Original article:

Current trends in multidrug-resistant AmpC beta-lactamase producing *Enterobacter cloacae* isolated from a tertiary care hospital

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Abstract:

Background: The emergence of AmpC beta-lactamase producing *Enterobacter cloacae* becomes a serious nosocomial menace due to wider resistance. The study aimed to know the existence of these superbugs in the hospital settings and to report the current trends in their antibiotic resistance. ***Methods:*** We chose a tertiary care pediatric hospital for this cross-sectional study and processed 27,000 clinical specimens for the isolation of *E. cloacae* using routine microbiological procedures. A total number of 96 *E. cloacae* isolates from various sources were screened for AmpC production with cefoxitin (30 µg) and confirmed by inhibitor based technique. The antibacterial drug resistance studied against various groups of antibiotics *in vitro*. ***Results :*** Boronic acid inhibitor based method revealed 63 (65.6%) pathogens as AmpC beta-lactamase producing *E. cloacae*. Most of the infected patients with AmpC producing *E. cloacae* were neonates (34; 54.0%) and infants (11; 17.5%). The primary source of AmpC producing *E. cloacae* was blood (43; 68.3%), and they were frequently distributed in the neonatal nursery unit (33; 52.4%) and medical ward (13; 20.6%). All of these bugs showed a high level of resistance (100%) against the co-amoxiclav and cephalosporin group.The organisms exhibited less resistance to levofloxacin, imipenem and colistin sulphate as 23 (36.5%), 20 (31.7%) and 17 (27.0%), respectively. ***Conclusion:*** The consistent emerging threat of Amp C harbouring *E. cloacae* could disseminate AmpC genes in other genera of the bacteria which lead to the therapeutic failure and leave the doctors with limited treatment options of levofloxacin, imipenem and colistin sulphate.

Keywords: *Enterobacter cloacae*; AmpC beta-lactamase; boronic acid confirmation; multi-drug resistance

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Introduction

Amp C beta-lactamases are clinically significant enzymes (cephalosporinases)encoded by the genes of various members of enterobacteria. Resistance against the extensive classes of antibiotics, particularly β-lactam drugs (ceftazidime, and ceftriaxone)is solely the over expression of chromosomal and plasmid-mediated AmpC beta-lactamases. Due to this multidrug resistance development against any of the infection caused by *Enterobacter aerogenes* and *Enterobacter cloacae*, get difficult to treat[1].

E.cloacae is a rod-shaped flagellated opportunistic

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pathogen found with different size ranges (0.3-0.6 x 0.8-2.0 µm)[2]. This facultative anaerobe is ubiquitous in terrestrial as well as the aquatic environment and also occurs as a commensal of humans and animals intestinal track[3]. *Enterobacter* species being Gram-negative bacteria have considered the fourth most common source of nosocomial infections[4]. Transmission route of this organism is mainly the fecal-oral route and also by the direct and indirect interaction with mucosal surfaces of infected individuals. *E. cloacae* cause several clinically significant and life-threatening infections (bacteremia, endocarditis, septic arthritis, and osteomyelitis) and have become a rapidly emerging clinical pathogen[5]. Different cytotoxins such as enterotoxins, hemolysins, and pore-forming toxins contribute to the major role in the pathogenicity of *E. cloacae*[3]. These infections are highly associated with immune-compromised patients[6]. *E. cloacae* show a high frequency of enzymatic resistance to broad-spectrum cephalosporins[7]. The development and emergence of multi-drug resistant pathogens are mainly due to the extensive and misuse of antibiotics[8]. AmpC beta-lactamases attribute in different from ESBLs (extended-spectrum beta-lactamases) for their potential to break down the cephamycins[9]. Resistance to a wide range of β-lactamase inhibitor (cefoxitin) represents them as clinically important enzymes. AmpC enzymes are associated with Class C as per ambler stratification, and in group 3 according to their functional properties[10]. *Enterobacter* species, in particular, *E. cloacae* and *E. aerogenes* are best treated by carbapenems[11]. Quinolones can also be an effective alternative[12]. The objectives of this study included the analysis of AmpC beta-lactamase production and current trends in multidrug resistance produced by *E. cloacae*.

Material and Methods

This prospective study held at the Children's Hospital, Lahorefor a period of six months, from June to November 2017. We processed 27,000 various clinical specimens of blood, cerebrospinal fluid (CSF), tracheal secretions, catheter tips, sputum, pus, and wound swabs. The specimens we reprocessed according to the conventional microbiological techniques and *E. cloacae* identified by the various biochemical and API 10S (bioMerieux). Only bacterial isolates of *E. cloacae* were included in the study while the rest of the cultures which showed no growth or other pathogenic growth were excluded from the study.

The selection criteria for the initial screening of AmpC beta-lactamase production was based on resistance to cefoxitin (30 µg) antibiotic disc. The cefoxitin disc placed on Muller Hinton agar plate, already inoculated and streaked with *E. cloacae.* AmpC beta-lactamase positivity in the screening test was evaluated by measuring the zone size of cefoxitin < 18 mm[13]. The further confirmation of AmpC β-lactamase was performed by the inhibitory based method by the use of 400 µg boronic acid on 30 µg cefoxitin antibiotic disc. The preparation of the solution included 120 mg of phenylboronic acid, 3 ml of dimethyl sulfoxide (DMSO) and addition of 3ml of sterile deionized water. Approximately, 20 µl stock solution of boronic acid pipetted on one of the cefoxitin disc placed on already streaked *E. cloacae* on the Muller Hinton agar plate. The zones of inhibition with and without the cefoxitin compared and an inhibition zone of ≥ 5mm was taken as a positive confirmatory test[14].An inhibitor based method with an enhanced zone size has been showing in Figure 1.

The antimicrobial resistance observed by the use of the Kirby Bauer technique using McFarland's (0.5) standard. Different drug groups such as cephalosporins (cefixime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime), co-amoxiclav, fluoroquinolone (ciprofloxacin, levofloxacin, moxifloxacin), aminoglycoside (gentamicin, tobramycin, amikacin), carbapenem (imipenem, meropenem), piperacillin-tazobactam, cefoperazone-sulbactam, chloramphenicol, colistin sulphate and co-trimoxazole used to see the resistance in Amp C harbouring strains of *E. cloacae*. The zone of inhibition against each of the antimicrobial agent observed after 18-22 hours of incubation at 35-37°C and interpreted[13].

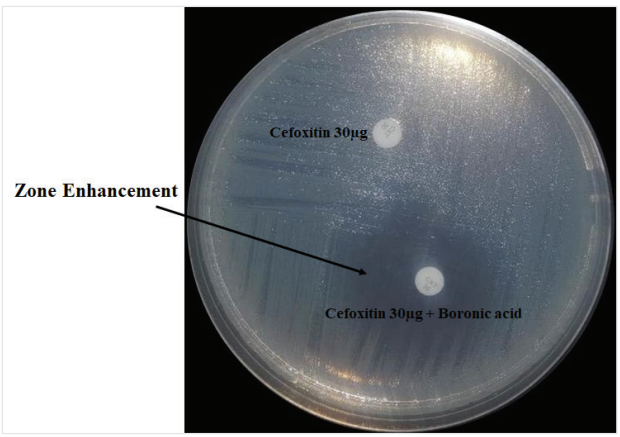

Figure 1: Phenotypic confirmation of AmpC production. Cefoxitin disc with boronic acid

showing an enhanced zone of inhibition (≥ 5mm)in comparison to cefoxitin alone.

Ethical clearance: The institutional ethical review panel ethically approved the study.

Results

We isolated 96 *E. cloacae* in total out of which 79 (82.3%) isolates were positive on screening with cefoxitin. Out of these 79 pathogens, 63 (65.6%) bacterial strains were confirmed as AmpC producers based on inhibitor based confirmatory test (Table 1).

Table 1: Frequency of AmpC producing strains among *E. cloacae* (n=96)

Characteristics	AmpC Screening	Inhibitor-Based Test
AmpC β-lactamase	79 (82.3%)	63 (65.6%)
Non-AmpC	17 (17.7%)	0

Amp C harbouring *E. cloacae* were found in 36 (57.1%) male and 27 (42.9%) female patients. The individuals with confirmed AmpC *E. cloacae* infection were divided into five groups. All the cases with *E. cloacae* were between neonates and 15 years of age. The majority of cases infected with AmpC producing *E. cloacae* included 34 (54.0%) neonates and 11 (17.5%) infants. The sources of AmpC producing *E. cloacae* primarily include 43 (68.3%) blood, 8 (12.7%) catheter tips and 6 (9.5%) pus and wound swabs. The majority of these pathogens were distributed in the neonatal nursery unit (33; 52.4%), medical ward (13; 20.6%), intensive care unit and surgery (5; 7.9%). The distribution of rests of the source is shown in Table 2.

The AmpC producing *E. cloacae* were 100% resistant to co-amoxiclav, ceftazidime, cefotaxime, cefuroxime, cefixime, ceftriaxone, and cefoxitin. The resistance of Amp C harbouring *E. cloacae* to different drugs revealed 50 (79.4%) to tobramycin, 49 (77.8%) to chloramphenicol, 47 (74.6%) to gentamicin, 44 (69.8%) to co-trimoxazole and 42 (66.7%) to amikacin. AmpC producing *E. cloacae* found to be less resistant to levofloxacin, imipenem and colistin sulphate as 23 (36.5%), 20 (31.7%) and 17 (27.0%), respectively (Table 3).

Table 2: General characteristics of AmpC producing *E. cloacae* (n=63)

Characteristics	Frequency	Percentage
Gender		
Males	36	57.0
Females	27	43.0
Age Groups		
Neonates (< 28 days)	34	54.0%
Infants (29 days to 1 year)	11	17.5%
1year-5 years	3	4.8%
5 years -10 years	9	14.3%
10 years -15 years	6	9.5%
Sources		
Blood	43	68.3
Catheter Tips	8	12.7
Pus and Wound Swab	6	9.5
Tracheal Secretions	3	4.8
CSF	2	3.2
Sputum	1	1.6
Wards		
Neonatal Nursery Unit	33	52.4
Medical Ward	13	20.6
Intensive Care Unit	5	7.9
Surgery	5	7.9
Nephrology	4	6.3
Cardiology Ward	3	4.8

Table 3: Antimicrobial resistance of AmpC producing *E. cloacae* (n=63)

Antibiotic	Resistant	Sensitive	Intermediate
	n (%)	n (%)	n (%)
Co-amoxiclav	63 (100)	0	0
Cefuroxime	63 (100)	0	0
Cefixime	63 (100)	0	0
Cefotaxime	63 (100)	0	0
Cefoxitin	63 (100)	0	0
Ceftazidime	63 (100)	0	0
Ceftriaxone	63 (100)	0	0
Tobramycin	50 (79.4)	12 (19.0)	1 (1.6)
Chloramphenicol	49 (77.8)	14 (22.2)	0
Gentamicin	47 (74.6)	16 (25.4)	0
Co-trimoxazole	44 (69.8)	19 (30.2)	0
Amikacin	42 (66.7)	21 (33.3)	0
Moxifloxacin	39 (61.9)	22 (34.9)	2 (3.2)
Cefoperazone-sulbactam	35 (55.6)	25 (39.7)	3 (4.8)
Meropenem	35 (55.6)	23 (36.5)	5 (7.9)
Piperacillin-tazobactam	35 (55.6)	23 (36.5)	5 (7.9)
Ciprofloxacin	32 (50.8)	24 (38.1)	7 (11.1)
Levofloxacin	23 (36.5)	37 (58.7)	3 (4.8)
Imipenem	20 (31.7)	42 (66.7)	1 (1.6)
Colistin Sulphate	17 (27.0)	46 (73.0)	0

Discussion

The rate of increased multidrug resistance is receiving full consideration globally. The lack of antibiotic regulation in most Asian countries, including Pakistan, is supposed to have involved the promptly increasing infections of multidrug-resistant pathogenic bacteria. The recurrent cause of resistance among Gram-negative organism is mainly due to the AmpC β-lactamase enzyme[15]. Clinical microbiologists and infectious disease specialists are agreed that MDR Gram-negative bacteria cause the ultimate risk to public health. The trend of resistance development is more in Gram-negative organisms; also the detection and development of new antibiotics to fight against these bugs are extreme rarer[16,17]. The growing resistance of Gram-negative is due to moveable genetic elements, thus readily spreading through the bacterial population[18,19].

A marked origination of β-lactam resistance in *Enterobacter* infections have been established[20]. AmpC enzymes are chromosomally encoded cephalosporinases that confers resistance to broad-spectrum antibiotics due to their overexpression by mutations[1]. The extensive use of cephalosporin antibiotics and the transmission of AmpC producing strains through invasive procedures could be the reason for the dissemination of multidrug-resistant pathogens[21]. They can hydrolyze cephamycins and are resistant to clavulanate and other β-lactamases inhibitors[9]. In the current study, 65.6% *E. cloacae* were AmpC β-lactamase positive. AmpC β-lactamase positive isolates have been reported as 64.2% in India and 51.6 % in across Europe and Israel which is comparable to the present study.

The present study showed that a high percentage of 57.1% of AmpC producing *E. cloacae* found in male patients. Lee *et al*. reported 53.1% of AmpC β-lactamases in malesfrom a tertiary care teaching hospital in Taiwan. In our study, high incidence rate(34; 54.0%) of AmpC producing *E. Cloacae* were found in neonates[22]. Kothari *et al.* reported a 35.6% frequency of AmpC producing *E. cloacae*[23]. The incidence of AmpC producing *E. cloacae* was different in different specimens. A higher number of AmpC strains have been reported from the cases of bacteremia as in our findings[14,24]. Neonatal Nursery Unit had the highest isolation rate (52.4%) of AmpC *E. cloacae* which is comparable with a similar observation in another study where neonatal wards (52%) showed maximum isolation[24].

We reporta variable multidrug-resistant pattern of AmpC producing *E. cloacae* against cephalosporins antibiotics. Imipenem and colistin sulphate reported as the least resistant drugs and which can be chosen for the treatment in the specific clinical condition of the patient. The resistance against carbapenem drugs is reported to be a risk factor for higher mortality[25]. Mohamudha *et al.* reported that 53.8% AmpC enzyme harbouring *E. cloacae* isolates were resistant to third-generation cephalosporins including cefoxitin, amikacin, gentamicin, and co-trimoxazole[26]. Poor hand washing practices contribute to the dissemination of multidrug-resistant pathogens[27,28]. Different educational activities for the healthcare personnel, personal aseptic guidelines for patients and attendants should be organized to evade the occurrence of infections[29,30].

Conclusion

Amp C producing *E. cloacae* are multi-drug resistant primarily isolated from the blood source. Their existence in the blood stream infections could be due to the invasive procedures lead to the nosocomial transmission of these bugs. Neonates are the most susceptible age group which could be due to the handling of the neonates with contaminated hands of the healthcare staff. The presence of these highly resistant strains is a menace towards dissemination of AmpC genes in the other genera of the bacteria which lead to the therapeutic failure and leave the doctors with limited treatment options of levofloxacin, imipenem and colistin sulphate which should be used if they are enormously necessary. Early detection by using the boronic acid technique could help to reduce the morbidity of the infected patients to implement effective strategies to reduce the transmission of AmpC producing *E. cloacae*.

Limitations: The study could not include the molecular characterization and sequencing of AmpC genes due to financial and time constraints.

Conflict of interest: Nothing to declare

Authors' Contributions:

MR, HE, AZ, HJ, DAAF, and SN conceive the idea and designed study. MR, HE, AH, MI, SY, and MK gathered data gathering and performed experiments. DAAF, SY, KJ and HE analyzed and interpreted data. MR, SY, and DAAF wrote the manuscript. MR, HE, KJ and DAAF final approval of the edited manuscript.

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