Prevalence, Haemolytic Activities and Flouroquinolones Susceptibility Profiles of *Moraxella catarrhalis, Streptococcus pneumoniae and Haemophilus influenzae* Associated with Acute Otitis Media.

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Abstract: This study was designed to determine the prevalence, haemolytic activities and flouroquinolones susceptibility profiles of *Moraxella catarrhalis, Streptococcus pneumoniae and Haemophilus influenzae* associated with acute otitis media. 272 middle-ear swabbed samples were aseptically collected and analyzed using standard microbiological and disk diffusion methods for antibiotic susceptibility. Haemolytic activities were determined using blood agar. Significant difference at ($p<0.05$) was observed among 124 (45.6%) male patients and 148 (54.4%) female patients with acute otitis media. 51(18.8%) *Moraxella catarrhalis*, 58 (21.3%) *Haemophilus influenzae* and 47(17.3) *Streptococcus pneumoniae* were identified. The flouroquinolones susceptibility of these bacteria showed that 80-84% of the *Moraxella catarrhalis* were sensitive to ciprofloxacin, levofloxacin and moxifloxacin, while 29.4% of *Moraxella catarrhalis* were resistant to ofloxacin. The resistance rate of *Streptococcus pneumoniae* to the flouroquinolones varied from 29.8 % for levofloxacin to 42.6% for ciprofloxacin. 42 (26.9%) of the isolates were resistant to at least 2 antibiotics with multiple antibiotics resistance index ranging between 0.25 to 0.75 in *Moraxella catarrhalis* and 0.25 to 1.00 in both *Haemophilus influenzae* and *Streptococcus pneumoniae* . Haemolytic activities of these isolates showed that for *Moraxella catarrhalis*, 7 strains (13.7%) showed α-haemolysis, 27 (52.9%) β- haemolysis, and ($\alpha + \beta$) hemolysis occurred in 3 (5.9%). *Haemophilus influenzae* and *Streptococcus pneumoniae* showed 9 (15.5%), 28 (59.6%) α-haemolysis, respectively. *Haemophilus influenzae* also showed 21 (36.2%), 2 (3.4%) and 26 (44.8%) β, $\alpha + \beta$ and γ. respectively, while 3 (6.4%) *Streptococcus pneumoniae* showed β- haemolysis. Despite the effectiveness of flouroquinolones especially levofloxacin and moxifloxacin against these bacterial isolates, prudent use of the antibiotics is strongly recommended.
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Key Words: flouroquinolones, *Moraxella catarrhalis, Streptococcus pneumoniae*, *Haemophilus influenzae*, Otitis media, Susceptibility.

INTRODUCTION:

Otitis media is an inflammation of the middle ear and may be associated with a collection of liquid in the middle ear (Todd, 1995). The inflammation of the middle ear is due to pathogenic micro-organisms that are resident there (Ekpo *et al*., 2009; Akinjogunla and Enabulele, 2010). Otitis media may be acute otitis media (AOM), acute suppurative otitis media (ASOM) or chronic suppurative otitis media (CSOM) (Bluestone, 1998). Sources of infection in acute otitis media is solely dependent on the route by which infection reaches the middle ear and the chief route by which this occurs is through the eustachian tube (Daly,1997). The eustachian tube normally drains middle ear secretions, protect the middle ear from nasopharyngeal secretion, sound pressure, and ventilate the middle ear to equilibrate air pressure. (Bluestone, 1983; Todd, 1995). The physiology of children's eustachian tubes can predispose them to otitis media because their eustachian tube is shorter, straighter, made up of more flaccid cartilage, more horizontal than adults and also they have not developed the same resistance to bacteria as found in adults (Bluestone and Klein, 2001; Akinjogunla and Enabulele, 2010; Ihsan *et al*., 2010).

Over two billion dollars of the annual cost of health care in the United States of American is attributable directly or indirectly to otitis media, where as in Nigeria the importance of otitis media infections have not been fully established (Akinjogunla and Enabulele, 2010). The patients with otitis media present the classic "earache", pain that is severe, continuous and is often accompanied by fever (Damoiseaux, 2005; Ekpo *et al*., 2009). Otitis media is continuum of acute and chronic manifestations that can have long term sequelae for the patients. The long term sequelae of otitis media include: mild to moderate hearing loss, impaired cognitive

development, tympanic membrane perforations, facial paralysis, meningitis, and brain abscess (Damoiseaux, 2005; Rovers *et al.*, 2006).

Moraxella catarrhalis is a Gram-negative non-encapsulated diplococcal bacterium belonging to the family *Moraxellaceae*. The genus *Moraxella* actually comprises both coccoid and rod-shaped bacteria, and the classification of *M. catarrhalis* has been rather complex, the bacterium being alternatively named *Branhamella catarrhalis*, *Moraxella* (*Branhamella*) *catarrhalis*, and now the preferred *Moraxella catarrhalis* (John, 2009). *M. catarrhalis* is generally associated with upper respiratory tract infections in children and lower respiratory tract infections in adults. *M. catarrhalis* has been regarded as the third most important bacterial agent of acute otitis media in children, after *S. pneumoniae* and *H. influenzae* (Bluestone *et al.* 1992; Turner *et al.*, 2002; Gunasekera *et al.*, 2008). *Streptococcus pneumoniae* is Gram-positive coccus, 1-2 um in diameter, typically in pairs. *S. pneumoniae* causes serious invasive infections such as septicaemia and mild upper respiratory infections. It belongs to the normal nasopharyngeal microbial flora that consists of bacteria with physiologic and genetic properties suitable for colonization and multiplication under certain conditions. *Haemophilus influenzae* is a Gram-negative pathogen causing respiratory tract infection and also an etiologic agent of morbid-mortality among children under five years old, both in developed countries and in Latin American countries. (Giebink, 1989; Peltola, 2000; Nicholson *et al.*, 2002)

Recent development in the treatment of patients with acute otitis media include the evidence of the efficacy of newer topical fluoroquinolones (Bearden and Danziger, 2001; Loy *et al.*, 2002**)**. The introduction of the Fluoroquinolones (FQs) about three decades ago provided clinicians with a class of broad-spectrum agents applicable to a range of bacterial infections (Hooper, 1998). Flouroquinolone antibiotics target DNA gyrase and topoisomerase IV by disrupting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication. Recently, several treatment failures with fluoroquinolones have been reported due to decreased susceptibility to ciprofloxacin. The mechanisms of resistance predominate with flouro-quinolones are: alterations in target enzymes, bacterial cell permeability, and drug efflux mechanisms (Bearden and Danziger, 2001). There is paucity of information especially in this environment on the efficacies of flouroquinolones in treating acute otitis media. Thus, this study was aimed at determining the prevalence, heamolytic activities and susceptibility to flouroquinolones of *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* isolated from acute otitis media in order to update data and evaluate best therapeutic indications for the antimicrobial drugs.

MATERIALS AND METHODS

COLLECTION OF PATIENTS SAMPLES:

Non duplicate middle-ear swabbed samples were collected under aseptic conditions from 272 patients (124 males and 148 females) with acute otitis media attending University of Uyo Teaching Hospital; General Hospital Ikot- Ekpene; St. Luke Hospital, Anua and Nekede Paediatric Hospital in Akwa Ibom State between January, 2009 and December, 2010.

PREPARATION OF MEDIA AND BACTERIOLOGICAL EXAMINATION:

The middle-ear swabbed samples were cultured aerobically on Blood agar (BA), Chocolate agar (CA) and MacConkey agar (MCA). The powdered medium was mixed with distilled water and steamed to dissolve the agar. The mixture was then sterilized in an autoclave at 121° C and subsequently allowed to cool to about 45° C. About 15 to 20 mls of the sterilized molten agar medium were poured into sterile Petri dishes and left undisturbed until the agar were set. Blood agar was made by mixing sterilized molten nutrient agar at about 45°C- 50°C with 2mls of blood before pouring into the plates, while Chocolate agar was made by heating blood agar to 70°C - 80°C until it became chocolate brown in colour. The middle-ear swabbed samples were inoculated into broth cultures for 4-6hrs and later inoculated onto plates of Blood agar (BA), Chocolate agar (CA) and MacConkey agar (MCA). The plates were incubated aerobically at 37° C for 24 hours. After overnight incubation, the colonies on the positive plates were sub-cultured onto nutrient agar slants and routine conventional laboratory techniques including Gram staining, motility, oxidase, catalase, coagulase, urease production, indole production, citrate utilization, methyl red, DNAse Production, Voges-proskauer tests and carbohydrate fermentation tests such as mannitol, sucrose, glucose and lactose were carried out.

ANTIBIOTIC SENSITIVITY TESTING

The antibiotic sensitivity of these bacterial species isolated from the acute otitis media samples was performed by disk diffusion method (DDM) on Muller-Hinton agar plates as described by the National Committee for Clinical Laboratory Standards. 0.1 ml of each bacterial isolates was seeded into each of the Petri dishes containing Mueller-Hinton agar and were allowed to stand for 30 mins to enable the inoculated

organisms to pre-diffuse. The commercially available discs containing the following antibiotics**:** Ofloxacin (Ofl,5ug), ciprofloxacin (Cip, 5ug), levofloxacin (Lev, 5ug) and Moxifloxacin (Mox,5ug) (Oxoid, UK) were aseptically placed at reasonable equidistance on the surfaces of the Muller-Hinton agar plates with a sterile forceps and were incubated for 18 - 24hrs at 37°C. Zones of inhibition after incubation were observed and the diameters of inhibition zones were measured in millimeters. The interpretation of the measurement as sensitive and resistant was made according to the manufacturer's standard zone size interpretive manual. The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR' was* percentage resistance, '*a*' was the number of resistant isolates and '*b*' was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS' was* percentage sensitivity, '*c*' was the number of sensitive isolates and '*d*' was the number of isolates tested with the antibiotic.(Akinjogunla et al 2010)

MULTIPLE ANTIBIOTIC RESISTANCE INDEX (MAR)

Multiple antibiotic resistance index (MAR) was determined using the formula MAR=*x/y*, where *x* was the number of antibiotics to which test isolate displayed resistance and *y* is the total number of antibiotics to which the test organism has been evaluated for sensitivity (Akinjogunla and Enabulele, 2010).

HAEMOLYTIC ACTIVITIES OF THE BACTERIAL ISOLATES

The haemolytic activity of the bacterial species was identified by the presence of clear (β-hemolysis) or diffuse (α-hemolysis) halos around the colonies on blood agar plates prepared with Mueller-Hinton agar (MHA, Biolife, Italy) and blood . Observation of the haemolysis zone around colonies and the type of haemolysis was recorded as *α, β,* γ and double (*α + β*) after incubation for 24 h at 37ºC.

STATISTICAL ANALYSIS OF RESULTS

Graphs and tables were used for data presentation while frequencies and percentages were calculated for study variables. Chi-square (χ2) test was used to calculate probabilities and determine significance. A p-value of less than or equal to 0.05 was considered to be statistically significant (*p≤0.05*), while p-value more than 0.05 was considered to be statistically not significant (NS).

RESULTS:

The results of the morphological and biochemical characteristics of the isolates are shown in Table 1. 51 (18.8%) *Moraxella catarrhalis,* 58 (21.3%) *Haemophilus influenzae* and 47 (17.3) *Streptococcus pneumoniae* and other bacterial isolated were also obtained from a total of 272 middle ear samples collected from patients with acute otitis media during eighteen months of study (Table 2). Significant difference at ($p<0.05$) was observed among 124 (45.6%) male patients and 148 (54.4%) female patients with acute otitis media (Table 2). Table 3 showed the distribution of study population by age-group and also by rate of isolation of *Moraxella catarrhalis, Haemophilus influenzae* and *Streptococcus pneumoniae* isolated from the patients with acute otitis media. The flouroquinolones susceptibility profiles of *Moraxella catarrhalis, Haemophilus influenzae and Streptococcus pneumoniae* isolated from patients with acute otitis media are shown in (Figures 1, 2 and 3). 80-84% of the *Moraxella catarrhalis* were sensitive to ciprofloxacin, levofloxacin and moxifloxacin while 29.4% of *Moraxella catarrhalis* were resistant to ofloxacin (Fig.1). *Haemophilus influenzae and Moraxella catarrhalis* showed highest susceptibility against moxifloxacin. *Streptococcus pneumoniae* showed moderate sensitivity to moxifloxacin and ofloxacin. The resistance rate of *Streptococcus pneumoniae* to the flouroquinolones used varied from 29.8 % for levofloxacin to 42.6% for ciprofloxacin (Fig.3). Table 4a showed the highest sensitivity and resistance for *Moraxella catarrhalis, Haemophilus influenzae* and *Streptococcus pneumoniae* against flouroquinolones while Table 4b shows the estimation of susceptibility by inhibition zone diameter measurements. The inhibitory zone diameters of the flouroquinolones used ranged between 0 mm and 42 mm for all the isolates tested. The highest inhibitory zone diameter was obtained with moxifloxacin (42 mm). The results of this study also showed that 42 (26.9%) of the isolates were resistant to at least 2 flouroquinolone antibiotics and 3 (1.9%) of isolates were resistant to all 4 used flouroquinolones with multiple antibiotics resistance (MAR) ranging between 0.25 to 0.75 in *Moraxella catarrhalis* and 0.25 to 1.00 in both *Haemophilus influenzae and Streptococcus pneumoniae* (Table 5)

Haemolysis patterns of the *Moraxella catarrhalis, Haemophilus influenzae and Streptococcus pneumoniae* isolated on blood agar plates are shown in Table 6. For *Moraxella catarrhalis,* 7 strains (13.7%) showed α-haemolysis, 27 (52.9%) β-haemolysis, and double hemolysis (*α + β*) occurred in 3 (5.9%). *Haemophilus influenzae* and *Streptococcus*

pneumoniae showed 9 (15.5%), 28 (59.6%) α-haemolysis, respectively. *Haemophilus influenzae* also showed 21 (36.2%), 2 (3.4%) and 26 (44.8%) β, *α + β* and γ. respectively, while 3 (6.4%) *Streptococcus pneumoniae* showed β-haemolysis (Table 6).

Table 1: Morphological and Biochemical characteristics of *Moraxella catarrhalis, Haemophilus influenzae and Streptococcus pneumoniae* isolated from acute otitis media.

PARAMETERS	*M. catarrhalis*	*H. influenzae*	*S. pneumoniae*
Grams reaction	- /cocci	- /cocci	+/cocci
Catalase test	+	+	-
Citrate test	-	-	-
Oxidase test	+	-	-
Coagulase test	NA	NA	-
Indole test	-	-	-
Urease activity	-	-	-
Glucose	-	+	+
Lactose	-	-	-
Sucrose	-	-	-
Mannitol	-	+	+
Motility	+	+	+
Methyl Red	+	+	+
Voges Proskauer	-	-	-
DNAse production	+	NA	NA

NA: Not Applicable

Table 2: Relative frequency of *Moraxella catarrhalis, Haemophilus influenzae and Streptococcus pneumoniae* isolated from 272 patients (124 males and 148 females) with Acute Otitis Media.

Bacterial. spp.	Gender		Total
	Males No (%)	Females No (%)	No (%)
Moraxella catarrhalis	20 (16.1)	31(20.9)	51 (18.8)
Haemophilus influenzae	24 (19.4)	34 (23.0)	58 (21.3)
Streptococcus pneumoniae	19 (15.3)	28 (18.9)	47 (17.3)
Total	63 (50.8)	93 (62.8)	156 (57.4)

p≤0.05

Table 3: Distribution of study population by age-group and also by rate of isolation of *Moraxella catarrhalis, Haemophilus influenzae* and *Streptococcus pneumoniae* isolated from 272 patients with Acute Otitis Media

Age (Years)	No. of Samples Collected	No. (%) Positive for *M. catarrhalis*	No. (%) Positive for *H. influenzae*	No. (%) positive for *S. pneumoniae*	Total (%)
≤ 10	84	14 (16.7)	16 (19.0)	12 (14.3)	4(50.0)
11-20	46	9 (19.6)	9 (19.6)	12 (26.1)	30(65.2)
21-30	32	6 (18.8)	7 (21.9)	6 (18.8)	19 (59.4)
31-40	29	4 (13.8)	5 (17.2)	5 (17.2)	14 (48.3)
41-50	31	6 (19.4)	8 (25.8)	3(9.7)	17 (54.8)
51-60	17	5 (29.4)	5 (29.4)	4 (23.5)	14 (82.4)
≥ 61	12	2 (16.7)	4 (33.3)	1 (8.3)	7 (58.3)
USP	21	5 (23.8)	4 (19.0)	4 (19.0)	13 (61.9)
Total	272	51 (18.8)	58 (21.3)	47 (17.3)	156 (57.4)

Key: USP: Unspecified

Table 4a: The Highest Sensitivity and Resistance for *Moraxella catarrhalis, Haemophilus influenzae* and *Streptococcus pneumoniae* against Flouroquinolones.

Bacterial spp	Highest Sensitivity (Percentage)	Highest Resistance (Percentage)
M. catarrhalis	Moxifloxacin (84.3%)	Ofloxacin (29.4%)
H. influenzae	Moxifloxacin (77.6%)	Ofloxacin, Ciprofloxacin, Levofloxacin (31.0%)
S. pneumoniae	Levofloxacin (70.2%)	Ciprofloxacin (42.6%)

Table 4b: Estimation of Susceptibility by Inhibition Zone Diameter Measurements.

Flouroquinolones (Antibiotics)	Code	Disc Potency	Diameter of zone of inhibition (mm)		
			Susceptible	Intermediate	Resistant
Ofloxacin	OFL	5ug	≥ 21	16-20	≤ 15
Ciprofloxacin	CIP	5ug	≥ 21	16-20	≤ 15
Levofloxacin	LEV	5ug	≥ 17	13-16	≤ 12
Moxifloxacin	MOX	5ug	≥ 17	15-16	≤ 14

Table 5: Multiple Antibiotic Resistance (MAR) Index of Bacteria Isolated from acute otitis media

MAR Index	*Moraxella catarrhalis* No (%)	*Haemophilus influenzae* No (%)	*Streptococcus pneumoniae* No (%)
0.25	20 (39.2)	31 (51.7)	26 (55.3)
0.50	9 (17.6)	10 (17.2)	12 (25.5)
0.75	1 (2.0)	4 (6.9)	3 (6.4)
1.00	--	1 (1.7)	2 (4.3)

Table 6: Haemolytic Activities of *Moraxella catarrhalis, Haemophilus influenzae and Streptococcus pneumoniae* Isolated from acute otitis media

Bacterial spp.	No	Heamolysis α (%)	β (%)	α + β (%)	γ (%)
Moraxella catarrhalis	51	7 (13.7)	27 (52.9)	3 (5.9)	14 (27.5)
Haemophilus influenzae	58	9 (15.5)	21 (36.2)	2 (3.4)	26 (44.8)
Streptococcus pneumoniae	47	28 (59.6)	3 (6.4)	1 (2.1)	15 (31.9)
Total	156	44 (28.2)	51(32.7)	6 (3.8)	55 (35.3)

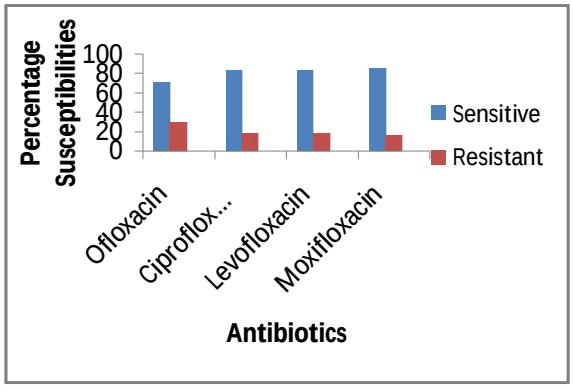

Fig 1: Flouroquinolones Susceptibility Profile of *Moraxella catarrhalis* Isolated from Acute Otitis Media

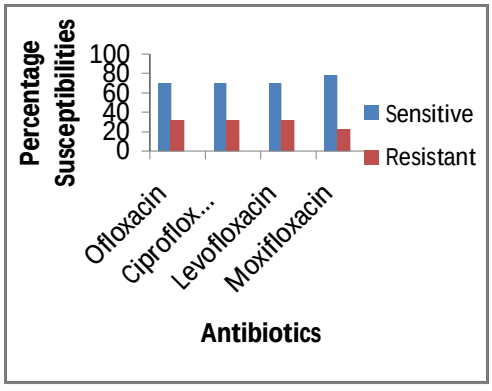

Fig 2: Flouroquinolones Susceptibility Profile of *Haemophilus influenzae* Isolated from Acute Otitis Media

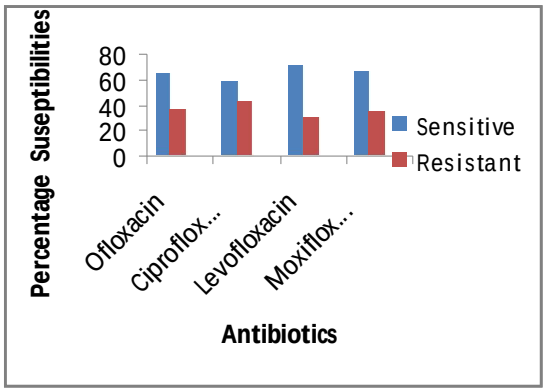

Fig 3: Flouroquinolones Susceptibility Profile of *Streptococcus pneumoniae* Isolated from Acute Otitis Media

Discussion

The results obtained from the morphological and biochemical characterization of the isolates revealed *H. influenzae, S. pneumoniae* and *M. catarrhalis* as the three most commonly encountered pathogens playing a role in etiopathogenesis of acute otitis media. Confirmation of the identity of *M. catarrhalis* in our study depended on the ability of the isolate to produce DNAse. This is similar to recommendation by Peiris et al (1993) reported that DNAse production should be tested for the definitive identification of *M. catarrhalis.* The occurrence of *H. influenzae, S. pneumoniae* and *M. catarrhalis* as the three most commonly encountered bacteria in this study is in agreement with Canter (1997), Siegel and Bien (2004), Rovers et al (2004). The results obtained are also similar to the reports in the United States of American (USA) where the most frequent pathogens were *Haemophilus* spp. and *Streptococcus* spp. (Giebink, 1987; Hoffman et al., 2003; Schleiss et al., 2004). Yamanaka et al. (2008); Bluestone and Klein (2001) showed that *H. influenzae* and *Streptococcus pneumonia* are the most prevalent organisms responsible for acute otitis media. This result differs from the reports of Oni et al. (2002) in which the predominant organisms isolated from acute otitis media were *Pseudomonas* spp. and *Proteus* spp. The isolation of *M. catarrhalis* in the middle ear of patients suffering from patient with acute otitis media in this research is also in conformity with the report by Ekpo et al. (2009). Some European studies have equally found *Haemophilus influenzae* to be the most common organism associated with acute otitis media , followed by *Streptococcus pneumoniae* and *Moraxella* (*Branhamella) catarrhalis* (Canter, 1997).

Pathogenicity of *H. influenzae, S. pneumoniae* and *M. catarrhalis* in acute otitis media are attributable to virulence factors such as haemolysis produced by these organisms and the occurrence of these virulence factors especially the haemolytic activities in these bacteria is in conformity with Cheesbrough (2004).

The activities of flouroquinolone antibiotics against *H. influenzae, S. pneumoniae* and *M. catarrhalis* isolated from acute otitis media patients attending the three hospitals showed the varied levels of susceptibility. The results of the antibiotics susceptibility in this study showed that *H. influenzae, S. pneumoniae* and *M. catarrhalis* were highly sensitive to levofloxacin and moxifloxacin. The high sensitivity of *S. pneumoniae* to both levofloxacin and moxifloxacin is in agreement with Ken et al (2008). Although, Force et al. (1995) reported ciprofloxacin to be an effective and safe therapy for acute otitis media but widely varying percentages of ciprofloxacin resistance have been reported with a global trend of increasing resistance. The *S. pneumoniae* obtained in this study showed a moderately high resistance to both ofloxacin and ciprofloxacin. The very high ciprofloxacin resistance (up to 35%) in *S. pneumoniae* may be due to the use of ciprofloxacin in ear, nose and throat infections. This resistance of bacterial spp to fluoroquinolones is predominantly associated with mutation in the chromosomal genes for DNA gyrase (gyrA) or topoisomerase IV (topo IV), and these are usually targets of action by the quinolone class. Ferra´ndiz et al. (2000) have also suggested that interspecies horizontal gene transfer between *S. pneumoniae* and viridans streptococci is a mechanism by which these bacteria gain flouroquinolone resistance.

Conclusion: This study provides the baseline reference data on the frequency of *H. influenzae, S. pneumoniae* and *M. catarrhalis* isolated from the middle ear of patients with acute otitis media in Uyo and the susceptibility of these bacteria to second and fourth generations of flouroquinolones. The management of acute otitis media and treatment failure is becoming more difficult due to increased resistance of acute otitis media pathogens. Thus, (1) newer fluoroquinolones which have excellent penetration into the middle ear fluid (MEF) and their efficacy should be assessed in various clinical trials; (2) a combination of the pharmacodynamics and pharmacokinetics of these fluoroquinolones, site of infection, and the Minimum Inhibitory Concentration (MIC) is needed to predict the in-vivo efficacy and best clinical applicability.

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