of horizontal gene transfer from an environmental bacterium in the rhizosphere. The chromosomal homologs of the CTX-M genes in different species of *Kluyvera* have been mobilised into *Klebsiella* spp and *E.coli* on different occasions and global locations, which gave rise to 6 sublineages often referred to as “groups” (CTX-M-1,2,8,9,25 and KLUC named after the archetypal enzymes of each group), as show in Figure 1. The indisputable proof of this evolutionary route came from work in Paris by Poirel and Naas that showed that the neighbouring sequences of *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-5</sub> and Toho-1 were identical to those in *Klyvera* (Humeniuk et al. 2002). Subsequently work in China revealed that SHV and TEM type ESBLs were not the most common but another CTX-M variant, CTX-M-14, with smaller numbers of CTX-M-3 (Chanawong et al.,2002 **5**). Simultaneously with the description of CTX-M-14; CTX-M-15 was described in Paris from six of isolates from New Delhi in India. These two particular genotypes have assumed worldwide dominance with reports from studies in India and China showing these to be the dominant CTX-M, and in the case of India CTX-M-15 being the only genotype present in the entire country (Ensor et al., 2006 **9**) Hawkey 2008 **13**). We now have a position in the world where CTX-M is the most dominant ESBL and indeed in some locations is the most dominant  $\beta$ -lactamase in Gram-negative bacteria. The situation has been likened to a pandemic and it is not apparent at what rate in human isolates ESBL carriage will finally stabilize. CTX-M ESBL genes are now widely dispersed not only in human gut flora but also that of food

animals which has implications for both the colonisation of people and spread via food across the globe. The wider environment, particularly rivers, have been exposed to CTX-M ESBL producing *E.coli* and *Klebsiella* spp via human sewage and animal manure creating a further reservoir and route for colonisation/infection of humans. The phylogenetic groups of *E.coli* carrying CTX-M genes are varied; however the B2 extra-intestinal pathogenic strains carry CTX-M genes more frequently. This is particularly true of the ST131 lineage which typically carries CTX-M-15 which is the most common genotype of CTX-M in the world. The expansion of CTX-M ESBL has driven the use of antibiotics that retain activity against them which generally means carbapenems (e.g. meropenem, ertapenem etc.) This selection pressure has given rise to the emergence of a whole family of diverse carbapenemases which represent a new major threat to modern medicine.

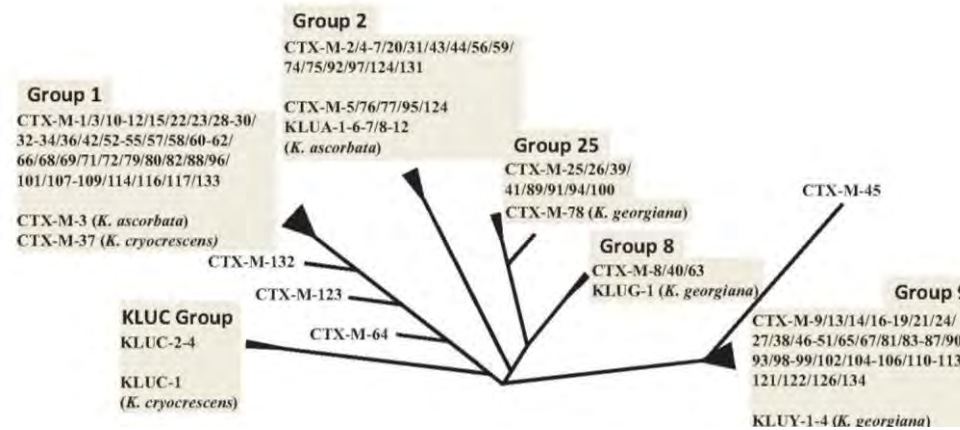


Figure 1. Tree diagram showing the similarity among enzymes of the CTX-M lineage and clustering of members of different CTX-M groups. The tree was constructed with the TREEVIEW program on the basis of amino acid sequence alignment of available sequences of CTX-M and cognate proteins from *Kluyvera* spp. Available at the Lahey Clinic Website (<http://www.lahey.org/Studies/>). Figure after D'Andrea et al (2013) IJMM, 303:305-317.

## 2.0 Contextual significance of the publications presented in this thesis

My research on the CTX-M genetic family of extended spectrum  $\beta$ -lactamases is integrated by addressing a number of specific areas which are interrelated. These are used as sub-headings to group my publications and explain their significance in relation to other publications and the state of knowledge at the time that I wrote the papers.

My papers that I present for this thesis are referred to in the text and on the reprints at the back of the thesis by number (1-33). That number correlates with the list of publications (Section 3.0), the text following in 3.1. The additional references by other workers cited in this review are listed in the reference section 4.0.

### 2.1 Molecular evolution, genetic mobility and expression of CTX-M $\beta$ -lactamases

My involvement with the CTX-M  $\beta$ -lactamases dates from my first visit to China in 1998 to run a course on the detection of antibiotic resistant bacteria for the WHO Emerging Pathogens Initiative. I went to Guangzhou in Southern China, in 1998. The course was based at the First Municipal Peoples Hospital of Guangzhou. Dr Jian Hui Xiong had carried out a survey of resistance of gram negative bacilli to various antibiotics using NCCLS methodology and approved media. The ESBL production rate was 33% for *E.coli* and 37% for *Klebsiella pneumoniae*. I appreciated that this was an exceptional finding and these isolates needed molecular characterisation for which facilities were not available in China at that time. I therefore arranged for the isolates to be transported to my laboratory and examined in detail. There had only been a single confirmed report of SHV-2 in *Klebsiella spp.* and *Enterobacter spp.* in 1994 (Cheng 1994) A total of 15 isolates of *Enterobacteriaceae* including 8 *E.coli* and 3 *Klebsiella pneumonia* were fully

characterised. SHV ESBL genes (SHV-11 & SHV-12) were found in 8 isolates but none of the other commonly recognised, at that time, ESBL genes were found using PCR. I thought this may be a rare ESBL gene so in view of the strong cefotaximase activity designed a number of consensus PCR primers for various rarer ESBL genes including CTX-M. I was delighted to get product from the PCR primers for CTX-M for 13 of the 15 isolates. (Chanawong et.al 2002 5).

Subsequent transfer of plasmids and full sequencing with cloning revealed 3 novel CTX-M genes for which we were allocated the genotype numbers 13,14 and 15. Whilst the paper was in proof I was contacted by a French microbiologist suggesting that our CTX-M-15 was identical to CTX-M-9 reported by Sabate and colleagues in Spain. We had carefully checked our sequence and it was one base different to that in in Genbank for CTX-M-9. However, when we rechecked it in Genbank it appeared that the entry had subsequently been re-edited to the same sequence that we had, hence we amended our paper to refer to CTX-M-9 rather than CTX-M-15. Ironically the number went back into the pool and was allocated to a *bla*<sub>CTX-M</sub> gene found in some Indian isolates characterised in Paris which is now recognised as the most common *bla*<sub>CTX-M</sub> worldwide.

Whilst we were characterising these isolates a paper appeared from the Ron Jones group looking at ESBL *Klebsiella* spp. from Beijing but only using isoelectric focusing on the  $\beta$ -lactamases (Shen et al. 2001). They found 4/14 isolates to produce a  $\beta$ -lactamase with a PI of 8.2 which is remarkably close to the value of 8.1 which we found for CTX-M-14 and CTX-M-13. I am sure these isolates reported by Shen carried CTX-M-14 (we only found CTX-M-13 in a single isolate). Our identification of CTX-M-14 was the first description of what subsequently became the world's second most common CTX-M ESBL. Most

importantly we identified *ISEcpI* as the likely mobilising element for *bla*<sub>CTX-M-14</sub> as I knew it had been reported by P.D. Stapleton in an ICAAC abstract in 1999 as mobilising the chromosomal AmpC to become CMY-4 plasmid mediated AmpC β-lactamase. Our Southern blots clearly showed the presence of the *bla*<sub>CTX-M-14</sub> gene on both the chromosome and plasmids. We also demonstrated the migration (in some strains) to the chromosome of recipient strains following conjugation. This was the first description of the mobilisation of *bla*<sub>CTX-M</sub> between replicons. It was the first study revealing the extent of the problem of ESBLs in China and identifying CTX-M-14 as the most important enzyme. I wrote a concluding sentence in the paper which turned out to be prophetic, “The ease with which such bacteria can be isolated should be a cause for grave concern and indicates the need for more detailed surveillance and epidemiological surveys in this region, which has increasing contact with the rest of the world.”

On moving to Birmingham in 2002 I was approached by Dr. Nigel Brenwald of City Hospital to characterise a collection of *Klebsiella pneumoniae* that had been causing what transpired to be the first nosocomial outbreak of CTX-M producing *Enterobacteriaceae* in the UK (Brenwald et al 2003 4). The Open Reading Frame of the *bla*<sub>CTX-M-8</sub> gene had 99% of homology with *bla*<sub>CTX-8</sub> genotype group. I initiated a detailed study of our gene which was designated *bla*<sub>CTX-M-26</sub> and was sent an example of CTX-M-25 from Canada by Dr. Mike Mulvey to characterise at the same time (Munday et al. 2004a 22). We identified *ISEcpI* as the mobilising element in *bla*<sub>CTX-M-26</sub> which lacked the ceftazidimase activity of CTX-M-25 and I identified the amino acid substitution probably responsible for this substrate profile change.

There has been considerable debate on the susceptibility of strains of *Enterobacteriaceae* producing difference genotypes of CTX-M  $\beta$ -lactamase to  $\beta$ -lactamase inhibitors (BL/BLI). The level of expression of the CTX-M  $\beta$ -lactamase would be expected to cause variation in susceptibility to BL/BLIs. Prof. Nancy Hanson made an observation that *E.coli* from urine samples had elevated levels of mRNA if they produced CTX-M-15 compared to CTX-M-14 production. She asked me to collaborate to study my global collection of CTX-M-15 and 14 producers and look at protein levels in both genotypes. Surprisingly we saw a good correlation between mRNA transcripts and protein production for CTX-M-14 but not in the case of CTX-M-15 (Geyer et al 2016 JAC 71:607-16 **12**). Our observation suggests that reduced susceptibility to Beta Lactam/Beta Lactamase Inhibitor combinations is due to an as yet unrecognised mechanism(s) for CTX-M-15. This finding may well be important in the future as we will rely on new BL/BLI combinations to treat MRGNB such as infections as ceftazidime/avibactam is not only active against carbapenemases but also CTX-M ESBL producers.

## **2.2 Detection and genotyping of CTX-M ESBLs**

During the course of strain typing and genotyping by PCR based sequencing of the first UK outbreak of CTX-M ESBL producing *Enterobacteriaceae* susceptibility studies I suggested that cefpodoxime would be the best  $\beta$ -lactam substrate to detect genotypes with both cefotaximase (CTX-M-14 9 etc.) and those with additional ceftazidimase activity (Brenwald et al.2003 **4**). This was the first report of the use of cefpodoxime to detect CTX-M  $\beta$ -lactamases and became widely used in the UK, leading to one of the best ESBL surveillance situations in any European country.

In order to undertake large scale surveillance I felt we needed a fast and cheap method for the detection and placing of CTX-M ESBLs in the appropriate genotype groups. The biggest obstacle was identifying consensus sequences to design PCR primers to give both specificity for the different genotype groups whilst retaining sensitivity to detect the full range of individual genotypes. Working with Dr Xu Li we produced a rapid and single tube multiplex assay (Xu et al.2005 **30**). The “holy grail” is to be able to determine individual genotypes without full sequencing so very large numbers of strains can be examined rapidly and cheaply. When we designed our PCR assay we linked it to the technique used to detect single nucleotide polymorphisms (SNP) in cancer genotyping, denaturing high performance liquid chromatography (HPLC) (dHPLC). I pioneered the use of dHPLC for PCR amplicon sizing for strain typing *Mycobacterium tuberculosis* in the 1990s, but now applied the heteroduplex ADS BIOTECH dHPLC WAVE methodology in this paper as proof of principle. We went on to develop methods to cover all the commonly encountered CTM-X genotypes (Xu et al. 2007 **31**). We were the first to apply and validate this technology and have used it in many studies as have other groups. It has the advantage that every new specific genotype encountered will produce a novel trace which following DNA sequencing can be added to the library.

In parallel with my dHPLC WAVE approach I also developed a very different methodology for specific CTX-M genotyping based on the oligotyping was being used for strain typing of *M. tuberculosis*. This involves generating a large number of specific oligonucleotide probes which can detect specific SNPs and immobilising them on a matrix. The unknown field strain has a CTX-M specific PCR applied to it to generate single stranded segments of the *bla*<sub>CTX-M</sub> ORF which are also labelled during the PCR



process. Depending on the SNP present, a unique probe binding pattern will be seen which translates into genotype. This was the first time reverse-line hybridization was applied to  $\beta$ -lactamase characterisation (Ensor et.al 2007 **8**). We used it for several years ultimately finding that dHPLC WAVE was cheaper and higher throughput.

It is important in epidemiological studies to identify the genotype of the strain of *E.coli* or *Klebsiella pneumoniae* carrying the CTX-M  $\beta$ -lactamase. Whilst Multi Locus Sequence Typing (MLST) is helpful in grouping key strains a more discriminatory method is needed. In the 2000's this was pulse field gel electrophoresis (PFGE) as the use of whole genome sequencing to determine SNP type had not been introduced. I was asked to collaborate with a Dutch group to explore the use of Raman spectroscopy for strain typing *Klebsiella* spp. We did identify some clusters by Raman spectroscopy using our collection of CTX-M producing *K.pneumoniae* from humans and chicken meat. Although we identified new MLST types of *K.pneumoniae* many of the MLST types were grouped in the same Raman cluster. We found a low level of discrimination, supporting the view that the method was not particularly useful (Overdest et al. 2014 **26**).

### **2.3 Worldwide epidemiology of CTX-M ESBL (excluding UK and Asia)**

Identifying the threat of CTX-M  $\beta$ -lactamases in China long before they reached the UK stimulated me to take an interest in the worldwide distribution and incidence in these  $\beta$ -lactamases. The most comprehensive global surveillance for resistant in *E.coli* and *Klebsiella* spp. is run by the Merck pharmaceutical company as the Study for Monitoring Antimicrobial Resistance Trends (SMART) worldwide study. It supports a network of globally distributed hospital centres that every year collect *Enterobacteriaceae* from significant intra abdominal infections. The isolates are forwarded to a central laboratory

using internationally recognised methodology to determine antibiotic susceptibilities and ESBL production. From time to time sample sets of data from an individual year in a global region are published. I was invited with Professor Yeuda Carmeli to analyse the European data for 2008 and produce a paper (Hawser et al 2011 **16**). This was the first time EUCAST break points were used for a published SMART study and the rapid rise of ESBLs in some countries such as Italy and the UK were identified.

I was always struck by the occurrence of particular genotypes in certain regions e.g. CTX-M14 in China, CTX-M-15 as the only genotype in India/Pakistan, CTX-M-3 in South America, CTX-M-1 in Poland, Russia and Italy/Libya. A phylogenetic analysis suggested that the mobilization from *Kluyvera* spp chromosome happened twice for CTX-M-2, at least 3 times for CTX-M-whereas, CTX-M-9, CTX-M-8 and CTX-M-25 all only mobilised once (Barlow et al. 2008). In a review I authored (Hawkey & Jones 2009**15**) I advanced the hypothesis that an emergence in particular locations of CTX-M was favoured by high rates of antibiotic usage and poor health infrastructure (particularly sewage treatment). The subsequent spread of those evolved genotypes was via the movement of people. I realised there are clear cultural/historical connections with some countries. e.g. India/UK; Italy/Libya. The widespread movement of the Chinese often carrying with them CTX-M-14 and people from India/Pakistan with CTX-M-15 seemed to be the driver for the global spread of the CTX-M genotypes. The global pie chart of genotypes in that review became widely used by many lecturers and the review is one of the Journal Antimicrobial Chemotherapy's top 10 cited papers of all time

In addition to the first recognition of CTX-M as the dominant ESBL in China (Chanawong et al 2002 5) and CTX-15 in India (Ensor et.al. 2006 9). I have undertaken first genotyping surveys in two other countries.

There was very little data on Arabian gulf countries so I was pleased to be able to collaborate with microbiologists in Kuwait. (Ensor et.al.2009 7). We found the CTX-M-15 genotype to be totally dominant (27/29 isolates only 2 CTX-M-9). There are very large numbers of guest workers, largely from India and Pakistan. We also found CTX-M ESBLs to be more common in non- Kuwaiti Arabs with a history of recent travel.

Although very close geographically and culturally to the UK the first genotyping study in Ireland demonstrated some significant differences between the two countries (Morris et al. 2009 21). A total of 812 isolates of Enterobacteriaceae from throughout Ireland were collected of which 506 from 462 patients were ESBL positive. A single isolate from each patient was studied in more detail and all were subjected to PFGE analysis, a total of 371/462 being positive by our PCR method for CTX-M.(Xu et.al 3005 30). Each PFGE type had a representative isolate genotyped by dHPLC for *bla*<sub>CTX-M</sub> genotype. *bla*<sub>CTX-M15</sub> was found in 177 isolates, *bla*<sub>CTX-M-14</sub> in 78 isolates and 3 isolates for *bla*<sub>CTX-M-9</sub> and a single *bla*<sub>CTX-M-1</sub>. The occurrence of CTX-M14 was much higher than in the UK which may reflect the greater involvement of the population with beef and milk production. In England CTX-M13 and 14 are roughly equally distributed in cattle Veterinary Laboratory Agency UK (VLA-unpublished data).

#### 2.4 **Epidemiology of CTX-M in China & Asia.**

My work leading to the identification of CTX-M 14 and 13 in China was referred to earlier in this thesis and represents arguably one of the most important pieces of early

work on MRGNB in China (Chanawong et.al 2002 **5**). I was invited by the European Society of Clinical Microbiology and Infectious Diseases to be a faculty member at their first meeting on extended spectrum  $\beta$  lactamases in Venice in 2007. They commissioned me to review all of the literature relating to ESBLs in Asia. I presented my findings at the meeting and then produced a much condensed manuscript for publication (Hawkey 2008 **13**). My review of the situation in China showed that initially reports were confined to SHV-5, 2 and 9. The description of CTM-13 which was not found in any subsequent surveys was important as in the last few years it has begun to emerge as a significant genotype in some Chinese surveys. There were subsequent studies particularly the one by Yu and colleagues which showed that 14 was dominant with a smaller number of CTMX-3 which we had found in some later studies particularly the one around Wuhan (Munday et.al 2004 **24**). Interestingly Taiwan very much reflected the genotype distribution in China which bearing in mind the cultural and ethnic links could be explained by movement of population. Japan and South Korea from genotype terms are very different to China which again may fit with the movement of population and interactions. The first descriptions of CTX-M were subsequently realised to be CTX-M group  $\beta$  lactamases when the widely distributed TOHO-1 and 2 were sequenced they seem to be confined to Japan. They were subsequently given the designation CTX-M-44 and 45 respectively. TOHO-1 is very closely related to CTX-M-2, and subsequent surveys in Japan showed that CTX-M-2 is the dominant CTX-M genotype in Japan but the second most common type TOHO-2 (CTX-M-45) is a distant relative of CTX-M-9. Korea has a similar picture to Japan but with more impact made by CTX-M-14 and remarkably a very small number of CTX-M-15 which in this period of time was more associated with India. With regard to India the presence of phenotypically described ESBL's had been recorded for a number

of studies since the 1990's however, no one had genotyped isolates from that country. I undertook the first study which showed only CTX-M-15 to be present at a very high rate (Ensor et.al 2006 **9**).

Other Asian countries had very little data with the exception of Thailand where an earlier study by my group had demonstrated the presence of a single isolate carrying VEB-1 the dominant ESBL type were SHV ESBLs 5 and 12. (Chanawong et al. 2001) That latter study was from Khon Kaen in Northern Thailand with isolates collected in late 1994-1996 and a study published on isolates from 2003 showed the presence of CTX-M-9 and CTX-M-14 in addition to VEB (36% of ESBLs) in Southern Thailand(Chanawong et al. 2007). This presumably represents the spread into Thailand of Chinese and Vietnamese strains carried in the gut of travellers. Following our initial description of CTX-M-13, and 14 Dr Yusong Yu in Shanghai carried out a limited survey as did two separate groups in Beijing. They demonstrated probably because of the very small numbers of strains examined, CTX-M-3 together with CTX-M-14 in Beijing. The opportunity arose to undertake a study on ESBLs from Wuhan which is geographically remote from the three major cities in China, being located in Central China. This survey showed the presence of CTX-M-14 but also that approximately 25% of the strains were CTX-M3 reinforcing the notion that 3 also emerging in the Asia specific region and spread early on in the dissemination of CTX-M, ESBL's. (Munday et.al 2004c **24**). By the mid 2000's there had been no English language published survey of resistance in MRGNB across China other than that written by myself and Dr Craig Munday (Munday et.al 2004 c **24**) which summarised four published studies, which consisted of small published individual local city surveys and reported on a 5<sup>th</sup> larger survey in Wuhan. I felt we needed a better handle on what was

going on in China with regards to the prevalence of CTX-M  $\beta$ -lactamases, particularly in the community. I was able to persuade Merck to support a study of antimicrobial susceptibility of community isolates. The majority were from urine or sputum from those already identified as community patients across 23 individual laboratories in 7 regions focused on Beijing, Guangzhou, Shanghai, Hong Kong and the Provinces of Hunan, Wuhan and Zhejiang between 2002 and 2003. A total of 2099 non duplicate clinical isolates were collected of which 1615 were *Enterobacteriaceae*. (Ling et.al 2006 **18**). The 953 *E.coli* isolates were collected from the community in which a resistance rate of 14.4% was seen to cefotaxime and 2.7% to ceftazidime, interestingly the ciprofloxacin resistance rate was 50.6% and the gentamicin resistance rate was 39.4%. This illustrates the high levels of resistance rates present in Chinese isolates, even in the early 2000's. In the case of *Klebsiella* spp. the cefotaxime resistance rate was 15.4% and 8.1% to ceftazidime. The ciprofloxacin resistance rate was 25.2% which probably reflects the intensive use of quinolones in the community. The less intensive use of quinolones in a hospital setting results in less selective pressure on *Klebsiella* spp. which is more associated but are there is wide spread availability in the community where *E.coli* is exposed to ciprofloxacin. A small sample of *E.coli* and *Klebsiella* spp. were subsequently checked by PCR for the presence of different genotypes when CTX-M-14 followed by 3 were found to be the common genotypes (this data was never published).

With the very high rate of CTX-M, ESBLs in China and the fact that our earlier study was from 1997 where we found CTX-M-14 was the dominant type it occurred to me that it would be helpful to have a detailed genotyping study on a large number of isolates from a large city other than Guangzhou. We therefore initiated a project which involved three

major hospitals across Changsha the capital city of Hunan province, the city has a population of over 10 million people. Between October 2004 and July 2005 425 non-duplicate clinical isolates of *Enterobacteriaceae* were selected. (Liu et al. 2009 **19**). The overall ESBL rate was 33.4% (1424/425) and 109/142 were found to carry CTX-M; PCR dHPLC backed up by DNA sequencing was used to determine genotype. 47% were CTX-M-14, 29.4% were CTX-M-3 but a great surprise was that 17.4% were CTX-M-15. This was the first report of CTX-M-15 in China and perhaps represented the importation from India of CTX-M-15 producing strains. CTX-M-15 was found in both *E.coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* suggesting there had been substantial spread in different bacterial hosts within the city. CTX-M-15 was also found in two of the three hospitals. More recently, I was interested in whether or not CTX-M-15 had begun to take over from CTX-M-14 and CTX-M-3 in China and we undertook a further study between November 2013 and January 2014 across the whole of Hunan province by sampling from 8 different geographical centres healthy subjects in the community that were submitting pre-employment faeces samples.(Zhong et.al 2015 **33**). We sampled 563 subjects 287 of which (51.0%) carried ESBL producing *E.coli* all of which were CTX-M positive. The genotype distribution was interesting: CTX-M-14 (48.4%), CTX-M15 (27.5%), CTX-M27 (15.0%), the remaining 9.9% were made up small numbers of CTX-M 65, 24,3,104 and 101. This study dramatically demonstrated the success of CTX-M-15 in displacing CTX-M-3 totally and reducing CTX-M-14, but also the rise of CTX-M-27 which more recently has been identified in Chinese pig herds. This finding leads me to speculate that this may be a very important source of CTX-M genes for transfer into human adapted strains of *E.coli*. Interestingly, only 11.1% of the 287 CTX-M positive isolates belonged to the ST131 clone although one must bear in mind that this was a survey of healthy

individuals. Within that clone the 016-ST131 isolates were dominant and contained the *fimH41* allele. The *fimH* genes encode type 1 fimbriae that enhance uropathogenicity. The most commonly encountered ST131 strains carry either *fimH30*, a genetic variant which is particularly associated with resistance to fluoroquinolones and production of CTX-M. The *fimH41* allele has only recently been identified and has not previously been associated with CTX-M production which was shown in our paper. The remaining isolates belonged to 025b, ST131. PFGE analysis showed that the ST131 isolates formed one large group at a 64% similarity level and could be further sub-divided. This study shows most importantly that 016-ST131 isolates have now emerged from China and form the predominant type of ST in faecal isolates of *E.coli* from healthy individuals carrying CTX-M. At the time of writing this was the first study to identify 0.16-ST131 as the predominant type of a clonal group in a collection of ST 131 and this may presage the emergence of interesting potentially virulent clones in the general community within China. As mentioned in the earlier section on worldwide epidemiology ESBL producing *Enterobacteriaceae* had been long recognised phenotypically in India with reports from the end of the last century but no genotyping other than the very first report of CTX-M-15 which involved the study of a small number of isolates in Paris which came from six Indian patients at the Betra Hospital , New Delhi (Karim et al. 2001) ESBL rates reported in South Asia vary widely from 12-71% , but most studies reported a prevalence rate of around 50% and upwards. I managed to identify an Indian collaborator in Aligarh who collected strains from 3 centres across India 111 isolates from Aligarh, 10, from Varanasi, 9 from Karnataka S. India) and came to the UK to work under my supervision. The dramatic finding he made was from further analysis of the 130 non duplicate clinically significant from *E.coli* and *K pneumoniae* isolate resistant to third generation



cephalosporins, was that 73% carried CTX-M and that they were all CTX-M-15. We undertook further analysis of these isolates looking at the location of IS26 and identified a number of variants suggesting that there has been considerable evolution over time of these genes of the genetic environment of the CTX-M-15 (Ensor et al. 2006 9). Our data which was the first systematic molecular large scale survey on characterizing CTX-M in India lends support to the belief that CTX-M-15 emerged in India sometime in the late 1980's and that it then dispersed across the world.

## 2.5 Epidemiology and occurrence of CTX-M in the UK

In the course of undertaking routine surveillance whilst at the University of Leeds I discovered a single isolate from a six year old child in May 2000. The isolate was from a routine stool screening sample from a haematology patient in which an ESBL phenotype *Klebsiella oxytoca* was recovered. The MIC's of cefotaxime were 32 mg/L and ceftazidime 4mg/L, which were consistent with an ESBL with marked cefotaximase activity. PCR amplification and DNA sequencing revealed the presence of *bla*<sub>CTX-M-9</sub>. This was the first ever report of CTX-M in the UK. Upon moving to Birmingham I maintained an interest in surveillance for CTX- ESBLs and as described previously was responsible for characterising isolates in an outbreak at the City Hospital caused by CTX-M-26. In September 2001 I had just moved to Birmingham and received a call from Dr. Rod Warren at Shrewsbury saying that he thought he had found 4 isolates of *E.coli* which were producing an ESBL and could I characterise them. We did this and discovered that two were both producing CTX-M-15. We subsequently learnt that the isolates had also been sent to Colindale who had initially mis-sequenced them as a different genotype. We contacted the reference lab and asked them if they were absolutely certain of their sequencing and they rechecked and confirmed that our sequencing was correct. These isolates subsequently were further characterised with others and published by the reference laboratory (Karisik et al. 2006). It seemed to me that the bowel flora was the most important reservoir of these organisms both in the community and in hospital as it had been well established in outbreaks of *Klebsiella pneumoniae* in the late 1970s and 1980s. I therefore suggested that we should undertake a survey of faecal carriage in the general population. The only readily accessible samples being those submitted from

patients with diarrhoea or GI illness. We had a laboratory in York which wished to undertake a project on ESBL's and they between October and December 2003 examined 1000 consecutive, non- duplicate faeces samples of which 565 were from the community. I found an overall carriage rate of ESBL phenotype *Enterobacteriaceae* of 1.9% and interestingly identified five CTX-M-15, three CTX-M-14 and nine CTX-M-9 producing strains of *Enterobacteriaceae* confirming the importance of the faecal reservoir both in hospitals and in out-patients. This was to my knowledge the first observation of CTX-M in faecal flora in the UK (Munday et.al 2004 **23**) In September 2004 the BSAC organised an extraordinary meeting to bring an awareness of the importance of CTX-M to the broader microbiology community. I contributed to this meeting with our experience and also reported on my earlier work in Asia as well as reminding the audience that cefpodoxime as we had shown earlier is one of the best single substrates particularly when tested as the only cephalosporin on urinary isolates to ensure that ESBLs were detected in all isolates of *Enterobacteriaceae*. Together with Professor David Livermore we wrote a short review of that meeting which also contained some observations on the importance and the way in which CTX-M was beginning to dominate the UK microbiology scene. (Livermore and Hawkey 2005 **20**). The earlier study referred to above on the CTX-M-15 in the UK very much gave the impression that CTX-M-15 was the only important CTX-M and that this was strongly associated with ST131 lineage of *E.coli*. This was because many of the isolates came from a single hospital. Whilst we must accept that this is a most important clone, some of my informal surveillance activity suggested there was a greater diversity of both host *E.coli* and genotype of CTX-M circulating in the West Midlands. We were able to involve 13 different centers across the West Midlands region which has a population of approximately 6 million people. The laboratories for a period of two months

in 2006 collected 370 consecutive non duplicate isolates which were presumed to be ESBLs. These were screened for the presence of CTX-M, genotyped and also differentiated by pulse field gel electrophoresis (Xu et.al 20011 **32**). We found that 96.6% of the isolates carried a CTX-M-15 with the remainder carrying most commonly CTX-M-9 and 14. By using an ST131 specific PCR 66% of CTX-M positive isolates belonged to the ST 131 lineage. Our PFGE analysis show that the largest number belonged to strain A. Strain A was previously identified by Woodford and colleagues. This was the first regional detailed epidemiological study of CTX-M in the UK and showed that there had been considerable spread of the *bla* *CTX-M* particularly of CTX-M-15 into non ST 131 clones and that strain A was considerably more heterogeneous than when first described. We showed it had acquired a much higher level of gentamicin resistance probably by transposition of gentamicin resistance genes into the resident CTX-M plasmids. Approximately a third of the producers identified were unrelated strains suggesting that the genes had become dispersed rapidly in the *Enterobacteriaceae* over a 3 year period since the initial outbreak. This study was undertaken between April and May 2006 just 3 years after the first published report of the occurrence of CTX-M-15 in the UK. Following our work in India to identify a CTX-M-15 genotype, being the only CTX-M carried in that country, I wished to understand the degree of penetration by these genes into the local Birmingham population. To go out and recruit individuals in the community, distribute individual questionnaires and obtain detailed information on them and then culture their faeces, would have required considerable funding which was not available at the time. I therefore hit on the notion of using a computer database and programme called OriginsInfo which ascribes a cultural ethical and linguistic origin to an individual based on their name. It has been validated and successfully used in a variety of

health care settings including cancer epidemiology and healthcare delivery audits. We sampled stool samples from 732 individuals within the general community around Heartlands hospital in 2010 and screened them for ESBL production using chromogenic agar (Wickramasinghe et.al 2012 **29**).

Their names were fed through the data base and described as either European origin, or Middleeastern/South Asia (MESA) origin, a very small number of other groups such as Chinese and African were also identified. The finding was very striking in that there was a statistically significant difference ( $p < 0.001$ ) in carriage in the European group (8.1%) and the MESA group (22.8%). There was also high rate of carriage of CTX-M-15 producing *E.coli* in MESA subjects ( $p < 0.001$ ). This was the first demonstration anywhere in the world that cultural and ethnic origins would result in differences in the rate of carriage of CTX-M ESBL producing *E.coli* across a single community. The findings were important and have been widely cited as this lends credence to the concept of the movement of people being the main driver for the spread of CTX-M across the world. In a collaborative study with Professor Neil Woodford's group at Colindale we sought funding for and were funded to look at the diversity of plasmids in ESBL *E.coli* and diversity of plasmid addiction systems by them carried by *E.coli* in the UK. A large sample of plasmids were characterized in detail, interestingly quite a significant number 48/118, could not be electroporated or transformed into a laboratory *E.coli* background suggesting that there was some degree of fixation of plasmids in particular host lineages. We did manage to demonstrate that 70/118 plasmids were transformable and that this is an important route in the UK for the dissemination of CTX-M ESBLs. We also wished to understand the nature of plasmid addiction systems and found a wide variety of addiction

systems which had not been appreciated in previous studies in the UK. (Doumith et.al 2012 6).

## **2.6 The environmental resistome in relation *bla*<sub>CTX-M</sub>**

The largest reservoir of *Enterobacteriaceae* carrying *bla*<sub>CTX-M</sub> is the gut of mammals both human and food/companion/wild animals. Faeces are discharged into the environment and the subsequent contamination of water, food and the environment is an important route for their spread and therefore a crucial area for control. Antibiotics used in human and animal medicine/husbandary exert a variable selection as part of the transmission dynamics which are summarised in Figure 2. The role of mobile elements in the resistome is also very important and I have a long standing interest in this area.

Group 9 CTX-M genes are most frequently associated with class one integrons which are complex structures enabling the expression of genes imported by horizontal gene transfer into bacteria that lack a potent promoter. They also carry genes encoding resistance to quaternary ammonium compounds which I have always felt may act as a selection indirectly for antibiotic resistance gene cassettes contained within the class 1 integrons. There has been a lack of controlled studies demonstrating the impact of quaternary ammonium compounds, which are widely used as fabric softeners, detergents and in hospitals in disinfectants, on the carriage of class 1 integrons. On moving to Birmingham I was able to establish a research collaboration with Professor Liz Wellington at the University of Warwick who is an environmental microbiologist and I suggested to her that we should undertake some environmental studies on the impact QACs have on the incidence of integrons and potential antibiotic resistance gene maintenance.

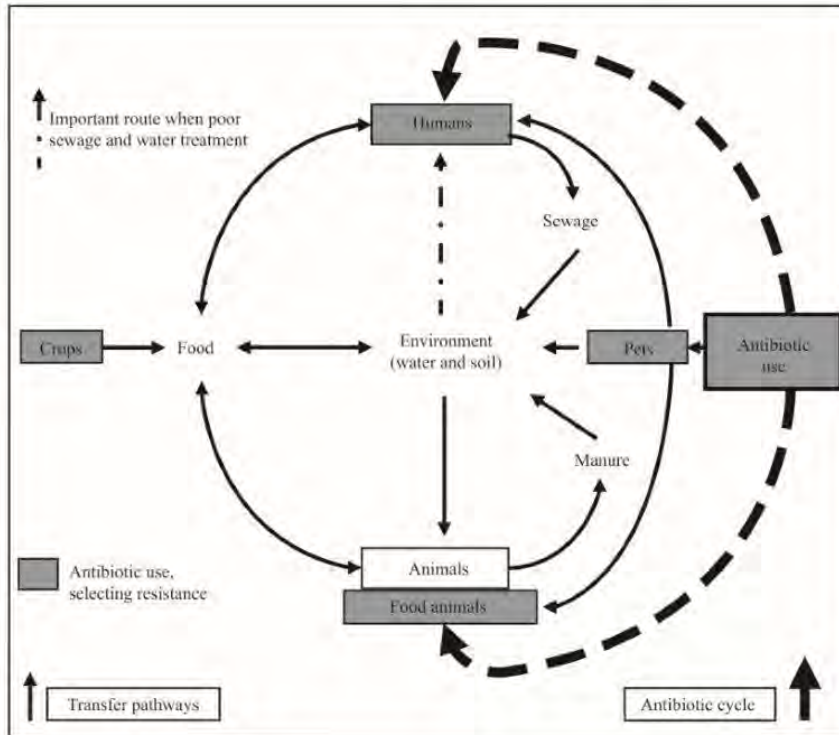


Figure 2. Principle transfer pathways for antibiotic resistance genes in humans, animals, food and the environment. (Reproduced with permission from The Joint Work Group Defra's Antimicrobial Resistance Co-operation (DARC) and Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infections (ARHAI) ESBLs. A threat to human and animal health?

I previously worked in Yorkshire where I had access to a fabric finishing mill which used large quantities of QACs (1-.2% of the fabric weight processed), which was then discharged into a large treatment system using reed beds, before finally being discharged into the river Aire.(Gaze et al 2005)

We were able to set up a study whereby we sampled river sediment and water at all of the relevant parts of the mill and reed bed system including the outlet to the sewage works and river. No antimicrobials or human sewage were passed into this system.

This study was the first time we were able to demonstrate that the level of occurrence of class 1 integrons was very high compared to other less contaminated environmental sites such as farm soil and relatively pristine water courses. (Gaze et al. 2005 **10**). As part of our continuing collaboration we went on to later study the effect of quarternary ammonium compounds in a variety of agricultural wastes, de-watered sewage sludge and soils. For the first time we were able to demonstrate the impact that amended sludge containing low levels of antimicrobials in an agricultural environment has on the prevalence of class one integrons. Again we demonstrated a substantial increase in integron carriage rates which in turn would enable those bacteria to acquire resistance cassettes on exposure to other bacterial strains with transferable antimicrobial resistance. (Gaze et al 2011 **11**)

As a result of my work on faecal carriage in particular ethnic groups in Birmingham I decided it would be interesting to embark on a study of sewage effluent and the effect of modern sewage treatment on the persistence of *bla*<sub>CTX-M</sub> genes in the effluent from sewage treatment works. The only previous study in river waters was one which did not



find *bla*<sub>CTX-M-15</sub> and only found a very low frequency of occurrence of *bla*<sub>CTX-M-14</sub> (Dhanji et al. 2011). Working with my collaborator Liz Wellington and a joint post doc, Dr Will Gaze, I suggested that we look at the sewage works serving Coventry which takes all of the domestic and industrial sewage from that city which has quite a high level of ethnic diversity with a large Bangladeshi population (Amos et al 2014 2) . We looked at three sampling points upstream of the sewage works and three sampling points downstream of the sewage works at 300, 600 and 900 metre intervals. The treatment plant serves approximately 500,000 people and processes 120 million litres of raw sewage per day using primary, secondary and tertiary treatments. We demonstrated there was a highly statistically significant difference in counts of *E.coli* producing CTX-M downstream compared to upstream of the discharge point. The genes we recovered were largely *bla*<sub>CTX-M-15</sub> as might be expected from our work of faecal carriage and the diversity of the population served by this works. What was particularly concerning was the very high rate of the isolation of both *E.coli* and using metagenomic methods, a diversity of environments for the *bla*<sub>CTX-M</sub> particularly in the non culturable microbiome. We also found in the exogenous isolated *Enterobacteriaceae* that a large number of rearrangements compared to those normally seen in clinical strains environment for CTX-M-15 were identified. This leads me to believe that the act of sewage treatment puts a severe pressure (physiological and antimicrobial) on the genes in the microbial environment of the sewage leading to genetic re-arrangements, some of which may be more easily dispersed than the parental types. This was the first study to show the importance of the dissemination of CTX-M through sewage treatment works. (Amos et al 2014a 2) We went on to undertake a much more detail metagenomic library analysis of the river course from the previous study and were able to demonstrate a huge diversity of

evolution of particularly aminoglycoside inactivating genes but also  $\beta$ -lactamase genes, further supporting the impact that sewage works have on releasing antibiotic resistance genes into the environment following treatment in waste water treatment plants. (Amos et al 2014 b 3). I suggested to Professor Liz Wellington that it would be a good idea to bring some of our data and other workers data to the attention of the medical community because of the increasing interest in antimicrobial resistance. She agreed and she first authored with myself and a number of others a highly cited review paper to which I contributed all of the medical and CTX-M commentary and data. (Wellington et al 2013 28).

## **2.7 CTX-M $\beta$ -lactamases and their occurrence in food**

*E.coli* particularly and to and lesser extent *Klebsiella pneumoniae* carrying CTX-M are found in the gut of humans from when they can have acquired them by ingestion from environmental contamination but also potentially from contaminated food. The wide spread use of modern intensive agriculture, particularly for poultry and fish production, with the extensive use of antimicrobials is a now a well- recognised threat to the control of antimicrobial resistance. During my visits to China in the early 2000s I had discussions with their State Drug Administration and it became clear that very large amounts of antimicrobials were being used in both poultry and fish production in China. Talking to other vets and experts as a result of my membership of the Department for Environment Food and Rural Affairs (DEFRA) Antimicrobial Resistance Committee I suggested that surveillance should be instituted for CTX-M genes by the Veterinary Medicines Directorate (VMD) in England. This surveillance was instituted in the late 2000s and interestingly found in dairy cattle and subsequently in poultry and pigs the presence of

surprisingly high levels of CTX-M  $\beta$ -lactamase producing *E.coli*. In dairy cattle the predominant genotype of CTX-M was CTX-M-14 and whereas in chicken initially only low levels of CTX-M-1 were found, this has increased more recently. I decided it would be valuable to undertake a survey particularly of imported chicken meat as well as UK produced meat as my knowledge of the characteristic genotype distribution of CTX-M might give a clue and/or correlate with the source of the imported chicken meat. I collaborated with Dr Rod Warren in the public health laboratory in Shrewsbury and together we collected 129 fresh and frozen chicken breast fillets from retail outlets and processed them for the presence of CTX-M ESBLs. Of the 62 UK reared chicken samples only 1 yielded CTX-M producing *E.coli* which was a CTX-M-1 producing *E.coli*. In contrast 4 of the 10 Brazilian samples produced CTX-M-2 which is the genotype most frequently encountered in that part of the world and we also had 2 Dutch samples producing CTX-M-2. We identified 5/40 unknown samples of chicken meat which had been processed at a minimum of 2 UK cutting stations, our suspicions being that this had been raised potentially in Thailand, by extrapolation of the presence of CTX-M-14. This was the first study to demonstrate that chicken meat may represent an important source for the movement of CTX-M genes around the globe. (Warren et al 2008 **27**).

As a consequence of our publication I was contacted by a Dutch group who were also interested in doing a comprehensive study of a small town in Holland to ascertain what the distribution was amongst food samples, clinically significant infections and faecal carriage in the normal population with regard to CTX-M genotypes and sequence type of *E.coli*. Our study (Overdeest et al 2011 **25**) proved to be a seminal work demonstrating the same genotypes that were most common in the normal population in the towns studied

were present in the chicken and other meat and caused bacterial infections. CTX-M-1 was also the dominant (58.1% ) genotype in chicken samples available in retail centres. The same genotype was also responsible for 28% of bacteremia in the same town. Whilst we could not make an absolute link between the individual strains this study stimulated an enormous interest both in the Netherlands and elsewhere and has driven further more detailed studies, one of which from Flagstaff Arizona was presented in preliminary form at ECMID 2016 suggests that about 15% of all urinary tract infections arise from the consumption of meat carrying human pathogenic *E.coli*.

My detailed understanding from many visits to China of the use of antibiotics there has led me to establish a collaboration with the College of Veterinary Medicine at the South China Agricultural University in Guangzhou to study the occurrence of CTX-M producing *E.coli* in Chinese farmed fish. Fish meat in China is often very lightly cooked and carries significant numbers of *E. coli* at the point of consumption. Therefore is likely to be a good vector for disseminating *bla*<sub>CTX-M</sub> and/or other multi resistance strains of MRGNB. I therefore decided to design a study with Dr Hong-Xia Jiang to look at retail fish prior to consumption for both the presence of *bla*<sub>CTX-M</sub> and plasmid mediated quinolone resistance. It is my impression from talking to experts in the Ministry of Health in China that large amounts of quinolones are used in fish production and not third generation cephalosporins. There is also little or no sewage contamination of fish farms as there are separate means available for the disposal of human sewerage. We found relatively low numbers of *bla*<sub>CTX-M</sub> but all of the genotypes seen frequently in the Chinese population ie. *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-79</sub>., 19/112 strains carried ESBL genes. We did however, find very high rates of Plasmid Mediated Quinolone Resistance (PMQR),

identifying these genes in 59/80 strains picked from non-selective media. This was the first study to demonstrate the existence in farmed fish in China of high levels of PMQR genes as well as the presence of *bla*<sub>CTX-M</sub> ESBL genes. The study will be an important indicator of the considerable selective impact that the use of antimicrobials in fish farming will potentially have on human carriage of CTM producing *E.coli*. Hopefully this and other studies will lead to a change in policy.

## 2.8 Conclusions and future work

I have been fortunate in being responsible for the first identification in 1997 in China of CTX-M-14, one of the world's most important ESBLs. Prior to that time CTX-M  $\beta$ -lactamases were seen as one of the many minority ESBL types, although they were recognised as important in South America. It quickly became clear to me and my Chinese collaborator that because about 30% of *E.coli* in the survey we undertook in Guangzhou were cefotaxime resistant, with an ESBL phenotype, that this was a very important observation. We reasoned that the fact that CTX-M-14 was plasmid mediated and transferable and that examination of a small number of isolates from Beijing and Shanghai showed the same genes, that this resistance marker was spread at high frequency across the whole of China. The success of this resistance coupled with its frequent carriage in isolates of *E.coli* and other *Enterobacteriaceae* that are multiply antibiotic resistant suggested a potential for worldwide spread. This prompted us to look in the UK and whilst in Leeds we only found a single CTX-M-9 carrying isolate in 2003. We subsequently uncovered the beginning of the UK endemic of CTX-M ESBLs which we discovered was caused by CTX-M-15. This in turn suggested that India was likely to be the source, particularly as some of our earliest cases were from South Asia. The first

survey for CTX-M in India confirmed our worse fears with very high rates of CTX-M both in clinical isolates and faecal carriage in the general population. This demonstrated that there was a global pandemic. The most important consequence of this pandemic was the increased usage of carbapenem antibiotics and the emergence of carbapenemases. My work focussed on improving detection and molecular characterisation methods to enable us to understand the global epidemiology. This provides basic information to design control strategies and ultimately control and hopefully reduce the frequency of CTX-M ESBLs. We will see a lower usage of carbapenems and maybe reductions in carbapenemase producing *Enterobacteriaceae*.

The consequences of the rise in CTX-M ESBLs, in terms of the environmental resistance are potentially huge. Unlike MRSA multiply antibiotic resistant *Enterobacteriaceae* have a number of ecological niches in the mammalian gut and water courses. It is also apparent, that they can survive in soils and the phytosphere, particularly when carried by *Klebsiellae*. This has an irony in that the CTX-M gene was almost certainly mobilized from the *Kluyvera* spp. chromosome in the rhizosphere as *Kluyvera* spp. are the most important phosphate solubilizing bacteria. This widespread contamination of many environmental niches with CTX-M genes creates a huge reservoir of genes to be further distributed back to humans and food animals. I encapsulated this circulation in a diagram (Figure2) which I produced for a joint report on ESBLs for ARHAI and DEFRA.

Future research is needed to more accurately delineate the size and complexity of human colonisation and environmental reservoirs. As our work on sewage has shown both amplification and release into the environment of CTX-M genes and their host plasmids following even high level treatment. Further work to reduce this contamination is

urgently needed. If we can identify novel strategies to eliminate plasmid carriage or the host *E.coli* this would reduce the reservoir of CTX-M in healthcare settings and potentially reduce transmission. Finally the development of novel alternative agents will reduce the pressure to use both third generation cephalosporin antibiotics and carbapenems.

### 3.0 List of Publications

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### **3.1 Text of Publications**

1. Alobwede, I., M'Zali, F.H., Livermore, D.M., Heritage, J., Todd, N., & Hawkey, P.M. 2003. CTX-M extended-spectrum beta-lactamase arrives in the UK. *J.Antimicrob.Chemother.*, 51, (2) 470-471

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## Correspondence

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### **CTX-M extended-spectrum $\beta$ -lactamase arrives in the UK**

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Keywords: extended-spectrum  $\beta$ -lactamase, cefotaxime resistance



2. Amos, G.C., Hawkey, P.M., Gaze, W.H., & Wellington, E.M. 2014a. Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. *J.Antimicrob.Chemother.*, 69, (7) 1785-1791



## Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment

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**Objectives:** Multidrug-resistant Enterobacteriaceae pose a significant threat to public health. We aimed to study the impact of sewage treatment effluent on antibiotic resistance reservoirs in a river.

**Methods:** River sediment samples were taken from downstream and upstream of a waste water treatment plant (WWTP) in 2009 and 2011. Third-generation cephalosporin (3GC)-resistant Enterobacteriaceae were enumerated. PCR-based techniques were used to elucidate mechanisms of resistance, with a new two-step PCR-based assay developed to investigate *bla*<sub>CTX-M-15</sub> mobilization. Conjugation experiments and incompatibility replicon typing were used to investigate plasmid ecology.

**Results:** We report the first examples of *bla*<sub>CTX-M-15</sub> in UK river sediment; the prevalence of *bla*<sub>CTX-M-15</sub> was dramatically increased downstream of the WWTP. Ten novel genetic contexts for this gene were identified, carried in pathogens such as *Escherichia coli* ST131 as well as indigenous aquatic bacteria such as *Aeromonas media*. The *bla*<sub>CTX-M-15</sub> gene was readily transferable to other Gram-negative bacteria. We also report the first finding of an imipenem-resistant *E. coli* in a UK river.

**Conclusions:** The high diversity and host range of novel genetic contexts proves that evolution of novel combinations of resistance genes is occurring at high frequency and has to date been significantly underestimated. We have identified a worrying reservoir of highly resistant enteric bacteria in the environment that poses a threat to human and animal health.

**Keywords:** antibiotic resistance,  $\beta$ -lactamases, CTX-M, environmental pathogens, carbapenem resistance

### Introduction

Growing evidence suggests anthropogenic activities such as agriculture contribute to environmental reservoirs of resistant bacteria that can directly or indirectly transfer to humans.<sup>1,2</sup> Waste water treatment plants (WWTPs) process waste from several sources, including human, animal and industrial waste, providing a hotspot for horizontal gene transfer to occur between bacteria from many origins. Few studies have demonstrated the impacts of liquid WWTP effluent on antibiotic resistance loads in rivers, particularly with reference to third-generation cephalosporin (3GC) resistance.<sup>3</sup> The most common mechanism conferring resistance to 3GCs is the production of plasmid-mediated extended-spectrum  $\beta$ -lactamases (ESBLs), of which the most prevalent

are the CTX-M enzymes encoded by *bla*<sub>CTX-M</sub>.<sup>4</sup> Evidence suggests insertion sequence elements *ISEcp1* and *IS26* mobilized progenitors of *bla*<sub>CTX-M</sub> onto plasmids from the chromosome of *Kluyvera* species, a common rhizosphere organism.<sup>5</sup> Subsequently, plasmid-borne *bla*<sub>CTX-M</sub> genes have disseminated throughout the Enterobacteriaceae and Gammaproteobacteria.<sup>6</sup> Currently, there are >145 different genotypes of *bla*<sub>CTX-M</sub> (<http://www.lahey.org/studies>), which are often region specific, with *bla*<sub>CTX-M-15</sub> being the most prevalent in humans worldwide and in the UK.<sup>4</sup> Rivers are routinely used for the release of WWTP effluent and are a repository for sewage through storm drain overflow. A surveillance study found *bla*<sub>CTX-M-14</sub> in UK rivers; however, to date, the most clinically important ESBL *bla*<sub>CTX-M-15</sub> has not been found.<sup>7</sup> Environmental reservoirs of antibiotic-resistant bacteria

are likely to represent a significant exposure risk to humans, through direct contact or indirectly through contaminated drinking water or irrigation of crops. We hypothesize that waste water disposal methods are a contributing factor in resistance gene dissemination in rivers. The current study involves a comparative analysis of 3GC-resistant Enterobacteriaceae upstream and downstream of a WWTP effluent point.

## Materials and methods

### Sampling

Sampling took place in December 2009 and January 2011. Sediment core samples were taken from a river in the UK midlands at three sites in triplicate, 300, 600 and 900 m upstream of a WWTP and three sites in triplicate, 300, 600 and 900 m downstream of a WWTP. The treatment plant served ~500 000 people and processed >120 million litres of raw sewage per day using a primary settlement tank, secondary activated sludge treatment and final tertiary filtration. Upstream of the treatment plant, geospatial analyses had indicated no other WWTP for  $\geq 10$  km. All samples were immediately stored at 4°C and processed within 24 h.

### Viable counts

Sediment from one of each downstream sample site (300, 600 and 900 m) was pooled in equal parts (1 g total) and resuspended in 1 mL of PBS buffer. This was repeated for upstream sediment samples. In total, each of the triplicate sediment samples was pooled to form three downstream samples and three upstream samples. Chromocult Coliform Agar (Merck) was prepared in accordance with the manufacturer's instructions and amended with cefotaxime (2 mg/L) or ceftazidime (16 mg/L). Downstream and upstream samples were plated (200  $\mu$ L) in triplicate for each antibiotic and unamended Chromocult, before incubating for 24 h at 30°C. Viable plate counts were taken; blue colonies indicated presumptive *Escherichia coli* and pink colonies indicated other coliforms termed presumptive coliforms excluding *E. coli* (PCEs). Reference strains of *E. coli*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Pseudomonas fluorescens* and *Aeromonas media* were used to evaluate the performance of Chromocult at 30°C.

### Bacterial isolation

PCEs and *E. coli* were picked and streaked to obtain pure cultures. The number of isolates obtained for each site differed due to different resistance gene prevalences between sample sites.

### Antimicrobial susceptibility determination

MICs of cefotaxime (1–2048 mg/L) and imipenem (1–32 mg/L) were determined using a broth microdilution method based on CLSI and EUCAST standards as previously described.<sup>8</sup>

### DNA extractions

Isolates were incubated overnight at 30°C in Luria broth (LB) and DNA was extracted using a Nucleospin Blood Kit (Macherey-Nagel) in accordance with the manufacturer's instructions.

### Identification of bacteria

Bacteria were first identified by sequencing PCR products obtained using the universal 27F and 1525R 16S rRNA primers.<sup>9</sup> Further identification of Enterobacteriaceae was performed by partial sequencing of *dnaJ* as

previously described.<sup>10</sup> *Aeromonas* spp. were identified using partial sequencing of *gyrB*.<sup>11</sup> *E. coli* strains were typed using the Achtman scheme.<sup>12</sup>

### Detection of 3GC resistance genes in isolates

PCR amplification of *bla* genes, including *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub>, was performed as previously described.<sup>13</sup>

### Analysis of *bla*<sub>CTX-M-15</sub> flanking regions

Characterization of the regions flanking *bla*<sub>CTX-M</sub> was performed with PCR as previously described.<sup>14</sup>

Further analysis of flanking regions unidentified by conventional PCR was done by modifying the two-step gene-walking method (please see the Supplementary data at JAC Online)<sup>15</sup> and newly designed primers (CTXD-F, 5'-TCACCCAGCCTCAACCTAAG-3'; and CTXD-R, 5'-CGCTCATCAGC ACGATAAG-3') were used to detect duplications (please see the Supplementary data at JAC Online).

### Conjugation assays

*E. coli* DH10B (Str<sup>R</sup>) with induced rifampicin resistance was used as a recipient strain for solid conjugal mating assays with positive *bla*<sub>CTX-M-15</sub> strains as donors. Transconjugants were selected using LB plates amended with streptomycin (100 mg/L), rifampicin (100 mg/L) and cefotaxime (2 mg/L). Positive transconjugants were confirmed using the PCR primer pair CTX-F and CTX-R.

### Plasmid replicon typing

Plasmid replicon types in *bla*<sub>CTX-M-15</sub> positive strains were identified using a PCR-based method.<sup>16</sup> Strains with identical replicon types were further analysed using restriction fragment length polymorphism (RFLP) (please see the Supplementary data at JAC Online).

### Statistical analysis

All statistics were performed using Genstat 15th edition SP1 (VSN International). For comparison of means, log counts were checked for normal distribution using the Shapiro–Wilk test followed by analysis using a paired-sample t-test. Proportions were compared using Fisher's exact test.

## Results

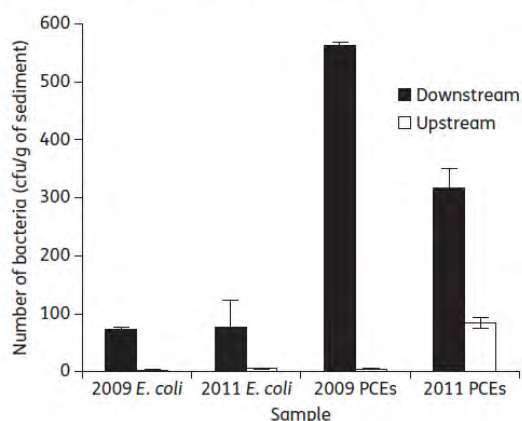
### Viable plate counts

There was a significant increase in the numbers of 3GC-resistant presumptive *E. coli* and PCEs in the river sediment downstream of effluent discharge in both 2009 and 2011 (Figure 1) (*t*-test  $P < 0.0001$  in all cases). No significant difference was recorded in numbers of 3GC-resistant *E. coli* in downstream samples between 2009 and 2011; however, there was a significant increase in numbers of 3GC-resistant PCEs in upstream samples between 2009 and 2011 (*t*-test  $P < 0.0001$  in all cases). The mean average number of total coliforms in 2011 was  $4 \times 10^5$ /g of wet sediment downstream and  $2 \times 10^5$ /g of wet sediment upstream. The mean average number of *E. coli* in 2011 was  $8 \times 10^3$ /g of wet sediment downstream and  $4 \times 10^3$ /g of wet sediment upstream. From this, we can calculate that 0.95% of *E. coli* were resistant to 3GCs downstream compared with 0.13% of *E. coli* upstream. Similarly,

for coliforms there were 0.079% resistant downstream compared with 0.042% upstream.

### Identification of bacteria

All isolated 3GC-resistant presumptive *E. coli* ( $n=41$ ) were confirmed as *E. coli* by sequencing *dnaJ* PCR products (Table 1). Isolated 3GC-resistant PCE isolates ( $n=19$ ) were also identified using *dnaJ* (Table 1). In both downstream and upstream samples, a proportion of PCEs (18.2% downstream and 50% upstream) were identified as members of the *Aeromonas* genus, species *A. media*. Multilocus sequence typing (MLST) analysis of *E. coli* isolates revealed uncharacterized sequence types (STs) particularly from upstream samples (80%), indicating the existence in this environment of novel STs. Downstream of the WWTP, the human-associated ST3103 and ST38 were codominant in 2009, but neither of these STs was detected in 2011 samples, which were dominated by the well-recognized human disease-associated types ST131 (20%) and ST167 (25%) [Table 2 and Table S1 (available as Supplementary data at JAC Online)].



**Figure 1.** Counts of 3GC-resistant presumptive *E. coli* and PCEs from samples collected downstream and upstream of a WWTP in 2009 and 2011. Error bars are  $\pm$  standard errors of biological replicates.

### Detection and characterization of $\beta$ -lactamases in resistant isolates

All *E. coli* were positive for  $bla_{CTX-M}$  and  $bla_{TEM}$ , but negative for  $bla_{SHV}$  (Table 1). Sequencing revealed all  $bla_{CTX-M}$ -bearing isolates in 2011 carried  $bla_{CTX-M-15}$  and 54.5% of isolates in 2009 carried  $bla_{CTX-M-15}$  with the remainder carrying  $bla_{CTX-M-1}$ .

### Analysis of genetic variation in $bla_{CTX-M}$ flanking regions

A total of 11 different genetic contexts were found in association with  $bla_{CTX-M-15}$  (Figure 2, Table 2 and Table S1), 10 of which were novel and denoted I–R in keeping with the nomenclature as previously described.<sup>14</sup> A total of five genetic contexts were found upstream of the WWTP and nine genetic contexts were found downstream of the WWTP. Three of the genetic contexts were downstream and upstream of the WWTP simultaneously: Group A was the most prevalent and accounted for 67% of the total context type in 2009 and 29% in 2011; Group N was found in *A. media* in upstream samples, but in *E. coli* and *K. oxytoca* in downstream samples; and Group K was found in *A. media* and *E. coli* in upstream samples, but in *E. coli* and *C. freundii* in downstream samples. Three groups carried multiple copies of  $bla_{CTX-M-15}$ : Group K was the only group recovered downstream of the WWTP that consisted of a repeated  $bla_{CTX-M-15}$ ; and the other two contexts (Groups L and R) both came from upstream of the WWTP. The CTXD-F and CTXD-R primers allowed for detection of  $bla_{CTX-M-15}$  repeats found in Groups K, L and R; however, several groups were unresolved even after two-step gene walking, due to multiple copies of  $bla_{CTX-M-15}$  and repeat regions. Aside from Group A, Group I was the only other group recorded in both 2009 and 2011, but in *E. coli* in 2009 and *K. oxytoca* in 2011.

Several of the groups contained new elements not previously associated with  $bla_{CTX-M-15}$  flanking regions, such as IS1, putative phage proteins, toxin genes and resistance genes to other antibiotics (Figure 2).

### Relationship between genetic context and MIC

Isolates containing seven of the new genetic contexts had MICs of cefotaxime  $>1024$  mg/L. Significantly higher MICs were characteristic of isolates found in downstream samples compared with

**Table 1.** Prevalence of different  $\beta$ -lactamases determined by PCR screening

Sample site	Organism	Number isolated	$bla_{CTX-M}$ prevalence (%)	$bla_{TEM}$ prevalence (%)	$bla_{SHV}$ prevalence (%)
Downstream 2009	<i>E. coli</i>	11	100	100	0
Downstream 2011	<i>E. coli</i>	20	100	100	0
Downstream 2011	<i>K. oxytoca</i>	3	100	100	0
Downstream 2011	<i>C. freundii</i>	3	100	100	0
Downstream 2011	<i>C. braakii</i>	1	100	100	0
Downstream 2011	<i>Raoultella ornithinolytica</i>	1	0	100	0
Downstream 2011	<i>A. media</i>	2	0	0	0
Downstream 2011	<i>P. fluorescens</i>	1	100	100	0
Upstream 2011	<i>E. coli</i>	10	100	100	0
Upstream 2011	<i>K. oxytoca</i>	1	0	0	0
Upstream 2011	<i>C. freundii</i>	3	33.3	66.6	33.3
Upstream 2011	<i>A. media</i>	4	50	100	0

**Table 2.** Molecular characterization of 52 *bla*<sub>CTX-M</sub>-positive isolates

CTX-M genotype (genetic context group)	Composition of isolates in each genetic context	Associated plasmid Inc replicon types	Cefotaxime MIC (mg/L)	Transfer through conjugation
<b>2009 isolates</b>				
CTX-M-1	downstream: <i>E. coli</i> ST38 (5)	FIB	1024–2048	yes
CTX-M-15 international environment (Group A)	downstream: <i>E. coli</i> ST3103 (4)	F, K, IL/IY	>2048	yes
CTX-M-15 Group I	downstream: <i>E. coli</i> ST New (1)	FIB, I1/IY	64	yes
CTX-M-15 Group J	downstream: <i>E. coli</i> ST3103 (1)	FIB, I1/IY	>2048	yes
<b>2011 isolates</b>				
CTX-M-15 international environment (Group A)	downstream: <i>C. braakii</i> (1), <i>C. freundii</i> (1), <i>E. coli</i> incl. ST131 (3), ST167 (1) and ST New (2) upstream: <i>E. coli</i> incl. ST131 (1) and ST New (3)	FIB, K, HI2, A/C, FIIA	16–2048	yes
CTX-M-15 Group I	downstream: <i>K. oxytoca</i> (2)	FIIA, HI2	>2048	yes
CTX-M-15 Group K	downstream: <i>C. freundii</i> (2), <i>E. coli</i> incl. ST1060 (1) and ST167 (1) upstream: <i>A. media</i> (1) and <i>E. coli</i> ST New (1)	FIA, FIB, K, IL/IY, A/C	1024–2048	yes
CTX-M-15 Group L	upstream: <i>C. freundii</i> (1)	FIA	64	yes
CTX-M-15 Group M	downstream: <i>E. coli</i> ST New (1)	HI2	16	yes
CTX-M-15 Group N	downstream: <i>E. coli</i> ST167 (3) and <i>K. oxytoca</i> (1) upstream: <i>A. media</i> (1)	FIB, K	1024–2048	yes
CTX-M-15 Group O	downstream: <i>E. coli</i> ST1421 (1)	FIB, IL/IY	>2048	yes
CTX-M-15 Group P	downstream: <i>E. coli</i> ST New (1)	F, K	128	yes
CTX-M-15 Group Q	downstream: <i>E. coli</i> incl. ST131 (1) and ST New (2)	FIB, FIIA, HI2, K	>2048	yes
CTX-M-15 Group R	upstream: <i>E. coli</i> ST New (1)	FIB, HI2	16	no
CTX-M-15 unidentified groups	downstream: <i>E. coli</i> ST New (3) and <i>P. fluorescens</i> (1) upstream: <i>E. coli</i> incl. ST410 (1) and ST New (3)	F, FIA, FIIA, FIB, K	32–512	yes

Downstream: isolates recovered downstream of WWTP. Upstream: isolates found upstream of WWTP. ST is the result from MLST with New referring to an isolate with no MLST type matching the MLST database. GenBank accession numbers: Group I, KF155153; Group J, KF155154; Group K, KF155155; Group L, KF155156; Group M, KF155157; Group N, KF155158; Group O, KF155159; Group P, KF155160; and Group Q, KF155161.

upstream samples ( $t$ -test  $P=0.024$ ); however, promoter analysis (Figure S1, available as Supplementary data at JAC Online) revealed that all but one of the contexts shared the same promoter region of *bla*<sub>CTX-M-15</sub> as Group A. One *E. coli* strain had a high level of resistance to imipenem (>32 mg/L), though this was not conferred through *bla*<sub>CTX-M-15</sub> (Table S1).

#### Plasmid diversity and relationships to genetic contexts

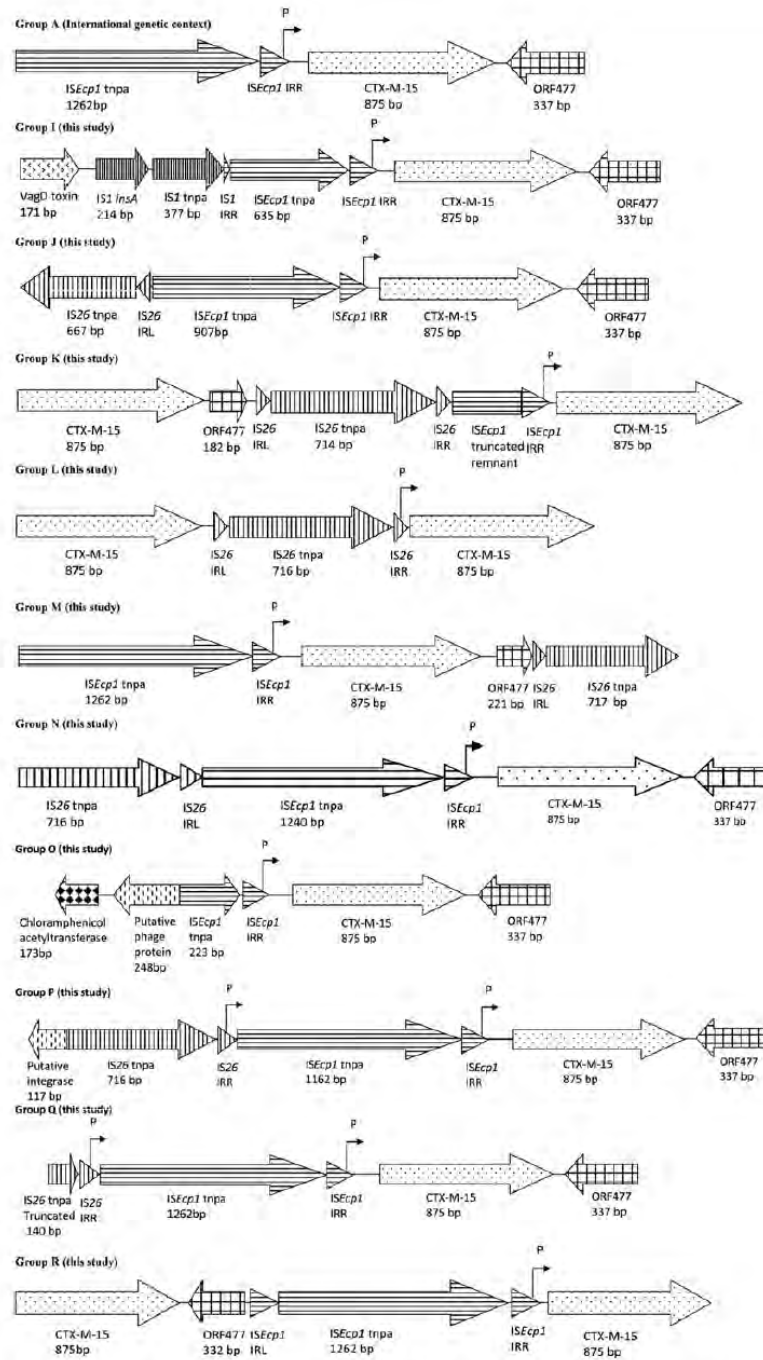
In both years, a number of different replicon types were associated with CTX-M-carrying strains (Figure 3, Table 2 and Table S1). IncF was the most prevalent replicon type both downstream and upstream of the WWTP. The same genetic context was regularly recorded in strains carrying different replicon types, with the Group A context being associated with the most diverse range of replicons (seven types; Table 2). PFGE analysis was used to compare isolates with the same *bla*<sub>CTX-M-15</sub> context and plasmid replicons (Table S1). Group N was carried by the same plasmid upstream and downstream of the WWTP across different families; initially upstream in *A. media* and downstream in *K. oxytoca* and *E. coli*.

Conjugation experiments revealed all but two of the *bla*<sub>CTX-M</sub>-bearing isolates could transfer this gene at frequencies between  $10^{-3}$  and  $10^{-7}$ , with expression of cefotaxime resistance

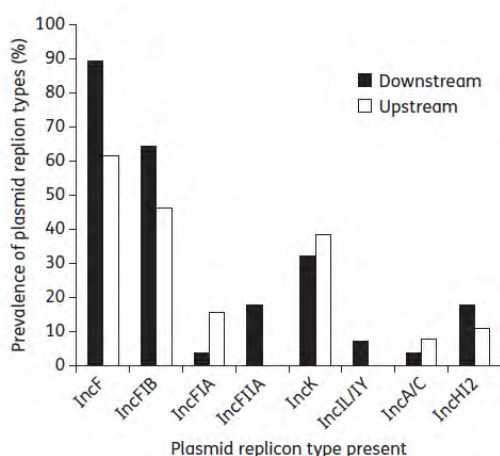
$\geq 2$  mg/L in transconjugants. Conjugation rates varied depending on the host background and the plasmid composition (Table S1).

#### Discussion

Tertiary treatment by WWTPs is the most rigorous level of waste water treatment in the UK as set out by the Water Services Regulation Authority (Ofwat).<sup>17</sup> We demonstrated that even with this level of treatment, WWTP effluent has a significant impact on numbers of 3GC-resistant bacteria in river sediment communities. As well as an increase in the total numbers of 3GC-resistant bacteria, the WWTP had an impact on the prevalence of 3GC resistance in bacteria with a 7-fold increase in the prevalence of 3GC-resistant *E. coli*. The cause of 3GC resistance predominantly resulted from the dissemination of *bla*<sub>CTX-M-15</sub>. This is the first report of *bla*<sub>CTX-M-15</sub> in UK river waters and represents a worrying trend as this gene is the most common ESBL in *E. coli* and *Klebsiella* spp. causing clinical disease.<sup>4</sup> Novel hosts were isolated across the Gammaproteobacteria, including *Citrobacter braakii*, *A. media* and *P. fluorescens*, none of which has previously been reported as a carrier of *bla*<sub>CTX-M-15</sub>. Many of the resistant bacteria were pathogens, such as *E. coli* ST131, ST167 and ST38, *C. freundii* and *K. oxytoca*. In particular, the



**Figure 2.** Flanking regions of *bla*<sub>CTX-M-15</sub> recovered in isolates obtained during this study as confirmed by two-step PCR and sequencing. Nomenclature is an extension of a previously defined typing system.<sup>14</sup>



**Figure 3.** Prevalence of different plasmid replicon types as validated by PCR for 41 *bla*<sub>CTX-M-15</sub>-bearing isolates from river sediment downstream of WWTP effluent and upstream of WWTP effluent in 2011.

finding of *bla*<sub>CTX-M-15</sub> in the pandemic pathogen *E. coli* ST131 as a viable and significant reservoir in environmental samples represents a serious threat to human health. This supports recent findings of the threat that rivers pose to human health highlighted by a study in which one-third of people swimming in areas of the River Thames suffered gastrointestinal illness.<sup>18</sup> In addition, we report the first finding of an imipenem-resistant *E. coli* in a UK river, an indication of the emerging spread of carbapenem resistance in the environment, which is a great cause for concern.

We demonstrated that there was high genetic diversity in *bla*<sub>CTX-M-15</sub> carriage and hypothesize that such an unprecedented diversity can be attributed to the direct introduction of bacteria by WWTP effluent possibly combined with *in situ* selection either in the river or WWTP. Selection is likely to be aided by antibiotic and detergent residues that have previously been detected in WWTP effluent as well as the high density of bacteria present in WWTPs, which will facilitate cell-to-cell contact.<sup>3,19</sup> This hypothesis is supported by plasmid analyses as replicon typing revealed eight types present in isolates carrying *bla*<sub>CTX-M-15</sub>. Of particular concern is the frequency (46%) at which multiple plasmid replicons were colocalized in one isolate. This would allow for interplasmid transfer of *bla*<sub>CTX-M-15</sub> through transposition and homologous recombination and each different genetic context of *bla*<sub>CTX-M-15</sub> may be indicative of a transfer event.<sup>20</sup>

The carriage of multiple plasmid incompatibility groups will contribute to higher conjugation rates. This resulted in conjugation frequencies that were higher than reported for similar studies conducted with clinical strains and plasmids.<sup>21</sup> The extensive mobility of plasmids was further emphasized by the recovery of identical plasmids in diverse backgrounds. Of concern was the pool of plasmids shared between hosts regarded as clinical bacteria and those regarded as indigenous to the river environment.

We have demonstrated repeated evidence of the significant introduction of clinically relevant ESBL-producing bacteria by WWTP effluent into a UK river. Many of the pathogens had novel *bla*<sub>CTX-M-15</sub> flanking regions, including *E. coli* ST131-carrying

Group Q. The prevalence of human-associated bacteria with a high diversity of *bla*<sub>CTX-M-15</sub> flanking regions downstream of the WWTP supports the hypothesis that community carriage is more extensive than currently thought.<sup>22</sup> The change in prevalence of *E. coli* STs between 2009 and 2011, with ST131 becoming one of the most dominant STs, is likely a reflection of the clonal spread of this ST in the human population.<sup>22</sup>

An increase in the number of 3GC-resistant coliforms upstream between 2009 and 2011 is potentially a consequence of faecal contamination from surrounding farm environments. This time-scale coincides with the detection of the ESBL gene *bla*<sub>CTX-M-1</sub> in UK cattle, chickens, turkeys and most recently dogs.<sup>23</sup> The gene *bla*<sub>CTX-M-15</sub> has been detected throughout Europe in companion animals and a diverse range of wild birds.<sup>24</sup> Whilst it is not possible to determine the direction of spread from humans to animals, the significant environmental reservoir in rivers will impact both. In conclusion, we report a reservoir of *bla*<sub>CTX-M-15</sub> in a UK river with clear evidence of extensive recombination of the gene within plasmid populations. A growing environmental reservoir presents a risk to human health, with evidence implicating WWTP effluent as a major contributor to the formation of this reservoir.

Further research is needed into sewage treatment systems that result in minimal introduction of resistant bacteria and selecting agents such as antibiotic residues and quaternary ammonium compounds. Stricter regulations and higher levels of treatment are needed if we are to halt the rise of antibiotic resistance in the environment.

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## Transparency declarations

None to declare.

## Author contributions

G. C. A. A., W. H. G. and E. M. W. designed the research, G. C. A. A. performed the research, all authors analysed the data and all authors contributed to writing the paper.

## Supplementary data

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

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## Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes

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### ABSTRACT

The environment harbours a significant diversity of uncultured bacteria and a potential source of novel and extant resistance genes which may recombine with clinically important bacteria disseminated into environmental reservoirs. There is evidence that pollution can select for resistance due to the aggregation of adaptive genes on mobile elements. The aim of this study was to establish the impact of waste water treatment plant (WWTP) effluent disposal to a river by using culture independent methods to study diversity of resistance genes downstream of the WWTP in comparison to upstream. Metagenomic libraries were constructed in *Escherichia coli* and screened for phenotypic resistance to amikacin, gentamicin, neomycin, ampicillin and ciprofloxacin. Resistance genes were identified by using transposon mutagenesis. A significant increase downstream of the WWTP was observed in the number of phenotypic resistant clones recovered in metagenomic libraries. Common  $\beta$ -lactamases such as *bla*<sub>TEM</sub> were recovered as well as a diverse range of acetyltransferases and unusual transporter genes, with evidence for newly emerging resistance mechanisms. The similarities of the predicted proteins to known sequences suggested origins of genes from a very diverse range of bacteria. The study suggests that waste water disposal increases the reservoir of resistance mechanisms in the environment either by addition of resistance genes or by input of agents selective for resistant phenotypes.

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### 1. Introduction

The growing number of bacteria resistant to multiple antibiotics pose a great risk to both animal and human health, yet despite this the role of the environment in the

dissemination of antibiotic resistance genes is still largely unknown (Wellington et al., 2013). Anthropogenic activities such as agriculture increase the load of environmental antibiotic resistant bacteria with recent reports of diverse resistance genes present in farm environments (Zhu et al., 2013) and increased levels of antibiotic resistance genes in soil following the application of manure (Byrne-Bailey et al., 2011). Waste water treatment plants (WWTPs) are a hotspot for resistance gene transfer between bacteria from different origins due to mixing of urban, industrial, clinical and agricultural waste (Rizzo et al., 2013). The subsequent disposal of effluent and solids from WWTPs can increase loads of antibiotic resistant genes in the environment for

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example via the application of sludge to land as fertilizer (Gaze et al., 2011) and effluent to rivers which could act as a reservoir of resistance genes (Korzeniewska et al., 2013). The overall impact of WWTPs on environmental resistome is poorly studied and has potentially far reaching impacts of escalating resistance gene dissemination.

The application of metagenomics has revealed antibiotic resistance gene diversity in the uncultured bacterial fraction of environmental samples is much higher than observed in cultured isolates and resistance genes associated with pathogens can be found in soil metagenomes (Forsberg et al., 2012; Pehrsson et al., 2013). The mobilisation of *bla*<sub>CTX-M</sub> from *Kluyvera* sp. and *qnr* genes from *Shewanella* sp. provide evidence of resistance gene flow from non-clinical populations to clinical environments where they have had a significant role in conferring antibiotic resistance to animal and human pathogens (Nordmann and Poirel, 2005; Olson et al., 2005). Functional metagenomics has been used to investigate antibiotic resistance in a variety of environments including Alaskan soil (Allen et al., 2009), apple orchards (Donato et al., 2010), seabirds (Martiny et al., 2011), and sludge (Parsley et al., 2010). Research is needed to quantify the impacts of anthropogenic activities on the environmental resistome.

In this study we aimed to investigate the impact of WWTP effluent on the resistome of a river, with the hypothesis that antibiotic resistance gene abundance and diversity would be significantly impacted by the effluent outflow. River sediment samples were taken from downstream and upstream of an effluent pipe and functional metagenomics was used to investigate resistance gene abundance and diversity.

## 2. Materials and methods

Sediment core samples were taken on January 23rd 2011 in triplicate from a river 300 m, 600 m and 900 m upstream and downstream of a large WWTP in the UK Midlands. The WWTP served a large urban catchment of approximately 500,000 people.

### 2.1. Construction of metagenomic libraries

DNA was extracted from samples using FASTDNA Spin kit for soil (MP biomedical, UK) as per the manufacturer's instructions. DNA concentration was measured using spectrophotometry and standardised to the same concentration across all samples. Downstream samples (DS) were pooled into 1 ml final volume to create the library. Upstream samples (US) were also pooled into 1 ml final volume to create the library. DS and US DNA was initially purified using gel fractionation; 1% agarose (Helena Laboratories, UK) was prepared, samples were loaded and gel electrophoresis was performed at 40 V for 18 h. DNA was selected from the size range 5 kb–20 kb and recovered via electroelution. Gel purified DNA was blunt-ended and phosphorylated using an End-Repair kit (Epicentre, USA), then purified and concentrated using Microcon centrifugal filters (Millipore, UK). Blunt DNA was ligated into vector pCC1 (Epicentre, USA) using Fast-link

DNA ligase with an extended 16 °C incubation overnight. The ligation was desalted via drop dialysis using 'V' series membranes (Millipore, UK), and electroporated into Transformax Epi300 *Escherichia coli* (Epicentre, USA). Libraries were titred using a dilution series.

### 2.2. Metagenomic library analysis

Both libraries were amplified in LB broth amended with chloramphenicol (12 mg l<sup>-1</sup>) by incubating overnight at room temperature. Chloramphenicol was used to maintain the pCC1 vector. Resulting amplified libraries were stored at 4 °C for analysis. Ten clones were selected from each library and plasmid extractions were performed using a Miniprep kit (Qiagen, UK). Plasmids were cut with BamHI (New England Biolabs, USA) to excise inserts with resulting digests analysed using gel electrophoresis. Banding patterns were analysed to calculate the mean insert size for each library.

### 2.3. Quantification of total bacterial numbers

The total quantity of bacteria in the DS and US samples was analysed using qPCR for the 16S rRNA gene as previously described (Gaze et al., 2011).

### 2.4. Metagenomic library screening for antibiotic resistance phenotype

Both entire libraries were screened on the aminoglycoside antibiotics amikacin, gentamicin, neomycin, the fluoroquinolone ciprofloxacin and the  $\beta$ -lactam ampicillin. Concentrations for screening were selected by testing the minimum inhibitory concentrations (MICs) in *E. coli* Epi300 strain with an empty pCC1 vector, and choosing a concentration a tenth higher than this MIC to ensure a minimal amount of false positives. The Epi300 strain with an empty pCC1 vector was subsequently used as a negative control. Positive clones were selected and plasmid extractions were performed (Qiagen, UK). Restriction fragment length polymorphism (RFLP) utilising restriction enzymes BamHI, EcoRI and EcoRV was used for analysis of clones to determine the positive number of clones resistant to each antibiotic.

### 2.5. Characterisation of resistant clones

A selection of unique clones were chosen for further characterisation. Unique positive clones were tested for resistance at the clinical breakpoints of each selected antibiotic using published methods (Andrews, 2001). Resistance genes were elucidated using transposon mutagenesis with the EZ-Tn5 kit (Epicentre, USA) which inactivated the resistance gene allowing for selection of clones by loss of phenotype. Clones were then sequenced using the Kan-3 Fp-2 primer forward primer and Kan-3 RP reverse primer. Sequence data was analysed with NCBI blast (Camacho et al., 2009) and NCBI ORF finder (Available at: <http://www.ncbi.nlm.nih.gov/gorf/>, html [last accessed 31/01/14]) to identify the resistance gene and flanking regions.

**Table 1**  
Summary of metagenomic libraries made from DS and US samples.

Sample	Average insert size	Average number of clones	Library coverage
DS	4.2 kb	$2 \times 10^6$	8.4 Gb
US	4.7 kb	$2 \times 10^6$	9.4 Gb

### 2.6. Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium). Differences in gene prevalence were tested for significance using a  $\chi^2$ -test for the comparison of two proportions (from independent samples). The average *E. coli* genome size (4.6 Mb) was used to estimate how many genomes were in each library by dividing number of DNA Mb in the library by 4.6. The estimated proportion of bacteria in the sediment resistant to each antibiotic was calculated by dividing the number of resistant unique clones by the average number of genomes in the library, this was then expressed as a percentage.

### 2.7. Phylogenetic analysis of resistance genes

DNA sequences were trimmed and aligned with ClustalW (Thompson et al., 1994) using MEGA 5.2 (Kumar et al., 1994). Phylogenetic trees were constructed using the Neighbour-Joining method (Saitou and Nei, 1987). A bootstrap consensus tree inferred from 1000 replications was generated, evolutionary distances were computed using the *p*-distance method (Tamura et al., 2011). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

## 3. Results

### 3.1. Metagenomic libraries

Estimated sizes of the libraries were 8.4 Gb for DS and 9.4 Gb US (Table 1). Using the average genome size of *E. coli* (4.6 Mb) it can be estimated that the DS library consisted of approximately 1826 bacterial genomes and the US library consisted of approximately 2043 bacterial genomes. The total bacterial abundance in each sediment sample was calculated by qPCR to be  $9.11 \times 10^7$  bacteria  $g^{-1}$  sediment DS and  $6.78 \times 10^7$  bacteria  $g^{-1}$  sediment US. From this we can calculate that the DS library captured approximately

0.002% of the bacteria in a gram of river sediment downstream of the WWTP, and the US library captured approximately 0.003% of the bacteria in a gram of river sediment upstream of the WWTP.

### 3.2. Resistance gene abundance

Antibiotic resistant clones were selected and RFLP was performed on extracted plasmid DNA to determine the number of unique clones conferring resistance to each antibiotic (Table 2). Several hundred clones conferring ampicillin resistance were recovered in both the DS and US libraries; analysis of a subset of these revealed more than 50 unique clones indicating that at least one in every 36 genomes carried an ampicillin resistance gene downstream. There was a significantly higher number of antibiotic resistant clones from the DS library compared to the US library for antibiotics neomycin (14 DS vs. 6 US) (Chi Square 4.232  $P=0.0397$ ), amikacin (4 DS vs. 0 US) (Chi Square 23.040  $P<0.0001$ ) and ciprofloxacin (4 DS vs. 1 US) (Chi Square 18.181  $P<0.0001$ ). All unique clones were resistant at clinically significant breakpoints (Table 2) using published methods (Andrews, 2001).

### 3.3. Antibiotic resistant gene identities

Transposon mutagenesis was performed on a subset of antibiotic resistant clones selected at random in order to investigate the genes responsible for conferring resistance (Table 3). The  $\beta$ -lactamase gene *bla*<sub>TEM</sub> was identified in multiple ampicillin resistant clones in both DS and US libraries. The flanking regions of each *bla*<sub>TEM</sub> were unique proving they were from different host backgrounds. Resistance to gentamicin was conferred by genes bearing similarity to clinically important genes such as aminoglycoside 3'-adenylyltransferase (90%) found in clinical pathogens such as *Yersinia pestis*. Genes previously not associated with gentamicin resistance were also recovered (Table 3). Ciprofloxacin resistance was attributed to the proteins RecA (74%) and RecX (33%), which have to date not been associated with resistance to fluoroquinolones. Other unusual genes were recovered for neomycin and amikacin resistance.

### 3.4. Diversity of aminoglycoside resistance genes

Three clones conferring resistance to aminoglycosides contained highly divergent acetyltransferases with 36–59% protein similarity to known proteins. Phylogenetic

**Table 2**  
Analysis of resistant clones in DS and US libraries.

Antibiotic	MIC of <i>Epi300</i> strain (mg L <sup>-1</sup> )	Number of resistant clones DS	Number of resistant clones US	Proportion of bacteria carrying resistant gene downstream (%)	Proportion of bacteria carrying resistant gene upstream (%)	MIC tested for resistant clones (mg L <sup>-1</sup> )
Ampicillin	8	>50	>50	2.74	2.45	16
Gentamicin	3	15	9	0.82	0.44	6
Neomycin	8	14	6	0.76	0.29	16
Amikacin	4	4	0	0.22	0	16
Ciprofloxacin	0.25	4	1	0.22	0.05	1

The estimated proportion of bacteria in the sediment resistant to each antibiotic was calculated by dividing the number of resistant unique clones by the average number of genomes in the library, this was then expressed as a percentage.

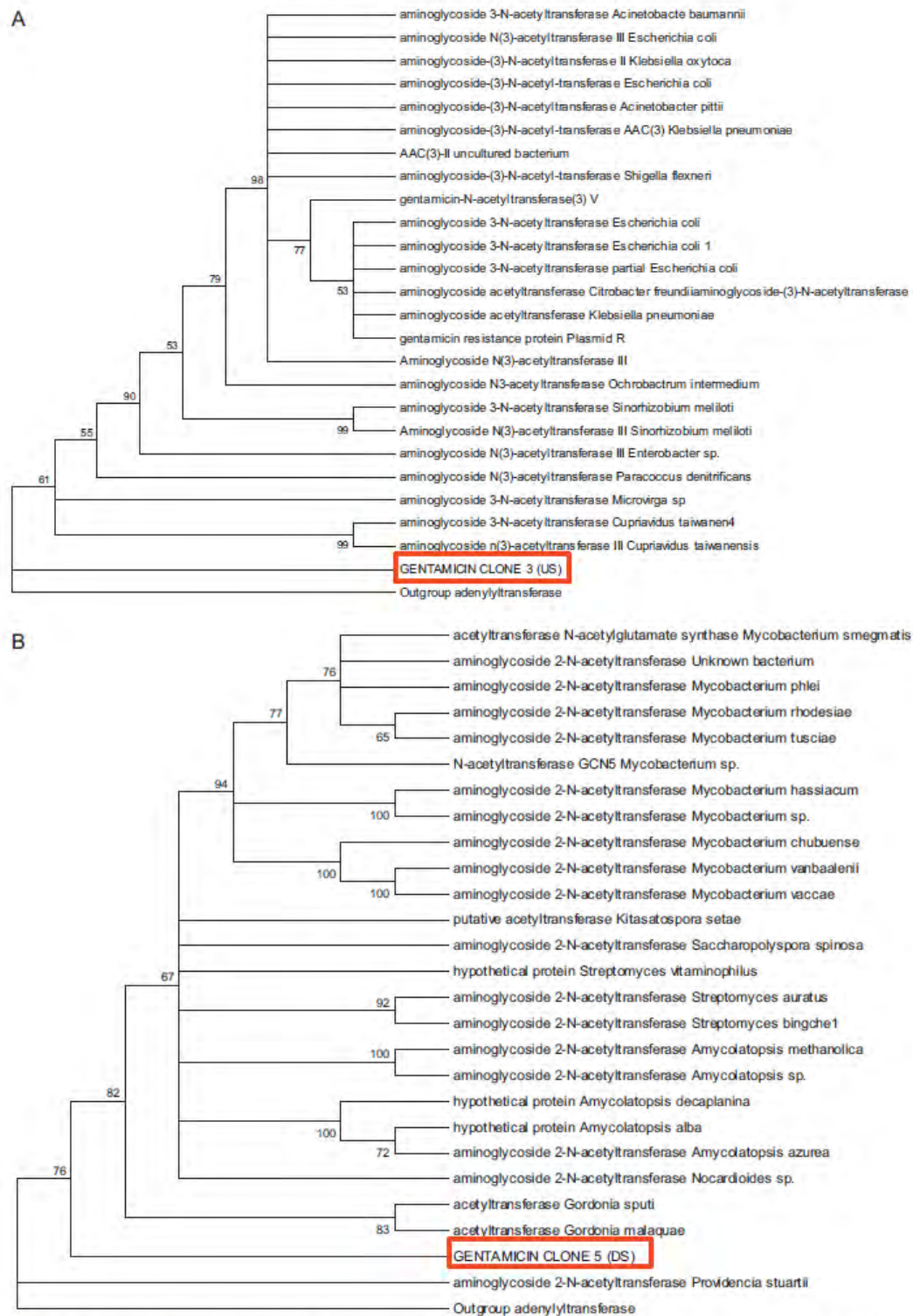


Fig. 1. Phylogenetic relationships of acetyltransferases recovered US and DS. (A) Gentamicin clone 3 US. (B) Gentamicin clone 5 DS and (C) Amikacin clone 1 DS. Bootstraps inferred from 1000 replications. Branches < 50% bootstraps were collapsed. The evolutionary distances were computed using the p-distance method (Tamura et al., 2011) and are in the units of the number of amino acid differences per site.

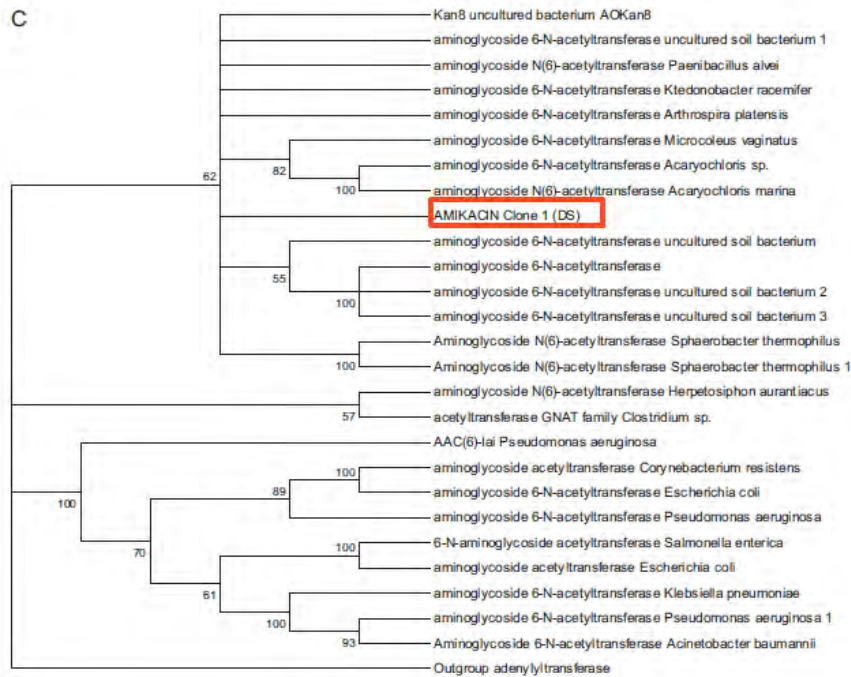


Fig. 1. (Continued).

analysis revealed the three genes encoded for proteins related to aminoglycoside 3'-N-acetyltransferases (Gentamicin clone 3 US), aminoglycoside 2'-N-acetyltransferases (Gentamicin clone 5 DS) and aminoglycoside 6'-N-acetyltransferases (Amikacin clone 1 DS) (Fig. 1). Gentamicin clone US3 was not recovered in the aminoglycoside 3'-N-acetyltransferase clade and formed an outlier with the root. The two other clones were similar to genes present in environmental bacteria such as actinobacteria and cyanobacteria.

#### 4. Discussion

This study revealed that WWTP effluent increased the abundance of bacterial resistance to clinically important antibiotics in river sediments. The presence of clinically relevant resistance genes in the environment has previously been reported (Forsberg et al., 2012), however this study illustrates a potential dissemination route via WWTPs. Although sequence based studies have suggested that areas impacted by WWTP effluent contain elevated numbers of sequences of resistance genes (Port et al., 2012), this is the first comparative study of the functional river resistome before and after the addition of effluent.

The number of unique resistance clones in DS libraries and US libraries were recorded, and the most prevalent phenotype was ampicillin resistance. We can estimate a prevalence of  $\sim 2 \times 10^5$  ampicillin resistance genes in the downstream sediment and  $1.65 \times 10^5$  ampicillin

resistance genes in the upstream sediment which indicates the widespread resistance to this antibiotic. This is not unexpected as a number of studies have illustrated the abundance of resistance to semisynthetic  $\beta$ -lactams (Allen et al., 2009). The amikacin resistance population showed the largest amplification in numbers for downstream samples, with an estimated sevenfold increase of resistance genes at  $2 \times 10^4$  DS and  $3 \times 10^3$  US. This level of amplification was closely followed by ciprofloxacin and neomycin. Prevalence data here suggests an input of resistance determinants or selective agents through the WWTP effluent.

Aminoglycoside resistance genes recovered were extremely diverse, however some encoded for acetyltransferases, the same family of genes conferring resistance in clinical bacteria (Chevereau et al., 1974). All these genes recovered when expressed in the current study gave clinically relevant MICs to at least one aminoglycoside antibiotic. A number of environmental studies concerned with reservoirs of resistance genes have recovered similarly diverse genes (McGarvey et al., 2012) however none have done estimates of prevalence or investigated potential routes of dissemination. In addition our study revealed a novel gentamicin resistance gene which appeared to have an independent phylogeny to other acetyltransferases. This study supports previous research which indicated resistance gene diversity in uncultured environmental bacteria is much higher than that studied in clinically relevant bacteria (Forsberg et al., 2012).

**Table 3**  
Identities of resistance genes and predicted proteins from clones analysed by transposon mutagenesis.

Antibiotic resistance conferred (library)	Predicted size of protein (amino acids)	Predicted domains	Nearest sequence identity (bacteria identity)
Gentamicin clone 1 (US library)	420	Potassium transporter superfamily	77% Potassium transport protein ( <i>Janthinobacterium</i> sp.)
Gentamicin clone 2 (US library)	58	None	75% Hypothetical protein ( <i>Escherichia coli</i> )
Gentamicin clone 3 (US library)	264	Aminoglycoside 3-N-acetyltransferase	59% Aminoglycoside-(3)-N-acetyltransferase ( <i>Escherichia coli</i> )
Gentamicin clone 4 (US library)	329	Thiamine pyrophosphate family	88% Pyruvate dehydrogenase subunit E1 ( <i>Janthinobacterium</i> sp.)
Gentamicin clone 5 (DS library)	178	Aminoglycoside 3-N-acetyltransferase	36% Acetyltransferase (GNAT) family protein ( <i>Providencia rettgeri</i> )
Gentamicin clone 6 (DS library)	77	DUF4111	90% Aminoglycoside 3'-adenylyltransferase ( <i>Yersinia pestis</i> )
Gentamicin clone 7 (DS library)	88	Aminoglycoside 3'-phosphotransferase (APH)	100% Aminoglycoside 3'-phosphotransferase ( <i>Pseudomonas putida</i> )
Amikacin clone 1 (DS library)	185	Aminoglycoside 3-N-acetyltransferase	58% Aminoglycoside N(6)-acetyltransferase ( <i>Gloeocapsa</i> sp.)
Amikacin 2 (DS library)	119	Nucleotidyl transferase superfamily	62% Methionyl-tRNA synthetase ( <i>Halicomonobacter hydrossis</i> )
Ampicillin clone 1 (US library)	289	Beta-lactamase2 superfamily	99% Beta-lactamase TEM ( <i>Bacillus subtilis</i> )
Ampicillin clone 2 (DS library)	289	Beta-lactamase2 superfamily	99% Beta-lactamase TEM ( <i>Bacillus subtilis</i> )
Ampicillin clone 3 (DS library)	289	Beta-lactamase2 superfamily	99% Beta-lactamase TEM ( <i>Bacillus subtilis</i> )
Neomycin clone 1 (DS library)	107	TroA like superfamily FepB BC-type Fe <sup>3+</sup> -hydroxamate transport system, periplasmic component	48% Hypothetical protein ( <i>Streptomyces</i> sp.)
Neomycin clone 2 (DS library)	162	Glycosyltransferase family 25	36% Glycosyltransferase 25 family member 1 ( <i>Agrobacterium</i> sp.)
Ciprofloxacin clone 1 (DS library)	145	RecX (recombination regulator)	33% Regulatory protein RecX ( <i>Listeria seeligeri</i> )
	154	RecA (bacterial DNA recombination protein)	74% Recombinase A ( <i>Geobacter lovleyi</i> )

Identities of resistance genes and predicted proteins from clones analysed by transposon mutagenesis. Predicted size of proteins were generated using ORF finder by NCBI. Translated nucleotide identities were used to generate protein sequence from which predicted domains and sequence identity were obtained using NCBI BLASTP.

Significant increases in the number of aminoglycoside resistant clones observed DS compared to US combined with the sequence data is the first evidence that anthropogenic pollution such as WWTP effluent increases the abundance of environmentally diverse resistance genes.

Sequence analysis of some resistance genes showed similarity to genes assigned to housekeeping functions. This may be evidence for evolutionary origins of resistance genes (Aminov and Mackie, 2007). One mechanism for ciprofloxacin resistance involved the recombination system RecA and RecX, RecX facilitates recombination repair by modulating RecA, thus could be a possible mechanism to repair the damage done by ciprofloxacin inhibition of the DNA gyrase (Cardenas et al., 2012). Amongst the other genes recovered, transporters were evident, but more obscure mechanisms of resistance attributed to enzymes such as glycosyltransferase and pyruvate dehydrogenase were also recovered, for both of which the resistance function is unknown. Our data demonstrates the diversity of genes recovered by expression analysis which can confer antibiotic resistance and indicates that there is a significant environmental reservoir of diverse mechanisms encoding protection against antibiotics (Martiny et al., 2011).

The impact of effluent discharge on the number of clones conferring resistance to clinically important antibiotics such as ciprofloxacin and amikacin is a cause for concern. Rivers are a vital part of the ecosystem and provide an essential source of drinking water for wild animals as well as being used for crop irrigation and recreational activities. High levels of antibiotic resistance in rivers resulting from WWTP pollution may explain why an increasing number of studies are reporting resistance genes in both wild and domestic animals. Several studies have demonstrated resistance genes in livestock such as the clinical *bla*<sub>CTX-M-15</sub> in UK cattle (Watson et al., 2012), chickens, turkeys (Randall et al., 2011) and dogs (Timofte et al., 2011). Increasing levels of antibiotic resistant genes in wild animals such as wolves (Goncalves et al., 2013) and birds (Dolejska et al., 2011; Ewers et al., 2010) could be through drinking contaminated water, particularly for seagulls which often feed and drink near sewage treatment plants (Fricker, 1984).

In conclusion, functional metagenomics provides a valuable resource when analysing resistance mechanisms in bacteria often revealing genes which sequenced-based analysis would not detect. WWTPs create a large reservoir of resistance genes which potentially can contribute to clinical cases of resistant bacteria in animals and humans through

direct exposure from contaminated river water. Further research is needed to improve waste disposal methods in order to reduce environmental reservoirs of antibiotic resistance and ultimately lower the clinical burden.

### Conflicts of interest

We declare that we have no conflicts of interest.

### Acknowledgements

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## Correspondence

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**An outbreak of a CTX-M-type  
 $\beta$ -lactamase-producing *Klebsiella pneumoniae*:  
the importance of using cefpodoxime to detect  
extended-spectrum  $\beta$ -lactamases**

Nigel P. Brenwald<sup>1</sup>\*, Gail Jevons<sup>1</sup>, Jenny M. Andrews<sup>1</sup>,  
Jian-Hui Xiong<sup>2</sup>, Peter M. Hawkey<sup>2</sup> and Richard Wise<sup>1</sup>

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Keywords: CTX-M-type  $\beta$ -lactamase, *Klebsiella pneumoniae*



5. Chanawong, A., M'Zali, F.H., Heritage, J., Xiong, J.H., & Hawkey, P.M. 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. *Antimicrob. Agents Chemother.*, 46, (3) 630-637

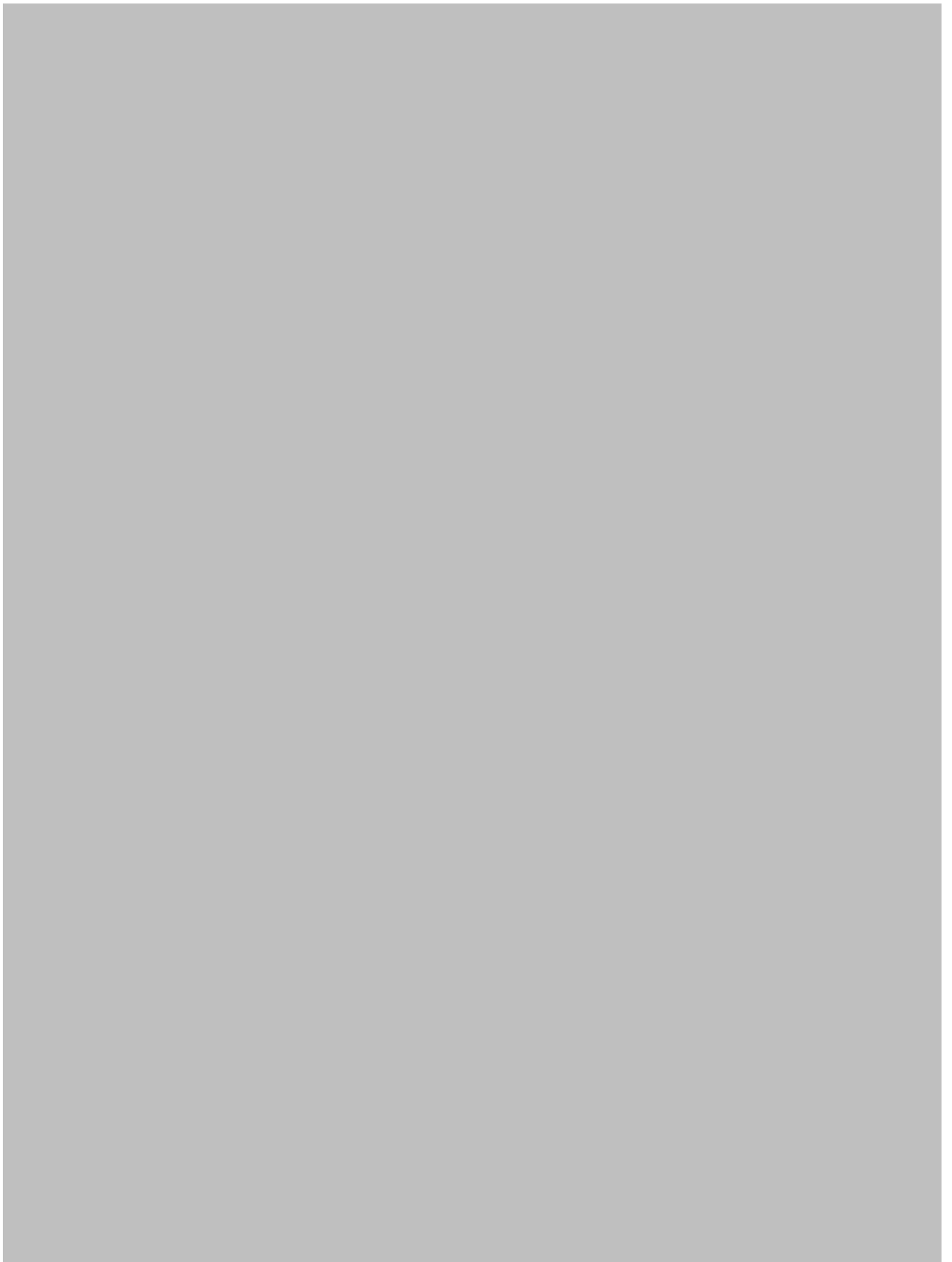
## Three Cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China

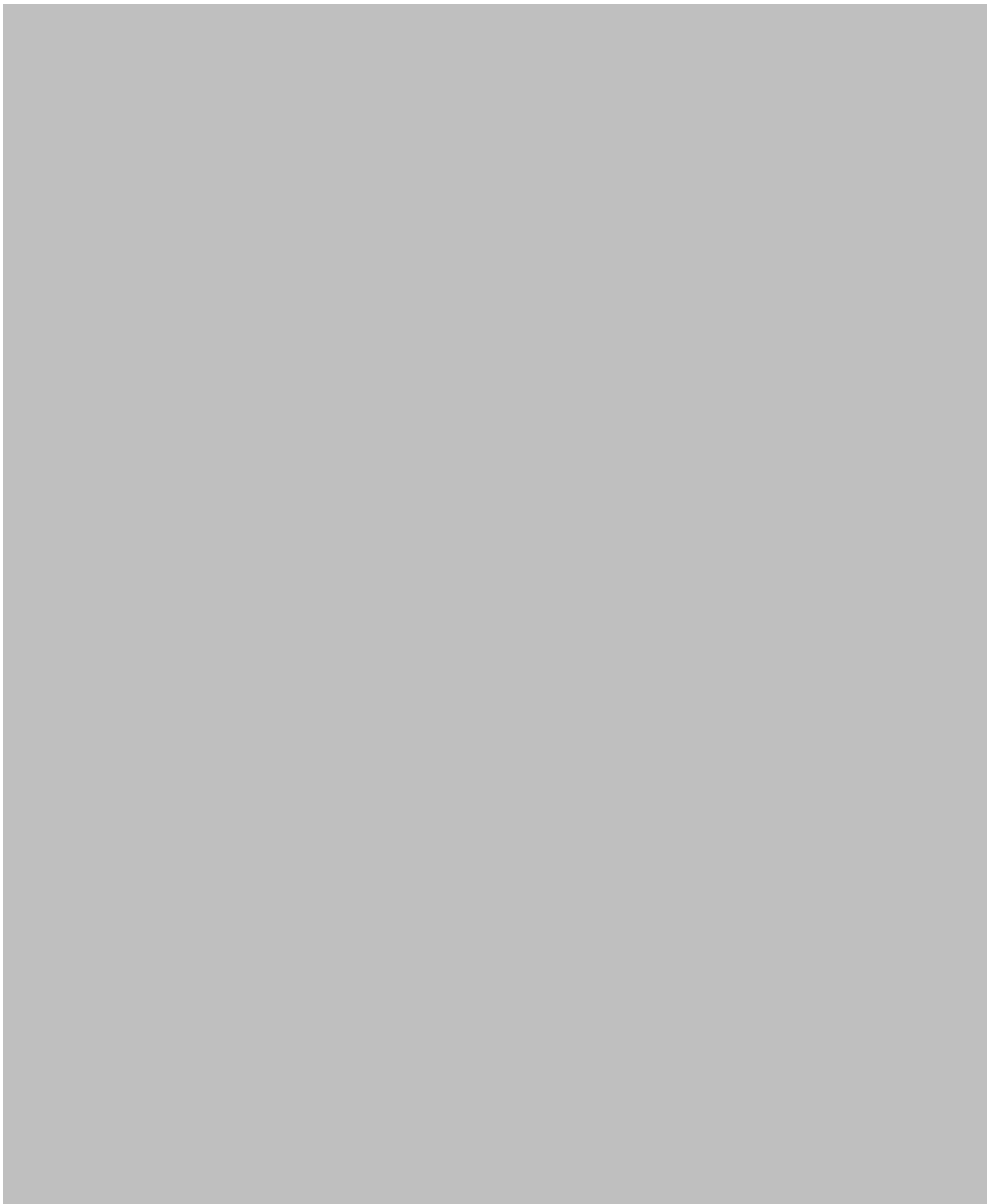
Aroonwadee Chanawong,<sup>1</sup> Fatima Hannachi M'Zali,<sup>1</sup> John Heritage,<sup>1</sup> Jian-Hui Xiong,<sup>2,3</sup> and Peter Michael Hawkey<sup>1,2\*</sup>

Division of Microbiology, University of Leeds, Leeds LS2 9JT,<sup>1</sup> and Department of Immunity and Infection, The Medical School, University of Birmingham, Birmingham B15 2TT,<sup>2</sup> United Kingdom, and First Municipal People's Hospital of Guangzhou, Guangzhou 510180, People's Republic of China<sup>3</sup>

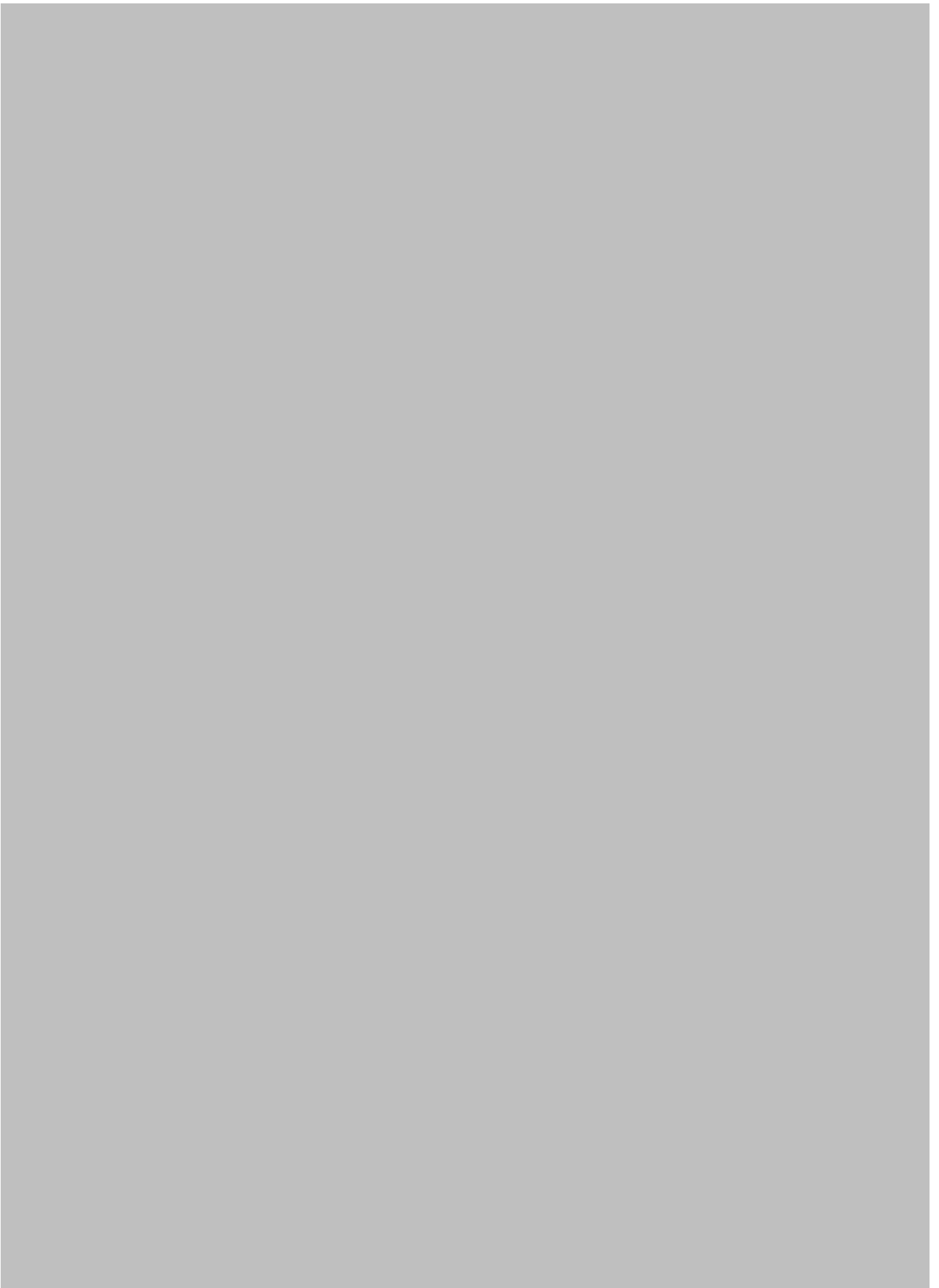
Received 11 April 2001/Returned for modification 1 August 2001/Accepted 30 October 2001

Of 15 extended-spectrum  $\beta$ -lactamase (ESBL)-producing isolates of the family *Enterobacteriaceae* collected from the First Municipal People's Hospital of Guangzhou, in the southern part of the People's Republic of China, 9 were found to produce CTX-M ESBLs, 3 produced SHV-12, and 3 produced both CTX-M and SHV-12. Eleven isolates produced either TEM-1B or SHV-11, in addition to an ESBL. Nucleotide sequence analysis of the 12 isolates carrying *bla*<sub>CTX-M</sub> genes revealed that they harbored three different *bla*<sub>CTX-M</sub> genes, *bla*<sub>CTX-M-9</sub> (5 isolates), *bla*<sub>CTX-M-13</sub> (1 isolate), and *bla*<sub>CTX-M-14</sub> (6 isolates). These genes have 98% nucleotide homology with *bla*<sub>Tob-2</sub>. The *bla*<sub>CTX-M</sub> genes were carried on plasmids that ranged in size from 35 to 150 kb. Plasmid fingerprints and pulsed-field gel electrophoresis showed the dissemination of the *bla*<sub>CTX-M</sub> genes through transfer of different antibiotic resistance plasmids to different bacteria, suggesting that these resistance determinants are highly mobile. Insertion sequence *ISEcp1*, found on the upstream region of these genes, may be involved in the translocation of the *bla*<sub>CTX-M</sub> genes. This is the first report of the occurrence of SHV-12 and CTX-M ESBLs in China. The presence of strains with these ESBLs shows both the evolution of *bla*<sub>CTX-M</sub> genes and their dissemination among at least three species of the family *Enterobacteriaceae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, isolated within a single hospital. The predominance of CTX-M type enzymes seen in this area of China appears to be similar to that seen in South America but is different from those seen in Europe and North America, suggesting different evolutionary routes and selective pressures. A more comprehensive survey of the ESBL types from China is urgently needed.

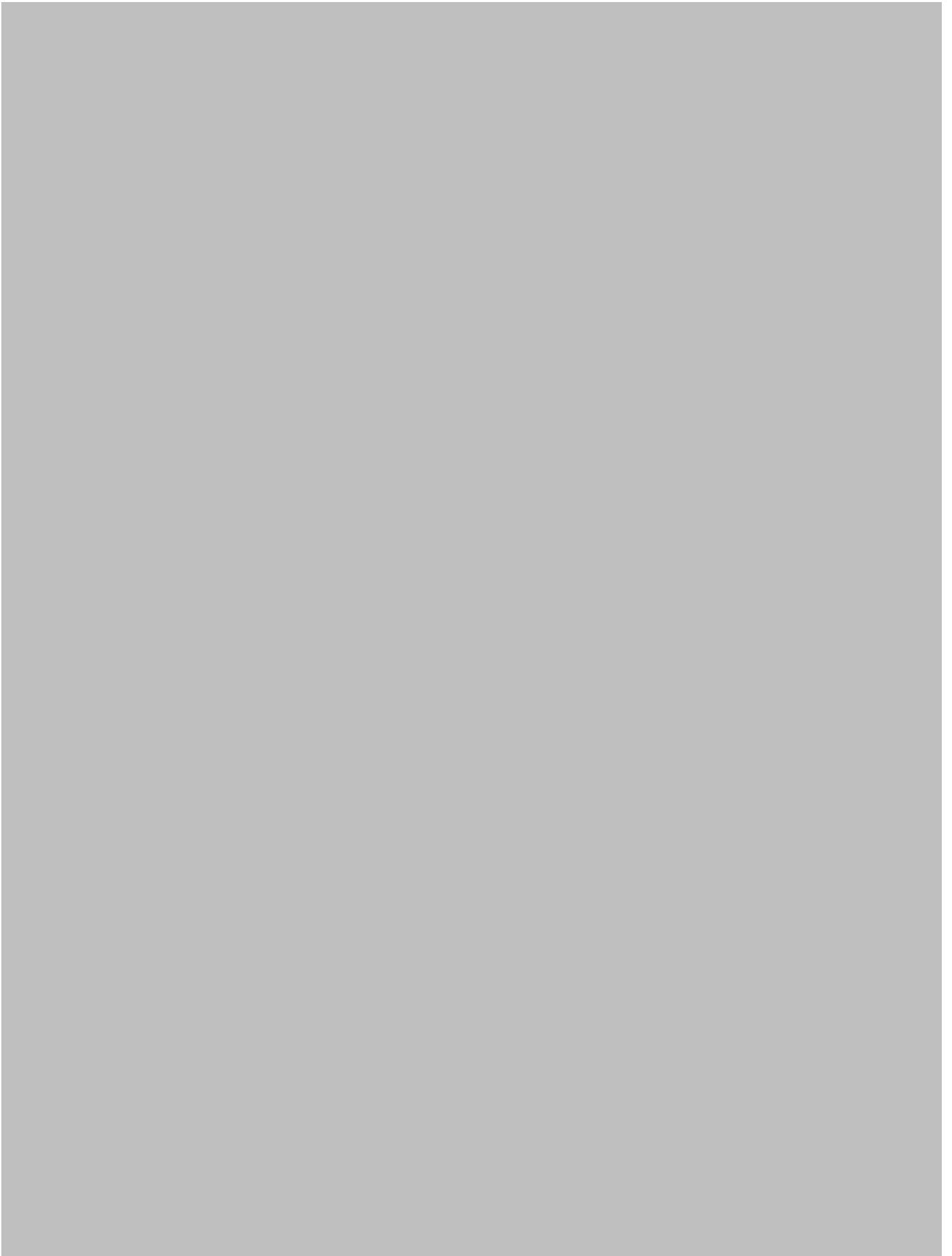
















6. Doumith, M., Dhanji, H., Ellington, M.J., Hawkey, P., & Woodford, N. 2011. Characterization of plasmids encoding extended-spectrum beta-lactamases and their addiction systems circulating among *Escherichia coli* clinical isolates in the UK. *J.Antimicrob.Chemother.* 67:878-885

## Characterization of plasmids encoding extended-spectrum $\beta$ -lactamases and their addiction systems circulating among *Escherichia coli* clinical isolates in the UK

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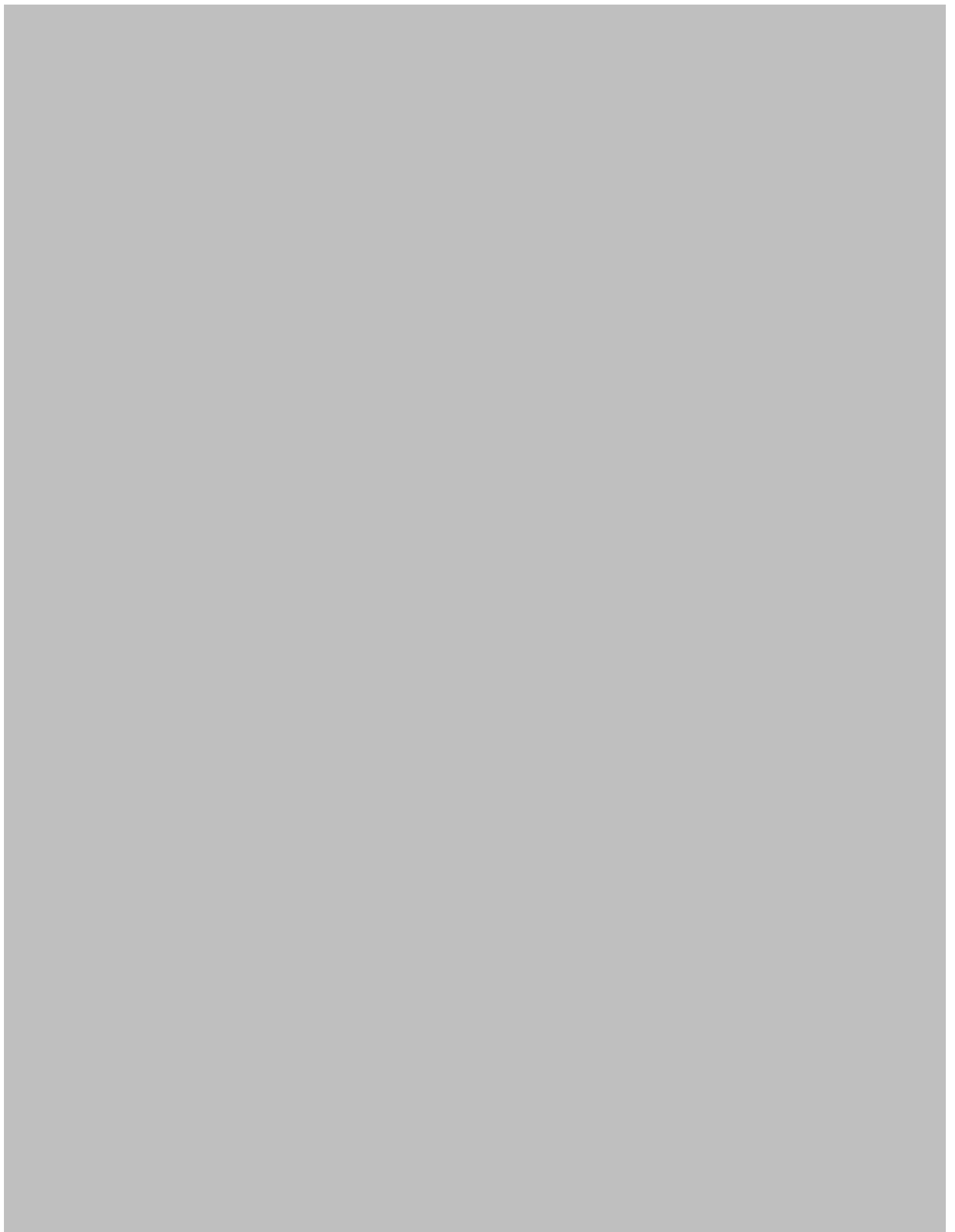
**Objectives:** To characterize plasmids encoding extended-spectrum  $\beta$ -lactamases (ESBLs) from a recent UK collection of clinical *Escherichia coli* isolates.

**Methods:** The isolates comprised 118 ESBL producers referred from 54 laboratories. Plasmids were transferred by electroporation, and their incompatibility groups, associated addiction systems and resistance genes with the flanking genetic environments were identified by PCR or sequencing.

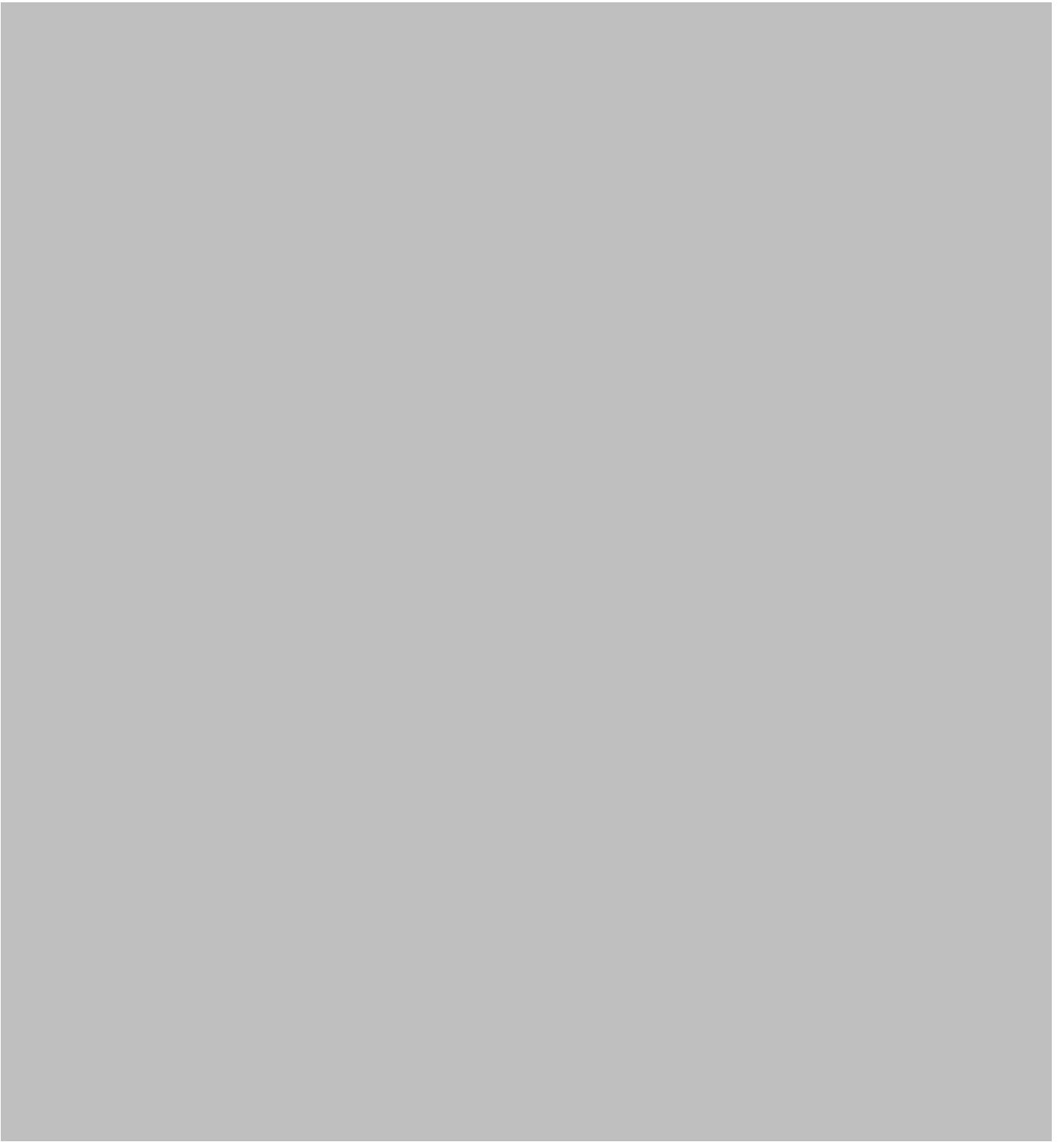
**Results:** Seventy isolates had plasmids encoding CTX-M-15 ( $n=53$ ), CTX-M-14 ( $n=9$ ), CTX-M-27 ( $n=1$ ), CTX-M-3 ( $n=2$ ) and SHV-12 ( $n=5$ ) ESBLs that were transformable; non-transformable ESBLs were mainly CTX-M enzymes (42/48). Most transformable  $bla_{CTX-M-15}$  genes (43/53) were harboured on single replicon or multireplicon IncF plasmids, with IncFIA4-FIB1-FII31 ( $n=11$ ) and IncFIA1-FII2 ( $n=15$ ) being most frequent; the latter included eight pEK499 plasmids, typical of UK epidemic strain A. Plasmids harbouring  $bla_{CTX-M-14}$  belonged variously to IncF, Inc11 and IncHI2 types, and 16 encoding CTX-M or SHV enzymes were non-typeable. Only IncF plasmid types carried the addiction systems sought and those with  $bla_{CTX-M-15}$  frequently harboured  $bla_{OXA-1}$  and  $aac(6')-Ib-cr$ , and often transferred trimethoprim and tetracycline resistance; those with  $bla_{CTX-M-14}$  encoded trimethoprim, sulphonamide, streptomycin and tetracycline resistance. Most ESBL genes were associated with the well-known mobile elements ISEcp1 and IS26, but nearly half (23/55) of the ISEcp1 sequences upstream of  $bla_{CTX-M-15}$  were interrupted by an IS26 at various positions.

**Conclusions:** Most ESBLs (70/118) were encoded by transformable plasmids, although a sizable minority could not be transformed. The majority of transformable plasmids (51/70; 72.9%) were diverse multiresistant IncF types possessing multiple addiction systems. The spread of  $bla_{CTX-M-15}$  can be attributed not just to clonal expansion, but also to the horizontal dissemination of related plasmids.

**Keywords:** replicon typing, toxin–antitoxin systems, multiresistance, ST131





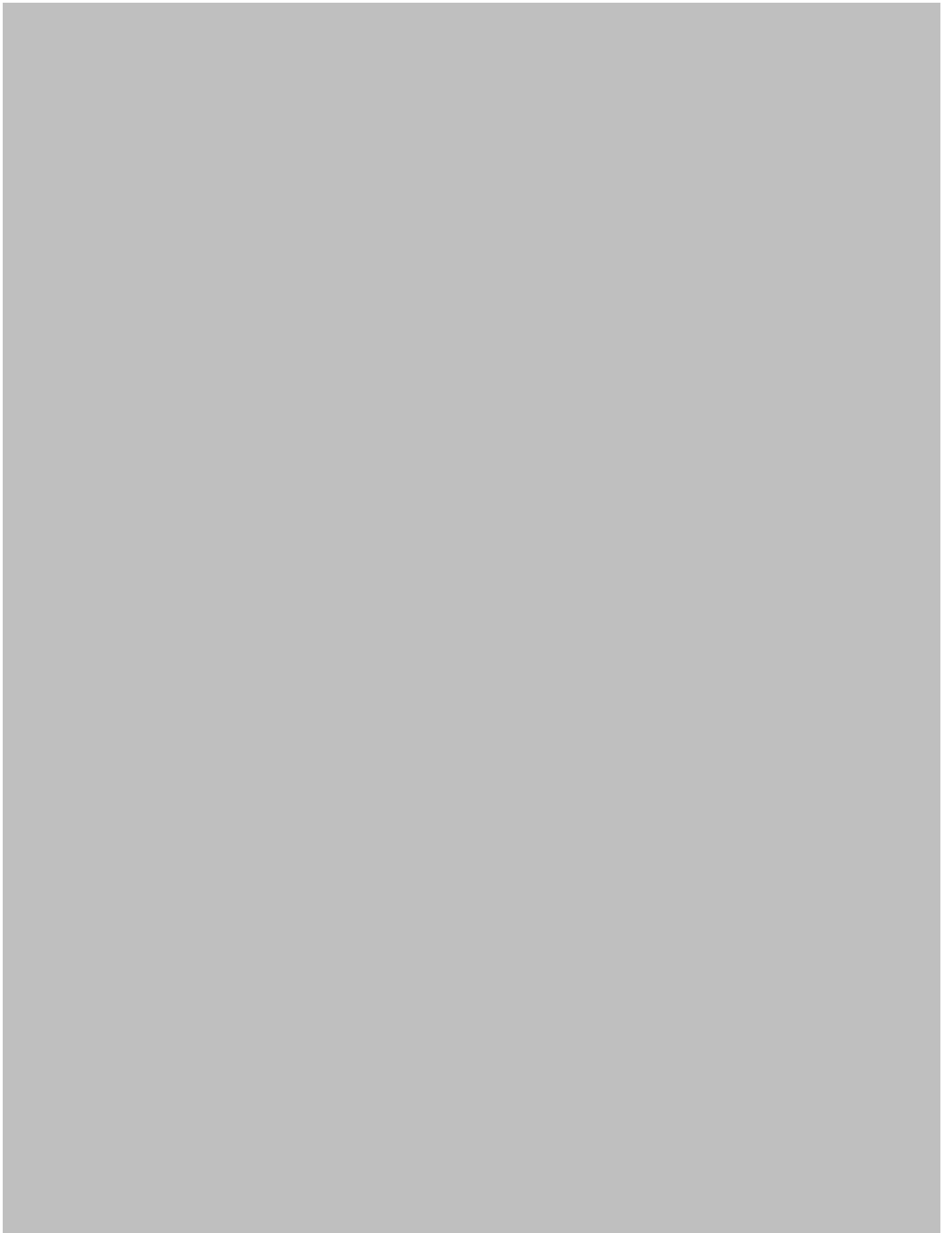












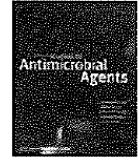
7. Ensor, V.M., Jamal, W., Rotimi, V.O., Evans, J.T., & Hawkey, P.M. 2009. Predominance of CTX-M-15 extended spectrum beta-lactamases in diverse *Escherichia coli* and *Klebsiella pneumoniae* from hospital and community patients in Kuwait. *Int.J.Antimicrob.Agents.*, 33, 487-489



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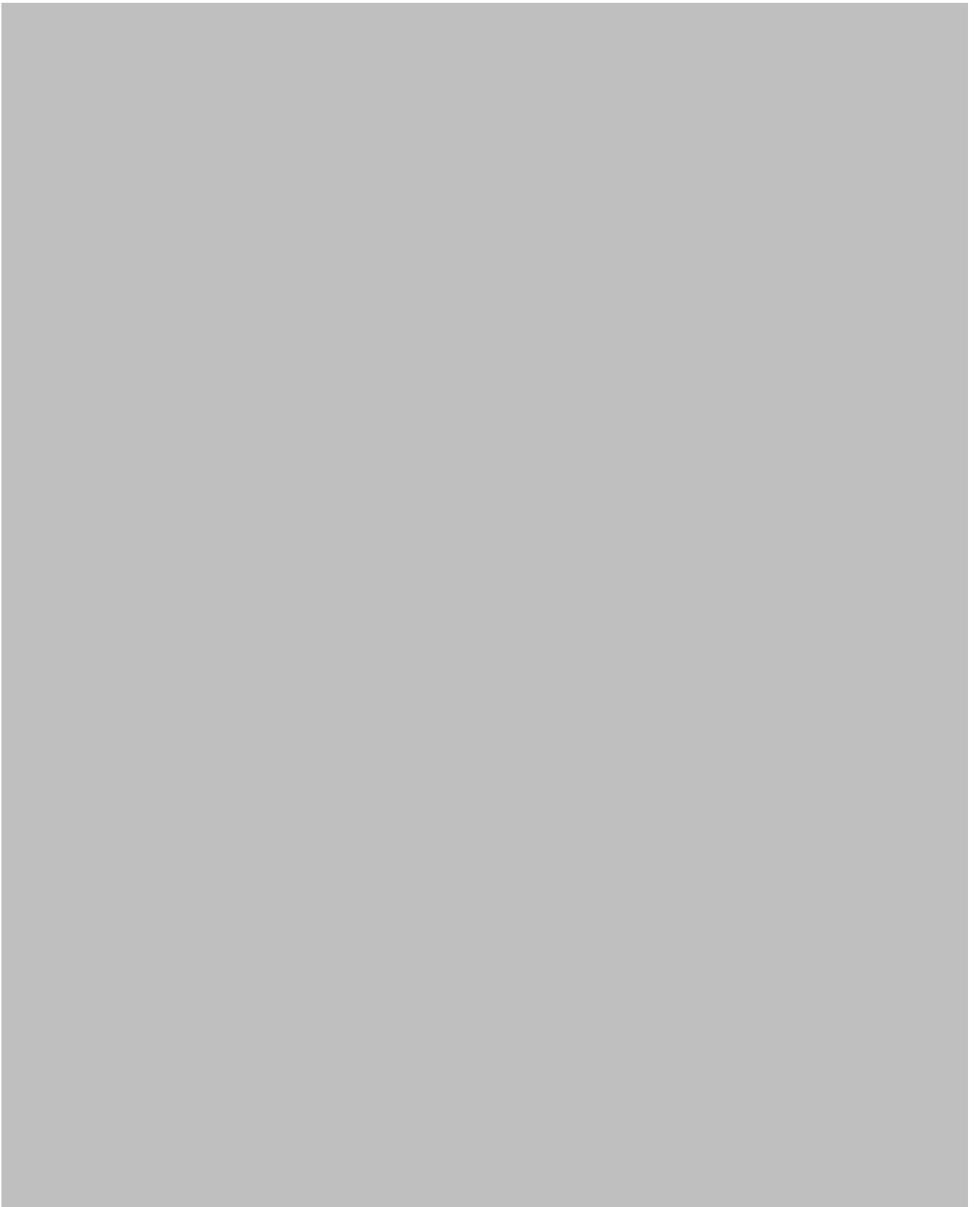
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### Letters to the Editor

**Predominance of CTX-M-15 extended spectrum  $\beta$ -lactamases in diverse *Escherichia coli* and *Klebsiella pneumoniae* from hospital and community patients in Kuwait**







8. Ensor, V.M., Livermore, D.M., & Hawkey, P.M. 2007. A novel reverse-line hybridization assay for identifying genotypes of CTX-M-type extended-spectrum  $\beta$ -lactamases. *J.Antimicrob.Chemother.*, 59, (3) 387-395

## A novel reverse-line hybridization assay for identifying genotypes of CTX-M-type extended-spectrum $\beta$ -lactamases

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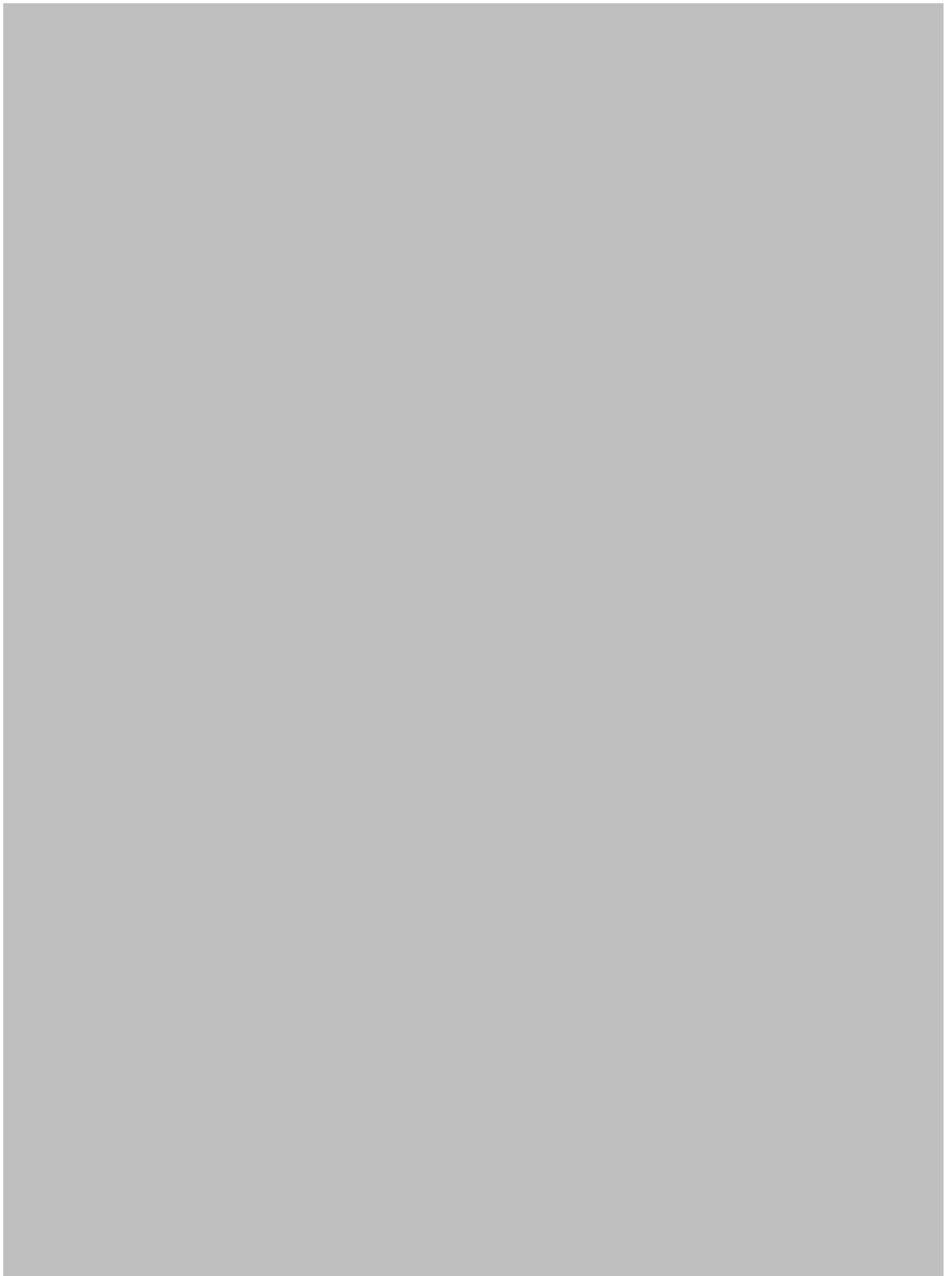
**Objectives:** To develop a reverse-line hybridization assay to identify CTX-M genotypes, potentially useful for large-scale investigation of surveillance collections.

**Methods:** Isolates carrying previously characterized *bla*<sub>CTX-M</sub> genes were used to develop the method. In addition, 334 isolates from five separate surveys were used to validate the method. CTX-M group was known from an independent multiplex PCR for 122 isolates and genotype was confirmed for 80 isolates by DNA sequencing. A multiplex PCR was designed to amplify a genotype-specific region within the *bla*<sub>CTX-M</sub> open-reading frame. Oligonucleotides were designed to hybridize to regions within each amplicon, covering mutations that distinguish among *bla*<sub>CTX-M</sub> genotypes.

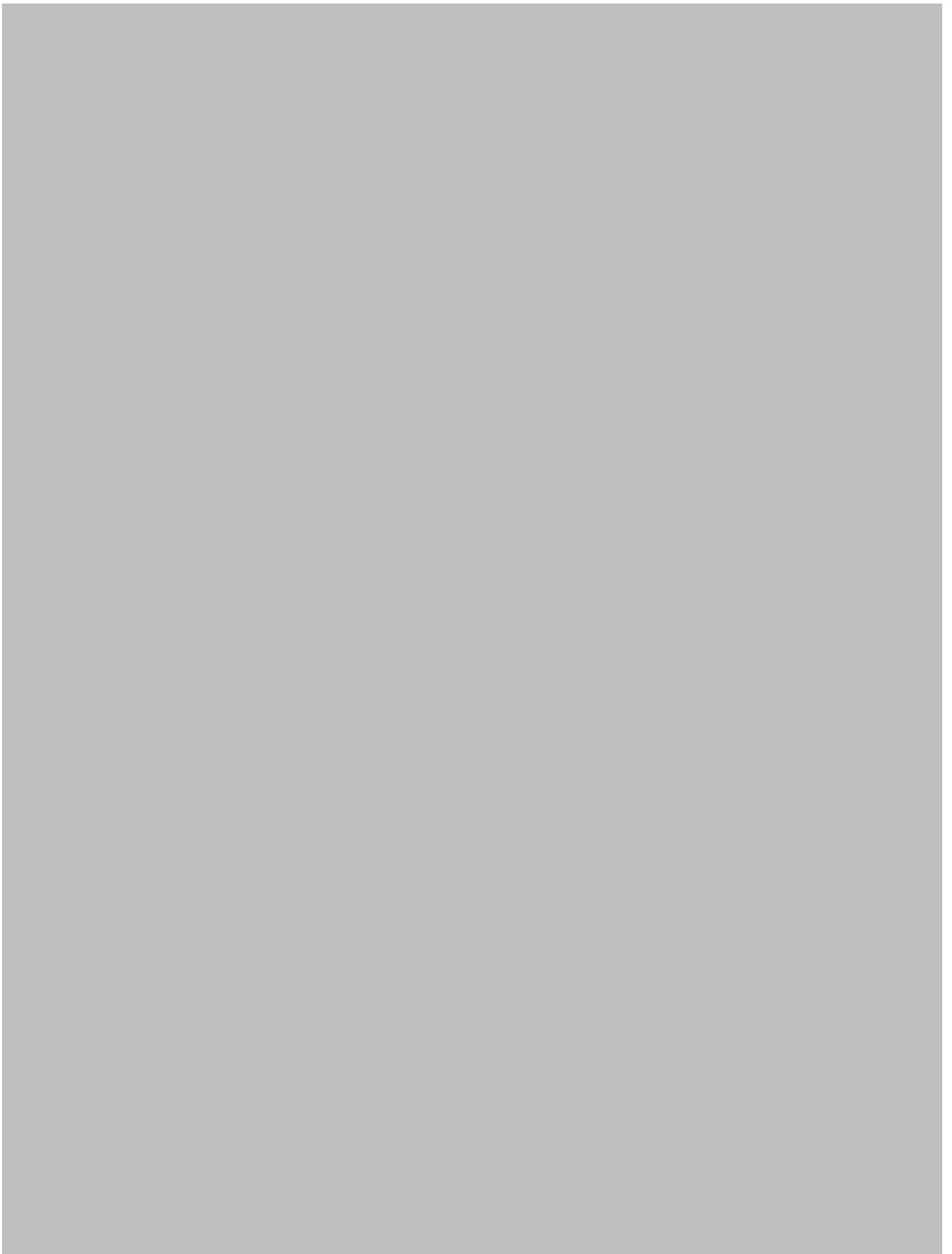
**Results:** CTX-M phylogenetic groups were identified by the multiplex PCR with 100% concordance. The reverse-line hybridization assay specifically identified commonly-reported variants within these groups (98.7% concordance).

**Conclusions:** The hybridization method enabled precise identification of CTX-M genes, rather than just to group level, without the need for DNA sequencing. In its present format, the method enables 43 isolates to be processed per membrane, giving results within one working day. It is a useful tool for the epidemiological investigation of *bla*<sub>CTX-M</sub> genes among survey collections of Enterobacteriaceae.

Keywords: ESBLs, genotyping, multiplex PCR, molecular epidemiology





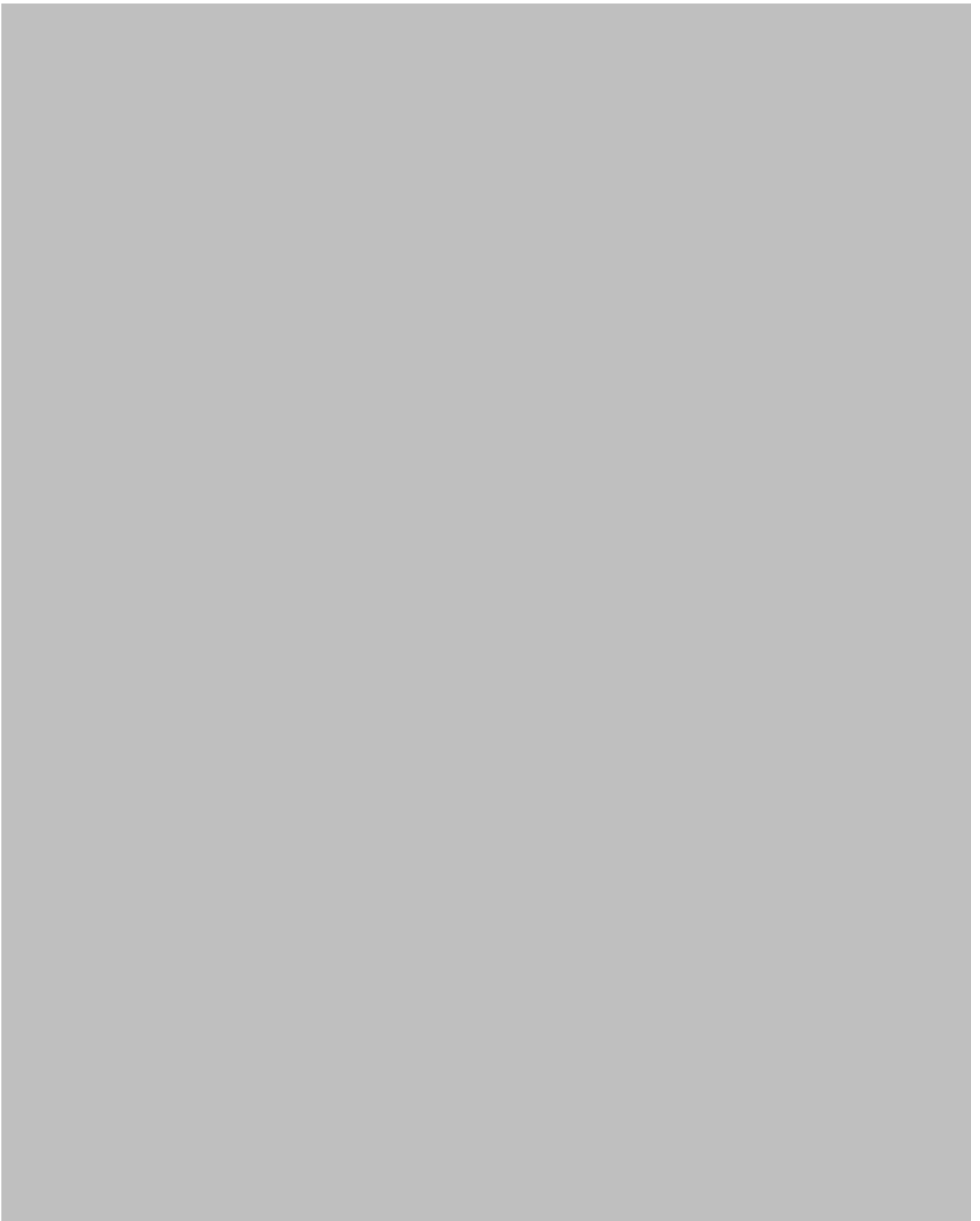
















9. Ensor, V.M., Shahid, M., Evans, J.T., & Hawkey, P.M. 2006. Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in *Enterobacteriaceae* from Indian hospitals. *J.Antimicrob.Chemother.*, 58, (6) 1260-1263 available from: PM:17071956

## Occurrence, prevalence and genetic environment of CTX-M $\beta$ -lactamases in Enterobacteriaceae from Indian hospitals

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**Objectives:** To determine occurrence, prevalence and CTX-M genotypes produced by Enterobacteriaceae from clinical samples from three geographically distant Indian hospitals and to detect linkage of IS26 with *bla*<sub>CTX-M</sub> and map its precise insertion position.

**Methods:** A total of 130, non-duplicate *Escherichia coli* and *Klebsiella pneumoniae* resistant to a third-generation cephalosporin (3GC) from three Indian centres were screened for extended-spectrum  $\beta$ -lactamase (ESBL) production using phenotypic detection methods. All isolates were screened for *bla*<sub>CTX-M</sub> using multiplex PCR. Precise CTX-M genotype was identified using reverse-line hybridization. All CTX-M-producing isolates were screened for linkage of IS26 with *bla*<sub>CTX-M</sub>. DNA sequencing was used to map the exact insertion position of this mobile element.

**Results:** Ninety-five of 130 3GC-resistant (73%) (73% of total *E. coli*, 72% of total *K. pneumoniae*) isolates were found to carry *bla*<sub>CTX-M-15</sub>. No other CTX-M genotype was detected. IS26 linkage with *bla*<sub>CTX-M-15</sub> was detected in 31% of isolates carrying *bla*<sub>CTX-M-15</sub>. DNA sequencing revealed variable insertion of this mobile element within *tnpA* of ISEcp1. RAPD-PCR typing demonstrated great diversity in isolates carrying *bla*<sub>CTX-M-15</sub>; no predominant clone was identified.

**Conclusions:** In contrast with other studies where greater diversity exists, CTX-M-15 was the only CTX-M ESBL produced in this Indian collection of unrelated *E. coli* and *K. pneumoniae*. This is the first systematic survey report from India detecting CTX-M-type  $\beta$ -lactamases. This is also the first report indicating such high mobility/diversity of insertion of IS26 in close association with *bla*<sub>CTX-M</sub> in a single bacterial collection.

**Keywords:** ESBLs, IS26, CTX-M-15, India









10. Gaze, W.H., Abdousslam, N., Hawkey, P.M., & Wellington, E.M. 2005. Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob. Agents Chemother.*, 49, (5) 1802-1807

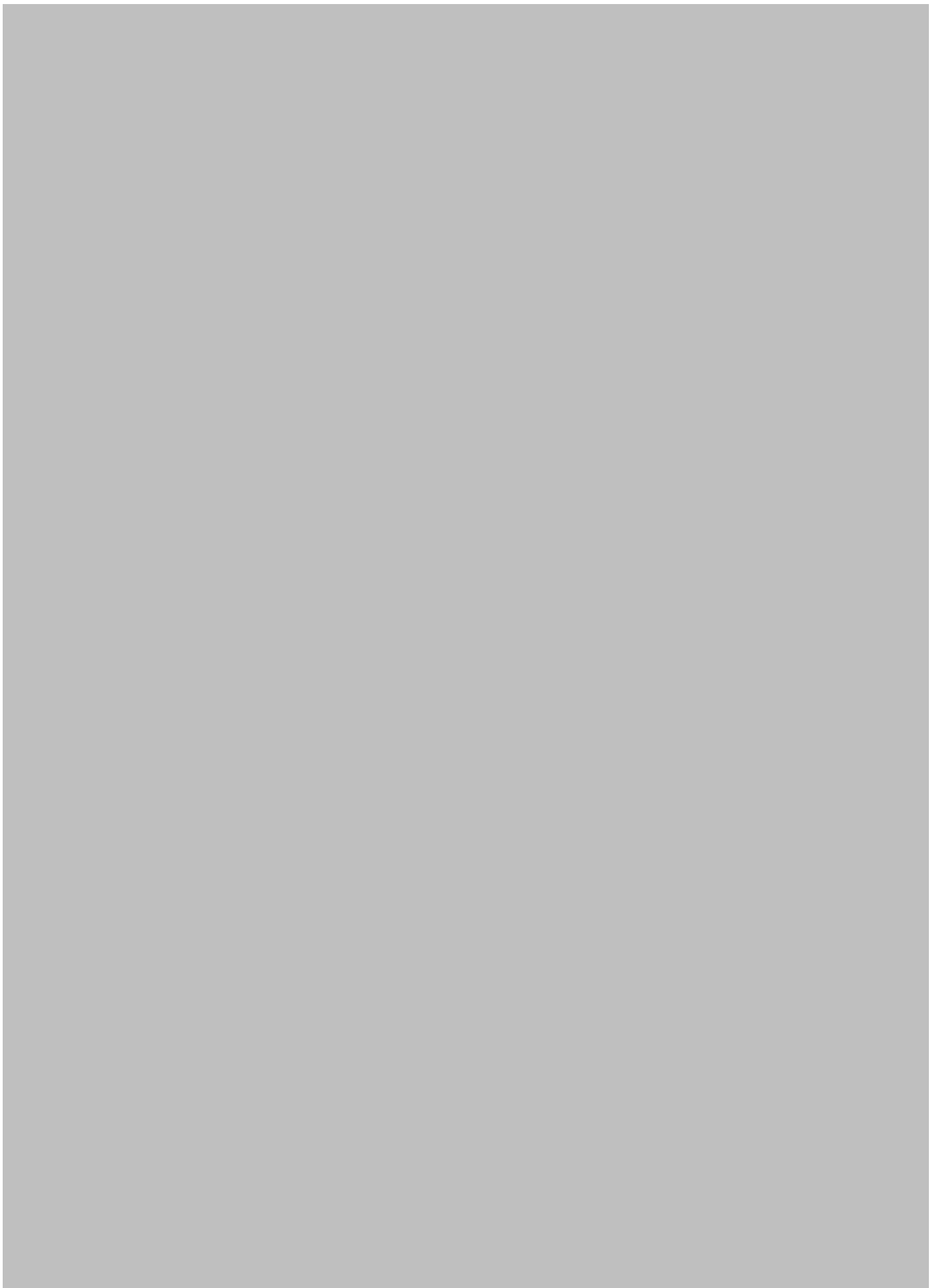
## Incidence of Class 1 Integrons in a Quaternary Ammonium Compound-Polluted Environment

W. H. Gaze,<sup>1</sup> N. Abdousslam,<sup>1</sup> P. M. Hawkey,<sup>2</sup> and E. M. H. Wellington<sup>1\*</sup>

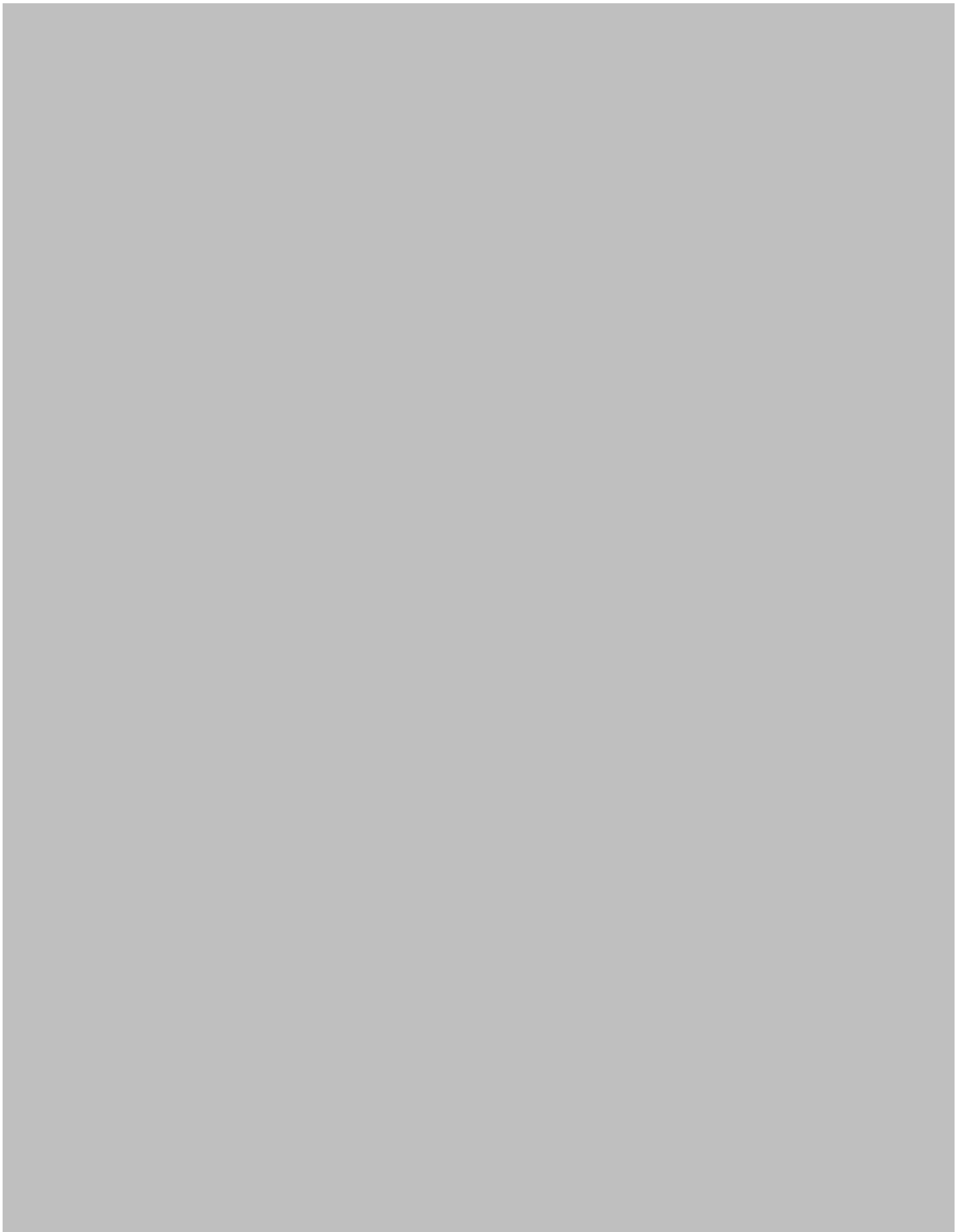
*Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom,<sup>1</sup> and  
University of Birmingham, Division of Immunity and Infection, Edgbaston,  
Birmingham B15 2TT, United Kingdom<sup>2</sup>*

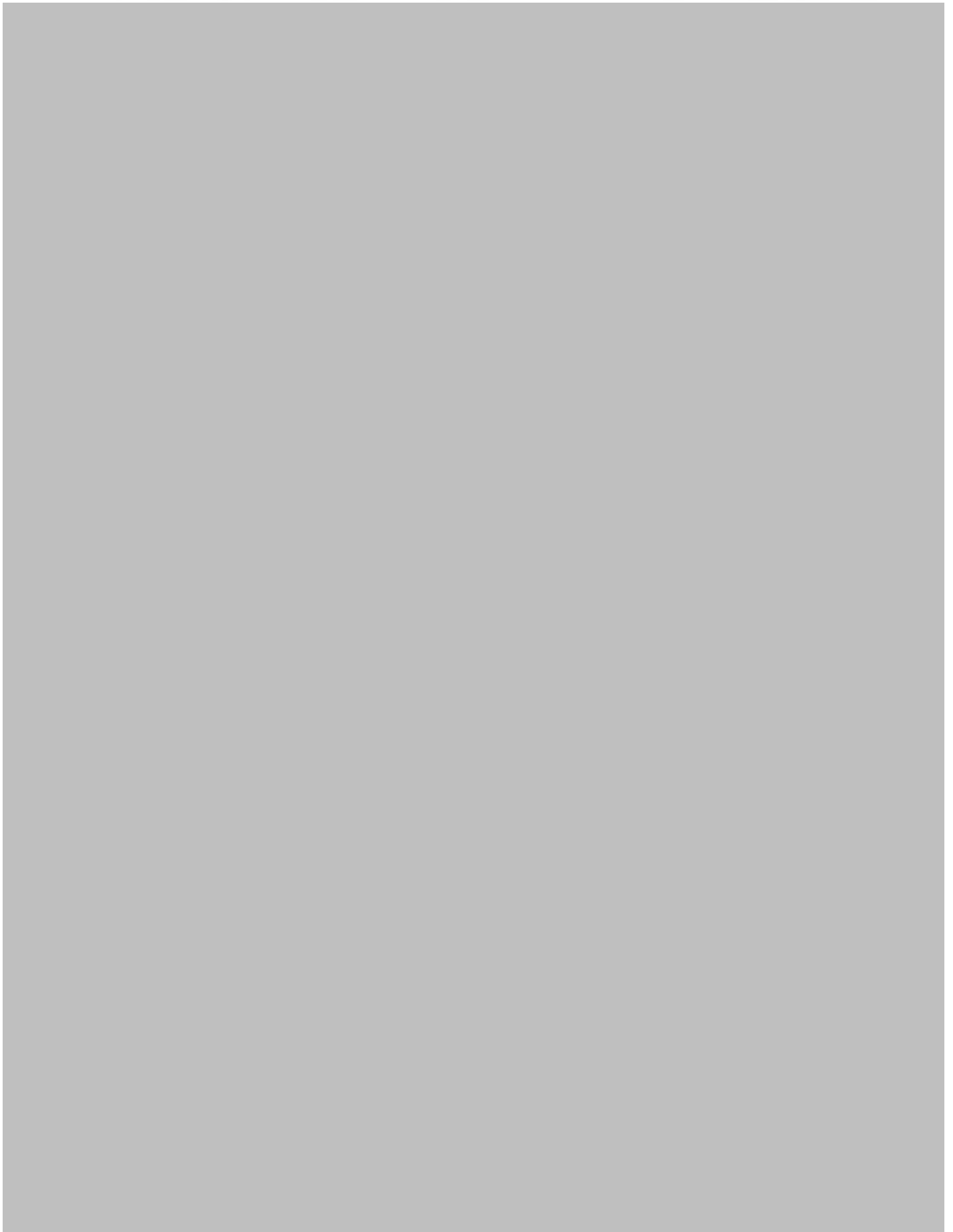
Received 21 July 2004/Returned for modification 12 September 2004/Accepted 30 January 2005

Samples of effluent and soil were collected from a reed bed system used to remediate liquid waste from a wool finishing mill with a high use of quaternary ammonium compounds (QACs) and were compared with samples of agricultural soils. Resistance quotients of aerobic gram-negative and gram-positive bacteria to ditallowdimethylammonium chloride (DTDMAC) and cetyltrimethylammonium bromide (CTAB) were established by plating onto nutrient agar containing 5 µg/ml or 50 µg/ml DTDMAC or CTAB. Approximately 500 isolates were obtained and screened for the presence of the *intI1* (class 1 integrase), *qacE* (multidrug efflux), and *qacEΔ1* (attenuated *qacE*) genes. QAC resistance was higher in isolates from reed bed samples, and class 1 integron incidence was significantly higher for populations that were preexposed to QACs. This is the first study to demonstrate that QAC selection in the natural environment has the potential to coselect for antibiotic resistance, as class 1 integrons are well-established vectors for cassette genes encoding antibiotic resistance.















11. Gaze, W.H., Zhang, L., Abdousslam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A.B., & Wellington, E.M. 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME.J.*, 5, (8) 1253-1261  
available from: PM:21368907

ORIGINAL ARTICLE

# Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment

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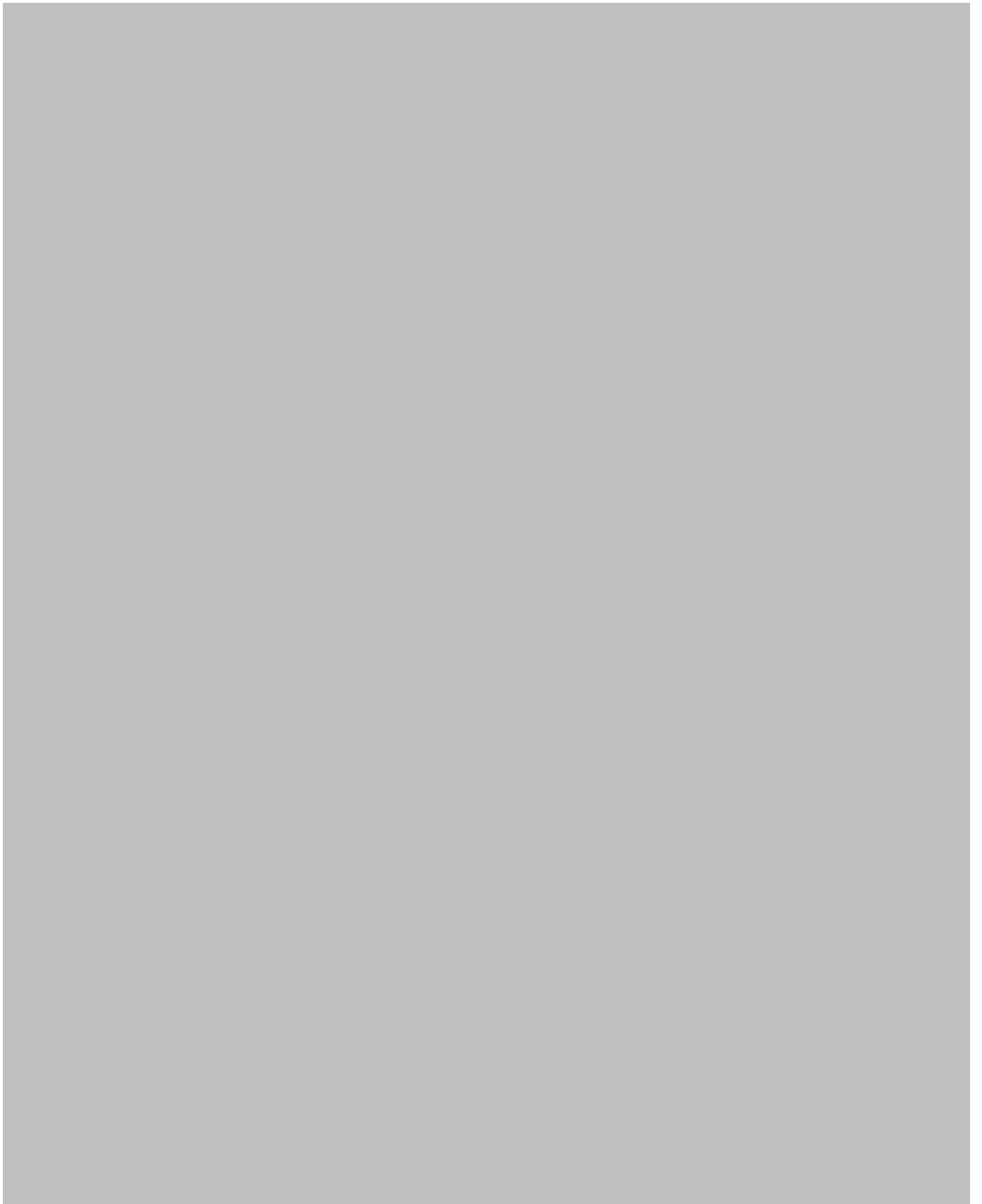
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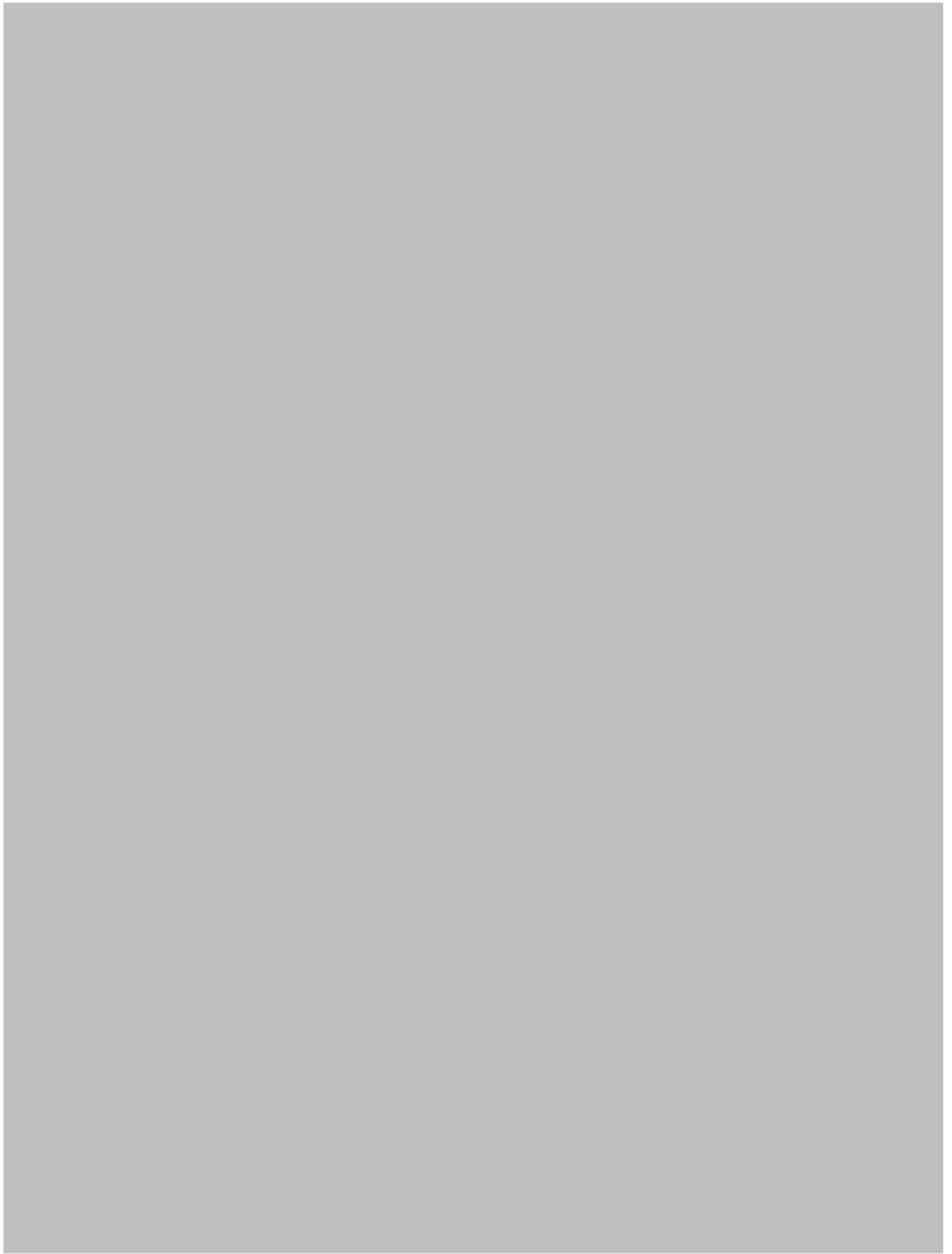
The impact of human activity on the selection for antibiotic resistance in the environment is largely unknown, although considerable amounts of antibiotics are introduced through domestic wastewater and farm animal waste. Selection for resistance may occur by exposure to antibiotic residues or by co-selection for mobile genetic elements (MGEs) which carry genes of varying activity. Class 1 integrons are genetic elements that carry antibiotic and quaternary ammonium compound (QAC) resistance genes that confer resistance to detergents and biocides. This study aimed to investigate the prevalence and diversity of class 1 integron and integron-associated QAC resistance genes in bacteria associated with industrial waste, sewage sludge and pig slurry. We show that prevalence of class 1 integrons is higher in bacteria exposed to detergents and/or antibiotic residues, specifically in sewage sludge and pig slurry compared with agricultural soils to which these waste products are amended. We also show that QAC resistance genes are more prevalent in the presence of detergents. Studies of class 1 integron prevalence in sewage sludge amended soil showed measurable differences compared with controls. Insertion sequence elements were discovered in integrons from QAC contaminated sediment, acting as powerful promoters likely to upregulate cassette gene expression. On the basis of this data,  $> 1 \times 10^{15}$  bacteria carrying class 1 integrons enter the United Kingdom environment by disposal of sewage sludge each year.

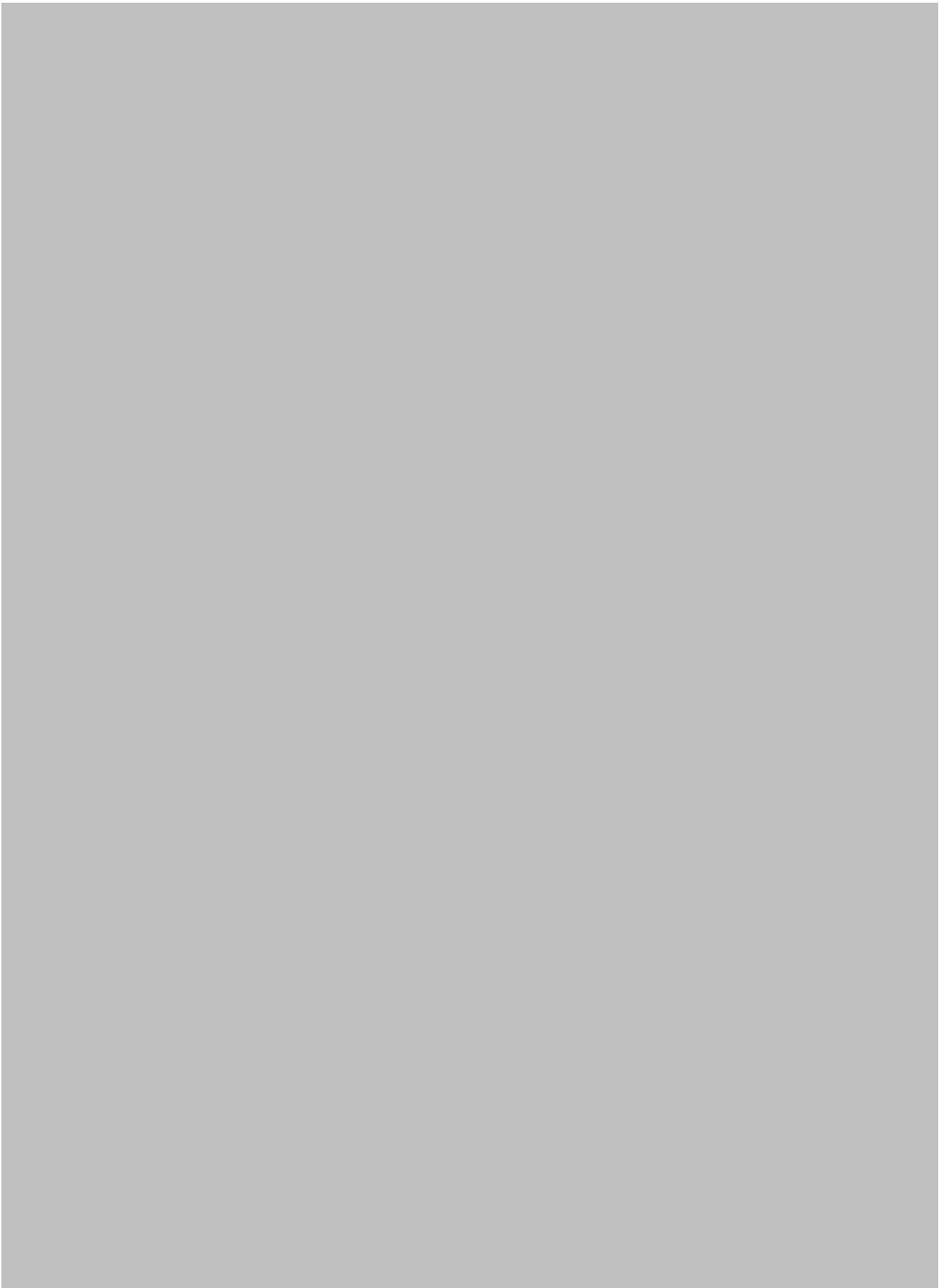
*The ISME Journal* advance online publication, 3 March 2011; doi:10.1038/ismej.2011.15

**Subject Category:** microbial population and community ecology

**Keywords:** integron; pollution; sewage; agriculture; horizontal gene transfer; antibiotic resistance

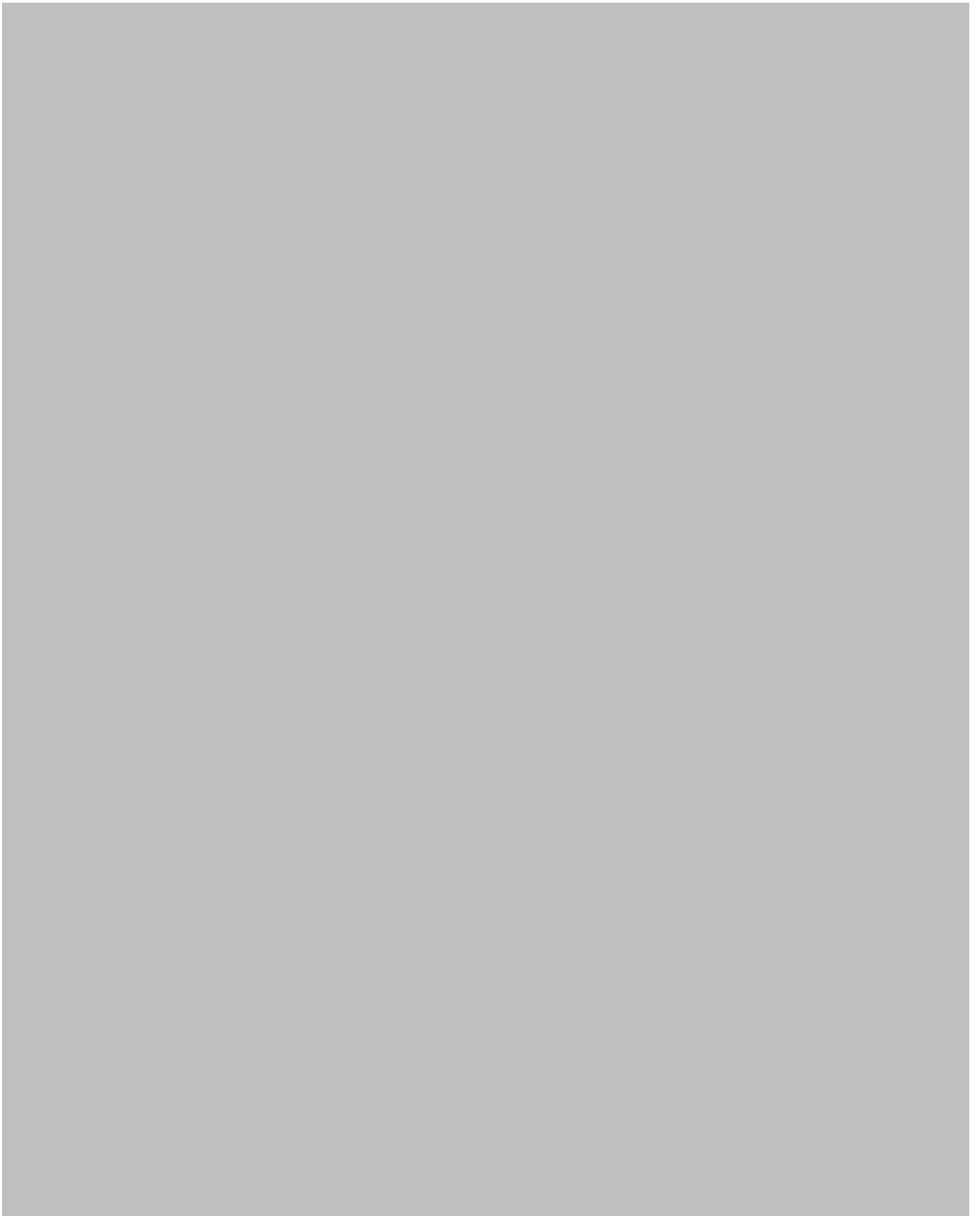




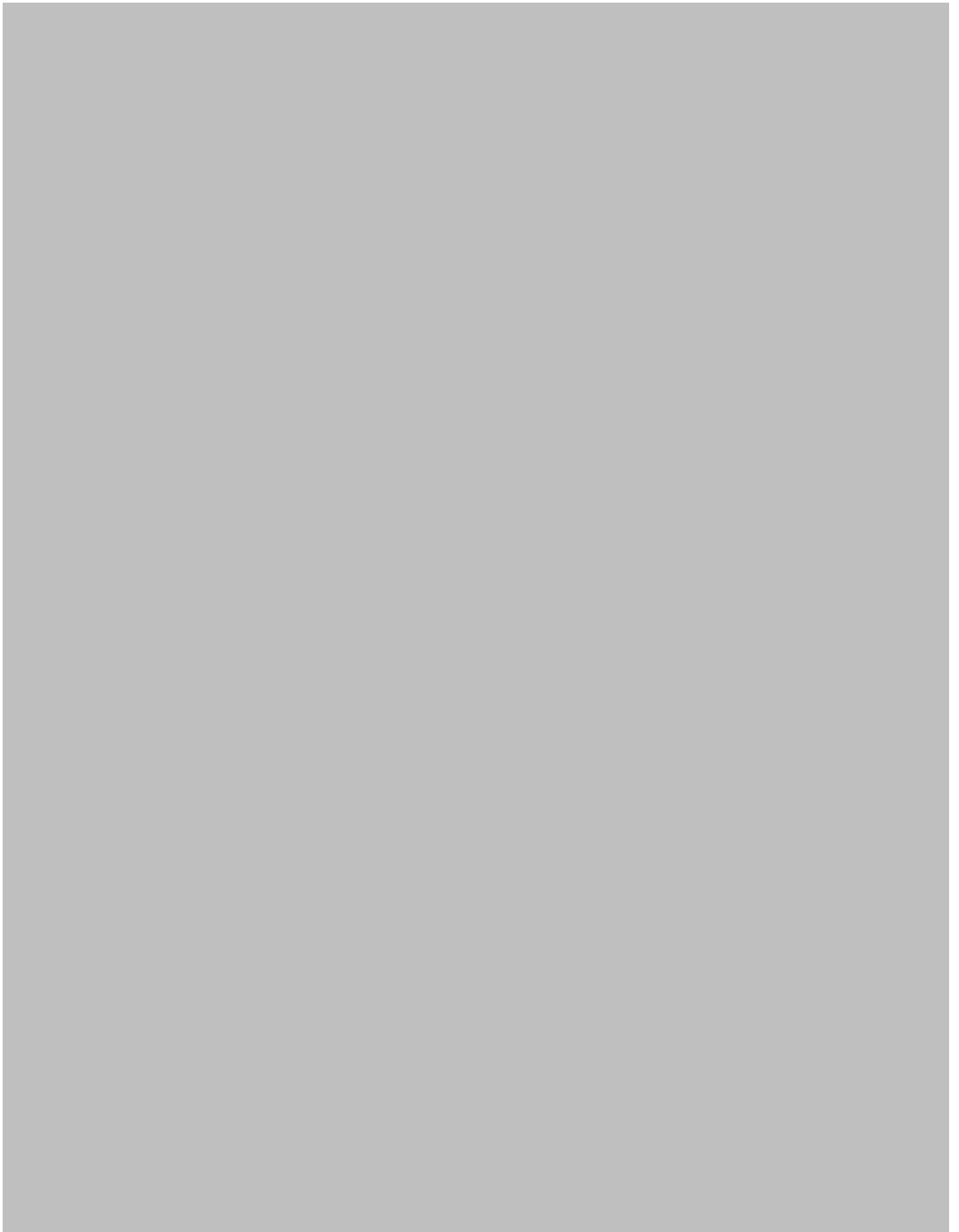


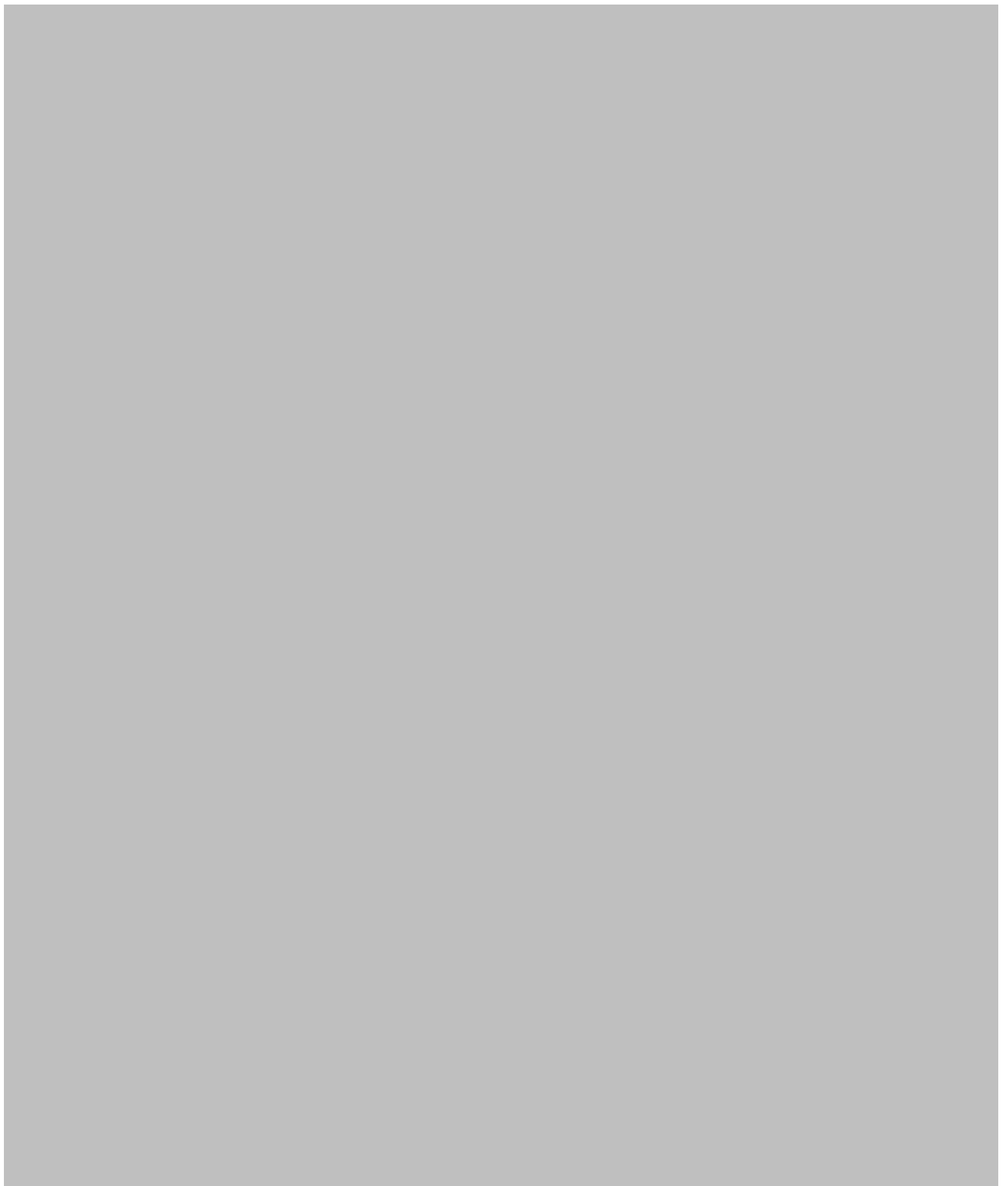












12. Geyer, C.N., Fowler, R.C., Johnson, J.R., Johnston, B., Weissman, S.J., Hawkey, P., & Hanson, N.D. 2016. Evaluation of CTX-M steady-state mRNA, mRNA half-life and protein production in various STs of *Escherichia coli*. *J.Antimicrob.Chemother.*, 71, (3) 607-616 available from: PM:26612874

## Evaluation of CTX-M steady-state mRNA, mRNA half-life and protein production in various STs of *Escherichia coli*

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**Objectives:** High levels of  $\beta$ -lactamase production can impact treatment with a  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combination. Goals of this study were to: (i) compare the mRNA and protein levels of CTX-M-15- and CTX-M-14-producing *Escherichia coli* from 18 different STs and 10 different phylotypes; (ii) evaluate the mRNA half-lives and establish a role for chromosomal- and/or plasmid-encoded factors; and (iii) evaluate the zones of inhibition for piperacillin/tazobactam and ceftolozane/tazobactam.

**Methods:** Disc diffusion was used to establish zone size. RNA analysis was accomplished using real-time RT-PCR and CTX-M protein levels were evaluated by immunoblotting. Clinical isolates, transformants and transconjugants were used to evaluate mRNA half-lives.

**Results:** mRNA levels of CTX-M-15 were up to 165-fold higher compared with CTX-M-14. CTX-M-15 protein levels were 2–48-fold less than their respective transcript levels, while CTX-M-14 protein production was comparable to the observed transcript levels. Nineteen of 25 *E. coli* (76%) had extended CTX-M-15 mRNA half-lives of 5–15 min and 16 (100%) CTX-M-14 isolates had mRNA half-lives of <2–3 min. Transformants had mRNA half-lives of <2 min for both CTX-M-type transcripts, while transconjugant mRNA half-lives corresponded to the half-life of the donor. Ceftolozane/tazobactam zone sizes were  $\geq 19$  mm, while piperacillin/tazobactam zone sizes were  $\geq 17$  mm.

**Conclusions:** CTX-M-15 mRNA and protein production did not correlate. Neither *E. coli* ST nor phylotype influenced the variability observed for CTX-M-15 mRNA or protein produced. mRNA half-life is controlled by a plasmid-encoded factor and may influence mRNA transcript levels, but not protein levels.

### Introduction

In Gram-negative bacteria,  $\beta$ -lactamase production is the most common mechanism identified conferring resistance to  $\beta$ -lactams.<sup>1</sup> Development of  $\beta$ -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam provides an effective method for evading this resistance mechanism. These inhibitors have minimal antibiotic activity against enteric bacilli when used alone; however, a synergistic effect is created when administered in combination with a penicillin or cephalosporin. Currently there are four penicillin/inhibitor combinations approved for clinical

use in the USA, including ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin/clavulanate and piperacillin/tazobactam.<sup>2</sup> Recently, the FDA approved the use of ceftolozane/tazobactam for the treatment of complicated urinary tract infections. By irreversibly binding to the enzyme, the  $\beta$ -lactamase inhibitor protects the  $\beta$ -lactam antibiotic from being hydrolysed by the  $\beta$ -lactamase. Such  $\beta$ -lactamase inhibitor combinations are highly active against most class A  $\beta$ -lactamases, but are poorly active against classes C and D, and inactive against class B  $\beta$ -lactamases.<sup>2</sup> The clinical efficacy of a  $\beta$ -lactamase inhibitor/ $\beta$ -lactam combination depends on many factors, including concentration of inhibitor used in the

formulation, amount of  $\beta$ -lactamase produced by the bacterial cell and the concentration of antibiotic that enters the periplasmic space. Emergence of resistance to  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations can severely impact the ability to treat serious respiratory tract, urinary tract and bloodstream infections. Therefore, the ratio of  $\beta$ -lactam/ $\beta$ -lactamase inhibitor used in combinations is critical because use of an inappropriate amount of inhibitor may impact the therapeutic value of the drug.<sup>3</sup>

CTX-M-producing *Escherichia coli* are predominately the pandemic ST131 clone and frequently cause urinary tract infections.<sup>4,5</sup> The rapid spread of these strains has led to the CTX-M pandemic.<sup>6</sup> Two major genotypes of CTX-M have become established worldwide, CTX-M-15 and CTX-M-14. These CTX-M producers have contributed to both hospital- and community-acquired urinary tract infections.<sup>6–11</sup> *E. coli* represents 50% of infections leading to uroseptic shock in hospitalized patients and the majority of uroseptic infections in these patients originate from the community.<sup>12,13</sup>  $\beta$ -Lactam/ $\beta$ -lactamase inhibitor combinations can be an effective treatment for infections caused by CTX-M-producing organisms.<sup>2,6,10,13,14</sup>

Recently, our laboratory has documented elevated levels of CTX-M-15 mRNA, in comparison with CTX-M-14 mRNA levels, in *E. coli* from human urine samples.<sup>15</sup> This difference in steady-state mRNA expression between CTX-M-15 and CTX-M-14 producers was observed from isolates collected from various geographical locations worldwide indicating that this observation was not due to a local clonal population of isolates (Table 1). Steady-state mRNA levels take into account both the rates of mRNA synthesis and degradation (mRNA half-life).<sup>16</sup> Thus, the observed differences in expression levels between  $bla_{CTX-M-14}$  and  $bla_{CTX-M-15}$  could be due to an extended mRNA half-life for  $bla_{CTX-M-15}$  transcripts compared with  $bla_{CTX-M-14}$  transcripts. It is possible that elevated levels of CTX-M-15 transcripts could lead to elevated levels of CTX-M-15 protein production negating the effect of  $\beta$ -lactamase inhibitors. The goal of this study was to compare the mRNA expression and protein levels of CTX-M-14 and CTX-M-15  $\beta$ -lactamases among clinical *E. coli* isolates of varying STs and phylotypes from human urine samples. In addition, we evaluated the mRNA half-life of these transcripts and the susceptibility of these clinical isolates to piperacillin/tazobactam in addition to evaluating the zone of inhibition for ceftolozane/tazobactam.<sup>17</sup>

## Methods

### Bacterial isolates and susceptibility testing

The study population comprised 57 CTX-M-producing *E. coli* clinical isolates chosen from 18 geographic locations to ensure that the data did not represent a local point-source clonal outbreak (Table 1). In addition to harbouring a CTX-M-14 or CTX-M-15  $\beta$ -lactamase, most isolates possessed other  $\beta$ -lactamases such as TEM-1-like or OXA-1, as determined by family-specific PCR.<sup>18</sup> Twenty-five CTX-M-15-producing and 16 CTX-M-14-producing *E. coli* were used to evaluate mRNA half-life. The donor strains used in conjugation studies included XQ12 (CTX-M-15), XQ35 (CTX-M-15), C15 (CTX-M-15), D14 (CTX-M-14) and XQ10 (CTX-M-14). J53 (sodium azide resistant) *E. coli* served as the first recipient strain in the conjugation strategy and SalLT2 (*Salmonella enterica* serovar Typhimurium LT2) was the intermediate recipient. The final recipient strains used for conjugation included J53 *E. coli*, K12 MG1655 (WT *E. coli*) and FHM16 (ST131 *E. coli* without a CTX-M enzyme). Vectors used for cloning and

construction of transformants included pCR<sup>®</sup>2.1, pJET1.2/blunt, pSP-*luc+*, pUCP26, pACYC184 (chloramphenicol promoter intact), pMDR009 (chloramphenicol promoter deleted) and pMP220 (Table S1, available as Supplementary data at JAC Online). Disc diffusion assays were performed as previously described and piperacillin/tazobactam susceptibilities were interpreted using CLSI guidelines.<sup>19</sup> In addition, zone sizes were determined for ceftolozane/tazobactam against 129 total isolates. No disc diffusion breakpoints for Enterobacteriaceae are currently available for ceftolozane/tazobactam; therefore, only zone sizes can be reported.

### Conjugation assays and clinical plasmid isolation

Conjugation studies were performed using a combination of broth and filter mating. Transconjugants were created to evaluate the possibility that a factor(s) encoded on the clinical plasmid was influencing the differential expression of CTX-M-14 and CTX-M-15 mRNA. Donor strains, XQ12 (CTX-M-15), XQ35 (CTX-M-15), C15 (CTX-M-15), D14 (CTX-M-14) and XQ10 (CTX-M-14) were conjugated with J53 *E. coli* using brain heart infusion (Difco, Thermo Scientific) broth mating. A donor to recipient ratio of 1:2.5 was used in all conjugation experiments. Transconjugants were selected on LB agar with sodium azide (NaN<sub>3</sub>) 100 mg/L and cefotaxime 24 mg/L. J53 transconjugants were mated with SalLT2 and plated on MacConkey agar with cefotaxime at 24 mg/L. Following transfer into SalLT2, the CTX-M-harboring plasmid was moved by filter mating into MG1655 and FHM16 with transconjugants selected using MacConkey agar supplemented with 24 mg/L cefotaxime. Plasmid transfer was confirmed via agarose gel electrophoresis (data not shown).<sup>20</sup>

### Cloning and sequencing

Transformants were constructed by amplifying the  $bla_{CTX-M-14}$  and  $bla_{CTX-M-15}$  structural genes and promoter regions by PCR, ligation of the PCR products into vector MDR009 (chloramphenicol promoter removed) and transformation into MG1655, J53 and FHM16 *E. coli*. The structural genes encoding CTX-M-14 and CTX-M-15 were sequenced from various clinical isolates along with their upstream promoter regions using primers listed in Table S2. All clones and subclones were sequenced. To create the CTX-M MG1655 transformants, pMRD009 was the final destination vector. For the luciferase clones, the CTX-M promoter regions were fused to the luciferase gene and cloned into vector pMP220, while CTX-M genes driven by the chloramphenicol or *lacZ* promoter were cloned into pACYC184 (ATCC) and pUCP26, respectively.

### Primer efficiency evaluations and determination of copy number using PCR

Because different primer sets were used for expression of CTX-M-14 and CTX-M-15, comparisons of the data obtained using these primers could not be made unless the primers amplified with the same efficiency. Total DNA was extracted using the Qiagen DNeasy<sup>®</sup> Blood and Tissue kit. Ten-fold serial dilutions were prepared ranging from 250 ng to 0.025 ng. The master mix included 1 $\times$  Rotor-Gene SYBR Green buffer (Qiagen, Valencia, CA, USA), 10 pmol of each forward and reverse CTX-M-specific real-time primer and RNase-free H<sub>2</sub>O for a total reaction volume of 40.5  $\mu$ L. A separate master mix was prepared using the endogenous control primer set (*frr* or 16S rRNA) and these genes were used to normalize the expression data. A standard curve was constructed for both the CTX-M and endogenous control primer sets for each isolate. The C<sub>t</sub> values were plotted against the logarithm of the DNA concentration. Each standard curve was generated from a linear regression of the plotted points. Using the slope of each standard curve, the PCR amplification efficiency (E) was calculated using the equation  $E = 10^{-1/\text{slope}} - 1$ ,<sup>21</sup> while the efficiency percentage was calculated using the equation  $E = (10^{-1/\text{slope}} - 1) \times 100$ . The R<sup>2</sup> value ranged from 0.97 to 0.99. The target [CTX-M-15, CTX-M-14

**Table 1.** Characteristics, expression data and susceptibility data (zone sizes in mm) for CTX-M-15- and CTX-M-14-producing *E. coli* isolates used in this study

Strain	Geographical location of isolation	<i>bla</i> <sub>CTX-M</sub> allele	ST	Phylotype	Relative fold change in expression ± SD	Relative fold change in protein ± SD	Zone diameter (mm)	
							piperacillin/tazobactam	ceftolozane/tazobactam
D14	Omaha, NE	CTX-M-14	ST405	D2	1	1 ± 0.15	22	21
CUMC 247	Omaha, NE	CTX-M-15	ST131	B2	72 ± 12.56	2.5 ± 0.39	23	21
XQ12	Seattle, WA	CTX-M-15	ST131	B2	21 ± 5.12	10.3 ± 3.6	22	22
XQ35	Seattle, WA	CTX-M-15	ST131	B2	8 ± 0.87	1.06 ± 0.87	20	25
A15	Omaha, NE	CTX-M-15	ST44	D2	15 ± 1.28	ND		
C15	Omaha, NE	CTX-M-15	ST405	B1	48 ± 0.70	3.9 ± 2	23	22
H15	Omaha, NE	CTX-M-15	ST205	B2	20 ± 0.68	12.2 ± 5.0	17	23
W15	Omaha, NE	CTX-M-15	ST131	B2	29 ± 1.43	2.6 ± 0.25	17	21
FHM6	India	CTX-M-15	ST131	B2	48 ± 8.41	3.2 ± 1.21	21	19
RS059	UK	CTX-M-15	ST131	B2	77 ± 32.47	10.0 ± 2.43	23	20
RS061	UK	CTX-M-15	ST131	B2	52 ± 3.65	7.7 ± 2.33	24	20
RS007	UK	CTX-M-15	ST131	B2	1 ± 0.09	ND		
RS120	UK	CTX-M-15	ST131	B2	1 ± 0.14	ND		
F010	UK	CTX-M-15	ST69	D1	26 ± 2.51	3.5 ± 1.5	20	25
F024	UK	CTX-M-15	ST2076	D1	46 ± 2.30	ND		
F076	UK	CTX-M-15	ST182	D1	74 ± 6.92	ND		
RS135	UK	CTX-M-15	ST131	B2	165 ± 27.76	22.32 ± 10.7	18	20
RS153	UK	CTX-M-15	ST617	A2	37 ± 2.75	8.1 ± 1.27	25	30
CMB106	Minneapolis, MN	CTX-M-15	ST131	B2	51 ± 8.17	2.1 ± 0.46		
JJ2052 S	Evanston, IL	CTX-M-15	ST90	B1	32 ± 1.58	2.5 ± 1.97		
JJ2052 F	Evanston, IL	CTX-M-15	ST90	B1	32 ± 3.90	2.4 ± 1.80		
JJ2053	Evanston, IL	CTX-M-15	ST131	B2	-14 ± 0.04 <sup>a</sup>	UD		
JJ2131	Minneapolis, MN	CTX-M-15	ST167	A2	47 ± 10.83	2.9 ± 1.79		
JJ2235 S	Houston, TX	CTX-M-15	ST167	A2	39 ± 11.27	15.4 ± 2.75		
JJ2235 F	Houston, TX	CTX-M-15	ST167	A2	62 ± 17.32	4.4 ± 2.41		
JJ2236	New York, NY	CTX-M-15	ST648	D2	65 ± 10.88	14.1 ± 4.86		
JJ2241	New Brunswick, NJ	CTX-M-15	ST131	B2	14 ± 3.97	-2 ± 0.10 <sup>a</sup>		
JJ2242	Rochester, NY	CTX-M-15	ST131	B2	6 ± 0.44	UD		
JJ2243	Milwaukee, WI	CTX-M-15	ST131	B2	1 ± 0.90	UD		
JJ2244	Detroit, MI	CTX-M-15	ST131	B2	29 ± 4.22	1.7 ± 0.62		
JJ2246 S	Ewa Beach, HI	CTX-M-15	ST410	A2	60 ± 15.83	5.1 ± 1.50		
JJ2246 F	Ewa Beach, HI	CTX-M-15	ST410	A2	155 ± 41.89	28.1 ± 2.48	20	22
JJ2247	Galveston, TX	CTX-M-15	ST131	B2	48 ± 7.02	-1.3 ± 0.42 <sup>a</sup>	20	22
JJ2251	New York, NY	CTX-M-15	ST131	B2	49 ± 5.06	1.6 ± 0.93	20	26
JJ2253 SW	Minneapolis, MN	CTX-M-15	ST410	A2	36 ± 5.94	2.4 ± 0.57		
JJ2253 RG	Minneapolis, MN	CTX-M-15	ST410	A2	34 ± 5.91	12.1 ± 3.10		
JJ2267	Salt Lake City, UT	CTX-M-15	ST648	D2	42 ± 25.83	9.5 ± 1.50		
JJ2431	New York, NY	CTX-M-15	ST131-like	B2	39 ± 9.42	23.8 ± 3.15		
MHHB54	Spain	CTX-M-15	ST131	B2	49 ± 2.40	6.2 ± 2.10		
MHVlab2	France	CTX-M-15	ST131	B2	59 ± 15.05	16.1 ± 3.49		
QU015	Queensland, Australia	CTX-M-15	ST131	B2	14 ± 3.85	1.9 ± 0.55		
C14	Omaha, NE	CTX-M-14	ST648	D2	5 ± 2.08	5.0 ± 0.82	21	21
La14	Omaha, NE	CTX-M-14	ST648	D2	4 ± 1.68	6.2 ± 0.10	26	28
Lo14	Omaha, NE	CTX-M-14	ST405	D2	5 ± 0.39	3.1 ± .81		
N14	Omaha, NE	CTX-M-14	ST3856	A2	3 ± 0.40	6.2 ± 3.3		
F044	UK	CTX-M-14	ST131	B2	1 ± 0.15	-1.4 ± 0.09 <sup>a</sup>	25	24
NL217	UK	CTX-M-14	ST131	B2	1 ± 0.19	1.6 ± 0.75	26	23
XQ10	Seattle, WA	CTX-M-14	ST38	D	1 ± 0.40	ND		
XQ13	Seattle, WA	CTX-M-14	ST68	D	2 ± 0.72	1.5 ± 0.90	23	21
XQ22	Seattle, WA	CTX-M-14	ST156	B2	1 ± 0.73	ND	23	23

Continued

Table 1. Continued

Strain	Geographical location of isolation	<i>bla</i> <sub>CTX-M</sub> allele	ST	Phylotype	Relative fold change in expression ± SD	Relative fold change in protein ± SD	Zone diameter (mm)	
							piperacillin/tazobactam	ceftolozane/tazobactam
XQ24	Seattle, WA	CTX-M-14	ST10	A	1 ± 0.17	ND	22	23
JJ2339	Ewa Beach, HI	CTX-M-14	ST38	D2	2 ± 0.64	2.1 ± 0.66		
JJ2354	Orange County, CA	CTX-M-14	ST354	D1	2 ± 0.76	1.4 ± 0.47		
JJ2356	Seattle, WA	CTX-M-14	ST46	A1	1 ± 0.26	3.7 ± 1.17		
FS-ESBL013	Denmark	CTX-M-14	ST10	D2	2 ± 0.53	1.3 ± 0.42		
FS-ESBL014	Denmark	CTX-M-14	ST38	A2	3 ± 1.55	13.9 ± 4.32		
FS-ESBL062	Denmark	CTX-M-14	ST10	D1	2 ± 0.47	11 ± 1.06		

ND, not determined; UD, undetected.

All transcript levels are relative to D14, which was used as the comparator and set to 1.

Fold changes in protein are normalized values.

<sup>a</sup>Down-regulation of gene or protein production.

or luciferase (*luc*) and reference (*frr*, 16S rRNA) primer sets did not vary more than 10% from each other.

#### *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-14</sub> relative copy number determination

Relative gene copy number quantification was calculated using the equation of Skulj et al.<sup>22</sup> copy number = (Ec average)  $C_e^c$  / (Ep average)  $C_p^p$ , where *E* is the primer efficiency, *c* is the normalization gene and *p* is the gene of interest. Copy number studies were completed on select CTX-M-14- and CTX-M-15-producing clinical isolates in Tables 2 and 3.

#### MLST and phylotyping

Seven-locus MLST was done according to the Achtman system (<http://mlst.ucc.ie/mlst/dbs/Ecoli>). Major *E. coli* phylogenetic groups (A, B1, B2 or D) were determined using an established triplex PCR method.<sup>23</sup>

#### RNA isolation and mRNA expression assays

RNA was isolated using TRIzol<sup>®</sup> Max<sup>™</sup> (Invitrogen) from 1.5 mL of mid-logarithmic phase culture grown in Mueller–Hinton broth (OD<sub>600</sub> of ~0.5).<sup>24</sup> Genomic DNA was removed by RQ1 DNase (Promega, Madison, WI, USA) treatment. 250 ng of DNA-free RNA was used in 50 µL PCRs that consisted of the 1× concentration of QuantiTect<sup>®</sup> SYBR<sup>®</sup> Green RT-PCR master mix, RT mix (Qiagen, Hilden, Germany) and 25 pmol of each primer. RT-PCR was performed on the Rotor Gene Q 5plex high resolution melt system (Qiagen, Valencia, CA, USA) that included RT activation at 50°C for 40 min, HotStart Taq DNA polymerase activation at 95°C for 15 min and three step cycling conditions for 40 cycles. Cycling parameters included denaturation at 95°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 30 s. Relative mRNA expression for CTX-M-14 and CTX-M-15 was calculated using the 2<sup>-ΔΔCt</sup> method with D14 as the comparator.<sup>24,25</sup> The single copy gene, *frr* was used to normalize the data. Three independent RNA isolations and three individual RT-PCR assays were completed with a coefficient of variation of <10%.

#### Evaluation of mRNA half-lives

Cultures were grown to mid-logarithmic phase (OD<sub>600</sub> of ~0.5) in Mueller–Hinton broth and treated with 200 mg/L rifampicin (Sigma Aldrich, St Louis, MO, USA). Cells were harvested by centrifugation at 0, 2, 4, 6, 8,

10, 15, 20, 25 and 30 min post-rifampicin addition and RNA was isolated using TRIzol<sup>®</sup> Max<sup>™</sup>. Real-time RT-PCRs were done using 250 ng of DNA-free RNA. Data were normalized to the 16S rRNA gene of *E. coli*. The equation by Pfaffl<sup>26</sup> was used to determine the ratio of transcript that remained at each timepoint.

#### Immunoblot for CTX-M-14/CTX-M-15 detection

The linear response range of the Stain-Free fluorescence and the anti-CTX-M antibody for bacterial lysates was determined by performing a western blot on a dilution series of total protein ranging from 40 to 0.625 µg as previously described.<sup>27</sup> The protein linearity ranged from 5 to 40 µg (Figure S1) and the linear range of the anti-CTX-M antibody was from 2.5 to 40 µg (Figure S2). Therefore, 10 µg of total protein and an antibody dilution factor of 1:45000 were used for immunoblot analyses.

Whole cell protein lysate was prepared from clinical isolates listed in Table 1 as previously described.<sup>28</sup> Stain-Free SDS-PAGE, imaging and total protein normalization were carried out as previously described.<sup>27</sup> A custom polyclonal antibody specific for CTX-M-14 and CTX-M-15, directed toward the peptide sequence CAIPGDPRDIT was generated by GenScript (Piscataway, NJ, USA) and the secondary antibody (horseradish peroxidase-goat anti-rabbit IgG) was used with a dilution factor of 1:50000. The chemiluminescent signal intensity of CTX-M was normalized to the Stain-Free fluorescent signal intensity of total protein for each isolate. K12 MG1655 (WT *E. coli*) and FHM16 (clinical isolate without CTX-M) lysates were used as controls for cross-reactivity to proteins other than CTX-M. To ensure that the antibody recognized each protein with the same affinity, *E. coli* transformants expressing each CTX-M gene (*bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-14</sub>) from a common plasmid driven by the same promoter element were used to compare the efficiency of antibody detection (data not shown). Three biological replicates of each isolate were independently collected and the means of the normalized data sets were used to calculate the relative amount of CTX-M production.<sup>29</sup> Statistical significance of CTX-M protein levels between isolates was evaluated using a *t*-test (double-sided and paired) performed with GraphPad Prism software, version 4.0.

## Results

#### Relative levels of *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub> transcripts

The CTX-M β-lactamases originated from the *Kluyvera* species, a group of environmental, non-pathogenic organisms that contain

**Table 2.** Upstream region and mRNA half-life of selected strains of CTX-M-15 and CTX-M-14 producers

Strain	<i>bla</i> <sub>CTX-M</sub> allele	Upstream region	mRNA half-life (min)
XQ12	CTX-M-15	ISEcp1 <sup>a</sup>	8 or 10 <sup>c</sup>
C15	CTX-M-15	ISEcp1 <sup>a</sup>	5
XQ35	CTX-M-15	ISEcp1 <sup>a</sup>	<2
JJ2053	CTX-M-15	ISEcp1 <sup>a</sup>	9
JJ2241	CTX-M-15	ISEcp1-like <sup>b</sup>	8
JJ2242	CTX-M-15	ISEcp1-like <sup>b</sup>	<2
JJ2243	CTX-M-15	ISEcp1-like <sup>b</sup>	12
JJ2244	CTX-M-15	ISEcp1-like <sup>b</sup>	15
JJ2247	CTX-M-15	ISEcp1-like <sup>b</sup>	2
JJ2131	CTX-M-15	ISEcp1-like <sup>b</sup>	<2
JJ2235 S	CTX-M-15	ISEcp1-like <sup>b</sup>	<2
JJ2235 F	CTX-M-15	ISEcp1-like <sup>b</sup>	<2
JJ2236	CTX-M-15	ISEcp1-like <sup>b</sup>	10
JJ2253 RG	CTX-M-15	ND	11
MHHBS4	CTX-M-15	non-ISEcp1 <sup>b</sup>	<2, 7 or 12 <sup>c</sup>
MHVlab2	CTX-M-15	non-ISEcp1 <sup>b</sup>	9
QU015	CTX-M-15	non-ISEcp1 <sup>b</sup>	<2
JJ2251	CTX-M-15	non-ISEcp1 <sup>b</sup>	9
JJ2052 S	CTX-M-15	non-ISEcp1 <sup>b</sup>	2
JJ2052 F	CTX-M-15	non-ISEcp1 <sup>b</sup>	9
JJ2246 S	CTX-M-15	non-ISEcp1 <sup>b</sup>	<2 or 7 <sup>c</sup>
JJ2246 F	CTX-M-15	non-ISEcp1 <sup>b</sup>	7
JJ2267	CTX-M-15	ND	9
JJ2253 SW	CTX-M-15	ND	5
JJ2243	CTX-M-15	ND	<2
D14	CTX-M-14	ISEcp1 <sup>a</sup>	<2
XQ10	CTX-M-14	ISEcp1 <sup>a</sup>	<2
C14	CTX-M-14	ISEcp1 <sup>a</sup>	<2
La14	CTX-M-14	ISEcp1 <sup>a</sup>	<2
Lo14	CTX-M-14	ISEcp1 <sup>a</sup>	3
N14	CTX-M-14	ISEcp1 <sup>a</sup>	<2
F044	CTX-M-14	ISEcp1 <sup>a</sup>	<2
XQ22	CTX-M-14	ISEcp1 <sup>a</sup>	<2
XQ24	CTX-M-14	ISEcp1 <sup>a</sup>	<2
FS-ESBL014	CTX-M-14	ND	<2
JJ2339	CTX-M-14	ND	<2
JJ2354	CTX-M-14	ND	<2
JJ2356	CTX-M-14	ND	<2
NL217	CTX-M-14	ND	<2
FS-ESBL013	CTX-M-14	ND	3
FS-ESBL062	CTX-M-14	ND	3

Each mRNA half-life represents one experiment. Studies were not completed in triplicate due to cost.

Half-life calculated using the 2001 method of Pfaffl.<sup>26</sup>

<sup>a</sup>Confirmed by sequence analysis.

<sup>b</sup>Upstream region PCR mapped.

<sup>c</sup>Indicates the graph intersects the 50% transcript remaining line at more than one timepoint.

chromosomally encoded CTX-M β-lactamase genes.<sup>30</sup> Therefore, to evaluate the relative expression of both mRNA and protein levels among the *E. coli* CTX-M-producing isolates, an *E. coli* isolate (D14) that produced a low amount of CTX-M transcript compared

**Table 3.** Steady-state mRNA expression and mRNA half-lives for CTX-M-14 and CTX-M-15 transconjugants in different *E. coli* backgrounds

Clinical isolate donor/transconjugant	<i>bla</i> <sub>CTX-M</sub> allele	Relative mRNA levels ± SD	mRNA half-life (min) <sup>a</sup>
XQ12	CTX-M-15	20 ± 5.12	8–10
XQ12-J53	CTX-M-15	23 ± 10.58	7
XQ12-K12 MG1655	CTX-M-15	31 ± 4.41	9
XQ12-FHM16	CTX-M-15	26 ± 3.63	9
XQ35	CTX-M-15	4 ± 1.60	<2
XQ35-J53	CTX-M-15	7 ± 3.47	<2
XQ35-K12 MG1655	CTX-M-15	26 ± 7.21	<2
XQ35-FHM16	CTX-M-15	16 ± 3.12	<2
C15	CTX-M-15	56 ± 17.52	5
C15-J53	CTX-M-15	62 ± 6.89	5
C15-K12 MG1655	CTX-M-15	25 ± 12.73	5
C15-FHM16	CTX-M-15	59 ± 28.98	5 or 11
D14	CTX-M-14	1 ± 0.20	<2
D14-J53	CTX-M-14	1 ± 0.46	<2
XQ10	CTX-M-14	0.73 ± 0.48	<2
XQ10-J53	CTX-M-14	0.76 ± 0.39	<2

All fold changes are relative to D14.

<sup>a</sup>Calculated using the 2001 method of Pfaffl.<sup>26</sup>

with the other study isolates was used as the comparator (Table 1). mRNA analysis of CTX-M-specific transcripts relative to isolate D14 showed that *E. coli* isolates expressing CTX-M-15 had mRNA transcript levels that ranged from 14-fold lower to 165-fold higher than the comparator. However, most CTX-M-15 producers (32 of 40; 80%) showed 20–165-fold increases in *bla*<sub>CTX-M-15</sub> transcripts. When mRNA levels of the CTX-M-14 producers were compared with each other relative to the comparator strain, D14, the range in expression among these isolates was only 5-fold (Table 1). Among all the isolates evaluated, the trend observed for the elevated expression of CTX-M-15 transcripts and the lower level of CTX-M-14 transcripts were consistent among the isolates regardless of the geographic location from which the isolates were collected.

**Relationship between CTX-M mRNA expression and *E. coli* ST and phylotype**

Various STs and phylotypes were evaluated for CTX-M gene expression and protein production. No correlation was observed with respect to specific ST and/or phylotype of *E. coli* expressing CTX-M-14 or CTX-M-15 genes (Table 1). For example, strains RS135 and JJ2053 were both identified as ST131 phylotype B2 isolates. However, RS135 expressed the *bla*<sub>CTX-15</sub> gene 165-fold higher than the comparator D14 strain whereas strain JJ2053 expressed the *bla*<sub>CTX-15</sub> gene 14-fold less than the comparator. Twenty-one of 41 isolates were ST131 phylotype B2 with *bla*<sub>CTX-15</sub> gene expression ranging from a 14-fold decrease to a 165-fold increase in CTX-M-15 mRNA levels relative to the comparator strain, D14. Three ST405 isolates were evaluated for CTX-M gene expression. Two strains expressed CTX-M-14 and were both phylotype D2. Strain Lo14 expressed the *bla*<sub>CTX-14</sub>



gene 5-fold higher than the comparator strain, D14. The CTX-M-15-producing ST405 strain was of phylotype B1 and expressed  $bla_{CTX-M-15}$  48-fold higher than the comparator, D14. Four strains were phylotype D1, but were all different STs (ST69, ST2076, ST183 and ST354). ST354 expressed the  $bla_{CTX-M-14}$  gene only 2-fold higher than the  $bla_{CTX-M-14}$  mRNA expression of D14, but the  $bla_{CTX-M-15}$  mRNA expression observed in the D1 phylotypes ranged from 26- to 74-fold higher compared with D14.

#### Determination of CTX-M-14/CTX-M-15 mRNA half-lives from clinical isolates

The differential expression of mRNA was not attributed to the ST or the phylotype of the organism. Therefore, we wanted to determine whether the differential expression between these two types of CTX-M transcripts was occurring at the initiation of transcription or post-transcriptionally due to mRNA stability differences. The promoter regions of 29 strains encoding CTX-M-14 or CTX-M-15 were sequenced and most of the strains had identical upstream sequences that housed the insertion sequence *ISEcp1* (Table 2). When PCR mapping data were used to define the promoter, there was no difference in the trends of CTX-M-15 expression for *ISEcp1*-like upstream elements versus those strains that did not generate a specific amplicon for the *ISEcp1* element (for example, strains JJ2236 and JJ2246F; Table 2). Taken together, these data indicated that transcription initiation was probably not the cause of the differences observed in the mRNA levels.

mRNA half-life, i.e. the degradation rate of a transcript, can impact steady-state mRNA levels. Therefore, mRNA half-life studies were performed. The average half-life of an *E. coli* transcript is ~2–3 min.<sup>16</sup> Sixteen CTX-M-14-producing clinical *E. coli* isolates were evaluated for mRNA half-life. Each isolate had a half-life of  $\leq 3$  min (Table 2 and Figures S3–S7). However, 16 of 25 CTX-M-15-producing *E. coli* isolates had an extended half-life of between 5 and 15 min. The remaining nine CTX-M-15 clinical *E. coli* isolates had mRNA half-lives of  $\leq 2$  min. The difference in mRNA half-life observed for these isolates could be the result of chromosomal- or plasmid-encoded factors.

#### Factors controlling CTX-M mRNA half-life: chromosomal or plasmid encoded

To determine the contribution of chromosomal- or plasmid-encoded factors to the observed differences in the half-lives of the  $bla_{CTX-M}$  transcripts, candidate strains were chosen and transconjugants and transformants constructed (Table 3). DNA from isolates D14 and XQ12 were used as a template to construct the transformants. For the CTX-M-15-producing isolates, three clinical isolates representing three distinct mRNA half-lives were selected to be conjugation donors and included XQ12 (half-life of 8–10 min), XQ35 (half-life of <2 min) and C15 (half-life of 5 min). Three different recipient strains were used for the conjugations for the CTX-M-15-containing plasmid: J53, MG1655 and FHM16 (Table 3). For the CTX-M-14-producing isolates, two clinical isolates both having an mRNA half-life of <2 min were selected for conjugation studies and included D14 and XQ10.

Before the half-lives of the CTX-M transconjugant transcripts were determined, the gene copy number and CTX-M steady-state expression were evaluated. CTX-M gene copy number was determined for the D14, XQ10 and XQ12 transconjugants. The copy

number of the CTX-M genes for the transconjugants tested was identical to the gene copy number determined for the clinical donor strains, which was 1. D14 was used as the comparator strain when evaluating steady-state expression of the transconjugants to remain consistent with the initial expression data (Table 3). For most of the transconjugants, the mRNA expression levels were similar to those of the clinical isolate from which they were created (Table 3). Compared with D14, CTX-M-15 mRNA expression in the XQ12 clinical isolate was 20-fold higher and in the XQ12-J53, XQ12-MG1655 and XQ12-FHM16 transconjugants was comparable at 23-, 31- and 26-fold higher (Table 3). The steady-state mRNA expression level for the XQ35 clinical isolate was 4-fold higher than the comparator strain D14 and expression in the J53, MG1655 and FHM16 transconjugants was 7-, 26- and 16-fold higher, respectively (Table 3). Interestingly, the mRNA expression level of the XQ35-MG1655 and XQ35-FHM16 transconjugants was ~2- to 6-fold higher compared with the XQ35 clinical isolate and the J53 transconjugant. Steady-state mRNA expression levels for the C15 clinical isolate and its J53, MG1655 and FHM16 transconjugants were 56-, 62-, 25- and 59-fold higher, respectively when compared with D14. The CTX-M-15 expression level from the C15-MG1655 transconjugant was 2–3-fold lower compared with the J53 and FHM16 transconjugants (Table 3). The CTX-M-14 steady-state mRNA levels for XQ10 and D14 and their J53 transconjugants were both similar to those of the D14 clinical isolate (Table 3).

Variation in steady-state levels of mRNA expression was observed between some of the CTX-M transconjugants, but not all, when compared with the clinical isolate. These differences were not due to gene copy number (all isolates had one copy of the gene), but could be attributed to transcription initiation events or transcript half-life in these different genetic backgrounds. To test the influence of these genetic backgrounds on mRNA stability, the mRNA half-life of each of the transcripts expressed in the transconjugants was measured (Table 3 and Figures S3–S7). Despite the difference in genetic background (J53 versus MG1655 versus ST131), each of the transconjugant's mRNA half-lives reflected the mRNA half-life of the clinical isolate from which the plasmid was obtained. Specifically, for transconjugant XQ12 the CTX-M-15 transcript had an extended half-life of 7–9 min, the XQ35 transconjugant had a  $bla_{CTX-M-15}$  mRNA half-life of <2 min and the C15 transconjugant had a  $bla_{CTX-M-15}$  half-life of 5 min (Table 3). The same trend was seen with the CTX-M-14 transconjugants, each of which had a  $bla_{CTX-M-14}$  half-life of <2 min, similar to the parent strain. These data indicated that plasmid-encoded factors and not chromosomally encoded factors were contributing to the difference in mRNA half-life observed between  $bla_{CTX-M-14}$  and  $bla_{CTX-M-15}$ .

To verify further that a clinical plasmid-encoded factor(s) was responsible for mRNA stability, the mRNA half-lives of the CTX-M-14 and CTX-M-15 K12 transformants were evaluated. To create these constructs, the structural CTX-M-14 or CTX-M-15 gene and upstream promoter regions were amplified, cloned into pMDR009 and transformed into K12 MG1655 or FHM16 (ST131).<sup>31</sup> When mRNA half-lives were evaluated in the MG1655 transformants, both the CTX-M-14 and CTX-M-15 transcripts were <2 min (Figure S7). To determine whether the ST131 genetic background would influence the CTX-M-15 transcript half-life, the mRNA was evaluated in the transformant of ST131 *E. coli* strain, FHM16. The mRNA half-life of the CTX-M-15 transcript in

this transformant was also <2 min (Figure S7). These data substantiated that a plasmid-encoded factor(s), rather than the chromosomal background was responsible for the extended half-lives of the CTX-M-15 transcripts.

To rule out any contribution of the CTX-M native promoters or the structural genes themselves on CTX-M half-life, the half-lives of CTX-M promoter/luciferase fusions, *lacZ* promoter/CTX-M clones and chloramphenicol promoter/CTX-M clones were evaluated. When the promoter regions of the CTX-M-14 and CTX-M-15 genes were fused to luciferase, the half-life of the luciferase gene was <2 min for all clones (data not shown). Furthermore, the half-lives of the CTX-M-14 and CTX-M-15 transcripts driven by heterologous promoters (*lacZ* or chloramphenicol) were <2 min (data not shown). All clones discussed above included either the CTX-M promoter or structural gene, which had been removed from its native clinical plasmid and transformed into a K12 *E. coli* host.

**Relative levels of CTX-M-14 and CTX-M-15 protein**

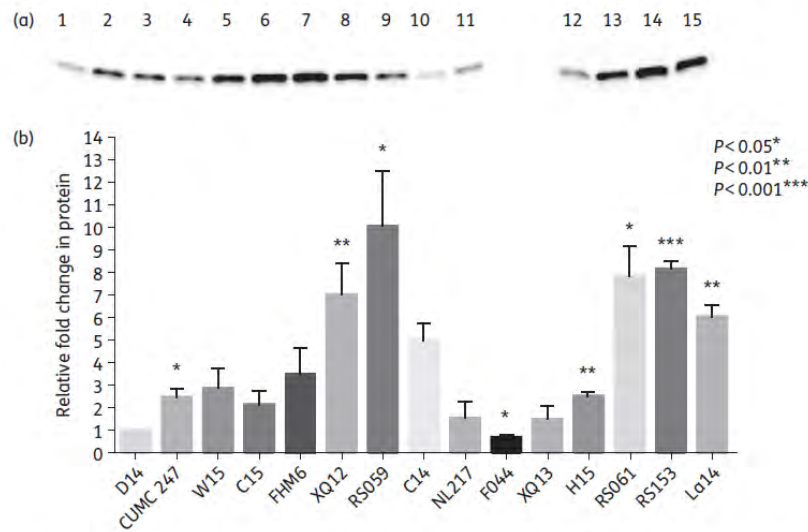
The elevated levels of the CTX-M-15 mRNA transcripts suggested that the level of CTX-M-15 enzyme produced by those *E. coli* isolates would also be elevated, perhaps jeopardizing the effectiveness of β-lactam/β-lactamase inhibitor combinations. Therefore, western blots were used to evaluate the relative level of CTX-M β-lactamase produced by the study isolates listed in Table 1.

An ~1:1 relationship between mRNA and protein production was observed for the CTX-M-14-producing isolates (Table 1 and Figure 1a and b). For example, *E. coli* isolate C14 expressed both its CTX-M-14 mRNA and protein at levels 5 times higher than the comparator isolate, D14. This trend in mRNA and protein

production was seen with all of the CTX-M-14-producing isolates with levels ranging between 1- and 6-fold higher compared with isolate D14 (Table 1). In contrast, this direct relationship was not observed for CTX-M-15-producing isolates. While mRNA levels for the CTX-M-15 transcript ranged from 14-fold lower to 165-fold higher than the CTX-M-14 mRNA levels of isolate D14, the corresponding protein levels ranged from undetectable to 10-fold higher (Table 1 and Figure 1a and b). In addition, the higher levels of CTX-M-15 mRNA did not always correlate with the highest levels of CTX-M-15 protein production. For example, isolate RS153 had an mRNA level 37-fold higher than the comparator strain, D14, but its protein level was only 8-fold higher. While a 77-fold increase in mRNA levels for strain RS059 was observed, only a 10-fold increase in protein production was noted. Isolate C15 had a 48-fold increase in mRNA compared with only a 2-fold increase in protein. Moreover, isolates JJ2246F and RS135 had the most pronounced increases for both mRNA and protein production of CTX-M-15 with a 155-fold and 165-fold increase in mRNA and a 28-fold and 22-fold increase in protein, respectively.

**Susceptibility to β-lactam/β-lactamase inhibitor combinations**

Protein production among the test isolates ranged from no detectable level of protein observed to a 28-fold increase in protein production for strain JJ2246F. The level of β-lactamase production is a key contributor to the β-lactam-resistant phenotype. Therefore given the range of CTX-M production in these isolates a subset of 23 isolates were tested to determine the impact CTX-M β-lactamase production had on piperacillin/tazobactam and



**Figure 1.** (a) Immunoblots for CTX-M-14 and CTX-M-15 from 15 representative clinical isolates. Protein levels were compared and analysed relative to *E. coli* strain D14. Lane 1, D14 (CTX-M-14); lane 2, CUMC 247 (CTX-M-15); lane 3, W15 (CTX-M-15); lane 4, C15 (CTX-M-15); lane 5, FHM6 (CTX-M-15); lane 6, XQ12 (CTX-M-15); lane 7, RS059 (CTX-M-15); lane 8, C14 (CTX-M-14); lane 9, NL217 (CTX-M-14); lane 10, F044 (CTX-M-14); lane 11, XQ13 (CTX-M-14); lane 12, H15 (CTX-M-15); lane 13, RS061 (CTX-M-15); lane 14, RS153 (CTX-M-15); lane 15, La14 (CTX-M-14). (b) Relative fold changes in CTX-M-15 and CTX-M-14 protein from the 15 isolates from (a) normalized to total protein using Stain-Free technology. All protein levels are relative to D14. Statistical significance of the isolates relative to D14 was evaluated using a *t*-test (two-tailed and paired) performed with GraphPad Prism 6.0.

ceftolozane/tazobactam zone size. Disc diffusion breakpoints for ceftolozane/tazobactam are currently set by the FDA and available for only *Pseudomonas aeruginosa*; therefore, susceptibility data for this drug combination for *E. coli* could not be determined. Only zone size comparisons could be made. In spite of the high-level expression from the CTX-M-15 gene, 7 of 15 isolates were susceptible to the piperacillin/tazobactam. Isolates W15 and H15 were resistant to piperacillin/tazobactam, while the remaining isolates were intermediate (Table 1). All of the CTX-M-14-producing isolates listed in Table 1 were susceptible to piperacillin/tazobactam. With respect to ceftolozane/tazobactam, all the isolates listed in Table 1 regardless of the level of protein production observed had zone sizes of  $\geq 19$  mm. An additional 126 isolates not evaluated for protein production, but possessing either CTX-M-14 or CTX-M-15  $\beta$ -lactamases, were also tested against ceftolozane/tazobactam. All 50 of the CTX-M-14-like-producing isolates had zone diameters for ceftolozane/tazobactam of  $\geq 21$  mm. Of the 76 additional isolates producing CTX-M-15-like enzymes, 73 showed zone diameters to ceftolozane/tazobactam of  $\geq 19$  mm, while 2 of 76 isolates had zone diameters of 18 mm and 1 of 76 had a zone diameter of 14 mm.

## Discussion

Previous studies evaluating the effectiveness of ceftolozane/tazobactam against isolates that produced CTX-M-14 or CTX-M-15 indicated that the concentration of tazobactam may play a role in the overall efficacy of this combination when used against these ESBL-producing bacteria.<sup>32,33</sup> When a breakpoint of 1 mg/L of ceftolozane plus 4 mg/L of tazobactam was used, 12% of isolates evaluated by Titelman et al.<sup>33</sup> were non-susceptible. A similar finding for ESBL-producing Enterobacteriaceae and ceftolozane/tazobactam has been reported by Livermore et al.<sup>32</sup> It is possible that the level of CTX-M production was responsible for the lack of susceptibility to ceftolozane/tazobactam in these isolates. Given our observation that CTX-M-15 isolates in general transcribed more mRNA than CTX-M-14 isolates, we wanted to evaluate the impact that this discrepancy in mRNA expression would have on CTX-M protein production and ultimately susceptibility to  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations. To our surprise, the level of CTX-M protein production for most of the isolates evaluated was similar for CTX-M-15 and CTX-M-14 producers despite the differences in mRNA expression. Therefore, the level of CTX-M production was most likely not responsible for the resistance observed in the 12% of isolates evaluated by Titelman.<sup>33</sup> The choice of breakpoint for ESBL-producing bacteria is controversial<sup>34</sup> and EUCAST and CLSI have set lower breakpoints for oxymino-cephalosporins to increase the probability of classifying isolates as non-susceptible. Our data show that the variation in susceptibility in ESBL producers for ceftolozane/tazobactam is not due to levels of  $\beta$ -lactamase production, so this criterion for establishing breakpoints maybe an oversimplification.

The evaluation of the CTX-M-14 and CTX-M-15 mRNA half-lives indicated that the prolonged CTX-M-15 half-life observed for many of the CTX-M-15 producers was controlled by a factor encoded on the CTX-M-15-harboring plasmid(s). This conclusion is substantiated by several experiments. First, transconjugants from CTX-M-14- or 15-producing donor strains had mRNA half-lives equivalent to the corresponding donor strains (Table 3).

Second, transformants of cloned CTX-M-14 and CTX-M-15 genes into identical plasmid vectors had  $bla_{CTX-M}$  mRNA half-lives of  $< 2$  min regardless of the genetic background, including a ST131 *E. coli* (FHM16). Third, native or heterologous promoters expressing  $bla_{CTX-M}$  alleles did not influence the mRNA half-life. These data also indicated that the plasmid backbones of CTX-M-15-encoding isolates were not identical since some of these isolates had mRNA half-lives of  $\leq 2$  min, whereas others had half-lives of  $\geq 5$  min. We speculate that the CTX-M-15-encoding plasmids that exhibited shorter half-lives have a similar composition to the CTX-M-14-encoding plasmids with respect to the encoded factor that determines mRNA half-life.

Steady-state levels of mRNA expression in the transconjugants indicated that for two of the three CTX-M-15-producing isolates (XQ12 and C15) evaluated, the genetic background did not influence promoter usage, plasmid copy number or transcript half-life. However, the steady-state level of mRNA for the CTX-M-15-producing XQ35 transconjugants in the FHM16 and MG1655 backgrounds was 4–6.5-fold higher compared with  $bla_{CTX-M-15}$  expression in the XQ35 clinical isolate. It is possible that when  $bla_{CTX-M-15}$  was expressed in the FHM16 and K12 MG1655 backgrounds, the copy number of the plasmid or transcription initiation events were altered compared with expression in the XQ35 and J53 backgrounds. Copy number has been shown to change even when the same plasmid vector is used, but transformed into different hosts (genetic backgrounds) of Enterobacteriaceae.<sup>35</sup>

Sequence analysis of CTX-M-14/CTX-M-15-containing plasmids has shown multiple open reading frames of hypothetical proteins that could encode for a factor that influences the CTX-M transcript half-life.<sup>36–38</sup> A protein that can modify mRNA half-life and is encoded by a plasmid has multiple implications. This factor could not only prolong the half-life of antibiotic resistance gene transcripts, as demonstrated in this study, but also influence the production of factors responsible for virulence or metabolic pathways, leading to increased fitness for strains that carry  $bla_{CTX-M-15}$ . Identification of this factor should be a priority, since it could serve as a potential target for the development of new antimicrobial agents to limit the production of proteins benefiting from a prolonged mRNA half-life.

The ability to evaluate  $\beta$ -lactamases in clinical isolates using hydrolysis data is difficult for isolates that carry multiple  $\beta$ -lactamases as in the ones evaluated in this study. Therefore, to evaluate the level of protein production of CTX-M  $\beta$ -lactamases only, immunoblot analysis was used. The use of an internal control to normalize the data is difficult using clinical isolates, as expression of any internal housekeeping protein may fluctuate in response to various selective pressures faced by a given organism. Therefore, the use of Stain-Free technology allows for a more accurate comparison of relative amounts of protein among clinical isolates.<sup>29</sup>

The difference observed between the levels of CTX-M-15 mRNA and protein suggests that these isolates are inefficient in translating all the transcribed mRNA into functional  $\beta$ -lactamase. Although some of the mRNA is translated, as evident from the immunoblot data, the level of CTX-M-15  $\beta$ -lactamase did not result in decreased zone sizes to either tested inhibitor combination. This suggests that the rate of penetration of antibiotics through the outer membrane may be important when evaluating CTX-M-producing resistant isolates. Even though there are no disc diffusion breakpoints for Enterobacteriaceae currently available

for ceftolozane/tazobactam, comparisons between MIC data and zone size suggest that a zone size of  $\geq 19$  mm could be considered susceptible (Cubist data on file). However, those data still contain some major errors between some isolates regarding MIC versus disc zone size that need to be resolved (Cubist data on file). None the less, most of the isolates in this study (126 of 129) had zone sizes to ceftolozane/tazobactam of  $\geq 19$  mm despite the elevated levels of protein production observed. Notably, isolate RS135 produced the highest level of mRNA and the second highest level of CTX-M-15 protein, yet showed a zone size of 19 mm for ceftolozane/tazobactam. In contrast, the highest protein production was noted for strain JJ2246F, but the ceftolozane/tazobactam zone of inhibition was 30 mm indicating that the level of  $\beta$ -lactamase did not contribute to smaller zone sizes for these isolates.

It was surprising that the levels of mRNA expression observed for most CTX-M-15-producing isolates were disproportional to protein production. This disproportional correlation between mRNA and protein production could be the result of a translational block due to an RNA binding protein or small RNA interference, or the result of decreased CTX-M-15 protein stability compared with CTX-M-14. The differential levels of expression between mRNA and protein production did not seem to be influenced by phylogenetic background since no correlations could be determined with ST or phylotype. As the selective pressure increases with  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations or carbapenems, a future concern is the selection of genomic or plasmidic mutations that will allow increased translatability of the CTX-M transcripts resulting in increased  $\beta$ -lactamase production. These events could lead to an emergence of CTX-M-producing organisms resistant to  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations and may increase the number of isolates resistant to the carbapenems through porin down-regulation. Fortunately, our current findings suggest that resistance in *E. coli* to  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations including ceftolozane/tazobactam is not dependent on the type or level of CTX-M  $\beta$ -lactamase produced by the *E. coli* isolate, but requires as yet unidentified resistance mechanisms.

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## Supplementary data

Tables S1 and S2 and Figures S1 to S7 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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## REVIEW

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### Prevalence and clonality of extended-spectrum $\beta$ -lactamases in Asia

P. M. Hawkey

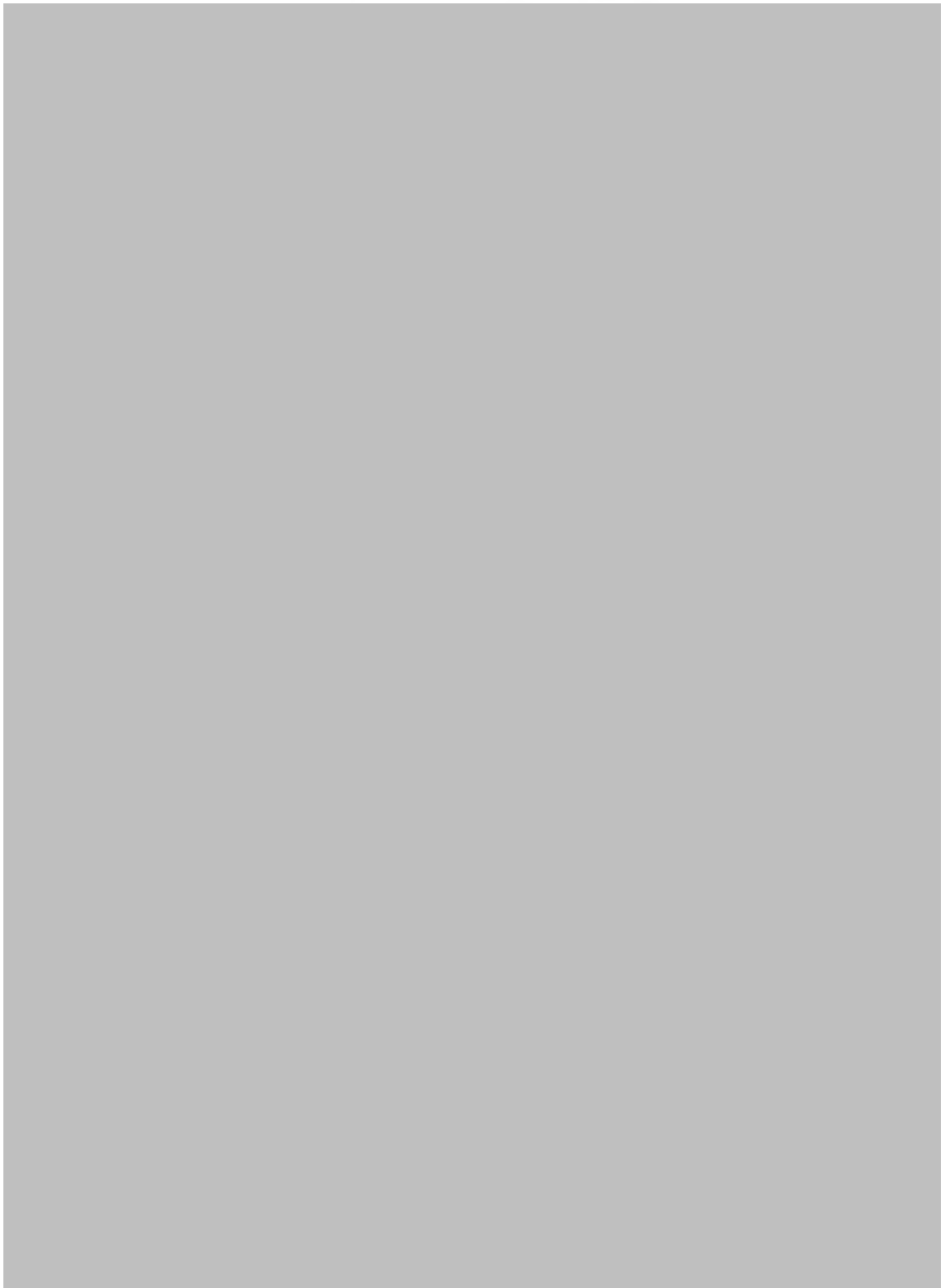
Division of Immunity and Infection, University of Birmingham, Birmingham and Health Protection Agency, West Midlands Public Health Laboratory, Birmingham Heart of England NHS Trust, Bordesley Green East, Birmingham, UK

#### ABSTRACT

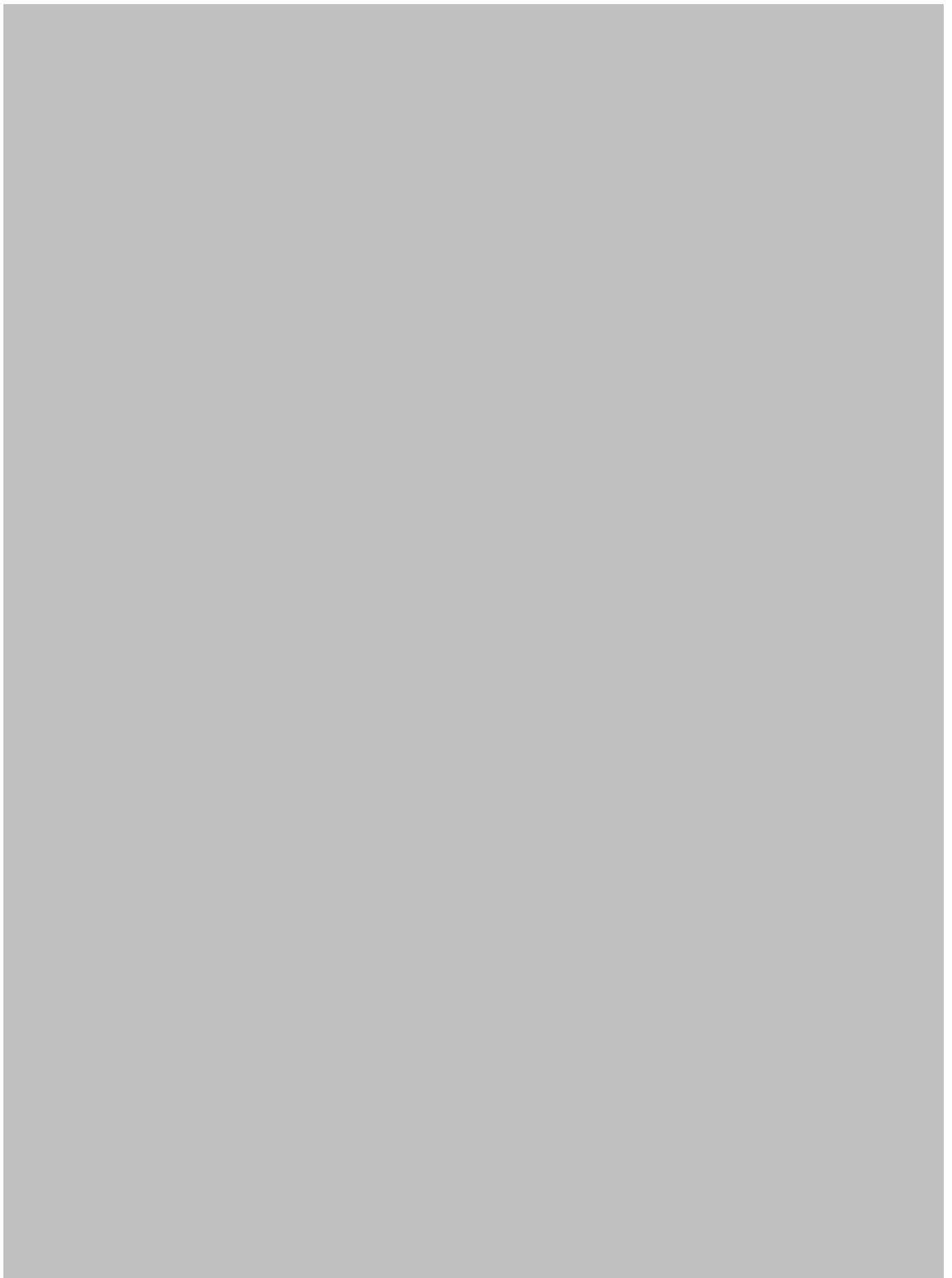
Asia is almost certainly a part of the world in which extended-spectrum  $\beta$ -lactamases (ESBLs) have emerged *de novo*, with some early antimicrobial resistance studies showing high levels of the ESBL phenotype, particularly among *Klebsiella*, and most notably in China, Korea, Japan and India. There is a lack of genotyping studies but work from the late 1990s suggests that SHV-5 and SHV-12 were most common then, with only very rare reports of TEM-related ESBL genes. As in other parts of the world, quite marked differences have since been seen in the pattern of ESBL genes, particularly in relation to the CTX-M family. The early emergence of TOHO CTX-M-2 in Japan contrasted with CTX-M-3 and -14 in China and many other parts of the Far East, suggesting the separate transfer of genes from the genome of *Kluyvera* spp. to mobile genetic elements in human-associated Enterobacteriaceae. ESBL production rates are now very high compared with Europe. In most countries, there are mixtures of CTX-M types, with VEB appearing significantly in Vietnam and Thailand, and ESBL isolates from India being completely dominated by the presence of *bla*<sub>CTX-M-15</sub> alone, with no other CTX-M types reported. With the total population of India and China being *c.* 2.4 billion and with faecal carriage rates of, probably, *c.* 10%, these countries represent major reservoirs of *bla*<sub>CTX-M</sub> genes. Increasing international travel and trade will lead to the movement of many of these ESBL genes. The high prevalence of ESBL genes in Asia means that the empirical treatment of serious infections with  $\beta$ -lactam antibiotics, except carbapenems, is seriously compromised.

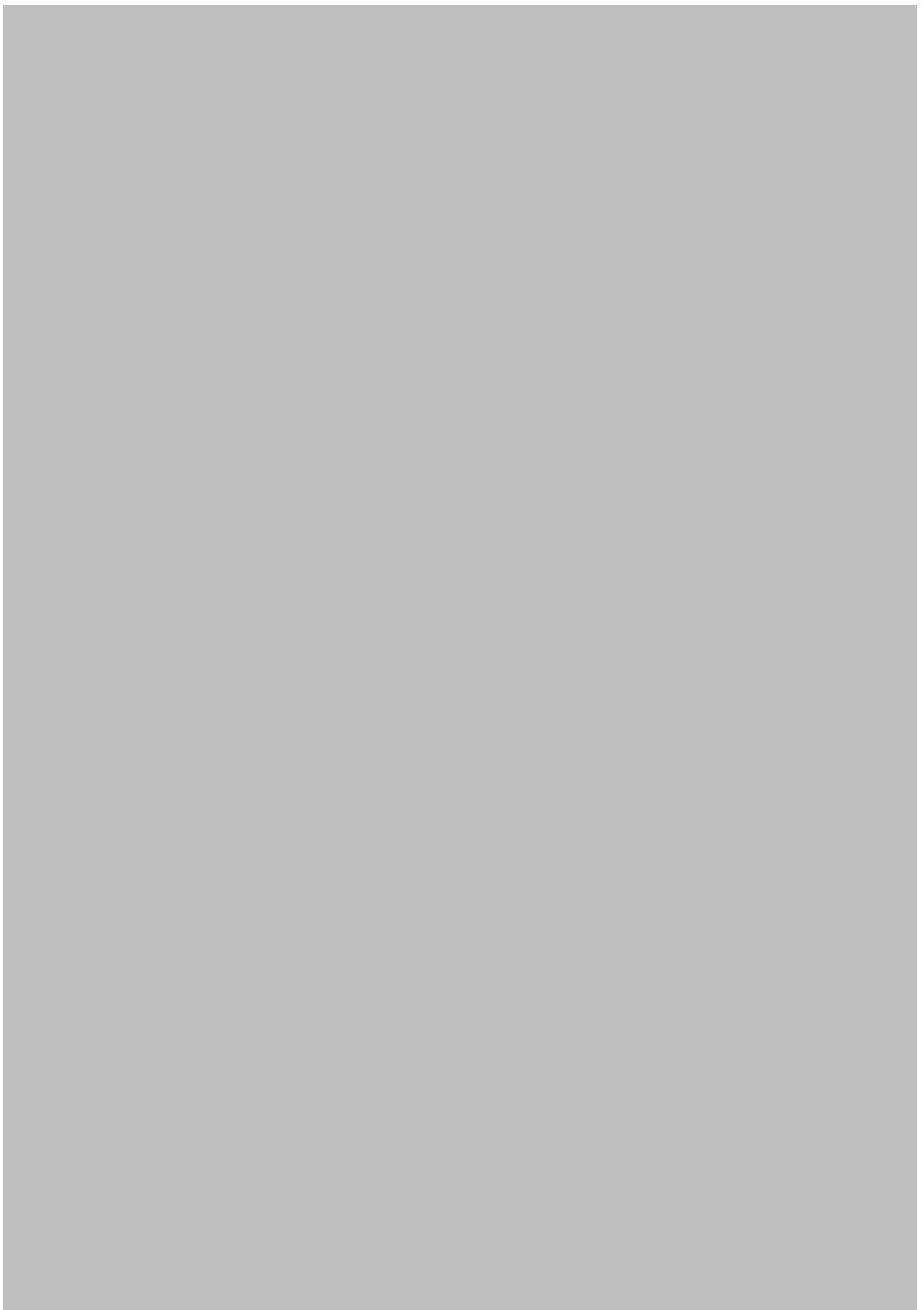
**Keywords** Asia, CTX-M, epidemiology, ESBL, integrons, review

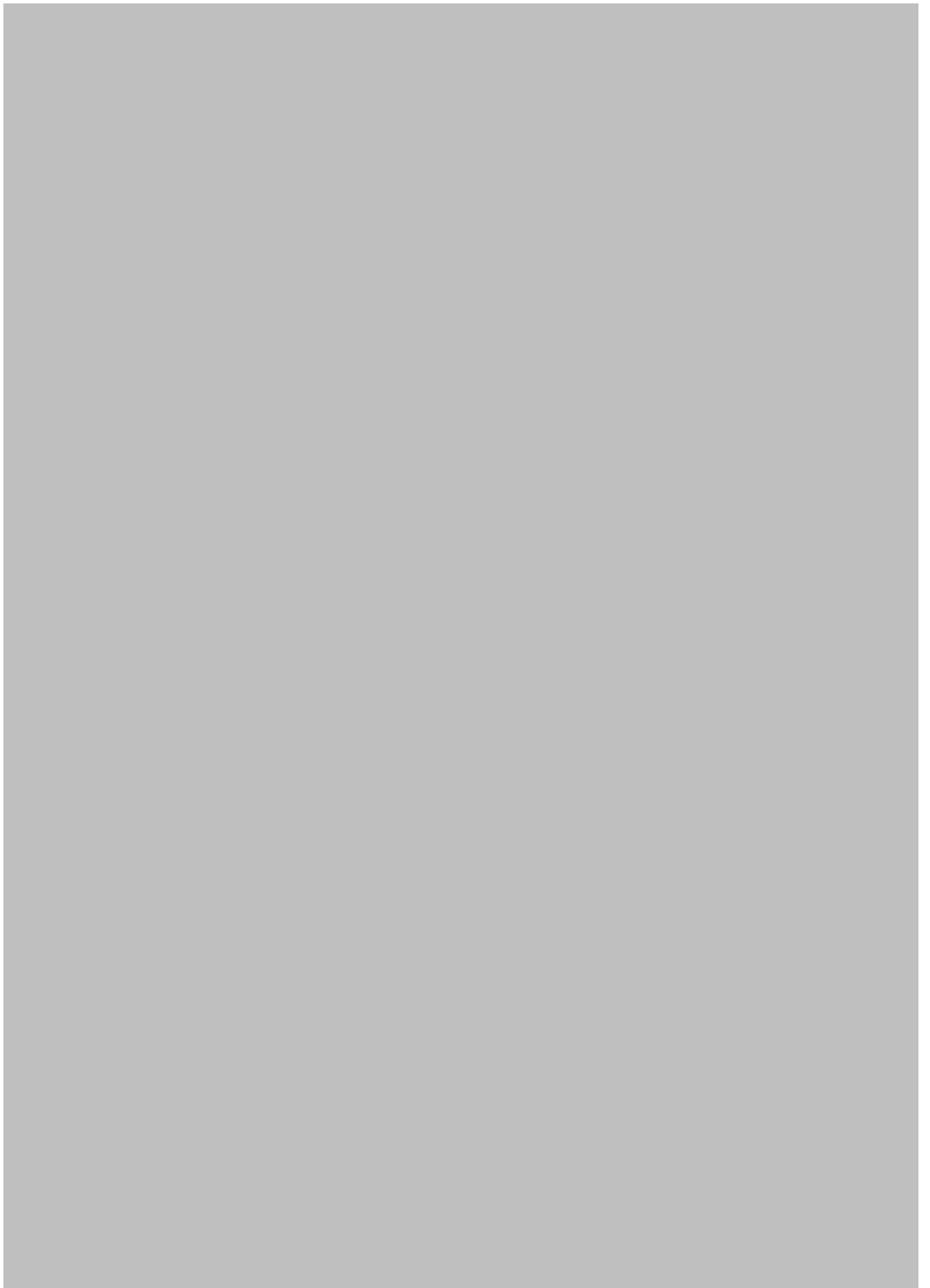
*Clin Microbiol Infect* 2008; 14 (Suppl. 1): 159–165

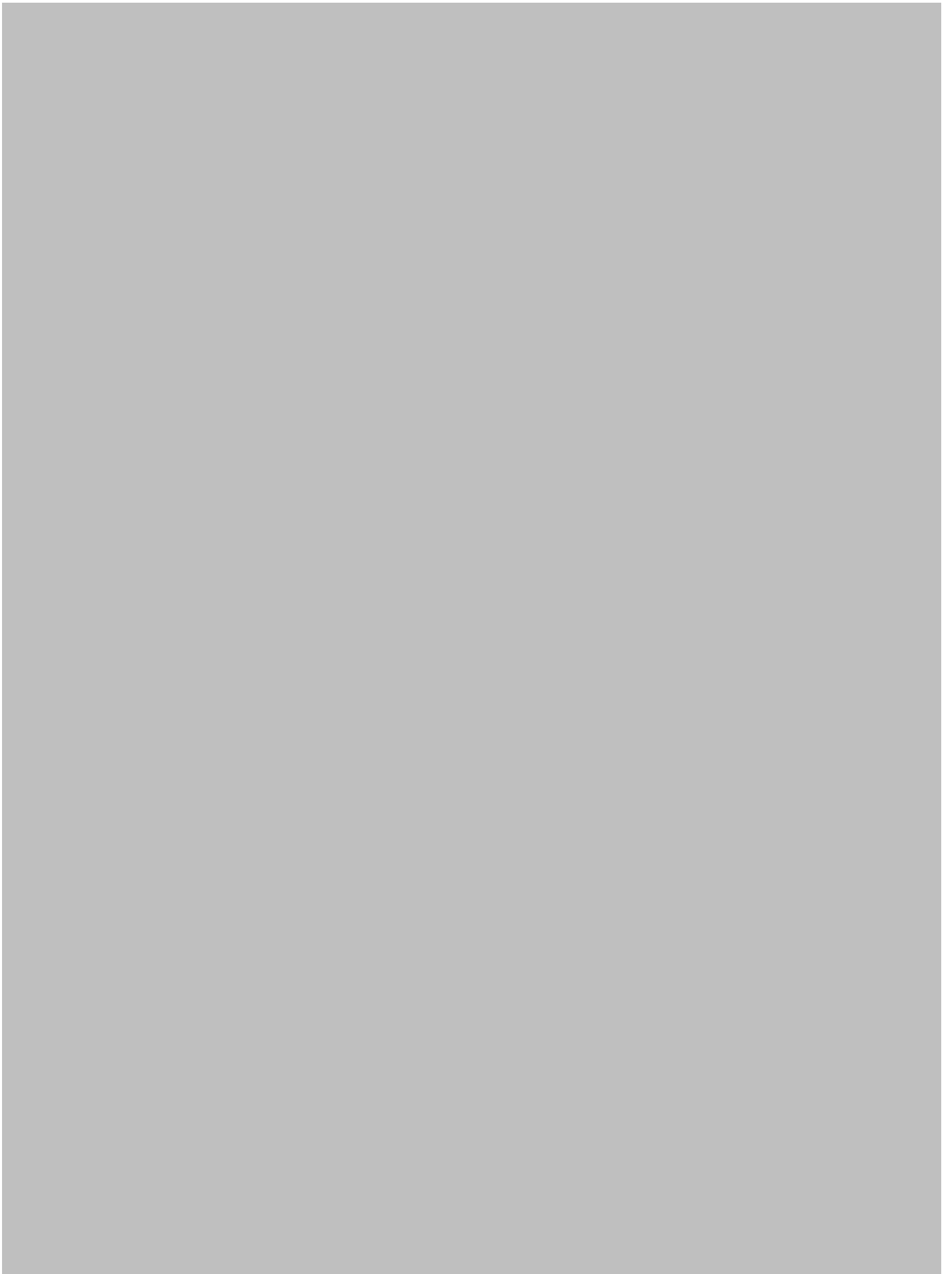


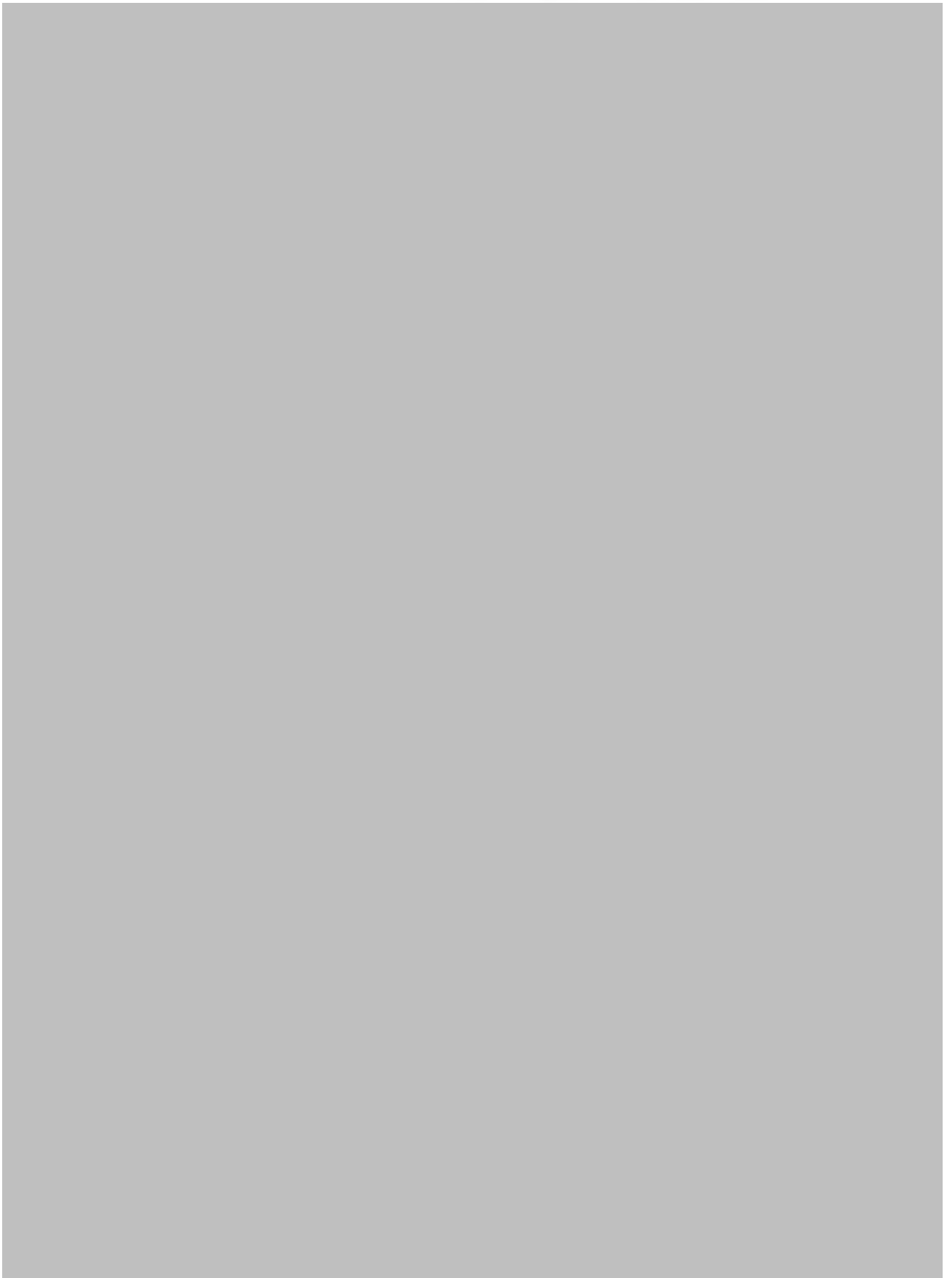












14. Hawkey, P.M. 2015. Multidrug-resistant Gram-negative bacteria: a product of globalization. *J.Hosp.Infect.*, 89, (4) 241-247 available from: PM:25737092



## Multidrug-resistant Gram-negative bacteria: a product of globalization

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### SUMMARY

Global trade and mobility of people has increased rapidly over the last 20 years. This has had profound consequences for the evolution and the movement of antibiotic resistance genes. There is increasing exposure of populations all around the world to resistant bacteria arising in the emerging economies. Arguably the most important development of the last two decades in the field of antibiotic resistance is the emergence and spread of extended-spectrum  $\beta$ -lactamases (ESBLs) of the CTX-M group. A consequence of the very high rates of ESBL production among Enterobacteriaceae in Asian countries is that there is a substantial use of carbapenem antibiotics, resulting in the emergence of plasmid-mediated resistance to carbapenems. This article reviews the emergence and spread of multidrug-resistant Gram-negative bacteria, focuses on three particular carbapenemases – imipenem carbapenemases, *Klebsiella pneumoniae* carbapenemase, and New Delhi metallo- $\beta$ -lactamase – and highlights the importance of control of antibiotic use.

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### Introduction

#### Global background

There has been a massive increase in global trade over the last 20 years, especially with the rapidly emerging nations of China and India. In 2008 more than US\$800 billion of trade

flowed between Asia and Europe and almost half the global trade in goods involved Europe.<sup>1</sup> This has had profound consequences for the evolution and the movement of antibiotic resistance genes. Of the top ten megacities (cities with  $\geq 10$  million population) eight are in Asia, the other two being Mexico City and New York. These megacities place huge demands on public health infrastructure, particularly in relation to sewage, drinking water, and overcrowding. In addition, the emerging economies are often heavy users of antimicrobials in both medicine and agriculture, which, combined with the deficits in public health infrastructure, has resulted in very high

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rates of resistance to antibiotics, especially among Gram-negative bacteria.

Global mobility has changed dramatically in the last 15 years. Travel by air has increased to the extent that, in 2012, >5000 billion revenue passenger-kilometres were recorded by the International Civil Aviation Organization.<sup>2</sup> Passengers carry Gram-negative bacteria in their bowel flora, particularly *Escherichia coli* and *Klebsiella* spp. Consequently there is increasing exposure of populations all around the world to resistant bacteria arising in the emerging economies. The role of the environment in the transmission of Gram-negative bacteria has been reviewed in detail elsewhere.<sup>3</sup> Unlike MRSA and enterococci, Enterobacteriaceae have a more fluid genome, mediated chiefly by the extensive carriage of conjugative plasmids that frequently carry antibiotic resistance genes as well as pathogenicity genes. The ability of antibiotic resistance genes to be transferred from environmental bacteria to medically relevant species of bacteria is well recognized.

#### Emergence of extended-spectrum $\beta$ -lactamases (ESBLs)

Arguably the most important development of the last two decades in the field of antibiotic resistance is the emergence and spread of extended-spectrum  $\beta$ -lactamases (ESBLs) of the CTX-M group.<sup>4</sup> Careful work by a group in Paris has shown that CTX-M resistance genes are present on the chromosomes of at least three species of *Kluyvera*, environmental bacteria closely associated with the rhizosphere (the complex microbial community surrounding plant rootlets). The resistance gene is inducible when on the chromosome but, once mobilized on to a plasmid with insertion sequences ISEcp1 and IS903, becomes highly mobile among the Enterobacteriaceae.<sup>5</sup> CTX-M was first recognized in 1989 in an isolate from a cancer patient of *E. coli* which was resistant to third generation cephalosporins. It was located on an 85 kilobase conjugative plasmid and the enzyme was originally designated MEN-1.<sup>6</sup> Four major groups of genetically distinct but related genotypes of CTX-M have emerged: 1, 2, 25, and 9.<sup>7</sup> Of the genotypes within these groups, two have been immensely successful, i.e. CTX-M 15 and to a lesser extent CTX-M 14.<sup>4</sup> CTX-M 15 is the only genotype present throughout India. Bearing in mind the early recognition there of the ESBL phenotype and the genotyping of strains from the early 2000s, it seems most plausible that this gene emerged in the Indian subcontinent and has then spread throughout the world.<sup>8</sup> CTX-M 14 was first described in Enterobacteriaceae from Guangzhou in Southern China in 1998.<sup>9</sup> The study indentifying CTX-M 14 observed a very high rate ( $\geq 35\%$ ) of ESBL production among *E. coli* in Guangzhou and, by reasoning similar to that applied to CTX-M 15 in India, it is most likely that CTX-M 14 emerged in China or an adjacent country during the mid-1980s, when locally produced cefotaxime was very widely used in hospital practice.

#### Spread of ESBLs

The movement of people around the world is a major consideration in the spread of multidrug-resistant (MDR) Gram-negative bacteria. Recent statistics for passenger movements at UK airports reveal that while the number of movements to or from the USA and Canada grew from 19.8 million in 2002 to 20.4

million in 2012, movements to or from India and Pakistan grew from 1.3 to 2.7 million over the same period, and to or from China and Hong Kong from 1.3 to 2.1 million. Total movements between the UK and the rest of the world grew from 39.5 to 50 million. Several studies have demonstrated the significance of this movement for antibiotic resistance. For example, Tham *et al.* found ESBL-producing bacteria in only two of 63 (3%) travellers returning to Sweden from European destinations, whereas they found ESBL producers in 50 of 138 (36%) of those who had travelled outside Europe.<sup>10</sup> The highest colonization rate was in those returning from India (11/14, 79%) closely followed by Egypt (19/38, 50%) and the Middle East (4/10, 40%). The genotypes of CTX-M identified in the travellers matched the regional distribution described by Hawkey and Jones.<sup>4</sup> All the ESBLs from travellers returning from India were CTX-M group 1 (most likely CTX-M 15), whereas all those returning from China were group 9 (most likely CTX-M 14). Egypt and Thailand had a mixture of groups 1 and 9, which is consistent with studies from those countries. Acquisition in these countries is at least partly attributable to the variable quality of sewage disposal and water treatment. In India, only 47% of households have a latrine.<sup>11</sup> Defecation in public places is a frequent occurrence, thus facilitating the spread of colonization among both local people and visitors. The situation in China is better, as 65% of the population have access to improved sanitation facilities (36% in India), but disposal of faeces can be of a variable standard especially in rural areas.<sup>12</sup> Even in those countries with level 3 treatment of sewage such as the UK, sewage treatment does not fully remove CTX-M-producing *E. coli*. A recent study from the English Midlands has shown that significant numbers of CTX-M-producing *E. coli* in treated effluent are discharged into water courses, where they may then be acquired by people during recreational activity in what appears to be a perfectly clean river, and also by livestock.<sup>13</sup> There is a paucity of data from many countries in Asia on resistance rates, but the SMART study (which samples *E. coli* and *Klebsiella* spp. causing significant intra-abdominal infections across a range of countries throughout the world and subjects them to standardized antimicrobial susceptibility testing and characterization) shows some interesting contrasts in different Asian countries. The data from 2008 showed an ESBL-producing *E. coli* rate for China of 59.1% and for India of 61.2%.<sup>14</sup> This contrasts with a rate of 22% in Hong Kong and 2.9% in Malaysia, which have better public health infrastructures as well as reasonably controlled antimicrobial prescribing in both human and animal medicine. Data on ESBL carriage rates in the community are even scarcer. Recently all of the available studies were summarized by Woerther *et al.*<sup>15</sup> Data on individuals with or without healthcare contact in the community are completely lacking from India. However, there are three studies of ESBL producers in healthy individuals in the community in China: Tien *et al.* reported a prevalence of ESBL-producing *E. coli* of 7% among 170 elderly people in Shenyang; by contrast, Li *et al.* reported a prevalence of 50% among 109 individuals in Fuzhou; and Zhong *et al.* found a prevalence of 51% in 567 healthy individuals with no healthcare contact in Hunan Province.<sup>16–18</sup> The high rate of carriage of CTX-M ESBLs in some countries is reflected in the carriage rate in those individuals in European countries with connections to those countries. Wickramasinghe *et al.* demonstrated this effect by showing that residents of Birmingham, UK, whose names indicated a global origin in either the Middle East or South Asia, had



a CTX-M ESBL faecal carriage rate of 23%, whereas those whose names indicated European origin had a carriage rate of 8%.<sup>19</sup> There was also a statistically significant association of carriage of CTX-M 15 among the Middle Eastern/South Asian individuals. A similar effect has been noted recently in Paris, where CTX-M-positive clinical isolates were much more prevalent in those whose birthplace was outside of mainland France.<sup>20</sup>

A consequence of the very high rates of ESBL production among Enterobacteriaceae in Asian countries is that there is a substantial use of carbapenem antibiotics, which has resulted with time in the emergence of plasmid-mediated resistance to that family of drugs. Of the five widely encountered carbapenemase genes – IMP, KPC, NDM, VIM, and OXA-48 – this review concentrates on IMP, KPC, and NDM.

### IMP carbapenemases

IMP carbapenemases, the first plasmid-mediated transferable carbapenemases to be recognized, emerged in Japan in the 1990s.<sup>21</sup> The carbapenemases, like the CTX-M ESBL enzymes, have a range of genotypes that are given sequential numbers (e.g. IMP-1, IMP-2, etc.), with each new carbapenemase differing from previously described genotypes by at least one amino acid. The first genotype described, IMP-1, is the most widely encountered carbapenemase in Japan. This emerged and spread rapidly among very different species of Enterobacteriaceae – *Acinetobacter* and *Pseudomonas* spp. Nine other genotypes were recognized between 1995 and 2001. One of those was IMP-4, first described from a single isolate of *Citrobacter youngae* from Guangzhou in Guangdong Province of Southern China.<sup>22</sup> IMP-4 was encoded on a cassette in a class 1 integron on a large plasmid and was transferrable to *E. coli*. An interesting feature of both IMP and VIM metallo- $\beta$ -lactamases in class 1 integrons is their ability to be expressed at high level in different hosts, and to be mobilized from one strain to another and one species to another.<sup>23</sup> Fascinatingly, almost simultaneous with this first description of IMP-4 in *Citrobacter youngae*, there was an outbreak in Hong Kong of *Acinetobacter baumannii* carrying the IMP-4 carbapenemase.<sup>24</sup> There were no further reports of IMP-4 until 2005, when there was a large outbreak in Melbourne, Australia, predominantly in *Serratia marcescens* but also in *Pseudomonas aeruginosa*, which subsequently spread to seven different species of Enterobacteriaceae and became endemic in eastern Australia.<sup>25</sup> Almost simultaneously Sydney experienced an outbreak of hospital cross-infection with Enterobacteriaceae producing IMP-4 in the same integron cassette array in which three other genes, *qacG2*, *aacA4* and *catB3*, were also present within the class 1 integron.<sup>26</sup> Interestingly the integron was found in a different genetic context and on a different plasmid from the original isolate from Guangzhou, suggesting movement either by homologous recombination or a CR1-associated mobilization. IMP-4 continues to be a problem within Eastern Australia. Following the initial reports in 2000, it was not until 2009 that IMP-4 was reported again (along with IMP-8 and KPC) in China, where it emerged as the most prevalent carbapenemase in a nationwide surveillance study involving 16 teaching hospitals.<sup>27</sup> More recent studies report the widespread occurrence of IMP-4 in southwest China, where a 2009–2010 study in a 3000-bed hospital in Chongqing revealed 26 isolates of carbapenem-

resistant Enterobacteriaceae of which 18 produced IMP-4 and one produced KPC.<sup>28</sup> Interestingly, 16 of the carbapenemases were in *Klebsiella pneumoniae*. A study from Fujian Medical University Union Hospital of faecal carriage among 303 randomly selected patients between November 2011 and January 2012 revealed a carriage rate of carbapenemase-producing Enterobacteriaceae of 2.6%.<sup>29</sup> Four isolates produced IMP-4, four produced KPC and one of the KPC isolates also produced NDM-1. In Europe and North America, IMP carbapenemases are currently rare, but their widespread presence in both China and Australia suggests that this carbapenemase may prove to be a problem in the future.

### KPC carbapenemase

Arguably one of the most widely distributed carbapenemases in the world is *K. pneumoniae* carbapenemase (KPC). It was first described in North Carolina, USA, in 1996, and re-emerged in New York City in the early 2000s, causing extensive outbreaks in intensive care units in the Brooklyn and Bronx districts.<sup>30</sup> KPC-1 and KPC-2 are identical: the initial difference was attributed to a single base pair error in sequencing and KPC-2 has been adopted as the correct DNA sequence. A number of other genotypes are recognized but none are as prevalent as KPC-2. KPC-3 has an enhanced activity against ceftazidime compared to cefotaxime. They are all serine-active  $\beta$ -lactamases which confer broad-spectrum resistance to all  $\beta$ -lactams except to high levels of temocillin. They are poorly inhibited by all the well-known  $\beta$ -lactamase inhibitors, but the new  $\beta$ -lactamase inhibitor, avibactam, when combined with ceftazidime shows excellent inhibitory activity against serine-active carbapenemases (KPC and OXA-48) as well as ESBLs. When KPC emerged, it was confined to ST-258 sequence type *K. pneumoniae* in the USA but emerged contemporaneously in *Pseudomonas aeruginosa* in Columbia and Puerto Rico. In the USA it was initially (in 2005) confined to the eastern seaboard with one or two states in the mid-west being affected, but the spread across the USA since then has been rapid. Data from the Centers for Disease Control and Prevention, Atlanta, on the situation in February 2014 showed that every state except Alaska and Idaho was affected. In many long-term care facilities, KPC is a considerable problem and is found in other Enterobacteriaceae. The global threat of KPC is exemplified by the experience in Israel. Because of a substantial problem since the late 1990s with CTX-M 2 ESBLs thought to have been imported by travellers from South America, carbapenems were heavily used. This created the ideal environment for the spread of KPC-producing *K. pneumoniae* that had likely been introduced to Israel by visitors from New York. First observed in Tel Aviv in late 2005, within two years it had subsequently spread to healthcare facilities throughout Israel.<sup>31</sup>

Greece has also had a substantial problem with KPC for a number of years, and, very recently, Italy has moved from a situation where carbapenemase genes were carried by less than 5% of *K. pneumoniae* isolates to one where, in 2011, they were carried by 25–50% of *K. pneumoniae* isolates.<sup>32,33</sup> This might be linked to Italy's position as one of Europe's heaviest prescribers of carbapenems and lowest users of alcohol hand-rub gel.<sup>33</sup> In China, KPC appears to be frequently co-carried with IMP-4 but it is not as prevalent, and isolates solely

producing KPC are recognized. Qi *et al.* at Zhejiang University first drew attention to the KPC problem in a study of carbapenemase genes in 95 isolates of *K. pneumoniae* from 13 different hospitals across five provinces in Eastern China.<sup>34</sup> All isolates are found to carry KPC-2 and many had the ST-11 sequence type (a single locus variant of ST-258), but other sequence types were identified and pulsed-field gel electrophoresis revealed considerable diversity of the host bacteria, suggesting extensive transmission via plasmids into different backgrounds. More recently, KPC has also been recognized in *E. coli* and in other locations within China.<sup>35</sup>

### NDM carbapenemase

In 2008, a new metallo-carbapenemase only distantly related to the IMP and VIM genes was identified in a Swedish patient of Indian origin who had returned to India for medical treatment and then been admitted to hospital in Sweden.<sup>36</sup> Metallo-carbapenemase-producing *K. pneumoniae* was isolated from his urine, and *E. coli* carrying the same metallo- $\beta$ -lactamase gene was isolated from his faeces. The carbapenemase, New Delhi metallo- $\beta$ -lactamase (NDM), was named after the location of his recent hospital care in India. Later sporadic findings of this new NDM gene in the UK could in at least 50% of cases be linked to receiving hospital care in India, Bangladesh, or Pakistan.<sup>37</sup> India is a frequent destination for medical tourism from the UK.<sup>38</sup> Data on the distribution of NDM in South Asia are scarce, but NDM is probably one of the most widely distributed carbapenemases in South Asia, along with OXA-48, which can be difficult to detect phenotypically. NDM has also spread to many other parts of the world, generally, as in the UK, as sporadic importations. Carbapenem-resistant *Klebsiella* spp. and to a lesser extent *E. coli* are increasingly documented in hospital facilities in India: for example, Saleem *et al.* have described increasing carbapenem resistance in *K. pneumoniae* causing late-onset sepsis in a neonatal intensive care unit in Karachi. Whereas, before 2010, isolates of *K. pneumoniae* from babies with late-onset sepsis were only sporadically resistant to imipenem, in 2010, 23% (6/26) of the isolates were imipenem resistant and, by 2011, 72% (13/18) were imipenem resistant.<sup>39</sup>

### Carbapenemase-producing Enterobacteriaceae in the UK

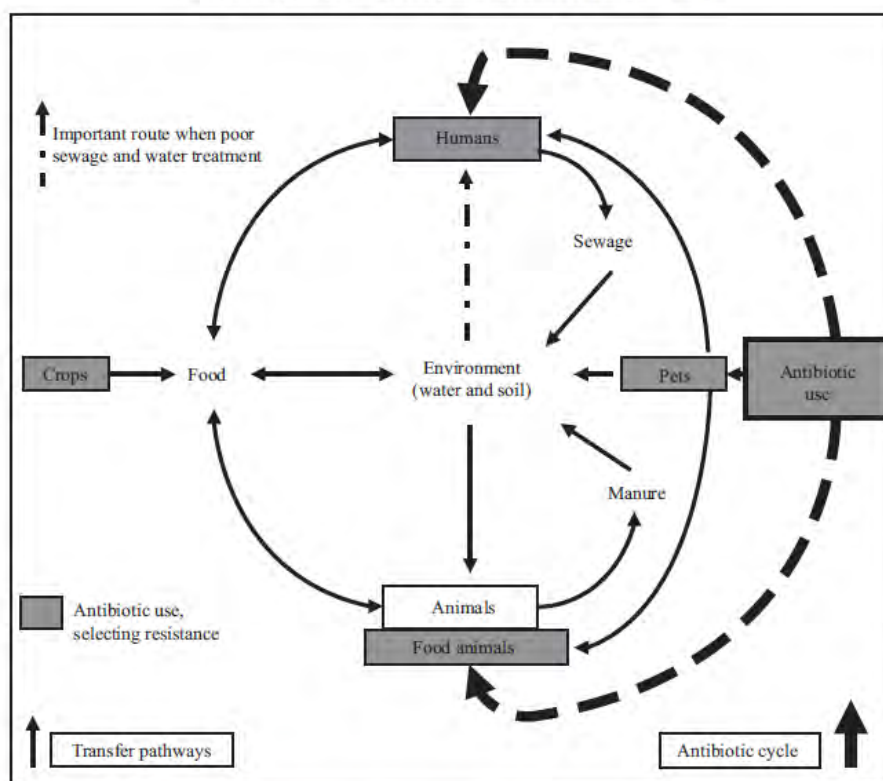
In the UK, all five major groups of carbapenemases, as well as rarer enzymes, have been reported by the reference laboratory. The UK picture is dominated by KPC, but most KPC-producing isolates were either from screening samples or from clinical specimens in northwest England. The next most frequently seen carbapenemase is NDM, which is distributed across the UK but with higher numbers in the London region. OXA-48 is also frequently encountered and many of these are attributable to episodes of cross-infection, particularly in intensive care units. I believe that the UK will see an increasing pressure in the future from imported NDM, which may, if not dealt with vigorously, cause local problems. The currently endemic KPC in northwest England is being controlled and may yet be eradicated, but we will experience further pressure from imported cases. In my view, the UK should be especially alert to future importations of NDM-1 and IMP-4 from Asia.

Moreover, OXA-48 may well become locally endemic, as its detection is extremely problematic.

### The environment and Gram-negative resistance

Faecal carriage by humans is a major source of MDR Gram-negative bacilli within the hospital environment and can be selected by antimicrobial therapy. Once MDR Gram-negative resistance genes become widespread in the community, the opportunity exists for bacteria to be more widely disseminated through the environment and establish a cyclical pattern of distribution.<sup>3</sup> River sediment has recently been shown to be a substantial reservoir of antibiotic resistance genes.<sup>40</sup> Using a metagenomic approach, the authors demonstrated that these were present both in culturable and unculturable bacteria. Even the use of level 3 sewage treatment in the UK does not prevent significant numbers of CTX-M-producing Enterobacteriaceae being released into the aquatic environment.<sup>13</sup> They can then be acquired by people engaged in water sports, as well by farm animals, which may explain the acquisition by dairy herds of CTX-M 14 and 15, the two most prevalent types in humans.<sup>41</sup> Once in the human food chain, the cycle can be completed (Figure 1). World production of poultry and pig meat has been increasing rapidly. Statistics from the UN's Food and Agriculture Organization in 2011 show that China produced 17.6 million tons of poultry meat and Europe produced 16.4 million tons.<sup>42</sup> Production of pig meat was 62.0 million tons in China, 26.8 million tons in Europe and 12.1 million tons in North and South America. Chinese production of pig meat represents 5% of total world production. The use of antibiotics in animal husbandry in China and many other Asian countries is substantial, although detailed data are not generally available. Xinhua news agency has published a report from the Ministry of Health and National Antibacterial Resistance Investigation Net on the production of antibiotics in China in 2007.<sup>43</sup> In all, 210,000 tons of antibiotics were produced that year, of which 30,000 tons were exported and 46% was used in food animals. The total agricultural and medical consumption of antibiotics thus amount to 136 grams per person per year. By contrast, antibiotic consumption in England in 2013 was 18 grams per person per year. Several factors driving antimicrobial use in medicine in both China and India need to be addressed urgently to reduce the overall selection pressure.

Aquaculture, including fish farming, is another aspect of environmental and agricultural usage of antibiotics that gives cause for concern. In Scandinavian countries, the use of antibiotics in aquaculture has been greatly reduced by the development of vaccines and alternative farming methods, but this is not true in warm water aquaculture where antimicrobials, especially quinolones, are extensively used. Chinese aquacultural production has risen from six million tons in 1990 to 32 million tons in 2008, when it represented 62% of world production.<sup>42</sup> The first study of antibiotic-resistant bacteria in farmed fish in China was published by Jaing *et al.* in 2012.<sup>44</sup> They studied farmed fish from 15 market outlets across Guangdong province (the Chinese province with the greatest production of farmed fish) and looked for ESBLs and plasmid-mediated quinolone resistance genes. *Escherichia coli* colonies were taken from non-selective media (one colony per sample) from the gut contents of each of 20 fish from 15 different markets. Eighty were ciprofloxacin resistant, of



**Figure 1.** Principal transfer pathways for antibiotic resistance genes in humans, animals, food, and the environment. (Reproduced with permission from The Joint Working Group of Defra's Antimicrobial Resistance Co-ordination (DARC) and Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infections (ARHAI). ESBLs – a threat to human and animal health? [https://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/215180/dh\\_132534.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/215180/dh_132534.pdf) [accessed 16.01.15]).

which 30 out of 80 carried *qnrB* genes and 16 out of 30 isolates carried *qnrS*. Other significant quinolone resistance-determining genes [*qnrD* and *aac(6)-Ib-cr*] were also found in the isolates, as well as a small number of ESBL genes (*bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-79</sub>), the most frequently occurring genotypes in the Chinese population.<sup>44</sup>

#### Future control of MDR Gram-negative bacilli around the world

Effective control requires good surveillance, and it is somewhat disquieting that, in the 2014 antimicrobial resistance report on surveillance from the World Health Organization, only 50% of member states returned data sets on nine key 'drug-bug' combinations.<sup>45</sup> Returns were highest in European states (38/53, 72%), but low in the Southeast Asian states (6/11, 55%). No data were available for the 2013 data collection exercise from either India or Indonesia, countries of populations of 1.2 billion and 400 million, respectively. Data are also lacking from many African countries. If we are to combat

the rise and global spread of MDR Gram-negative bacilli, all countries must contribute to accurate and timely surveillance. Surveillance is information for action: the only country yet to have successfully controlled carbapenemase-producing Enterobacteriaceae is Israel, which launched a concerted programme of screening and cohort isolation.<sup>46</sup> This level of intervention will be difficult to introduce in many parts of the world where there are weak laboratory facilities, poor infection control, and widespread prescribing of antibiotics. The threat for the future appears to come largely from the Asian region, where increasing wealth and intensification of both medical practice and farming results in increased usage of antibiotics. China's introduction of controls of antibiotic consumption is extremely welcome. Activity in India appears modest thus far. As African nations become wealthier and more agriculturally intensive, we may expect the same pattern to be repeated. If we are to avoid squandering the valuable resource of antimicrobials, we must act globally.

**Conflict of interest statement**  
None declared.

## Funding sources

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*J.Antimicrob.Chemother.*, 64 Suppl 1, i3-10

## The changing epidemiology of resistance

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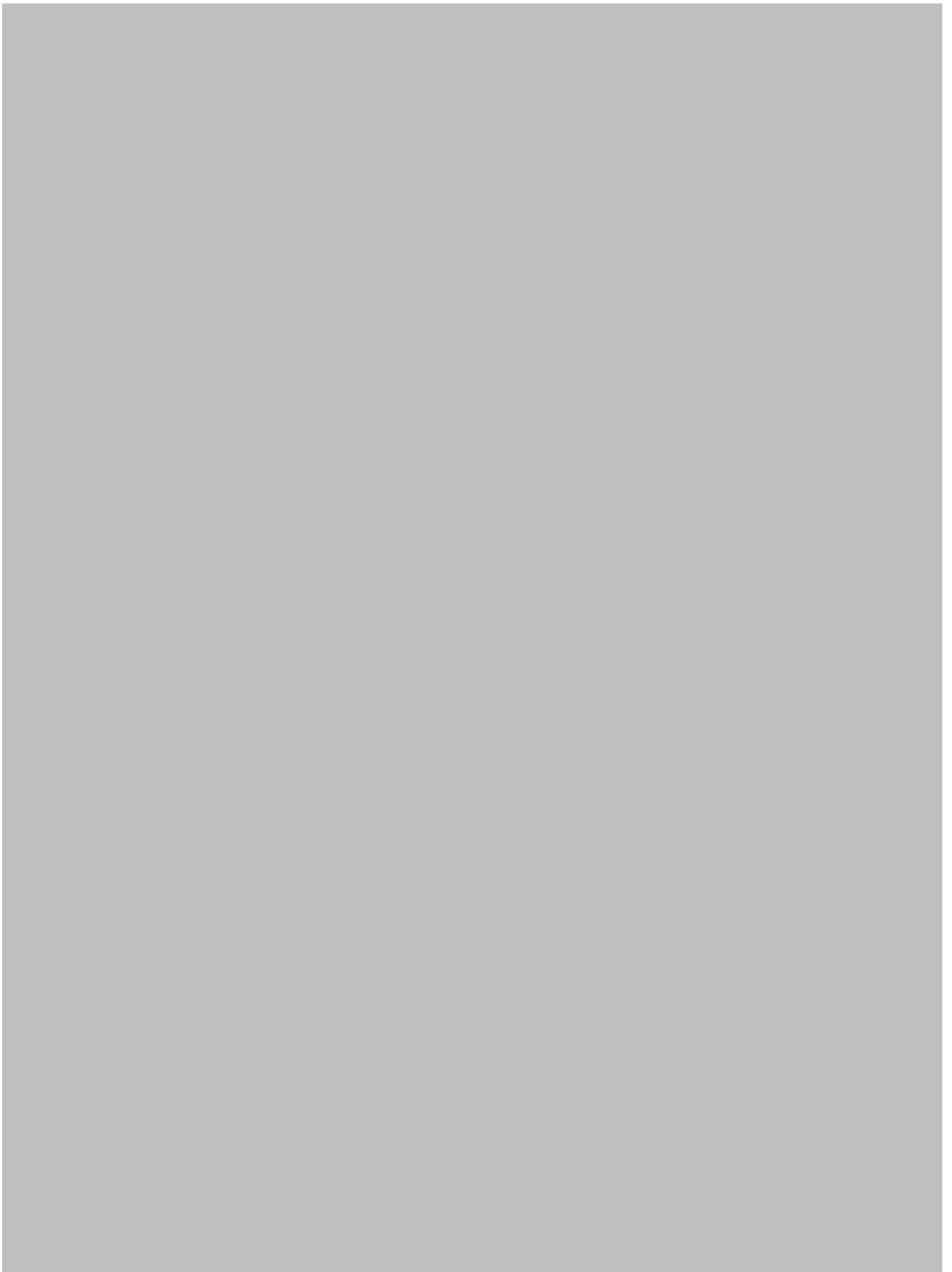
Antibiotic resistance is now a linked global problem. Dispersion of successful clones of multidrug resistant (MDR) bacteria is common, often via the movement of people. Local evolution of MDR bacteria is also important under the pressure of excessive antibiotic use, with horizontal gene transfer providing the means by which genes such as *bla*<sub>CTX-M</sub> spread amongst different bacterial species and strains.  $\beta$ -Lactamase production is a common resistance mechanism in Gram-negative bacteria, and the rapid dissemination of novel genes reflects their evolution under the selective pressure of antibiotic usage. Many Enterobacteriaceae now carry broad-spectrum  $\beta$ -lactamases such as CTX-M, with particular genotypes associated with different geographical regions. The spread of these enzymes has compromised the clinical utility of a number of  $\beta$ -lactam classes and with the spread of genes such as *bla*<sub>KPC</sub>, carbapenems may be increasingly compromised in the future. High-level fluoroquinolone resistance (mainly caused by *gyrA* mutations) has also been shown to be associated with CTX-M and CMY-type enzymes, commonly due to co-carriage on conjugative plasmids of the gene for the aminoglycoside-inactivating enzyme AAC-6<sup>1</sup>-Ib-cr and *qnr* genes (which confer low-level resistance), allowing the easy selection of *gyrA* mutants in the host strain. Resistance in Gram-positive bacteria is also widely distributed and increasing, with the emergence of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) blurring the distinction between hospital and community strains. Antibiotic use and environmental factors all have a role in the emergence and spread of resistance. This article reviews some of the new mechanisms and recent trends in the global spread of MDR bacteria.

Keywords: ESBLs, carbapenemases, Gram-positive bacteria, Gram-negative bacteria





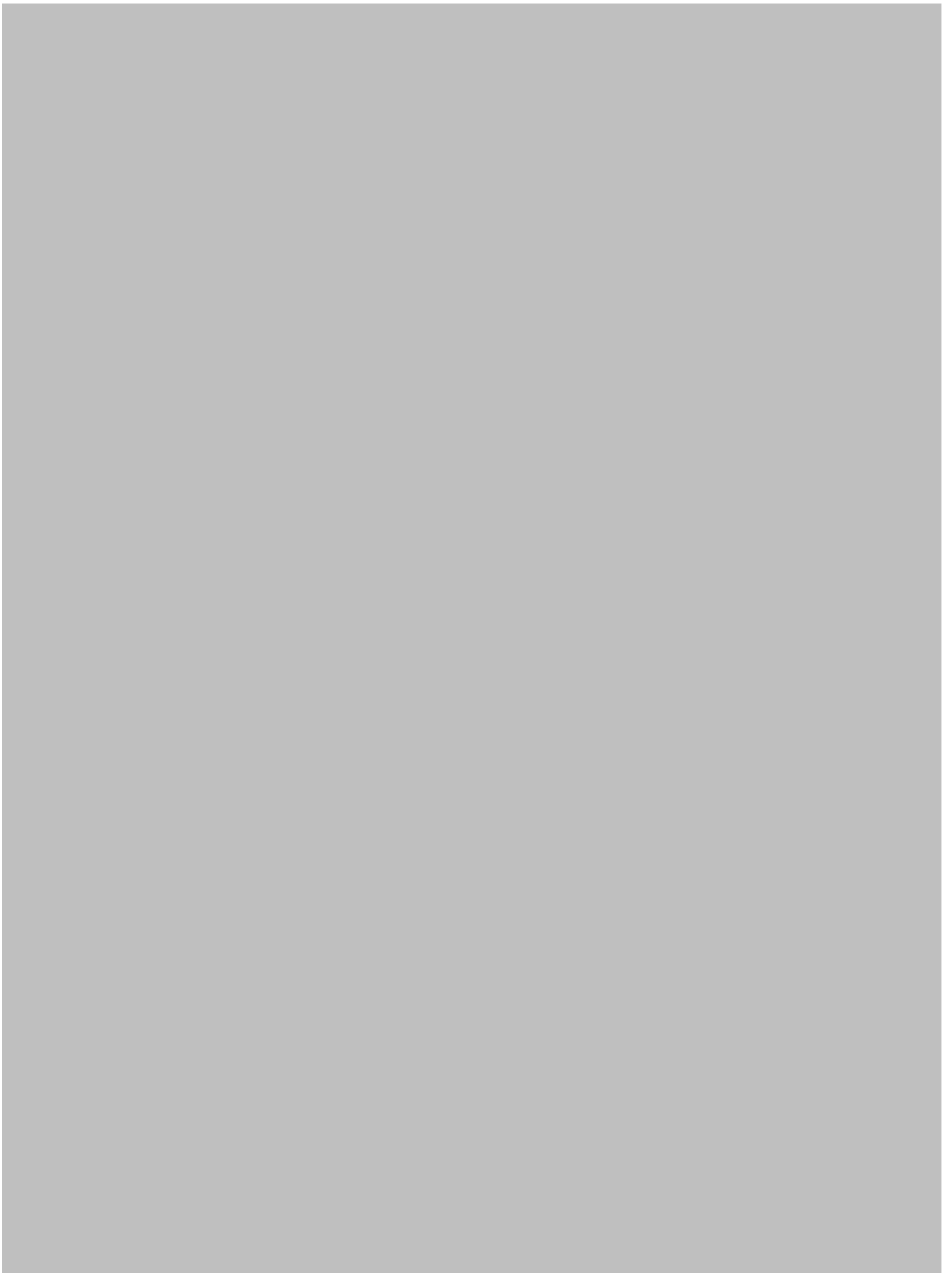












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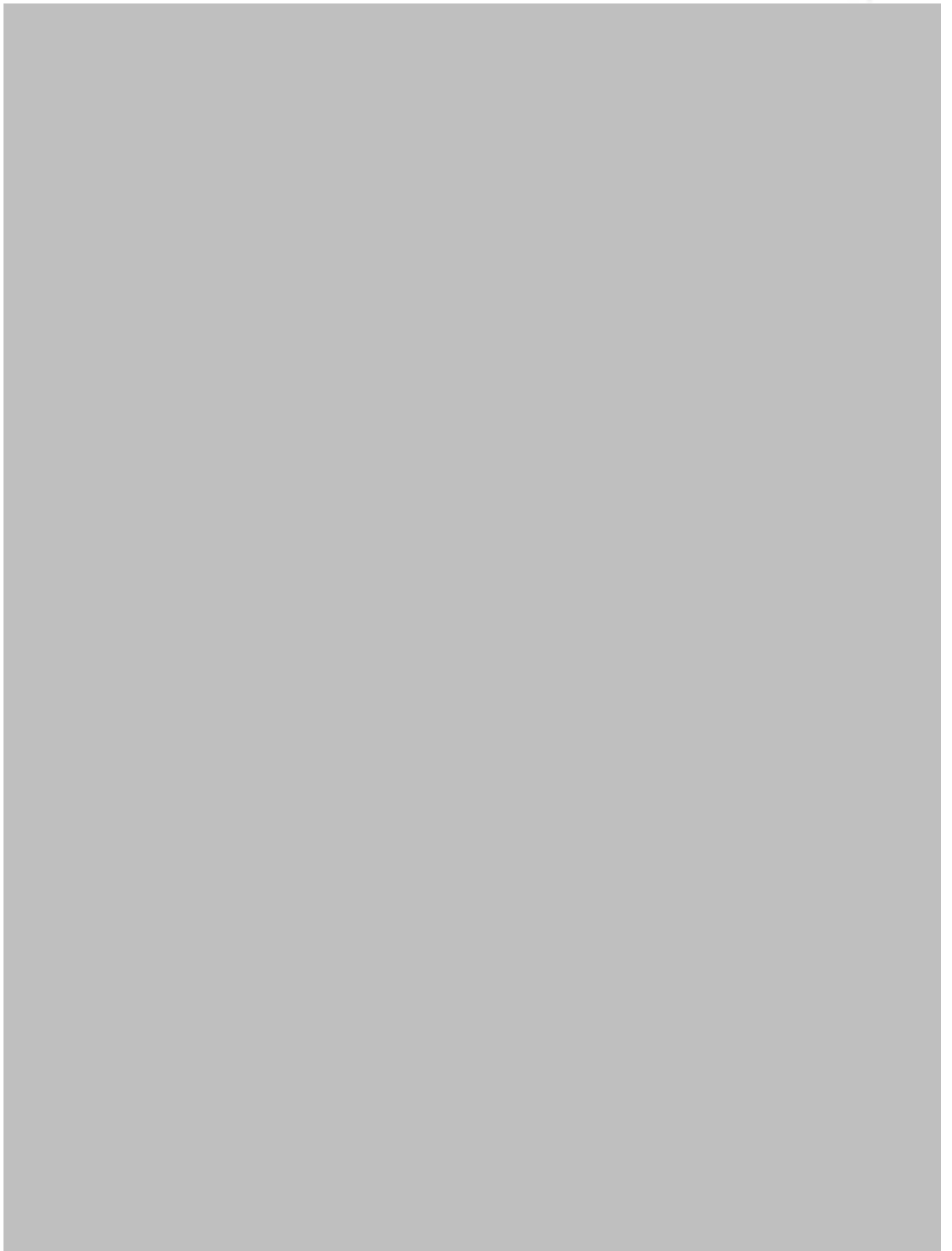
## **Antimicrobial susceptibility of intra-abdominal Gram-negative bacilli from Europe: SMART Europe 2008**

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Y. Carmeli · P. Hawkey**

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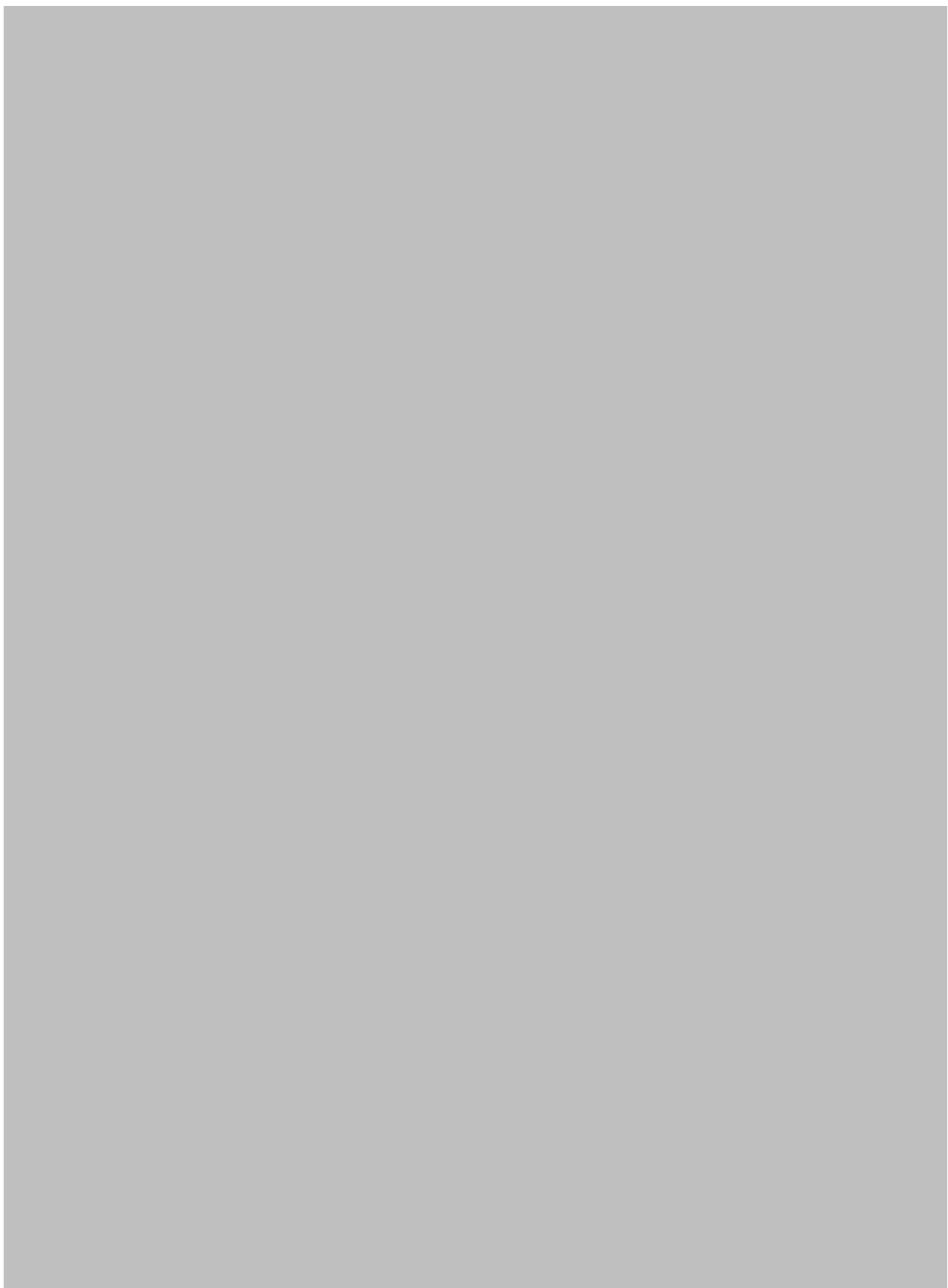




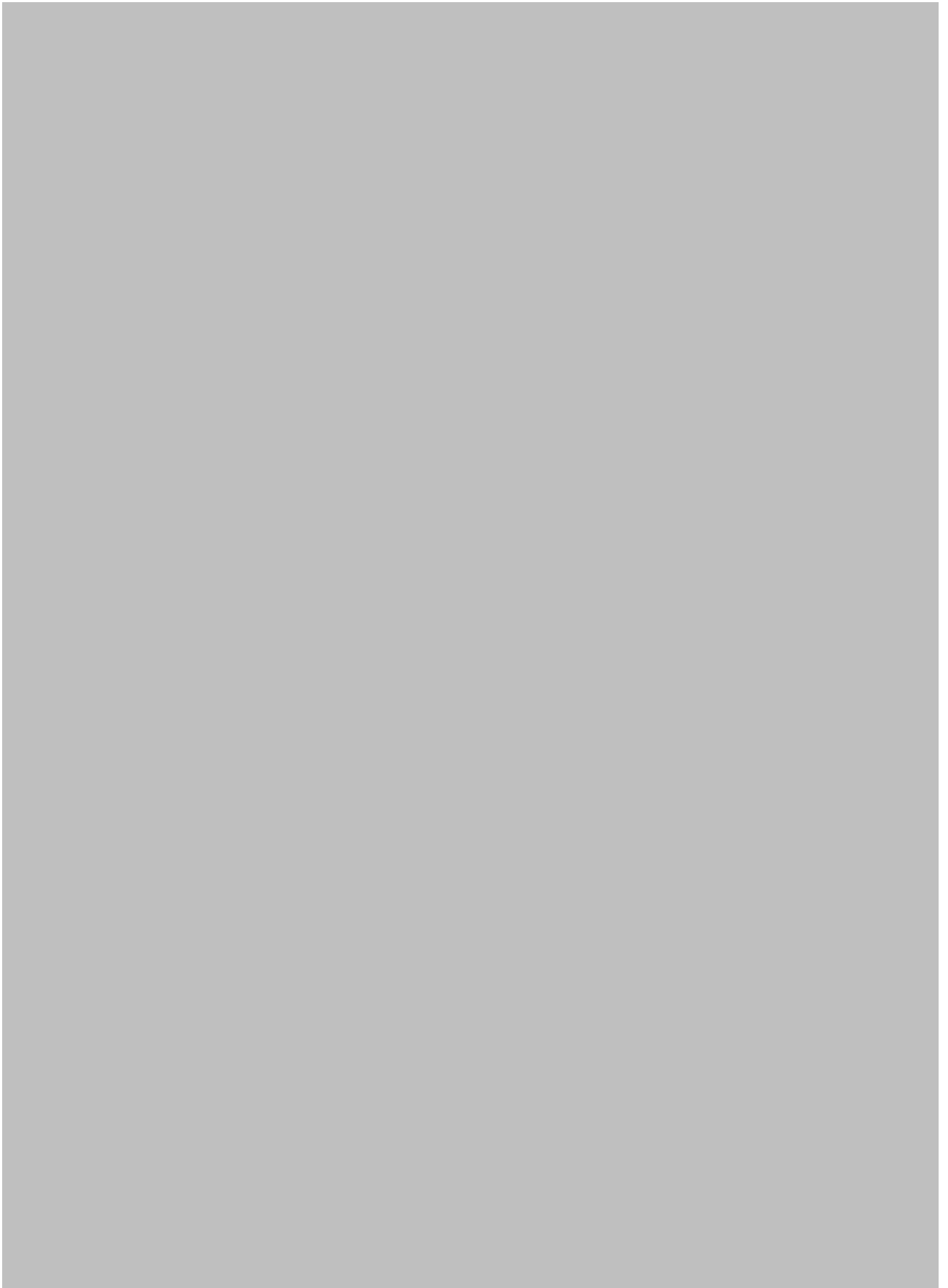












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## Prevalence and characteristics of $\beta$ -lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China

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**Objective:** To determine the molecular epidemiology of extended-spectrum  $\beta$ -lactamases (ESBLs) and plasmid-mediated quinolone resistance (PMQR) in *Escherichia coli* isolated from farmed fish in China.

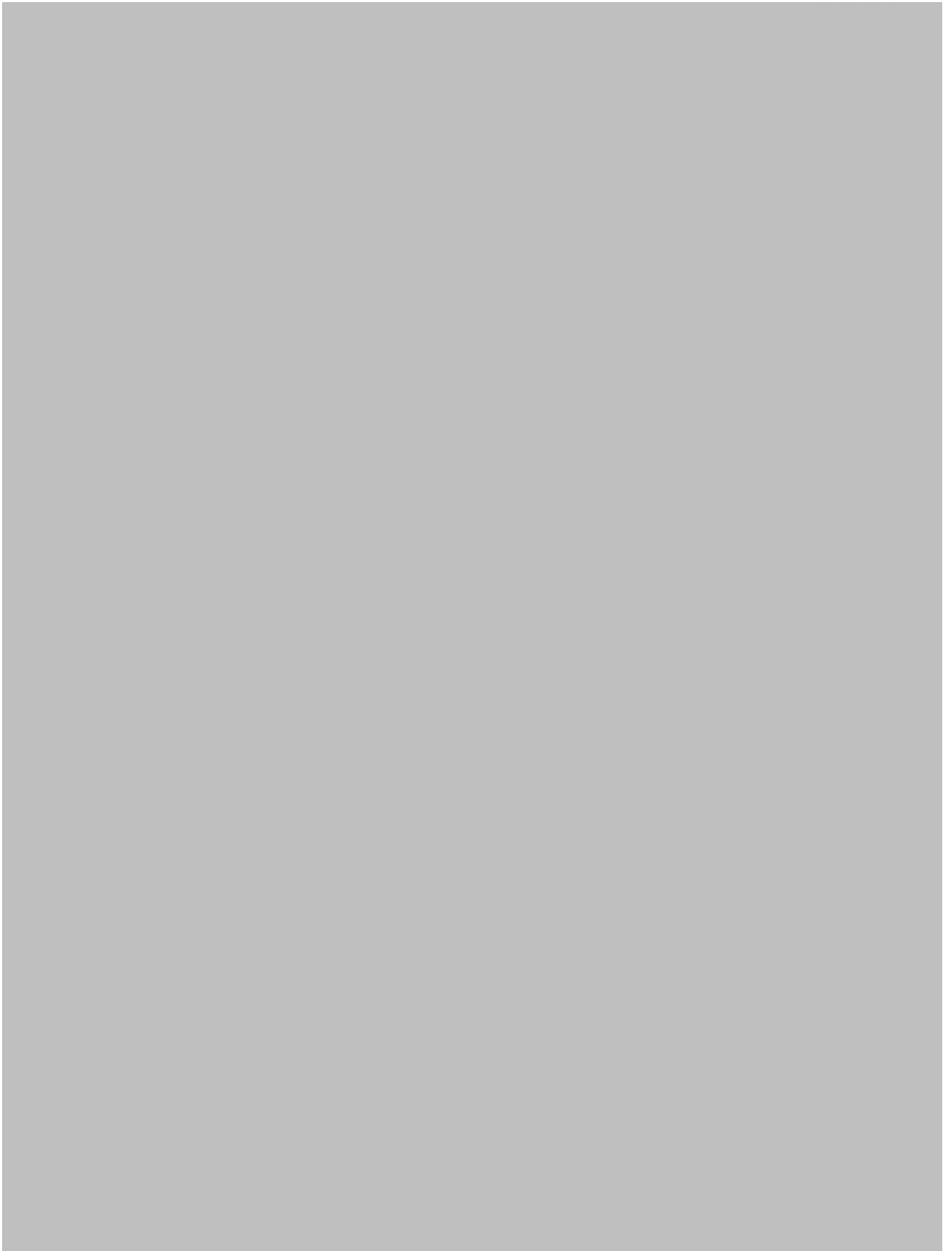
**Methods:** *E. coli* was isolated from fish gut samples from fish farmed throughout Guangdong province and tested for the presence of the  $\beta$ -lactamase genes and PMQR-encoding genes using PCR and DNA sequence analysis. Co-transfer of plasmids encoding for ESBLs as well as PMQR determinants was explored by conjugation into *E. coli*.

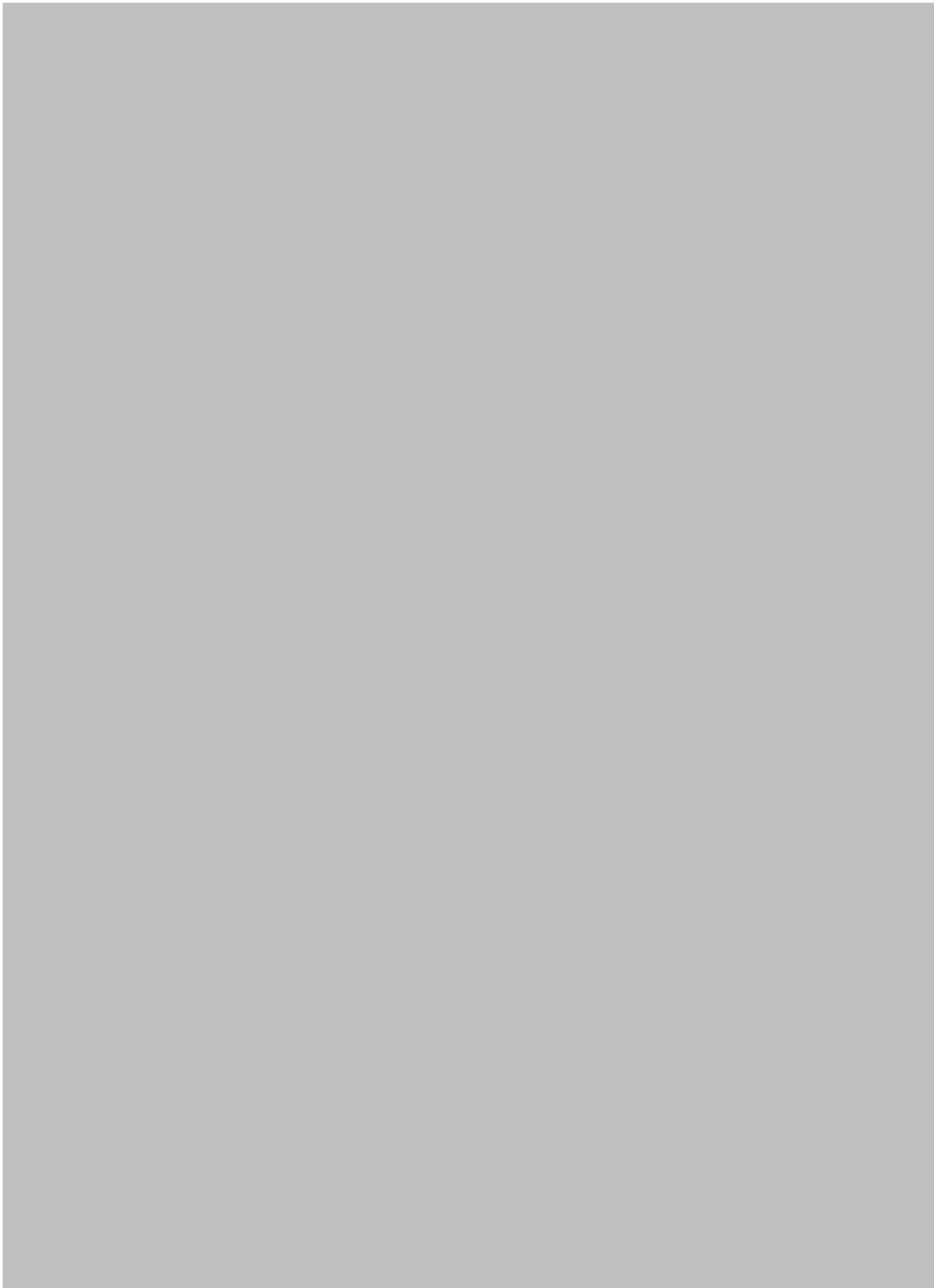
**Results:** A total of 218 non-duplicate *E. coli* were recovered from fish gut samples.  $\beta$ -Lactamase genes were identified in 19 (17%) of 112 strains with reduced susceptibility to ampicillin, and PMQR genes were identified in 59 (73.8%) of 80 strains with reduced susceptibility to ciprofloxacin. Only three ESBL genes were identified in three isolates: *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-79</sub> and *bla*<sub>SHV-27</sub>. PMQR gene screening identified *qnr* genes (*n*=59) as the most common, including *qnrB* (*n*=33), *qnrS* (*n*=21) and *qnrD* (*n*=5), with *aac(6)-Ib-cr* (*n*=6) being rarely found. The co-carriage of two or three PMQR genes in one strain was found in 7 (11.9%) isolates. The ESBL gene *bla*<sub>CTX-M-79</sub> was found to be co-carried with *qnrS*. Co-transfer of *qnrS* was observed with *bla*<sub>CTX-M-79</sub>.

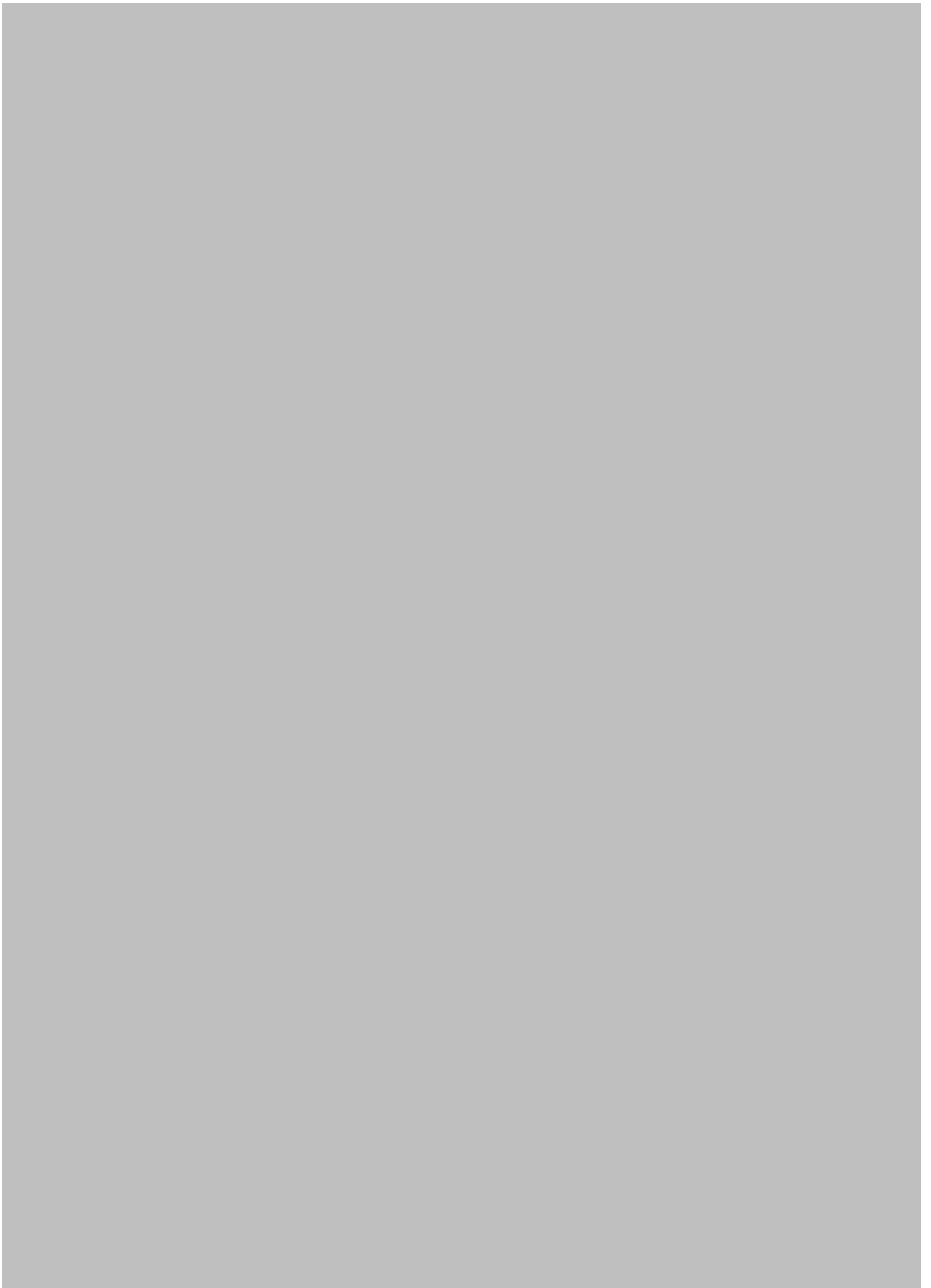
**Conclusions:** Our study is the first to demonstrate the existence of high levels of mobile genes conferring reduced susceptibility to fluoroquinolones as well as the presence of ESBL genes in fish produced in China, and identifies a significant reservoir of antibiotic resistance genes relevant to human medicine.

**Keywords:** plasmid-mediated quinolone resistance, ESBL, aquaculture, molecular epidemiology









18. Ling, T.K., Xiong, J., Yu, Y., Lee, C.C., Ye, H., & Hawkey, P.M. 2006. Multicenter Antimicrobial Susceptibility Survey of Gram-Negative Bacteria Isolated from Patients with Community-Acquired Infections in the People's Republic of China. *Antimicrob. Agents Chemother.*, 50, (1) 374-378

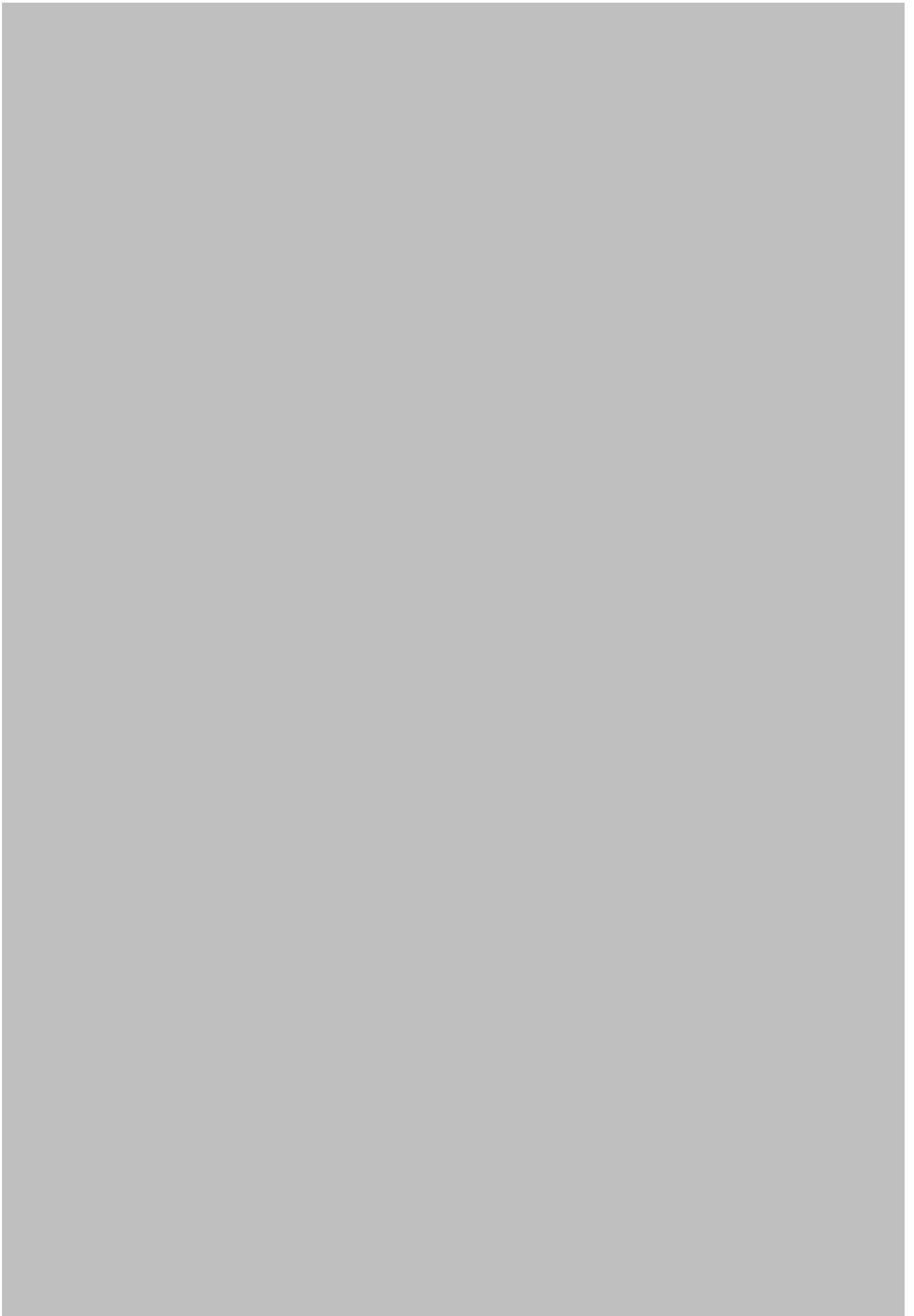
## Multicenter Antimicrobial Susceptibility Survey of Gram-Negative Bacteria Isolated from Patients with Community-Acquired Infections in the People's Republic of China

Thomas K. W. Ling,<sup>1\*</sup> Jianhui Xiong,<sup>2</sup> Yunsong Yu,<sup>3</sup> Ching Ching Lee,<sup>1</sup> Huifen Ye,<sup>4</sup>  
Peter M. Hawkey,<sup>2</sup> and The MK0826 China Study Group

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Received 15 June 2005/Returned for modification 12 August 2005/Accepted 17 October 2005

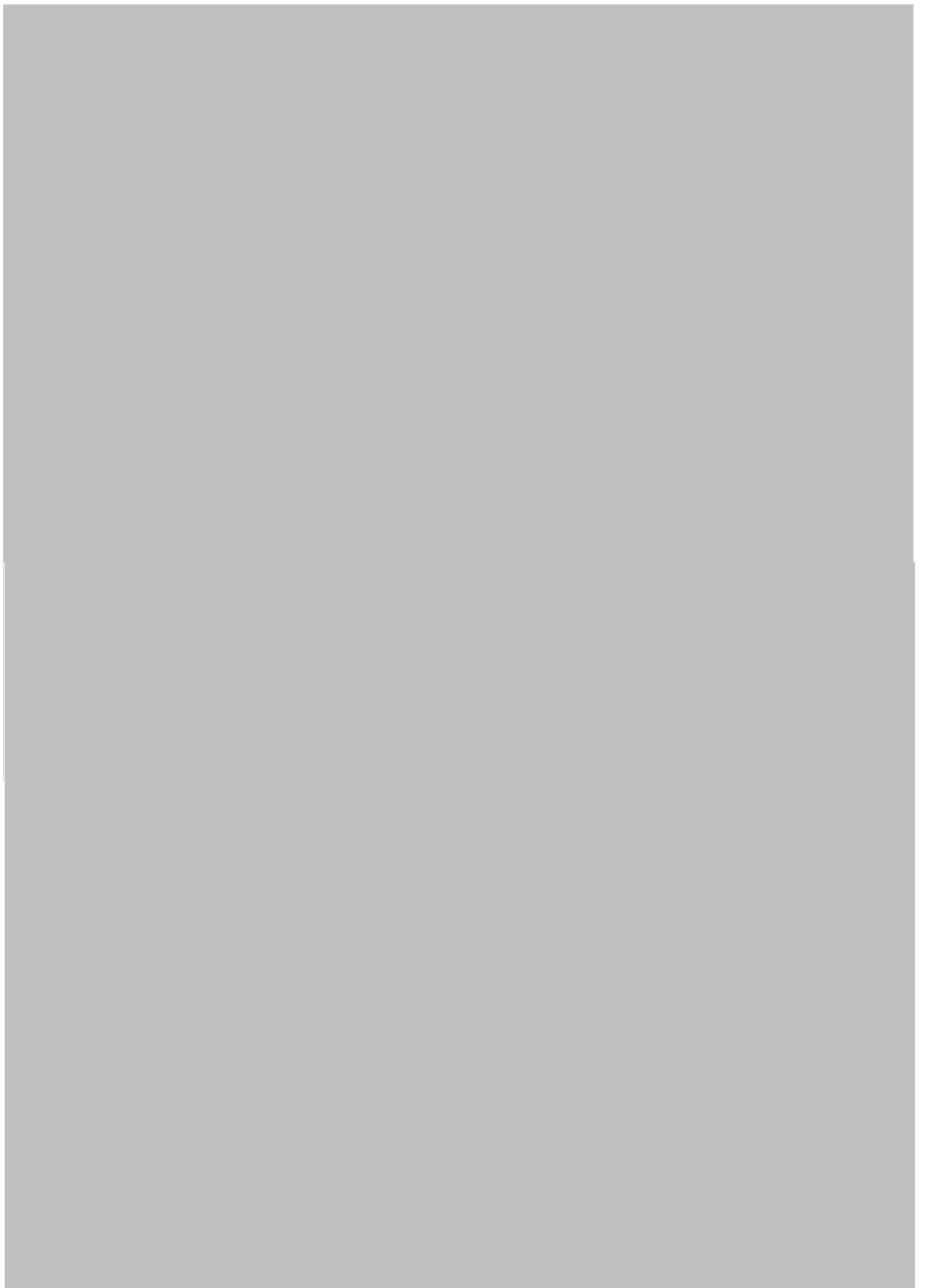
**A survey of 2,099 gram-negative bacilli from community infections at seven centers in the People's Republic of China is reported. The rates of resistance of 1,615 isolates of the family *Enterobacteriaceae* were as follows: 40.8% for ciprofloxacin, 32.2% for gentamicin, 0% for imipenem or ertapenem, and 14.7% for cefotaxime. The rates of extended-spectrum  $\beta$ -lactamase production were 16% for *Escherichia coli* and 17% for *Klebsiella*.**











19. Liu, W., Chen, L., Li, H., Duan, H., Zhang, Y., Liang, X., Li, X., Zou, M., Xu, L., & Hawkey, P.M. 2009. Novel CTX-M {beta}-lactamase genotype distribution and spread into multiple species of *Enterobacteriaceae* in Changsha, Southern China. *J.Antimicrob.Chemother.*, 63, (5) 895-900

## Novel CTX-M $\beta$ -lactamase genotype distribution and spread into multiple species of Enterobacteriaceae in Changsha, Southern China

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Received 3 November 2008; returned 16 December 2008; revised 14 February 2009; accepted 15 February 2009

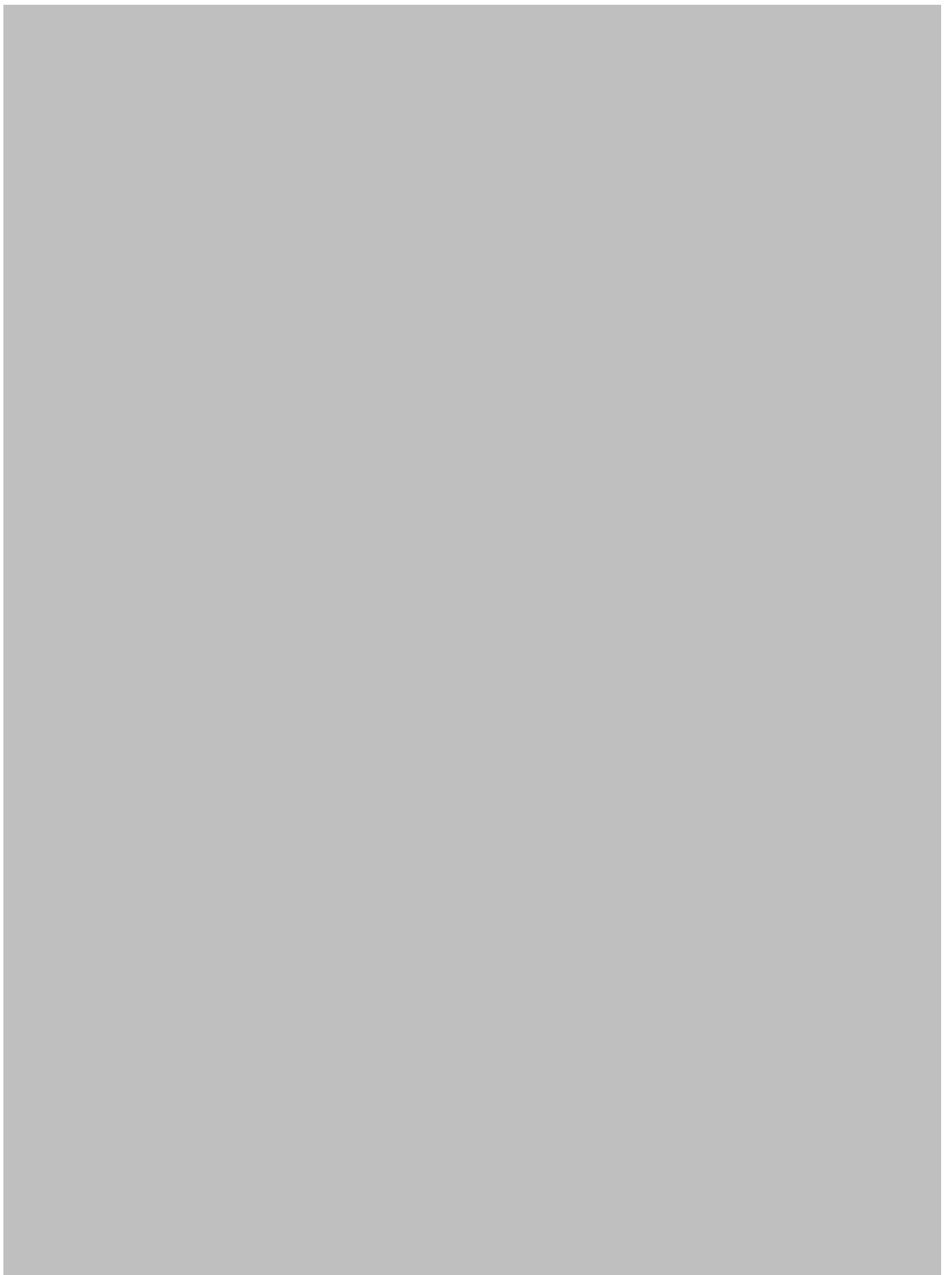
**Objectives:** The aim of this study was to undertake a survey of the occurrence of CTX-M and SHV extended-spectrum  $\beta$ -lactamase (ESBL) genotypes in Enterobacteriaceae from Hunan Province, China.

**Methods:** Clinical isolates (425) from three major hospitals in Changsha, Hunan Province, were collected between October 2004 and July 2005, and their antimicrobial susceptibilities of the genotype of *bla*<sub>CTX-M</sub> and *bla*<sub>SHV</sub> were determined. Random amplified polymorphic DNA was used to characterize the clonality of all of the isolates.

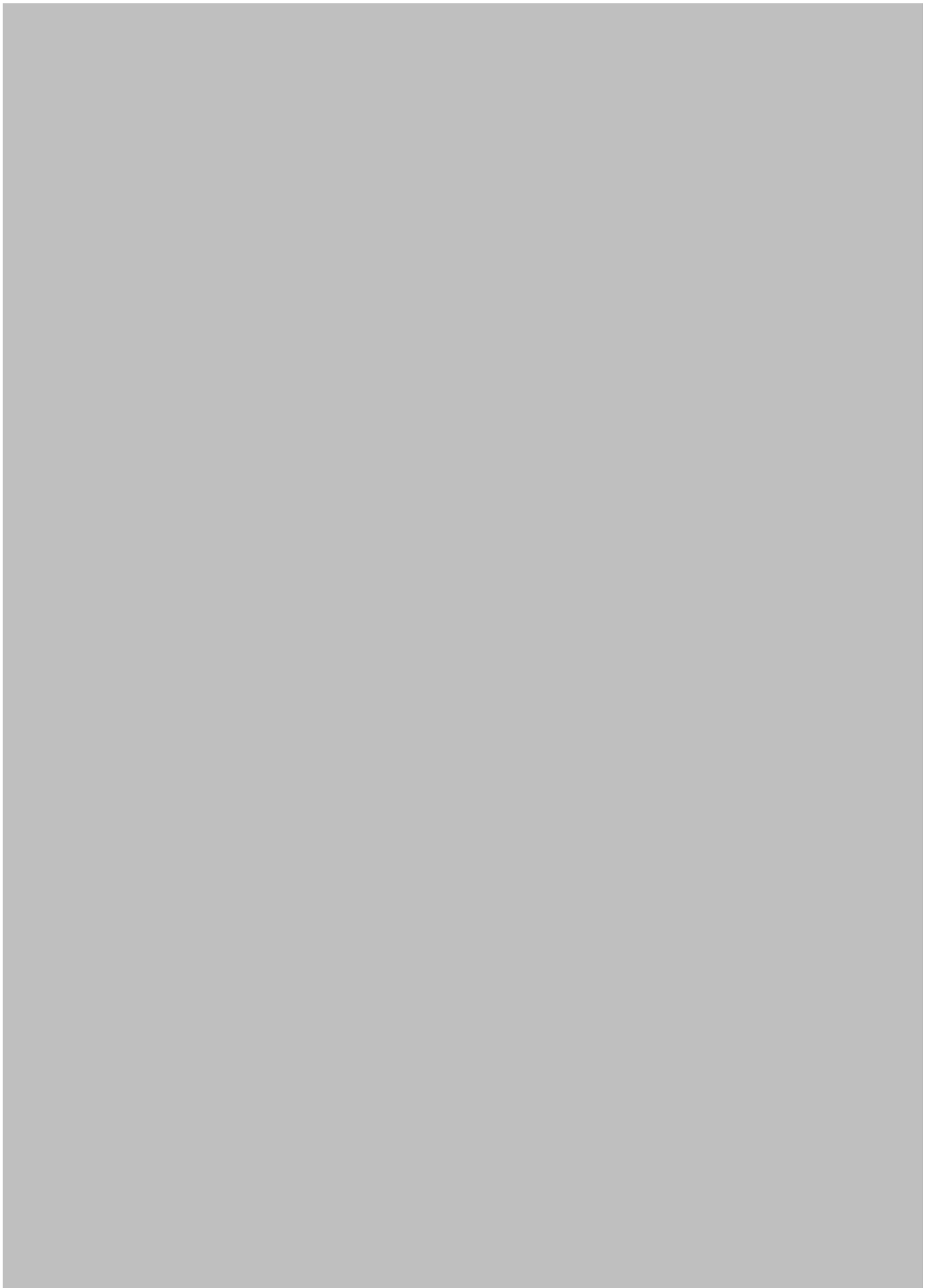
**Results:** The overall rate of ESBL-positive isolates was 33.4% (142/425). The dominant ESBLs were CTX-M types, and were found in 109/142 (76.8%) isolates comprising seven different genera/species, namely *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Proteus vulgaris* and *Providencia stuartii*. The most common *bla*<sub>CTX-M</sub> genotypes were *bla*<sub>CTX-M-14</sub> (47.7%), *bla*<sub>CTX-M-3</sub> (29.4%) and *bla*<sub>CTX-M-15</sub> (17.4%). A novel gene derived from *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-82</sub> (Ala-40→Pro), was identified.

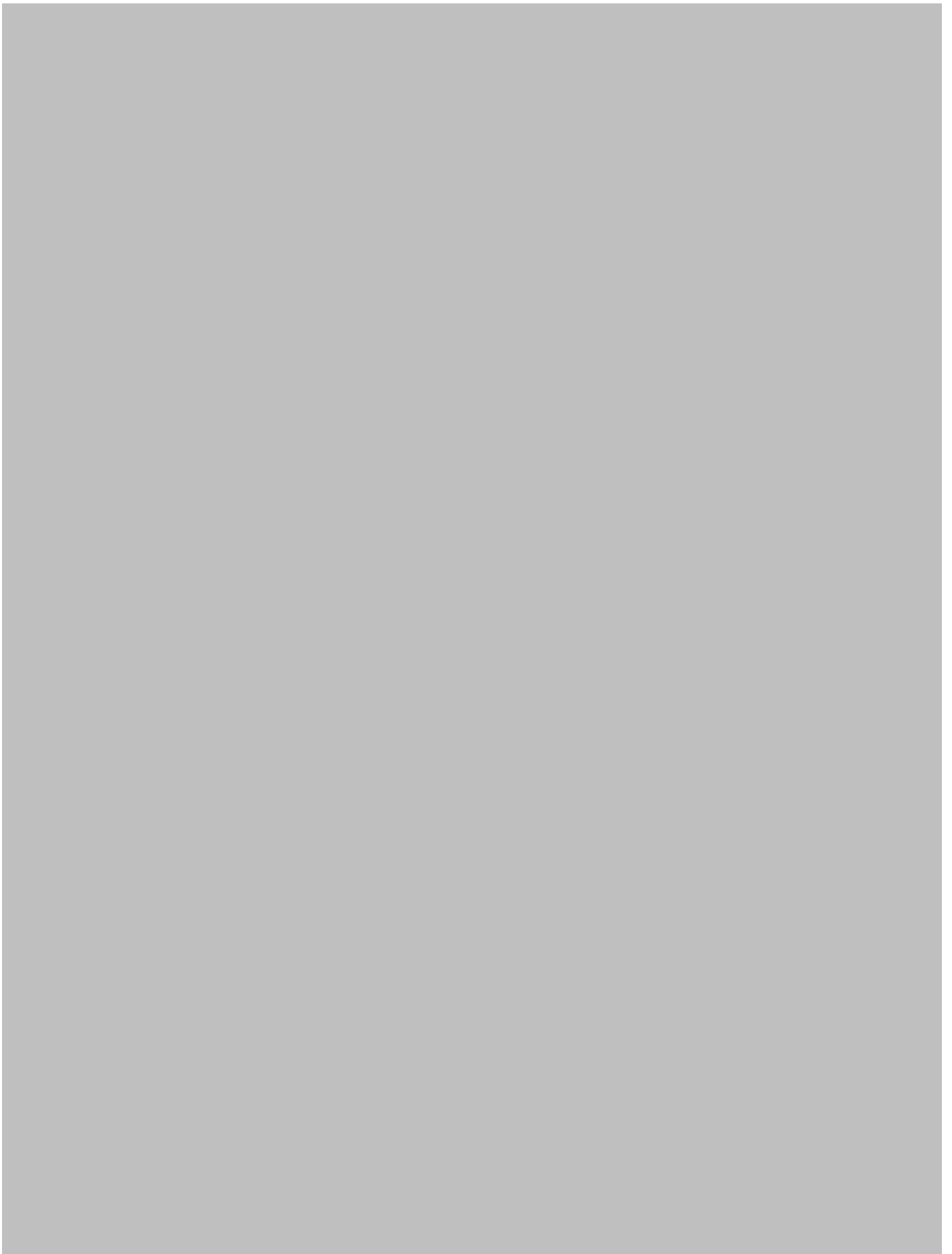
**Conclusions:** The dominant ESBL genotype in Hunan Province was *bla*<sub>CTX-M</sub>. The high prevalence (17.4%) of *bla*<sub>CTX-M-15</sub> has not previously been reported from China. Our results identify that an epidemic of *bla*<sub>CTX-M</sub> in Changsha, Hunan Province, has evolved with the appearance and spread of *bla*<sub>CTX-M-15</sub> against the dominant genotypes *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-3</sub>. The worldwide dominance of *bla*<sub>CTX-M-15</sub> could be poised to spread to China, displacing the current prevailing genotypes.

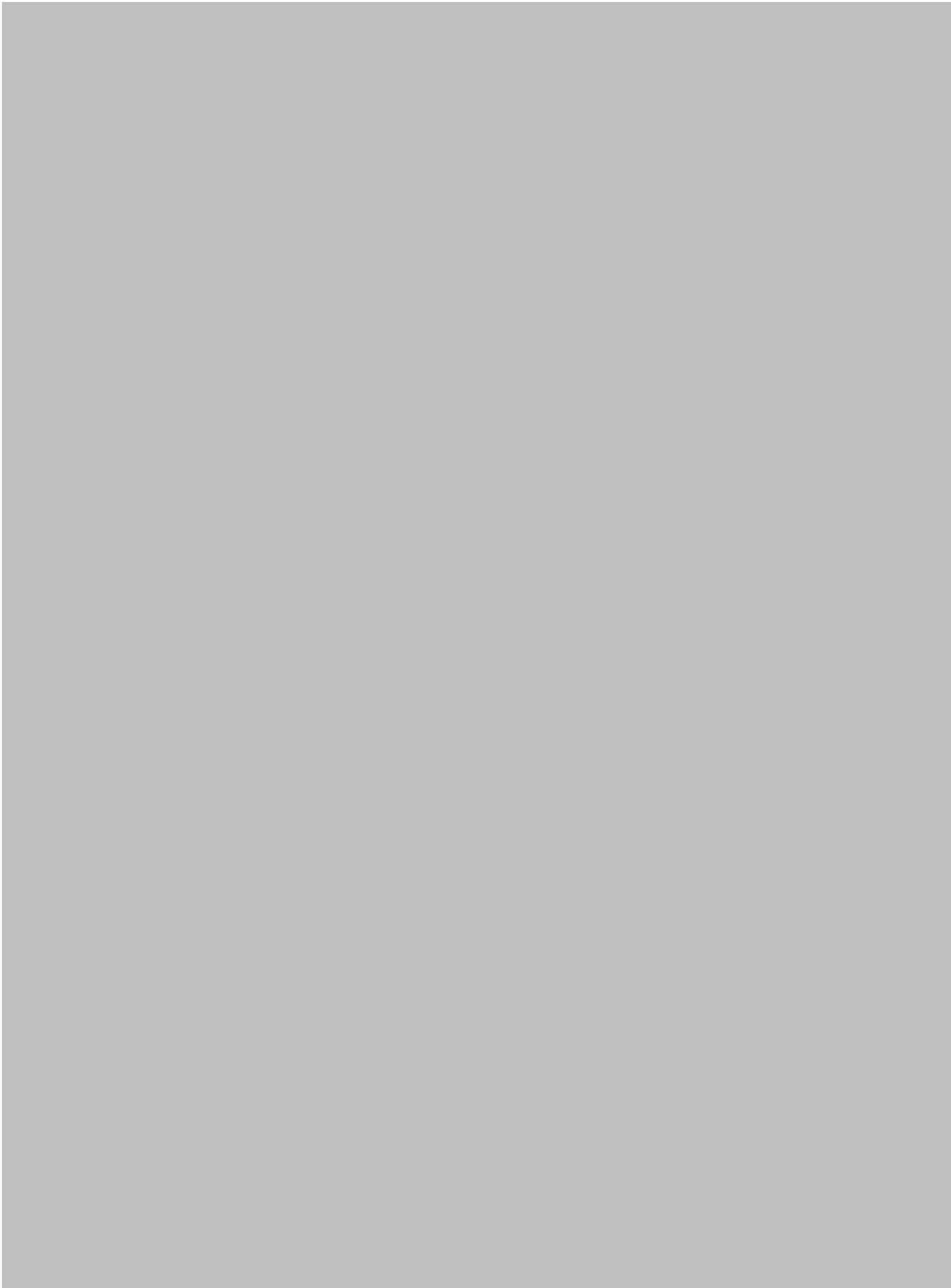
Keywords: ESBLs, *bla*<sub>CTX-M</sub>, Hunan Province













20. Livermore, D.M. & Hawkey, P.M. 2005. CTX-M: changing the face of ESBLs in the UK. *J.Antimicrob.Chemother.*, 56, (3) 451-454

## CTX-M: changing the face of ESBLs in the UK

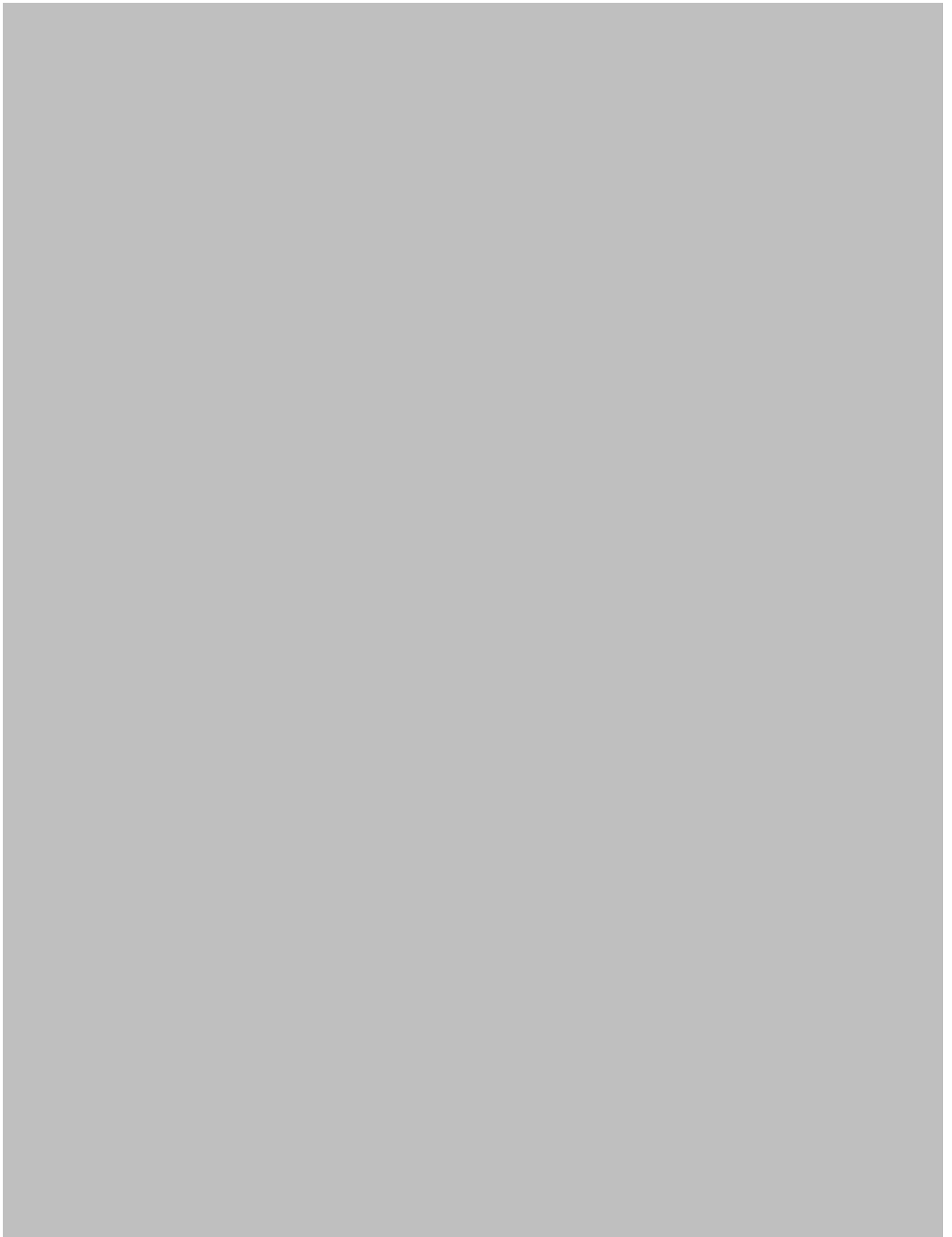
D. M. Livermore<sup>1</sup> and P. M. Hawkey<sup>2,3\*</sup>

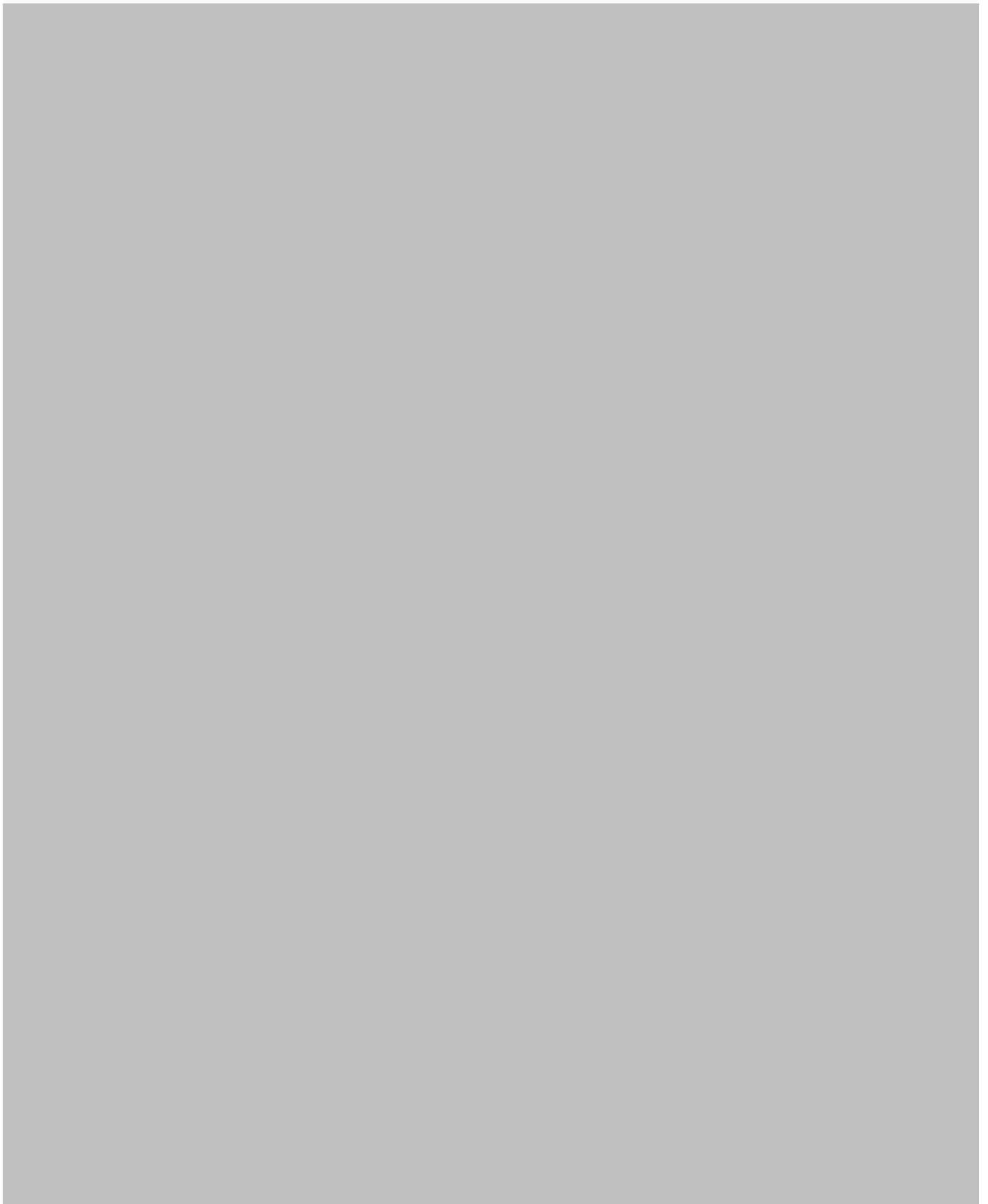
<sup>1</sup>Antibiotic Resistance Monitoring and Reference Laboratory, Centre for Infections, Health Protection Agency, 61 Colindale Avenue, London NW9 5HT, UK; <sup>2</sup>Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, UK; <sup>3</sup>Health Protection Agency West Midlands Public Health Laboratory, Birmingham Heartlands and Solihull NHS Trust, Bordesley Green East, Birmingham B9 5SS, UK

The UK has experienced a sudden rise in extended-spectrum  $\beta$ -lactamase (ESBL) rates, largely due to the appearance and spread of *Escherichia coli* producing CTX-M-15 type  $\beta$ -lactamase. The British Society for Antimicrobial Chemotherapy organized two update meetings during 2004 to report and discuss the recognition, clinical diagnosis, treatment and control of bacteria producing these  $\beta$ -lactamases. This paper reports the data and reviews made by contributors to the conferences. The historical distribution and emergence of ESBLs was reviewed along with the emergence of plasmid-mediated CTX-M ESBLs following their mobilization from the chromosome of *Kluyveraspp*. The first significant outbreak of CTX-M producers in the UK occurred in 2001 and involved *Klebsiella pneumoniae* with CTX-M-26 at one site, but by 2003, cloned and diverse *E. coli* with CTX-M-15 were widespread, with Shropshire one of the most affected regions. The specific experience in Shropshire was reported on and a comprehensive review made of the level of awareness of the need for ESBL detection in laboratories in England and Wales, together with a description of the variety of methods that may be applied, with recommendations for optimal methodology. The increased mortality associated with inappropriate treatment of infections caused by ESBL-producing strains was highlighted, together with discussion on potential control of cross-infection. The meeting concluded that the CTX-M genes have now become widespread in not only *E. coli* but other Enterobacteriaceae in the UK and this will represent a substantial threat to both the treatment of infections caused by these bacteria in the community and within hospitals.

Keywords: extended-spectrum  $\beta$ -lactamases, ESBLs, BSAC







21. Morris, D., Boyle, F., Buckley, V., Xu, L., Hanahoe, B., Hawkey, P., & Cormican, M. 2009. CTX-M enzymes are the predominant extended-spectrum beta-lactamases produced by Enterobacteriaceae in Ireland. *J.Antimicrob.Chemother.*, 64, (4) 864-866 available from: PM:19700476

*Journal of Antimicrobial Chemotherapy*  
doi:10.1093/jac/dkp297  
Advance Access publication 21 August 2009

**CTX-M enzymes are the predominant  
extended-spectrum  $\beta$ -lactamases produced  
by Enterobacteriaceae in Ireland**

Dearbháile Morris<sup>1\*</sup>, Fiona Boyle<sup>1</sup>, Victoria Buckley<sup>1</sup>,  
Li Xu<sup>2</sup>, Belinda Hanahoe<sup>3</sup>, Peter Hawkey<sup>2</sup> and  
Martin Cormican<sup>1,3</sup>

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University of Birmingham, Birmingham and Health  
Protection Agency, West Midlands Public Health Laboratory,  
Birmingham Heart of England NHS Trust, Bordesley Green*

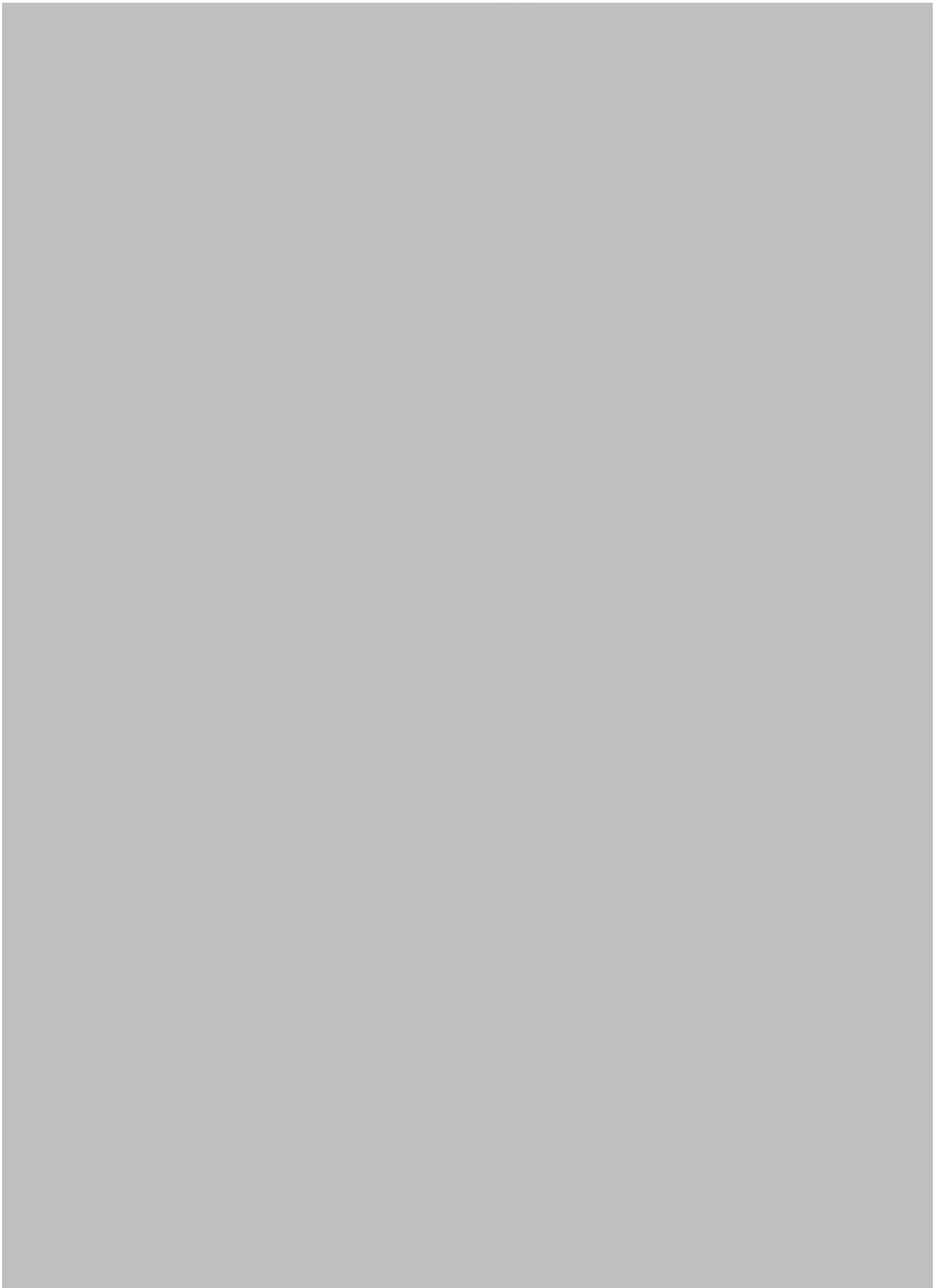
**Research letters**

*East, Birmingham, UK; <sup>3</sup>Department of Medical Microbiology, Galway University Hospital, Galway, Ireland*

Keywords: antimicrobial resistance, cefotaxime, plasmid-mediated

\*Corresponding author. Tel: 0035391544652;  
Fax: 0035391495514; E-mail: dearbhaile.morris@nuigalway.ie





22. Munday, C.J., Boyd, D.A., Brenwald, N., Miller, M., Andrews, J.M., Wise, R., Mulvey, M.R., & Hawkey, P.M. 2004a. Molecular and kinetic comparison of the novel extended-spectrum beta-lactamases CTX-M-25 and CTX-M-26. *Antimicrob. Agents Chemother.*, 48, (12) 4829-4834

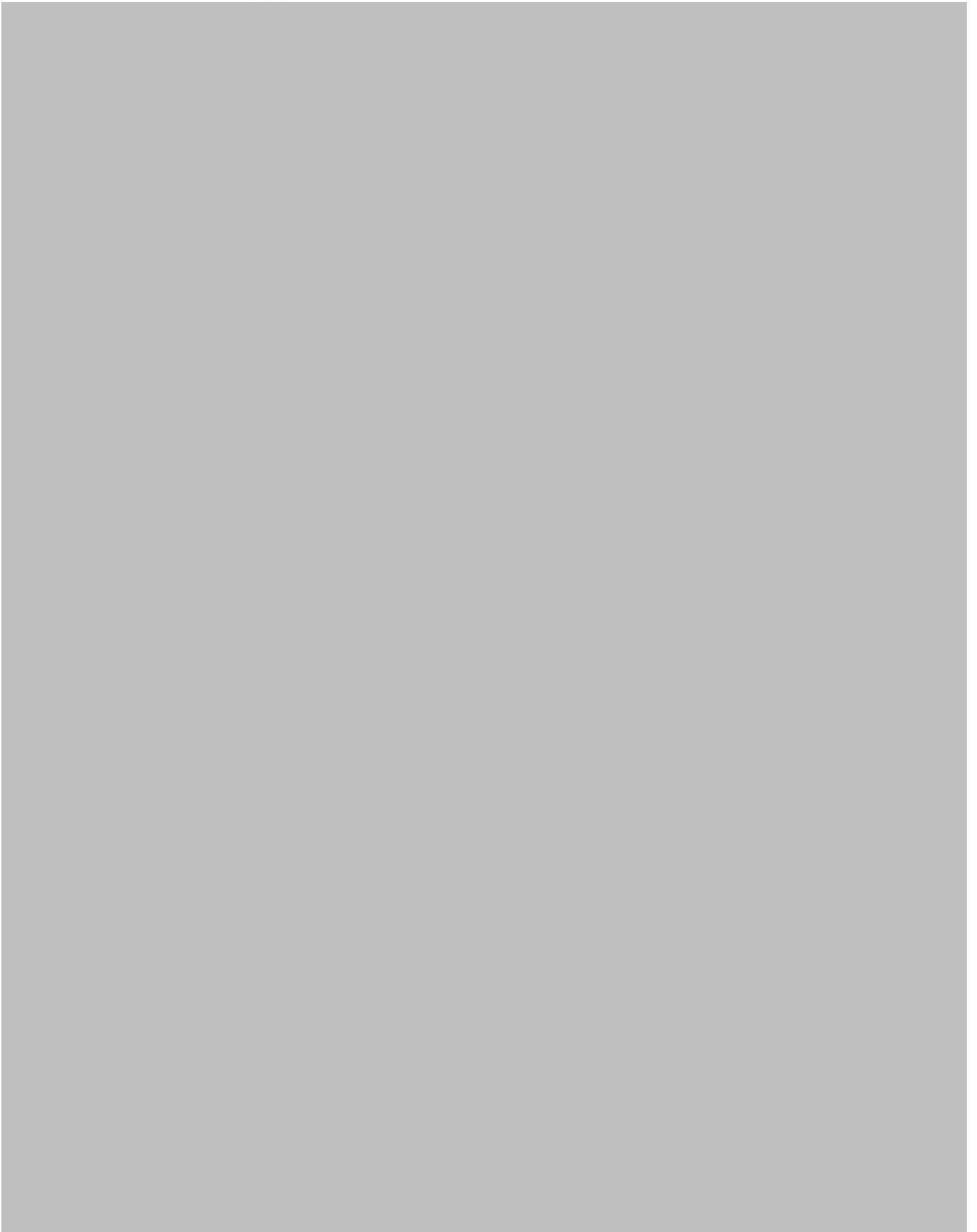
## Molecular and Kinetic Comparison of the Novel Extended-Spectrum $\beta$ -Lactamases CTX-M-25 and CTX-M-26

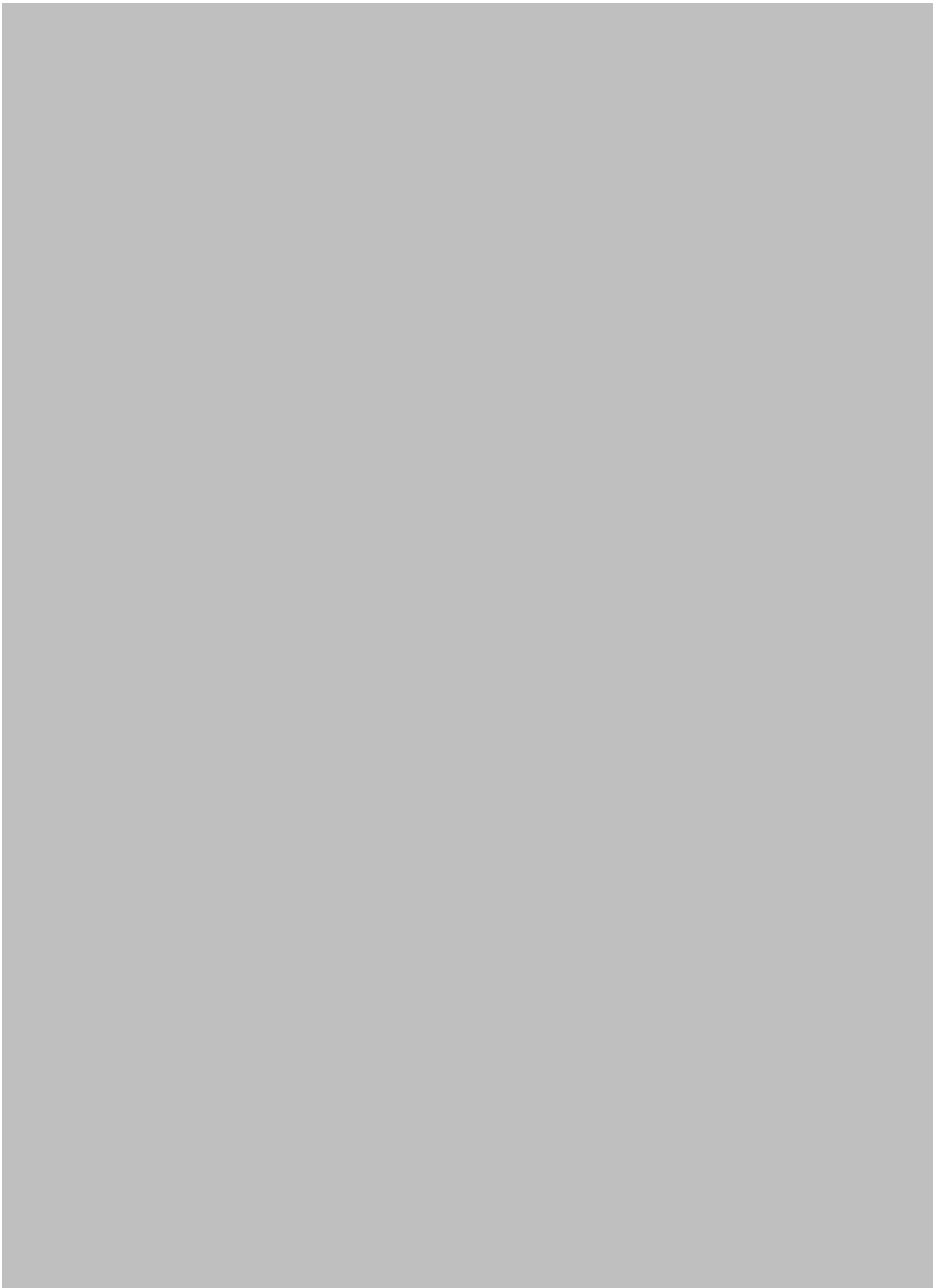
Craig J. Munday,<sup>1</sup> David A. Boyd,<sup>2</sup> Nigel Brenwald,<sup>3</sup> Mark Miller,<sup>4</sup> Jennifer M. Andrews,<sup>3</sup>  
Richard Wise,<sup>3</sup> Michael R. Mulvey,<sup>2</sup> and Peter M. Hawkey<sup>1\*</sup>

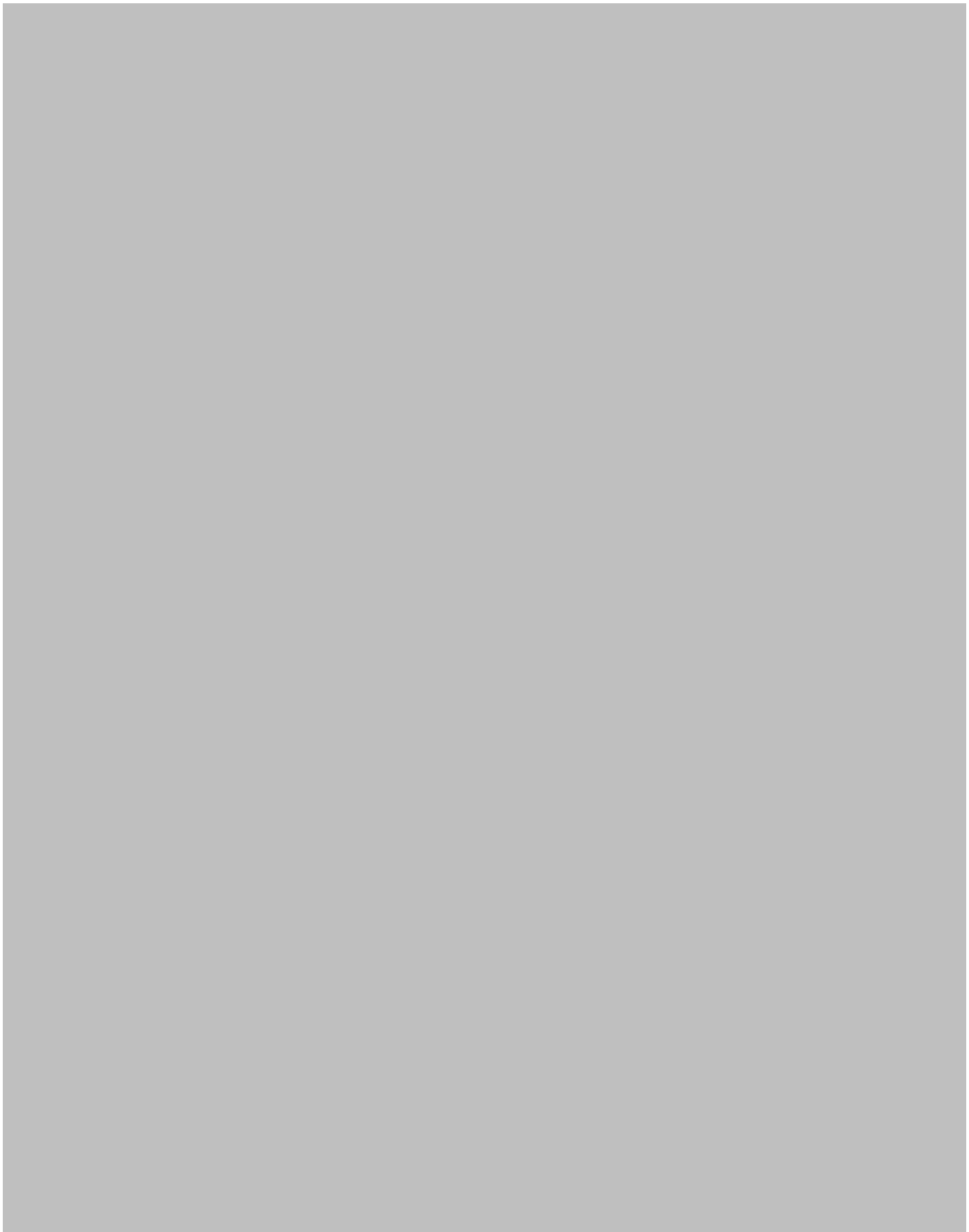
*Antimicrobial Agents Research Group, Division of Immunity and Infection, The Medical School, University of Birmingham,<sup>1</sup> and Department of Microbiology, City Hospital,<sup>3</sup> Birmingham, United Kingdom, and Nosocomial Infections, National Microbiology Laboratory, Health Canada, Winnipeg, Manitoba,<sup>2</sup> and SMBD-Jewish General Hospital, Montreal, Quebec,<sup>4</sup> Canada*

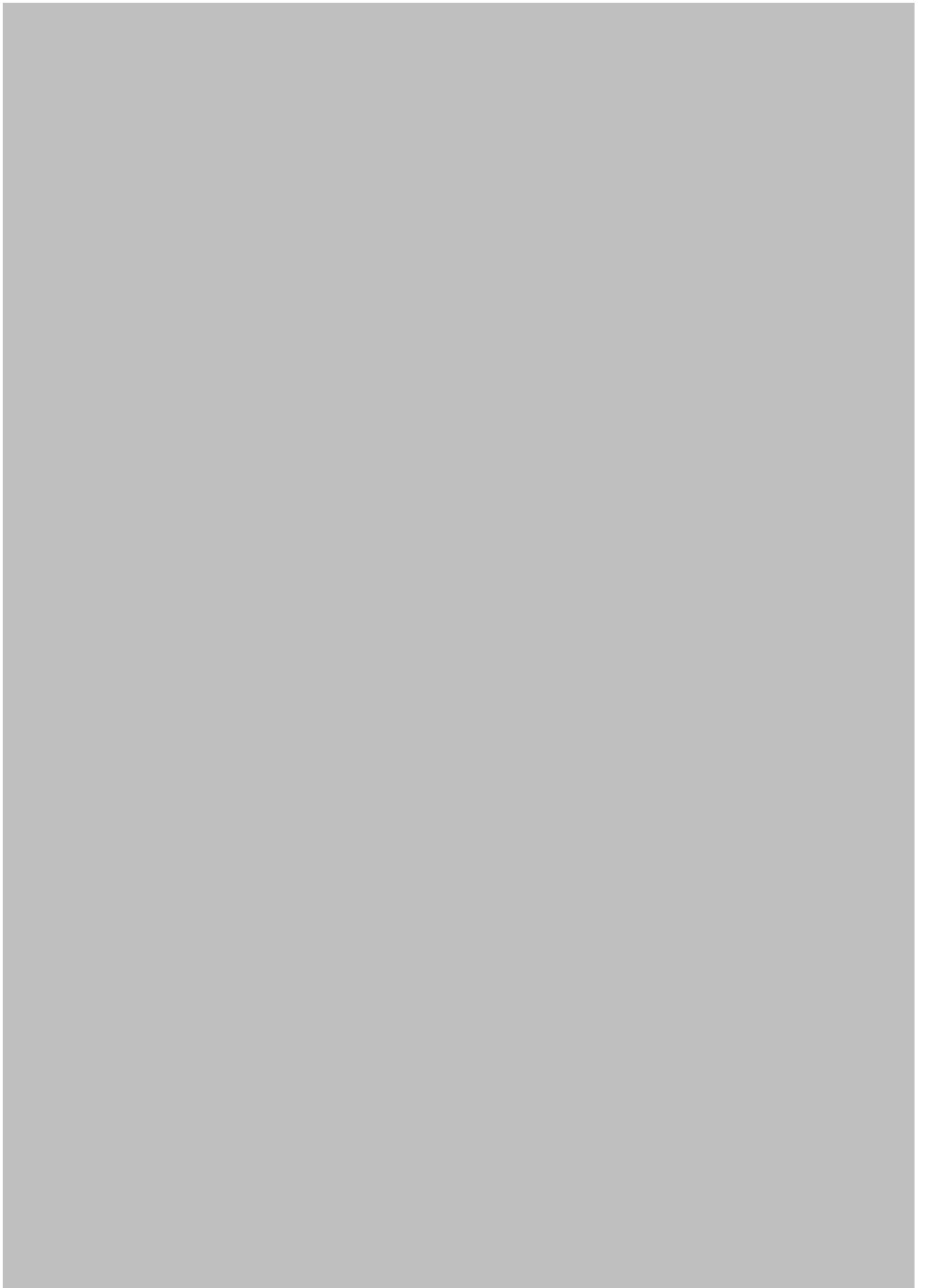
Received 6 January 2004/Returned for modification 4 April 2004/Accepted 11 August 2004

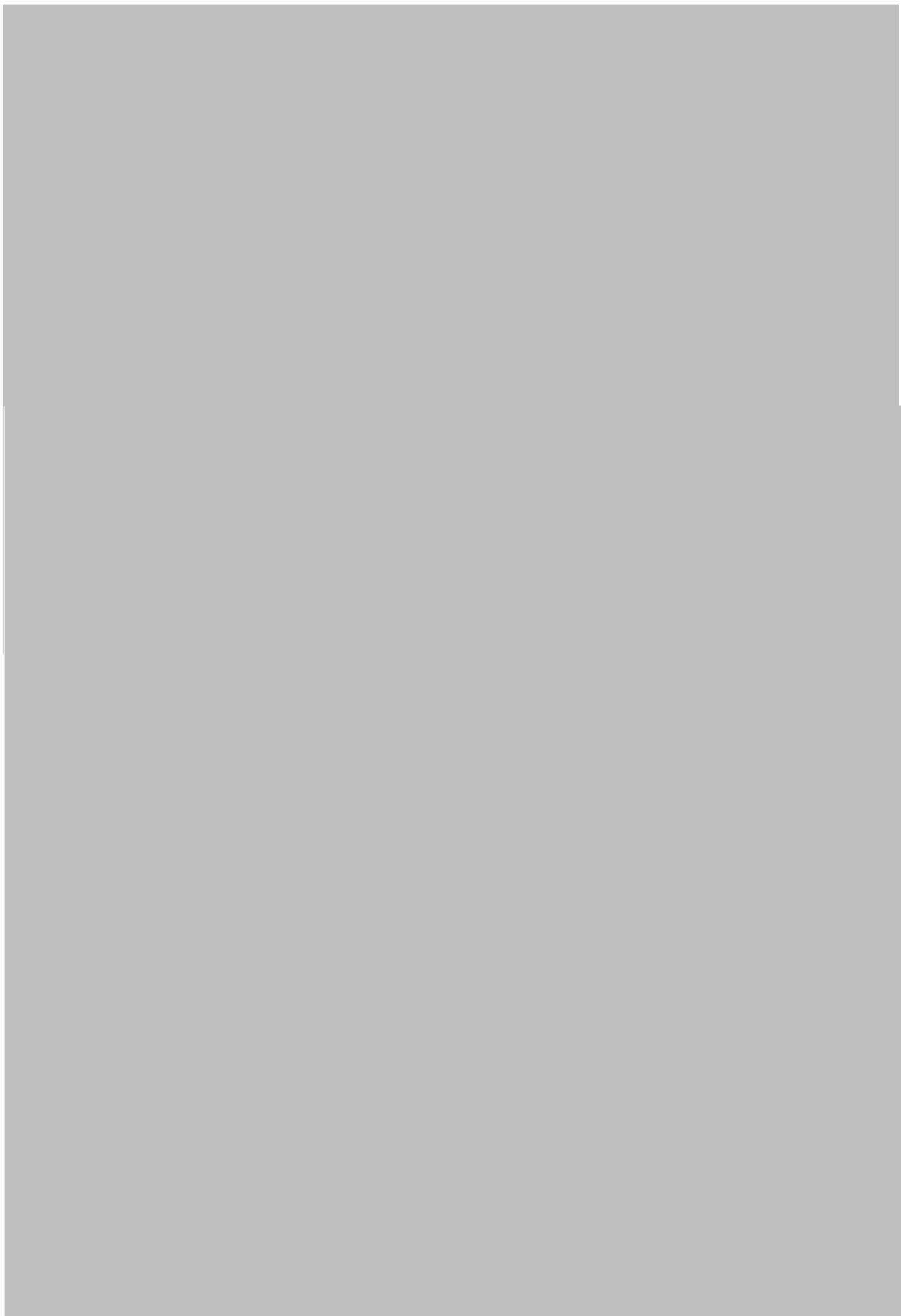
CTX-M-25 is a novel extended-spectrum  $\beta$ -lactamase isolated from a single Canadian *Escherichia coli* isolate. Susceptibility testing demonstrated that this enzyme confers resistance to both cefotaxime and ceftazidime, but the level of resistance was reduced with the addition of  $\beta$ -lactamase inhibitors. The *bla*<sub>CTX-M-25</sub> gene was detected on a 111-kb plasmid. It is a member of the CTX-M-8 group and has the closest amino acid identity (99%; three amino acid substitutions) with CTX-M-26. The *bla*<sub>CTX-M-26</sub> gene was detected on a 100-kb plasmid isolated from a *Klebsiella pneumoniae* strain from the United Kingdom, and plasmid profiling revealed that it showed some homology to the *bla*<sub>CTX-M-25</sub>-harboring plasmid. Both CTX-M genes were located downstream of *ISEcp1*, although the copy upstream of *bla*<sub>CTX-M-25</sub> was disrupted by *IS50-A*. Comparative kinetic studies of recombinant CTX-M-25 and CTX-M-26 enzymes showed that CTX-M-25 has a higher level of ceftazidime hydrolysis ( $k_{cat}$  values, 33 and 0.005 s<sup>-1</sup> for CTX-M-25 and CTX-M-26, respectively).













23. Munday, C.J., Whitehead, G.M., Todd, N.J., Campbell, M., & Hawkey, P.M. 2004b. Predominance and genetic diversity of community-and hospital-acquired CTX-M extended-spectrum  $\beta$ -lactamases in York, UK. *Journal of Antimicrobial Chemotherapy*, 54, (3) 628-633

## Predominance and genetic diversity of community- and hospital-acquired CTX-M extended-spectrum $\beta$ -lactamases in York, UK

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<sup>1</sup>Antimicrobial Agents Research Group, Division of Immunobiology and Infection, The Medical School, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT; <sup>2</sup>Department of Microbiology, York Hospital, Wigginton Road, York YO31 8HE; <sup>3</sup>Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA; <sup>4</sup>Birmingham Public Health Laboratory, Health Protection Agency, Heartlands Hospital, Bordesley Green East, Birmingham B9 5SS, UK

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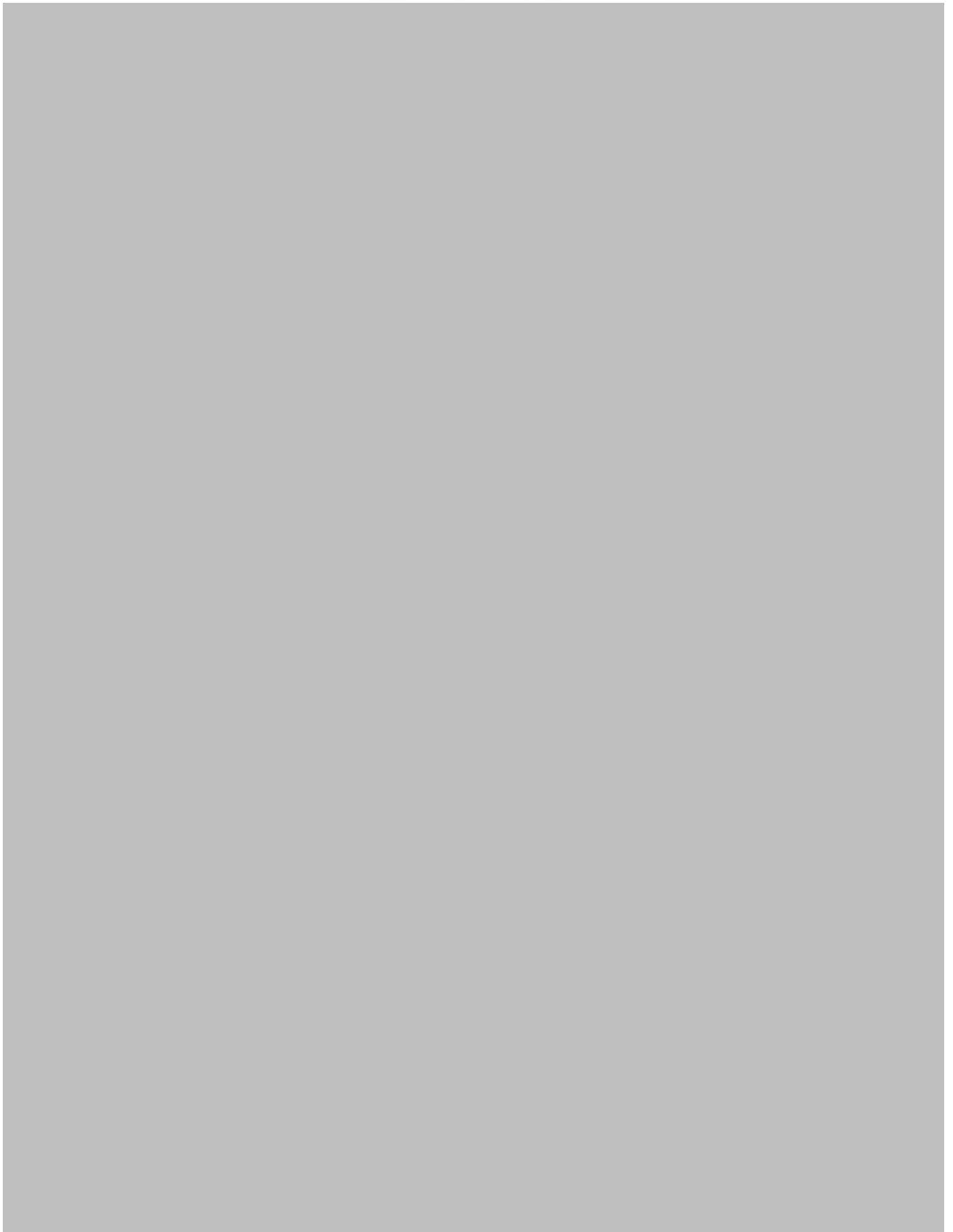
**Objectives:** This study was conducted to detect the presence of extended-spectrum  $\beta$ -lactamase (ESBL)-producing Enterobacteriaceae within the faecal flora of both community- and hospital-based patients in York and to characterize the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes present in these isolates.

**Methods:** One thousand faeces samples were collected and screened at York Hospital during October–December 2003. Ninety-five non-duplicate Enterobacteriaceae isolates resistant to third-generation cephalosporins were recovered; 22 isolates were selected for further study on the basis of a positive double disc diffusion test for ESBL production. Antibiotic susceptibility testing was performed to a range of antibiotics. The TEM, SHV and CTX-M genes were detected by PCR and the DNA sequenced.

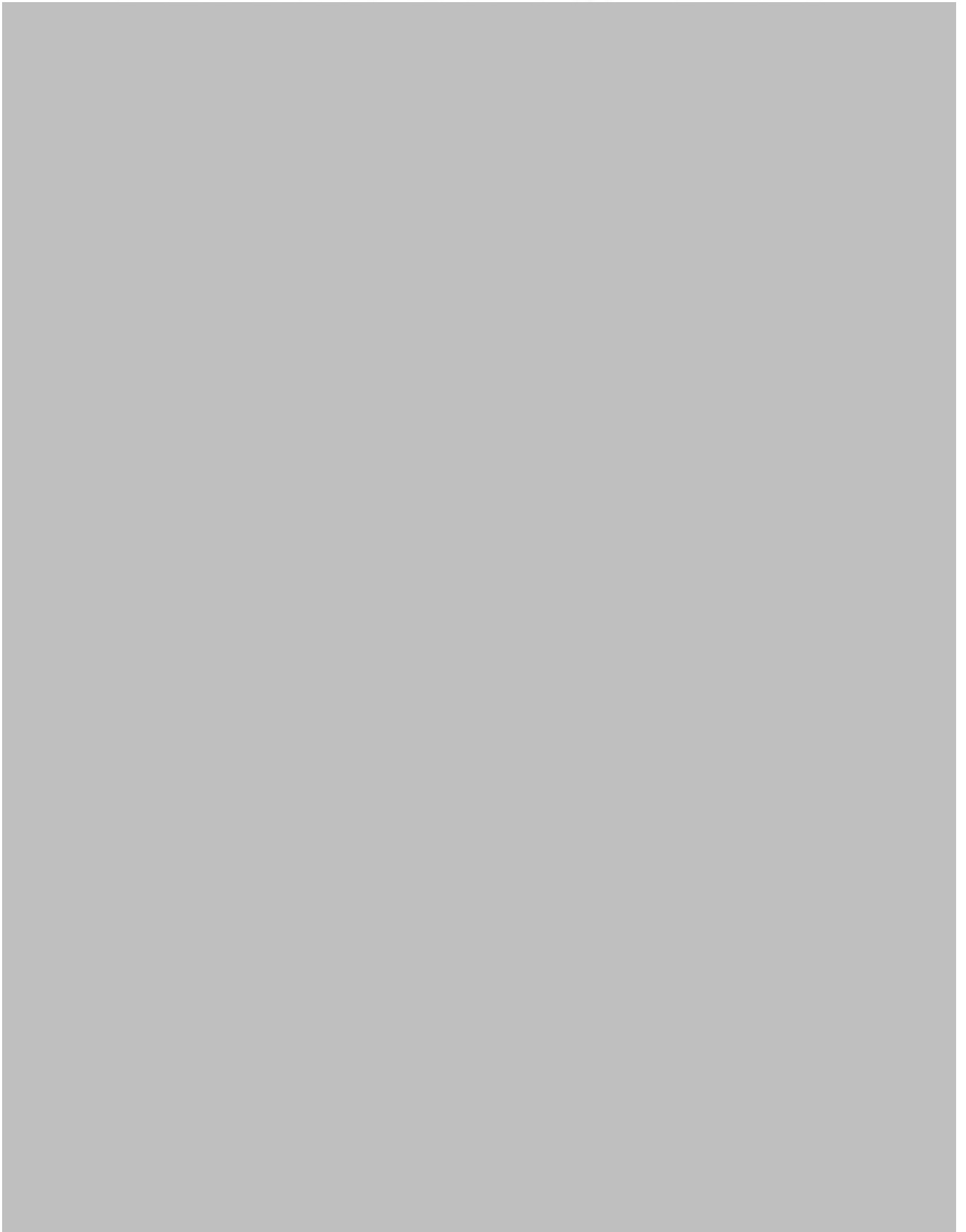
**Results:** The distribution of ESBL-positive isolates from the hospital and community was 1.4:1. These included nine *Escherichia coli*, seven *Enterobacter cloacae*, four *Citrobacter freundii* and a single isolate each of *Klebsiella* spp. and *Salmonella* spp. A total of 17 isolates contained *bla*<sub>CTX-M</sub> (five *bla*<sub>CTX-M-15</sub>, three *bla*<sub>CTX-M-14</sub> and nine *bla*<sub>CTX-M-9</sub>). *ISEcp1* was present in isolates expressing CTX-M-14 and -15, but was absent upstream of In60-associated *bla*<sub>CTX-M-9</sub>. *E. coli* isolates also contained either a *bla*<sub>TEM-1</sub> or *bla*<sub>TEM-2</sub>, whereas six of the *E. cloacae* carried *bla*<sub>SHV-12</sub> and the *Klebsiella* spp. *bla*<sub>SHV-36</sub> in addition to *bla*<sub>CTX-M-9</sub>. The single *Salmonella* spp. carried *bla*<sub>SHV-12</sub>.

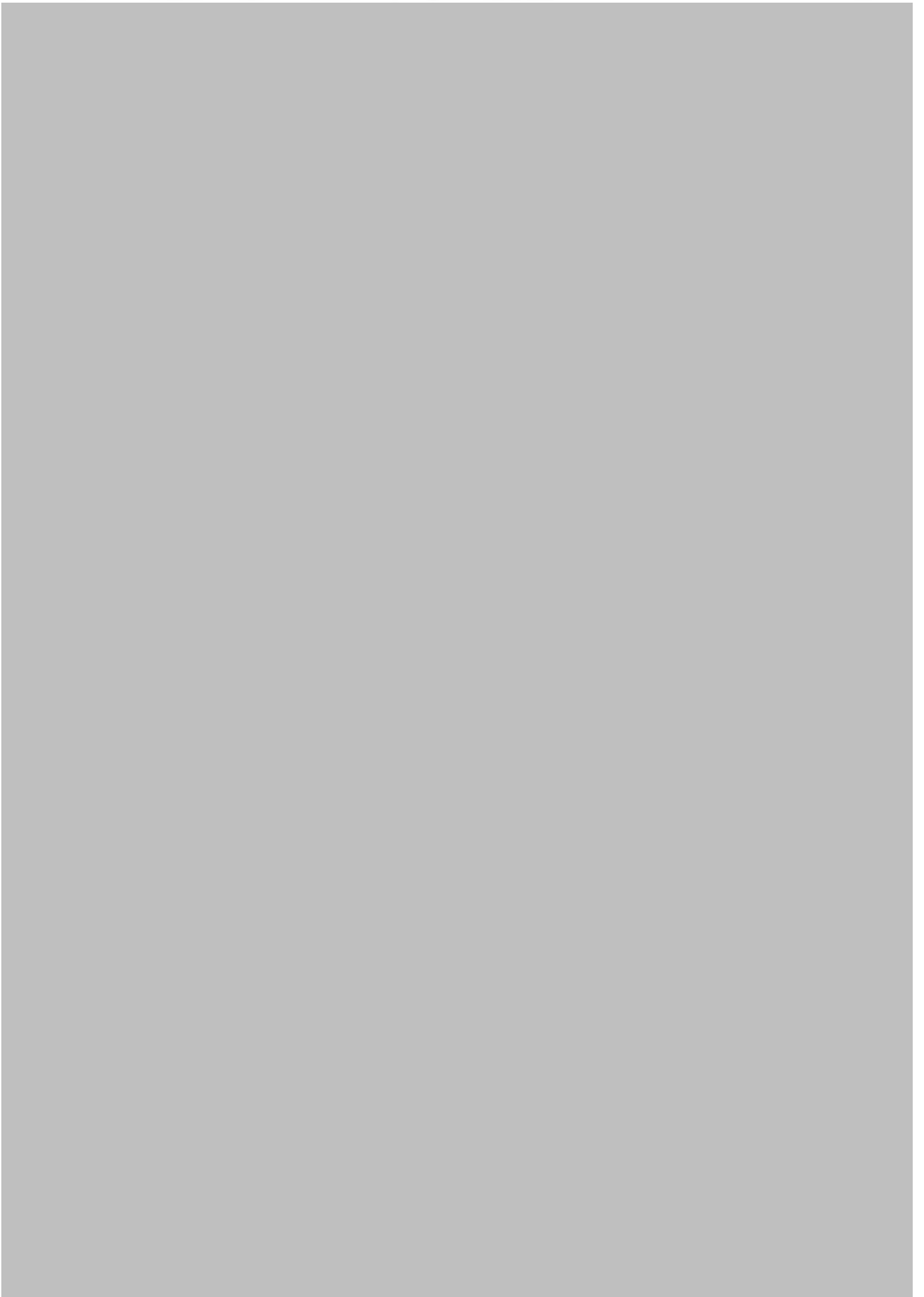
**Conclusions:** The overall prevalence of ESBL in isolates of Enterobacteriaceae from York was 1.9%. ESBL-producing isolates were found in both the community and hospital, with the CTX-M type most common. This is also the first report of an ESBL-producing *Salmonella* in the UK.

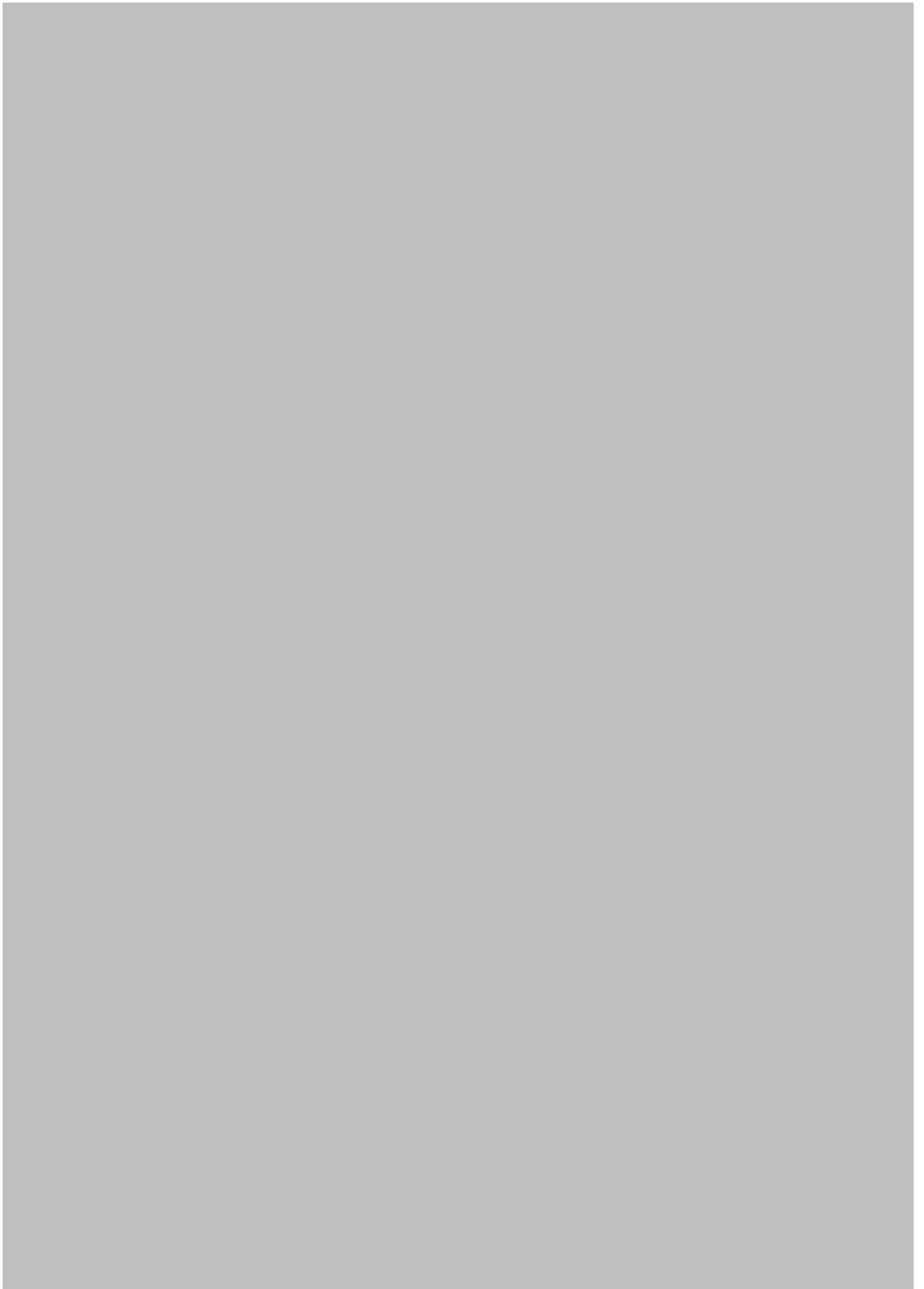
Keywords: ESBLs, *Salmonella*, Enterobacteriaceae











24. Munday, C.J., Xiong, J., Li, C., Shen, D., & Hawkey, P.M. 2004c.  
Dissemination of CTX-M type beta-lactamases in *Enterobacteriaceae* isolates in  
the People's Republic of China. *Int.J.Antimicrob.Agents.*, 23, (2) 175-180





## Dissemination of CTX-M type $\beta$ -lactamases in Enterobacteriaceae isolates in the People's Republic of China

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### Abstract

Previously there have been a number of reports of extended spectrum  $\beta$ -lactamase (ESBL) producing isolates of the family Enterobacteriaceae in Asia. We first reported the occurrence of *bla*<sub>CTX-M</sub> in Guangzhou, China, subsequently there have been reports of *bla*<sub>CTX-M</sub> from a number of other south Asian countries. Initial surveillance study data suggested that *bla*<sub>CTX-M</sub> might be widely distributed in China. This study examines the type of *bla*<sub>CTX-M</sub> occurring in other major population centres in China. Initial disk diffusion method susceptibility testing (NCCLS) selected ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from Beijing and near Wuhan, PRC. After screening in both China and the UK, 13 isolates producing CTX-M ESBLs were identified and studied, 11 also produced TEM-1, and 4 also produced SHV-1. Sequence analysis of the *bla*<sub>CTX-M</sub> containing isolates revealed these isolates contained two different *bla*<sub>CTX-M</sub>, three with *bla*<sub>CTX-M-3</sub> and 10 with *bla*<sub>CTX-M-14</sub>. After comparison with other previously published studies in the English language, we conclude that the most prevalent *bla*<sub>CTX-M</sub> so far reported in Asia are *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-3</sub>.

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**Keywords:** ESBL; CTX-M; Drug resistance; Plasmid

### 1. Introduction

The CTX-M-type  $\beta$ -lactamases represent a rapidly emerging series of extended spectrum  $\beta$ -lactamase (ESBL) with preferential hydrolysis of cefotaxime over ceftazidime [1].

They have a serine molecule at the active site and belong to the Ambler class A. The first description of CTX-M-1 (MEN-1) was made in 1990 [2,3]. It was not until 1998 that the third member, CTX-M-3 was described [4]. Subsequently the number of variants described have increased rapidly (30 as of June 2003).

The genes encoding these enzymes have been found in a diverse range of members of the family Enterobacteriaceae isolated from patients in an ever increasing number of countries. The group has become the most common type

of ESBL in Latin America [5] but is also becoming more common in Europe [1] and was recently reported in the UK [6]. Furthermore there has been a preliminary report of eight CTX-M producing isolates of *Escherichia coli* from patient specimens at five US hospitals in different states during 2001 (E. Smith Moland et al., Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., Abstr. LB-13, 2002).

The *bla*<sub>CTX-M</sub> genes have been found on a number of plasmids and are commonly associated with insertion elements, such as ISEcp1, found flanking the open reading frame region of the *bla*<sub>CTX-M</sub> genes [7]. In Asia, previous reports have identified CTX-M producing strains in Japan, Vietnam, Korea, Taiwan and southern and eastern China [8–16]. In this study, we report how ongoing monitoring for ESBL producing organisms in two hospitals in two mainland cities in the People's Republic of China, and subsequent molecular analysis has revealed the dissemination and spread of CTX-M genes.

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E-mail address: p.m.hawkey@bham.ac.uk (P.M. Hawkey).

## 2. Materials and methods

### 2.1. Clinical strains

Strains were collected as part of an ongoing antibiotic resistance-monitoring programme in Chinese hospitals in Wuhan and Beijing. Routine clinical isolates found to be resistant to cefotaxime in clinical testing using NCCLS methods [17] were collected. Isolates were then selected for further study on the basis of an initial positive test for ESBL-production by a double disc diffusion method [14]. They included three isolates from Beijing (one *E. coli* and two *Klebsiella pneumoniae*) collected in 1998 over a 2-month period, and 22 from Wuhan in 2001 (6 *E. coli* and 16 *K. pneumoniae*). The isolates were identified by conventional methods [18] and species identification of the isolates was achieved using API 20E test strips (BioMerieux S.A., Marcy l'Etoile, France).

### 2.2. Antibiotic susceptibility testing

A disk diffusion susceptibility test was performed on Iso-sensitest agar (Oxoid, Basingstoke, UK) by a comparative method [19]. Amikacin, aztreonam, cefotaxime, cefoxitin, cefpodoxime, cefpodoxime plus clavulanic acid, ceftazidime, ciprofloxacin, gentamicin, imipenem and piperacillin plus tazobactam were used. The MICs of aztreonam, cefotaxime, cefotaxime plus clavulanic acid (at a fixed concentration of 4 µg/ml), ceftazidime and ceftazidime plus clavulanic acid (also at a fixed concentration of 4 µg/ml), were determined by an agar dilution method [19]. Antibiotics were supplied as follows; aztreonam Bristol Myers Squibb, Hounslow, UK; cefotaxime, Aventis Pharma, West Malling, UK; clavulanic acid, Glaxo SmithKline, Uxbridge, UK, and ceftazidime, Glaxo SmithKline, Uxbridge, UK. The *E. coli* strain NCTC 10418 was used as an antibiotic sensitive control.

### 2.3. RAPD typing of bacteria

Template DNA was prepared from bacteria grown overnight at 37 °C on nutrient agar plates. Crude DNA extracts were obtained by suspending a colony in 50 µl of distilled water and boiling at 95 °C for 5 min. Each RAPD PCR reaction consisted of a Ready-To-Go PCR bead (Amersham Biosciences, Amersham, UK) 25 pmol of either DAF4 primer (5'-CGG CAG CGC C-3') for *E. coli*, or AP4 primer (5'-TCA CGA TGC A-3') for *K. pneumoniae* (MWG Biotech, GmbH, Ebersberg, Germany) and 2 µl of DNA suspension in a final volume of 25 µl. The amplification protocol was as follows; 5 min at 94 °C 40 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C. RAPD products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 mg/l) with TAE running buffer. A molecular size marker ladder, Hyperladder 1, was included on all gels (Biolone, London, UK). Banding patterns were evaluated

visually. Isolates of the same species were typed in the same batch and products run on the same gel. Where clusters were identified, an example of each was run on other gels to ensure accurate identification of clusters from gel to gel.

### 2.4. PCR amplification

All isolates were initially screened for the presence of *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub> and *bla*<sub>VEB</sub> by previously described PCR protocols [14,20]. Isolates were further screened for the presence of *bla*<sub>TOHO-1</sub> (780 bp), *bla*<sub>TOHO-2</sub> (906 bp) and *bla*<sub>CTX-M-1</sub> (604 bp) predicted product sizes in parenthesis as previously described [14] and newly designed primers to amplify *bla*<sub>CTX-M-8</sub> group genes using primers CTXM83 (5'-ATG GTA CGA CGA ATG ATA TC-3') and CTXM11 (5'-TAA TCA TAC AGA AGT CGC AG-3') to give a predicted product size of 246 bp. All these primers pairs will amplify only part of the ORF of the target gene. To derive the whole ORF, primers ISEcp1 U1 (5'-AAA AAT GAT TGA AAG GTG GT-3') and P2D (5'-CAG CGC TTT TGC CGT CTA AG-3') or ISEcp1 U1 and IS2 (5'-GTG AAG AAG GTG TTG CTG AC-3') were used for amplification of a ca. 1300 bp product containing the whole CTX-M-1 group ORF and Toho-2/CTX-M-9 group ORF, respectively [4,7]. All primers were amplified by MWG (MWG Biotech, GmbH, Ebersberg, Germany). Bacterial DNA was prepared by suspension of one or two fresh colonies in 50 µl of sterile distilled water and heating at 95 °C for 5 min. PCR amplification was carried out under the following conditions: 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

### 2.5. Nucleotide sequence determination

The sequence of the ca. 1300 bp fragments containing the whole ORF of the *bla*<sub>CTX-M</sub> genes and the *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> PCR products were determined using a Big Dye PCR reaction (Applied Biosystems Inc, Foster city, CA, USA) and analysed on a primus HT 3700 DNA analyser by the functional Genomics Laboratory, University of Birmingham, UK.

### 2.6. Plasmid DNA analysis, Southern blotting, and hybridisation

Plasmid DNA was extracted by a rapid method [21] and separated by agarose gel electrophoresis. Plasmids isolated from *E. coli* strains NCTC 50192 and NCTC 50193 were used as standard size markers. Plasmid DNA was then transferred from the agarose gel to a nylon membrane (Boehringer Mannheim, Roche Diagnostics, Lewes, UK) by the method of Southern [22] before hybridisation with digoxigenin-labelled *bla*<sub>CTX-M</sub> gene fragments with the PCR DIG detection system (Boehringer Mannheim).

## 2.7. Conjugation

Plasmid transfer of cefotaxime resistance markers was performed by a broth culture conjugation method [23]. Laboratory *E. coli* strain UB1637/R, carrying a rifampicin resistance mutation (*Lys*<sup>-</sup>, *His*<sup>-</sup>, *Trp*<sup>-</sup>, *Lac*<sup>-</sup>, *Rec A*<sup>-</sup>, *Rif*<sup>R</sup>) was used as the recipient [24]. The mating time was 4 h. Transconjugants were selected for on nutrient plates containing cefotaxime (4 mg/l; Sigma Poole Dorset) and rifampicin (128 mg/l; Sigma, Poole, UK).

## 3. Results

### 3.1. Bacterial strains

#### 3.1.1. Initial disc diffusion susceptibility and RAPD-typing

Twenty-six isolates preliminarily identified in China, as ESBL producers were used in susceptibility testing by the disc diffusion method. A total of 13 suspected ESBL producing isolates from Wuhan (six *E. coli*, five *K. pneumoniae*) and Beijing (two *K. pneumoniae*) exhibited resistance to cefotaxime and these were then subject to further testing (Table 1). Both *E. coli* and *K. pneumoniae* isolates were susceptible to imipenem, and all *E. coli* were susceptible to amikacin. Both isolates from Beijing were resistant to aztreonam, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin and gentamicin. There were 11 cefotaxime resistant isolates

from Wuhan, with varying susceptibility to the other antibiotics tested. In total, 13 isolates were cefotaxime resistant, as opposed to 11 ceftazidime resistant. All isolates showing different banding patterns by RAPD typing were considered different strains.

#### 3.1.2. Detection and identification of TEM, SHV and VEB type genes

Of the 13 isolates tested, one carried *bla*<sub>TEM</sub> alone, one carried *bla*<sub>SHV</sub> alone and the remaining 11 carried both *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>. No isolates contained *bla*<sub>VEB</sub>. Sequence analysis of the PCR products identified all *bla*<sub>TEM</sub> as *bla*<sub>TEM-1</sub> and all *bla*<sub>SHV</sub> as *bla*<sub>SHV-1</sub>.

#### 3.1.3. Detection and identification of the CTX-M genes

PCR was performed on all isolates using primers specific for *bla*<sub>TOHO-1</sub>, *bla*<sub>TOHO-2</sub>, *bla*<sub>CTX-M-1</sub> and *bla*<sub>CTX-M-8</sub> groups of CTX-M. With Toho-1 and CTX-M-8 primers, no amplification products were detected from any isolates. Using Toho-2 primers approximately 900 bp amplification products were detected in six *K. pneumoniae* and four *E. coli* isolates. DNA sequencing of these amplicons gave a sequence identical to the published CTX-M-14 sequence [14]. PCR amplification of isolates of *K. pneumoniae* and *E. coli* using ISEcp1 and IS903 specific primers produced ca. 1300 bp amplicons which were then used for further sequencing and confirmed as *bla*<sub>CTX-M-14</sub>. Using CTX-M-1 group primers, amplification products of approximately 600 bp were detected in two *K. pneumoniae* isolates and one *E. coli*. Subsequent PCR amplification and sequencing of the whole *bla*<sub>CTX-M</sub> ORF from these three isolates revealing all three to be *bla*<sub>CTX-M-3</sub>.

#### 3.1.4. Transfer of β-lactam resistance

Plasmid transfer of the ESBL phenotype to a laboratory *E. coli* strain was successful in six of 13 isolates (46%). Table 1 shows the number of strains capable of transferring the CTX-M gene. Two transconjugants derived from one *E. coli* and one *K. pneumoniae* parent isolate from Wuhan were also PCR positive for both *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> and one transconjugant, derived from a *K. pneumoniae* parent isolate, also from Wuhan, was PCR positive for *bla*<sub>TEM</sub>.

#### 3.1.5. Susceptibility of *bla*<sub>CTX-M</sub> carrying isolates

The MICs of β-lactams for CTX-M producing isolates were compared using BSAC breakpoints. All CTX-M producing isolates were resistant to cefotaxime (MIC range 16–256 mg/l). When a cefotaxime-clavulanic acid combination was used, only one *K. pneumoniae* producing CTX-M-14 was borderline resistant (MIC 2 mg/l), with the remaining isolates susceptible by BSAC methodology. Only the CTX-M-3 producing *E. coli* isolate was resistant to aztreonam and ceftazidime, but conversely, eight of the 10 CTX-M-14 producing isolates were resistant. All isolates were susceptible to a combination of ceftazidime-clavulanic acid.

Table 1

(a) Number of strains resistant to a range of antimicrobials using BSAC criteria. (b) The number of strains positive for the CTX-M group genes and in parenthesis the numbers transferred to *E. coli*. (c) Number of strains belonging to specific CTX-M gene types determined by DNA sequencing

	No. of resistant strains from Wuhan and Beijing (n = number of isolates)		
	Beijing <i>K. pneumoniae</i> (n = 2)	Wuhan <i>K. pneumoniae</i> (n = 6)	Wuhan <i>E. coli</i> (n = 5)
Antimicrobial agent(s)			
aztronam	2	2	5
cefotaxime	2	6	5
ceftazidime	2	3	4
cefoxitin	2	1	0
amikacin	0	3	0
ciprofloxacin	2	3	4
gentamicin	2	6	5
imipenem	0	0	0
piperacillin-tazobactam	0	6	2
CTX-M gene screen			
Toho-1 group	0	0	0
Toho-2 group	2	4 (3)	4 (1)
CTX-M-1 group	0	2 (1)	1 (1)
CTX-M-8 group	0	0	0
Specific CTX-M gene			
CTX-M-3	0	2	1
CTX-M-14	2	4	4

### 3.2. Plasmid DNA analysis

Using a *bla*<sub>CTX-M</sub> specific probe, hybridisation occurred with a plasmid of approximately 50–55 kb in all three *bla*<sub>CTX-M-3</sub> carrying isolates. In all 10 *bla*<sub>CTX-M-14</sub> carrying isolates plasmids were approximately 50–60 kb which also hybridised with the *bla*<sub>CTX-M</sub> specific probe.

### 4. Discussion

There has been an increase in the isolation of CTX-M producing bacteria globally, with reports from Europe, North and South America, Africa and Asia [1]. To date there are only four published reports in the English literature of the occurrence of *bla*<sub>CTX-M</sub> in mainland China. The distribution of these various CTX-M type enzymes is summarised on the map in Fig. 1. Our report shows the presence of CTX-M-3 (CTX-M-1 group) and CTX-M-14 (CTX-M-9 group) producing *E. coli* and *K. pneumoniae* in two geographically dispersed cities in China located in the central and northern parts of the country (Wuhan and Beijing). The CTX-M-type enzymes may be divided into four groups, dependent on amino acid homology [1]. Previous reports of CTX-M in Asia include *bla*<sub>CTX-M</sub> from the CTX-M-1 group (CTX-M-3, CTX-M-11, CTX-M-15 and CTX-M-22), CTX-M-2 group (CTX-M-5, Toho-1) and CTX-M-9 group (CTX-M-9, CTX-M-14, CTX-M-17 and Toho-2) [8–15,25–30], (Shibata N, Doi Y, et al., Abstr.

41nd Intersci. Conf. Antimicrob. Agents Chemother., Abstr. C2-2235, 2001; GenBank Nos.: AY013478; AF462635; AY005110). A recent report of the types of ESBLs identified over 1 year (1999) from a hospital in Shanghai, demonstrated by PCR the presence of CTX-M-1 group *bla*<sub>CTX-M</sub> in 13 of 58 ESBL producing *E. coli* and 41 of 81 ESBL producing *K. pneumoniae*; however, they did not find any CTX-M-2 group *bla*<sub>CTX-M</sub> and did not test for the presence of CTX-M-9 group *bla*<sub>CTX-M(15)</sub>. Another recent study from a different group in Shanghai, demonstrated the presence of CTX-M-3 in 83% of their ESBL producing isolates, but again only tested for this type of CTX-M [29]. We previously reported the presence of CTX-M-9, CTX-M-13, and CTX-M-14 from hospitals in Guangzhou, China, all of which belong to the CTX-M-9 group [14], which suggests that CTX-M type enzymes of this group would have been missed in the Shanghai studies.

There have also been reports of CTX-M-3 and CTX-M-14 from isolates of *E. coli* and *K. pneumoniae* from Taiwan and China's neighbouring countries of Korea and Vietnam [9,12,13], and would suggest that these two CTX-M types are the most prevalent in east Asia.

A ceftazidime MIC value of  $\leq 8$  mg/l in the presence of an ESBL type profile suggests CTX-M producers [12]. In our study, 5 of 13 CTX-M-3 and CTX-M-14 producing isolates had a ceftazidime MIC value of 16 mg/l. It has been reported that CTX-M-15 is the only CTX-M enzyme to hydrolyse ceftazidime efficiently [31], which would suggest an alternative mechanism of resistance to ceftazidime is present in



Fig. 1. Distribution of CTX-M type  $\beta$ -lactamases in south east Asia, which is based on English language published reports (2001–2003), where two pie charts are present (Guangzhou, Beijing) these are the results of two independent studies. Each pie represents the percentage of different CTX-M-type genes reported at each location. The number adjacent to each portion indicates the type of CTX-M gene present, e.g. 15 for CTX-M-15.

these isolates. As VEB-1, SHV or TEM type ESBLs were not found in these isolates, either another ESBL type or an efflux mechanism of resistance is present.

The spread of *bla*CTX-M in China has been suggested to involve several clones of *K. pneumoniae* and *E. coli* when it has been studied at distinct and separate geographical locations [14,15,29].

In this study, there was no one common strain of Enterobacteriaceae associated with the spread of *bla*CTX-M, with these genes distributed on a variety of different isolates from different geographical locations.

With the high prevalence of CTX-M-14 thus far reported in east Asia it is interesting to note there are relatively few differences between this enzyme and other members of the CTX-M-9 group found in this region. There is only one amino acid difference between the sequences of CTX-M-14 and CTX-M-9 (Val<sup>231</sup> → Ala) and CTX-M-17 (Glu<sup>289</sup> → Lys) respectively, and three amino acid differences between CTX-M-14 and CTX-M-13 (Val<sup>3</sup> → Met, Val<sup>52</sup> → Lys, Ala<sup>154</sup> → Glu). This suggests the molecular evolutionary events have occurred in East Asia, as all of the earliest reports are solely from this area. It is possible that in the future we may see convergent evolution of the *bla*CTX-M genes in a similar manner to the *bla*TEM and *bla*SHV resulting in an ever increasing number of variants [32,33]. Also, there is only one amino acid difference between the CTX-M-1 group enzymes CTX-M-3, found in China, and CTX-M-15, found in India (Asp<sup>240</sup> → Gly). This amino acid difference occurs in the omega loop region of CTX-M-15 resulting in increased ceftazidime hydrolysis and antibiotic resistance [31].

In conclusion, this study illustrates the dissemination of *bla*CTX-M genes by a broad range of genetic elements and highlights the need for routine surveillance for these newer ESBLs in east Asia.

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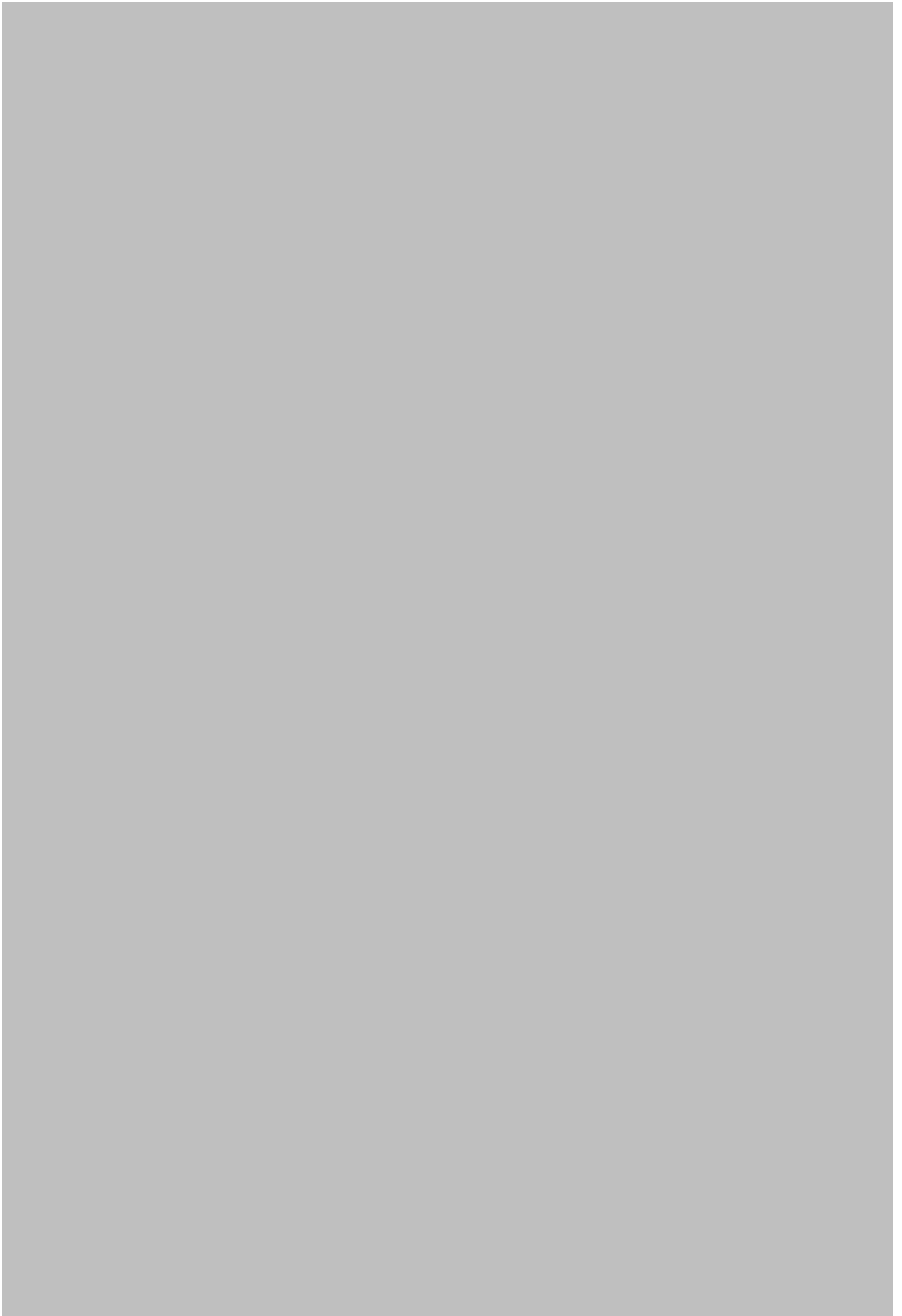
# Extended-Spectrum $\beta$ -Lactamase Genes of *Escherichia coli* in Chicken Meat and Humans, the Netherlands

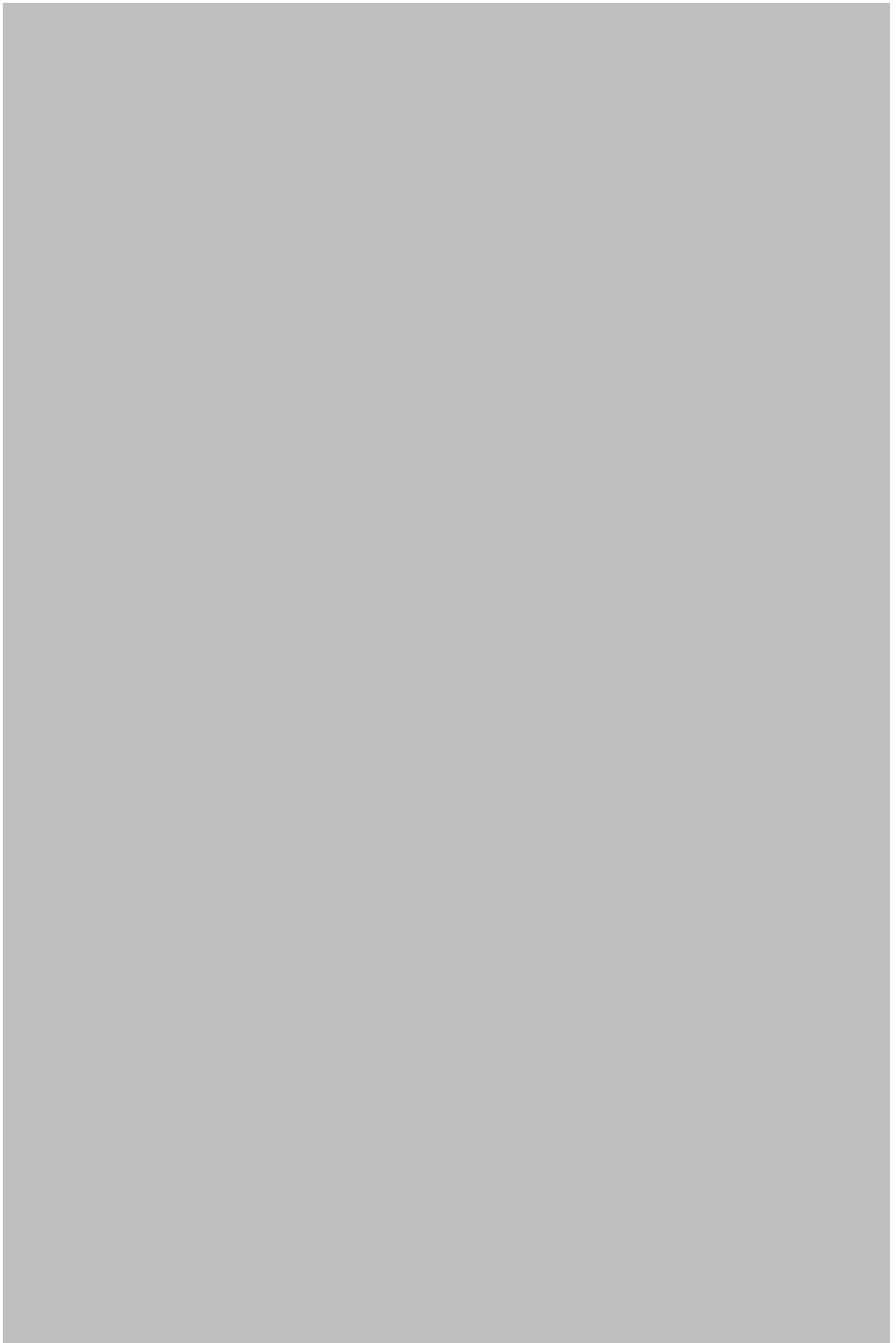
Ilse Overdeest, Ina Willemsen, Martine Rijnsburger, Andrew Eustace, Li Xu, Peter Hawkey, Max Heck, Paul Savelkoul, Christina Vandenbroucke-Grauls, Kim van der Zwaluw, Xander Huijsdens, and Jan Kluytmans

We determined the prevalence and characteristics of extended-spectrum  $\beta$ -lactamase (ESBL) genes of *Enterobacteriaceae* in retail chicken meat and humans in the Netherlands. Raw meat samples were obtained, and simultaneous cross-sectional surveys of fecal carriage were performed in 4 hospitals in the same area. Human blood cultures from these hospitals that contained ESBL genes were included. A high prevalence of ESBL genes was found in chicken meat (79.8%). Genetic analysis showed that the predominant ESBL genes in chicken meat and human rectal swab specimens were identical. These genes were also frequently found in human blood culture isolates. Typing results of *Escherichia coli* strains showed a high degree of similarity with strains from meat and humans. These findings suggest that the abundant presence of ESBL genes in the food chain may have a profound effect on future treatment options for a wide range of infections caused by gram-negative bacteria.

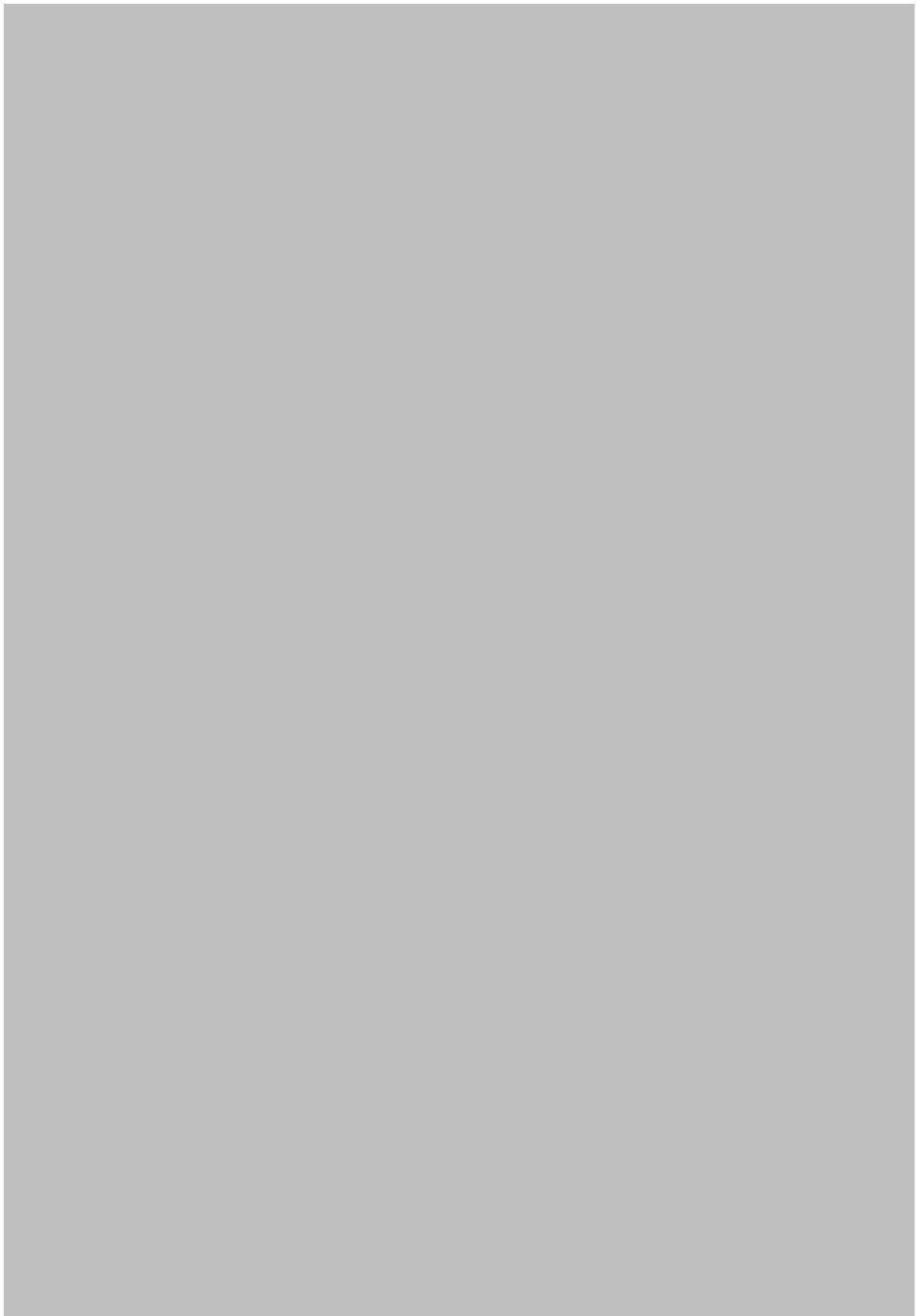


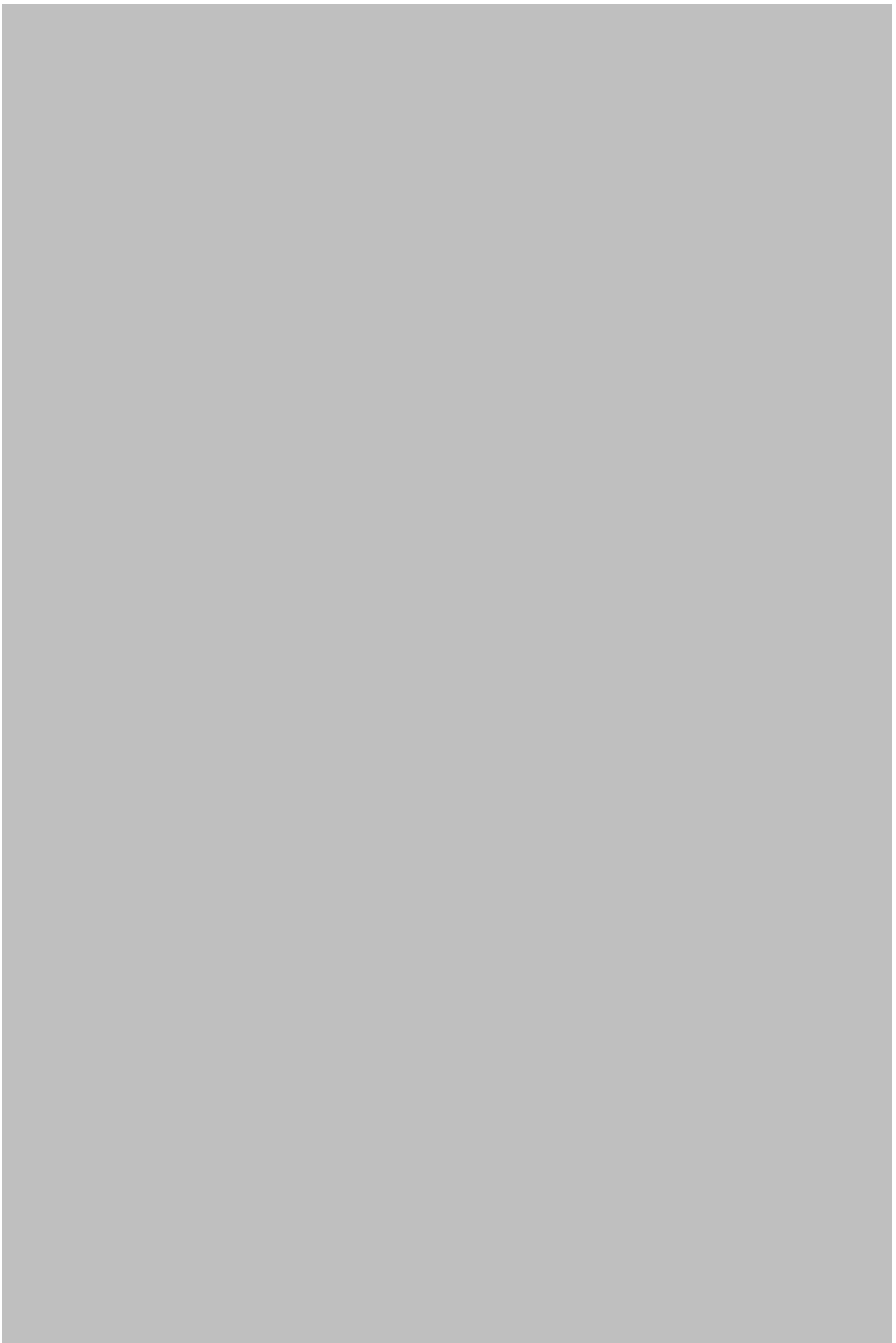












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## Extended-spectrum $\beta$ -lactamase producing *Klebsiella* spp. in chicken meat and humans: a comparison of typing methods

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1) Department of Medical Microbiology, St Elisabeth Hospital, Tilburg, 2) Department of Medical Microbiology and Infection Control, Amphia Hospital, Breda, 3) National Institute of Public Health and Environmental Protection, RIVM, Bilthoven, 4) Department of Medical Microbiology and Infection Control, VUmc University Medical Centre, Amsterdam, the Netherlands and 5) Heart of England National Health Service Foundation Trust, Birmingham, UK

### Abstract

Recently, chicken meat was identified as a plausible source of extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* in humans. We investigated the relatedness of ESBL-producing *Klebsiella* spp. in chicken meat and humans. Furthermore, we tested the performance of SpectraCell RA<sup>®</sup> (River Diagnostics), a new typing method based on Raman spectroscopy, in comparison with multilocus sequence typing (MLST) for *Klebsiella pneumoniae*. Twenty-seven phenotypically and genotypically confirmed ESBL-producing *Klebsiella* spp. isolates were typed with MLST and SpectraCell RA. The isolates derived from chicken meat, human rectal swabs and clinical blood cultures. In the 22 ESBL-producing *K. pneumoniae* isolates, CTX-M15 was the predominant genotype, found in five isolates of human origin and in one chicken meat isolate. With MLST, 16 different STs were found, including five new STs. Comparing the results of SpectraCell RA with MLST, we found a sensitivity of 70.0% and a specificity of 81.8% for the new SpectraCell RA typing method. Therefore, we conclude that SpectraCell RA is not a suitable typing method when evaluating relationships of ESBL-producing *Klebsiella* spp. at the population level. Although no clustering was found with isolates of chicken meat and human origin containing the same ESBL genes, MLST showed no clustering into distinctive clones of isolates from chicken meat and human origin. More studies are needed to elucidate the role of chicken meat in the rise of ESBL-producing *Klebsiella* spp. in humans.

**Keywords:** Extended-spectrum  $\beta$ -lactamase, *Klebsiella* spp., multilocus sequence typing, SpectraCell RA, typing

**Original Submission:** 23 January 2013; **Revised Submission:** 7 May 2013; **Accepted:** 11 May 2013

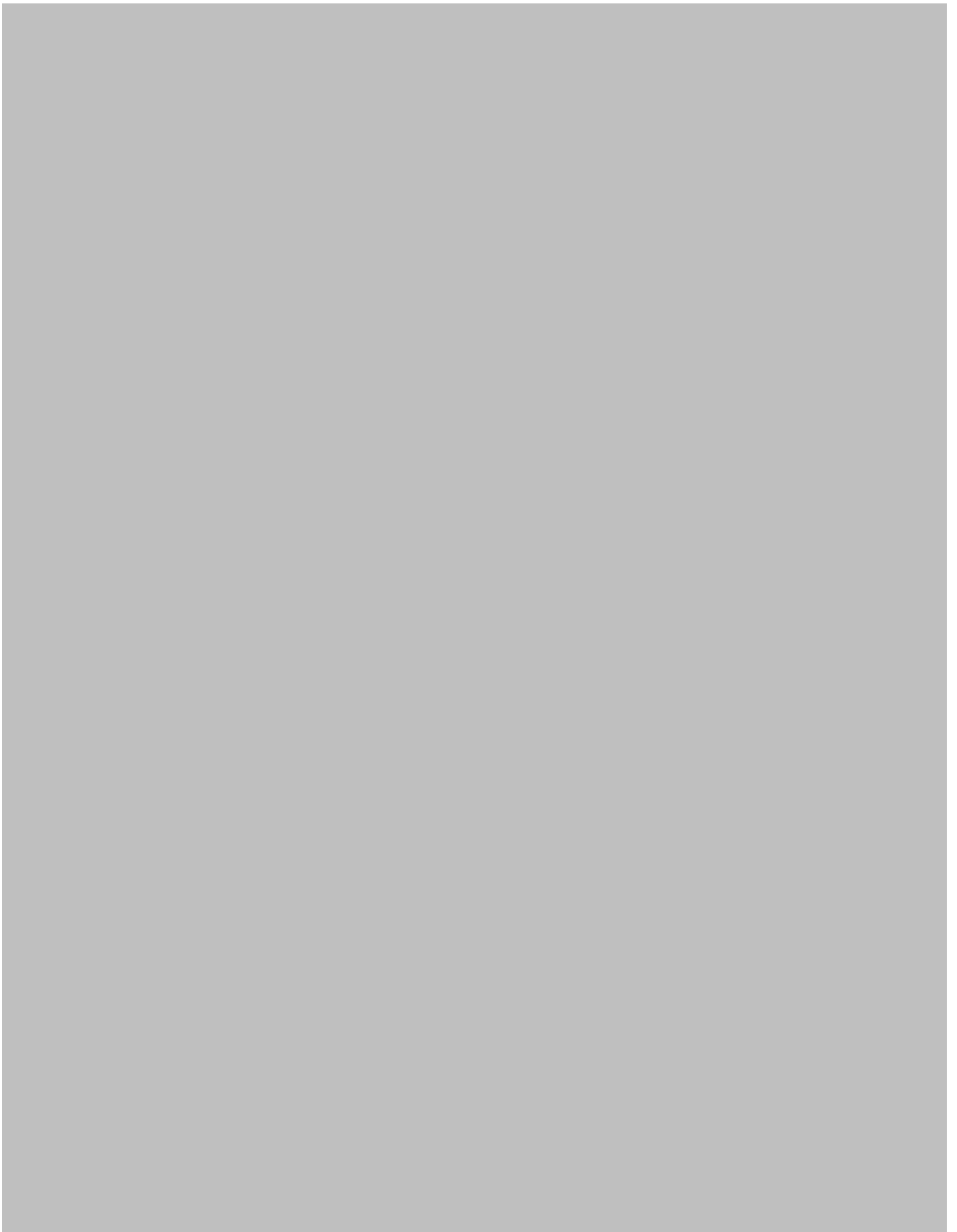
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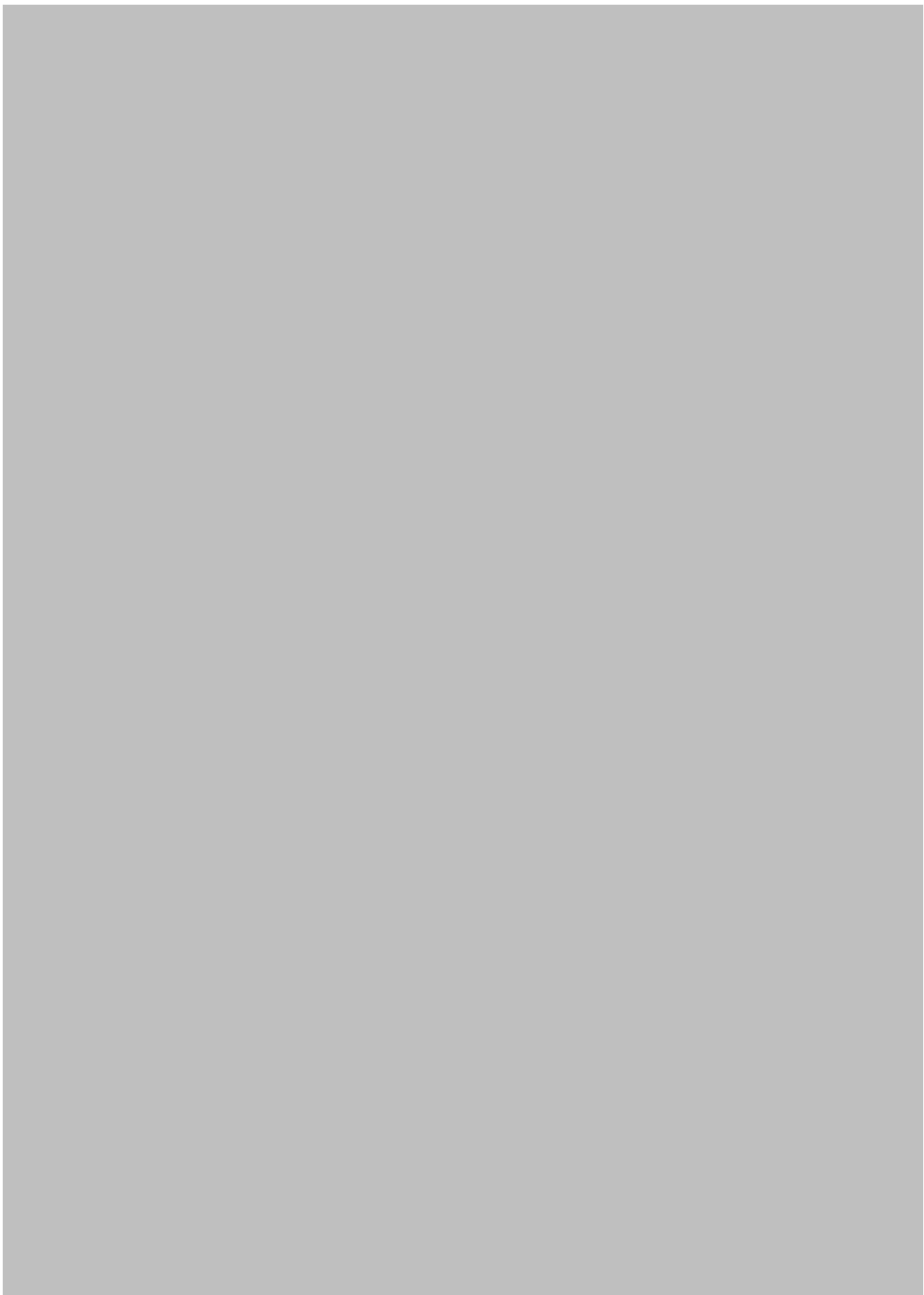
**Article published online:** 24 May 2013

*Clin Microbiol Infect* 2014; **20**: 251–255

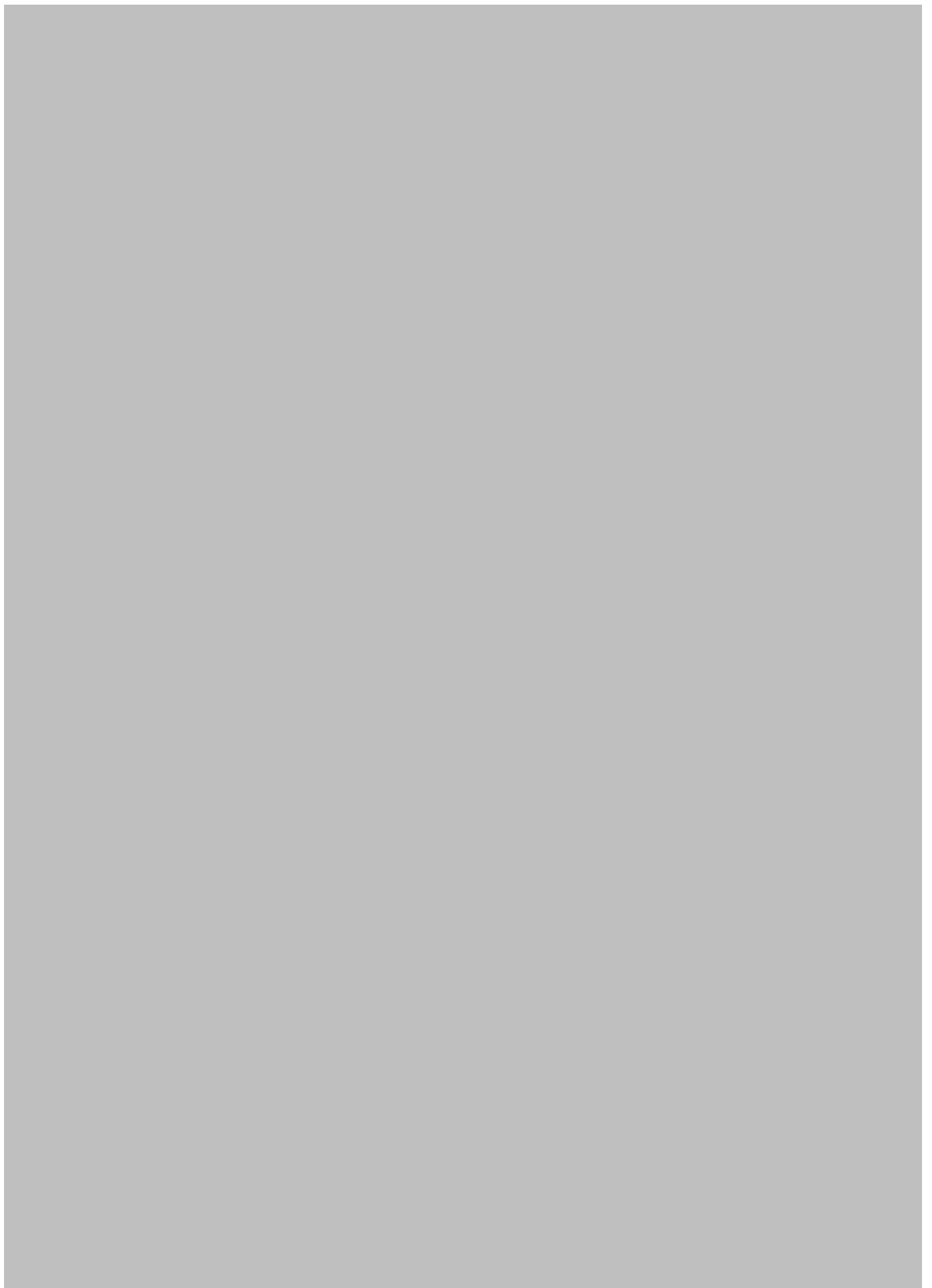
10.1111/1469-0691.12277











27. Warren, R.E., Ensor, V.M., O'Neill, P., Butler, V., Taylor, J., Nye, K., Harvey, M., Livermore, D.M., Woodford, N., & Hawkey, P.M. 2008. Imported chicken meat as a potential source of quinolone-resistant *Escherichia coli* producing extended-spectrum beta-lactamases in the UK. *J.Antimicrob.Chemother.*, 61, (3) 504-508 available from: PM:18222958

## Imported chicken meat as a potential source of quinolone-resistant *Escherichia coli* producing extended-spectrum $\beta$ -lactamases in the UK

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D. M. Livermore<sup>4</sup>, N. Woodford<sup>4</sup> and P. M. Hawkey<sup>2,3</sup>

<sup>1</sup>Microbiology Laboratory, Shrewsbury and Telford Hospital NHS Trust, Shrewsbury SY3 8XQ, UK; <sup>2</sup>Division of Immunity and Infection, The Medical School, University of Birmingham, Birmingham B15 2TT, UK; <sup>3</sup>West Midlands Health Protection Agency, Heart of England NHS Foundation Trust, Birmingham B9 5SS, UK; <sup>4</sup>Antibiotic Resistance and Monitoring Reference Laboratory, Health Protection Agency, 61 Colindale Avenue, London NW9 5EQ, UK

Received 23 September 2007; returned 4 October 2007; revised 4 December 2007; accepted 5 December 2007

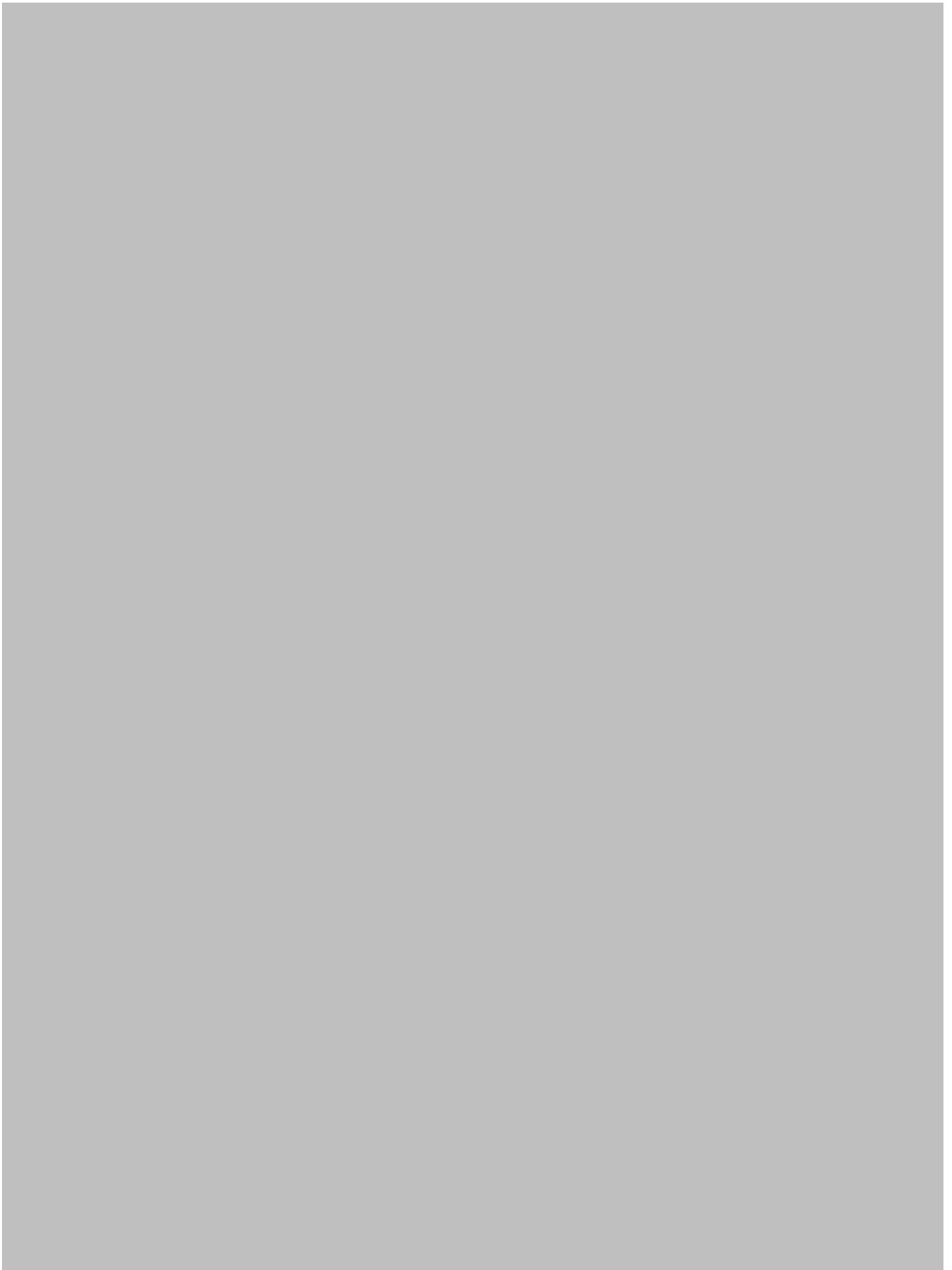
**Objectives:** *Escherichia coli* producing CTX-M-15 enzyme began to rapidly spread in the UK from around 2003 but other types also occur, notably CTX-M-14. We examined breasts from UK-reared ( $n = 62$ ) and imported ( $n = 27$ ) chickens as potential sources of quinolone-resistant *E. coli* with *bla*<sub>CTX-M</sub> genes. A further 40 samples for which the country of rearing could not be identified were examined.

**Methods:** During 2006, 129 fresh and frozen chicken breast fillets were purchased from retail outlets in the West Midlands. These were cultured for *E. coli* on CLED agar containing 8 mg/L ciprofloxacin and carrying a 10  $\mu$ g cefpodoxime disc. Resistant isolates were identified and typed by RAPD fingerprinting; *bla*<sub>CTX-M</sub> was identified by PCR and genotyped by reverse-line hybridization.

**Results:** The country of rearing was identified from the packaging for 89 of 129 purchased samples. Only one of the 62 UK-reared chicken samples carried *E. coli* producing a CTX-M-1 enzyme, whereas 10 of 27 samples reared overseas had *E. coli* with CTX-M enzymes. Specifically, 4/10 Brazilian, 3/4 Brazilian/Polish/French, and 2/2 Dutch samples had *E. coli* with CTX-M-2 enzymes. Six of 40 samples for which the country of rearing was not known had producers of CTX-M enzymes, 5 of them with CTX-M-14.

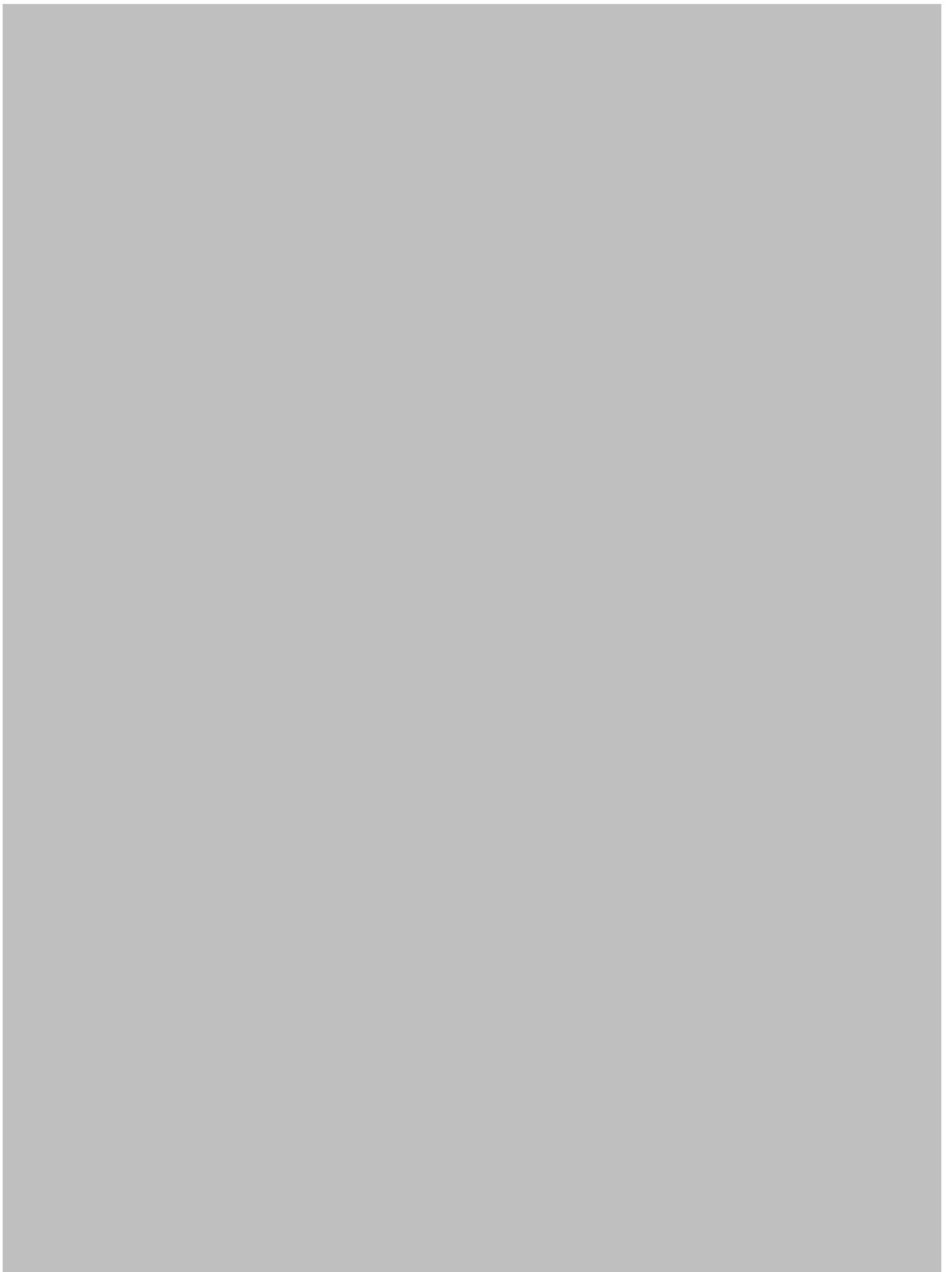
**Conclusions:** Quinolone-resistant *E. coli* with various CTX-M  $\beta$ -lactamase genes that are common in human infections worldwide were found in imported chicken breasts, indicating a possible source for gut colonization. Samples from Brazil were commonly positive for *E. coli* with CTX-M-2, the dominant *bla*<sub>CTX-M</sub> genotype from human infections in South America, which is currently rare in clinical infections in the UK. CTX-M-15, the dominant CTX-M type in human infections in the UK, was not found in chicken isolates, suggesting that the UK-reared chickens are not a reservoir of CTX-M-15.

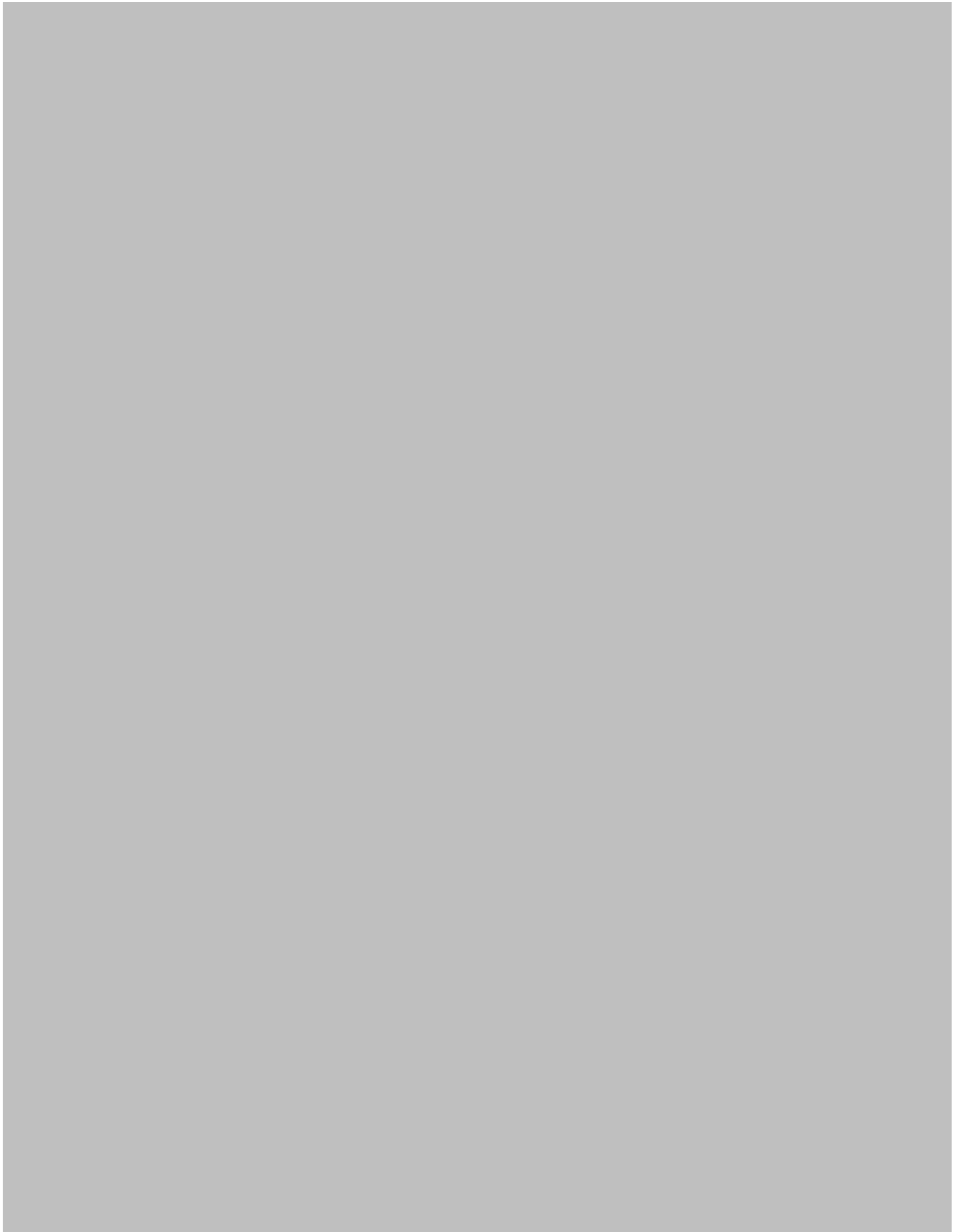
Keywords: ESBLs, food, quinolones, Enterobacteriaceae












28. Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M., & Williams, A.P. 2013. The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect.Dis.*, 13, (2) 155-165 available from: PM:23347633

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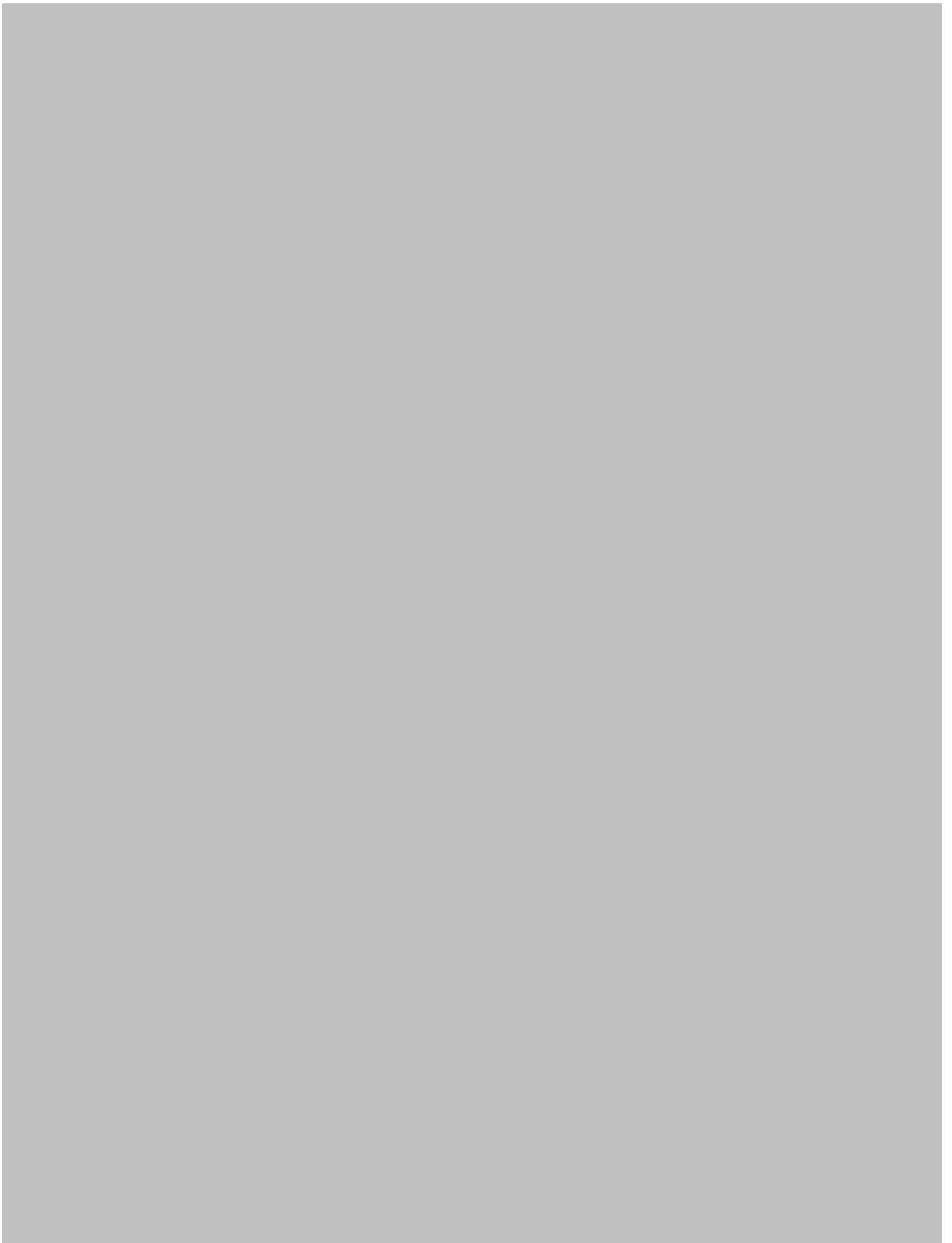
## The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria

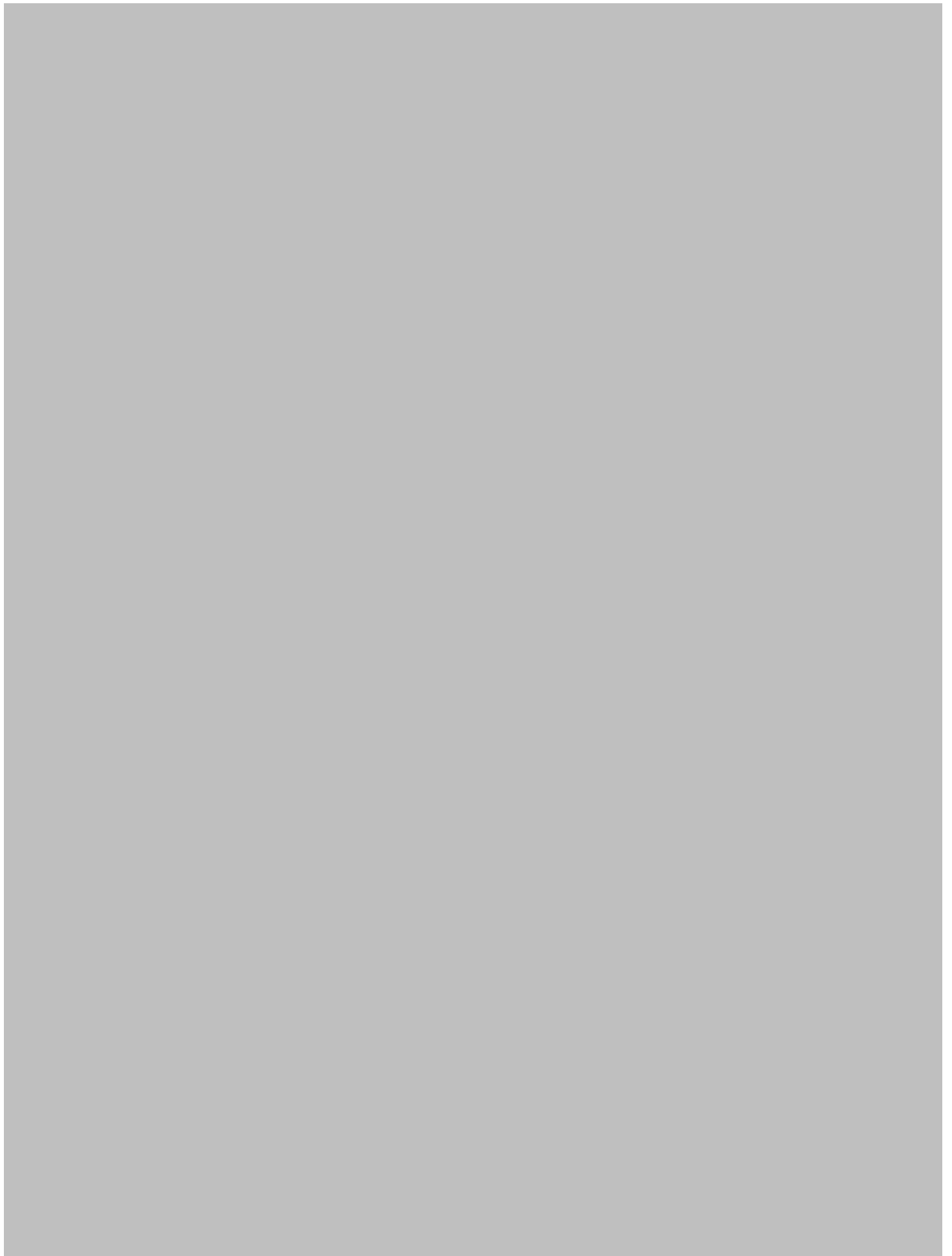
*Elizabeth M H Wellington, Alistair B A Boxall, Paul Cross, Edward J Feil, William H Gaze, Peter M Hawkey, Ashley S Johnson-Rollings, Davey L Jones, Nicholas M Lee, Wilfred Otten, Christopher M Thomas, A Prysor Williams*

During the past 10 years, multidrug-resistant Gram-negative Enterobacteriaceae have become a substantial challenge to infection control. It has been suggested by clinicians that the effectiveness of antibiotics is in such rapid decline that, depending on the pathogen concerned, their future utility can be measured in decades or even years. Unless the rise in antibiotic resistance can be reversed, we can expect to see a substantial rise in incurable infection and fatality in both developed and developing regions. Antibiotic resistance develops through complex interactions, with resistance arising by de-novo mutation under clinical antibiotic selection or frequently by acquisition of mobile genes that have evolved over time in bacteria in the environment. The reservoir of resistance genes in the environment is due to a mix of naturally occurring resistance and those present in animal and human waste and the selective effects of pollutants, which can co-select for mobile genetic elements carrying multiple resistant genes. Less attention has been given to how anthropogenic activity might be causing evolution of antibiotic resistance in the environment. Although the economics of the pharmaceutical industry continue to restrict investment in novel biomedical responses, action must be taken to avoid the conjunction of factors that promote evolution and spread of antibiotic resistance.



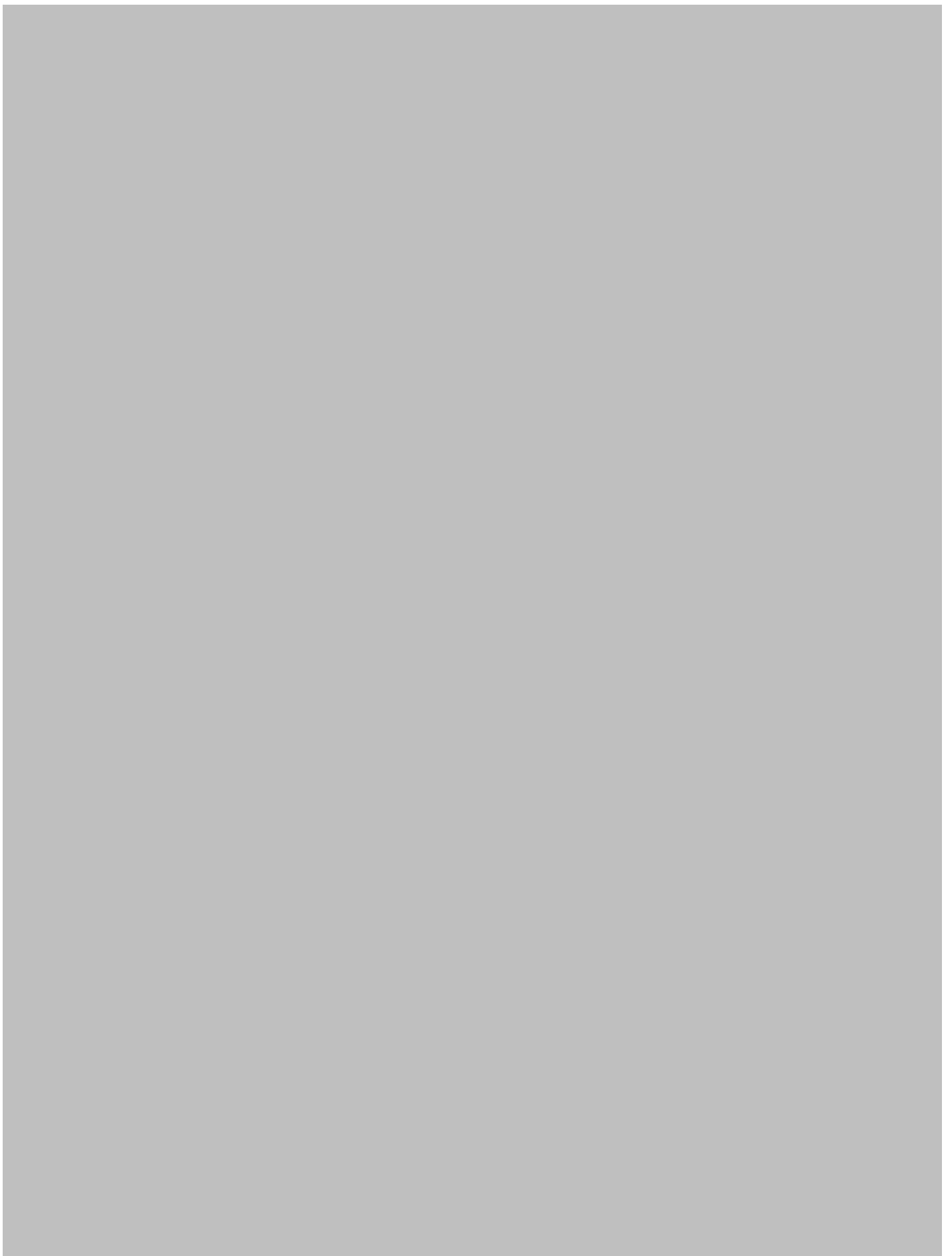




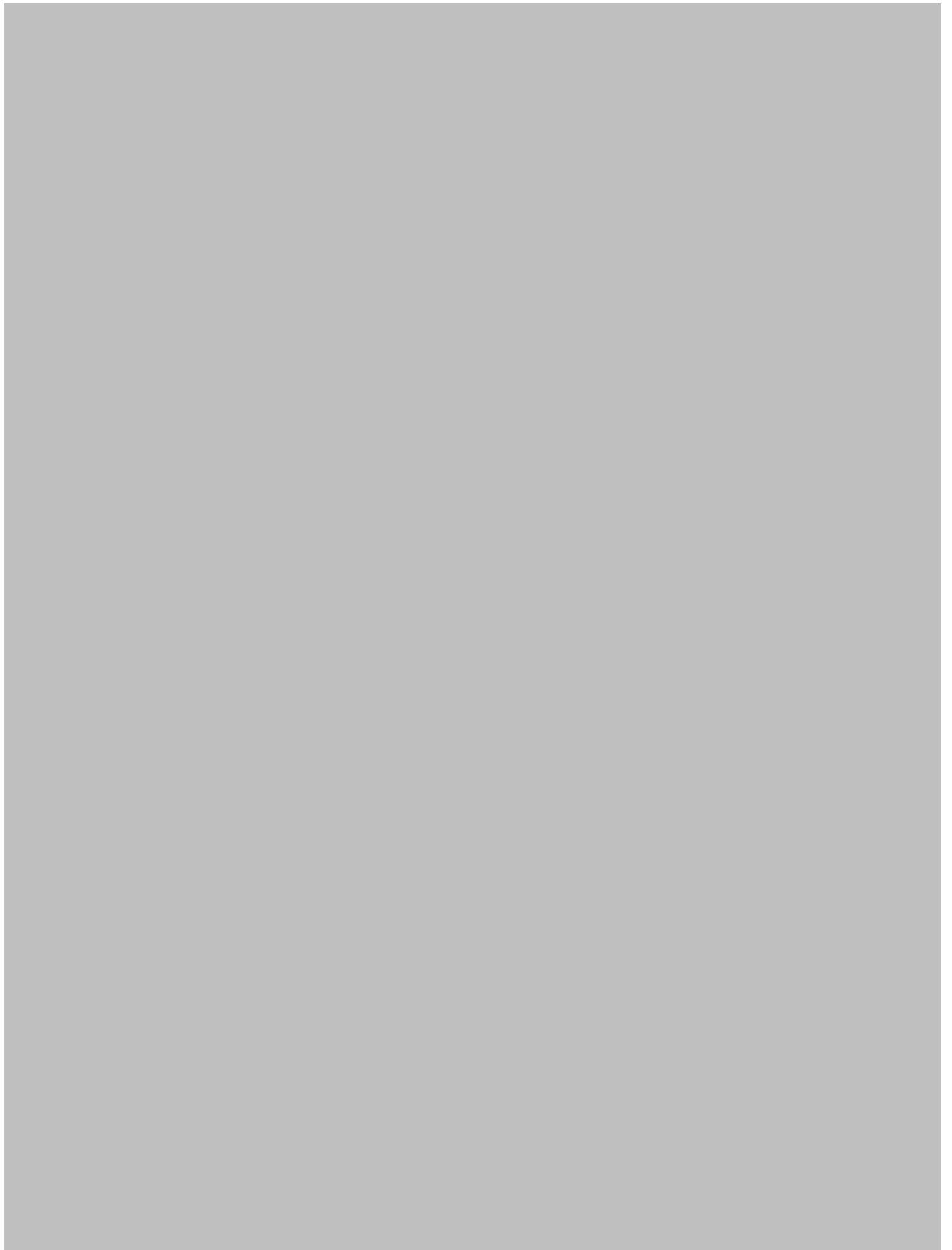




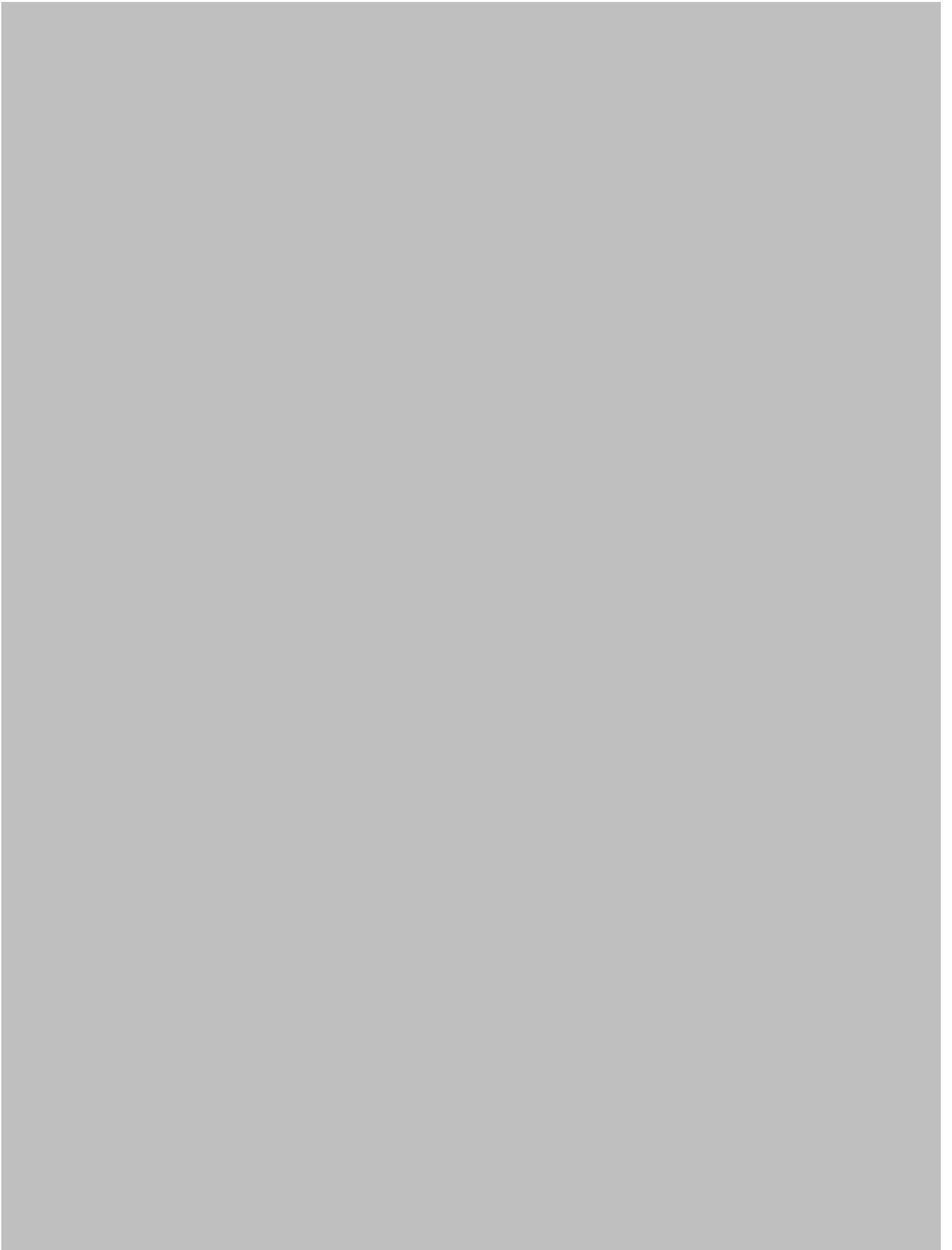












29. Wickramasinghe, N., Xu, L., Eustace, A., Shabir, S., Saluja, T., & Hawkey, P.M. 2012. High community faecal carriage rates of CTX-M ESBL-producing *Escherichia coli* in a specific population group in Birmingham, UK. *Journal of Antimicrobial Chemotherapy*, 67, 1108-1113

## High community faecal carriage rates of CTX-M ESBL-producing *Escherichia coli* in a specific population group in Birmingham, UK

Nimal H. Wickramasinghe<sup>1</sup>, Li Xu<sup>2,3</sup>, Andrew Eustace<sup>2</sup>, Sahida Shabir<sup>2</sup>, Tranprit Saluja<sup>4</sup> and Peter M. Hawkey<sup>2,3\*</sup>

<sup>1</sup>Department of Microbiology, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK; <sup>2</sup>HPA West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Birmingham, UK; <sup>3</sup>The School of Immunity and Infection, University of Birmingham, Birmingham, UK; <sup>4</sup>Department of Microbiology, University Hospital of North Staffordshire, Stoke-on-Trent, UK

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Received 9 September 2011; returned 27 September 2011; revised 13 January 2012; accepted 13 January 2012

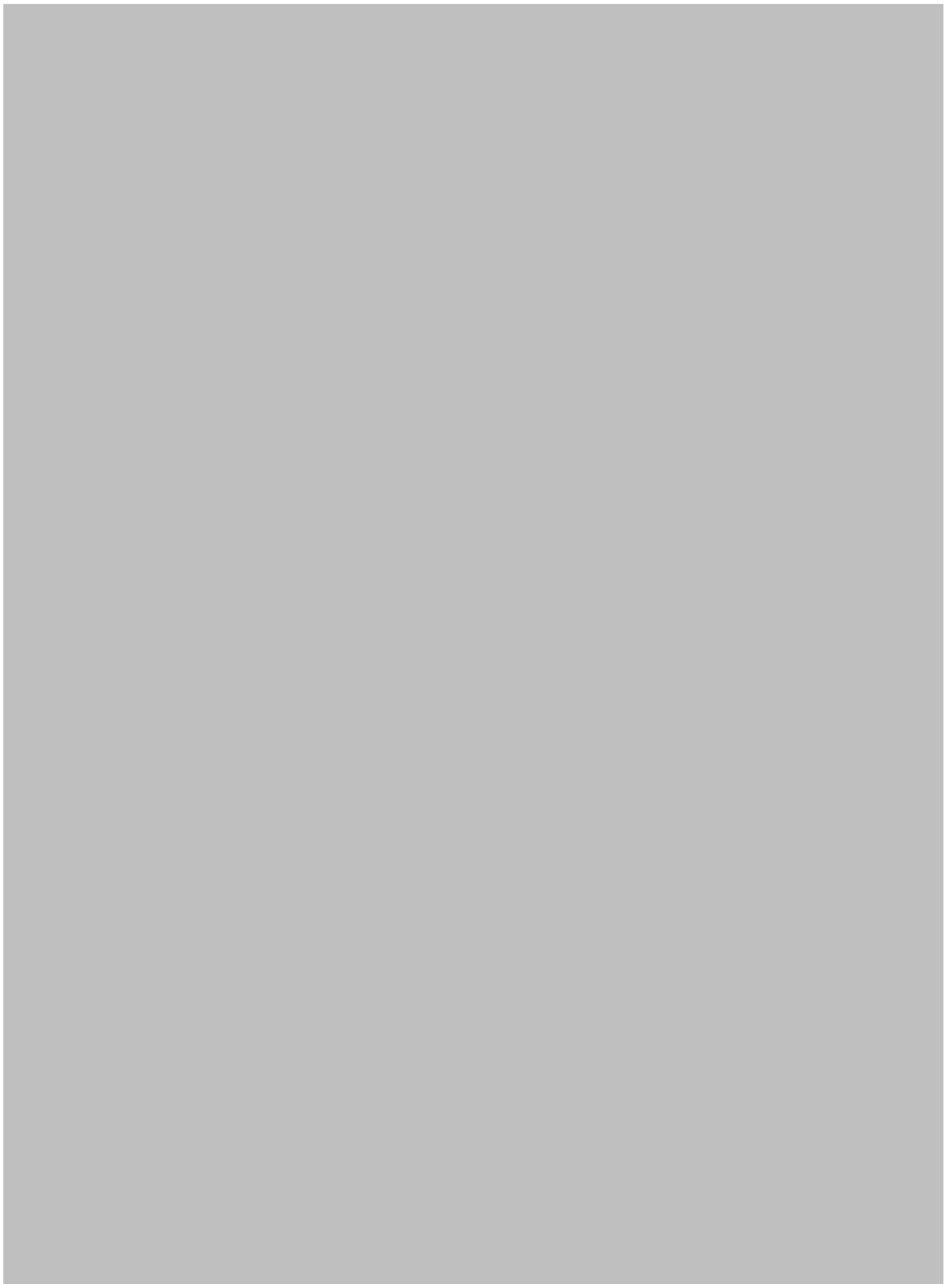
**Objectives:** To determine the proportion of *E. coli* carrying specific CTX-M extended-spectrum  $\beta$ -lactamase (ESBL) genotypes in a community population of East and North Birmingham.

**Methods:** General practice and outpatient stool samples from 732 individuals submitted for examination for faecal pathogens in 2010 were screened for ESBL-producing *E. coli* using chromogenic agar. Multiplex PCR, denaturing HPLC, DNA sequencing and PFGE were used to determine the CTX-M genotype and clonal subtype. Isolates from people were assigned to 'Europe', 'Middle East/South Asia' (MESA) or 'uncategorized' groups using software to determine probable global origin based on the subject's full name.

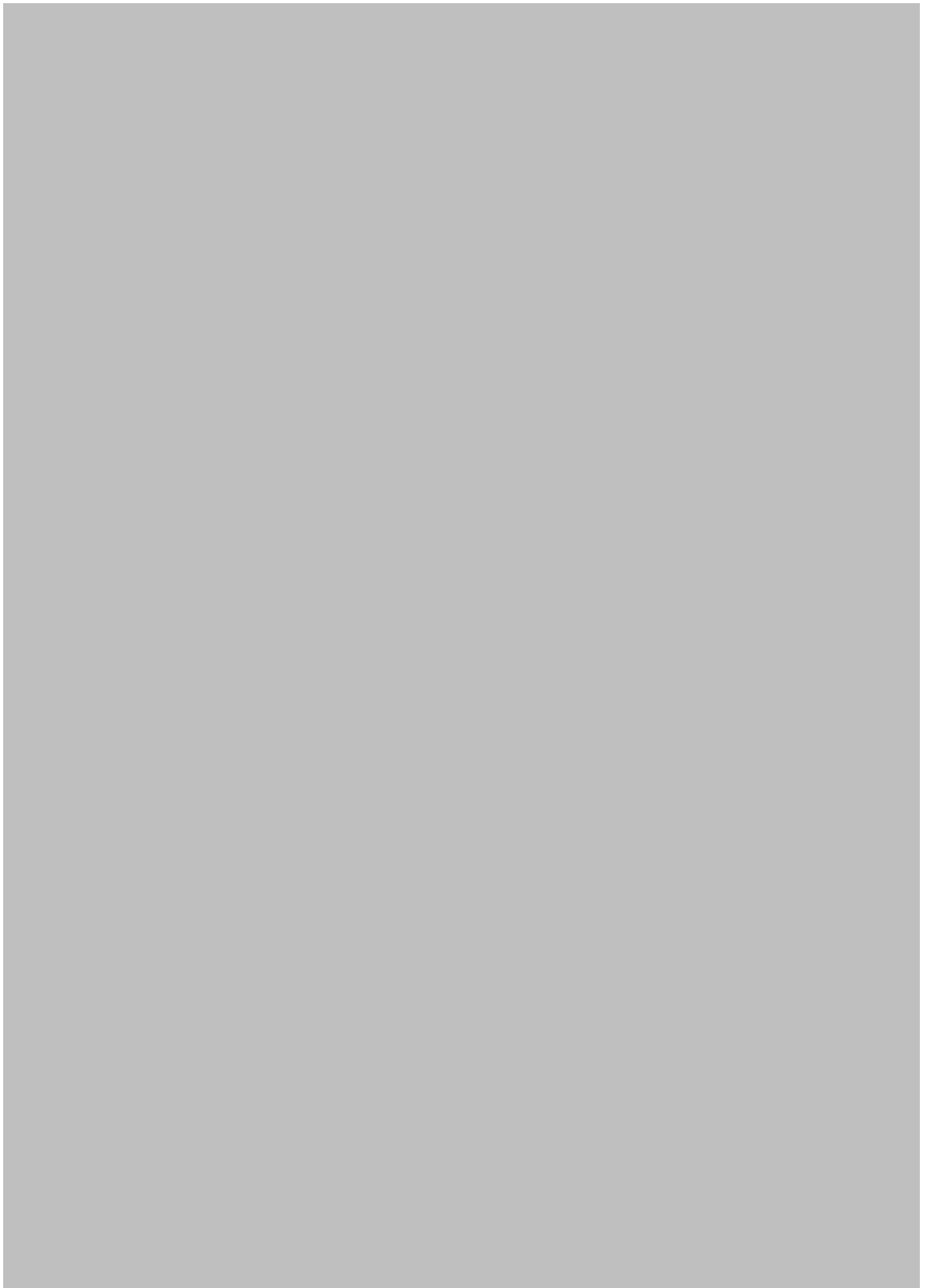
**Results:** Prevalence of CTX-M carriage in the sample population was 11.3%. There was a statistically significant difference ( $P < 0.001$ ) between carriage in the Europe group (8.1%) and the MESA group (22.8%). There was also a higher rate of carriage of CTX-M-15-producing *E. coli* ( $P < 0.001$ ) in MESA subjects.

**Conclusions:** The high community carriage rate and the significant difference in carriage between the Europe and MESA subjects may have important consequences for therapy. If the rising trend in carriage of bacteria producing ESBLs continues, guidelines for empirical therapy for patients presenting from the community may need to be modified. The findings also raise the concern that the pattern and routes of spread of CTX-M-15 may be replicated in the future by broader-spectrum  $\beta$ -lactamases, such as New Delhi metallo- $\beta$ -lactamase ('NDM-1').

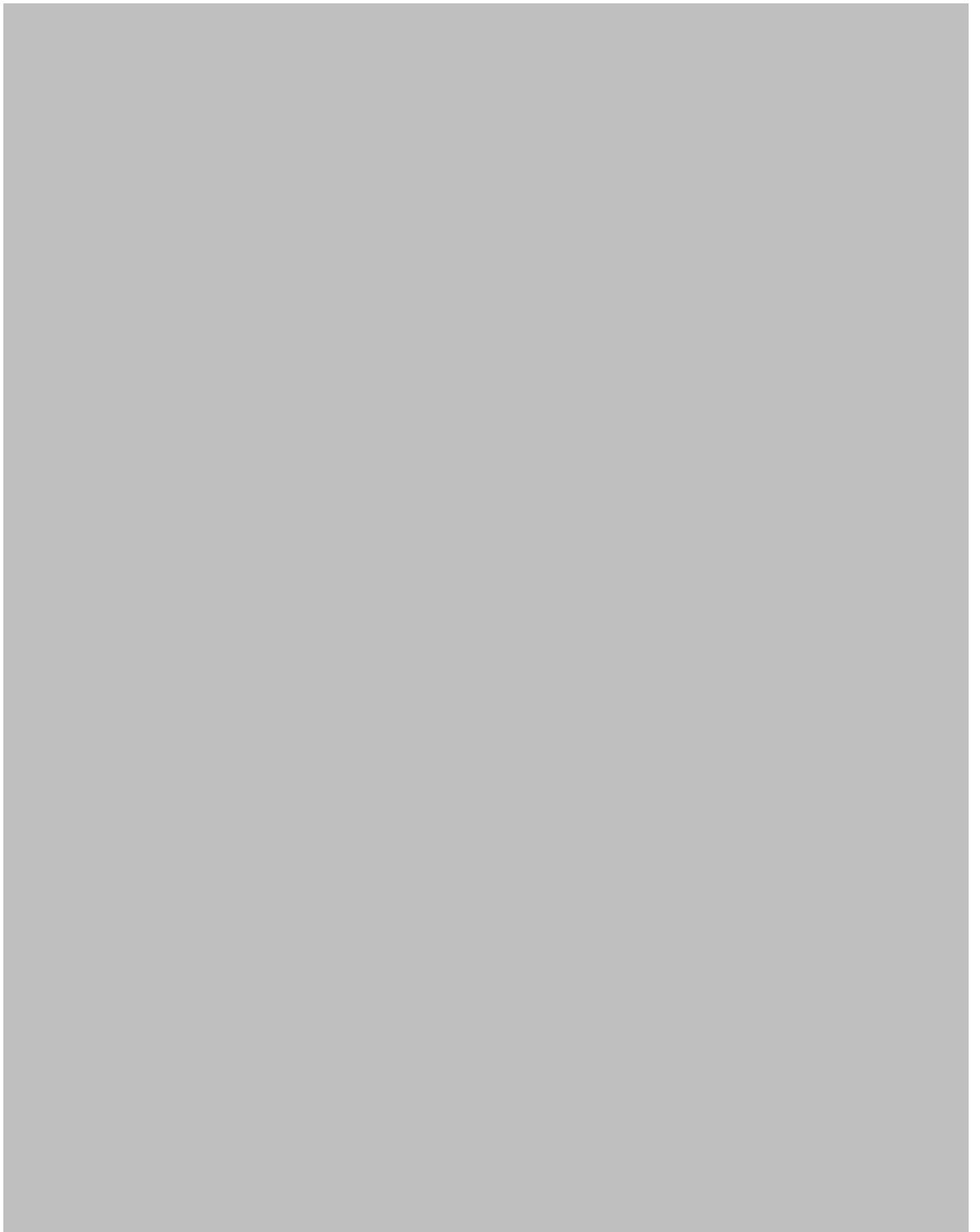
**Keywords:** CTX-M-15, ST131, ethnicity, clonal spread

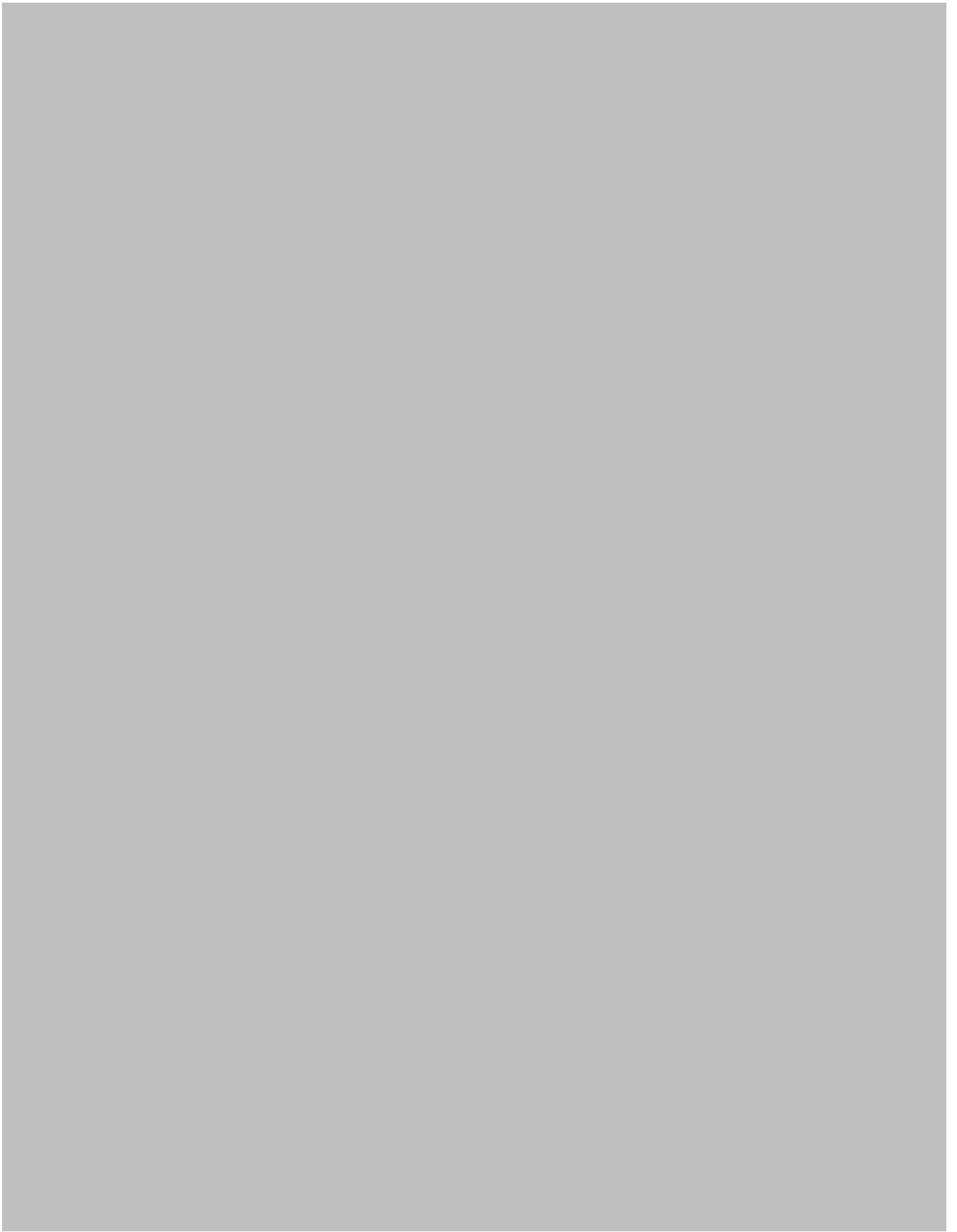












30. Xu, L., Ensor, V., Gossain, S., Nye, K., & Hawkey, P. 2005. Rapid and simple detection of blaCTX-M genes by multiplex PCR assay. *J.Med Microbiol.*, 54, (Pt 12) 1183-1187

## Rapid and simple detection of *bla*<sub>CTX-M</sub> genes by multiplex PCR assay

Li Xu,<sup>1,2</sup> Vicki Ensor,<sup>2</sup> Savita Gossain,<sup>1</sup> Kathy Nye<sup>1</sup> and Peter Hawkey<sup>1,2</sup>

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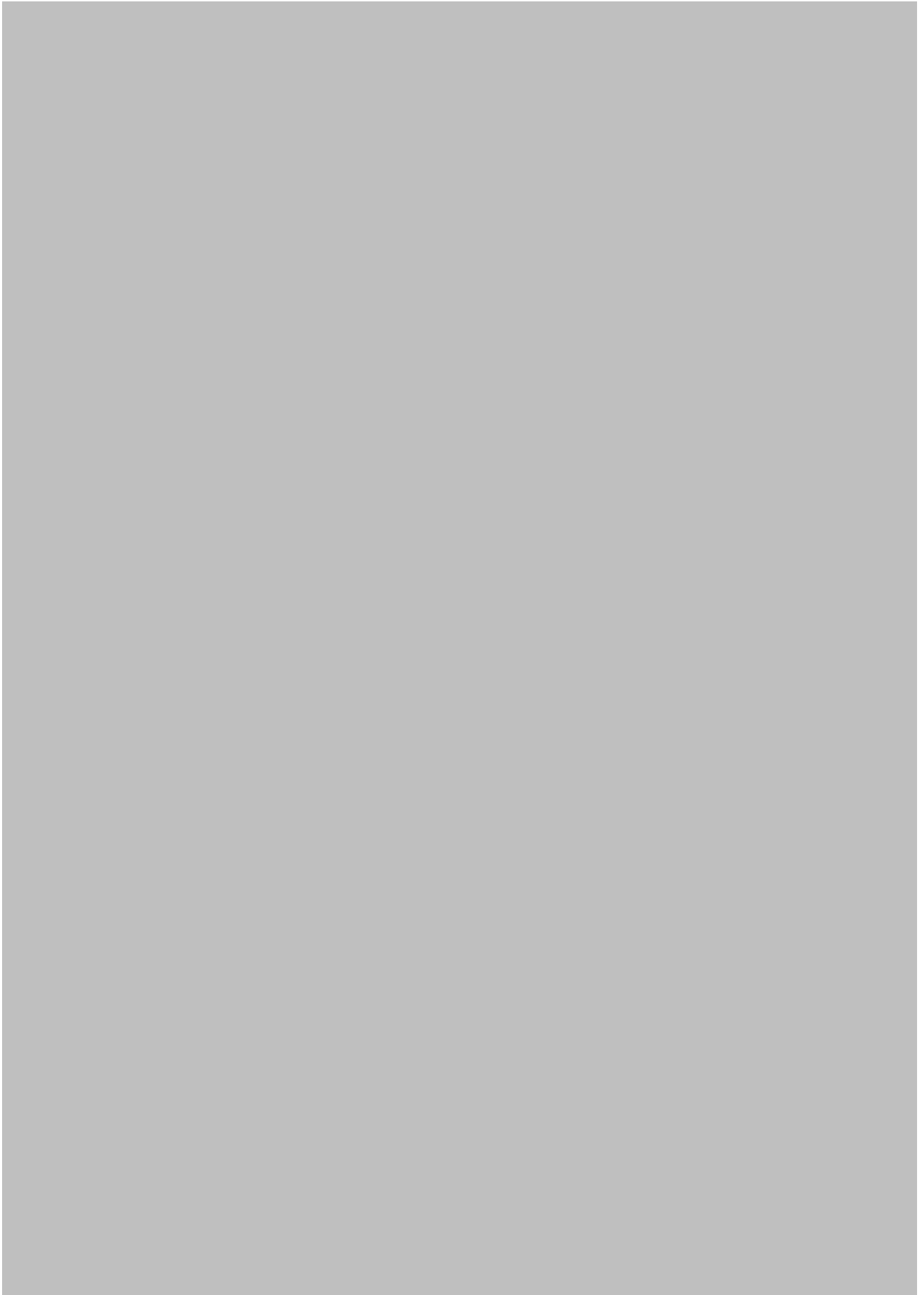
<sup>1</sup>Health Protection Agency West Midlands Public Health Laboratory, Birmingham Heartlands & Solihull NHS Trust, Birmingham B9 5SS, UK

<sup>2</sup>Division of Immunity and Infection, University of Birmingham, UK

Received 18 May 2005

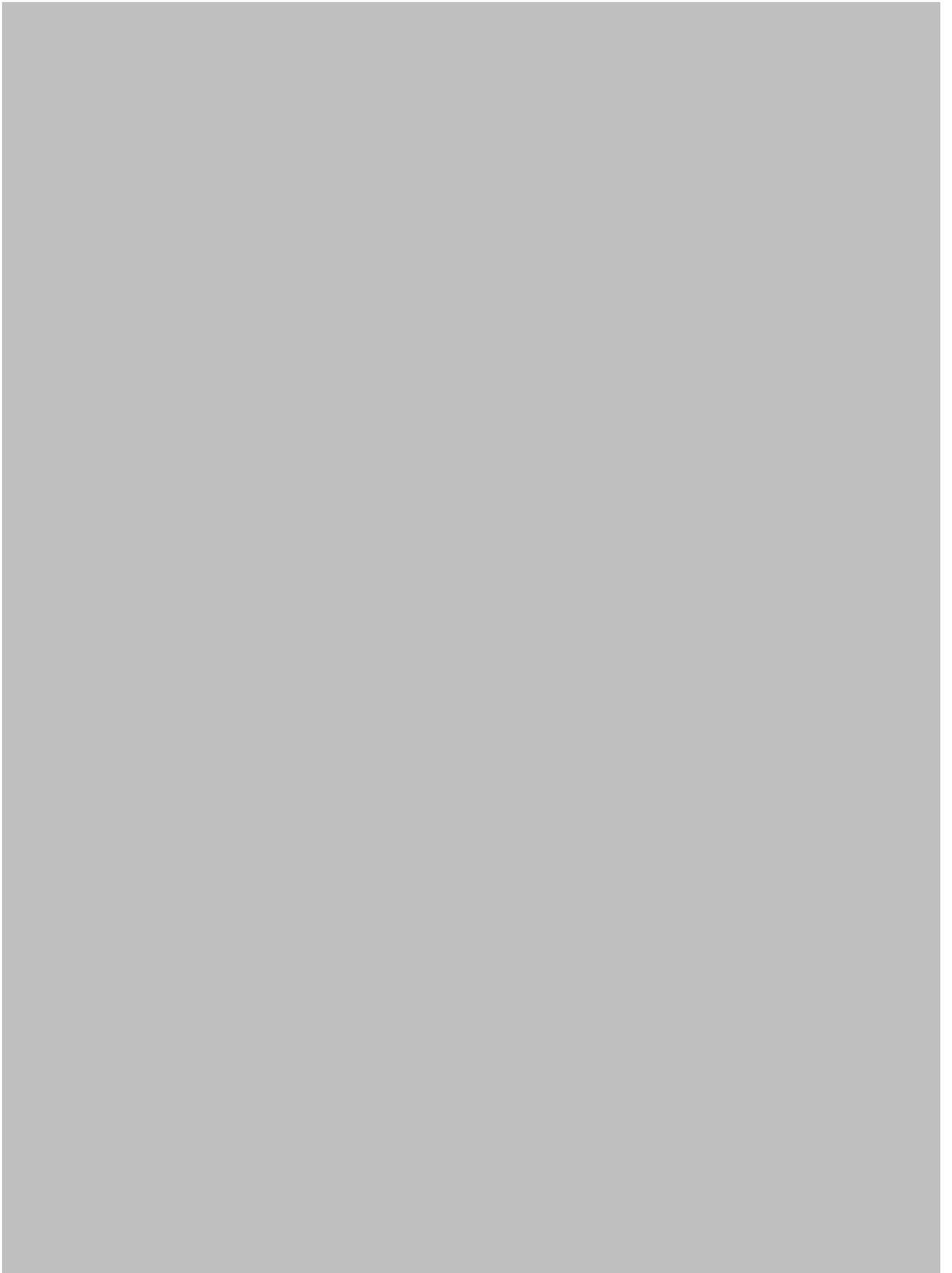
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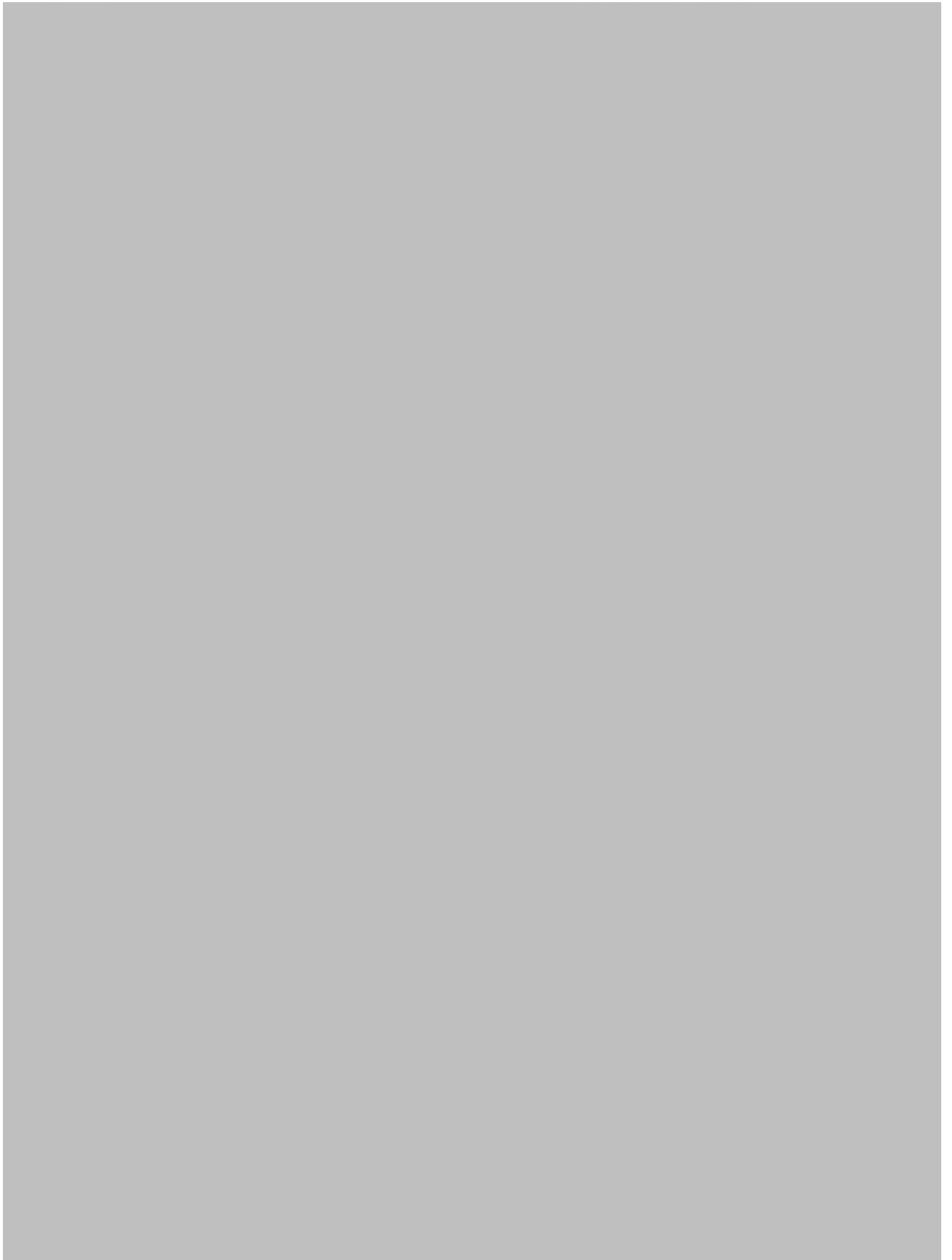
A novel multiplex PCR assay is described (CTX-Mplex PCR) that allows rapid detection of *bla*<sub>CTX-M</sub> genes and discrimination between groups 1, 2, 9 and 25/26. The specificity and sensitivity of the assay were evaluated with 10 control strains and then applied to 62 clinical isolates. The multiplex PCR detected and classified *bla*<sub>CTX-M</sub> genes with 100% accuracy. The utilization of a denaturing HPLC WAVE system to size the PCR products automatically from the multiplex PCR enhances the assay by saving time and costs.











31. Xu, L., Evans, J., Ling, T., Nye, K., & Hawkey, P. 2007. Rapid genotyping of CTX-M extended-spectrum beta-lactamases by denaturing high-performance liquid chromatography. *Antimicrob. Agents Chemother.*, 51, (4) 1446-1454

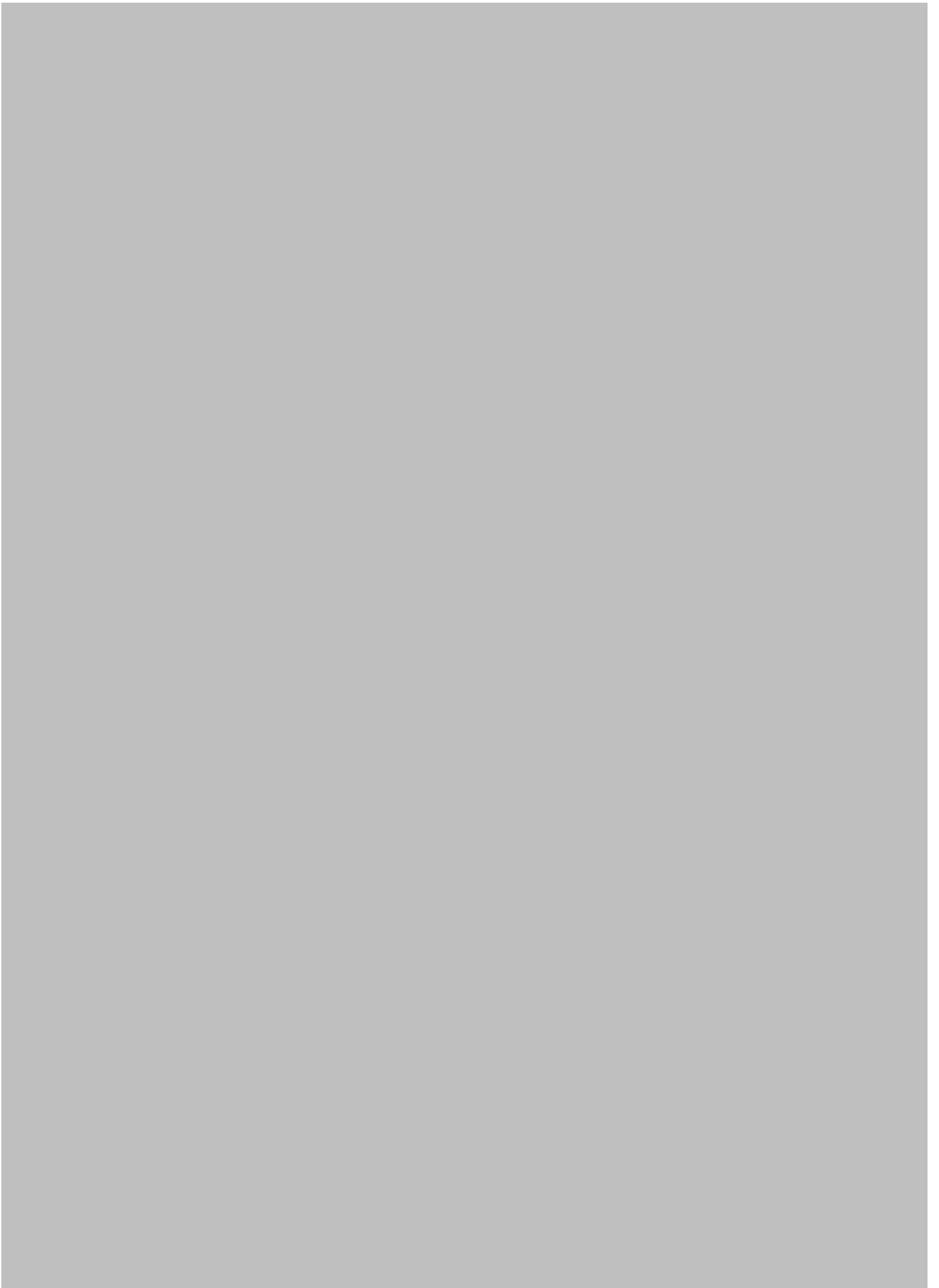
## Rapid Genotyping of CTX-M Extended-Spectrum $\beta$ -Lactamases by Denaturing High-Performance Liquid Chromatography<sup>V</sup>

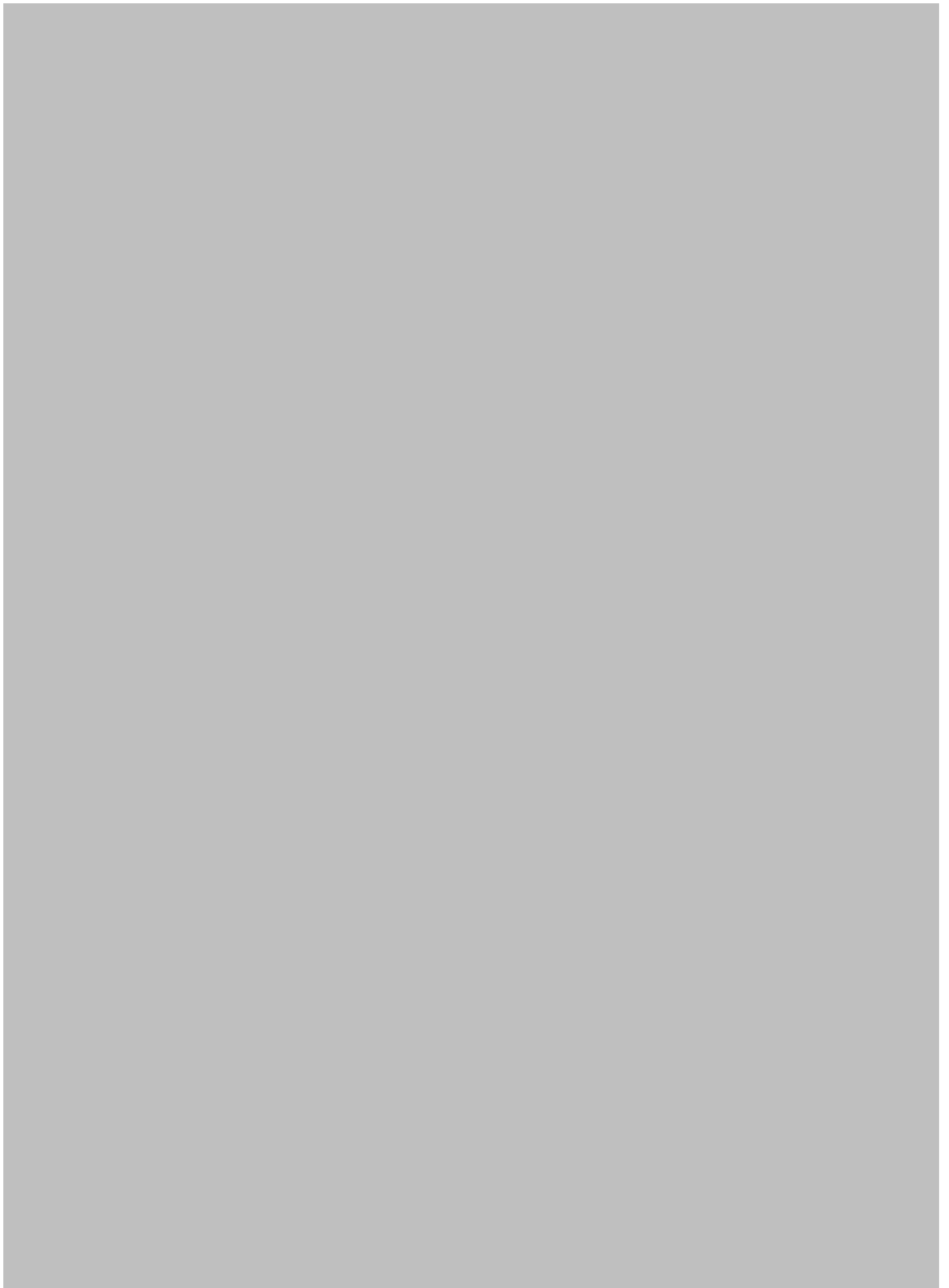
Li Xu,<sup>1,2</sup> Jason Evans,<sup>1,2</sup> Thomas Ling,<sup>3</sup> Kathy Nye,<sup>1</sup> and Peter Hawkey<sup>1,2\*</sup>

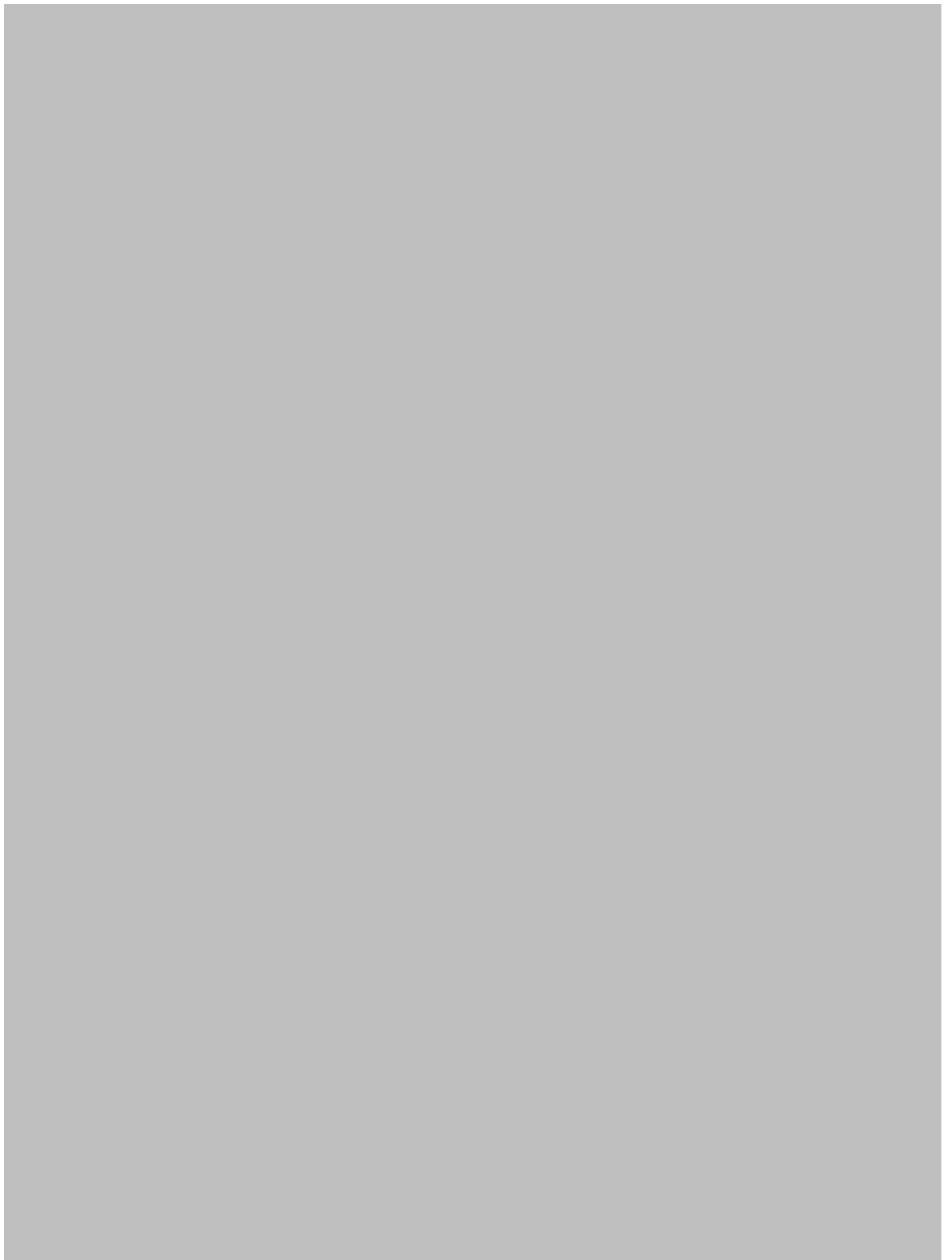
Health Protection Agency West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Birmingham B9 5SS, United Kingdom<sup>1</sup>; Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom<sup>2</sup>; and Prince of Wales Hospital, Hong Kong<sup>3</sup>

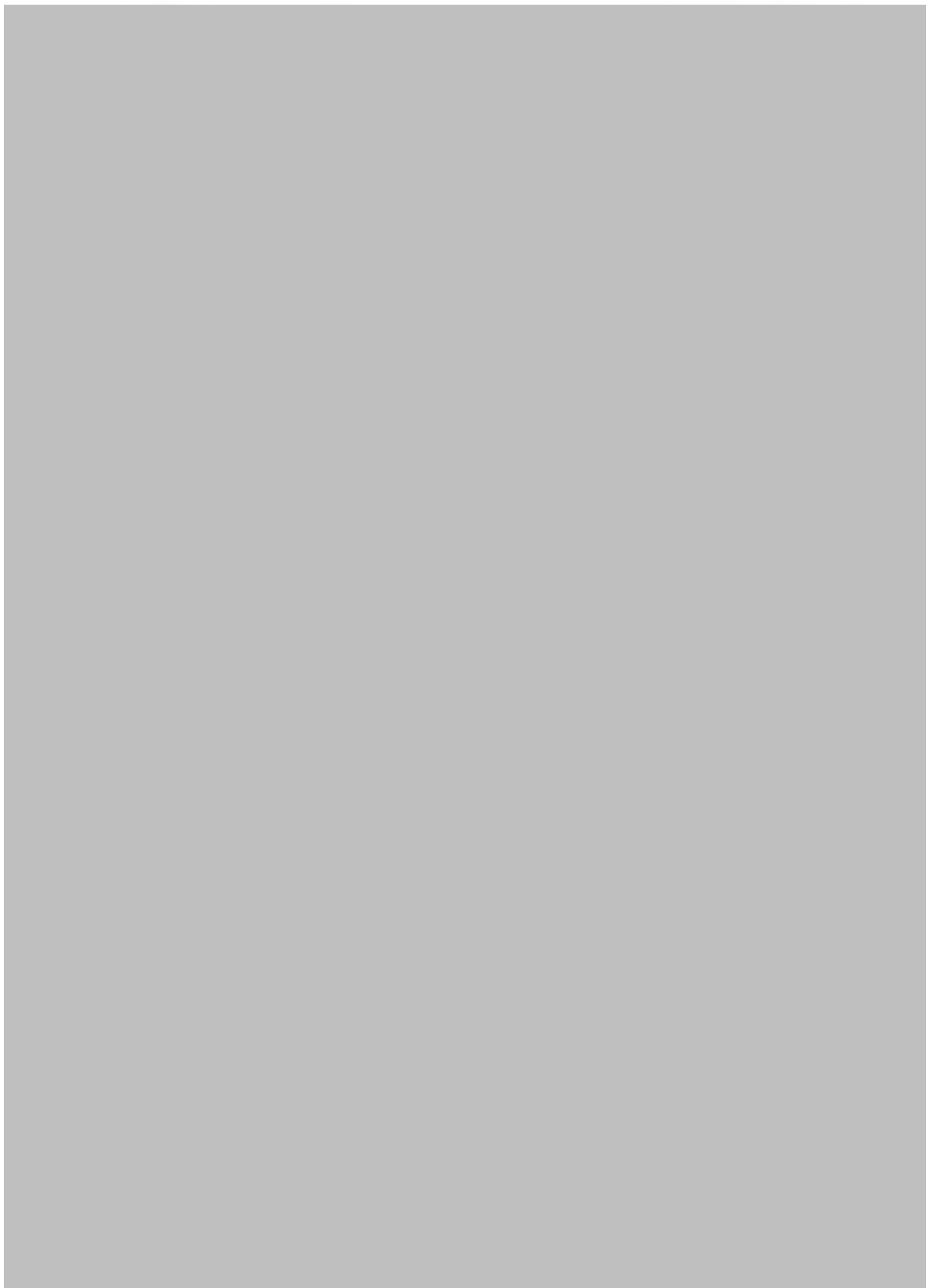
Received 29 August 2006/Returned for modification 27 October 2006/Accepted 21 December 2006

Denaturing high-performance liquid chromatography (dHPLC) is a powerful technique which has been used extensively to detect genetic variation. This is the first report of the application of dHPLC for rapid genotyping of bacterial  $\beta$ -lactamase genes. The technique was specifically developed to genotype members of all *bla*<sub>CTX-M</sub> DNA homology groups. Thirteen well-defined *bla*<sub>CTX-M</sub> extended-spectrum  $\beta$ -lactamase (ESBL)-producing strains were used to develop and optimize the dHPLC genotyping assay. Further evaluation was carried out with a blinded panel of 62 clinical isolates. The results of *bla*<sub>CTX-M</sub> genotyping achieved by dHPLC were comparable to the typing results obtained by DNA sequencing. Applying the newly developed dHPLC-based genotyping method, we successfully genotyped all 73 *bla*<sub>CTX-M</sub> ESBL-producing strains from the 4-month survey study. Furthermore, we found the first reported cases in the United Kingdom of clinically significant disease caused by CTX-M-14- and CTX-M-1-producing *Escherichia coli* strains. We conclude that the novel dHPLC assay is highly accurate, rapid, and cost-effective for the genotyping of *bla*<sub>CTX-M</sub>-producing ESBLs and has great potential for determining the clinical relevance of different and new *bla*<sub>CTX-M</sub> genotypes, as well as for epidemiological studies and surveillance programs.



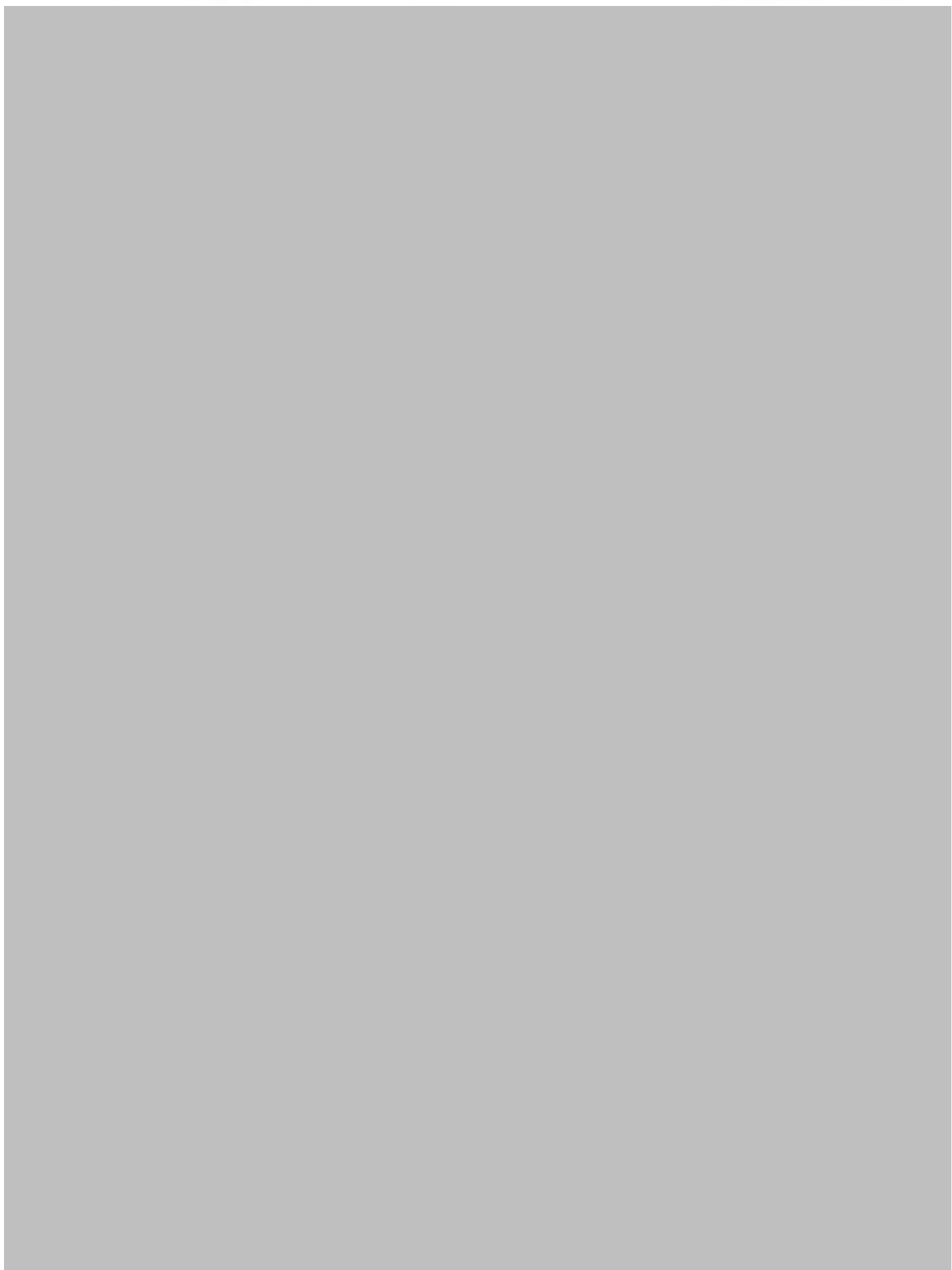


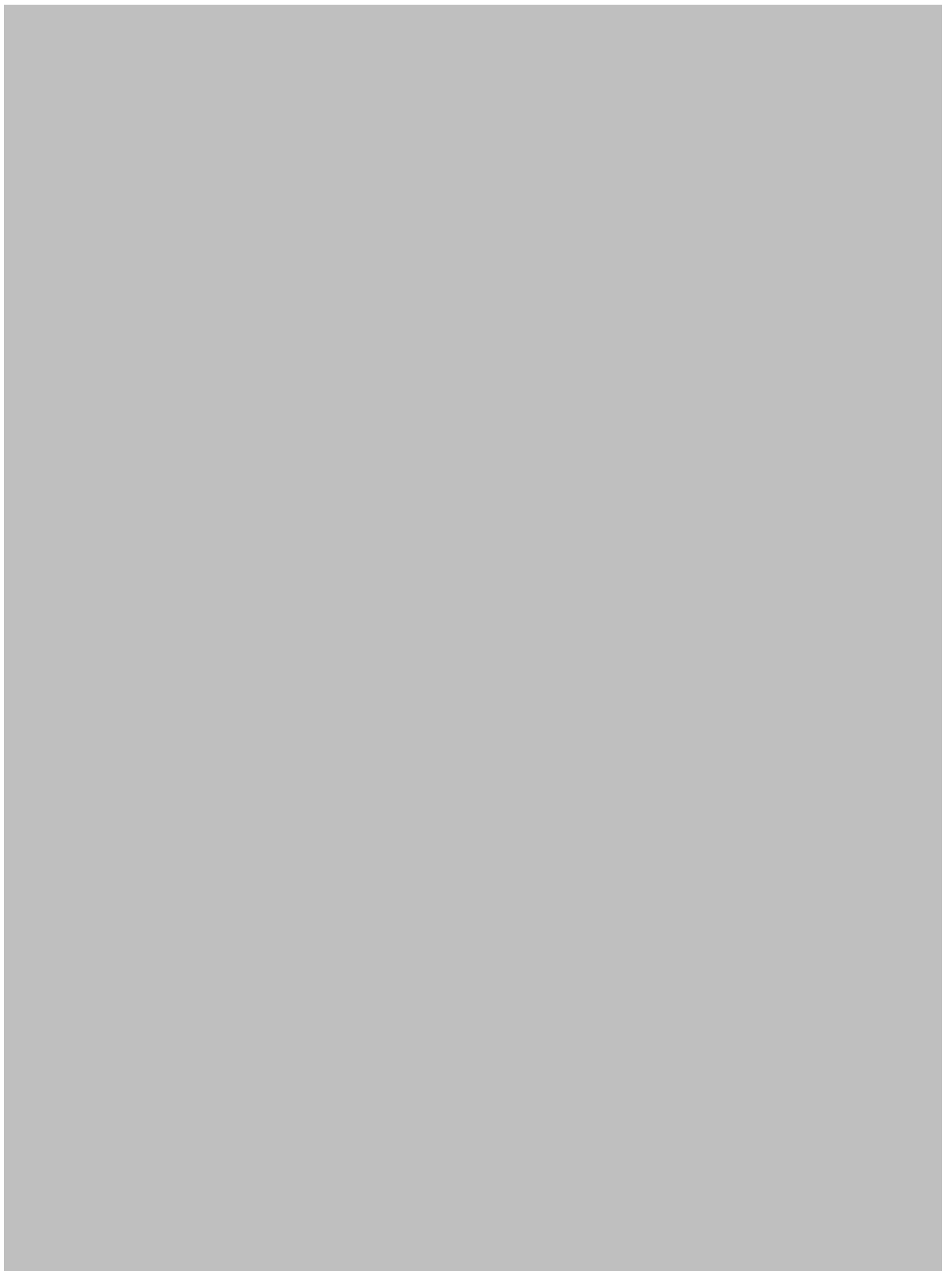


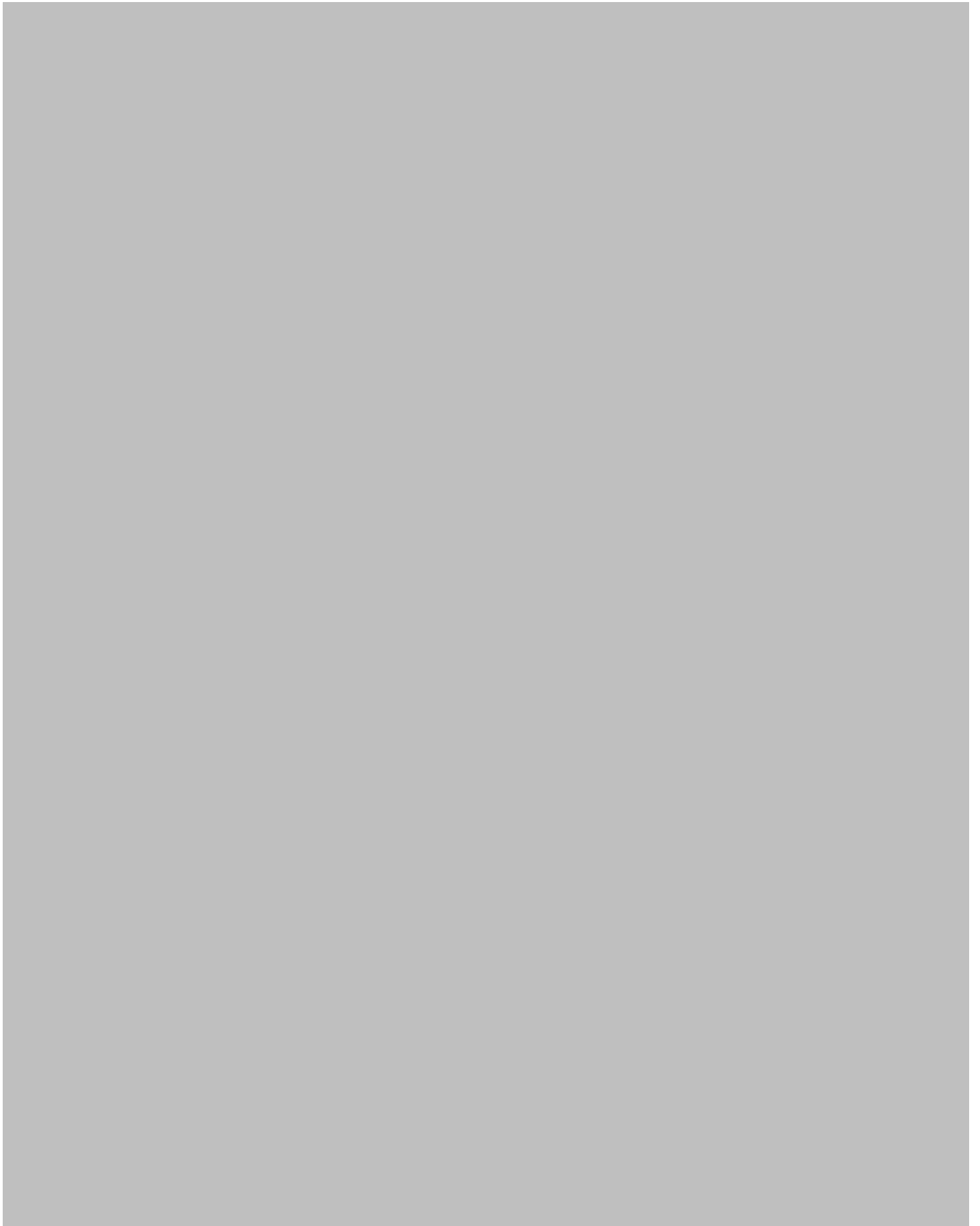












32. Xu, L., Shabir, S., Bodah, T., McMurray, C., Hardy, K., Hawkey, P., & Nye, K. 2011. Regional survey of CTX-M-type extended-spectrum beta-lactamases among *Enterobacteriaceae* reveals marked heterogeneity in the distribution of the ST131 clone. *J.Antimicrob.Chemother.*, 66, (3) 505-511 available from: PM:21183528

## Regional survey of CTX-M-type extended-spectrum $\beta$ -lactamases among Enterobacteriaceae reveals marked heterogeneity in the distribution of the ST131 clone

Li Xu<sup>1,2\*</sup>, Sahida Shabir<sup>1</sup>, Thomas Bodah<sup>1</sup>, Claire McMurray<sup>1,2</sup>, Katie Hardy<sup>1,2</sup>, Peter Hawkey<sup>1,2</sup> and Kathryn Nye<sup>1</sup>

<sup>1</sup>Health Protection Agency, West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Bordesley Green East, Birmingham B9 5SS, UK; <sup>2</sup>Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, UK

\*Corresponding author. Health Protection Agency, West Midlands Public Health Laboratory, Heart of England NHS Foundation Trust, Bordesley Green East, Birmingham, B9 5SS, UK. Tel/Fax: +44-(0)121-4241240; E-mail: l.mccrae@bham.ac.uk

Received 30 September 2010; returned 9 November 2010; revised 18 November 2010; accepted 18 November 2010

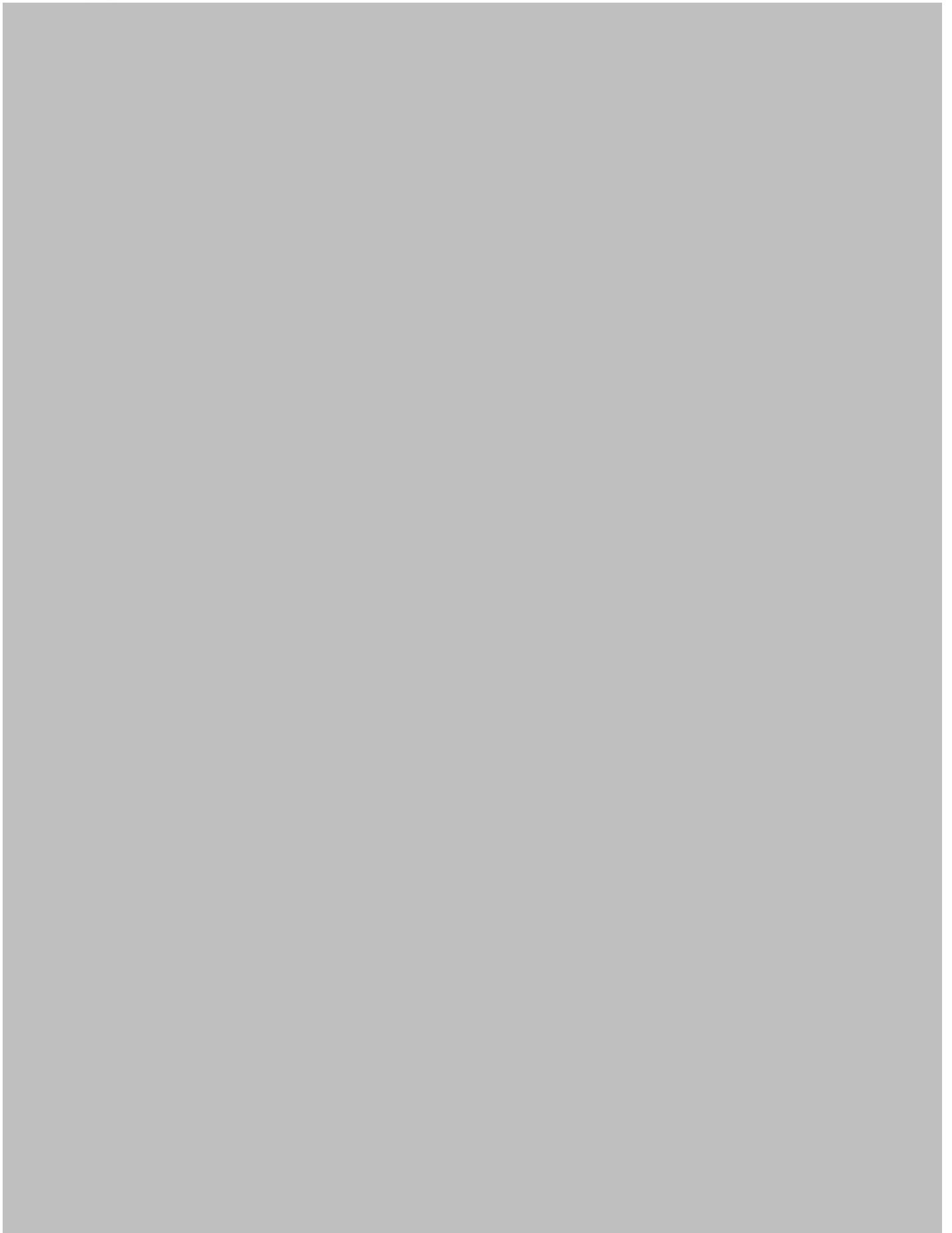
**Objectives:** To establish the prevalence and diversity of clinically significant extended-spectrum  $\beta$ -lactamase (ESBL)-producing Enterobacteriaceae harbouring *bla*<sub>CTX-M</sub> in the West Midlands region of the UK.

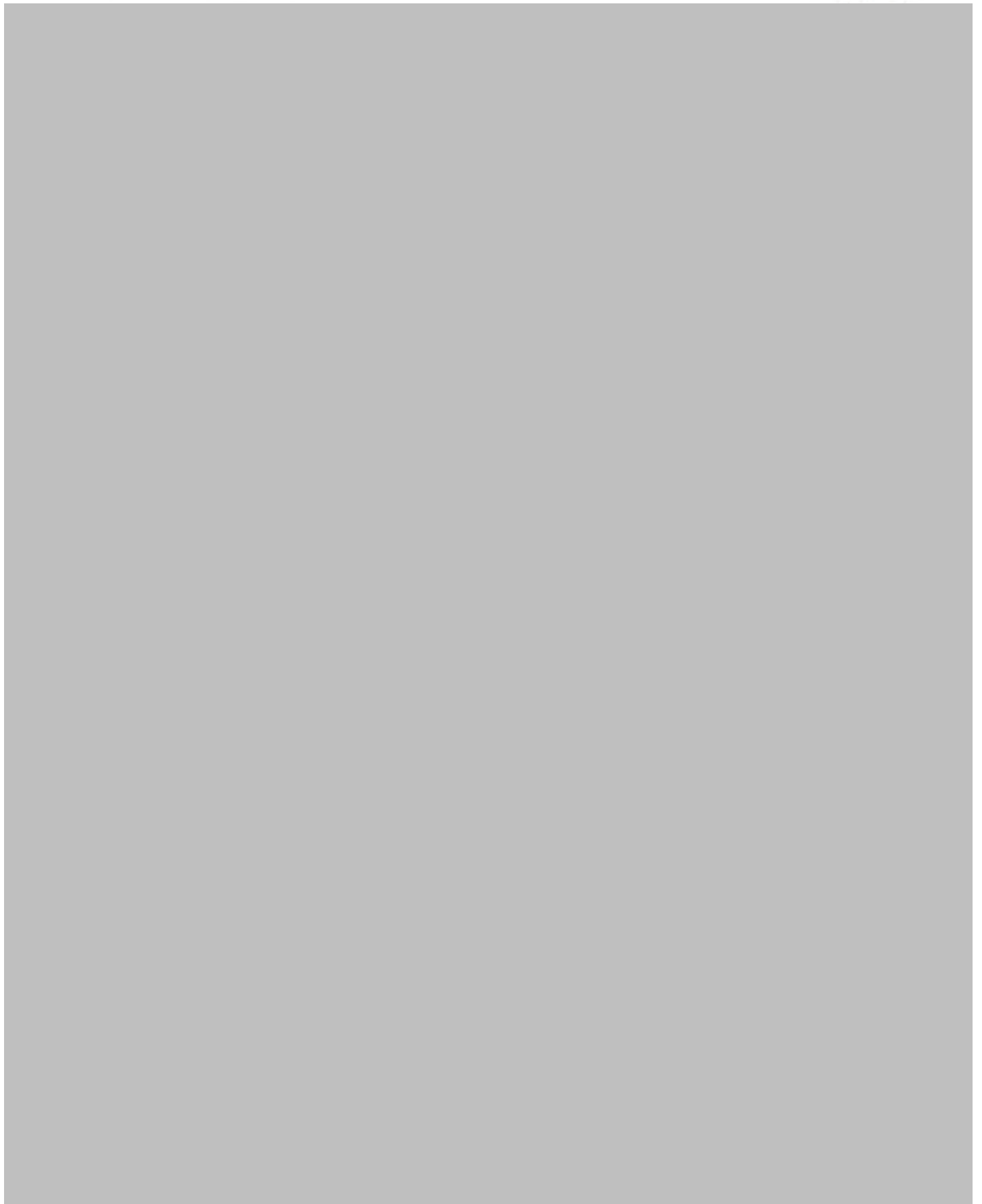
**Methods:** During a 2 month period, 370 consecutive, non-duplicate isolates were collected from 13 laboratories. Isolates were screened for the presence of *bla*<sub>CTX-M</sub> by multiplex PCR and genotyped using denaturing HPLC (DHPLC). Clonal relationships were studied by PFGE and O25b-ST131 *Escherichia coli* were identified by PCR.

**Results:** Two hundred and ninety-four out of 345 ESBL-producing isolates (85.2%) carried *bla*<sub>CTX-M</sub>. CTX-M group 1 enzymes were expressed in 284 (96.6%) isolates, with the other 10 carrying group 9, 2 and 25/26 genes. All group 1 isolates had *bla*<sub>CTX-M-15</sub> DHPLC profiles. The *bla*<sub>CTX-M</sub> *E. coli* were split into 23 PFGE clusters. The largest cluster (RE1) was indistinguishable from the previously described strain A and all but one harboured *bla*<sub>CTX-M-15</sub>. A total of 66% of *E. coli* were O25b-ST131 positive.

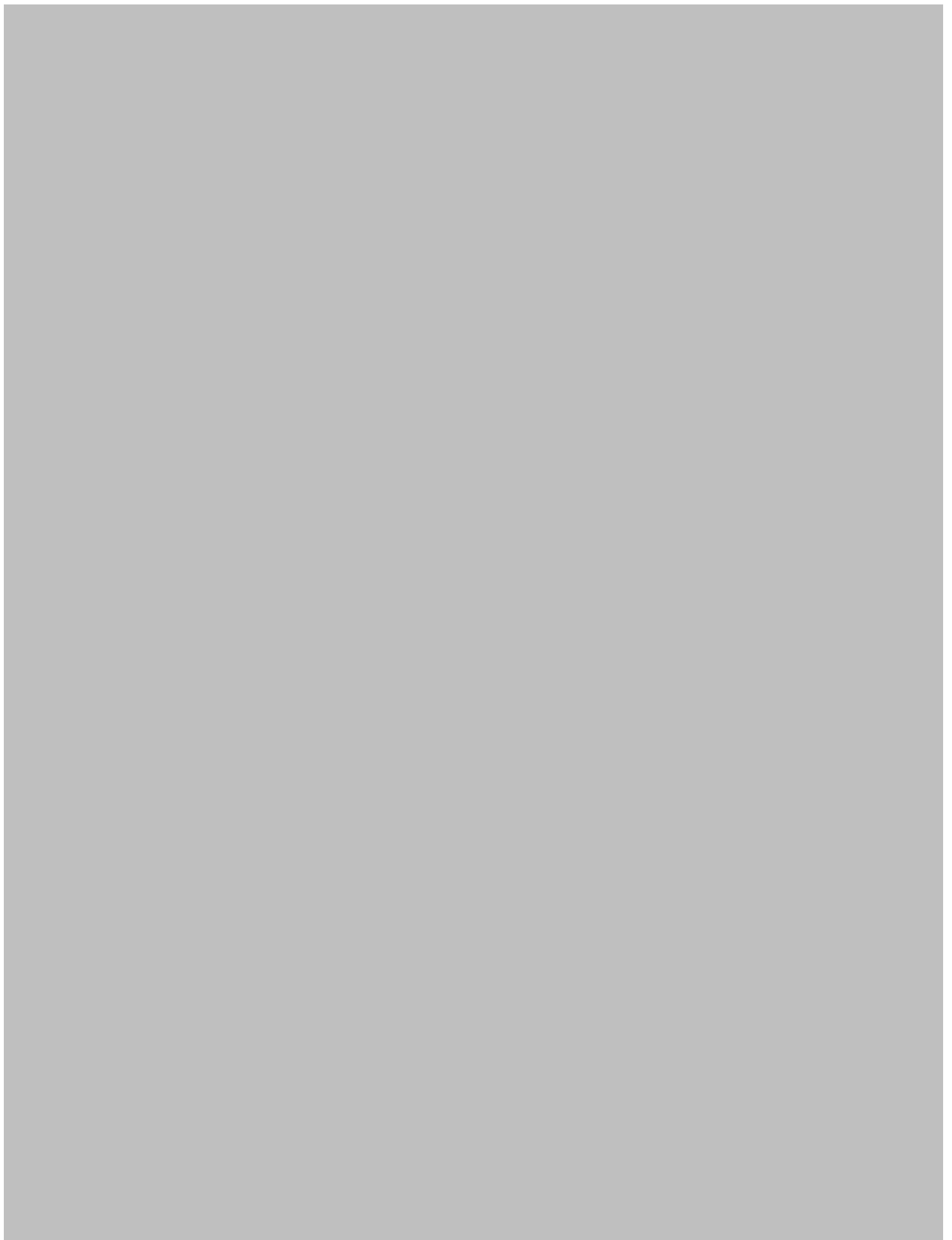
**Conclusions:** The CTX-M-15-producing RE1 clone (strain A) is the predominant clone in the West Midlands. This clone has spread throughout the region since its emergence in an outbreak 3 years earlier. Most, but not all, RE1 isolates belong to the O25b-ST131 lineage, providing further evidence that this lineage plays a pivotal role in the clonal dispersal of CTX-M-15-producing Enterobacteriaceae. Strain A was found to be considerably more heterogeneous than when first described and has acquired greater resistance to gentamicin. Approximately one-third of CTX-M producers represented a wide variety of unrelated strains. The study shows the rapid spread and diversification of CTX-M-producing Enterobacteriaceae over a 3 year period.

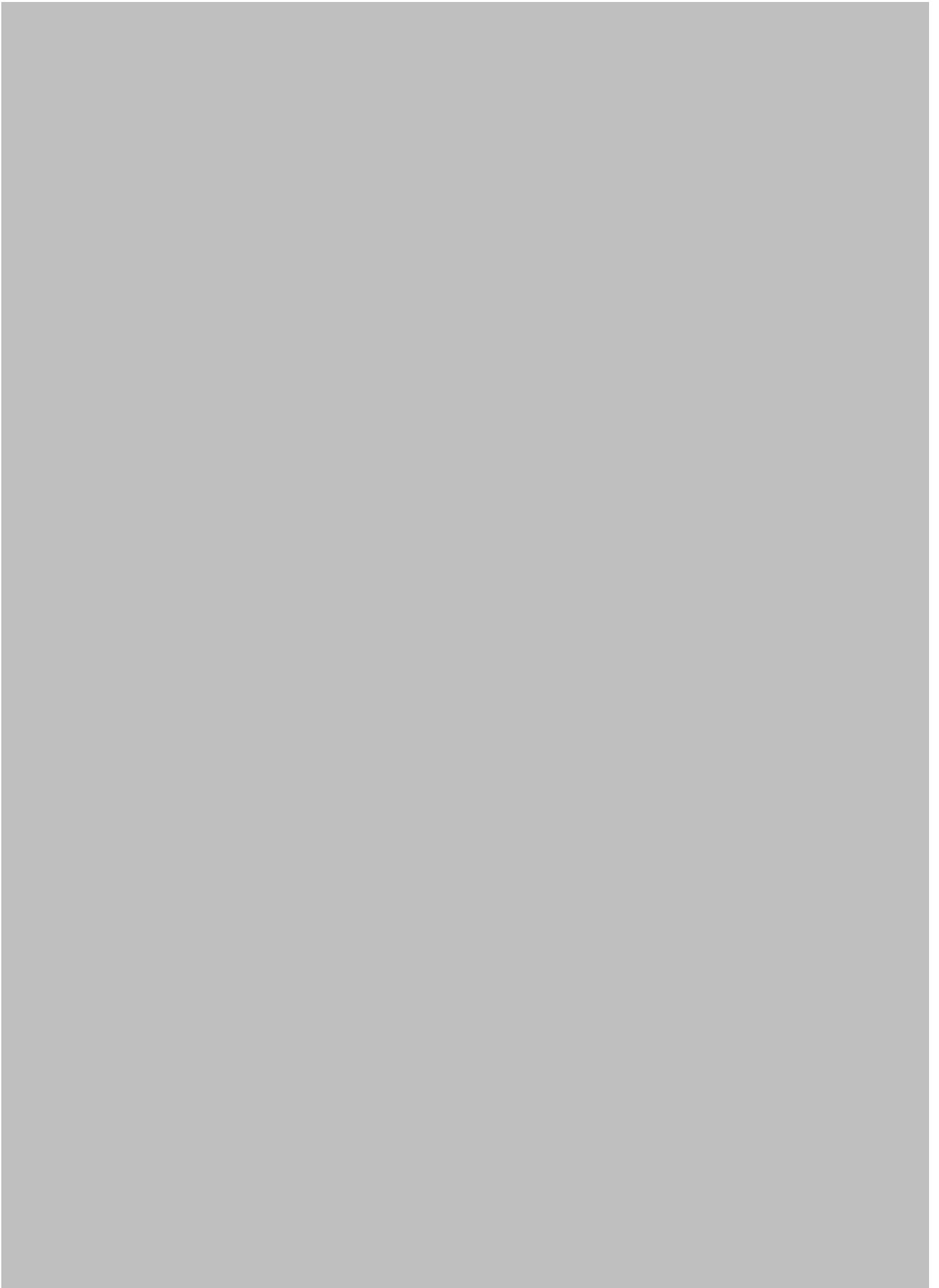
**Keywords:** ESBLs, molecular epidemiology, O25b-ST131















33. Zhong, Y.M., Liu, W.E., Liang, X.H., Li, Y.M., Jian, Z.J., & Hawkey, P.M. 2015. Emergence and spread of O16-ST131 and O25b-ST131 clones among faecal CTX-M-producing *Escherichia coli* in healthy individuals in Hunan Province, China. *J.Antimicrob.Chemother.*, 70, (8) 2223-2227 available from: PM:25957581

## Emergence and spread of O16-ST131 and O25b-ST131 clones among faecal CTX-M-producing *Escherichia coli* in healthy individuals in Hunan Province, China

Yi-Ming Zhong<sup>1</sup>, Wen-En Liu<sup>1\*</sup>, Xiang-Hui Liang<sup>1</sup>, Yan-Ming Li<sup>1</sup>, Zi-Juan Jian<sup>1</sup> and Peter M. Hawkey<sup>2,3</sup>

<sup>1</sup>Department of Clinical Laboratory, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China; <sup>2</sup>PHE Public Health Laboratory Birmingham, Heart of England NHS Foundation Trust, Birmingham B9 5SS, UK; <sup>3</sup>Institute of Microbiology and Infection, University of Birmingham, Birmingham B15 2TT, UK

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Received 19 December 2014; returned 14 January 2015; revised 3 April 2015; accepted 4 April 2015

**Objectives:** The objectives of this study were to determine CTX-M-producing *Escherichia coli* ST131 strain prevalence in stool specimens from healthy subjects in central China and to molecularly characterize clonal groups.

**Methods:** From November 2013 to January 2014, stool specimens from healthy individuals in Hunan Province were screened for ESBL-producing *E. coli* using chromogenic medium and CTX-M genotypes and phylogenetic groups were determined. ST131 clonal groups were detected by PCR and characterized for antibiotic resistance, *fimH*, *gyrA* and *parC* alleles, plasmid-mediated quinolone resistance determinants, virulence genotypes and PFGE patterns.

**Results:** Among 563 subjects, 287 (51.0%) exhibited the presence of faecal ESBL-producing *E. coli*, all of which produced CTX-M enzymes. The most common CTX-M genotypes were CTX-M-14 (48.4%), CTX-M-15 (27.5%) and CTX-M-27 (15.0%). Of the 287 CTX-M-producing isolates, 32 (11.1%) belonged to the ST131 clone. O16-ST131 isolates were dominant (75%) and contained the *fimH41* allele. The remaining eight (25%) ST131 isolates were of the O25b subgroup and contained *fimH30* or *fimH41*. Ciprofloxacin resistance was found in 100% of the O25b-ST131 isolates, whereas only 8% of the O16-ST131 isolates were resistant. All of the O25b-ST131 isolates except one showed *gyrA1AB* and *parC1aAB* mutations; most of the O16-ST131 isolates had *gyrA1A* and *parC1b* mutations. The virulence genotypes of O16-ST131 resembled those of the O25b-ST131 isolates. The 32 ST131 isolates formed one large group at the 64% similarity level. They comprised 15 PFGE groups (defined at ≥85% similarity).

**Conclusions:** O16-ST131 isolates have emerged as the predominant type of ST131 isolate in faecal CTX-M-producing *E. coli* in healthy individuals in China.

**Keywords:** *E. coli*, faecal carriage, ESBLs, genotypes

### Introduction

CTX-M-producing *Escherichia coli* are the most common type of ESBL-producing *E. coli*, causing hospital-associated and community infections with extensive dissemination in the environment.<sup>1</sup> There are few data on the community carriage of CTX-M ESBL-producing *E. coli* in China<sup>2</sup> and two studies describe greatly different carriage rates: 7% and 50.5%.<sup>3,4</sup> ST131 is the predominant extraintestinal pathogenic *E. coli* (ExPEC) clone and frequently carries CTX-M.<sup>5</sup>

The gut is the main reservoir of ExPEC,<sup>5</sup> although there are only a few studies of colonization by ST131, with differences in carriage rates.<sup>5,6</sup> Studies on ST131 carriage, with one exception,<sup>7</sup> either failed to distinguish the O16-ST131 clade from the more common O25b-ST131<sup>8</sup> or missed the O16 subgroup.<sup>4</sup> Therefore, further studies are necessary to characterize O16-ST131 colonization of the digestive tracts of healthy subjects.

The aim of this study was to determine the prevalence and genotype distribution of CTX-M-producing *E. coli* and the

prevalence of ST131 and the O16/O25b subgroups in healthy subjects across a province in China.

## Methods

### Participants

A cross-sectional survey was conducted on healthy subjects who had routine health screening at community health service centres (CHSCs) in Hunan Province between November 2013 and January 2014. A total of 11 CHSCs were selected using a multistage stratified sampling method. In the first stage, Hunan Province was divided into four different areas (northern, eastern, western and southern Hunan) according to economic development levels and geographical location. In the second stage, 11 counties were identified, representative by population, and a single CHSC from each was selected for study. Healthy individuals ( $\geq 18$  years of age) who were examined at the 11 CHSCs from November 2013 to January 2014 were given an attendee list number, which was used to identify participants on the day of visit. Every day, one individual from each centre was selected by matching one computer-generated random number to the attendance list at the centre and was invited to participate in the study. The proportion of refusals was 2.3%. Individuals were excluded if they had digestive tract diseases or had been exposed to a hospital environment or treated with antibiotics within 3 months prior to sample collection. The study was approved by the Ethics Committee of the Xiangya Hospital of Central South University (reference number ECCT 201310096). Participants provided fresh stools and informed consent on the day of visit.

### ESBL-producing *E. coli* identification, CTX-M type and phylogenetic group

Faecal samples were plated within 24 h onto chromogenic ESBL selective medium (ChromID, bioMérieux, Marcy-l'Étoile, France). *E. coli* were identified using API 20E (bioMérieux). Three to five pure colonies were examined for ESBL production by the double-disc synergy test according to CLSI guidelines.<sup>9</sup>

ESBL-producing *E. coli* isolates were investigated for the presence of *bla*<sub>CTX-M</sub> genes by multiplex PCR and sequencing as previously described.<sup>10,11</sup> The phylogenetic groups of *E. coli* were determined by quadruplex PCR.<sup>12</sup>

### Identification of ST131

The ST131 clonal group and O16/O25b variants were identified as previously described.<sup>13</sup> MLST was used to resolve discrepant results (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>).

### Antimicrobial susceptibility testing

For all of the ST131 isolates, three to five pure colonies were tested for antibiotic susceptibility by disc diffusion and the results were interpreted according to the breakpoints recommended by the CLSI.<sup>9</sup> The following antimicrobial agents were tested: ampicillin, ampicillin/sulbactam, cefazolin, ceftriaxone, ceftazidime, gentamicin, ciprofloxacin, piperacillin/tazobactam, imipenem, minocycline, nitrofurantoin, chloramphenicol and trimethoprim/sulfamethoxazole. *E. coli* ATCC 25922 was used as a quality control strain. Intermediate susceptibility was considered as resistant. The resistance score was calculated as previously described.<sup>7</sup> Isolates that were considered MDR were those resistant to at least three antimicrobial categories.

### Molecular characterization of ST131

The alleles of *fimH*, *gyrA* and *parC*, the H30-Rx subclones, plasmid-mediated quinolone resistance (PMQR) determinants [*qnrA*, *qnrB*, *qnrC*, *qnrS*, *aac(6)-Ib-cr* and *qepA*], virulence factor genes ( $n=32$ ) and virotypes were determined as previously described.<sup>14–17</sup>

ST131 isolates were subjected to PFGE analysis using XbaI digestion. A PFGE dendrogram was constructed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) according to the unweighted pair group method based on Dice coefficients. Isolates with a Dice similarity index  $\geq 85\%$  were considered to belong to the same PFGE group.

### Statistical analysis

Comparisons of proportions were performed using Fisher's exact test. Comparisons of antibiotic resistance scores were performed using the Mann-Whitney *U*-test. A *P* value  $< 0.05$  was considered to be statistically significant.

## Results

### Prevalence and genotype distribution of faecal CTX-M-producing *E. coli*

A total of 563 faecal samples, one sample from each participant, were analysed. The age range of the individuals (299 males and 264 females) recruited into the study was 18–83 (mean 44.3) years. Of the 563 samples collected, 287 were identified as ESBL-producing *E. coli*, with all strains harbouring *bla*<sub>CTX-M-type</sub> genes. The prevalence of CTX-M-producing *E. coli* was thus 51.0% (287/563 samples). Of the 287 isolates, 139 (48.4%) produced CTX-M-14, 79 (27.5%) CTX-M-15, 43 (15.0%) CTX-M-27, 11 (3.8%) CTX-M-65, 10 (3.5%) CTX-M-3, 2 (0.7%) CTX-M-24, 2 (0.7%) CTX-M-104 and 1 (0.3%) CTX-M-101.

Phylogenetic analysis of the 287 CTX-M-producing *E. coli* strains showed that 101 (35.2%) belonged to phylogroup D, 66 (23.0%) to phylogroup A, 63 (22.0%) to phylogroup B2, 36 (12.5%) to phylogroup B1, 11 (3.8%) to phylogroup F, 7 (2.4%) to phylogroup C, 2 (0.7%) to phylogroup E and 1 (0.3%) to clade I.

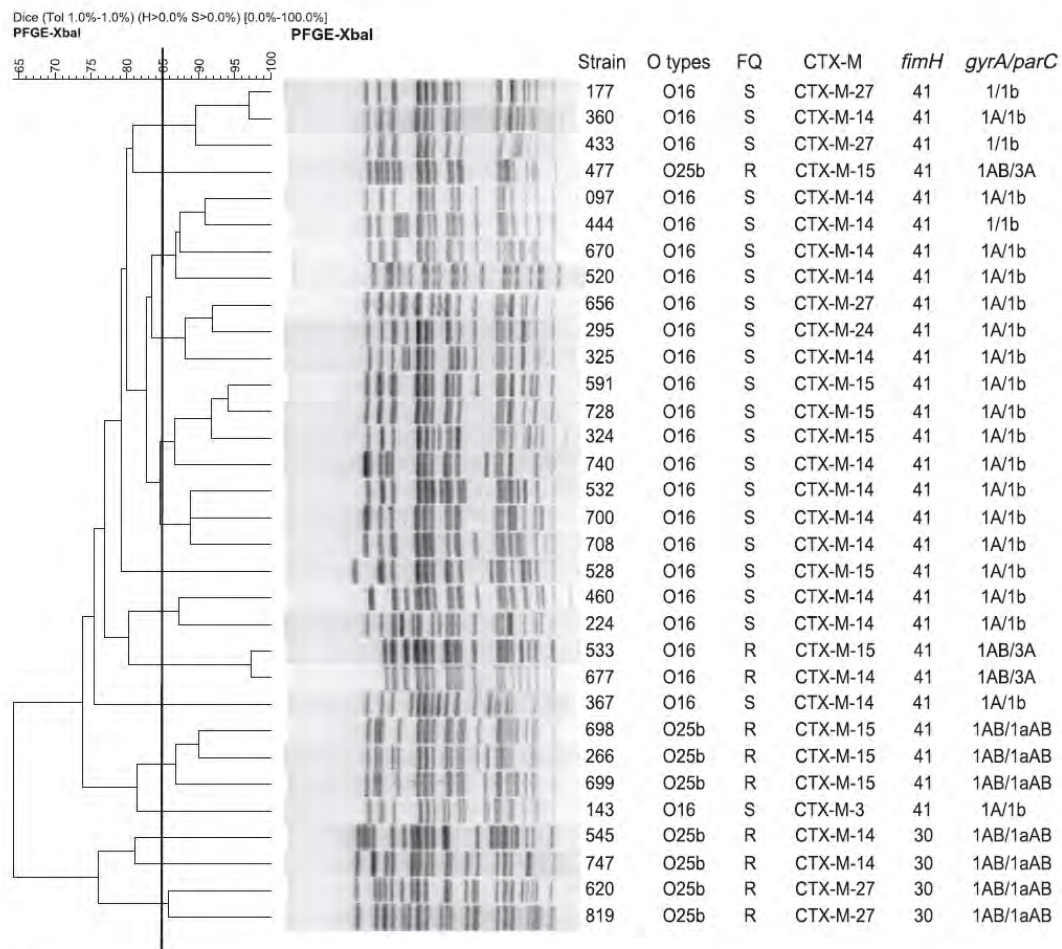
### *E. coli* ST131 clonal group and antimicrobial susceptibility

PCR analysis showed that 32 (11.1%) of the 287 CTX-M-producing isolates belonged to the ST131 clonal group, comprising 24 O16-B2-ST131 and 8 O25b-B2-ST131 isolates; only 2 isolates required full MLST.

All of the 32 ST131 isolates were found to be resistant to ceftriaxone, ceftazidime and ampicillin, 17 (53.1%) to ceftazidime, 10 (31.3%) to ciprofloxacin, 14 (43.8%) to gentamicin, 28 (87.5%) to ampicillin/sulbactam, 20 (62.5%) to trimethoprim/sulfamethoxazole, 8 (25.0%) to minocycline, 2 (6.3%) to piperacillin/tazobactam and 4 (12.5%) to chloramphenicol. None of the isolates was resistant to imipenem or nitrofurantoin.

### *E. coli* ST131 characterization

Characterization of the 32 *E. coli* ST131 isolates is shown in Figure 1. The *fimH* gene was carried by all 24 O16-ST131 isolates, which were H41 lineage. In contrast, only half of the eight



**Figure 1.** The XbaI PFGE-based dendrogram for the 32 *E. coli* ST131 isolates. The dendrogram was generated according to the unweighted pair group method based on Dice coefficients. O types, serogroup; FQ, fluoroquinolone phenotype (R, resistant; S, susceptible); *fimH*, allele of *fimH* (type 1 fimbrial adhesin); *gyrA/parC*, allele of *gyrA* and *parC* (encoding DNA gyrase and topoisomerase, respectively); 1/1b, *gyrA1* (WT) combined with *parC1b* (one silent mutation); 1A/1b, *gyrA1A* (one replacement mutation, Ser83Leu) combined with *parC1b*; 1AB/3A, *gyrA1AB* (two replacement mutations, Ser83Leu and Asp87Asn) combined with *parC3A* (a recombinant variant containing one replacement mutation Ser80Ile); 1AB/1aAB, *gyrA1AB* combined with *parC1aAB* (*parC1a* plus the replacement mutations Ser80Ile and Glu84Val). The 32 ST131 isolates formed one large group at the 64% similarity level. They comprised 15 PFGE groups (defined at  $\geq 85\%$  similarity).

O25b-ST131 isolates belonged to the H41 lineage. The remaining four isolates harbouring *fimH* belonged to the H30 lineage, with none representing the H30-Rx sublineage. All of the O25b-ST131 isolates except one showed *gyrA1AB* and *parC1aAB* mutations; most of the O16-ST131 isolates had *gyrA1A* and *parC1b* mutations. None of the PMQR determinants was detected in any of the 32 ST131 isolates. Nine PFGE clusters with a similarity index of  $\geq 85\%$  were identified. Unique PFGE profiles were seen in six isolates (Figure 1).

The virulence genotypes of O16-ST131 resembled those of the O25b-ST131 isolates (Table S1, available as Supplementary data

at JAC Online). All of the ST131 isolates were found to belong to virotype C.

#### Association of O16 and O25b subgroups with antimicrobial resistance and CTX-M type

As indicated in Table 1, the O25b-ST131 isolates had a significantly higher prevalence of resistance to ciprofloxacin compared with O16-ST131. The O16-H41 isolates were more likely to be positive for *bla*<sub>CTX-M-14</sub>. All of the O25b-H41 isolates produced CTX-M-15, which was not found in the O25b-H30 isolates.



**Table 1.** Antimicrobial resistance and CTX-M genotypes of the 32 *E. coli* ST131 isolates

	No. (%) of ST131 isolates			P value		
	O16-H41 (n=24)	O25b		O16-H41 versus O25b-H30	O16-H41 versus O25b-H41	O25b-H30 versus O25b-H41
		H30 (n=4)	H41 (n=4)			
<b>Antimicrobial resistance</b>						
ciprofloxacin	2 (8)	4 (100)	4 (100)	0.001	0.001	—
gentamicin	12 (50)	2 (50)	0	1.000	0.113	0.429
minocycline	4 (17)	1 (25)	3 (75)	1.000	0.038	0.486
piperacillin/tazobactam	1 (4)	1 (25)	0	0.270	1.000	1.000
ampicillin/sulbactam	22 (92)	2 (50)	4 (100)	0.086	1.000	0.429
trimethoprim/sulfamethoxazole	16 (67)	4 (100)	0	0.295	0.024	0.029
imipenem	0	0	0	—	—	—
nitrofurantoin	0	0	0	—	—	—
chloramphenicol	1 (4)	0	3 (75)	1.000	0.005	0.143
cefazolin	24 (100)	4 (100)	4 (100)	—	—	—
ceftriaxone	24 (100)	4 (100)	4 (100)	—	—	—
ceftazidime	10 (42)	3 (75)	4 (100)	0.311	0.098	1.000
ampicillin	24 (100)	4 (100)	4 (100)	—	—	—
MDR	11 (46)	4 (100)	3 (75)	0.102	0.596	1.000
Resistance score	3.4 (2–6) <sup>a</sup>	4.5 (3–6) <sup>a</sup>	4.5 (3–5) <sup>a</sup>	0.269	0.055	0.760
<b>CTX-M genotype</b>						
CTX-M-14	14 (58)	2 (50)	0	1.000	0.098	0.429
CTX-M-15	5 (21)	0	4 (100)	1.000	0.006	0.029
CTX-M-27	3 (13)	2 (50)	0	0.135	1.000	0.429
other CTX-M <sup>b</sup>	2 (8)	0	0	1.000	1.000	—

<sup>a</sup>The value denotes the mean and range.

<sup>b</sup>CTX-M-3 was found in one O16-ST131 isolate and CTX-M-24 was found in one O16-ST131 isolate.

## Discussion

We have found the colonization prevalence of CTX-M-producing *E. coli* in healthy individuals to be 51.0%, which is much higher than that reported from European countries (0.6%–11.3%).<sup>1,18</sup> Although a high prevalence (27.5%) of CTX-M-15 has not been observed in previous studies of community carriage from China,<sup>3,4</sup> our findings suggest that this worldwide dominant genotype is displacing the current genotypes (CTX-M-14 and CTX-M-27) in China. Our observation correlates with a recent report on clinical *E. coli* isolates across China showing 14.5% CTX-M-15 and 19.7% CTX-M-55 (a single-locus variant of CTX-M-15).<sup>19</sup> This emphasizes the importance of studying the human faecal resistome as many strains causing infection are derived from the gut.

O25b:H4 was considered the dominant ST131 clone serotype in almost all previous studies,<sup>5,6</sup> whereas O16:H5, a recently identified serotype of ST131 isolates, accounted for a small subset (1%–12%) in a few countries.<sup>5–7</sup> However, data from China are lacking and the present study is the first to show the presence of O16-ST131 in this country. Surprisingly, we found that O16-ST131 was the predominant subset (75%) among ST131 isolates, whereas this type only accounted for 8% in France.<sup>7</sup> These disparities may be due to geographical or host population differences. To our knowledge, our study is the first to report that the

O16 subset accounts for such a high proportion of the ST131 isolates. This finding implies that O16-ST131 may be poised to emerge as a major type of *E. coli* ST131 in the community in China.

All of the O16-ST131 isolates were found to belong to the *fimH*-based H41 subclone, as reported previously.<sup>7,13</sup> In contrast, the O25b-ST131 isolates carried the *fimH30* or H41 allele. This is the first study to show the presence of O25b-ST131 with the *fimH41* allele. Multiple previous studies have documented that most O25b-ST131 isolates contain the H30 allele, with a minority containing H22 and the remainder containing one of several rarer *fimH* alleles, but not *fimH41*.<sup>6,13</sup> The reason for the emergence of the *fimH41* allele in O25b-ST131 is not yet clear, but may be due to the homologous recombination of *fimH* alleles in O25b-ST131 isolates.<sup>20</sup> Further studies are required to elucidate and monitor the evolution of O25b-ST131 with the *fimH41* allele.

A limitation was that the small number of O25b-ST131 isolates restricted the broad generalizability of the characterization (especially statistical analyses) of O16-H41, O25b-H30 and O25b-H41 subgroups presented in this study. Thus, studies with larger sample sizes and more broadly distributed populations are required to further explore the characterization of these subgroups in the future.

In conclusion, to our knowledge, this is the first study to report O16-ST131 as the predominant type of clonal group in ST131. Our

findings indicate that future studies should focus more on O16-ST131 isolates, especially in high-incidence areas such as China.

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### Transparency declarations

None to declare.

### Supplementary data

Table S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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