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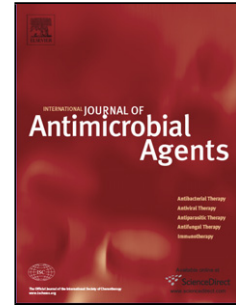
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## High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria

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## ABSTRACT

In Nigeria, quinolones and  $\beta$ -lactam antibiotics are widely used to treat bacterial infections. This study aimed to identify the prevalence of resistance to these drugs and to determine the mechanisms of resistance to these agents. In total, 134 non-duplicate, Gram-negative enteric isolates of 13 species from different hospitals were investigated for susceptibility to a panel of antibiotics, carriage of plasmid-mediated quinolone and  $\beta$ -lactam resistance genes, production of extended-spectrum  $\beta$ -lactamases (ESBLs), and mutations within topoisomerase genes. The level of resistance to all antibiotics tested was extremely high, with minimum inhibitory concentrations for 90% of the organisms ( $MIC_{90}$  values) of  $\geq 256$   $\mu\text{g/mL}$  for all drugs. Of the 134 isolates, 92 had mutations within the quinolone resistance-determining region (QRDR) of *gyrA* or within *gyrA* and *parC*. In addition, the plasmid-mediated quinolone resistance genes *qnrA*, *qnrB*, *aac(6')-Ib-cr* and *qepA* were identified. The *qnrD* allele, which has previously only been found in *Salmonella* isolates from China, was identified in two *Proteus* isolates and one *Pseudomonas* isolate. Of the 134 isolates, 23 (17.2%) carried *aac(6')-Ib-cr*, 11 (8.2%) carried a *qnr* variant and 5 (3.7%) were positive for *qepA*. Twenty-eight isolates (20.9%) produced ESBL variants, with a CTX-M variant being carried by 25 isolates (18.7%). In addition, six isolates (4.5%) carried *ampC* variants [ACT-1 (1 isolate), DHA-1 (4 isolates) and CMY-2 (1 isolate)]. This study demonstrates a very high level of multidrug resistance amongst Gram-negative enteric bacilli isolated from different sites from patients in Nigerian hospitals as well as the presence of a variety of plasmid-associated resistance genes, including some identified from Africa for the first time.

## 1. Introduction

Worldwide, ever increasing numbers of bacterial clinical isolates are being reported with resistance to therapeutically relevant antibiotics. This trend is set against a backdrop of diminished development of novel antibiotics, particularly those with activity against Gram-negative pathogens. In Nigeria, quinolones and  $\beta$ -lactam antibiotics are widely used as broad-spectrum antibiotics to treat infections caused by various Gram-negative pathogens. Whilst it was initially suggested that susceptibility to quinolones in Nigeria may remain high as these drugs are expensive and beyond the reach of most individuals, their use has increased and there have been sporadic reports of isolates with decreased quinolone susceptibility from Nigeria.

Chromosomal resistance to fluoroquinolones (FQs) due to amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (GyrA) and/or topoisomerase IV (ParC) or to efflux pump derepression/alteration in porin expression is well understood [1]. Plasmid-mediated mechanisms of resistance to FQs mediated by Qnr alleles (*qnrB*, *qnrS*, *qnrC* and *qnrD*), the *aac(6')-Ib-cr* variant and *qepA* have been identified from countries around the world [2]. Association of multiple antibiotic resistance genes on mobile genetic elements has been an important mechanism of dissemination of multidrug resistance and may explain in part the frequent association between FQ resistance and resistance to expanded-spectrum  $\beta$ -lactams in Enterobacteriaceae. In addition, the presence of multiple resistance genes on a plasmid expands the subset of drugs that may select for dissemination of multidrug resistance plasmids. A variety of extended-spectrum  $\beta$ -

lactamase (ESBL) enzymes have evolved and have been detected in Gram-negative pathogens in the last 30 years; most recently, the CTX-M group of enzymes have become the dominant ESBL detected worldwide [3].

In this study, an extremely high level of resistance to multiple antibiotics, including FQs and cephalosporins, was detected amongst a diverse panel of Gram-negative isolates from various Nigerian hospitals. This resistance was underpinned by the carriage of a wide variety of plasmid-borne quinolone resistance alleles and ESBL genes, including the first identification of the *qnrD* allele outside of China. This work gives a snapshot of the prevalence of antibiotic resistance in Nigeria where antibiotic use is unregulated and little data exist regarding resistance levels in this part of the world. The establishment of a pool of highly resistant strains in Nigeria may act as a reservoir for dissemination of resistant strains to other countries.

## 2. Materials and methods

### 2.1. Bacterial Isolates

In total, 134 clinical Gram-negative bacterial isolates of 13 species were obtained from 585 non-duplicate clinical specimens, including aspirates, ear swab, wound swab, throat swab, high vaginal swab, eye swab, sputum, urine, catheter tip, cerebrospinal fluid and blood culture, for the period 2005–2007. Single isolates from each specimen were retained. Isolates were from four teaching hospitals in southwestern Nigeria, namely University College Hospital (Ibadan), Obafemi Awolowo University Teaching Hospital (Ile-Ife), Ladoke Akintola University of Technology Teaching Hospital (Osogbo) and Olabisi Onabanjo University Teaching Hospital

(Sagamu). All isolates were identified using API 20E strips (bioMérieux, Marcy l'Etoile, France).

## 2.2. Determination of antibiotic susceptibility

Minimum inhibitory concentrations (MICs) of a panel of antibiotics were determined and interpreted using the agar dilution method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC) ([http://www.bsac.org.uk/susceptibility\\_testing/guide\\_to\\_antimicrobial\\_susceptibility\\_testing.cfm](http://www.bsac.org.uk/susceptibility_testing/guide_to_antimicrobial_susceptibility_testing.cfm)). The antibiotics tested were gentamicin, tetracycline, amoxicillin, amoxicillin/clavulanic acid (AMC), nalidixic acid, ciprofloxacin, pefloxacin, ofloxacin, sparfloxacin, ceftazidime and ceftriaxone. MIC breakpoints for defining ciprofloxacin susceptibility used were: susceptible, 1 µg/mL; intermediate susceptible, 2 µg/mL; and resistant, 4 µg/mL. All susceptibility testing runs included the control organisms *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662.

## 2.3. Genotyping of quinolone-resistance determining regions

The QRDRs of *gyrA*, *gyrB*, *parC* and *parE* were amplified and mutations were detected using denaturing high-performance liquid chromatography (HPLC) as described previously [4].

## 2.4. Amplification of antimicrobial resistance genes

Polymerase chain reaction (PCR) was used to detect genes encoding resistance to β-lactams (*bla*<sub>OXA</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub> and *bla*<sub>AmpC</sub>) as well as plasmid-mediated



quinolone resistance (PMQR) genes as previously described [5,6]. Multiplex PCR was used to amplify variants of *qnrA*, *qnrB* and *qnrS* in one reaction as previously described [6]. New primers were designed for amplification of *qnrD* (QnrDF, 5' GACAGGAATAGCTTGAAGG 3'; QnrDR, 5' CTGCTCTCCATCCAACCTTCA 3'). Amplimers resulting from these PCR reactions were sequenced to confirm the identity and specific variant of each gene identified and sequences were aligned to known reference sequences using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

#### 2.5. Phenotypic detection of $\beta$ -lactamases

Broth cultures of each test strain as well as ampicillin-resistant *E. coli* strain NCTC 10418 (carrying pUC18) were incubated overnight in 5 mL of Luria–Bertani broth. Then, 200  $\mu$ L of the resulting overnight culture was transferred into the wells of a microtitre tray; sterile broth was included as a negative control. Then, 10  $\mu$ L of nitrocefin solution (Fisher Scientific, Loughborough, UK) prepared according to the manufacturer's instructions was added to each well.  $\beta$ -Lactamase production was inferred when the broth turned red within 30 min of addition of nitrocefin as directed by the manufacturer.

#### 2.6. Detection of extended-spectrum $\beta$ -lactamases by the double-disk diffusion test (DDDT)

Suspensions of each test strain were prepared in sterile water to give an inoculum equivalent to a 0.5 McFarland standard before being used to inoculate the surface of Iso-Sensitest Agar plates [7]. ESBL-positive *Klebsiella pneumoniae* ATCC 700603

and ESBL-negative *E. coli* ATCC 25922 control strains were used in these experiments. A 30 µg ceftazidime disk (the best indicator for TEM- and SHV-derived ESBLs) was placed on the left of each plate and a 30 µg cefotaxime disk (the best indicator for CTX-M types) was placed on the right; an AMC (20/10 µg) (Oxoid Ltd., Basingstoke, UK) was placed in the centre between the other disks. The disks were placed 25–30 mm apart, centre-to-centre. Following overnight incubation in air at 37 °C, ESBL production was inferred when the zone of inhibition around the ceftazidime and cefotaxime disks was expanded by ≥5 mm by the presence of clavulanic acid.

### 2.7. Detection of AmpC

AmpC β-lactamase production was detected using cefepime and cefpodoxime disks in combination with clavulanic acid as previously described [8]. The plate and the inoculum were essentially the same as for ESBL detection described above. Following overnight incubation at 37 °C, AmpC β-lactamases were detected using a difference of ≥14 mm between cefepime/cloxacillin and cefpodoxime/cloxacillin disks.

## 3. Results

### 3.1. Antibiotic susceptibility

The MICs for all antibiotics and selected isolates using the agar dilution method are shown in Table 1. The results showed a high degree of resistance, with MIC<sub>90</sub> values (MIC for 90% of the organisms) of ≥256 µg/mL for all antibiotics.

### 3.2. Mechanisms of quinolone resistance

As is commonly seen in FQ-resistant mutants, substitutions in the topoisomerase genes *gyrA* and *parC* were identified. In total, 92 (68.7%) of the 134 isolates carried a mutation in the QRDR of *gyrA* or *parC*; no mutation was detected in *gyrB* or *parE* from any of the isolates studied. Mutations in *gyrA* were found at positions 83 and 87, whilst two substitutions were seen within *parC* at codons 80 and 87. All *parC* mutations were seen in strains that also carried a *gyrA* mutation. Eleven (8.2%) of the isolates carried a *qnr* gene, all of which had 100% sequence identity to previously described alleles. Five isolates (3.7%) were found to be *qnrA1*-positive, 3 (2.2%) were found to carry *qnrB* and 3 (2.2%) carried *qnrD*, previously only seen in Chinese *Salmonella* isolates [9]. The *qnrD*-carrying isolates comprised two *Proteus mirabilis* and one *P. aeruginosa*. The *qnrS* allele was not detected in any isolate. None of the *qnr*-positive isolates had more than one *qnr* gene. Twenty-three (17.2%) of the isolates were positive for *aac(6')-Ib-cr*, and 6 (54.5%) of the 11 *qnr*-positive strains also carried *aac(6')-Ib-cr*. Five isolates (3.7%) were found to carry the *qepA* efflux pump gene; no *qnr* or *aac(6')-Ib-cr* gene was detected in these isolates. The PMQR genes were well distributed among the isolates, hospitals and clinical specimens (Table 2). There were two isolates (L8 and SGm) with reduced FQ resistance (0.06–4 µg/mL) but no gyrase or topoisomerase IV mutations: L8 carried *aac(6')-Ib-cr*, and SGm carried *qnrB*. All other isolates carrying a PMQR gene also had a topoisomerase mutation(s).

### 3.3. Mechanisms of $\beta$ -lactam resistance

Of the 134 isolates, 111 (82.8%) were found to produce a  $\beta$ -lactamase by the nitrocefin assay; 85.7% of *K. pneumoniae* subsp. *pneumoniae*, 71.4% of *E. coli*, 69.2% of *P. aeruginosa* and 81.8% of *P. mirabilis* (Table 3). The rest of the strains were uniformly positive in the nitrocefin reactions. ESBL production was inferred using the DDDT; 28 isolates (20.9%) were found to be ESBL-positive by the DDDT (Table 3). Phenotypic detection of AmpC suggested that AmpC enzymes were present in 29 isolates (21.6%). Twenty-five AmpC-positive isolates were detected and were interpreted as positive using an increase of  $\geq 14$  mm in zone diameter around the antibiotic disk with added inhibitor compound between cefepime and cefpodoxime. Four further isolates were suspected of possessing an inducible AmpC where a defined increase in zone diameter around the antibiotic disk with added inhibitor compound compared with that with the antibiotic-containing disk alone was observed.

All 134 isolates were tested by PCR for the presence of  $\beta$ -lactamase genes. TEM variants were present in 109 isolates (81.3%), SHV variants in 33 (24.6%), OXA variants in 15 (11.2%) and CTX-M variants in 25 isolates (18.7%; 24 of these were CTX-M-15 and 1 isolate carried CTX-M-3); genes of all these groups were present in 6 isolates (4.5%) (data not shown). Multiplex PCR for detection of *ampC* genes confirmed six isolates as carrying non-chromosomal *ampC* enzymes and these genes were only found in isolates with CTX-M genes. The *ampC*-positive strains cut across most of the clinical sites of specimens, ranging from wound to ear, aspirate, blood culture and catheter tip. Of the six *ampC*-positive genes, one was amplified using EBC group primers, four by DHA group primers and one by CIT primers.

Sequencing identified these genes as ACT-1, DHA-1 and CMY-2, respectively. No enzymes belonging to ACC, FOX and MOX families were detected.

#### 4. Discussion

Isolates were collected for this study without any bias towards resistant strains. All isolates demonstrated an extremely high level of resistance to all the drugs tested, including the FQs for which most strains had MIC values as high as 256 µg/mL. There was no difference between the MIC<sub>50</sub> (MIC for 50% of the organisms) and the MIC<sub>90</sub> values of the isolates in this study, and clinical failure of FQ drug therapy is inevitable for strains with such high levels of resistance. Quinolone-resistant strains carried one or more mutations within *gyrA* and additional mutations within *parC*, as has commonly been seen in highly FQ-resistant mutants in other studies [1]. Carriage of a variety of transferable quinolone resistance alleles was detected in these strains, including *qnrA1*, *qnrB*, *qnrD*, *aac(6′)-Ib-cr* and *qepA*. This demonstrates that these alleles are truly global and have spread to West Africa; *qnrD* in particular has previously only been described in an isolate from China [9]. Identification of this allele in these Nigerian isolates of *Proteus* and *Pseudomonas* demonstrates its presence in another continent as well as in two species in addition to *Salmonella* where it was first described. Whether this represents global dissemination of this gene or identification of separate acquisition events from environmental species is unclear and further work is required to clarify this. Six (54.5%) of the eleven *qnr*-positive strains also carried *aac(6′)-Ib-cr*. Five isolates were detected to be carrying the plasmid-mediated quinolone resistance efflux pump gene *qepA*; interestingly, four of these isolates did not carry topoisomerase mutations but did demonstrate high-level resistance to quinolones. No other PMQR

genes were found in these isolates suggesting that *qepA* is contributing to this high-level resistance in these strains.

Of the 134 isolates, 28 (20.9%) were found to carry an ESBL. This rate of ESBL carriage is similar to that seen in other countries and is similar to the few previous reports from Nigeria, where a previous study found 8 (20%) of 40 *Enterobacter* isolates produced an ESBL by the DDDT [10]. In this study, the predominant ESBL genotype detected was CTX-M-15. There has been an increase in the isolation of CTX-M-producing bacteria in the last 20 years globally and these genes are spread worldwide. In this study, 25 isolates were CTX-M positive, of which 24 were CTX-M-15 and only 1 was CTX-M-3. CTX-M-15 has been described in Nigeria before but this study is the first report of CTX-M-3 in Nigeria.

The presence of *ampC* genes was indicated for a number of strains phenotypically and was confirmed in six isolates by PCR. These genes were found in isolates co-producing other ESBL genes such as CTX-M, TEM, SHV and OXA genes.

Sequencing identified these genes as ACT-1, DHA-1 and CMY-2. DHA-like enzymes (originally derived from *Morganella morganii*) were most common [4] in the isolates. These *ampC*-positive strains appeared to have low-level resistance to the third-generation cephalosporins used in this study but demonstrated high-level quinolone resistance.

In conclusion, there is a high level of resistance to many antimicrobials in different species of Gram-negative enteric bacilli, most critically to FQs and  $\beta$ -lactam antibiotics that are frequently used in Nigeria. Most of the strains carried genes

encoding resistance to both important classes of antimicrobial agents. A wide array of transferable  $\beta$ -lactamase and quinolone resistance alleles are present in Nigerian strains of various species. The uncontrolled use of antibiotics in Nigeria is likely to have contributed largely to this situation. The selection of such highly resistant isolates in countries such as Nigeria may act as a reservoir of resistant strains that can be transferred to other countries in the era of global travel.

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### **Competing interests**

None declared.

### **Ethical approval**

Not required.

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**Table 1**

Antimicrobial susceptibility of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates

Organism (no of strains)/antimicrobial agent	MIC ( $\mu\text{g/mL}$ )		
	MIC <sub>50</sub>	MIC <sub>90</sub>	Range
<i>E. coli</i> (n = 28)			
Ciprofloxacin	256	256	0.015–256
Pefloxacin	256	256	0.25–256
Ofloxacin	128	256	0.25–256
Sparfloxacin	256	256	0.25–256
Ceftazidime	32	256	0.25–256
Ceftriaxone	8	256	0.25–256
AMC	256	256	0.25–256
Amoxicillin	256	256	0.25–256
Gentamicin	256	256	0.25–256
Tetracycline	256	256	0.25–256
Nalidixic acid	>256	>256	1–256
<i>K. pneumoniae</i> (n = 63)			
Ciprofloxacin	256	256	0.015–256
Pefloxacin	256	256	0.25–256
Ofloxacin	256	256	0.25–256
Sparfloxacin	256	256	0.25–256
Ceftazidime	256	256	0.25–256
Ceftriaxone	256	256	0.25–256
AMC	256	256	0.25–256
Amoxicillin	256	256	0.25–256
Gentamicin	256	256	0.25–256
Tetracycline	256	256	0.25–256
Nalidixic acid	>256	>256	1–256
<i>P. aeruginosa</i> (n = 13)			
Ciprofloxacin	256	256	0.015–256
Pefloxacin	256	256	0.25–256

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Ofloxacin	256	256	0.25–256
Sparfloxacin	256	256	0.25–256
Ceftazidime	32	256	0.25–256
Ceftriaxone	16	256	0.25–256
AMC	256	256	0.25–256
Amoxicillin	256	256	0.25–256
Gentamicin	256	256	0.25–256
Tetracycline	256	256	0.25–256
Nalidixic acid	>256	>256	1–256

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AMC, amoxicillin/clavulanic acid; MIC, minimum inhibitory concentration; MIC<sub>50/90</sub>,

MIC for 50% and 90% of the organisms, respectively.

**Table 2**

Presence of plasmid-mediated quinolone resistance genes

Lab ID	Strain	Specimen	Source	<i>qnrA</i>	<i>qnrB</i>	<i>qnrS</i>	<i>qnrD</i>	<i>aac(6')-Ib-cr</i>	<i>qepA</i>
U2	<i>Proteus mirabilis</i>	Ear	In	–	–	–	–	+	–
U5	<i>P. mirabilis</i>	Wound	In	+	–	–	–	+	–
U7	<i>Klebsiella pneumoniae</i>	Aspirate	In	–	–	–	–	+	–
U10	<i>K. pneumoniae</i>	Wound	In	–	–	–	–	+	–
U17	<i>Pseudomonas oryzihabitans</i>	Wound	In	–	–	–	–	+	–
U20	<i>P. oryzihabitans</i>	Wound	In	–	–	–	–	–	+
U25	<i>P. oryzihabitans</i>	Wound	In	–	–	–	–	+	–
U33	<i>P. mirabilis</i>	Sputum	Out	–	–	–	–	+	–
U38	<i>P. mirabilis</i>	Wound	Out	–	–	–	–	+	–
U43	<i>K. pneumoniae</i>	Aspirate	In	–	–	–	–	+	–
U46	<i>Escherichia coli</i>	Wound	Out	–	–	–	–	–	+
U58	<i>P. mirabilis</i>	Wound	In	–	–	–	+	+	–
U59	<i>P. mirabilis</i>	Wound	In	–	–	–	+	+	–
U62	<i>P. oryzihabitans</i>	Urine	Un	+	–	–	–	+	–
U64	<i>Pseudomonas luteola</i>	Blood culture	In	–	–	–	–	+	–
U65	<i>E. coli</i>	Sputum	In	–	–	–	–	+	–
U72	<i>E. coli</i>	Wound	In	–	–	–	–	+	–
U73	<i>Morganella morganii</i>	Catheter tip	Out	–	–	–	–	+	–

U75	<i>K. pneumoniae</i>	Wound	In	-	-	-	-	+	-
U76	<i>Pseudomonas aeruginosa</i>	Wound	In	-	-	-	-	+	-
U77	<i>P. aeruginosa</i>	Wound	In	-	-	-	-	+	-
U79	<i>P. mirabilis</i>	Wound	In	-	-	-	-	+	-
U80	<i>Serratia odorifera</i>	Wound	In	-	+	-	-	+	-
U86	<i>M. morgani</i>	Wound	In	-	-	-	-	-	+
L7	<i>K. pneumoniae</i>	Urine	In	-	-	-	-	-	+
L8	<i>K. pneumoniae</i>	Urine	In	-	-	-	-	+	-
I5	<i>E. coli</i>	Urine	In	+	-	-	-	-	-
I6	<i>E. coli</i>	Sputum	In	+	-	-	-	-	-
I8	<i>E. coli</i>	CSF	In	-	-	-	-	-	+
I9	<i>E. coli</i>	Blood culture	Out	-	+	-	-	+	-
I11	<i>E. coli</i>	VP shunt	In	+	-	-	-	-	-
SGj	<i>P. aeruginosa</i>	Ear	Un	-	-	-	+	-	-
SGm	<i>Stenotrophomonas maltophilia</i>	Ear	Un	-	+	-	-	-	-

CSF, cerebrospinal fluid; VP, ventriculoperitoneal; In, inpatient; Out, outpatient; Un, unknown; +, positive; -, negative.

**Table 3**Carriage of  $\beta$ -lactamase ( $\beta$ L) genes

Species	N	Phenotypic detection [n (%)]		PCR-positive for $\beta$ L genes [n (%)]			
		$\beta$ L +ve <sup>a</sup>	ESBL +ve <sup>b</sup>	TEM	SHV	OXA	CTX-M
<i>Klebsiella pneumoniae</i>	63	54 (85.7)	8 (12.7)	47 (74.6)	9 (14.3)	1 (1.6)	4 (6.3)
<i>Escherichia coli</i>	28	20 (71.4)	7 (25.0)	24 (85.7)	10 (35.7)	5 (17.9)	9 (32.1)
<i>Pseudomonas aeruginosa</i>	13	9 (69.2)	1 (7.7)	12 (92.3)	4 (30.8)	2 (15.4)	3 (23.1)
<i>Proteus mirabilis</i>	11	9 (81.8)	3 (27.3)	10 (90.9)	3 (27.3)	3 (27.3)	3 (27.3)
<i>Pseudomonas oryzae</i>	6	6 (100)	2 (33.3)	6 (100)	3 (50)	0 (0)	1 (16.7)
<i>Burkholderia cepacia</i>	2	2 (100)	1 (50)	2 (100)	1 (50)	0 (0)	1 (50)
<i>Aeromonas hydrophila</i>	1	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (100)
<i>Enterobacter cloacae</i>	2	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)	0 (100)
<i>Morganella morganii</i>	3	3 (100)	2 (66.7)	2 (66.7)	1 (33.3)	1 (33.3)	1 (33.3)
<i>Pseudomonas luteola</i>	1	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
<i>Serratia odorifera</i>	1	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)
<i>Stenotrophomonas maltophilia</i>	2	2 (100)	0 (0)	2 (100)	1 (50)	1 (50)	1 (50)
<i>Citrobacter freundii</i>	1	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)

ESBL, extended-spectrum  $\beta$ -lactamase.<sup>a</sup> Positive by the nitrocefin test.<sup>b</sup> Positive for ESBL production by the double-disk diffusion text.