Canadian Journal of
pure&applied
sciences

ANALYSIS OF ANTIBIOTIC SUSCEPTIBILITY OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT CLINICAL SPECIMEN IN ENUGU STATE

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ABSTRACT

We analyzed the antibiotic susceptibility profile of one hundred and fifty (150) *Klebsiella pneumoniae* strains isolated from different clinical samples (urine= 72, high vaginal swab=12, sputum=50 and wound swab= 16) isolated from patients visiting University of Nigeria teaching hospital (UNTH) Enugu, during June 2008 – May 2009. All samples were analyzed and organism isolated using standard Microbiology techniques, antibiotic susceptibility testing was carried out as described in the manual of antibiotic susceptibility testing. Clonal relatedness of all the *K. pneumoniae* strains was determined by randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). Antibiotic susceptibility studies revealed that *K. pneumoniae* from wound samples were the most susceptible strains followed by HVS, sputum and urine. The overall susceptibility profile are as follows; imipenem (100%), amikacin (100%), cefoxitin (99.4%), aztreonam (98%), ceftazidime (98%), cefotaxime (96.7%), amoxicillin/clavulanic acid (96%), ciprofloxacin (96%), tobramycin (93.3%), kanamycin (90%), cefuroxime (86.7%), gentamicin (76%), sulphamethoxazole/trimethoprim (22%), chloramphenicol (15.4%) and ampicillin (5%). RAPD analysis to determine the clonal relatedness of resistance strains grouped them into two clusters (A and B) based on band patterns. All strains resistance to ampicillin and chloramphenicol showed 100% similarity in band patterns (clonal group A) while strains resistance to sulphamethoxazole/trimethoprim showed different band patterns (clonal group B). Our study revealed strains of *Klebsiella pneumoniae* belonging to two clonal groups based on their resistant to ampicillin, chloramphenicol and sulphamethoxazole/trimethroprim.

Keywords: *Klebsiella pneumoniae,* susceptibility, RAPD, clinical specimens.

INTRODUCTION

Klebsiella spps., particularly *Klebsiella pneumoniae* is a common hospital-acquired pathogen that causes nosocomial infections such as pneumonia (lung infections), wound infections, meningitis, abscesses, urinary tract infections and diarrhea (Paterson *et al.,* 2007). The population at risk is neonates and immune-compromised hosts. The genus *Klebsiella* comprises of five species, *K. pneumoniae, K oxytoca, K. planticola, K. terrigena* and *K. ornithinolytica* (Bruce, 1996), which are usually identified and differentiated according to their biochemical reactions. *Klebsiella pneumoniae* is the most common organism found in hospital patients and has been reported to cause out break of sepsis and death of newborns in the intensive care unit of a tertiary hospital in Brazil (Otman *et al.,* 2002) in the United Kingdom hospitals (Johnson *et al.,* 1992), in France (Arlet *et al.,* 1990). The discovery of antimicrobial agents had a major impact on the rate of survival from infections; however, the changing patterns of antimicrobial resistance caused a demand for new antimicrobial agents. Antimicrobial resistance is known to have a very serious impact in clinical and public health (Oteo *et al.,* 2002). The wide

spread use of broad-spectrum antibiotics had led to the emergency of nosocomial infections cause by drug resistant microbes (Chikere *et al.,* 2008). This is a world wide problem that is exacerbated by the limited number of new antimicrobial drugs (Spellberg *et al.,* 2004; Talbot *et al.,* 2006). Microbial resistance to antibiotics can be by the following ways; (a) drug inactivation by degradation or enzyme modification such as beta lactamases and aminoglycosides transferase (Kiratisin *et al.,* 2008) alteration of drug targets (Kusum *et al.,* 2004), emergency of a bypass pathway not inhibited by the drugs (Xiong *et al.,* 2002), reduced membrane permeability of the drug (Wang *et al.,* 2008). Resistance due to drug efflux can result in multi-resistance due to the presence of antibiotic resistance genes in the chromosome or plasmid within the intergrons which helps in horizontal transfer of resistance. Bacteria infections caused by *Klebsiella* spps. are often treated with beta-lactam antibiotics or alternatively with aminoglycosides or floroquinolones but prevalent of strains resistant to this selected antibiotics have been reported (Brui-Buisson *et al.,* 1987; Sirot *et al.,* 1988; Sekowska *et al.,* 2002). Bearing this in mind we therefore embarked on the present study to determine the antibiotic susceptibility of *K. pneumoniae* isolated from different clinical specimen in a University teaching hospital.

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MATERIALS AND METHODS

Study Population
Clinical samples were collected from a total of 390 patients (male 176, female 214) attending University of Nigeria Teaching Hospital (UNTH) Ituku Ozalla in Enugu capital city in South-Eastern Nigeria from June 2008 through May 2009. Clinical samples were obtained by informed consent of patients used for the study with the permission obtained from the ethical committee of the hospital.

Sample Collections
Non-repetitive clinical samples which include urine (126), high vaginal swab (68), wound swab (90) and sputum (106) were collected from 390 patients and were analyzed between 20- 45 mins of collection. These clinical samples were analyzed using standard routine Microbiology identification and characterization methods as described in Manual of Clinical Microbiology. After identification and characterization of the 390 samples, *K. pneumoniae* was isolated from 150 samples (urine 72, HVS 12, wound swab 16, sputum 50) and stored as a glycerol stock culture in a freeze at -20°C for further analysis (Farmer, 1999).

Antibiotic Susceptibility Testing
Antibiotic susceptibility of *K. pneumoniae* to commonly used antibiotics in the hospital was determined using the agar-diffusion methods on Mueller-Hinton as described in the Manual of antibiotic susceptibility testing (Coyle, 2005). Each organism was inoculated into 5ml of nutrient broth and incubated at 37°C for 18-24 hrs, the broth culture was diluted in sterile normal saline to 0.5 MacFarland equivalent standards which was uniformly inoculated on the surface of Mueller-Hinton agar plates using sterile cotton buds. The plates were inoculated with the following antibiotics after 15mins of inoculating the test organism; ampicillin, ceftazidime, cefotaxime, cefoxitin, cefuroxime, aztreonam, amoxicillin/clavulanic acid, imipenem, chloramphenicol, ciprofloxacin, sulphamethoxazole/trimethoprim, amikacin, kanamycin, tobramycin and gentamicin. Inoculated plates were incubated at 37°C for 18-24hrs after which the inhibition zone diameter was taken. Susceptibility and resistance was determined using the National Committee for Clinical Laboratory Standard breakpoints (CLSI, 2007).

DNA Isolation
Genomic DNA of all strains resistant to sulphamethoxazole/trimethoprim, ampicillin and chloramphenicol was prepared using the Nucleospin Kit (Macherey & Nagel, Germany) following manufacturer's instructions. An overnight culture in a fresh Luria Bertani broth incubated at 37°C for 18-24hrs was prepared of all *K. pneumoniae*. 1.5ml of this overnight broth culture was transferred into a reaction tube and centrifuged for 5 mins at 8,000 rpm and supernatant discarded. Pre-lysis was carried out by re-suspending the pellet in 180µl of T_1 buffer and 25µl of proteinase K, mixed vigorously and incubated at 56 °C for 30 mins with shaking. 200µl of B_3 buffer was added and incubated at 70°C for 10mins. 210µl of 96-100% ethanol was added into tube containing 200µl of B_3 buffer and was mixed vigorously until all insoluble particles becomes soluble. This solution was transferred into a Nucleospin column and centrifuged for 1 min at 11,000 rpm, flow through was discarded and the column was placed back into the collection tube. 500µl of BW buffer was added and centrifuged at 11,000rpm for 1 min, flow-through was discarded and 600µl of B_5 buffer was added, centrifuged for 1min at 11,000rpm, then flow through was discarded and the column was centrifuge again for 1 min at 11,000. Elution buffer was pre-incubated for 5mins at 70°C and 100µl was added into each column and centrifuged for 1min at 11,000 rpm to elute the total DNA. Eluted total DNA was stored at -20°C for further analysis.

Randomly Amplified Polymorphic DNA (RAPD) Analysis of Resistant *K. pneumoniae* Strains
RAPD was performed with all *K. pneumoniae* strains resistant to ampicillin, chloramphenicol and sulphamethoxazole/trimethoprim using a single primer. The PCR mixture contained 2.5µl each of buffer, 4.0mM each of dNTP, 2.5µM each of primer, 5.0µl each of genomic DNA, 2U each of Taq polymerase, 1.5µl of $MgCl_2$ and 9.5µl of water in a total of 25µl with the following PCR amplification protocol; initial denaturation at 94°C for 5 mins, followed by 34 cycles of denaturation at 94°C for 1 min, 36°C for 1 min, 72°C for 2mins and final extension step of 72°C for 8mins. Amplified PCR products were separated on 1.5% agarose gels at 75 volts, stained with ethidium bromide and visualized under UV illumination (Vogel *et al.,* 1999).

RESULTS

Table 1 shows the frequency of isolation of *K. pneumoniae* from various clinical specimen. Of the 390 clinical samples analysed for the presence of *K. pneumoniae* 150 were positive which includes urine = 72, HVS = 12, wound sample = 16 and sputum = 50. The overall prevalence of *K. pneumoniae* in the clincal samples were 38.5% with female 21.5% and male 16.9%. Strains of *K. pneumoniae* isolated from wound swabs were the most susceptible strains followed by HVS and sputum samples while those from urine samples were the least susceptible table 2. The overall susceptibility of *K. pneumoniae* to different antibiotics are as follows; imipenem (100%), amikacin (100%), cefoxitin (99.4%), ceftazidime (98%),aztreonam (98%), cefotaxime (96.7%), cefuroxime (86.7%), ampicillin (5%), amoxicillin/ clavulanic acid (96%), ciprofloxacin (96%), tobramycin (95.4%), kanamycin (90%), gentamicin (74%), sulphamethoxazole/trimethoprim (48%), and chloram-

phenicol (15.4%) (Fig. 1). *K. pneumoniae* strains were most highly resistant to ampicillin and chloramphenicol than with sulphamethoxazole/ trimethoprim. RAPD analysis to determine the clonal relatedness of resistance strains of *K. pneumoniae* from various clinical samples grouped our strains into two clonal groups (A and B) based on their band patterns. All strains resistant to ampicillin and chloramphenicol showed the same band patterns and were grouped as clone A, while those resistant to sulphamethoxazole/ trimethoprim showing different band patterns are grouped as clone B.

Table 1. Frequency of isolation of *K. pneumoniae* from different clinical specimen.

Clinical specimens	Number of specimen collected	Number of *K. pneumoniae* strains isolated
Urine	126	72
Sputum	100	50
High vaginal swabs	68	12
Wound swabs	90	16
Total	390	150

DISCUSSION

The rate at which antibiotic resistance is been reported in different parts of Nigeria is alarming. Adeyemo *et al.* (1994) reported the high resistance of *K, pneumoniae*, *E. coli, Pseudomonas* spp and *Proteus* spp isolated from children with UTI infections to ampicillin and co-trimoxazole. Omonigbo *et al.* (2001) reported on resistant urinary isolates of *Escherichia coli* and *K. pneumoniae* to Nalidixic acid, Onifade *et al.* (2005) reported bacteria susceptible to various classes of antibiotics isolated from pregnant women in ondo State, Aiyegoro *et al.*(2007) reported the presence of resistant bacteria isolated from children and adolescents in IIe-Ife, Nigeria, Chikere *et al.* (2008) reported resistant organisms isolated from patients in government hospital in Port-Harcourt, and Okonko *et al.*(2009) reported bacteria highly resistant to ampicillin, chloramphenicol and tetracycline isolated from clinical samples in Abeokuta. Their reports revealed a tremendous increase in antibiotic resistance in hospital form different parts of Nigeria and this can be inferred to the mis-use of antibiotics due to lack of adequate control body to regulate antibiotic use. In developing countries like Nigeria, in-appropriate use of antibiotics is common and

Table 2. Percentage susceptibilty of *k. pneumoniae* strains isolated from different clinical specimen.

Antibiotics Names

Amp	Ctx	Caz	Cxm	Ipm	Atm	Amc	Fox	Ak	Cn	K	Tob	Cip	Chl	Sxt
Klebsiella pneumoniae strains isolated from urine specimens														
0	95.8	98.6	84.7	100	98.6	93	100	100	76.4	88.8	76.5	98.6	13.8	44.4
Klebsiella pneumoniae strains isolated from sputum specimens														
6	94	100	88	100	100	94.1	98	100	74	90	81	90	24	50
Klebsiella pneumoniae strains isolated from wound swab specimens														
0	100	100	93.8	100	100	100	100	100	75	87.5	93	93.8	12.5	41.6
Klebsiella pneumoniae strains isolated from HVS specimens														
8.3	91.6	91.6	100	100	100	83.3	100	100	75	83.3	68	75	25	61.6

Keys: amp-ampicillin, ctx-cefotazime, caz-ceftazidime, cxm-cefuroxime, ipm-imipenem, atm-aztreonam, amc-amoxicillin/clavulanic acid, fox-cefoxitin, ak-amikacin, cn-gentamicin, k-kanamycin, tob-tobramycin, cip-ciprofloxacin, chl-chloramphenicol, sxt-sulphametoxazole/trimetroprim
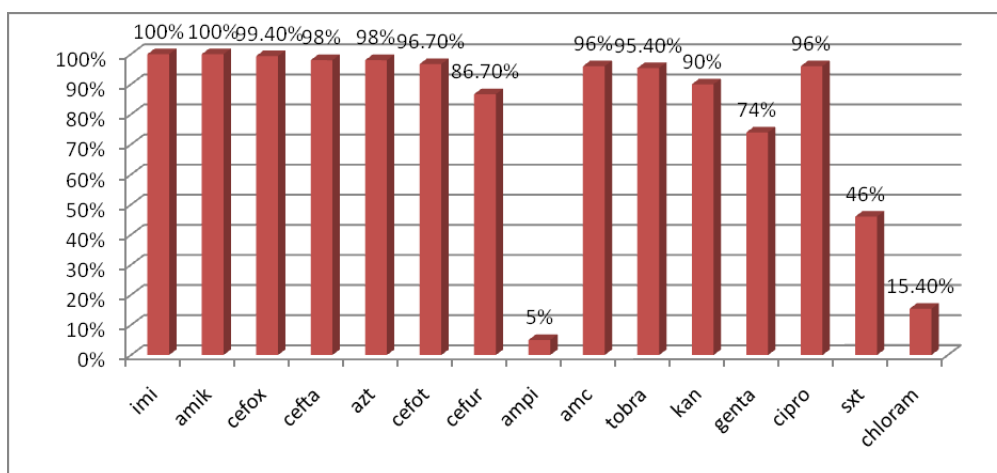

Fig. 1. Over-all Susceptibility Rate of *K. pneumoniae* Strains.

this usually leads to resistance development in bacteria previously known to be susceptible. Self medication is a common practice in Nigeria and this might probably be one major cause of antibiotic resistance in clinical isolates since patients only think of going to the hospital when they can no longer treat themselves. Taking of expired antibiotics, counterfeit drugs coupled with inadequate hospital control measures that are common practice can as well promote the development of resistance in clinical isolates (Chikere *et al.,* 2008).

A total of 150 clinical isolates of *K. pneumoniae* were isolated after analyzing 390 different clinical specimens which includes urine, wound swab, HVS and sputum collected from patients visiting UNTH Enugu during a twelve months study period. *K. pneumoniae* was predominantly isolated from urine samples 72(57.1%) followed by sputum 50(47.1%) wound swab 16(17.7%) and HVS 12(17.6%). Their susceptibility against 15 different antibiotics revealed that all strains of *K. pneumoniae* were susceptiblility imipenem and amikacin, over 95% of the strains were susceptible to cefoxitin, ceftazidime, aztreonam, cefotaxime, ciprofloxacin, amoxicillin/clavulanic acid, tobramycin, over 70% were susceptible kanamycin, cefuroxime, gentamicin. High resistant of *K. pneumoniae* strains to ampicillin (5%), chloramphenicol (15.4%) and sulphamethoxazole/ trimethoprim 48% was observed. This result is similar to that reported by Aiyegoro *et al.* (2007) who reported *Klebsiella* spps. resistance to amoxicillin and co-trimoxazole. Another study in Israel reported a multi-resistance *Klebsiella* spp in a neo-natal intensive care (Leavitt *et al.,* 2007). Resistance of *K. pneumoniae* to ampicillin is not surprising because of the intrinsic structure of *K. pneumoniae* cell (Farmer, 1999). *K. pneumoniae* being a gram-negative bacterium belonging to the enterobactericeae family are known for their high resistance to various antibiotics because of the presence of series of antibiotic resistance genes in their genetic make up which are easily transferred horizontally to other bacteria spps (Piddock, 1996), it has been implicated in series of nosocomial infection out break in hospitals (Chikere *et al.,* 2008; Lewis *et al.,* 2007). Amikacin and imipenem are the most effective antibiotics being 100% effective against *K. pneumoniae* strains, this may be that these antibiotics has not been extensively used to cause resistance developing against them by acquiring resistant genes. imipenem been a carbapenem are know to be active against some gram-negative organisms especially those not harbouring extended spectrum beta lactamases. Beta- lactam antibiotics are know to be the most widely used antibiotics but our strains were still highly susceptible to the 2nd ,3rd and beta lactam antibiotics used in the study. This will also be attributed to the fact that the organisms are not harbouring serious resistance genes like the extended spectrum beta lactamase enzymes but resistance to ampicillin, chloramphenicol and

sulphamethoxazole/trimethoprim may be attributed to the presence of beta lactamses enzymes that are know to detoxify the penicillins and other antibiotics. These antibiotics are cheap and can always be found over the counter in the pharmacy and the lack of control in the use of antibiotics in Nigeria may have lead to their mis-use both in the hospital and within the community as a result leading to the present resistance observed. The frequent un-controlled use of antibiotics in Nigeria has resulted to grave resistance development as have been reported in some previous studies (Onifade *et al.,* 2005; Aiyegoro *et al.,* 2007; Chikere *et al.,* 2008; Okonko *et al.,* 2009). Using RAPD PCR and response of *K. pneumoniae* strains to 15 different antibiotics gave us a method of presumptively identifying clonal groups. We identified two prevalent clones based on this method: one group with resistance to ampicillin and chlorapmphenicol and the other group with resistance to sulphamethoxazole/ trimethoprim. This was inferred based on band patterns appearance of resistance strains on agarose gel. It is also noteworthy that these resistance strains are not from one common source. The clustering of resistant strains into two different groups as observed could be worrisome and may pose some public health problems in future.

CONCLUSION

In conclusion, our present study revealed *K. pneumoniae* from different clinical specimen that are susceptible to a wide range of antibiotics but are highly resistant to ampicillin, chloramphenicol and sulphamethoxazole/ trimethoprim belonging to two different clones. This could be of serious public health implication because of the possibility of horizontal gene transfer to other bacteria spps. We therefore advocate for proper use of antibiotics in Nigeria both in the hospital and within the community and also requesting that government should provide a body that will be responsible for regulating the use of antibiotics.

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