OXA-Type-Beta-lactamases Among Extended-Spectrum-Cephalosporin Non-susceptible Pseudomonas Aeruginosa Isolates Collected from a Large Teaching Hospital in Cairo

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ABSTRACT:

Background: *Pseudomonas aeruginosa (P. aeruginosa)* is a leading cause of nosocomial infections, including pneumonia, urinary tract infections, and bacteremia and it is highly resistant to many antibiotics by many different mechanisms, including ß-lactamases' production, specially class D OXA-ß-lactamases.

Aim of work: The aim of this study was to determine the prevalence of OXA- ß-lactamases among extended-spectrum-cephalosporin (ESC) non- susceptible *P. aeruginosa* isolates.

Materials and Methods: Forty four ESC non- susceptible *P. aeruginosa* isolates were collected from a large teaching hospital , they were considered ESC non- susceptible if they were resistant or intermediately sensitive to one or more of ESC indicator antibiotics (ceftazidime, cefotaxime, ceftriaxone & cefepime) and aztreonam. They were examined for the presence of genes coding for OXA-group I, II & III using PCR based techniques and all isolates harboring OXA-group I genes were subsequently subjected to digestion by a group of restriction endonucleases to determine the OXA-group I type derivative of the isolates.

Results: Twenty-five isolates (56.8%) were found to harbour OXA-type enzymes. Seventeen were positive for OXA-group I and 4 were positive for OXA-group III. In addition, four isolates (16%) harboured two different OXA-type enzymes. Almost all isolates of OXA-group I were non-susceptible to ceftazidime and susceptible to cefepime while all OXA-group III isolates were susceptible to ceftazidime and most of them were non-susceptible to cefepime.

Key words: Extended-spectrum-cephalosporin non- susceptible *P. aeruginosa*, OXA- ß-lactamases, PCR & restriction endonucleases.

INTRODUCTION:

Pseudomonas aeruginosa is one of the bacterial species most frequently responsible for nosocomial infections and is notably resistant to many antibiotics, including β-lactams. *P. aeruginosa* is naturally susceptible to ureido- and caboxypenicillins, as well as to some extended-spectrum cephalosporins (ESCs) [(1)].

Resistance to extended-spectrum β-lactams in *P. aeruginosa* is associated mostly with the overproduction of chromosomal Amp C cephalosporinase, or with non-enzymatic mechanisms such as drug efflux or outer membrane impermeability [(2 & 3)].

Recently however, several class A, B and D extended-spectrum β-lactamases (ESBLs) have been reported in *P. aeruginosa* [(4)]. Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum cephalosporins (ESCs), such as cefotaxime, ceftriaxone, ceftazidime, and the monobactam aztreonam [(5)]. Such enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* but have been detected at low frequency in *Pseudomonas aeruginosa* [(6, 7, 8, 9 &10)].

Various Ambler's class D ESBLs, such as OXA-type ESBLs have been identified and encountered most commonly in *P. aeruginosa* [(11, 12 &13)]. OXA-type -lactamases have extreme sequence variation; with the identities varying from 16 to 99% between individual enzymes [(14)]. They fall into five groups (groups I–V). The OXA group I includes OXA-5, 7, 10, 13 and its extended spectrum derivatives (OXA-11, 14, 16, 17, 19). Group II includes OXA-2, 3, 15 and 20. Group III includes OXA-1, 4, 30 and 31. Whereas, group IV and group V include only OXA-9 and LCR-1 respectively. OXA-18 does not belong to any of these groups and it has low amino acid sequence homology with other groups [(15)]. Most OXA-type ESBLs detected belong to OXA-I or OXA-II derivatives [(16)].

Various tests have been developed to detect ESBLs. Most of the methods aim to detect ESBLs, of chromosomal or non-chromosomal origin, in Klebsiellae and other *Enterobacteriaceae*, e.g., *E. coli* and *Proteus mirabilis* [(17, 18, 19, 20 & 21)]. Detection of ESBLs in species with inducible chromosomal -lactamases, such as *Enterobacter* spp., proves to be a difficult task. In *P. aeruginosa* detection presents further difficulties; not only because it may have an inducible AmpC enzyme but also

because it has a much greater degree of impermeability than *Enterobacteriaceae*, as well as efflux-mediated resistance [5, 22, 23 & 24]. Moreover, most OXA-type ESBLs are only weakly inhibited by clavulanic acid and cannot be identified at routine microbiological laboratories; due to lack of standard phenotypic detection methods. Thus, the epidemiology of OXA-type ESBLs is not well known [12].

This study was performed to investigate the prevalence of OXA-β-lactamases and their extended-spectrum derivatives among nosocomial isolates of ESCs non-susceptible *P. aeruginosa.*

MATERIALS AND METHODS:

Bacterial isolates:

Forty four nosocomial ESC non-susceptible *P. aeruginosa* isolates were collected from a large teaching university hospital in the period between December 2006 and June 2007. The isolates were identified as *P. aeruginosa* by the conventional methods. The disc diffusion method was used to determine ESC non-susceptibility [25]. *P. aeruginosa* isolates were considered as ESC non-susceptible if they were intermediately sensitive or resistant to at least one of the following antibiotics; ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), cefepime (FEP) and aztreonam(ATM) [26].

The isolates were; 5 isolates from sputum (11.4%), 10 isolates from urine (22.7%), 19 isolates form wound (43.2%), 7 isolates from blood (15.9%), one isolate form endotracheal tube (2.3%) and two isolates form pleural fluid (4.5%). All isolates were stored at -20 °C in glycerol broth until tested for.

Molecular tests: All ESC non-susceptible *P. aeruginosa* isolates were exposed to PCR test to detect presence of β-lactamase OXA genes of OXA group I, II & III.

OXA-group I isolates were further subjected to restriction endonuclease (RE) analysis to determine the OXA-group I derivative type of these isolates.

PCR for detection of OXA ß-lactamase genes:

DNA extraction: The total DNA was extracted using the QIamp DNA Mini Kit, (Qiagen Corp., Valencia, CA, USA) according to manufacturer's protocol described by phenol–chloroform treatment followed by ethanol precipitation. The DNA pellet was re-suspended in 100 µL of double-distilled deionised water and was stored at -20 °C until PCR experiments were preformed.

Oligonucleotide primers: The following oligonucleotide primers were used to amplify certain genes specific to different OXA groups:

I. OXA-10F primer (TCT TTC GAG TAC GGC ATT AGC) and OXA-10R primer (CCA ATG ATG CCC TCA CTT TCC) which amplify a specific gene for OXA 10 (group I) with an amplicon size 760 bp [26].

II. OXA-2F primer (GCC AAA GGC ACG ATA GTT GT) and OXA-2R primer (GCG TCC GAG TTG ACT GCC GG) which amplify a specific gene for OXA 2 (group II) with an amplicon size 700 bp [27].

III. OXA-1F primer (AGC CGT TAA AAT TAA GCC C) and OXA-1R primer (CTT GAT TGA AGG GTT GGG CG) which amplify a specific gene for OXA 1 (group III) with an amplicon size 908 bp [28].

Amplification of OXA ß-lactamase genes: Appropriate precautions were taken to avoid cross contamination during sample preparation and performance of the PCR. PCR was carried out with 2µL of the template DNA, 0.5 µM of each primer, 10mM Tris–HCl, 100 µM dNTP and 1.5 U of Taq DNA polymerase (Ampli Taq Gold; Perkin-Elmer, Norwalk, CT, USA) in a total volume of 50 µL. Thermal cycling was initiated with a first activation step of 10 min at 95°C, followed by 40 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes using Perkin-Elmer Gene Amp PCR system 9600 thermal cycler. Samples were held at 4°C until the products could be analyzed.

Detection of the PCR products: PCR products were separated on agarose gels 1% using loading buffer (0.25% bromophenol blue, 0.25% xylene cyanolFF, 15% Ficol in water) and 100-base pair DNA ladder (Gibco-BRL) as molecular weight marker ladder. OXA-10 PCR products were then subjected to RE analysis with *Pvu II*, *HaeIII* and *AspLE I* [15, 29 &30].

RE analysis of OXA-group I PCR products:

RE analysis was performed on the OXA-group I amplified products as described by Vahaboglu *et. al* [29]. Amplified 760-bp products

were precipitated by sodium acetate (1:10 volume, 3 M) [pH 5.5])-cold ethanol (2 volumes) for 30 min at -70°C and centrifuged for 10 min at 12,000 rpm. The pellets were washed once with pure ethanol and once with 70% ethanol, air dried, and resuspended in 50 µl of SE buffer suitable for each enzyme.

The SE buffers: (SibEnzyme US LLC, West Roxbury, USA)

- For *Pvu II* enzyme: 10 mM Tris-HCl (pH 7.6 at 25°C); 10 mM $MgCl_2$; 50 mM NaCl; 1 mM DTT and BSA.
- For *Hae III* enzyme: 10 mM Tris-HCl (pH 7.6 at 25°C); 10 mM $MgCl_2$; 50 mM NaCl; 1 mM DTT.
- - For *AspLE I* enzyme: 50 mM Tris-HCl (pH 7.6 at 25°C); 10 mM $MgCl_2$; 100 mM NaCl; 1 mM DTT.

Purified products were digested with 10 U of *Pvu II*, *Hae III* or *AspLE I* (SibEnzyme US LLC, West Roxbury, USA) for 3 h at 37°C in 20-ml volumes. The reaction was stopped at 3 hours by addition of 5.0 µ of stop mixture (0.25% bromophenol, 0.25% xylene cyanole, 25.0% Ficoll 400). The digested products of restriction were separated on agarose gels 2% using loading buffer and 100-base pair DNA marker ladder.

Interpretation of the RE analysis results:

- *Pvu II*: differentiates OXA group I derivatives OXA-10, OXA-11, OXA-14, OXA-16 & OXA-17 (cleaved into two fragments 312 and 448 bp) from derivatives OXA-7 & OXA-13 (not digested) [15, 26, 27 & 29].
- *Hae III*: differentiates OXA group I derivatives OXA-10 & OXA-17 (cleaved into three fragments 198, 240 and 322 bp) from derivatives OXA-11, OXA-14 & OXA-16 (cleaved into fragments 198 and 562 bp) [15, 26, 27 & 29].
- *AspLE I*: differentiates OXA group I derivative OXA-10 (cleaved into two fragments 120 and 640 bp) from derivative OXA-17(cleaved into three fragments 120, 66 and 574 bp) [15, 26, 27 & 29].

RESULTS:

Forty-four *P. aeruginosa* isolates found to be intermediately sensitive or resistant (non-susceptible) to at least one of ESCs (ceftazidime, cefotaxime, ceftriaxone & cefepime) or aztreonam were included in this study.

PCR experiments using primers specific for three groups of the OXA-type β-lactamase genes were performed. Twenty-five out of the forty-four isolates (56.8%) were found to harbour OXA-type enzymes. The majority of the strains were positive for OXA group I, followed by group III. In addition, four isolates (16%) harboured two different OXA-type enzymes (Table 1) (Figures 1, 2 & 3).

A group of restriction endonuclease enzymes *(Pvu II*, *Hae III* & *AspLE I)* were used to digest the amplification products of OXA group I; to determine the OXA – I members and their extended spectrum derivatives. Eighteen isolates (40.9%) were OXA 10 derivative isolates and 2 (4.5%) were OXA 17 derivative isolates. The OXA – I type in the three isolates harbouring two different OXA-type enzymes was OXA – 10 (Table 1) (Figure 4).

Meanwhile isolates harboring OXA-group I genes were mostly non-susceptible to ceftazidime, cefotaxime, ceftriaxone & aztreonam; they were mostly susceptible to cefepime. On the other hand most of isolates harboring OXA-group III genes were non-susceptible to ceftriaxone & cefepime, marginally non-susceptible to cefotaxime & aztreonam but all of the isolates were susceptible to ceftazidime (Table 2).

Table 1: Prevalence of OXA-type-ß-lactamases among 44 P. aeruginosa isolates

OXA group	No. (%) of isolates
Group I - **OXA 10** - **OXA 17 (ESBL)**	20 (45.5%) 18 2
Group II	3 (4.5%)
Group III	6 (13.6 %)
Combined **Group I (OXA 10) & group II**	2
Group I (OXA 10) & group III	1
Group II & group III	1

Table 2: Comparison between antibiotic susceptibility of OXA-group I and OXA-group III isolates.

Antibiotics	OXA-group I (Total no. = 19)		OXA-group III (Total no. = 5)	
	Susceptible	Non-susceptible	Susceptible	Non-susceptible
CAZ	1 (5.3%)	18 (94.7%)	5 (100%)	0 (0%)
CTX	4 (21%)	15 (79%)	2 (40%)	3 (60%)
CRO	7 (36.8%)	12 (63.2%)	1 (20%)	4 (80%)
FEP	12 (63.2%)	7 (36.8%)	1 (20%)	4 (80%)
ATM	7 (36.8%)	12 (63.2%)	3 (60%)	2 (40%)

-There was an isolate containing both OXA-group I and OXA-group III genes and it was omitted from both groups.
-Abbreviations: CAZ: ceftazidime, **CTX:** cefotaxime, **CRO:** ceftriaxone, **FEP:** cefepime, **ATM:** aztreonam.

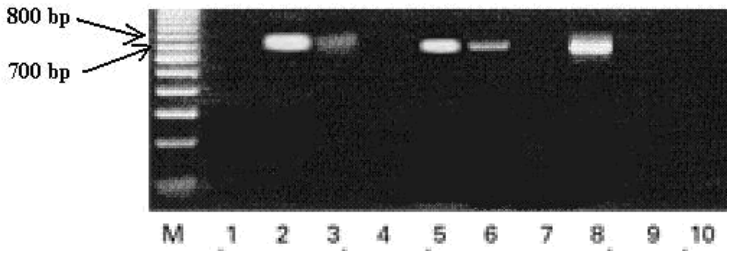

Figure 1: Agarose gel electrophoresis of the OXA 10 gene specific for OXA group I ESBLs; with amplicon size 760 bp showed that:

- Lane M: 100 bp DNA marker.
- Lane 4: negative control.
- Lanes 2, 3, 5, 6 & 8: positive cases of OXA group I ESBLs.
- Lanes 1, 7, 9 & 10: negative cases of OXA group I ESBLs.

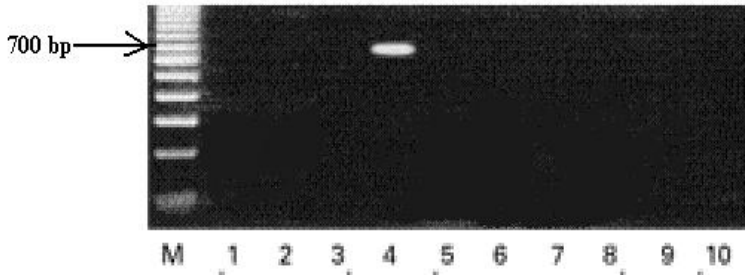

Figure 2: Agarose gel electrophoresis of the OXA 2 gene specific for OXA group II ESBLs; with amplicon size 700 bp showed that:

- Lane M: 100 bp DNA marker.
- Lane 5: negative control.
- Lane 4: a positive case of OXA group II ESBLs.
- Lanes 1,2, 3, 6, 7, 8, 9 & 10: negative cases of of OXA group II ESBLs.

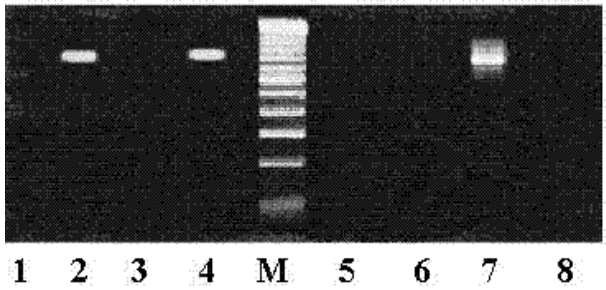

Figure 3: Agarose gel electrophoresis of the OXA 1 gene specific for OXA group III ESBLs; with amplicon size 908 bp showed that:

- Lane M: 100 bp DNA marker.
- Lane 5: negative control.
- Lane 2 & 4: positive cases of OXA group III ESBLs.
- Lanes 1,3 , 6, 7 & 8: negative cases of OXA group III ESBLs.

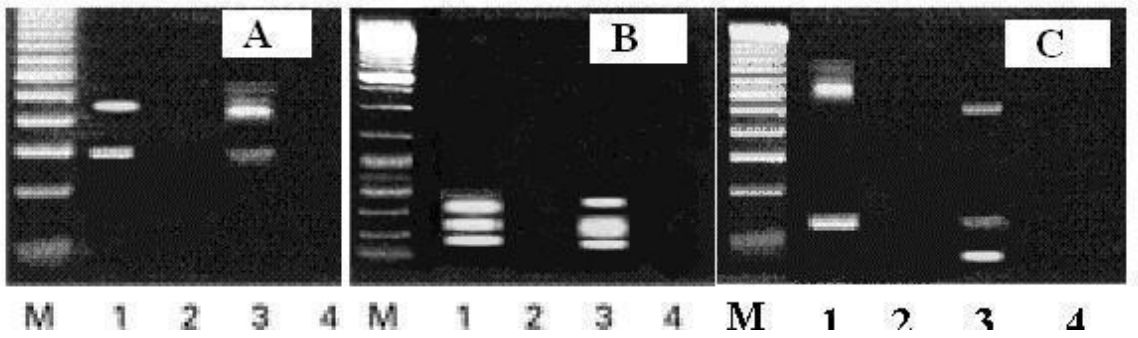

Figure 4: Agarose gel electrophoresis of amplification products of 2 isolates harbouring OXA group I genes after digestion by a group of restriction endonucleases showed that:

In fig. 4 A the 2 strains were digested by *Pvu II* restriction endonuclease into 2 bands 312 bp & 448 bp indicating that the 2 strains were either OXA-10, OXA-11, OXA-14, OXA-16 or OXA-17 derivatives.

In fig. 4 B the same 2 strains were digested by *Hae III* restriction endonuclease into 3 bands 198 bp, 240 bp & 322 bp indicating that the 2 strains were either OXA-10 or OXA-17 derivatives.

In fig. 4 C one strain was digested by *AspLE I* restriction endonuclease into 2 bands; 120 bp & 640 bp (lane 1), indicating that the strain was OXA-10 derivative. Whereas the other strain was cleaved into 3 bands; 120 bp, 66 bp & 574 bp (lane 3), indicating that the strain was OXA-17 derivative .

DISCUSSION:

Historically, the first characterized Class D -lactamases were also referred to as oxacillinases because they commonly hydrolyze the isoxazolylpenicillin oxacillin much faster than classical penicillins; i.e. benzylpenicillin. The designation, OXA, of Class D -lactamases, thus refers to their preferred penicillin substrate [(27)]. Currently, 121 variants of Class D -lactamases have been identified on the protein level and 45 of these exhibit carbapenem-hydrolyzing activities, in contrast to other Class D -lactamases [(31)].

The majority of the clinically relevant Class D β-lactamases are acquired enzymes; whose genes are residents on plasmids in gram-negative pathogens such as pseudomonads, acinetobacters and members of the *Enterobacteriaceae,* and contained within integrons or transposons. A number of extended-spectrum oxacillinases have been identified as variants of their restricted spectrum counterparts e.g., mutants of OXA-10 and OXA-2 [(32)].

ESBLs are being increasingly reported in *P. aeruginosa* worldwide [(5, 6, 8, 9, 10 & 24)]; among which OXA-type ESBLs have been encountered most commonly [(3, 11, 13, 16, &)].

In the present study, 44 *P. aeruginosa* strains, intermediately susceptible or resistant to at least one of the ESCs (ceftazidime,

cefotaxime, ceftriaxone & cefepime) and aztreonam, were examined for the presence of OXA-type β-lactamases; namely OXA-I, -II and –III by PCR. Twenty-five (56.8%) strains were found to harbour OXA-type enzymes. Seventeen (36.8%), 6 (13.6%) and 3 (6.8%) out of the 44 studied strains gave positive results with specific primers for OXA – I, - II and – III enzymes respectively. Moreover, 4 (9%) out of the 44 studied strains harboured two different OXA-group enzymes.

These results are comparable to the results of others [15, 16, 33 & 34]; where OXA-I was the most commonly detected among the OXA-type enzymes.

It has been reported that most OXA-type enzymes belong to group – I and – II derivatives [15 & 16]. However, the present study identified OXA – III production more than OXA – II. This is supported by the work of Lee *et al.* [7] where 13.5% of the studied *P. aeruginosa* strains possessed group I enzymes, 6.3% possessed group III enzymes and only 2.3% harboured group II enzymes.

Most Class D extended-spectrum enzymes are OXA – I derivatives; derived from OXA -10 by single or double base mutations. Simple methods for screening for these extended-spectrum derivatives, such as double-disk synergy test, can not be used since OXA -10 derived enzymes are poorly inhibited by β-lactamase inhibitors [29]. Currently, cloning and sequence determination are being used to distinguish OXA-10 extended derivatives from the rest of group I [32]. However, these methods are cumbersome and expensive, therefore poorly suited for epidemiological studies. An alternative method has been based on the analysis of the PCR products by digestion with restriction endonuclease enzymes chosen to detect restriction site changes generated at point mutations [15]. In *P. aeruginosa*, it has been limited to the detection of OXA-10 derived ESBLs using three different restriction enzymes; namely *PvuII, HaeIII* and *HhaI* [15 & 29].

In the present study, the PCR products positive for group I were subjected to digestion by *Pvu II, Hae III* and *AspLE I*. This method though highly specific [29], was rather time consuming and labour intensive because of the large number of PCRs and restriction digests needed to identify OXA-groups. *AspLE I* is an isoschizomer of the restriction enzyme *Hha I* having the same recognition sequence specificity [35], which was used instead of *HhaI*; owing to inavailability of the latter.

Digestion of the 760 bp OXA-10 derivatives amplification product of the 20 isolates by *Pvu II* produced two fragments; denoting that the alleles may be either OXA-10, 11, 14, 16 or 17. Digestion by *Hae III* cleaved the products into three fragments indicating that the alleles were either OXA-10 or OXA-17. Then digestion with *AspLE I* identified 18 (40.9% of the total strains) as OXA-10 producers and only 2 (4.5% of the total strains) as OXA-17 producers. In a study conducted in Korea; Lee *et al.* reported that the prevalence of OXA-10 was 13.1% and OXA-17 was 0.4 %. [7]. While Jing-Jou *et al.* reported that the prevalence of OXA-10 and OXA-17 in a university hospital in southern Taiwan was 0.6% and 14.3% respectively [33].

Classical OXA enzymes confer resistance to carboxypenicillins and ureidopenicillins but not to ceftazidime [7]. Among the OXA-10 derivatives, OXA-11, OXA-14 and OXA-16 confer a high level of resistance to ceftazidime and have a 157 Gly → Asp substitution; which may be critical to ceftazidime resistance. In contrast, OXA-17 has an Asn-73 → Ser substitution, which has minimal effects on ceftazidime [36].

Surprisingly, the present study revealed that 94.7% of OXA-I producers were non-susceptible to ceftazidime. A similar finding was reported by Vahaboglu *et al*, [29], where 66.6% of *P. aeruginosa* strains having an OXA-10 restriction pattern were found to be highly resistant to ceftazidime. This was explained by their further detection of a second enzyme; PER-1 type ESBL in all the ceftazidime resistant OXA-10 strains, known to confer high level of resistance to ceftazidime in *P. aeruginosa*. The present study did not attempt to search for such an enzyme, but the possibility of coexistence of other genes encoding drug resistance to ceftazidime was highly considered.

On the other hand, all OXA-III producers were susceptible to ceftazidime, whereas 80% of them were non-susceptible to cefepime. This agrees with different reports that state that OXA-III show a characteristically decreased susceptibility to cefepime but remain susceptible to ceftazidime [3 & 37].

In conclusion, OXA-type β-lactamases of diverse clonal origin, but not those derivatives with extended spectra of activity were found among nosocomial *P. aeruginosa* strains. Taking into account the threat of cross resistance potential and in vivo selection of ESBL, clinical efforts for early recognition of acquired -lactamase producing strains and rigorous infection control measures should be emphasized. Detection of Class D producing microorganisms poses an ongoing difficulty for scientists in clinical microbiology laboratories. Further investigations are needed in search for antibiotics which could function as potential markers of OXA-type β-lactamases in *P. aeruginosa*. Moreover, future development of novel beta-lactams resistant to hydrolysis by these versatile enzymes and the search for possible highly potent "second generation" beta-lactamase inhibitors is highly recommended.

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