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Occurrence and Detection of AmpC β-Lactamases among Enterobacteriaceae in a Tertiary Care Centre in Trivandrum, India

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A B S T R A C T

The prevalence of multi drug resistant gram negative bacteria has increased continuously over the past few years, of particular concern are strains producing AmpC β-lactamases. The irrational uses of antibiotics are the primary cause of the increase in drug resistant bacteria. Several studies have reported that AmpC producers are isolated mainly from patients with prolonged hospitalization, but certain recent studies have reported that AmpC producers was also isolated from long term care facilities and outpatient clinics, which indicate their presence in the community. All the organisms were identified according to standard methods. They were confirmed with Vitek 2. *E. coli* and *Klebsiella* spp were the most common gram negative organism while AmpC producers were found to be 11.2% of the isolated Enterobactericiae

Introduction

The prevalence of multi drug resistant gram negative bacteria has increased continuously over the past few years, of particular concern are strains producing AmpC β-lactamases (Soha, 2015; Jacoby, 2009).

The irrational uses of antibiotics are the primary cause of the increase in drug resistant bacteria. There is no doubt that the misuse of the drug in human beings has contributed to the increasing rates of the resistance, but recently the use of antibiotics in veterinary animals and agriculture and its consequent effects on resistance levels in peoples has also come under scrutiny (Randall, 2003). Several studies have reported that AmpC producers are isolated mainly from patients with prolonged hospitalization, but certain recent studies have reported that AmpC producers was also isolated from long term care facilities and outpatient clinics, which indicate their presence in the community (Arindam, 2015) AmpC β-lactamases are clinically significant because they may confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins, cephamycins and monobactams (Soha, 2015; Polsfuss, 2005).

A high rate of clinical failure among patients who were infected with AmpC β-lactamases producing bacteria and who received initial antimicrobial therapy, especially cephalosporin has been demonstrated. Therefore, detection of AmpC producing organisms is important to ensure effective therapeutic intervention and optimal clinical outcome (Tan, 2009).

There are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for AmpC detection. The detection of AmpC β – lactamases is a challenge for routine clinical microbiology laboratories in resource limited settings (Mona Waseef, 2013). An initial screen for reduced susceptibility to more than one of the five indicator cephalosporins followed by a confirmatory test can improve the sensitivity of detection. The further identification of specific genes associated with the production AmpC β-lactamases can be performed using specific nucleic acid-based assays. (Gupta, 2007). However these molecular assays are limited to resource-rich settings and are beyond the scope of routine microbiology laboratories. (Anandkumar Harwalkar, 2013)

This study aimed to find out the characterization of Enterobacteriaceae and antibiotic sensitivity patterns with special reference to detection of AmpC β-lactamases producing Enterobacteriaceae in patients in a tertiary care hospital in south Kerala.

Materials and Methods

Present study was conducted in the Microbiology laboratory of Sree Gokulam Medical College and Research Foundation over a duration of 3 months from Oct 2015 to Dec 2015.

All the samples received in bacteriology section of laboratory were inoculated on blood agar, MacConkey agar and incubated at 37°C for 48 h before being reported as sterile.

The isolates were identified based up on Colony characteristics, Gram staining, Motility and Conventional biochemical test like oxidase test, catalase test, indole, methyl red, Voges–Proskauer, citrate utilization, urease production oxidative fermentative test (Hugh-Leifson medium) for glucose, utilization of 10% lactose, gelatinliquefaction, lysine and ornithine decarboxylation, arginine dihydrolase test, growth at 42°C and 44°C, esculin hydrolysisand ONPG test (Win Washington, 2014).

The sensitivity test was performed by Kirby bauer disc diffusion method using commercially available discs (Himedia). The results were interpreted as per the CLSI guideline.

AmpC β-lactamases detection

Screening method for AmpC β-lactamases

Organisms showing resistance to Cefoxitin (zone size <18mm) should be considered as probable AmpC producer and should be confirmed by other methods (Supriya Upadhyay, 2011)

Modified double disc approximation test

Ceftazidime (30µg), Cefotaxime (30µg), are placed at a distance of 20mm from Cefoxitin (30µg) on a MHA plate inoculated test organisms as per CLSI.

Isolates showing blunting of Ceftazidime or Cefotaxime zone of inhibition adjacent to Cefoxitin disc or showing reduced susceptibility to either of the above drug and Cefoxitin are considered as screening positive. (Hemalatha, 2007).

Detection of AmpC by boronic acid disc test

Two types of Boronic acid discs can be used; one is commercially available with 250µg concentration and the other is home made with 400µg concentration (Mona Waseef, 2013).

Boronic acid disc preparation

Dissolve 120 mg of Phenyl Boronic Acid were added to 3ml of Dimethyl Sulfoxide (DMSO). Then 3milliliters of sterile distilled water were added. Twenty micro-litters of the stock solution were dispensed on to discs containing 30µg of Cefoxitin. Allow the disc to air dry for 60 minutes and store in an air tight container at 4ºC (Pai, 2004).

Combined disc method

Test organisms are inoculated to MHA as per CLSI. A disc containing 30µg of Cefoxitin and other 30µg of Cefoxitin impregnated with 400µg Boronic acid are placed on MHA plate and are incubated at 35ºC over night. An organism with a zone diameter of ≥ 5mm around the disc containing Cefoxitin + Boronic acid, than the zone diameter around Cefoxitin disc alone is consider as a positive test (Mona Waseef, 2013).

Results and Discussion

From October 2017 to January 2018, total 250 consecutive non-repetitive Enterobacteriaceae isolates were tested for detecting their antimicrobial susceptibility pattern and production of AmpC beta-lactamases by a screening and confirmatory testing.

The source of the isolates was from urine, sputum, pus, blood and CSF. The clinical spectrum represents out of 250 isolates, 145 are isolated from urine, 57 from pus, 45 from sputum, 2 from blood and 1 from CSF. Distribution pattern of isolated organisms shows that *E. coli* was the most commonly isolated organism followed by *Klebsiella*. Out of 250, 134 isolates were *E. coli*, after that *Klebsiella*, which were about 80 out of 250. Then *Proteus* (11), *Citrobacter* (10), *Enterobacter* (8), *Serratia* (4) and *Morganella* (3) respectively.

Overall antibiotic resistance pattern of the isolates showed high degree of resistance towards Ampicillin (86%), Piperacillin (80%), Amoxyclav (70%) and third generation Cephalosporins (50%). This was statistically significant with p value <0.001. Imipenam (6%), Meropenam (6%), Colistin (0%), Cefoperazone/Sulbactam (14%), Piperacillin/ Tazobactum (10%) and Amikacin (10%) had lower level of resistance

Drug resistance causes a therapeutic problem not only in the hospital settings, but also in the community as most of the bacteria have acquired resistance to multiple antibiotics. Various drug resistance mechanisms of Enterobacteriaceae include extended spectrum β-lactamases (ESBL) production, AmpC β-lactamases production, efflux mechanism, plasmids and porin deficiency. In clinical laboratory settings, commonly the commonly detected enzymes causing resistance are ESBLs and AmpC β-lactamases. Clinical relevance of AmpC β-lactamases lies in the fact that they confer resistance to both narrow and broad spectrum cephalosporins, β-lactam / β-lactamase inhibitor combinations and Astreonam.

Detection of AmpC β-lactamases from Enterobacteriaceae are important to improve the clinical management of patients suffering from infections and would also provide us with sound epidemiological data. However laboratory methods for screening and confirmation of ESBL should be accurate, simple and rapid. Whereas there are no Clinical and Laboratory Standard Institute

(CLSI) guidelines for detection of AmpC β-lactamase

In the present study, out of 250 isolates studied, 134(54%) are *E. coli*, and are the predominant isolates, followed by *Klebsiella* spp which are about 80(32%) cases and 8(3.2%) cases of *Enterobacter* and 10(4%) cases of *Citrobacter* were obtained. This result is comparable with a similar study conducted by Polsfuss *et al.,* (2005) in their study, out of 2518 samples, 1435(57%) were *E.coli*, 459(18.2%) were *Klebsiella*, 178(5.2%) were *Enterobacter* and 1(0.1%) case of *Citrobacter* were isolated (Polsfuss, 2005). But in the study conducted by Tapan Majumdar, Shibabrata Bhattacharya *et al.,* they found out of 200 tested strains of Enterobacteriaceae, 50 cases were reported as *E.coli*, 40 cases of *Klebsiella* spp, 12 cases of *Enterobacter* and 8 cases of *Citrobacter* were obtained (Tapan Majumdar, 2014). But in the study of Leslie Jose Selvaraj *et al.,* out of 993 Enterobacteriaceae Isolates, 510 cases were reported as *E. coli*, whereas 277 cases are *Klebsiella* spp and 168 cases are *Enterobacter* spp (Leslie Jose Selvaraj, 2008).

The present finding of antibiotic susceptibility pattern was out of 250 cases, antibiotic resistance were Ampicillin 85.4%, Cefuroxime 60.8%, Ceftazidime 52%, Cefepime 36.4%, Cefoxitin 31.6% and Imipenem 5% only.

From the analysis, data obtained, bases on the antibiotic resistance pattern, the drugs such as Ampicillin, Cefuroxime, ceftazidime, cefepime and Imipenem shows a significant sensitivity pattern that is, p<.0001

Similarly the Study of Tapan Majumdar *et al.,* it was obtained that over all antibiotic resistant pattern of the isolates showed high degree of resistance towards Amoxycillin, 3rd generation Cephalosporins and Tetracycline whereas Cefepime, Imipenam Aminoglycosides were showing less resistance (Tapan Majumdar, 2014). The study conducted by Anandkumar Harwalkar *et al.,* revealed that out of 153 isolates, (93.8%) was resistant to Ampicillin, 62(38%) were resistant to Trimethoprim / Sulfamethoxazole (Anandkumar Harwalkar, 2013) (Table 1–3).

Table.1 Distribution of organisms in different samples

Organism (n=250)	Urine	Pus	Sputum	Blood	CSF	Total
E.coli	108	19	4	2	1	134
Klebsiella	32	14	34	0	0	80
Enterobacter	2	3	3	0	0	8
Proteus	2	9	0	0	0	11
Citrobacter	1	7	2	0	0	10
Serratia	0	2	2	0	0	4
Morganella	0	3	0	0	0	3
Total	145	57	45	2	1	250

Table.2 Susceptibility pattern of Enterobacteriaceae

Antibiotics (N=100)	Resistant (%)	Sensitive (%)
Ampicillin	86	14
Piperacillin	80	20
Amoxyclav	70	30
Cephalexin	69	31
Cefuroxime	60	40
Cefotaxime	52	48
Ceftazidime	52	48
Cefepime	36	64
Gentamicin	32	67
Netilmicin	15	85
Amikacin	13	87
Ciprofloxacin	42	58
Norfloxacin	38	62
Ofloxacin	42	58
Nitrofurantoin	26	74
Cotrimoxazole	36	64
Tetracycline	41	59
Cefoperazone/sulbactum	14	86
Piperacillin/Tazobactum	12	88
Imipenem	6	94
Meropenem	6	94
Aztreonam	41	59
Cefoxitin	32	68
Colistin	6	94

Table.3 Distribution of AmpC among Enterobacteriaceae

Organism (n=250)	AmpC Positive (n=28)
E.coli	6 (21.4%)
Klebsiella spp	15 (53.6%)
Enterobacter spp	4 (14.3%)
Proteus spp	0 (0%)
Citrobacter	3 (10.7%)
Serratia	0(0%)
Morganella	0(0%)

Resistance to Tobramycin, amoxicillin / Clavulanate and Amikacin were noted in between 30% and 19.6% of isolates. Only 2(1.2%) strains showed resistance to Imipenem. In the similar study conducted by Rong Wang *et al.,* showed that 70.1% were resistant to Ampicillin, 44.2% were resistant to Cefuroxime, 31.2% were resistant to Ceftazidime, 20.8% were resistant to Cefepime, and 75.3% were resistant to Cefoxitin (Rong WANG, 2009).

Detection of AmpC beta-lactamases are based on a combination of sensitive screening and confirmation assays. Screening tests were done by the use of Double disc approximation test. And detection by Boronic acid disc test. In present study, *Klebsiella* represents 15 cases and *E.coli* 5 cases and *Enterobacter* 4 cases are detected as AmpC producers. Statistical analysis is done to test the association of distribution of AmpC on Enterobacteriaceae, the value obtained, Chi-square =292.5, p<0.0001 and Fischer's Exact statistic = 64.11, p<0.001.This implies that there exist a significant difference between the distribution of AmpC on different Enterobacteriaceae.

A similar study conducted by Mona Wassef *et al.,* shows *Klebsiella* spp represents 6 cases, 3 cases of *E. coli* and 2 cases of *Enterobacter* gave a positive Boronic acid Disc test. (Mona Waseef, 2013) But in the study of Shoorashetty *et al.,* shows out of 200 isolates, 6 *Klebsiella* spp, 6 *E. coli*, 6 *Enterobacter* and 8 *Citrobacter* gave a positive Boronic acid Disc test (Shoorashetty, 2011). Similar study conducted by Shanthi *et al.,* showed that, out of 77 isolates, 7 *E.coli* and 8 *Klebsiella* are gave a positive Boronic acid disc test for AmpC beta-lactamases.

β-lactam antibiotics are the most widely used chemotherapeutic agents. Commonest cause of resistant towards β-lactam antibiotics is the production of β-lactamases.

β-lactamases producers are an increasing cause of concern in hospitals as they produce a therapeutic confusion for the treating physician. In order to provide useful information for effective control and clinical therapy of infection, the resistant status and the rate of carrying AmpC β-lactamases of Enterobacteriaceae were investigated by phenotypic methods.

The study was conducted in a tertiary care hospital in Kerala, about 250 Enterobacteriaceae isolates were checked by Boronic Acid disc test to detect the resistance due to AmpC β-lactamases production in Enterobacteriaceae.

In the present study, first we concluded that from total Enterobacteriaceae isolates, the most commonly isolated organism was *E.coli* followed by *Klebsiella* spp. An overall antibiotic resistance pattern of the isolates showed high degree of resistance towards Ampicillin, Piperacillin, Amoxyclav and Third generation Cephalosporins and Fourth generation Cephalsporins. Whereas Imipenem, Meropenem, Colistin, Cefoperazone/Sulbactam, Piperacillin/ Tazobactum and Aminoglycosides were showing less resistance.

Amp C β-lactamase resistance was 11.2% (28/250) of circulating *Enterobacter*iciae in the hospital set up. Knowledge of the presence of Amp C is critical especially in patients with sepsis where in vivo in vitro sensitivity difference will be there for higher generation cephalosporins.

Characterization, Identification of the antibiotic susceptibility patterns and detection of AmpC β-lactamases aid in Hospital infection control and help the physician to prescribe the most appropriate antibiotics, thus decreasing the selective pressure, which generates antibiotic resistance.