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STUDIES ON *Streptococcus pneumoniae* ASSOCIATED WITH PULMONARY INFECTION AMONG HIV CLIENTS ATTENDING AMINU KANO TEACHING HOSPITAL (AKTH)

Mahmud,[1] A. H., Dutsinma, U. A.[1] and Idris, A. M. [2]
[1]Department of Microbiology, Faculty of Life Sciences, Bayero University, Kano Nigeria
[2]Department of Medical Microbiology and Parastology, Faculty of Clinical Science, Bayero University, Kano Nigeria
Tel: +2348103927920: abdulrahmanhabib01gmail.com

ABSTRACT
Respiratory infection is highly prevalent in HIV-infected patients and S. pneumoniae was a leading cause. The aim of this study was to isolate and identify Streptococcus pneumoniae associated with pulmonary infection among HIV Clients Attending Aminu Kano Teaching Hospital (AKTH). A total of 300 samples comprising sample from 180 HIV seropositive patients and 120 HIV seronegative patients as a (control) were enrolled in this study.) For the 180 HIV seropositive patients, 105(58.3%) were females, and 75(41.7%) were males. Among the HIV negative subjects 64(53.3%) were males and 56(46.7%) were females. The incident of Streptococcus pneumoniae isolates associated with pneumonia in HIV/AIDS seropositive patients in relation to age group in this study showed the higher prevalence of 15(51.72%), among the age groups of 30-39, had the highest of prevalence of 15(51.72%), which occur more common in HIV infected patients while for HIV seronegative patients age group 10-19 had the highest incidence of 7(29.17%).Result showed no significant difference in the prevalence of S .pneumoniae among the HIV seropositive and seronegative patients (P > 0.05) .Antimicrobial susceptibility showed that Ciprofloxacin 25(86.2%), was found to be most active invitro against the isolates followed by Oxacillin 21(72.4%), Augmentin19 (65.5), Chloramphenicol 15(51.7%). Tetracycline, 14(48.3%), Clindamycin 14(48.3) and Erythromycin 10(34.5%). Result showed no significant difference in the levels of antibiotic susceptibilities and resistance observed (p > 0.05). The CD_4 cells counts for the HIV seropositve patients were 161(89.4%) 350 cells/ml and 19(10.6%) <350 cells/m recorded as normal and low respectively. There was significant difference (p <0.05) for the CD4 cells counts of the study population with the prevalence of Streptococcus pneumoniae isolates among the HIV seropositive patients (P 0.000 <0.05). PCR amplification for Streptococcus pneumoniae penicillin resistance genes showed that Only 2(33.3%) out of 6 resistant isolates possed the pneumococcal pbp2b resistance gene. According to the findings, the pbp2b gene can play a role of fundamentalimportance in the resistance of S. pneumoniae. Modify heterogeneity in antibiotic use, which several antibiotics are taken in a rotation against taking just 1 antibiotic such that isolates resistant to 1 antibiotic are killed when the subsequent antibiotic is taken.

Keywords: HIV/AIDS, Streptococcus Pneumoniae, Antibiotic Resistance, Minimal Inhibitory Concentration (MIC), Pbp2b CD4 cells count.

INTRODUCTION

Human immunodeficiency virus (HIV) infection and AIDS increase the risk of invasive pneumococcal disease (IPD) (Cohen *et al*., 2010). Underlying HIV infection is an important risk factor for pneumonia morbidity and mortality (Theodoratou *et al*., 2014). *Streptococcus pneumoniae* is the leading bacterial opportunistic infection in HIV positive Over two million People die each year worldwide and it is estimated that up to 1 million of these deaths are caused by the bacteria *S. pneumoniae,* and over 90% of these deaths take place in developing countries. The incidence of bacterial pneumonia in HIV-infected patients individuals (Thornhill *et al*., 2014).It is well known that patients with HIV have an increased risk of respiratory tract infections. Pneumonia carries a high mortality rate in immune compromised patients (Adeleye *et al*., 2008). Pneumonia is common, occurring in all age groups, and is a leading cause of death among the young, the old, and the chronically ill patients (Rano *et al*., 2008).

ranges from 1.93 to 19.2% cases per 100 patients per year. The effect of HIV on the immune system is monitored by measuring the CD4 (T-helper) lymphocyte count in the blood. Depletion of CD4 cell count is a hallmark of disease progression in AIDS. CD4 cell count is essential to decide about the timing of initiation of antiretroviral therapy and for prophylaxis of opportunistic infections (Huang *et al.*, 2010). The pandemic has lead to increased morbidity and mortality and created widespread resistance of bacteria to antibiotics among populations. Pneumonia continues to be an important problem in HIV infected patient (Ochei and Kolkhtar, 2008). The objective of the study is to determine the prevalence of *Streptococcus pneumoniae* associated with pulmonary infection among HIV/AIDS seropositive clients attending Aminu Kano Teaching Hospital (AKTH).

MATERIALS AND METHODS

Sampling Population and Ethical Clearance

Ethical clearance was obtained from the Ethical committee of Aminu Kano Teaching Hospital (AKTH). HIV patients were recruited from Imam Wali Medical Center for virology (AKTH) Kano state Nigeria. Control were healthy employees with negative serology or no known risk factors for HIV infection but suspected to be Pneumonia patients.

Sample Size

Sample size was calculated to be 300 (180 from HIV/AIDS seropositive patients and 120 samples from HIV negative seronegative patients as a control) based on previously published studies by Ojo and Oluyege (2014) using Open Epi version 2.3 statistical software.

Inclusion Criteria

For the HIV seropositive patient (i) Consented to participate in the study (ii) Coughing
For the seronegative patients (i) Consented to participate in the study (ii) Coughing

Culture and Processing of Sample

Using a sterile microbiological loop a muco purulent part of the sputum sample was picked and inoculated onto blood agar and chocolate agar. The plates were incubated overnight at 37^0C. The colonies were identified using their growth patterns and colonial qmorphology on the agar plates. The organisms were identified by the appropriate biochemical tests such as Optochin test, Bile solubility test.

Antibiotic Susceptibility Testing

The following antibiotic disks were used:- Tetracycline (30µg), Chloramphenicol (30µg), Erythromycin (15µg), Ciprofloxacin (5µg), Clindamycin (2 µg), Oxacillin (1µg) and Augmentin (30µg). Antibiotics susceptibility testing was performed according to standard procedures by CLSI (2010) (Disc agar diffusion method).

Disk diffusion

All isolates were tested for antibiotic susceptibility using the disk diffusion method according to CLSI standard with seven antimicrobial disks(Oxoid) Identified bacterial colonies were emulsified in sterile distilled water to conform to 0.5 McFarland turbidity standard, which was then diluted ten times to give a density of approximately 106 CFU per ml for inoculation on Mueller Hinton agar (OXOID, UK). Using a sterile cotton swab, the suspension was uniformly swabbed on Muller Hintoin agar (MH) (Pronadisa, Conda Laboratories, S.A).

Polymerase Chain Reaction (PCR)

Isolates of *S. pneumoniae* found to be resistant to oxacillin antibiotics were removed from the culture medium through scraping with a platinum spatula and placed into a tube with 50 ml of distilled water for extraction of bacterial DNA. Oligonucleotide initiators (primers) were used in the selection of initiators derived from the lytA gene of *S. pneumoniae* and from the genes encoding pbp2b of oxacillin antibiotics . Described as follows: 5 ml of the buffer specific for Taq DNA polymerase, 2 mM of $MgCl_2$, 1 U of Taq DNA polymerase 5 U/ml (Gibco, Gaithersburg, MD, USA),200 mM of an equimolar mixture of nucleotide triphosphates and 50pmol of the forward primer (pbp2b-F) CTTTGTCCCAGGTTCGGTTG and Reverse primer (pbp2b-R) CCCAAGCCATATTCGCCAAA of the two pairs of primers. For the preparation of the sample to be amplified positive controls, were used.Two 2 ml of the DNA harvested from *S. pneumoniae* were mixed with 48 ml of the master mixture. Regarding the amplification cycles, the samples prepared with the respective positive and negative controls were placed in the thermo cycler and submitted to 35 cycles, after an initial denaturation at 94°C for 3 minutes. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 2 minutes. After the 35 amplification cycles. Agarose gel stained with 0.05(%) ethidium bromide was prepared with wells and mounted in an electrophoretic tank.

Visualization of the bands was done using ultraviolet transilluminator (UVP, Upland USA) and pictures of the gel were taken using transmitted illumination camera fitted with a black and white Polaroid film (Sedigheh, *et al.*, 2017).

Primers used for Amplification of Resistance genes of Penicillin pbp2b. The pbp2b Gene was Successfully Amplified by PCR.

Gene	Primer Sequence (5'!3')	Product, Length, bp
pbp2b-F	CTTTGTCCCAGGTTCGGTTG	320
pbp2b-R	CCCAAGCCATATTCGCCAAA	

Key: pbp2b-F **=** Forward primer
pbp2b-R = Reverse primer

Data Analysis

The data generated in this study was analyzed using Statistical Package for Social Sciences(SPSS) (Version 25.0 IBM, Huz). Chi-square analysis (2 X 2 Statistical Analysis was used to determine the Prevalence of *Streptococcus Pneumoniae* isolates between HIV seropositive patients and seronegative patients used as a control for the Study. Chi-square analysis for antimicrobial resistance and susceptibility profile patterns of *Streptococcus pneumoniae* isolates between HIV/AIDS seropositive and HIV seronegative patients, while correlation was used for the CD4 cells counts of the study population with the prevalence of *Streptococcus pneumoniae* isolates in HIV seropositive patients.

RESULTS

Gender Distribution of Subjects

Three hundred (300) samples used for the study, made up of 180 HIV seropositive and 120 HIV seronegative patients as a control. Highest number of participants were obtained between the age groups 30 – 39 years 75 (41.7%), 10- 19 years 7(29.17%), 40 – 59 years 48 (26.7%) and 25 (20.8%) in both HIV seropositive and HIV seronegative respectively. Married participants from both groups have the highest number with 93(51.7%), 74(61.7%) respectively, while the least number were found in widow participants with 15(8.3%), 5(4.2%) respectively. 145(80.6%) were already on treatment for HIV seropositve patients. Also for cough Related infection, both HIV seropositive and HIV seronegative patients almost all patients were reported with a cough related infection as follows 179 (99.4%) 1 (0.6%) 120 (100.0%) respectively. Lastly the CD_4 cells counts for the HIV seropositve patients were 161(89.4%) >350 cells/ml and 19(10.6%) <350 cells/m recorded as normal and low respectively (Table 1).

Among the HIV seropositive patients, (29) bacterial isolates of *Streptococcus pneumoniae,* were isolated. Highest prevalence were obtained in the age group 30-39 15(51.72), 40-49 4(13.79), 50-59 4(13.79), 20-29 3(10.34), 60-69 2(6.81), 10-19 1(3.45) and 70-79 0(0.00) respectively . Among the HIV seronegative patients, (24) bacterial isolates of *Streptococcus pneumoniae,* were isolated were obtained in the age group. Highest prevalence were 10-19 7(29.17), 40-49 6(25.00), 20-29 4(16.67), 50-99 4(16.67), 60-69 2(8.33), 30-391(4.17) and 70-79(0.00) respectively (Table 2)

Percentage of antibiotics sensitivity profile for *S. pneumoniae* isolates for HIV seropositive patients. It shows that Ciprofloxacin 25(86.2%), was most active invitro against the isolates followed by Oxacillin 21(72.4%),Augmentin19 (65.5), Chloramphenicol 15(51.7%), Tetracycline,14(48.3%), Clindamycin14(48.3) and Erythromycin 10(34.5%). Also the resistance profile for *S. pneumoniae* isolates for HIV seropositive patients. From the result obtained isolates showed a high rate of resistance of erythromycin 17(58.6%), followed by Chloramphenicol,12(41.4%), Tetracycline9(31.0%), Clindamycin 8(27.6), Augmentin7(24.1) and Oxacillin 6(20.7%) while the least was Ciprofloxacin 1(3.5%). Lastly the result for the intermediate antibiotics profile for *S. pneumoniae* isolates from HIV seropositive patients. From the result it shows that Clindamycin 7(24.1) Tetracycline 6(20.7%), Ciprofloxacin3(10.3%) Augmentin 3(10.3) was found out to be more effective than Chloramphenicol 2(6.9%), Erythromycin 2(6.9%) and Oxacillin 2(6.9%) respectively (Table 3).

Percentage of antibiotics sensitivity profile for *S. pneumoniae* isolates for HIV seronegative patients. It showed that Oxacillin20(83.3%), Augmentin 20(83.3%), Ciprofloxacin18(75.0%), Clindamycin 15(62.5), Erythromycin13(54.2%) Tetracycline 11(45.8%) were highly sensitive while the least was Chloramphenicol l7(29.2%) in HIV seronegative patients, respectively. Also the resistance profile for *S. pneumoniae* isolates from HIV seronegative patients.

It also showed that Chloramphenicol has a highly rate of resistance 10(41.7) followed by Tetracycline 8(33.3), Erythromycin 7(29.2) Clindamycin 7(29.2) Ciprofloxacin 4(16.7) while the least were Oxacillin 3(12.5) and Augmentin 3(12.5) respectively. Lastly the result for the intermediate antibiotics profile for *S. pneumoniae* isolates from HIV seronegative patients. From the result it showed that Tetracycline 5(20.8), Chloramphenicol, 7(29.2), Erythromycin,4(16.7) were more intermediate than Oxacillin, 3(12.5), Ciprofloxacin 2(8.3) Clindamycin 2(8.3) and Augmentin 1(4.2) in HIV seronegative patients respectively. (Table 4)

The pbp2b gene was successfully amplified by PCR. Only two(2) (33.3%) out of six(6) resistant isolates were observed penicillin resistance genes that detect the pneumococcal pbp2b genes. Primers were used in the selection of initiators derived from gene of *S. pneumoniae* and from the genes encoding pbp2b of penicillin-resistant gene of *S. pneumoniae.* The positive samples produced a visible band in the following sizes: pbp2b, 320 primers Figure I

Table 1 : Demographic information of subject enrolled among (HIV Seropositive and Seronegative Patients)

Variables	HIV Pos No (%) N=180	HIV Neg No (%) (Control) N=120
Age Group (years)		
10 – 19	1 (0.6)	33(27.5)
20 – 29	14 (7.8)	16 (13.3)
30 – 39	75 (41.7)	12 (10.0)
40 – 49	48 (26.7)	25 (20.8)
50 – 59	29 (16.1)	17 (14.4)
60 – 69	13 (7.2)	15 (12.5)
70 – 79	0 (0.0)	2 (1.7)
Sex		
Male	75 (41.7)	64 (53.3)
Female	105 (58.3)	56 (46.7)
Marital Status		
Single	72 (40.0)	41 (34.2)
Married	93 (51.7)	74 (61.7)
Widow	15 (8.3)	5 (4.2)
Cough Related infection		
Yes	179 (99.4)	120 (100.0)
No	1 (0.6)	0 (0.0)
On Treatment		
Yes	145 (80.6)	120 (100.0)
No	35 (19.4)	0 (0.0)
CD_4 Count		
Normal	161 (89.4)	Nil
Low	19 (10.6)	Nil

Key: Pos = Seropositive No= Number Neg= Negative % = Percentage

Table 2: Distribution of *S. pneumoniae* in relation to age groups between of HIV seropositive and seronegative patients (control)

Age groups (years)	Number	(%) (+VE)	Number	(%)(-VE)
10-19	1	3.45	7	29.17
20-29	3	10.34	4	16.69
30-39	15	51.72	1	4.17
40-49	4	13.79	6	25.00
50-59	4	13.79	4	16.67
60-69	2	6.81	2	8.33
70-79	0	0.00	0	0.00
Total	**29**	**100**	**29**	**100**

.Key
No=Number
% =Percentage

Table 3: Antimicrobial Susceptibility Patterns of *Streptococcus pneumoniae* Isolates from HIV/AIDS Seropositive Patients at AKTH

Antibiotics (µg)	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Tetracycline (30)	14 (48.3)	6 (20.7)	9 (31.0)
Cholrampinicol (30)	15 (51.7)	2 (6.9)	12 (41.4)
Erythromycin (15)	10 (34.5)	2 ((6.9)	17 (58.6)
Ciprofloxacin (5)	25 (86.2)	3 (10.3)	1 (3.5)
Oxacillin (1)	21 (72.4)	2 (6.9)	6 (20.7)
Augmentin (30)	19 (65.5)	3(10.3)	7(24.1)
Clindamycin (2)	14(48.3)	7(24.1)	8(27.6)

Table 4: Antimicrobial Susceptibility Patterns of *Streptococcus Pneumoniae* Isolates From HIV/AIDS Seronegative Patients At AKTH. (Control)

Antibiotics (µg)	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Tetracycline (30)	11(45.8%)	5(20.8)	8(33.3)
Chlorampinicol(30)	7(29.2%)	7(29.2)	10(41.7)
Erythromycin (15)	13(54.2%)	4(16.7)	7(29.2)
Ciprofloxacin (5)	18(75.0%)	2(8.3)	4(16.7)
Oxacillin (1)	20(83.3%)	3(12.5)	1(4.2)
Augmentin (30)	20(83.3)	1(4.2)	3(12.5)
Clindamycin (2)	15(62.5)	2(8.3)	7(29.2)

Key: No=Number
% =Percentage

Amplification of Resistance Genes Pbp2b by PCR

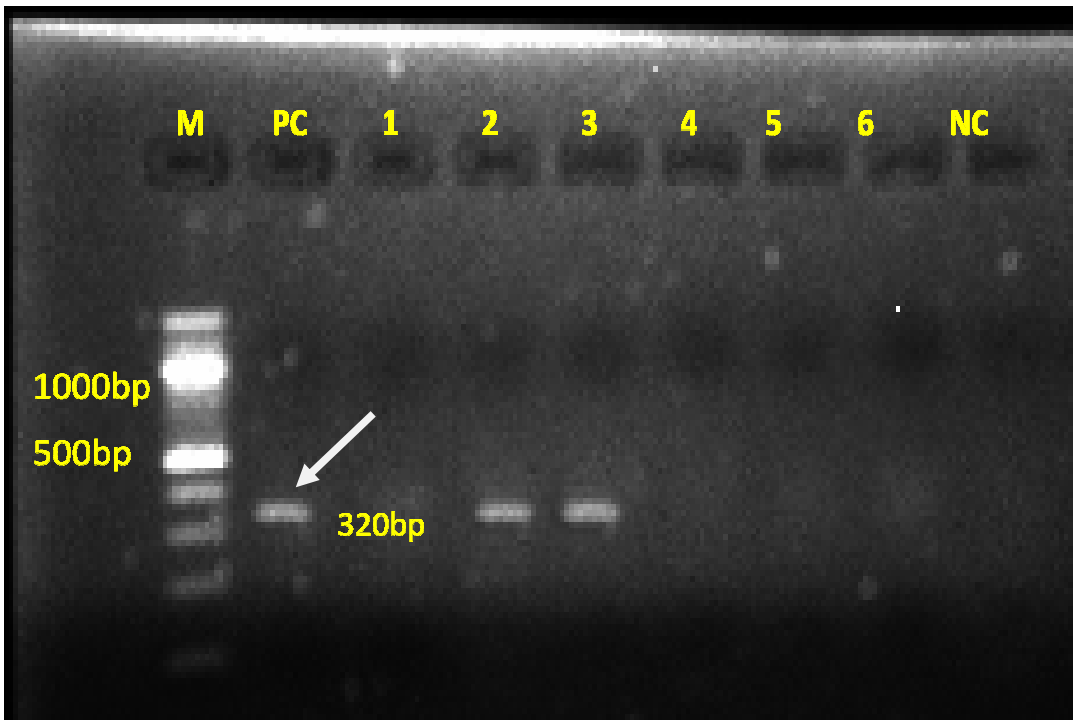

Plate III. Agarose Gel Electrophoresis for PCR - Amplified Fragments of the Pbp2b Genes
Key
M = Ladder 1000
Bp; PC = Positive Control
NC = Negative Control

DISCUSSION

Pneumonia is a common illness in all parts of the world. *Streptococcus pneumoniae* remains a leading cause of morbidity and mortality in HIV infected patients (Obaro, 2009). The prevalence of *Streptococcus pneumoniae* isolates used for this study in both HIV seronegative and HIV seropositive patients were 29(16.1%) and 24(20.0%) respectively. There was no significant difference with the prevalence of *S .pneumoniae* among the HIV seropositive and seronegative patients ($P > 0.05$). Shailaja *et al*. (2004) however had a higher prevalence of 44.28% amongst 100 HIV seropositive patients.The prevalence value here is much higher considering the fact that they studied HIV seropositive patients who were not on any antibiotic prophylaxis and also worked on 30 HIV seronegative patients as controls but failed to report the prevalence value in this category of patients.

The antibiotic susceptibility profile patterns for both HIV seropositive patients and HIV seronegative patients tested in this present study showed that Ciprofloxacin 25(86.2%), was most active invitro followed by Oxacillin 21(72.4%), Augmentin 19(65.5), Chloramphenicol 15(51.7%), Tetracycline, 14(48.3%), Clindamycin 14(48.3) and Erythromycin 10(34.5%) for HIV seropositive patients respectively. While for HIV seronegative patients antibiotic susceptibility profile showed that Oxacillin 20(83.3%), was most active followed by Augmentin 20(83.3%), Ciprofloxacin 18(75.0%), Clindamycin 15(62.5), Erythromycin 13(54.2%) Tetracycline 11(45.8%) in HIV seronegative patients, respectively. There was no significant difference in the levels of antibiotic susceptibilities and resistant observed ($p > 0.05$). These imply that the HIV status of patients did not influence antimicrobial susceptibility of the *Streptococcus pneumoniae* isolates among the HIV/AIDS seropositive and HIV seronegative Patients. This also agreed with work described by Ndip *et al*. (1995) who had previously reported that sensitivity to penicillin and erythromycin by *S. pneumoniae* isolates in 1995. Also in hospital-based study by Akanbi *et al.* (2004) in 2002 in Ilorin, Nigeria, the recovered *S. pneumoniae* isolates were resistant to the tested antibiotics, including penicillin (83%), erythromycin (56.6%), ceftriaxone (28%), ciprofloxacin (20%) and ampicillin (73.8%).

From the study is was found out that the CD_4 cells counts for the HIV seropositive patients were 161(89.4%) 350 cells/ml and 19(10.6%) <350 cells/m recorded as normal and low respectively. HIV-infected persons with low CD4 counts may be at highest risk for colonization and consequently for invasive disease. CD4 counts less than 350 have been associated with increased risk of invasive disease in some studies as reported by (Feikin *et al,*. 2004). In this study, t- test analysis was carried out to correlate the CD4 cells counts of the study population with the prevalence of *Streptococcus pneumoniae* isolates among the HIV seropositive patients. There was significant difference ($p <0.05$) for the CD4 cells count of the study population with the prevalence of *Streptococcus pneumoniae* isolates among the HIV seropositive patients. In Contrary to the publicized reports by Nicoletti *et al.*(2007)who found that neither the CD4 nor the level HIV of viral load was significantly associated with pneumococcal infection among the HIV patients.

In our study, only two (2) (33.3%) out of six(6) resistant isolates were observed to posses penicillin resistant genes that detect the pneumococcal pbp2b. The pbp2b gene was not detected in four (4) resistant pneumococci in our study, the source of resistance to penicillin in these strains can be caused by mutations in other genes that code the residual PBPs.

Penicillin resistant *streptococcus pneumoniae* (PRSP) isolates containing the pbp2b gene were the most frequently found.. It was found by Habibian *et al.* (2013) that the pbp2b gene was found in 4 of the total 10 samples (Habibian *et al*., 2013). Also Kotevska *et al*., (2009) reported that of the total 40 resistant/ intermediate resistant *S.pneumoniae,* 22 genes pbp2b and/or pbp2x were verified . This is higher than 4% from Sweden (Skovbjerg *et al.,* 2013), 7.2% from Germany (Imohl *et al.,* 2015), 7.8% reported from India (Song *et al.,* 2004), but less than 36.9% from Spain (Fenoll *et al.,* 2000), 27.95% from South Korea (Song *et al.,* 2015), 22.8% from France (Lee *et al,.* 2010), with the least being 94.5% reported from Hamadan, Iran (Cohen *et al,.* 2002). Previous researches have obviously pointed out that the three penicillin–binding proteins (PBP 1a, 2x and 2b) are important for the resistance to beta-lactams.

CONCLUSION

In this study, there was no significant difference in the prevalence of *S .pneumoniae* among the HIV seropositive and seronegative patients ($P > 0.05$). But the age group (30-39) years was recorded the highest prevalence of *Streptococcus pneumoniae* isolates in HIV seropositive patients and the age group (10-19) years recorded the highest prevalence of *Streptococcus pneumoniae* isolates in HIV serongative patients.

This age group are active and therefore exposed to various predisposing factor(s). Result also showed that *Streptococcus pneumoniae* isolates was susceptible to the antibiotics tested in order Ciprofloxacin > Oxacillin > Augmentin >Chloramphenicol > Tetracycline> Clindamycin and Erythromycin. On the other hand the isolates are resistant to antibiotics in order Erythromycin > Chloramphenicol, Tetracycline, >Clindamycin, >Augmentin > Oxacillin > Ciprofloxacin. There was no significant difference in the levels of antibiotic susceptibilities and resistant observed ($p > 0.05$). The CD_4 cells counts for the HIV seropositve patients were 161(89.4%) 350 cells/ml and 19(10.6%) <350 cells/m recorded as normal and low respectively. There was significant difference ($p < 0.05$) for the CD4 cells counts of the study population with the prevalence of *Streptococcus pneumoniae* isolates among the HIV seropositive patients. PCR implication revealed out that Only two(2) (33.3%) out of six(6) resistance isolates were observed penicillin resistance genes pneumococcal pbp2b genes

RECOMMENDATIONS

1. Most of the *Streptococcus pneumoniae* associated infection in AIDS clients, go undetected hence the importance of this study. Patients presenting with respiratory failure should undergo adequate analysis and examined before critical care for treatment and support to know exact causative agent especially in immune compromised individuals.
2. Antibiotic susceptibility testing should be done routinely in all *S.pneumoniae* clinical isolates from HIV patients, regardless of their source.
3. Patients with <350 CD4 cells are at a greater risk for pneumococcal disease and are less likely to have an adequate antibody response to pneumococcal immunization. Therefore pneumococcal vaccination should be given as early as possible after HIV infection is diagnosed .
4. In order to curb the problem of antibiotic resistance, indiscriminate use of antibiotics and over the counter sales of antibiotics should be discouraged and avoided.

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