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RESEARCH ARTICLE

PHENOTYPIC DETECTION OF AmpC β-LACTAMASE IN CLINICAL ISOLATES OF *KLEBSIELLA SPP.* BY AN INHIBITOR BASED METHOD USING BORONIC ACID

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ABSTRACT

Klebsiella spp. are commonly encountered bacterial pathogens. β-lactamases produced in *Klebsiella spp* are mainly AmpC and Extended spectrum β-lactamases (ESBLs). ESBLs, confer resistance to penicillins, cephalosporins and monobactams but are inhibited by clavulanic acid. AmpC β-lactamases confer resistance to oximino cephalosporins, alpha methoxy beta lactams and monobactams. In the present study, a total of 100 isolates of *Klebsiella spp.* from various clinical specimenswere processed in the Department of Microbiology, Bangalore Medical College and Research Institute, Bengaluru for detection of antibiogram pattern, ESBL and AmpC production. Antimicrobial susceptibility testing by Kirby-Bauer technique was done according to Clinical Laboratory Standards Institute (CLSI) 2012 recommendations and detection of ESBL production was performed by Phenotypic confirmatory method recommended by CLSI. AmpC production was initially screened using Cefoxitin resistance and later confirmed by the use of an inhibitor based method employing Boronic acid. 32 *Klebsiella spp.* isolates were found to be pure AmpC producers and 2 isolates were noted to have co-existence of ESBL and AmpC. The study revealed a high prevalence of AmpC producing *Klebsiella spp.* in our hospital, being most susceptible to Piperacillin - Tazobactam. A multidisciplinary approach to infection control with greater emphasis on detection of antimicrobial resistance among Gram negative bacteria accompanied by rational antimicrobial usage is needed to counter the infections of ESBL and AmpC producing pathogens.

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INTRODUCTION

Klebsiella spp. are members of family *Enterobacteriaceae*, encountered frequently as opportunistic pathogens in clinical settings. The common sites for nosocomial infections caused by *Klebsiella spp.* include the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites. People at high risk are middle-aged to older men with alcoholism, diabetes, or chronic bronchopulmonary disease. Cephalosporins are considered as first line drugs against *Klebsiella spp*. Inappropriate use of β-lactams against Gram negative bacteria has resulted in the widespread development of drug resistance which is transferred among the bacteria by various genetic mechanisms. β-lactamases confer resistance to most β-lactam antibiotics. In *Klebsiella spp.,* the β-lactamases mainly produced are AmpC and Extended spectrum β-lactamases (ESBLs). ESBLs, belonging to group 2be according to Bush-Jacoby-Medeiros classification confer resistance to penicillins, cephalosporins and monobactams but are inhibited by clavulanic acid. AmpCβ-lactamases, belonging to group I of Bush's functional classification confer resistance to oximino cephalosporins, alpha methoxy beta lactams and monobactams. (Shoorashetty *et al*., 2011) The present study was carried out over a period of two months from June to July 2013 in the Department of Microbiology, Victoria Hospital, a tertiary care hospital attached to Bangalore Medical College and Research Institute, Bengaluru to report the antimicrobial susceptibility pattern, to detect AmpC and ESBL mediated resistance to antibiotics and to screen for co-existence of AmpC and ESBL in *Klebsiella spp.* isolated from clinical specimens.

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MATERIALS AND METHODS

This prospective study carried out over a period of two months from June to July 2013 in the Department of Microbiology, Bangalore Medical College and Research Institute, Bengaluru

involved processing of clinical specimens obtained from patients attending the tertiary care hospitals attached to BMCRI. Collection of all clinical samples was done by appropriate methods under aseptic precautions. Urine and sputum samples were collected in sterile containers, pus samples by sterile swabs and blood samples by venepuncture and samples were sent to the laboratory immediately. The clinical specimens were processed for isolation and phenotypic identification of bacterial pathogens following standard microbiological protocols. A total of 100 consecutive non repetitive non enteric clinical isolates of *Klebsiella spp.* obtained from various patient sources were considered for the study.

Antimicrobial susceptibility testing of these isolates were done by Kirby-Bauer disk diffusion technique as per recommendations of Clinical and Laboratory Standards Institute (CLSI). The antimicrobial drugs tested (in µg) were Ceftazidime (30), Cefotaxime (30), Ceftriaxone(30), Ciprofloxacin (5), Amikacin (30), Piperacillin - Tazobactam (100/10), Ertapenem/Imipenem (10) and Aztreonam (30).

The protocol followed was as follows: Mueller-Hinton Agar plates was used. Inoculum was prepared in sterile saline solution and turbidity was adjusted to 0.5 McFarland standard. Mueller Hinton Agar plate was inoculated by lawn culture. Antimicrobial disks were placed on the inoculated surface using a sterile forceps. The reporting of antimicrobial susceptibility of these isolates was done after 18 hours of incubation at 35^0C +/- 2^0C by measuring the zones of inhibition of the isolate for these disks and comparing the zone size with the interpretative zones recommended by CLSI. (Hemalatha *et al*., 2007; Clinical and Laboratory Standards Institute, 2012)

ESBL screening

Detection of ESBL production was as per CLSI recommendations [Phenotypic confirmatory method]. Ceftazidime and Cefotaxime disks with and without Clavulanic acid were used. Disks of Ceftazidime (30 µg) and Ceftazidime - Clauvulanic acid (30/10 µg) were placed on the medium at a distance of 30 mm. An organism exhibiting 5 mm or greater zone size increase around the Ceftazidime - Clavulanic acid disk compared to the Ceftazidime disk was considered as indicative of ESBL production. (Clinical and Laboratory Standards Institute, 2012)

Detection of AmpC and co-existence of ESBL: Inhibitor based test

The Boronic acid disk test was performed by inoculating *Klebsiella* isolates on Mueller-Hinton agar and placing a disk containing 30 µg of Cefoxitin and a disk containing 30 µg of Cefoxitin and 400 µg of Boronic acid onto the agar (standard disk diffusion method). Simultaneously discs of Ceftazidime (30 µg) and Ceftazidime - Clauvulanic acid (30/10 µg) were placed on the medium at a distance of 30 mm. Inoculated plates were incubated overnight at 35°C. An organism that demonstrated a zone diameter around the disk containing Cefoxitin and Boronic acid, that is 5 mm or greater than the zone diameter around the disk containing Cefoxitin was considered an AmpC producer and an organism exhibiting 5 mm or greater zone size increase around the Ceftazidime - Clavulanic acid disk compared to the Ceftazidime disk was considered indicative of ESBL production. (Hemalatha *et al*., 2007; Philip E. Coudron, 2005; Anand Manoharan *et al*., 2012)

Klebsiella ATCC 700603 and *E.coli* ATCC 25922 were used as control organisms for ESBL screening. (Clinical and Laboratory Standards Institute, 2012)

Data collected during the study was analysed using Microsoft Excel and Statistical Package for Social Sciences (SPSS) software for statistical significance. Ethical committee clearance was obtained before the start of the study.

RESULTS

Out of the 100 isolates of *Klebsiella spp.* obtained from clinical specimens during the present study, 34 isolates were observed to be AmpC producers and 2 were ESBL producers. The 2 ESBL producers also produced AmpC (Table 1). Pure AmpC production was seen in 32 isolates. The Boronic acid detection method identified 31 AmpC isolates out of 61 isolates screened positive by Cefoxitin resistance method and 3 out of 39 isolates found to be negative by the latter method (Table 2). Isolates are most sensitive to Piperacillin-Tazobactam (70%) followed by Amikacin, Gentamicin, Ciprofloxacin and least sensitive to Ceftazidime. The antimicrobial susceptibility pattern of *Klebsiella spp.* isolates in the present study is tabulated in Table 3.

Table 1. Comparison of CLSI, Boronic Acid Disc and Cefoxitin-Boronic Acid Test

Result	CLSI PCT for ESBL CTX+CA	CTX + BA for AmpC	Cefoxitin Resistance		Cefoxitin-Boronic Acid Test	
			Sensitive	Resistant	Positive	Negative
Pure AmpC (n =32)	0	32	3	29	32	0
ESBL+AmpC (n= 2)	2	2	0	2	2	0
Non-producers	0	0	36	30	0	66
Total (n=100)	2	34	39	61	34	66

Table 2. Comparison of Cefoxitin screening and Boronic acid detection for AmpC

Clinical isolates of *Klebsiella spp.* (n = 100)	Positive AmpC detection using boronic acid (n = 34) (%)
Cefoxitin screening positive for AmpC (n = 61)	31 (50.81)
Cefoxitin screening negative for AmpC (n = 39)	3 (7.69)

Table 3. Antimicrobial susceptibility pattern of *Klebsiella spp.* isolates

Antimicrobial	Overall (n = 100)		AmpC producers (n = 34)	
	Number of isolates sensitive	%	Number of isolates sensitive	%
Piperacillin-Tazobactam	70	70	26	76.9
Cefoxitin	33	33	4	11.8
Ceftriaxone	26	26	9	26.4
Ceftazidime	21	21	6	17.6
Cefepime	30	30	10	29.4
Cefotaxime	35	35	11	32.4
Aztreonam	32	32	12	35.2
Ertapenem	30	30	7	20.5
Imipenem	35	35	11	32.4
Ciprofloxacin	47	47	16	47.1
Amikacin	62	62	20	58.8
Gentamicin	49	49	17	50

DISCUSSION

Currently Clinical and Laboratory Standards Institute (CLSI) recommends confirmatory method for ESBL detection but there are no established guidelines for detection of AmpC β-lactamases. Several phenotypic methods like three dimensional tests, disk potentiation tests, cefoxitin agar medium based tests, etc., have been employed for the detection of AmpC but are of less value. In addition, it is not currently possible to detect ESBL production in AmpC producing bacteria by the confirmatory method as AmpC is resistant to clavulanic acid. Many studies have reported a high prevalence of AmpC in *Klebsiella spp.* and have advocated the use of inhibitor based method for detection of AmpC in Gram negative bacteria, which involves use of boronic acid derivative, a reversible inhibitor of AmpC enzymes. Studies have advocated the use of boronic acid along with clavulanic acid to detect ESBL in AmpC producing Gram negative bacteria. (Shoorashetty *et al*., 2011; Hemalatha *et al*., 2007)

Various studies from India showed a wide range of prevalence of AmpC production among *Klebsiella* isolates. Reports from Karnataka and Delhi showed 3.3 and 20.7 per cent of Gram negative bacteria (GNB) to be AmpC producers based on phenotypic tests. In 2005, the figures from Delhi and Kolkata were 36.06 and 6.7 per cent, respectively for GNB showing AmpC production. Further, based on molecular techniques, a report from Delhi showed the figures to be as high as 50 per cent. In 2007, from Chennai the figures were 47.3 per cent. Recently a study showed high level of AmpC β-lactamases in cases with complicated urinary tract infection. Black *et al*. showed 31 per cent of AmpC production in cefoxitin nonsusceptible strains by AmpC disc test.

There have been reports of ESBL's from major hospitals in India and some of them have recorded the incidence to be as high as 60-68%. The prevalence of AmpC producing *Klebsiella spp.* isolates in the present study shows a significant increase as compared to an earlier study in the same institute by R.M. Shoorashetty *et al*. and this pattern is similar to the trend observed in other studies. The prevalence of 34% AmpC production in the present study correlates with the studies of Manchanda *et al*. Black *et al*. Dutta *et al*. and Naveen Grover *et al*. Widespread inappropriate use of higher antibiotics might be the reason for the rise of AmpC production. (Shoorashetty *et al*., 2011; Ratna *et al*., 2003; Manchanda and Singh, 2003; Singhal *et al*., 2005; Arora and Bal, 2005; Manchanda *et al*., 2006; Hemalatha *et al*., 2007; Taneja *et al*., 2008; Mathur *et al*., 2002; Jones *et al*., 2002; Dutta *et al*., 2014; Naveen Grover *et al*., 2013)

The Cefoxitin resistance in isolates which did not exhibit any significant enhancement with boronic acid reflects mechanisms other than AmpC production.As noted in the present study, Dutta *et al*. have reported co-existence of AmpC and ESBL in isolates of *Klebsiella spp.* in their study conducted in Assam. The co-existence can be confirmed using novel methods involving combinations of Cefotaxime or Ceftazidime with Clavulanic acid and Boronic acid and comparing the enhancement. This co-existence could possibly be due to occasional dissemination of plasmid mediated AmpC enzyme among Enterobacteriaceae in combination with ESBL. (Shoorashetty *et al*., 2011; Shoorashetty *et al*., 2011)

A study by Knudsen JD *et al* reported significant reduction of ESBL/AmpC producing *Klebsiella spp.* following a multidisciplinary intervention with a main focus on antimicrobial stewardship in North Dakota, United States of America. (Knudsen and Andersen, 2014)

Conclusion

From the present study, we conclude that there is a higher prevalence of AmpC producing *Klebsiella* isolates as compared to a previous study in the same institutions.

Overall, the susceptibility of isolates of *Klebsiella spp.* encountered in the present study was maximum to Piperacillin - Tazobactam (70%), followed by Amikacin, Gentamicin, Ciprofloxacin. Ceftazidime was found to be least effective against these isolates. AmpC producing isolates of *Klebsiella spp.* encountered in the present study were also found to be most susceptible for Piperacillin - Tazobactam, followed by aminoglycosides. Further studies involving larger sample size are required to confirm the findings. Rational use of antibiotics and improved diagnostic methods as part of multidisciplinary intervention is the need of the hour for effective management of cases and to counter the threat of antimicrobial resistance.

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