Erythromycin Resistance in Bacterial Isolates from Patients with Respiratory Tract Infections in Ikere-Ekiti, Nigeria

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Abstract

The occurrence of erythromycin resistance in bacteria causing respiratory tract infections varies among populations and is associated with inappropriate antibiotic usage. This study was undertaken to determine erythromycin resistance in bacterial isolates from patients with respiratory tract infections in Ikere-Ekiti. Bacteria were isolated from sputum specimens using standard microbiological protocols while the antibiotic susceptibility pattern of isolates was determined using the Kirby-Bauer method. Erythromycin resistance genes: mefA, ermA, and ermB were detected using multiplex polymerase chain reaction (PCR). Out of the 157 bacterial isolates identified using standard microbiological methods, Klebsiella spp. 40 (25%) was the most frequent followed by Staphylococcus aureus 34 (22%) and Proteus spp. 34 (22%) while Streptococcus spp., Escherichia coli, and Bacillus spp. had 19 (12%), 20 (13%), and 10 (6%) respectively. Most of the bacterial isolates were resistant to penicillin 148 (94%), cloxacillin 147 (93.6%), amoxicillin-clavulanic acid 141 (90%), erythromycin 141 (90%), and tetracycline 124 (79%). However, the isolates were least resistant to trimethoprim-sulfamethoxazole 57 (36%). Out of the 48 bacterial isolates investigated using multiplex PCR amplification for erythromycin resistance genes, 10 (20.8%) were positive for mefA, 3 (6.3%) were positive for ermA, and 2 (4.2%) were positive for ermB. The association between the presence of mefA, ermA, and ermB genes and erythromycin resistance was not significant (p=0.464). The presence of erythromycin resistant genes in pathogenic bacterial isolates from the respiratory tract was revealed in this study. Awareness of the risk of self-medication and abuse of antibiotics should be emphasized.

Keywords: Erythromycin, mefA, ermA, ermB, Resistance, Nigeria
1.0 Introduction

The anatomical features of the human respiratory system are subdivided into the upper respiratory tract and the lower respiratory tract (Tu et al., 2013). Most respiratory infections affect the upper respiratory tract while the lower respiratory tract involves only 5% (Velso et al., 2012). Constant exposure to airborne microorganisms causes respiratory infections. Inhalation of infectious pathogens introduced into the air via sneezing, coughing, or talking from an infected person, indirect transmission via sharing of cups, and other eating utensils with an infected person has also been implicated (Jafari et al., 2009). Respiratory tract infections (RTIs) are considered an important public health problem as they are responsible for over 50 million deaths annually in health care and community settings (Aguilar et al., 2010) which is widespread across people of all ages. The most frequently documented and common RTIs include pharyngitis, laryngitis, sore throat, bronchitis, tuberculosis, whooping cough (pertussis), emphysema, pneumonia, mastoiditis, otitis media, and the common cold, etc. Most of these infections are usually disregarded by people due to their mild, transient, and self-limiting characteristics.

Bacteria are responsible for at least 10% of upper respiratory tract infections while viruses are accountable for the remaining 90% (Lau et al., 2006). The bacterial species that are commonly implicated in RTIs include Streptococcus spp., Staphylococcus spp., Haemophilus influenzae, Klebsiella spp., Proteus spp., Acinetobacter spp., Enterobacter spp., and Pseudomonas spp. (Aly and Balkhy, 2012). Others include Corynebacterium diphtheriae, group C beta-hemolytic Streptococci, and Neisseria gonorrhoeae. Antibiotics are usually prescribed in the treatment of RTIs caused by bacteria (Gorse et al., 2009) in most cases before the final laboratory reports are available thereby exacerbating antibiotic abuse. The extensive use of antibiotics in human and veterinary medicine has increased resistance to all major antibiotic groups (Morel and Mossialos, 2010) including erythromycin (a Macrolide). The spectrum of activity of macrolides is restricted to Gram-positive bacilli and intracellular bacteria (Rickettsia and Chlamydia species), to Gram-positive and Gram-negative cocci. Generally, Gram-negative bacilli are resistant to macrolides except for Legionella spp., Bordetella pertussis, Helicobacter, and Campylobacter (Leclercq, 2002; Alli et al., 2015). The emergence of drug resistance in many pathogenic bacteria has seriously compromised the use of erythromycin. There are several mechanisms of bacteria resistance which include drug inactivation, efflux, or target site alterations. The target site for macrolides is the large ribosomal subunit (50S). Alteration of specific nucleotides in 23S rRNA within the large ribosomal subunit can be linked to macrolide resistance (Tu et al., 2005). Resistance to erythromycin can be due to drug efflux or specific site alteration by an rRNA-methylating enzyme (Coutinho et al., 2010).

The emergence of antimicrobial resistance among the common bacterial pathogens has currently complicated the therapeutic use of macrolides. Therefore, a definitive bacteriological investigation and susceptibility pattern would be necessary for efficacious treatment (Aguilar et al., 2010). The burden of respiratory diseases is not well studied particularly in developing countries where treatment of respiratory tract infection is based on empirical therapy, probably due to increased cost of laboratory services and high levels of poverty. Also, ignorance and distribution of fake drugs have likewise contributed to antimicrobial resistance (El-Astal, 2004; Osewwe et al., 2017).

Investigation of etiologic agents of RTIs and their sensitivities to available drugs are of great importance to the selection and appropriate use of antimicrobial agents (El-Astal, 2004). The study aimed to isolate and identify the bacteria that are associated with respiratory tract infections, determine their current antibiotic susceptibility patterns to the commonly used antibiotics, and to detect the erythromycin resistance genes in Ikere-Ekiti, Nigeria.

2.0 Experimental

Sample area/collection

All age groups (200 patients) with symptoms of respiratory tract infections (RTIs) attending the Medical Laboratory Department in the State Specialist Hospital, Ikere-Ekiti were enrolled in the study. Patients were instructed to take deep breaths before giving a deep cough to produce sputum samples into a well labeled, wide-mouthed screw cap sterile universal containers.

Isolation and Identification of Bacterial Isolates

The sputum samples were processed within 1 hour after collection. The samples were aseptically cultured on blood, chocolate, and MacConkey agar plates. The blood agar plates with optochin disks (5 µg) placed in the middle of secondary inoculation were used for the presumptive identification of alpha-hemolytic Streptococcus pneumoniae while chocolate agar plates were used to screen for Haemophilus influenzae. The MacConkey agar plates were used to differentiate the lactose and non-lactose fermenters. The blood agar and MacConkey agar plates were incubated aerobically at 37 °C for 24 hours while the chocolate agar plates were incubated anaerobically in an anaerobic jar at 37 °C for 24 hours. The bacterial isolates were characterized by standard microbiological methods that involved colony and cultural morphology, Gram staining reaction, and biochemical tests (Cheesbrough, 2006).

Antimicrobial Susceptibility Testing

The antibiotic susceptibility of one hundred and fifty-seven bacterial isolates was tested by spreading freshly prepared inoculum of 0.5 McFarland suspension of the bacterial isolates on Mueller-Hinton agar using the Kirby-Bauer method as described by Clinical Laboratory Standard Institute (CLSI, 2018). The antibiotics included are amoxicillin-clavulanic acid (20/10 µg), erythromycin (15 µg), tetracycline (30 µg), cinoxacin (5 µg), gentamicin (10 µg), trimethoprim/sulfamethoxazole (25 µg), chloramphenicol (30 µg) and penicillin (10 units) purchased from Oxoid Ltd. (Basingstoke Hampshire, U.K.). Plates were incubated aerobically at 37 °C for 24 hours and the inhibition zones of the isolates were measured in millimeters. Results interpretation was done according to CLSI (2018).
Detection of Erythromycin Resistance Genes

A total of forty-eight bacterial isolates showing antibiotic resistance were selected for DNA extraction using the Jena Bioscience Bacteria DNA Preparation Kit while the concentration and purity of the extracted DNA were evaluated using a NanoDrop (ND 1000) Spectrophotometer (Thermo Scientific, USA). The ermA, ermB and mefA genes were detected by multiplex PCR amplification, using previously published primers (Sutcliffe et al., 1996; Seppälä et al., 1998). The primers were used as ermA (F) 5’-AGAAGGTTA TAATGAAACGA-3’ and ermA (R) 5’-GGCATGA CATAAAACCTTCCAT-3’ (210 bp); ermB (F) 5’-GAAAAGGTACCAACC AATAA-3’ and ermB (R) 5’-AGTAAAGGTTACTAAATGTGTTAC-3’ (640 bp); and mefA (F) 5’- AGTCATCAAATCAGATGGC-3’ and mefA (R) 5’-TTCTCTGGATTCT AAAAGTG-3’ (350 bp).

The polymerase chain reaction including the amplification of the 16S rRNA gene (27 F and 515 R) was carried out using the Solis Biodyne 5X Hot Fire Pol Blend Master mix with the primer pair 27 F (AGA GTTG TGA TCC TGG CTC AG) and 515 R (TTA CCG CGG CKG CTG GCA C). After electrophoresis, DNA bands were visualized with a UV transilluminator by ethidium bromide staining and the 100 bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. The PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (thos with and without amplification) were then subjected to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products (those with and without amplification) were then sent to GATC Biotech Germany for Sanger sequencing and all resulting PCR products. The percentage susceptibility of bacterial isolates from sputum samples to antibiotics showed that the isolates were highly resistant to penicillin 148 (94%) and cloxacillin 147 (93.6%) followed by amoxicillin-clavulanic acid 141 (90%), erythromycin 141 (90%) and tetracycline 124 (79%) while the least resistance was recorded against trimethoprim-sulfamethoxazole 57 (36%) followed by gentamicin 65 (41%).

Klebsiella spp. 40 (25%) and E. coli 20 (13%) were highly resistant (100%) to amoxicillin-clavulanic acid. Also, Klebsiella spp. and E. coli were 36 (90%) and 20 (100%) resistant to tetracycline respectively. However, E. coli was least resistant to gentamicin 2 (10%) and trimethoprim-sulfamethoxazole 2 (10%).

The percentage susceptibility of bacterial isolates from sputum samples to antibiotics showed that the isolates were highly resistant to penicillin 148 (94%) and cloxacillin 147 (93.6%) followed by amoxicillin-clavulanic acid 141 (90%), erythromycin 141 (90%), and tetracycline 124(79%) as shown in Figure 1. However, trimethoprim-sulfamethoxazole 100 (64%) was the most effective antibiotic against the isolates as revealed in Figure 1.

The 16S rRNA gene (27 F and 515 R) from resistant bacterial isolates was amplified. Out of the 48 bacterial isolates analyzed with multiplex PCR amplification, 15 (13.3%) carried the erythromycin resistance genes with 10 (20.8%) positive for mefA, 3 (6.3%) positive for ermA, and 2 (4.2%) for ermB genes. Plates 1A and 1B showed samples 11, 30, 55, 56a, 56b, 57, 58 and 65a were positive for mefA gene with 350 bp while samples 19b and 53b were positive for ermA gene with 210 bp. Plate 2 showed that samples 72b and 77a were positive for ermB gene with 640 bp, sample 80 was positive for ermA gene with 210 bp and samples 93b and 93c were positive for mefA gene with 350 bp.

Statistically, there was no association between the presence of mefA, ermA, and ermB gene and erythromycin resistance in the bacterial isolates ($\chi^2=0.927$, p=0.464). Also, no association was found between the presence of mefA and erythromycin resistance in the bacterial isolates ($\chi^2=0.269$, p=0.604). Similarly, there was no association between the presence of ermA ($\chi^2=0.068$, p=0.794) and erythromycin resistance in the bacterial isolates, neither for ermB ($\chi^2=0.044$, p=0.833). The blast results of the nucleotide sequences of the bacterial isolates are presented as a phylogenetic tree as shown in Figure 2. Two out of the forty-eight bacterial isolates did not yield a significant result after blasting.
Table 1: The demographic and clinical characteristics of 200 enrolled patients.

| Characteristics       | Number (%) |
|-----------------------|------------|
| Gender                |            |
| Male                  | 124 (62)   |
| Female                | 76 (38)    |
| Age (years)           |            |
| <10                   | 4 (2)      |
| 11-20                 | 16 (8)     |
| 21-30                 | 38 (19)    |
| 31-40                 | 42 (21)    |
| 41-50                 | 38 (19)    |
| 51-60                 | 26 (13)    |
| 61-70                 | 20 (10)    |
| 70-above              | 16 (8)     |
| Diseases/ Symptoms    |            |
| Cough                 | 41 (20.5)  |
| Difficulty in breathing| 24 (12)  |
| LRI                   | 117 (58.5)|
| Others                | 18 (9)     |

Note: LRI: Lower respiratory infections. Others included weak patients, sore throat, and otitis media.

Table 2: Antimicrobial resistance profiles of bacterial isolates from sputum samples

| Bacterial Isolates    | Antibiotics |
|-----------------------|-------------|
|                       | AMC  | ERY  | TET  | CXC  | GEN  | TMX/SMX | CHL  | PEN  |
| Streptococcus spp.    | 19 (12)|      |      |      |      |         |      |      |
|                       | 12 (63)| 14 (74)| 14 (74)| 19 (100)| 12 (63)| 14 (74)| 17 (89)|      |
| Escherichia coli      | 20 (13)|      |      |      |      |         |      |      |
|                       | 20 (100)| 20 (100)| 20 (100)| 2 (10)| 2 (10)| 2 (10)| 20 (100)|      |
| Proteus spp.          | 34 (22)|      |      |      |      |         |      |      |
|                       | 32 (94)| 34 (100)| 29 (85)| 34 (100)| 14 (41)| 9 (26)| 11 (32)| 34 (100)|
| Staphylococcus aureus | 34 (22)|      |      |      |      |         |      |      |
|                       | 27 (79)| 32 (94)| 25 (74)| 27 (79)| 16 (47)| 16 (47)| 25 (74)| 34 (100)|
| Klebsiella spp.       | 40 (25)|      |      |      |      |         |      |      |
|                       | 40 (100)| 38 (95)| 36 (90)| 40 (100)| 11 (28)| 11 (28)| 27 (68)| 40 (100)|
| Bacillus spp.         | 10 (6) |      |      |      |      |         |      |      |
|                       | 10 (100)| 3 (30)| 0 (0)| 7 (70)| 10 (100)| 7 (70)| 10 (100)| 3 (30)|
| Total                 | 157 (100)| 141 (90)| 141 (90)| 124 (79)| 147 (93.6)| 65 (41)| 57 (36)| 89 (57)| 148 (94)|

KEY: AMC: Amoxicillin-clavulanic acid, ERY: Erythromycin, TET: Tetraacycline, CXC: Cloxacillin, GEN: Gentamicin, TMX/SMX: Trimethoprim-sulfamethoxazole, CHL: Chloramphenicol, PEN: Penicillin; Figures in brackets are percentages.
Figure 1: Percentage susceptibility of bacterial isolates from sputum samples to antibiotics

Plates 1 A and B: Multiplex PCR Products from analyses of Bacteria DNA with mefA, ermA, and ermB Primers. NB: Lane 1 represents M 100 bp DNA ladder molecular weight marker; Lane 2 represents the negative control (NC); Samples 11, 30, 55, 56a, 56b, 57, 58 and 65a were positive for mefA erythromycin resistance gene with 350 bp, and Samples 19b and 53b were positive for ermA erythromycin resistance gene with 210 bp.
Plate 2: Multiplex PCR Products from the Analyses of Bacteria Isolates DNA with mefA, ermA, and ermB Primers. NB: Lane 1 represents Marker; Samples 72b and 77a were positive for ermB erythromycin resistance gene with 640 bp; Sample 80 was positive for ermA erythromycin resistance gene with 210 bp and Samples 93b and 93c were positive for mefA erythromycin resistance gene with 350 bp.

Figure 2: Phylogenetic tree of the genetic relatedness of the different bacterial isolates. Alignment of sequences and phylogenetic tree were constructed using the Neighbor-Joining method in MEGA7 software. The percentage of the tree topology was tested by bootstrap (1000 replicates) and are indicated at the branches. The scale bar indicates units of the number of base substitutions per site.
4.0 Discussion

The occurrence of antibiotic resistance in pathogenic bacteria associated with respiratory tract infections cannot be overemphasized. This study revealed the distribution of pathogenic bacterial isolates from patients with Respiratory Tract Infections (RTIs) as well as their antibiotic susceptibility profile. A previous study in Ekiti, Nigeria revealed that 10% of the total emergency cases were respiratory infections (Desalu et al., 2011). This study screened a total of 200 patients having respiratory infections 124 (62%) were males and 76 (38%) were females. In a similar study, Osevwe et al. (2017) also reported higher male patients (62.2%) than female patients (38.8%). The finding attributed the involvement of males in different activities like consumption of alcohol and smoking which may aggravate the risk of acquiring RTIs (Grau et al., 2014). The age range of 31–40 years had the highest number of patients 42 (21%) while the lowest age range was between 0-10 years. This is similar to the study by Motayo et al. (2012) that reported the highest occurrence in the age range of 31-45 years. Previous studies had reported the highest occurrence among the age group 20-29 years (Taura et al, 2013) and 21-30 years (Osevwe et al., 2017) which is in contrast to this study. The occurrence of respiratory diseases/symptoms of patients recruited in the study showed that lower respiratory tract infections (LRIs) 117 (58.5%) were the most common followed by cough 41 (20.5%) and difficulty in breathing 24 (12%). In contrast to this study, a similar study reported pulmonary tuberculosis (25%) as the most common respiratory condition diagnosed followed by upper respiratory tract infections (23.7%) and lower respiratory tract infections (15.8%) (Motayo et al., 2012).

The distribution of bacteria causing respiratory tract infections may vary among populations in geographical locations, isolates sources, clinical samples among others. The prevalence of Klebsiella spp. (25%) in this study had been previously reported as the most common bacteria isolated from RTIs in other studies (Egbe et al., 2011; Osevwe et al., 2017). Haemophilus influenzae was reported by Nwaze et al. (2012) as the most frequently isolated pathogen from the respiratory tract of children in southeastern Nigeria which is in contrast to this study. Several researchers have proposed that the prevalence of resistance to a particular antibiotic does not always reflect the antibiotic consumption in a given locality (Brown et al., 2005). Apart from antibiotic stress, horizontal gene transfer is also an important factor in the occurrence of antibiotic resistance in clinical isolates (Brown et al., 2005).

The percentage susceptibility of bacterial isolates to different antibiotics revealed that trimethoprim-sulfamethoxazole 100 (64%) had the highest potency followed by gentamicin 92 (59%). The efficacy of gentamicin as the second drug of choice may be related to its multiple mechanisms of action. Chloramphenicol also showed a sensitivity of 68 (43%) which is similar to a study by Taura et al. (2013) who revealed moderate sensitivity to chloramphenicol. High resistance was recorded against penicillin 148 (94%), doxacillin 147 (93.6%), amoxicillin-clavulanic acid 141 (90%), erythromycin 141 (90%), and tetracycline 124 (79%). Similar studies by previous investigators have also reported high resistance to erythromycin (86.5%) and tetracycline (100%) (Motayo et al., 2012) which is in agreement with this study. A high level of resistance recorded by erythromycin is an indication of the acquisition of erythromycin resistance genes by bacterial isolates. Streptococcus spp. displayed a high resistance against all the antibiotics tested in this study. A similar study by Motayo et al. (2012) had previously recorded a high resistance of Streptococcus pneumoniae to erythromycin (37.5%). However, in this study, 74% of Streptococcus spp. exhibited resistance to erythromycin which is a manifestation of a possible acquisition of erythromycin resistance genes by this organism. Also, Osevwe et al. (2017) had also reported resistance of Streptococcus pneumoniae to gentamicin (100%), cloxacillin (71.4%), and chloramphenicol (85.7%) which is in agreement with this study. E. coli was 90% sensitive to gentamicin, trimethoprim-sulfamethoxazole, and 100% resistant to amoxicillin-clavulanic acid, and tetracycline. In a similar study, Taura et al. (2013) reported that E. coli was sensitive to gentamicin, Motayo et al. (2012) also reported 25% of E. coli isolates were sensitive to gentamicin. Proteus spp. was highly resistant to amoxicillin-clavulanic acid 32 (94%), and tetracycline 29 (85%). However, trimethoprim-sulfamethoxazole 25 (74%) and gentamicin 20 (69%) were effective against Proteus spp. The high sensitivity of Proteus spp. to gentamicin and its resistance to tetracycline reported by previous studies (Motayo et al., 2012; Taura et al., 2013) is similar to this study.

S. aureus and Klebsiella spp. were sensitive to gentamicin and trimethoprim-sulfamethoxazole but resistant to all other antibiotics tested in this study. In a similar report by Taura et al. (2013) revealed that S. aureus was moderately sensitive to chloramphenicol and however showed resistance to amoxicillin-clavulanic acid, erythromycin, tetracycline, gentamicin, and trimethoprim-sulfamethoxazole. Also, Motayo et al. (2012) reported a high resistance (100%) by Klebsiella spp. against tetracycline which is similar to the finding of this study. Bacillus spp. was sensitive to tetracycline 10 (100%), erythromycin 7 (70%), and penicillin 7 (70%) but resistant to all other antibiotics tested in this study. Most bacterial isolates in this study were highly resistant to amoxicillin-clavulanic acid, erythromycin, tetracycline, cloxacillin, and penicillin. All the bacterial isolates displayed variable antimicrobial resistance profiles as detailed in Table 2.

Macrolide resistance has been shown previously to be associated with the rate at which these antibiotics were used. Also, reports have shown that a reduction in macrolide use led to a decline in the incidence of macrolide resistance (Leclercq, 2002). A predominant mechanism of resistance to erythromycin is the specific site modification mediated by the presence of an RNAerm methylase that alters a site on 23S rRNA (Sutcliffe et al., 1996; Ogbolu et al., 2018). The bacterial isolates were analyzed for erythromycin resistance genes, the mefA was the most frequent gene 20.8% followed by ermA and ermB with 63% and 4.2% respectively. In a similar study, 29.2% of mefA gene was reported in pneumococcal isolates from the respiratory tract (Syriopou-lopoulos et al., 2000). In a study conducted in the southwestern part of Nigeria, the absence of ermA and ermB genes was reported (Vitali et
while in another study the presence of \(ermB\) gene was the least prevalent (11.1%) while \(ermA\) gene was not found in the erythromycin resistant isolates tested (Ogbolu et al., 2018). None of the isolates showed the presence of multiple erythromycin resistant genes and there was no association (\(p>0.05\)) between the presence of erythromycin resistant genes, \(mefA,\) \(ermA,\) \(ermB,\) and erythromycin resistance in the bacterial isolates tested in this study.

Conclusion

The occurrence of resistance to many frequently prescribed antibiotics coupled with the presence of erythromycin resistance genes in bacterial isolates requires increased surveillance of bacteriological investigation of respiratory tract infections. The presence of \(mefA,\) \(ermA,\) and \(ermB\) genes in the bacterial isolates may be responsible for erythromycin resistance. Further investigation on the prevalence of these genes and other genes coding for erythromycin resistance circulating in other parts of Nigeria will be of immense value in the eradication of erythromycin resistance.

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Declaration of Conflict of Interests

The authors declare no conflict of interests.

Authors’ Contributions

Conception: [OMK and OOI]
Design: [OMK and OOI]
Execution: [OOI]
Interpretation: [OMK and OOI]
Writing the paper: [OMK and OOI]

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