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CMY-2-type plasmid-mediated AmpC β-lactamase finally emerging in Argentina

Sir,

Shigella spp. are an important causative agent of diarrhoea around the world. In Argentina, *Shigella flexneri* is the main species recovered from patients with shigellosis [1]. Third-generation cephalosporin resistance in *Shigella* spp. is still an infrequent event. In our country, it accounted for only 10 of 9033 clinical isolates of *S. flexneri* recovered in a surveillance study of 55 hospitals carried out during 1995–2003. Third-generation cephalosporin resistance in these 10 isolates was due only to different types of extended-spectrum β-lactamases (ESBLs) [1].

Plasmid-mediated AmpC β-lactamases are enzymes derived from chromosomal, typical class C β-lactamases and their detection in organisms naturally lacking these types of enzymes is increasing worldwide [2]. To date, plasmid-mediated AmpC in *Shigella* spp. has only been documented in Taiwan during an outbreak of *Shigella sonnei* harbouring CMY-2 AmpC β-lactamase [3]. In Argentina, plasmid-mediated AmpC was only previously reported in 1994 in a *Klebsiella pneumoniae* isolate harbouring FOX-1 [4].

Here we report the emergence of clonally related CMY-2-producing *S. flexneri* isolates in Argentina.

Between April and June 2006, three isolates of *S. flexneri* showing resistance to cephamycins (cefoxitin) were recovered from bloody stool samples of unrelated paediatric patients at the Hospital Interzonal Especializado Materno Infantil in Mar del Plata City (Buenos Aires province). One of the patients had never been admitted to hospital before, whilst the other two were hospitalised for 2 days just before and 1 month prior to *S. flexneri* isolation. None of the three patients had received antibiotics at least 1 year before the episode. The isolates were sent for further characterisation to the Antimicrobial Agents Division (National Reference Laboratory) and were confirmed to be *S. flexneri* 3a by usual serotyping methods.

Susceptibility testing was performed both by disk diffusion and agar dilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines [5]. All three isolates (M7896, M9016 and M9017) were highly resistant to aminopenicillins, narrow-spectrum cephalosporins and cefoxitin, had decreased susceptibility to third-generation cephalosporins and remained susceptible to cefepime (Table 1). With the exception of trimethoprim/sulfamethoxazole and tetracycline, all isolates were susceptible to the non-β-lactams tested, including fluoroquinolones, aminoglycosides, chloramphenicol, nitrofurantoin and minocycline (data not shown). Third-generation cephalosporin susceptibility was not restored by the addition of clavulanic acid (Table 1), consistent with a class C enzyme. To elucidate the mechanism involved in third-generation cephalosporin non-susceptibility, a synergy test was implemented by placing a disk containing 3-aminophenylboronic acid (APB) (300 μg/disk) between commercial disks of cefoxitin and either ceftazidime or cefotaxime [6]. APB showed positive synergy in all three cases. The minimum inhibitory concentrations (MICs) of cefotaxime and ceftazidime decreased 32–64 times and those of cefoxitin decreased 8 times when APB was added (300 mg/L) (Table 1).

Analysis of clonal relatedness, assessed by *Xba*I pulsed-field gel electrophoresis (PFGE) as described previously [1], showed that the three isolates belonged to the same clonal type. To investigate the mobility of the cefoxitin resistance determinant, a biparental conjugation was performed using M7896 and nalidixic acid-resistant *Escherichia coli* ER1793 as the donor and recipient strains, respectively [1]. After selection on tryptic soy agar plates supplemented with nalidixic acid (50 mg/L) plus ampicillin (20 mg/L), the transconjugant *E. coli* M7896-TC was selected. The β-lactam susceptibility profile of *E. coli* M7896-TC resembles that of the donor strain (Table 1). Resistances to trimethoprim, sulfisoxazole and tetracycline were not co-transferred to *E. coli* M7896-TC (data not shown). Using the Kieser method [7], a plasmid of ca. 150 kb was observed both in *S. flexneri* M7896 and *E. coli* M7896-TC. Enzymatic activity against cefoxitin in *S. flexneri* M7896 and *E. coli* M7896-TC was confirmed both by a microbiological assay [8] and by isoelectric focusing of crude β-lactamase extracts [1], which revealed a unique band at an isoelectric point (pI) of 8.2.

A multiplex polymerase chain reaction (PCR) for the detection of plasmid-mediated AmpC genes of different groups was performed on the three *S. flexneri* isolates and *E. coli* M7896-TC [9]. A 462-bp amplicon was obtained for the four isolates when using primers directed against the CIT group (LAT-1 to LAT-4, CMY-2 to CMY-7 and BIL-1). The PCR product of *S. flexneri* M7896 was sequenced and showed 100% identity with $bla_{\text{CMY-2}}$ (Genbank accession no. X91840).

To the best of our knowledge, this is the first report of CMY-2-type plasmid-mediated AmpC in *S. flexneri*. Quiet recently, CMY-2 was detected in *K. pneumoniae*

Table 1
β-Lactam susceptibility profiles of *Shigella flexneri* isolates, *Escherichia coli* recipient strain ER1793 and *E. coli* (TC)

Antimicrobial	MIC (mg/L)				
	S. flexneri M7896	*S. flexneri* M9016	*S. flexneri* M9017	*E. coli* ER1793	*E. coli* M7896-TC
Cefotaxime	16	8	8	≤0.03	8
Cefotaxime/APB	0.25	0.25	0.25	NP	0.5
Ceftazidime	16	16	16	0.125	32
Ceftazidime/APB	0.25	0.25	0.5	NP	1
Ceftazidime/CLA	8	8	8	NP	32
Cefepime	0.125	0.06	0.06	≤0.5	0.25
Cefepime/CLA	0.06	0.03	0.03	NP	0.125
Cefoxitin	128	128	128	4	128
Cefoxitin/APB	16	16	16	NP	16

MIC, minimum inhibitory concentration; APB, 3-aminophenylboronic acid (300 mg/L); CLA, clavulanic acid (4 mg/L); NP, not performed.

and *Citrobacter koserii* co-producing CTX-M-2 in our country [10].

Data from our study were used to include an APB disk-based test in the routine disk diffusion susceptibility testing protocol of the laboratories belonging to the WHONET–Argentina network (WAN) (67 hospitals) in order to confirm unusual β-lactam resistance profiles. Since this modification up to the present time (6 months), 15 of 25 000 enterobacterial isolates (unpublished data from WAN), including *Proteus mirabilis* ($n = 8$), *K. pneumoniae* ($n = 1$), *Salmonella* spp. ($n = 1$), *S. flexneri* 2a ($n = 1$) and *E. coli* ($n = 4$), were sent to the National Reference Laboratory from six distantly located hospitals in Argentina because of a positive synergy test with APB disks. All these strains were confirmed by multiplex PCR to produce plasmid-mediated AmpC β-lactamase belonging to the CIT group.

Usually, plasmid-mediated AmpC enzymes remain undetected, especially when there is co-expression of multiple β-lactamases (e.g. combination of AmpC plus ESBL or multiple ESBLs) [9]. The use of cefoxitin resistance as a screening tool for plasmid-mediated AmpC expression is extremely unspecific, since reduction in outer membrane permeability can result in a similar resistance phenotype [2,11]. Other methodologies including APB have been used to detect plasmid-mediated AmpC [11]. However, there is no standardised methodology. Moreover, many plasmid-mediated AmpC-producing isolates may not be properly categorised by conventional expanded-spectrum cephalosporin CLSI breakpoints. In fact, the three *S. flexneri* isolates studied here could be categorised as intermediate/susceptible to third-generation cephalosporins according to CLSI criteria, with the concern of potential treatment failure as has already been documented [11]. Prudent use of third-generation cephalosporins in these pathogens is extremely important, particularly in countries such as Argentina, where high-level resistance to first-line antibiotics (chloramphenicol, tetracycline, trimethoprim/sulfamethoxazole, ampicillin) is a frequent event in the treatment of shigellosis [1].

In summary, the use of an APB disk for synergy assays was shown to be a reliable methodology for plasmid-mediated AmpC recognition. Detection of these resistance determinants is as critical as ESBL detection, since both are plasmid-mediated mechanisms that can easily spread to other organisms within the hospital setting.

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References

[1] Andres P, Petroni A, Faccone D, Pasteran F, Melano R, Rapoport M, et al. Extended-spectrum β-lactamases in *Shigella flexneri* from Argentina: first report of TOHO-1 outside Japan. Int J Antimicrob Agents 2005;25:501–7.

[2] Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother 2002;46:1–11.

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

[4] Gonzalez Leiza M, Perez-Diaz JC, Ayala J, Casellas JM, Martinez-Beltran J, Bush K, et al. Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated β-lactamase with two molecular variants. Antimicrob Agents Chemother 1994;38:2150–7.

[5] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Seventeenth informational supplement. M100-S17. Villanova, PA: CLSI; 2007.

[6] Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamane K, Doi Y, et al. Practical methods using boronic acid compounds for identification of class C β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. J Clin Microbiol 2005;43:2551–8.

[7] Kieser T. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 1984;12:19–36.

[8] Melano R, Corso A, Petroni A, Centron D, Orman B, Pereyra A, et al. Multiple antibiotic-resistance mechanisms including a novel combination of extended-spectrum β-lactamases in a *Klebsiella pneumoniae* clinical strain isolated in Argentina. J Antimicrob Chemother 2003;52:36–42.
[9] Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 2002;40:2153–62.
[10] Radice M, Cittadini R, Stortz M, Ruggiero M, Gutkind G, Vay C. Emergence of plasmid-mediated AmpC β-lactamases in ESBL-producing enterobacteria in Buenos Aires, Argentina. In: Program and Abstracts of the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC); 17–20th September 2007. Chicago, IL. Washington, DC: ASM Press; 2007. Abstract C-2 1512.
[11] Doi Y, Paterson DL. Detection of plasmid-mediated class C β-lactamases. Int J Infect Dis 2007;11:191–7.

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Emergence of highly fluoroquinolone-resistant *Salmonella enterica* serovar Typhi in a community-based fever surveillance from Kolkata, India

Sir,

Typhoid fever caused by *Salmonella enterica* serovar *typhi* (*S.* Typhi) remains a major health problem in developing countries [1]. Traditional drugs such as chloramphenicol, ampicillin and co-trimoxazole were most effectively used as first-line drugs for the treatment of typhoid cases [1,2]. However, during the late 1980s and early 1990s the occurrence of multidrug-resistant *S.* Typhi strains, i.e. resistant to chloramphenicol, ampicillin and co-trimoxazole, led to the widespread use of fluoroquinolones (FQs) [1–3]. In recent years, nalidixic acid-resistant strains associated with reduced susceptibility to ciprofloxacin (minimum inhibitory concentration (MIC) ≥0.5 μg/mL) emerged and treatment failure with ciprofloxacin became a serious global concern [1,4]. Subsequently, isolation of highly FQ-resistant *S.* Typhi strains has been reported occasionally from other developing countries such as India and Bangladesh [5–7].

In this report, we document the isolation of two highly FQ-resistant (resistant to ciprofloxacin and ofloxacin) *S.* Typhi strains from blood samples of two patients included in a population-based fever (≥3 days) surveillance conducted in two urban slums of Kolkata, India, during the period May 2003 to December 2004. Isolation and identification of *S.* Typhi strains were carried out by standard microbiological culture using BD BACTEC™ 9240 blood culture systems and later confirmed by biochemical tests and agglutination with specific antisera (Becton Dickinson, Franklin Lakes, NJ) for O, H and Vi antigens. Antimicrobial susceptibility was tested by disk diffusion using commercially available antimicrobial disks (Becton Dickinson) and, for resistant strains, MICs of antimicrobials were determined by Etest strips (AB BIODISK, Solna, Sweden). Results were interpreted following Clinical and Laboratory Standards Institute guidelines [8]. Direct sequencing of the quinolone resistance-determining regions (QRDRs) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) subunit genes from both FQ-resistant *Salmonella* isolates was performed with an ABI Prism™ Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) on an automated sequencer. Mutations were determined by comparison with the DNA sequence of standard susceptible isolates available in GenBank. Pulsed-field gel electrophoresis (PFGE) using *Xba*I restriction enzyme (Takara Shuzo, Otsu, Japan) for DNA digestion was performed following a standard protocol [9]. Digested DNA was electrophoresed on a 1% agarose gel using CHEF-DR III (Bio-Rad, Hercules, CA) with switch times of 2.2–63.8 s at 6 V/cm for 16 h at 14 °C along with λ ladder PFGE molecular weight marker (New England Biolabs, Beverly, MA). Macrorestriction fragment patterns were analysed visually according to the criteria of Tenover et al. [10].

The two patients, a 3-year-old male and a 5-year-old female, from whom the two FQ-resistant *S.* Typhi strains were isolated on 26 July 2004 belonged to the same family and presented with enteric fever. Both isolates showed high-level resistance to nalidixic acid (MIC > 256 μg/mL) as well as to FQs such as ciprofloxacin and ofloxacin (MICs of both drugs = 16 μg/mL). The isolates were also resistant to tetracycline and co-trimoxazole, but susceptible to traditional drugs such as chloramphenicol and ampicillin and newer drugs such as amoxicillin/clavulanic acid, ceftriaxone, aztreonam and amikacin. Sequence data of the QRDR of the two FQ-resistant *S.* Typhi strains revealed three mutations: double mutations (Ser83Phe and Asp87Gly) in the *gyrA* gene and a single mutation (Ser87Ile) in the *parC* region. None of the nalidixic acid-susceptible strains were reported to have a mutation in these regions. Our study has the limitation that, in addition to QRDR mutations, the possible role of efflux mechanism for the development of FQ resistance in *S.* Typhi could not be explored; however, the three mutations described are