

Full Length Research Paper

Plasmid encoded amoxicillin resistance in common bacterial pathogens from pneumonia patients in University of Benin Teaching Hospital (UBTH), Benin City, South Western Nigeria.

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In order to investigate the mechanism of antibiotic resistance of some bacterial pathogens from the sputum of pneumonia patients, 160 sputum samples were collected from patients within 1-30 years of age attending the outpatients department of the University of Benin Teaching Hospital, Benin City. 101 (63.1%) out of this number gave growth for bacterial pathogens. The bacteria isolated included *Staphylococcus aureus*, *Staphylococcus* spp., *Klebsiella pneumoniae*, *Streptococcus viridans* and *Moraxella catarrhalis*. The highest in occurrence was *S. viridians* (51.5%) and the least was *Staphylococcus* spp (2.9%). The age group 1-5 years had the highest bacterial count (69.9%) while age group 21-25 years had the least count (50%). The isolates were highly resistant to the various antibiotics used. The resistance rate was highest for streptomycin (94.1%) and lowest for gentamycin (20.8%). 89(88.1%) out of the total number, showed the presence of amoxicillin resistance (*amx^r*) gene. When these resistant isolates were subjected to curing in the presence of 10% sodium dodecyl sulfate, 73(82.01%) lost their *amx^r* gene which showed that this gene was probably located on a plasmid in these strains.

Keywords: Plasmid encoded resistance, sputum, pneumonia, bacterial pathogens, antibiogram, amoxicillin resistance, *Streptococcus* spp.

INTRODUCTION

Pneumonia is an illness, usually caused by infection, in which the lungs become inflamed and congested, reducing oxygen exchange and leading to cough and breathlessness (Anthony *et al.*, 2008). It affects individuals of all ages but occurs most frequently in children and the elderly. Among children, pneumonia is the most common cause of death worldwide (Bryce *et al.*, 2005; Wardlaw *et al.*, 2006). The world health organization estimates that there are 150.7 million cases of pneumonia each year in children younger than 5 years with as many as 20 million severe enough to require

hospitalization (Rudan *et al.*, 2004). Many developing countries have mortality rates of 60-100 per 1000 children under 5 years of age (UNICEF, 2007).

Pneumonia can be caused by bacterial, viral or parasitic infections as well as by non-infectious agents (Anthony *et al.*, 2008; Mason and Nelson, 2005). Although bacteria and viruses can cause pneumonia independently, influenza and *Streptococcus* can interact to exacerbate severe pneumonia (O' Brian, 2000). The influenza neuraminidase protein facilitates pneumococcal invasiveness by stripping sialic acid from receptors on the epithelial cells lining the respiratory tract (Madhi and Klugman, 2004).

Sputum is a heterogeneous mixture which is coughed out from the lungs containing mucus, saliva and a variety

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of bacterial species. There has been reports from various workers on the prevalence of different bacterial species in sputum (Pye *et al.*, 1995; Nabeetha *et al.*, 2005; Verenkar *et al.*, 1993). Pye *et al.* (1995) reported the presence of *Pseudomonas aeruginosa*, *Branhamella catarrhalis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Nabeetha *et al.* (2005) recovered same organisms including some Gram negative enteric bacteria while Verenkar *et al.* (1993) recovered some Gram positive cocci and Gram negative rods in their sputum samples. Reports have shown that the best sputum samples are those that contain the least saliva since its presence contaminates sputum (Nabeetha *et al.*, 2005).

Antibiotic resistance is inevitable. Therapeutic exposure of human bacterial pathogens to antibiotics exerts a continuous selection pressure on pathogens present both in infection and carriage (Ball *et al.*, 2002). Bacteria have developed resistance to antibiotics through enzymatic degradation, alteration of bacteria proteins, efflux of antibiotics, childcare/crowded living conditions and indiscriminate use of antibiotics (Samore *et al.*, 2001; Tenover *et al.*, 2006). Presences of resistance plasmids (R-plasmids) in cells make the cells resist the effect of antibiotics and makes chemotherapy of infections very difficult. Plasmids are extrachromosomal genetic elements that are transferred from one cell to another through horizontal gene transfer (Sheikh *et al.*, 2003). They carry a considerable variety of genes including those that confer antibiotic resistance, virulence factors and functions that enhance the capacity to repair DNA damage (Wang *et al.*, 2004). Cells lose their R-plasmids (in a process known as curing) when treated with sub-inhibitory concentrations of chemical dyes.

Klebsiella pneumoniae has been recognized as a nosocomial pathogen for many years and has been implicated in severe (often fatal) pneumonia (Gupta, 2002; Ewing, 1986). *Klebsiella* species exhibit simultaneous resistance to multiple drugs. Its resistance to ampicillin has been reported to be as high as 100% (Loh *et al.*, 2007). *M. catarrhalis* has been recognized as the third most common respiratory tract pathogen in children and adults and its nasopharyngeal carriage has been reported to be a risk factor for serious respiratory tract infections (Slevogt *et al.*, 2006; Lalitagauri *et al.*, 2006). An amoxicillin resistance rate as high as 60% has been reported against this organism (Wallace *et al.*, 2000). It has been reported that the wide variety of multidrug resistance genes carried by *S. aureus* is responsible for the high prevalence of resistance among the *Staphylococcus* (Shakibare *et al.*, 2002; Charlebois *et al.*, 2002). High rates towards amoxicillin (70%), methicillin (76%) and augmentin (65%) have been reported for this organism (Diano and Akano 2009).

The present study was undertaken to determine the frequency of occurrence of some bacterial pathogens from the sputum of pneumonia patients, their antibiogram

and antibiotic resistance mechanisms with a view to identifying an effective drug of choice for treatment.

MATERIALS AND METHODS

Sample collection

A total of one hundred and sixty sputum samples were collected in sterile screw capped containers from pneumonia patients attending the University of Benin Teaching Hospital. These were analysed within 6 hours of collection for the prevalence of pathogens involved in pneumonia cases.

Isolation and identification

The methods of Pye *et al.* (1995) and Nabeetha *et al.* (2005) were used for isolation. Sputum samples were homogenized with 100µg/ml of dithiothreitol and incubated at 37°C for 15 minutes to aid homogenization. 1ml of sample was diluted in sterile saline (0.9% w/v sodium chloride) to give 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} . Three dilutions (10^{-3} , 10^{-4} and 10^{-5}) were inoculated onto blood, chocolate and MacConkey agar and incubated at 37°C in an atmosphere of 7% CO₂ and examined for growth after 24 hours and 48 hours. Optochin disc was placed onto the streak of these isolates to differentiate the species of *Streptococci*. Coagulase test was performed according to the method of cheesbrough (2002) and used to identify *Staphylococcus aureus*.

Antibiotics susceptibility test

This was performed using the standard disk diffusion test as described by Kirby and Bauer (1966) and NCCLS (2004). The antibiotics disk (optodisc) used contained the following antibiotics: chloramphenicol (30µg), augmentin (30µg), amoxicillin (30µg), sparfloxacin (10µg), perfloxacin (30µg), ciprofloxacin (10µg), gentamicin (30µg), streptomycin (30µg), ofloxacin (30µg) and cotrimoxazole (30µg). The zones of inhibition were measured and interpreted according to the criteria of NCCLS (2004).

Plasmid curing

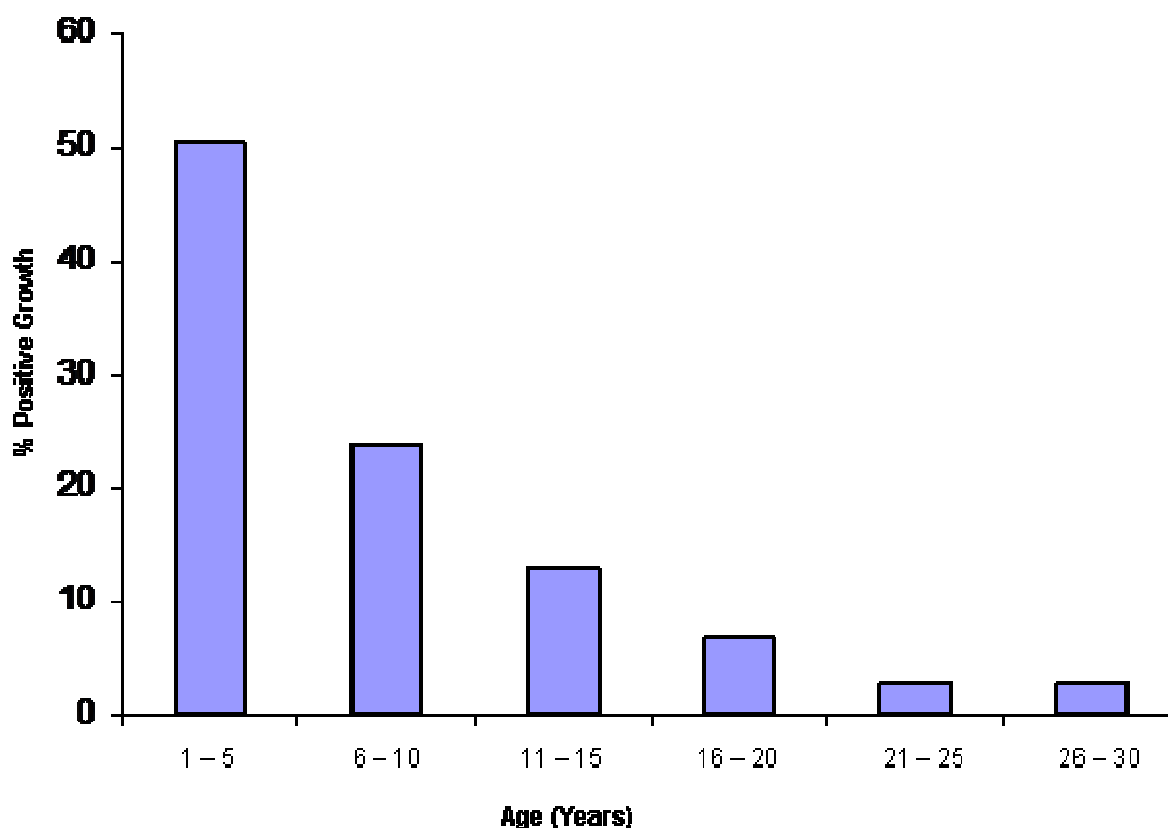
The method of Yah *et al.* (2008) was used. Sub-inhibitory concentration of sodium dodecyl sulfate (SDS) was used for plasmid curing. Antibiotic resistant isolates were grown at 37°C for 24 hours in nutrient broth containing 10% SDS. The broth was agitated to homogenize the content and a loopful subcultured onto Mueller Hinton agar (MHA) plates. The plates were incubated at 37°C for 24 hours after which colonies were screened for antibiotic resistance by the disk diffusion method. Cured markers were determined by comparison between the pre- and post-curing antibiograms of isolates. Loss of resistance markers gave an indication that those markers were probably located on a plasmid and not on the chromosome.

RESULTS

In this study, cultures that showed 10^5 bacterial colonies per ml of sputum were regarded as having significant growth. Table 1 and figure 1 show the frequency of

Table 1: Incidence of bacterial isolates in relation to age (%)

Age	Total no. examined	No. with growth		Total (%)
		Mixed growth (%)	Single growth (%)	
1 – 5	73	50	1	51 (50.5)
6 – 10	38	23	1	24 (23.8)
11 – 15	26	13	---	13 (12.9)
16 – 20	13	6	1	7 (6.9)
21 – 25	6	3	---	3 (3.0)
26 – 30	4	2	1	3 (3.0)
Total	160	97	4	101 (100)

**Figure 1:** Percentage incidence of bacterial isolates in relation to age

bacterial growth in relation to age. Out of a total of 160 sputum samples, 101(63.1%) showed significant bacterial growth. The age bracket 1-5 years had the highest bacterial count (50.5%) and age bracket 21-30 years had the least (3%). The bacterial growth count obtained was statistically significant ($p < 0.05$).

In the frequency of occurrence of bacterial pathogens (table 2), *Streptococcus viridans* appeared to be the most frequent (51.5%). This was followed by *Staphylococcus aureus* (21.8%), *Klebsiella pneumoniae* (13.9%), *Moraxella catarrhalis* (9.9%) and *Staphylococcus* spp. (2.9%). The prevalence rate of these bacterial pathogens was statistically significant ($p < 0.05$). Antibiotic

susceptibility test result (table 3 and figure 2) revealed that the highest resistance rate was recorded against streptomycin (94.1%) and least against gentamycin (23.8%). Resistance of isolates against the quinolones (ciprofloxacin, perfloxacin, ofloxacin and sparfloracin) was quite high.

Eighty – nine (88.1%) of the total isolates displayed high resistance rate against amoxicillin antibiotic (table 4). The rate of resistance was highest for *Streptococcus viridians* (56.2%) and lowest for *Staphylococcus* spp. (2.2%). The 89 amoxicillin resistant (*amx*^r) isolates were subjected to curing in the presence of 10% SDS (table 5). Seventy-three (82%) were cured of their *amx*^r gene. Loss of

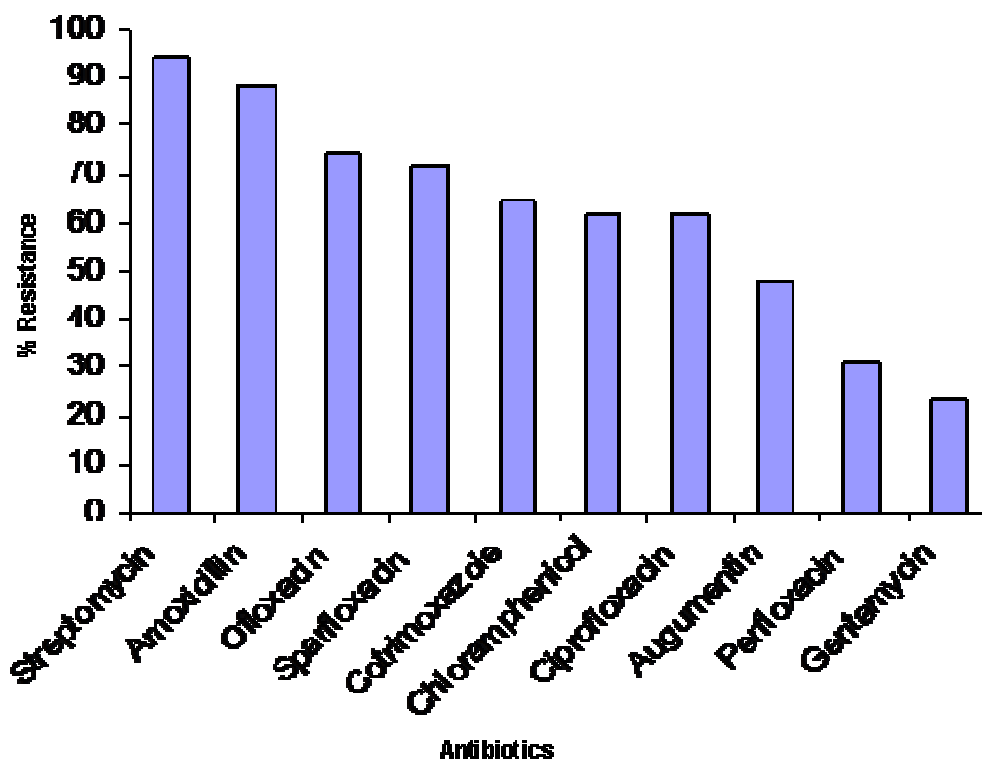


Figure 2: Percentage resistance of isolates to antibiotics

Table 2: Incidence of bacterial isolates from sputum (%)

Bacterial isolates	No. of isolates (%) n = 101
<i>S. viridans</i>	52 (51.5)
<i>S. aureus</i>	22 (21.8)
<i>K. pneumoniae</i>	14 (13.9)
<i>M. catarrhalis</i>	10 (9.9)
<i>Staphylococcus spp.</i>	3 (2.9)
Total	101 (100)

resistance gene was total in 10 (11.2%) out of the total isolates and variable in 63 (70.8%). This gave an indication that the *amx'* gene was located on a plasmid.

DISCUSSION

This study revealed that the frequency of occurrence of pneumonia was highest in children within age group 1-5 years. This is consistent with the result of other workers (Rudan *et al.*, 2004; Scott *et al.*, 2000; Williams *et al.*, 2002). Rudan *et al.* (2004) reported an over 25% mortality in children below 5 years of age. Scott *et al.* (2000) reported 5-23% mortality rate in the same age group and Williams *et al.* (2002) reported a 21% mortality rate. The high incidence of pneumococcal organisms within this age may be attributable to improper development of the innate immune system, malnutrition and micronutrient deficiencies (Scott *et al.*, 2000). It may also be due to frequent hospitalizations and poor intercoastal muscle development which decrease resistance to airflow (Anthony *et al.*, 2008; Scott *et al.*, 2000). The decrease in the frequency of occurrence of pneumonia with increasing age observed in this study might be due to a well developed innate immune system, balanced diet and reduced indoor air pollution (Anthony *et al.*, 2008).

Table 3: Resistance of isolates to antibiotics

Antibiotics	No. resistant (%) n = 101
Streptomycin	95 (94.1)
Amoxicillin	89 (88.1)
Ofloxacin	75(74.3)
Sparfloxacin	72 (71.3)
Seprtin	65(64.4)
Chloramphenicol	62(61.4)
Ciprofloxacin	62 (61.4)
Augumentin	48(47.5)
Perfloracin	31(30.7)
Gentamycin	24(23.8)

Table 4: Prevalence of amoxicillin resistant isolates (%)

Bacterial isolates	Total no. of isolates	No. resistant (%)
<i>S. viridans</i>	52	50(56.2)
<i>S. aureus</i>	22	19(21.3)
<i>K. pneumoniae</i>	14	11 (12.4)
<i>M. catarrhalis</i>	10	7 (7.9)
<i>Staphylococcus spp.</i>	3	2(2.2)
Total	101	89 (100)

Table 5: Plasmid curing of resistant isolates

Bacterial isolates	No. subjected to curing	No. cured of only <i>amx^r</i> gene	No. cured of all resistance markers	Total no. cured (%)
<i>S. aureus</i>	19	15	1	16 (22.0)
<i>Staphylococcus spp.</i>	2	1	---	1 (1.4)
<i>K. pneumoniae</i>	11	8	2	10 (13.7)
<i>S. viridians</i>	50	35	5	40 (54.8)
<i>M. catarrhalis</i>	7	4	2	6 (8.2)
Total	89	63	10	73 (100)

The bacterial species isolated in this study included *Streptococcus viridans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Staphylococcus spp.* These were similar to those reported by earlier workers (Pye *et al.*, 1995; Nabeetha *et al.*, 2005; Verenkar *et al.*, 1993; Anthony *et al.*, 2008; Enabulele *et al.*, 2008). Pye *et al.* (1995) reported the presence of *Pseudomonas aeruginosa*, *Branhamella catarrhalis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus* in their sputum samples. Verenkar *et al.* (1993) and Nabeetha *et al.* (2005) reported the presence of same organisms including some gram positive cocci and gram negative enteric rods. Variations in the types of organisms isolated could be attributable to factors such as media, culturing method, time and method of sampling. Period of transportation and storage before culturing can also be causative factors.

The resistance rate was high for the quinolones (perfloracin, ciprofloxacin, ofloxacin, sparfloxacin) as observed in this study. This is in accordance with the findings of Enabulele *et al.* (2006) in Benin City, South-South Nigeria but disagrees with that of Adamu *et al.* (2009) who observed a low resistance rate of isolates against the quinolones. This goes to show that regional differences could play a role in the resistance profile of bacteria and further justifies the need to undertake regular antibiotic susceptibility studies on bacterial isolates from different geographical areas. The high resistance rate (94.1%) observed for streptomycin in this study is in agreement with the high resistance rate (79%)

reported by Iwalokun *et al.* (2001) but disagrees with the relatively low resistance rate (54%) reported by Sheikh *et al.*, 2003. The high level of resistance against streptomycin antibiotic is probably due to abuse of the drug since it is frequently recommended for the treatment of respiratory tract infections and is readily available across the counter in tablet form. This has led to decrease in its effectiveness with subsequent increase in resistance rate.

The observed rate of resistance of isolates to gentamycin was quite low (23%). This agrees with the reports of Yah *et al.* (2007) and Akortha and filgona (2009) who reported low resistance rates of 17.9% and 17.7% respectively for same antibiotic. This low resistance rates reported for gentamycin may be due to the fact that this drug is available in ampules and can only be administered intravenously making intake not as palatable as oral antibiotics. The results of this study confirms the *in vitro* bacteriological efficacy of gentamycin as reported by earlier workers (Olukoya and Oni, 1990 and Iwaiokun *et al.*, 2001). Gentamycin therefore remains the drug of choice for the treatment of pneumonia as most pneumococcal agents are susceptible to it. Generally high resistance rates were observed for nearly all the antibiotics tested. This has serious implications for the empiric therapy of respiratory tract infections. There is a general abuse of commonly used antibiotics by patients through the purchase of expired drugs, purchase of incomplete doses and taking of antibiotics without prescription.

The high amoxicillin resistance rate (88.1%) observed

in this study is comparable to that of other workers. A resistant rate of 92.2% and 70% were reported by Akortha and Egbule (2008) and Diano and Akano (2009) respectively. When these isolates were cured in presence of 10% SDS, 82% lost their *amx^r* gene. This is comparable to the findings of various researchers. Gonzalec *et al.* (1991) reported the curing of some Enterotoxigenic *Escherichia coli* (ETEC) with the use of acridine orange while Enabulele *et al.* (1993) reported a curing rate of 13.6% among some Gram negative bacteria using same curing agent. Similarly, Akortha and Egbule (2008) and Akortha and Filgona (2009) reported a curing rate of 79% and 76% respectively with the use of 75µg/ ml acridine orange. Other types of curing agents which had been successfully used by researchers include proflavin, phenolic compounds and ethidium bromide (Sheikh *et al.*, 2003).

Elimination of plasmids with dyes inhibits plasmid replication and results in plasmid free segregants during subsequent cell division (Akortha and Aluyi, 2002). Curing of resistance plasmids with dyes gives an indication that the mechanism of resistance is plasmid-mediated. This resistance mechanism is of great importance in the chemotherapy of infections since high rate of antibiotic resistance is usually caused by of horizontal transfer of resistance determinants.

Pneumonia with a global burden on high mortality rate is a continuous and tangible threat in which prompt action and research are urgently required. Antibiotic resistance is on the increase and rapid diagnosis, adequate and timely treatment can avoid the spread of resistance agents thereby reducing morbidity and mortality rates.

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