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ORIGINAL PAPER

Occurrence of extended-spectrum β-lactamases in *Escherichia coli* isolated from piggery farms in Imo State, Nigeria

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Abstract A total of 600 *Escherichia coli* isolates recovered from pig wastes from three Senatorial Zones of Imo State, Nigeria, were tested for production of extended-spectrum β-lactamases (ESBLs) by the double-disk (DD) potentiation method. Of the numbers of isolates made, 190 (32%) were positive for the ESBLs production. Results of testing 190 positive isolates for ESBL production by several recommended methods were as follows (percentage detection in parentheses): DD method with aztreonam (91), ceftazidime (82), ceftriaxone (84), or cefpodoxime (94); broth microdilution method with ceftazidime (77) or cefotaxime (96) alone or in combination with clavulanate; and the standard disk diffusion method with new breakpoints and standard concentrations of aztreonam (69), ceftazidime (81), ceftriaxone (77), or cefpodoxime (98) or a novel concentration (5 μg) of ceftazidime (84). These data indicate that ESBLs occur at a relatively high incidence in our piggery farms and that the standard disks diffusion method with cefpodoxime and the DD method with several β-lactams are practical and cost-effective methods for detecting ESBL-producing isolates of *E. coli.*

Keywords β-Lactamases · *Escherichia coli* · Pig wastes · ESBL · *Enterobateriaceae* · DD test

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Introduction

In recent years, bacterial resistance to β-lactam antibiotics has risen dramatically (Katayama et al. 2004). Contributing to this increase has been the spread of extended-spectrum β-lactamases (ESBLs), enzymes that hydrolyze the expanded-spectrum cephalosporins, like ceftazidime and cefotaxime, and/or the monobactam aztreonam (Davies et al. 2008). Resistance to extended-spectrum cephalosporins (ESCs) has emerged worldwide since 1988. By 2004, 43 countries had reported this public health problem (Arlet et al. 2006).

Unfortunately, the resistance to expanded-spectrum cephalosporins of many of these strains is not detected by routine susceptibility testing methods that follow current National Committee for Clinical Laboratory Standards (NCCLS) breakpoints (Brinas et al. 2005). Standard disk diffusion testing of cefpodoxime appears to be a promising method for screening *Klebsiella pneumoniae* and *Escherichia coli* (Thomson 2001), but this drug is not routinely tested in many laboratories. A 5–μg ceftazidime disk has recently been proposed for use to discriminate between ESBL-producing and non-ESBL-producing strains of *E. coli* and *K. pneumoniae* (Carter et al. 2000). In addition, a clavulanate double-disk potentiation procedure has improved detection (Datta et al. 2004).

Few studies have compared the abilities of different laboratory methods to detect ESBL-producing organisms among members of the family *Enterobacteriaceae.* No study has been conducted to detect the ESBL-producing members of this family in farm animals. Therefore, this work was designed to determine the occurrence of ESBLs in *E. coli* isolated from fresh pig wastes in farms in Imo State, Nigeria. Determination of true prevalence would require testing all isolates with labor-intensive procedures

including isoelectric focusing (IEF). Therefore, isolates were screened for ESBLs with the double-disk (DD) method. Isolates that were DD-positive were examined further for ESBL production with definitive procedures. The abilities of different methods to detect ESBLs in these organisms were also compared.

Materials and methods

A total of 600 *E. coli* isolates were recovered from fresh pig wastes got from the three Senatorial Zones of Imo State, Nigeria (200 isolates each from Owerri, Okigwe and Orlu zone). Mueller_Hinton agar was inoculated from a blood agar plate culture grown overnight, as recommended for the standard disk diffusion test (NCCLS 2004). Disks containing the standard 30 μg of aztreonam, ceftazidime and ceftriaxone were placed 15 mm (edge to edge) from an amoxicillin–clavulanic acid disk (20 and 10 μg, respectively). They were also tested by the DD method with cefpodoxime (10 μg). Inoculated media were incubated overnight at 35°C. An enhanced zone of inhibition between any one of the β-lactam disks and the disk containing clavulanic acid was interpreted as evidence for the presence of an ESBL. Isolates with this pattern were recorded as DD-positive. In addition to recording enhanced zones of inhibition from the DD plate, standard zone diameters were determined by measuring the zone directly, or, if necessary, by measuring the radius and multiplying by two.

MICs of ceftazidine and cefotaxime with and without 2 μg clavulanic acid per ml (fixed concentration) were determined by the standard microdilution broth method (NCCLS 2004). *Escherichia coli* ATCC 25922 was used as control. Some isolates were also tested by the standard disk diffusion method with disks containing 5 μg of ceftazidine (CAS-5), which were prepared by adding ceftazidine to blank disks. Zone diameters were recorded.

Results

Of the 600 *E. coli* isolates tested by the DD method, enhanced zones of inhibition were observed with 190 isolates (Table 1). Of the 190 ESBL-producing isolates tested by the DD method, the following numbers of isolates gave clavulanate enhanced zones with the β-lactam disk indicated: aztreonam, 173 (91%); ceftazidine, 155 (82%); ceftriaxone, 160 (84%); and cefpodoxime, 179 (94%) (Table 2). ESBLs were indicated in the tests with all isolates with two disks, aztreonam and ceftriaxone or aztreonam and cefpodoxime.

Some investigators have used a fourfold difference in MICs between a β-lactam tested alone and in combination with an inhibitor to classify organisms putative ESBL-producing isolates (Jett et al. 1995). Therefore, the MICs of ceftazidine and cefotaxime alone and in combination with 2 μg of clavulanate per ml were determined for the 190 ESBL-producing isolates. All MICs for one or both of the β-lactams with clavulanic acid were at least fourfold less than the MICs of the same agent without clavulanic acid (Table 2).

Table 1 Numbers of isolates that produced extended-spectrum β-lactamases (ESBLs)

Location	No. of isolates tested	ESBL-producing isolates		
		No.	%(ESBL)[a]	% (Loc.)[b]
Owerri	200	78	41	39
Okigwe	200	46	24	23
Orlu	200	66	35	33
Total	600	190	100	

[a] Percentage of all isolates that produced ESBLs

[b] Percentage of isolates within each location that produced ESBLs

Guidelines for disk susceptibility tests state that some ESBLs may confer high-level resistance to certain β-lactams and may be detected by disk diffusion testing, but that other isolates may display a lower level of resistance (NCCLS 2004). The latter isolates may not reach standard breakpoints for resistance, and therefore, the following breakpoints were recommended for testing ESBL-producing *E. coli* and *Klebsiella* species: aztreonam, <28 mm; ceftazidime, <23 mm; ceftriaxone, <26 mm; and cefpodoxime, <23 mm (NCCLS 2004). With these breakpoints, the following numbers of the 190 ESBL-producing isolates were identified: aztreonam, 131 (69%); ceftazidime, 154 (81%); ceftriaxone, 146 (77%); and cefpodoxime, 186 (98%) (Table 2).

Jacoby and Han (1996) have proposed that a CAZ-5 disk be used to detect ESBL-producing members of the family *Enterobacteriaceae*. The members of 190 ESBL-producing isolates with zones of inhibition of <18 and <21 mm were 155 (82%) and 160 (84%), respectively.

Discussion

A number of studies have assessed the occurrence of ESBLs among members of the family *Enterobacteriaceae* (Singhal et al. 2004; Rahal et al. 2002; Navon-Venezia et al. 2003; Spanu et al. 2002; De Champs et al. 2000; Perilli et al. 2002). Most, however, have focused primarily on *E. coli* and *K. pneumoniae* (Lautenbach et al. 2001; Lin et al. 2003; Du et al. 2002; Kim et al. 2002; Ho et al. 2002), and many involved hospitals (Singh et al. 2002; Aubert et al. 2005; Shiroto et al. 2005). In one Italian

Table 2 Numbers of ESBL-producing isolates that were detected by different method

Locations (No. of isolates)	No. of ESBL-producing isolates detected by:											
	DD[a]				MD[b]		SDD[c]				CAZ-5[d]	
	ATM	CAZ	CRO	CPD	CAZ	CTX	ATM	CAZ	CRO	CPD	<18	<21
Owerri (78)	75	64	78	75	71	78	69	69	75	75	64	69
Okigwe (46)	45	38	42	38	38	38	42	45	45	45	45	45
Orlu (66)	53	53	40	66	38	66	20	40	26	66	46	46
Total (190)	173	155	160	179	147	182	131	154	146	186	155	160

[a] Double-disk (DD) potentiation method. Disks contained standard concentrations of aztreonam (ATM), ceftazidime (CAZ), ceftriaxone (CRO), and cefpodoxime (CPD)

[b] Microdilution method (MD). Wells contained ceftazidime (CAZ) and cefotaxime (CTX) with and without clavulanate. Differences between MICs with and without inhibitor were at least fourfold

[c] Standard disk diffusion method. Disks contained standard concentrations of β-lactams, and zone diameters were with breakpoints for detecting ESBL-producing isolates

[d] Disk diffusion method. Each disk contained 5 μg of ceftazidime (CAZ-5). Inhibition zone diameters of <18 and <21 mm were measured

study, a total of 8,015 strains were isolated from ten Italian laboratories and 509 (6.3%) of these were designated ESBL producers from the results of a double-disk synergy test (Bonfiglio et al. 2007). In this study, disks were placed 30 mm apart (center to center) and results were confirmed by more definitive testing. The detection of 190 ESBL-producing isolates from 600 isolates during the present study represents an occurrence around twenty one times higher than that reported in the earlier Italian study. Clearly, the relatively high occurrence of ESBL-producing organisms underscores the importance of testing *E. coli* and thus, all *Enterobacteriaceae* from pig wastes for this characteristic to reduce the transfer of the characteristic genes to clinical strains.

A number of methods for the detection of ESBL production among the Enterobacteriaceae are currently under development (Wiegand et al. 2007; De Gheldre et al. 2003; Komatsu et al. 2003). The reliability of these methods and the DD test has not yet been thoroughly evaluated. Although the current study did not assess the ability of a variety of tests to detect ESBL production in all the 190 *E. coli* isolates studied by the DD test, results obtained with DD-positive isolates did reveal differences and limitations.

In this study, the inability of standard interpretive criteria applied to results of disk diffusion tests to detect ESBL production in *E. coli* has been amply demonstrated. Although the percentages of isolates detected with the new interpretive criteria were higher, the tests with cefpodoxime were the highest (98%). These results agree with those reported previously by Thomson (2001) who showed that cefpodoxime was the most reliable for detecting ESBL production among *Enterobacteriaceae*. Lautenbach et al. (2001) tested *K. pneumoniae* and *E. coli* strains with the CAZ-5 disk and reported that a zone of inhibition of <18 or <21 mm could be used to detect ESBL production in these two species. In the present study, this method was used to detect ESBL-producing isolates.

The ability of other tests to detect ESBL production in the isolates showed that the inclusion of 2 μg of clavulanate per ml in MIC tests with cefotaxime indicated the presence of ESBLs in the majority of the isolates. It was also shown that ceftazidime was not as reliable an indicator of ESBL production in these tests as cefotaxime. Overall, the DD test was the most practical and least expensive test to perform in this study. Unlike other methods, the DD test utilizes more than one test agent, and this combination approach allows maximal detection of ESBL production among all the species of *Enterobacteriaceae.*

In summary, 31.7% of *E. coli* isolated from piggery farms in Imo State, Nigeria produced ESBLs. This finding is significant, because many of these isolates did not appear to be resistant to expanded-spectrum cephalosporins in routine susceptibility tests. The optimal laboratory test for detection of ESBL production has not been identified. However, results of this study indicate that the DD test can be very useful, and disk diffusion tests with cefpodoxime warrant further study.

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