ANTIBIOTIC SUSCEPTIBILITY PROFILING AMONG GRAM POSITIVE AND GRAM NEGATIVE PATHOGENS IN NORTH BIHAR, INDIA

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ABSTRACT

This study was undertaken to determine the prevalence of Gram negative and Gram positive in various clinical samples collected during study and to analyze the antibiotic susceptibility patterns of various drugs against these isolates to find which drug offers the best solution against multidrug resistant Gram negative and Gram positive pathogens. In the current study, a total of 741 isolates were isolated from different clinical specimens between October 2018 to January 2019. Antibiotic susceptibility testing were carried out according to the recommendations of Clinical Laboratory Standards Institute (CLSI) guidelines. Out of 741 clinical isolates, 575 (77.59%) were of Gram negative and 166 (22.40%) were of Gram positive. Among Gram negative pathogens, (n=575), the highest occurrence of pathogens was found in urine samples (81.57%) followed by stool (17.04%), pus (0.35%) and others 1.04%, whereas Gram positive (n=166) isolates were more dominant in sputum (24.70%) followed by pus/swab (23.49%), throat (18.07%), vagina (9.04%), synovial fluid (1.2%), each of ear swab, aural fluid, conjunctival fluid contributed to 0.6% and others making 21.69%. Further analysis of pathogens (n=741), Escherichia coli (63.43%) was the most dominant pathogen followed by Streptococcus pyogenes (9.18%), Streptococcus species (8.77%), Enterobacter species (8.64%), Proteus species (3.78%), Streptococcus haemolyticus (3.10%), Klebsiella species (1.48%) Staphylococcus species (1.35%) and Pseudomonas species (0.27%). Our susceptibility data revealed that Potentox was the most active antibacterial agent with the majority of isolates displaying 88.66% susceptibility, which is 20.10-54.25% high compared to other tested drugs. Levofloxacin appeared to be second most active agent (68.56%) followed by cefepime plus tazobactam (58.30%), meropenem (45.75%) and amikacin (34.41%). From the above results, it is evident that Potentox has enhanced in-vitro antibacterial activity and exhibited 20 to 54 % superiority over other drugs. Therefore, it can be a better choice to treat the infections caused by drug-resistant Gram negative and Gram positive pathogens in the clinical settings, and can be an important empiric consideration as a drug of choice to carbapenem.
Introduction

Antibiotic resistance is a global health crisis which has emerged as one of the principal public health problems of the 21st century and it must be managed with the utmost urgency. According to a study of The US Centers for Disease Control and Prevention (CDC) estimates, antibiotic resistance is causing more than 2 million infections and 23,000 deaths each year in the United States at a direct cost of $20 billion and additional productivity loss of $35 billion (Neil, 2014; Vasoo et al., 2015). It can occur as a natural selection process where nature empowers all bacteria with some degree of low-level resistance (Levy et al., 2007; Neil, 2014; WHO, 2015). The overuse and misuse of antibiotics contributed the more rapid emergence of antibiotic-resistant bacteria and antibiotic resistant genes (ARGs), reducing their therapeutic potential against human and animal pathogens (Wright, 2010).

Aminoglycosides (AG) are broad spectrum antibiotics with high potency and have been used to treat many serious Gram-negative and some Gram-positive infections (Hermann, 2007). They exert their antibacterial activity by inhibiting protein synthesis via binding to the 16S rRNA and by disrupting the bacterial cell membrane integrity (Shakil et al., 2008). Gram-negative bacteria are responsible for more than 30% of hospital-acquired infections and more than 40% of infections in patients in intensive care units (Peleg and Hooper, 2010; Kallen et al., 2010). However, over the past few years, the emergence of resistant strains of Pseudomonas species, Escherichia coli, Klebsiella species, Acinetobacter species has reduced the potential of aminoglycosides in empiric therapies (Gad et al., 2011; Randhawa et al., 2004; Shahid and Malik, 2005). There are a number of aminoglycoside resistance mechanisms that include reduced uptake or decreased cell permeability (Garneau-Tsodikova and Labby, 2016), alteration of the ribosomal binding site by rRNA methylases (Galimand et al., 2012), overexpression of efflux pump (Poole, 2004) and production of aminoglycoside-modifying enzymes (AMEs) (Miró et al., 2013). In Gram negative organisms, resistance to aminoglycosides such as amikacin, tobramycin and gentamycin has been reported to vary from 32.6% to 83.6% (Shahid and Malik 2005).

Cefepime is a fourth generation cephalosporin antibiotic, has broad spectrum activity and is less affected by the non hydrolytic barrier mechanism of resistance (Shrivastava and Chaudhary, 2009). However, in the past few years, it is being threatened because of increasing resistance (Dua et al., 2011). Chong et al., (2010) have reported 35.3% resistance of cefepime against gram-negative isolates. Chaudhary and Payasi (2015) have highlighted 55-74% resistance of cefepime against gram-negative pathogens.

With the global rise of antibiotic-resistant pathogens and failure of monotherapy, an alternative to ineffective monotherapy is combination therapy that uses two or more drugs to broaden the antibacterial spectrum and prevent the development of resistance (Hughes et al., 1997). A combination of antibiotics provides a broader spectrum of coverage than any single antibiotic alone. A well-chosen combination should be synergistic, provide an antibacterial spectrum greater than the sum of their individual activities and allow the use of smaller doses of each of the combined drugs. Combination therapy may reduce the likelihood that resistance will emerge during therapy, since only bacteria with mutations providing resistance to both antibiotics will be able to survive and grow (Mouton et al., 1999). The combination of aminoglycosides with beta-lactams has been documented to be
synergistic (Sanz et al., 2002; Bliziotis et al., 2005; Chaudhary and Payasi, 2015).

In view of the above background, Venus Medicine Research Centre, India has developed a new antibiotic adjuvant entity (AAE) which is named as Potentox, and is a combination of fourth-generation cephalosporins and aminoglycoside amikacin in a ratio of 4:1, respectively along with antibiotic resistance breakers (ARBS).

Therefore, present study is aimed to evaluate the antibiotic susceptibility patterns of Potentox (cefepime+amikacin), in comparison with other drugs against different clinical isolates of Gram-positive and Gram negative.

Materials and Methods

Sample collection

Various clinical specimens including urine, stool, pus/swab, sputum, vagina, throat, synovial fluid, ear swab, aural fluid, conjunctival fluid, blood etc were collected from indoor and outdoor patients at Sharma Diagnostic, Darbhanga, Bihar (India).

Isolation and identification of microbes

All the samples were collected aseptically in a sterile robust leak proof containers in sufficient amount. The samples were transported immediately to microbiology lab for further processing. The samples were inoculated on blood agar and Mac Conkey’s agar.

The collection and processing of the specimens were done as per Standard Operating Procedures. The growth obtained was identified by the colony characteristics, gram staining and by standard biochemical reactions.

Antibiotic susceptibility testing

The Kirby-Bauer disc diffusion method was performed to determine the susceptibilities of the different antibiotics and the results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2019).

In brief, similar colony of the overnight grown cultures of bacterial isolates on Mueller-Hinton agar (MHA) plate was diluted in saline to adjust the turbidity of the bacterial suspension to 0.5 McFarland standard (approximately 10^8 cfu/ml). Soon after, a sterile cotton swab was dipped into the bacterial suspension and streaked it across the surface of the MHA plate. For even distribution of inoculum, the swab was streaked two more times at 60° over the agar surface. The plates were dried at room temperature for 15 min before applying the discs containing different antibiotics. The discs were applied in such a way to ensure a minimum distance of 24 mm from center to center. These plates were then incubated at 37°C in an incubator for 18–24 hours. All determinations were made in duplicate. Zone diameter end points were measured and recorded. Control strains of Escherichia coli ATCC 25922 and Klebsiella pneumoniae ATCC 700603 were used for quality control of susceptibility testing. Positive growth controls of each isolate (bacteria in medium) were incubated under the same conditions. Negative control for each plate was medium only.

Results and Discussion

This study reports on rates of antimicrobial resistance/susceptibility among Gram negative and Gram positive pathogens collected from Sharma Diagnostic, Darbhanga, Bihar, India between October 2018 to January 2019. This study
demonstrated change in the patterns of antibiotic resistance. The antimicrobial resistance against antibiotics varies according to geographical areas and depends upon various factors such as abuse, availability and consumption of antibiotics (Miriagou et al., 2010). In recent years, increasing occurrence of aminoglycoside resistant strains have imposed a major threat not only because of their ability to cause serious infections but also because of their increasing resistance to antimicrobial agents. Combination antibiotic therapy is appropriate and desirable; however, it should be used wisely. Therefore, present study is conducted to evaluate the efficacy of combination of cefepime and amikacin in comparison with other drugs.

Out of 741 clinical isolates, 575 (77.59%) were of Gram negative and 166 (22.40%) were of Gram positive. Many studies have also revealed that Gram negative bacteria is major opportunistic and frequent pathogens and are extremely prevalent in hospital-associated infections which favours recent study (Neil, 2014; Ejaz et al., 2006). Among Gram negative pathogens, (n=575), the highest occurrence of pathogens was found in urine samples (81.57%) followed by stool (17.04%), pus (0.35%) and others (1.04%) whereas Gram positive (n=166) isolates were more prevalent in sputum (24.70%) followed by pus/swab (23.49%), throat (18.07%), vagina (9.04%), synovial fluid (1.2%), each of ear swab, aural fluid, conjuctival fluid contributed to 0.6% and others making 21.69% (Table 3).

Morphological and biochemical characterization of the pathogens (n=741) showing bacterial growth revealed presence of each of 4 different Gram negative organisms and Gram positive organisms. The detailed profile of various organisms used in the study is shown in Figure 1. Among the identified Gram negative bacteria, Escherichia coli (63.43%) was found to be the most dominant pathogen followed by Enterobacter species (8.64%), Proteus species (3.78%), Klebsiella species (1.48%) and Pseudomonas species (0.27%). The findings is comparable with other studies indicating 49.2% to 68.8% prevalence of E. coli (Sikka et al., 2012; Dash et al., 2013; Patil et al., 2013; Sachdeva, 2016). In another study performed by Hamdan et al., (2011) reported E. coli as the most common pathogen about 77.7% among Gram negative isolates. Proteus spp. (3.78 %) contribute nonsignificantly in the present study, same as reported by Ejaz et al., (2006) and Sachdeva (2016) where they reported low prevalence of P. mirabilis (1.0 %) and 1.5%. Contrary to previous studies (Bagga, 2015; Sachdeva, 2016), we have noticed least prevalence of Pseudomonas species and Klebsiella species. Among, Gram positive bacteria, Staphylococcus spp. and Streptococcus spp. being most commonly isolated pathogens. In this study, Streptococcus pyogenes (9.18%) was the most commonly isolated followed by Streptococcus species (8.77%), Streptococcus haemolyticus (3.10%) and Staphylococcus species (1.35%) (Figure 1).The incidence of Staphylococcus species and S. haemolyticus in this study was quite lower compared to earlier study (Gupta et al., 2015; Amutha and Viswanathan, 2015; Sarkar et al., 2015). Prevalence of Streptococci in India ranges from 4.2% to 23.7%, which are comparable to the current study (Rao Sadanand and Shaker Venkatesh, 2018; Sarkar et al., 2015).

The overall susceptibility pattern of antibiotics against tested pathogens is presented in Figure 2. The susceptibility pattern in this study revealed that Potentox was the most active antibacterial agent with the majority of isolates displaying 88.66% susceptibility, which was 20.10-54.25% high compared to other tested drugs. This may be due to synergism of aminoglycosides with β-
lactams which enhanced the intracellular uptake of aminoglycosides by enhancing bacterial cell permeability. Furthermore, Potentox synergistically is assumed of having protein kinase inhibitor activity to inhibit the aminoglycoside modification through ATP-dependent O-phosphorylation, catalysed by aminoglycoside kinases particularly aminoglycoside phosphotransferases (Aphs). The enhanced susceptibility of Potentox is consistent with our previous studies where it has been demonstrated to have noticeable antibacterial activity (Chaudhary et al., 2013; Chaudhary and Payasi, 2014). Its antibacterial activity has also been proved in animal model (Chaudhary et al., 2011; Dwivedi et al., 2009). Although, Potentox showed very low resistance (2.34%) compared to other drugs.

Although fluoroquinolones are the potent antibiotics and have been useful in the treatment of a range infections, reports in a number of countries indicates an increase in microbial resistance (Dalhoff, 2012; Redgrave et al., 2014). Resistance to these agents is multifactorial and can be via one or a combination of target-site gene mutations, increased production of multidrug-resistance (MDR) efflux pumps, modifying enzymes, and/or target-protection proteins (Redgrave et al., 2014). Besides these, another possible reason for this could be indiscriminate use of antibiotics in hospital.

Carbapenems are broad-spectrum antibiotics which possess stability against hydrolysis by ESBL and AmpC chromosomal β-lactamase enzymes and are often reserved to treat the most serious infections (Zhanel et al., 2007). However, carbapenem-resistance among Gram negative bacteria has been reported increasingly throughout the world including India (Gupta et al., 2006; Shah and Narang, 2005; Hu et al., 2012).

In the current study, susceptibility to meropenem was 45.75% which is similar to previous study (Chaudhary et al., 2015, Turner, 2008). The reduced to meropenem in these isolates probably results from reduced accumulation of drug or over activation of efflux pump (Sinha and Srinivasa, 2007). Several authors have highlighted the reduced susceptibility od penems (Chaudhary and Payasi, 2012; Chaudhary et al., 2013).

Table 1

| Sr. No. | Pathogen             | Selected media                             |
|---------|----------------------|--------------------------------------------|
| 1       | E. coli              | EMB agar medium                            |
| 2       | Proteus spp.         | EMB agar and Mcconkey's agar               |
| 3       | Staphylococcus spp.  | Mannitol Salt Agar                         |
| 4       | Pseudomonas spp.     | Citrimide agar                             |
| 5       | Klebsiella spp.      | Hicrome Klebsiella selective agar base medium |
| 6       | Streptococcus spp.   | Streptococci Selective Agar                |
**Table.2** Comparative zone diameters used for interpreting as susceptible, intermediate or resistant

| Drugs | Microorganisms | Zone of diameter (mm) |
|-------|----------------|-----------------------|
|       |                | Susceptible | Intermediate | Resistance |
| Potentox (Cefepime/Amikacin) | Enterobacteriaceae | ≥ 19 | 16-18 | ≤15 |
| Cefepime/Tazobactam | | ≥ 25 | 19-24 (SDD) | ≤18 |
| Amikacin | | ≥ 17 | 15-16 | ≤14 |
| Levofloxacin | | ≥ 17 | 14-16 | ≤13 |
| Meropenem | | ≥23 | 20-22 | ≤19 |
| Potentox | Pseudomonas spp. | ≥ 18 | 15-17 | ≤14 |
| Cefepime/Tazobactam | | ≥ 18 | 15-17 | ≤14 |
| Amikacin | | ≥ 17 | 15-16 | ≤14 |
| Levofloxacin | | ≥ 17 | 14-16 | ≤13 |
| Meropenem | | ≥19 | 16-18 | ≤15 |
| Potentox | Staphylococcus spp. | ≥ 18 | 15-17 | ≤14 |
| Cefepime/Tazobactam | | - | - | - |
| Amikacin | | - | - | - |
| Levofloxacin | | ≥ 19 | 16-18 | ≤15 |
| Meropenem | | - | - | - |
| Potentox | Streptococcus spp. | ≥ 18 | 15-17 | ≤14 |
| Cefepime/Tazobactam | | ≥ 24 | - | - |
| Amikacin | | - | - | - |
| Levofloxacin | | ≥ 17 | 14-16 | ≤13 |
| Meropenem | | - | - | - |

**Table.3** A profile of clinical samples used as a source of the pathogenic isolates

| Sr. No. | Specimen | Gram Negative | Gram Positive |
|---------|----------|---------------|---------------|
| 1       | Urine    | 469 (81.57)   | 0             |
| 2       | Stool    | 98 (17.04)    | 0             |
| 3       | Pus/Swab | 2 (0.35)      | 39 (23.49)    |
| 4       | Sputum   | 0             | 41 (24.70)    |
| 5       | Vagina   | 0             | 15 (9.04)     |
| 6       | Throat   | 0             | 30 (18.07)    |
| 7       | Synovial fluid | 0   | 2 (1.20)       |
| 8       | Ear swab | 0             | 1 (0.60)      |
| 9       | Aural fluid | 0           | 1 (0.60)      |
| 10      | Conjunctival fluid | 0 | 1 (0.60) |
| 11      | Other    | 6 (1.04)      | 36 (21.69)    |
| Total (n) | 741 | 575           | 166           |
Cefepime/tazobactam is a new promising combination and it can be used as an alternative to therapeutic option to carbapenems against fermenters and nonfermenters for the treatment of moderate-to-severe infections (Agarwal et al., 2019; Sharma et al., 2012; Ghafur et al., 2012). Combination of a fourth-generation cephalosporin with a β-lactamase inhibitor has the theoretical advantage of additional activity
against Amp C and possibly OXA enzymes over a third-generation cephalosporin-BLI combination (Ghafur et al., 2012). Our study revealed 58.14% susceptibility of this combination which is little lower to the study of Agarwal et al., (2019) who reported 68 % susceptibility of cefepime plus tazobactam.

Though aminoglycoside drugs are being widely prescribed to patients in India, in the current study, amikacin appeared to be least susceptible (34.41%). Less susceptibility of amikacin could be due to a decreased uptake and/or accumulation of the drug in bacteria and the expression of aminoglycoside modifying enzymes (AMEs) that eventually inactivate the drugs (Gad et al., 2011). Studies from different parts of India show increasing resistance to aminoglycoside drugs among the bacterial isolates and more and more AMEs genes are being identified and found responsible for molecular mechanism of the drug resistance (Mir et al., 2016). This study displayed 64.51 % resistance to amikacin against tested pathogens which is comparable to other studies conducted in Turkey and India which have detected 49.7% and 55.1% resistance of Gram negative organisms to amikacin in India and Turkey, respectively (Shahid and Malik, 2005; Over et al., 2001). A study done by Estahbanati et al., (2001) reported 53.3% of clinical isolates from Iranian burn patients were resistant to amikacin.

Wattal et al., (2010) observed increasing prevalence of carbapenems resistance, varying from 13 to 51% in E. coli and Klebsiella spp. in New Delhi, India hospitals. Similarly, Gupta et al., (2006) also demonstrated high prevalence of resistance, varying from 17 to 22% to various carbapenems among Enterobacteriaceae strains.

The strength of this study was that it had included a large number of pathogens recovered from patients clinical specimens including both community and hospital acquired infections, thereby being able to present the susceptibility behaviour of pathogens in a broader way.

Conclusion of the study is as follows:

From the above results, it is evident that Potentox has enhanced in-vitro antibacterial activity and exhibited 20 to 54 % superiority over other drugs. Therefore, it can be a better choice to treat the infections caused by drug-resistant Gram negative and Gram positive pathogens in the clinical settings, and can be an important empiric consideration as a drug of choice to carbapenem.

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