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Detections of mefA, ermB, and mphA Macrolides Resistant Genes in Bacteria Isolated from Covid-19 Patients from Selected Health Facilities in Ibadan, Nigeria

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Abstract

Background: COVID-19 is a disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Epidemiological data indicated that bacterial complications in COVID-19 would decrease clearance rate of the infecting agent and increase mortality rate. Macrolides such as Azithromycin are usually administered to COVID-19 patients as palliative treatments. Currently, a considerable number of bacterial strains have developed resistance to various antibiotics, especially macrolides. Resistance is reported to be due to possession of mefA, ermB, and mphA genes by Gram positive and Gram negative bacteria. Therefore, this study determined antibiotic resistance patterns and identify mefA, ermB and mphA macrolide-resistant genes in bacterial pathogens isolated from COVID-19 cases in Ibadan, Nigeria. **Methods:** 400 Nasopharyngeal samples were collected from symptomatic cases before antibiotic medication; structured questionnaires were administered to collect socio-demographic data of participants. Samples were cultured on Blood, Chocolate, MacConkey and Mannitol salt agar at 37˚C for 48 hrs. Bacterial identification was performed using VITEK 2.0 ID cards and API 20E for Gram positive and negative bacteria respectively. Antibiotic Susceptibility Testing was performed using Kirby Bauer disc diffusion methods and VITEK 2.0 AST card kits. DNA of multidrug resistant bacterial isolates was extracted; resistant genes were determined using a polymerase chain reaction with specific primers. Amplified genes were detected using agarose gel electrophoresis. **Results:** 240 (60%) had bacterial growth and 97 (22.2%) yielded no growth. From the 240 bacterial isolates, 38 (15.83%) were multi-drug resistant including resistance to macrolides (Azithromycin) 20 (52.63%) of which were positive

for either mefA or ermB, and none (0.0%) possess mphA gene; 14 (36.8%) isolates had mefA gene, 10 (26.3%) isolates carried ermB gene. **Conclusion:** Multi-drug bacterial resistance including macrolides and quinolones was detected. Only mefA and ermB genes were detected in the bacterial isolates, especially in Gram positive organisms. The detection of mefA and ermB genes in the MDR bacterial isolates raised concern on the use of azithromycin as palliative treatment for COVID-19 symptomatic patients.

Keywords

SARS-CoV-2, Bacterial Co-Infection, API 20E, VITEK 2.0 and Resistant Genes

1. Introduction

Antibiogram when properly performed and interpreted, is an important source of information for health care providers. It is the overall profile of antimicrobial susceptibility or resistance of a microbial species to a battery of antimicrobial agents [1]. Data from antibiogram are most useful when initiating empiric therapy and when tracking antimicrobial resistance trends over time within a hospital or health care system. Most SARS-CoV-2 infected persons are put on antimicrobial treatment as palliative treatment, which might lead to an increase in antimicrobial resistance. Reports on 102 patients from critical and non-critical care in China indicated that 101 (99%) received antibacterial therapy [2] Also, 87 out of the 102 COVID-19 patients (85%) received quinolone therapy, 34/101 (33%) cephalosporin's, and 25/102 (25%) carbapenems [2]. It was indicated that no bacterial nor fungal co-infection was reported, because it is difficult to distinguish bacterial or fungal infections from existing viral pneumonia based on clinical and radiological performance [2]. There are increasing reports on the co-occurrence of respiratory viruses like influenza epidemics/pandemic, and secondary bacterial and invasive fungal infections that resulted in poor patient's outcome with consequently, high mortality rates [3]. Therefore, there is a critical demand for urgency with a special focus on the possibility of possession of antibiotic resistant genes that could be transferred and render antibiotic palliative treatments useless.

Due to widespread resistance to many common first-line antibiotics, carbapenems, polymyxins, and tigecycline were more recently considered to be the drugs of choice; however, resistance to these drugs has been reported. Despite this, they are still being used in areas where resistance has not yet been reported [4] [5]. The use of β-lactamase inhibitors such as sulbactam has been advised in combination with antibiotics to enhance antimicrobial action even in the presence of a certain level of resistance [4] [5]. Combination therapy after rigorous antimicrobial susceptibility testing has been found to be the best course of action in the treatment of multidrug-resistant *P. aeruginosa* [4] [5]. As fluoroquino-

lones are one of the few antibiotic classes widely effective against *P. aeruginosa*, in some hospitals, their use is severely restricted to avoid the development of resistant strains [4] [5].

Reports demonstrated that a combination of hydroxyl-chloroquine and Azithromycin was effective for a large proportion of COVID-19 patients [6]. It is hard to estimate how often this combination is prescribed, but such a rate would be high enough to cause a shortage of Azithromycin. However, 30% - 40% of common types of bacterial agents are already resistant to Azithromycin, and overuse could render this or other antibiotics even less effective [7].

One factor involved in the antibiotic resistance in bacterial co-infection is the widespread use of antibiotics in COVID-19 patients. Emerging data show that more than 90% of COVID-19 patients receive antibacterial drugs [8]. This rapid increase in antibiotic administration can cause a strong selective pressure on bacterial pathogens to evolve resistance leading to the increased incidence of drug-resistant bacterial infections in the years subsequent to the COVID-19 pandemic [9]. It was estimated that 10 million people could die from antibiotic-resistant bacterial infections in the year 2050 [9]; such prediction may be altered and worsened due to the devastating impact of the COVID-19 pandemic on the usage of antibiotics, so this timeline will almost have to be modified [10]. Nevertheless, concerted efforts must be made to better understand antibiotic administration in COVID-19 patients. Antibiotics do not directly act on viral infections but viral respiratory infections often lead to bacterial co-infections [11]. The current pandemic highlights the necessity for understanding the complex relationship between viral and bacterial infections. Of note, patients who have been treated with high dose antibiotics may have more co-infections with drug resistant bacteria and a recent clinical trial conducted demonstrated that the use of broad-spectrum antibiotics (which led to depleting gut microbiota) decreased and impaired the immune system's ability to generate antibodies [12].

Nevertheless, acquired macrolide resistance is an increasingly recognized problem. The development of acquired resistance towards azithromycin and other related macrolides is associated with active macrolide efflux pumps produced by the bacteria [13]. Macrolides are antibacterial substances which have a central lactone ring as their basic structure. Lincosamides are structurally different from macrolides, but their binding sites overlap. The binding site of streptogramin B overlaps that of macrolides and lincosamides. Modification of the bacterial target site of these molecules typically leads to cross-resistance between macrolides, lincosamides and streptogramin B (MLSB resistance phenotype). Macrolide antibiotics are mainly active against Gram positive bacteria such as Staphylococcus aureus. Acquired resistance to macrolide antibiotics has been extensively studied in these bacteria and is generally due to N-6 dimethylation of a specific adenine residue in 23S rRNA. Members of the family Enterobacteriaceae, like most Gram-negative organisms, are intrinsically resistant to low levels of erythromycin A, probably by efflux pump [14]. Macrolides are used

for treatment of diseases that are common in food-producing animals and for medication of large groups of animals (mass medication). Lincosamides are more limited in indications, and the number of products is lower. Macrolides have been categorized as critically important and lincosamides as highly important for veterinary medicine in the list of antimicrobials of veterinary importance [14]. Macrolides and streptogramins are classified as critically important in human medicine [15].

Azithromycin and other macrolides have been largely used to treat infections from Gram-positive microorganisms, including *Streptococcus pneumoniae*, methicillin-sensitive *Staphylococcus aureus*, and group A, B, C, and G Streptococcus, but Azithromycin also possesses satisfactory activity against different gram-negative microorganisms, including *Haemophilus spp.*, *Moraxella catarrhalis*, *Escherichia coli*, *Salmonella spp.*, *Yersinia enterocolitica*, *Shigella spp*, *Campylobacter jejuni*, *Vibrio cholerae*, *Neisseria gonorrhoeae*, and *Helicobacter pylo*ri [16]. In fact, Azithromycin is active against atypical pneumonia pathogens, including *Legionella pneumophila*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* [17]. Nevertheless, acquired macrolide resistance is an increasingly recognized problem. The development of acquired resistance towards azithromycin and other related macrolides is associated with active macrolide efflux pumps produced by the bacteria [18] Active macrolide efflux pumps are encoded by the macrolide efflux genes MSRA and MSRB. These efflux pumps are part of the bacterial systems involved in the extrusion of molecules from bacteria to the environment, including bacterial products such as siderophores, as well as toxic compounds and macrolide antibiotics. The first mechanism of resistance genes is the efflux of the drug from the bacteria which is encoded by mefA; the second is alteration of the ribosomal target by a ribosomal methylase which is encoded by the erm gene; and the third is mphA which is mutations in the genes encoding ribosomal proteins L4 and L22 in 23S rRNA) [19]. Chromosomal efflux pumps are bacterial systems involved in the extrusion of molecules from bacteria to the environment, including bacterial products such as siderophores as well as toxics and antibiotics. In this line, chromosomal efflux pumps are involved in intrinsic and acquired Azithromycin resistance [19]. Additionally, target amino acid substitutions in the L4 (rplD) and L22 (rplV) ribosomal proteins and in 23S rRNA (rrlH) have also been involved in macrolide resistance. Nonetheless, the most relevant mechanisms of azithromycin resistance in Enterobacteriaceae are those encoded in mobile elements. Different Macrolide Resistance Genes (MRGs) have been described, leading to resistance through different pathways such as target modifications produced by rRNA methylases encoded in erm genes or macrolides-inactivation, mediated by esterases such as those encoded by ere(A) or ere(B) genes or by phosphorylases such as those encoded in the mph(A) and mph(B) genes. Additionally, transferable genes such as msr(A), mef(A) or mef(B) have been reported to encode macrolide-efflux pumps [20] [21] [22].

The direct effect on drug-resistant bacteria as a result of enhanced antibiotic

administration, the transmission of drug-resistant bacteria in COVID-19 conditions is therefore very important. Findings from this study could help experts' advice on using the antibiotics in COVID-19 patients and help them to better understand the spread of co-infections in hospitals and the mechanism of antimicrobial resistance in bacteria and SARCOV-2 coinfections.

2. Materials and Methods

Study Design and Ethical Considerations

This is a cross-sectional, purposeful, hospital-based experimental study carried out in all COVID-19 government isolation center in Oyo state, Nigeria from July 2020 to April 2021. Questionnaires and informed consent were employed to gather data from infected individuals after obtaining their informed consent.

Sample Size: 400 samples of SARS-CoV-2 positive individuals were purposively enrolled into the study before antibiotic medication. Informed consent of participants were obtained. Thirty-eight (38) multi-drug resistant phenotypically of Gram negative and Gram positive bacterial confirmed from the 240 pathogenic bacteria isolated from nasopharyngeal samples of COVID-19 patients were used.

Laboratory Procedures

DNA was extracted from the isolated multidrug resistant bacteria using NIMR Bioteck Commercially prepared kit as described by the manufacturer. DNA Concentration and Purities were measured using Nanodrop Spectrophotometer (Thermo Scientific), mefA, ermB and mphA genes were detected using polymerase chain reaction as described below:

5 µL of the prepared cDNA of each sample was used in the multiplex reaction using PCR master mix from Inqaba biotech and 1st round primers for mefA, ermB and mphA to make a 50 µL reaction mix as described in table. The PCR master mix contains a premix of PCR buffer, Magnesium chloride, dNTPs, and Taq Polymerase enzyme in optimized concentrations. Nucleotide sequence of the primers is as shown in table. Micro amps tubes containing the PCR reaction mixes were placed in a thermal cycler (Master cycler gradient Eppendorf, Hamburg, Germany) programmed to run as follows: There was an activation of the Taq polymerase enzyme at 95˚C for five minutes followed by 34 cycles of denaturation of the double stranded DNA at 95˚C for 30 seconds, primer annealing at 55˚C for 60 seconds, and an elongation of 60 seconds at 72˚C for 5 minutes. Amplicons were identified using 2% agarose Gel Electrophoresis.

3. Results

Table 1 and Table 2 showed the primer sequence for the detection of the macrolide resistance genes while Table 3 showed culture results of the nasopharyngeal swabs indicating 240 out of 400 SARS COV-2 infected participants (60.0%) had bacterial co-infection while 63 (15.5%) had fungal co-infection. Table 4 showed that Gram Negative Bacteria were the most predominant isolates

Table 1. Multiplex primers used for ermB and mefA macrolides resistance gene.

Primers	Sequences (5' to 3')	Size	References
ermB	F: GAAAAGGTACTCAACCAAATA R:AGTAAGGGTACTTAAATTGTTTAG	639	[23]
mefA	F: AGTATCATTCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	348	[23]

Table 2. Simplex primer used for mphA macrolide resistance gene.

Primers	Sequence (5' to 3')	Size	References
mphA	F: GTGAGGAGGAGCTTCGCGAG R:TGCCGCAGGACTCGGAGGTC	403	[24]

Table 3. Nasopharyngeal swab culture results of COVID-19 respondents.

Variable	Frequency (N = 400)	Percentage
Bacteria	240	60.0
Fungi	63	15.8
No growth/Contaminant	97	22.2
Total	400	100.0

Table 4. Gram reaction results of Bacteria isolates from COVID-19 samples.

Variable	Frequency (N = 240)	Percentage
GRAM (n = 240)		
GNB	124	51.7
GPC	86	35.8
GNB + GPC	30	12.5
Total	240	100.0

accounting for 124 of the 240 (51.7%) while Gram Positive Cocci accounted for 86 (35.8%). The frequency and percentage of all isolated Gram negative bacteria identified by API 20E is shown in Figure 1 and the isolates were *Acinetobacter baumanii* 7 (4.5%), *Citrobacter freundii* 1 (0.6%), *Citrobacter koseri* 3 (1.9%), *Cronobacter specie* 1 (0.6%), *Enterobacter aerogenes* 23 (14.9%), *Enterobacter cloacae* 27 (17.5%), *Escherichia coli* 8 (5.1%), *Klebsiella oxytoca* 5 (3.3%), *Klebsiella pneumoniae* 35 (22.7%), *Kluyevera* specie 3 (1.9%), *Proteus mirabilis* 4 (2.6%), *Proteus vulgaris* 1 (0.6%), *Pseudomonas aeruginosa* 10 (6.5%), *Pseudomonas luteola* 3 (1.9%), *Raoultella ornithinolytica* 6 (3.9%), *Serratia ficaria* 6 (3.9%), *Serratia liquefaciens* 2 (1.3%), *Serratia marscensces* 6 (3.9%) and *Serratia rubidea* 3 (1.9%) respectively. Gram positive cocci isolated were *Enterococcus faecalis* 4 (3.6%), *Staphylococcus xylosus* 1 (0.9%), *Staphylococcus aureus* 36 (32.4%), *Methicillin Resistant Staphyloccus aureus* 1 (0.9%), *Staphylococcus equorum* 4 (3.6%), *Staphylococcus gallinarium* 1 (0.9%), *Staphylococcus lentus* 16 (14.4%), *Staphylococcus saprophyticus* 35 (31.5%), *Staphylococcus scuiri* 11 (9.9%) and *Streptococcus agalactiae* 2 (1.9%) as shown in Figure 2.

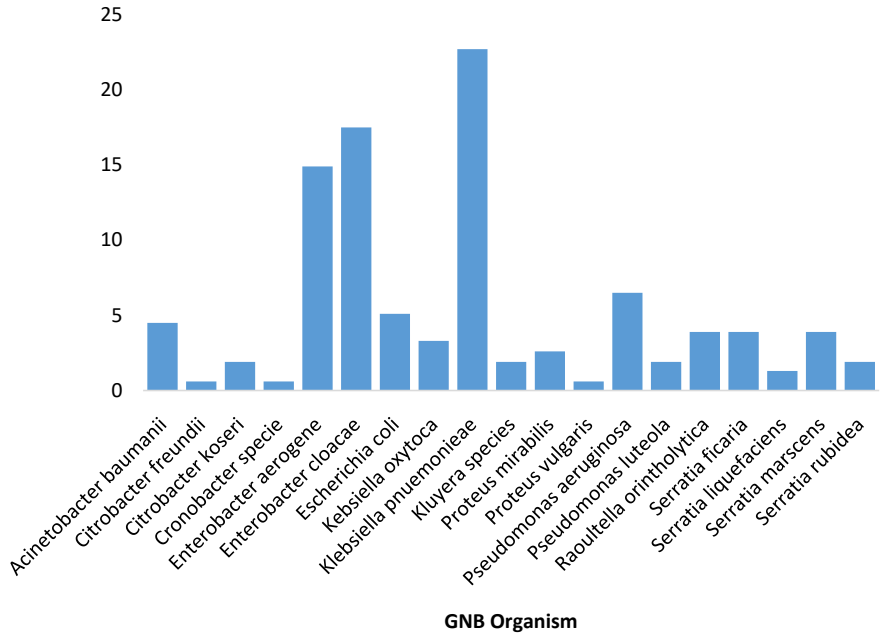

Figure 1. Identified gram negative bacterial isolates using API 20E.

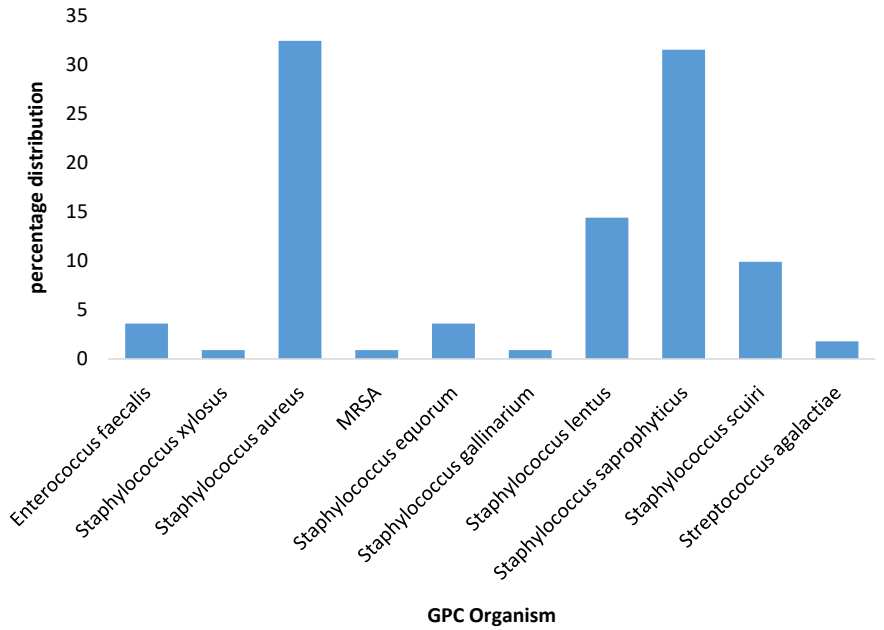

Figure 2. Identification of the isolated gram positive cocci using VITEK 2.0.

Figure 3 showed percentage distribution of antibiotics susceptibility pattern of Gram negative bacteria; isolates were mostly susceptible to Levofloxacin (89%), Amikacin (88%), Ceftazidime (79%), Ciprofloxacin (66%), Azithromycin (51%), Cefuroxime (11%), Sulbactam (11%), Ceftriaxone (8%), Gentamycin (6%) respectively. Figure 4 showed Gram positive cocci isolates indicating susceptibility to Vancomycin (74.10%), Ciprofloxacin (71.30%), Levofloxacin (70.40%), Cefoxitin (69.40%), Gentamycin (45.40%), Clindamycin (29.60%), Oxacillin (23.10%) and macrolides that is, Erythromycin (42.60%) and Azithromycin (33.3%).

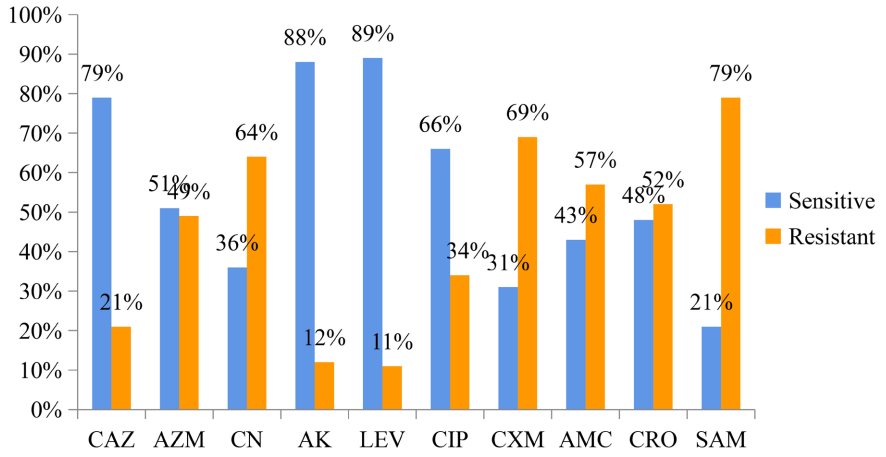

Figure 3. Antibiogram susceptibility pattern of GNB isolates using Kirby Bauer disc diffusion and VITEK 2.0 system. CAZ = Ceftazidime; AZM = Azithromycin; CN = Gentamicin; AK = Amikacin; LEV = Levofloxacin; CIP = Ciprofloxacin; CXM = Cefuroxime; AMC = Augmentin; CRO = Ceftriaxone. Cefoxitin (69.40%), Gentamycin (45.40%), (29.60%); SAM = Sulbactam.

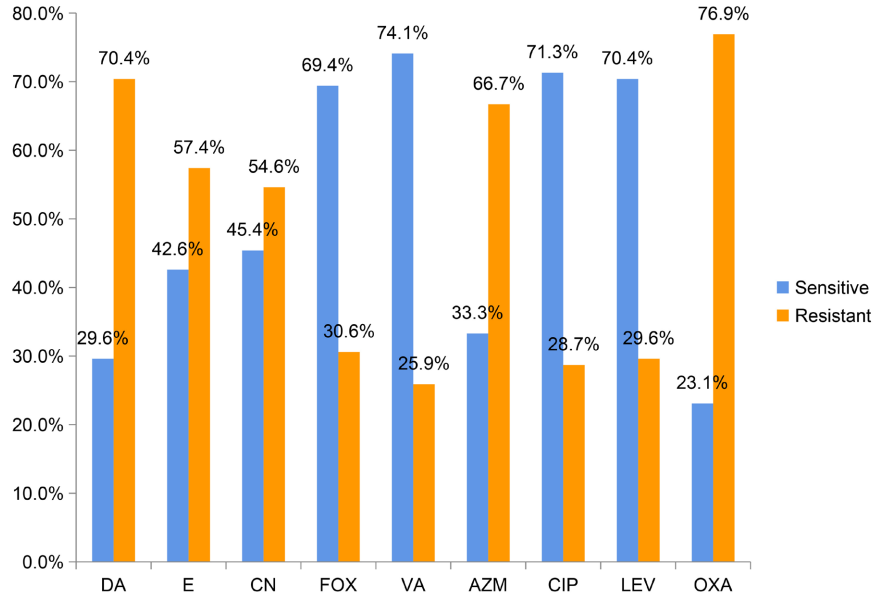

Figure 4. Antibiogram susceptibility pattern of GPC isolates using Kirby Bauer and VITEK 2.0. Key: DA = Clindamycin; E = Erythromycin; FOX = Cefoxitin; VA = Vancomycin'; AZM = Azithromycin; OXA = Oxacilin.

Table 5 showed the occurrence and distribution of macrolides resistant genes among *the multidrug-resistant Enterococcus faecalis*, *Stapylococcus saprophytics*, *Staphylococcus lentus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter cloacae and Klebsiella pneumoniae* isolates. The *mefA*, *ermB* genes were detected with varying frequencies in the macrolide-resistant bacterial isolates. Figure 5(a) and Figure 5(b) showed mefA and ermB macrolides genes with 348 bp and 639 bp respectively. However, *none* of the isolates had mphA macrolide gene as indicated in Figure 6.

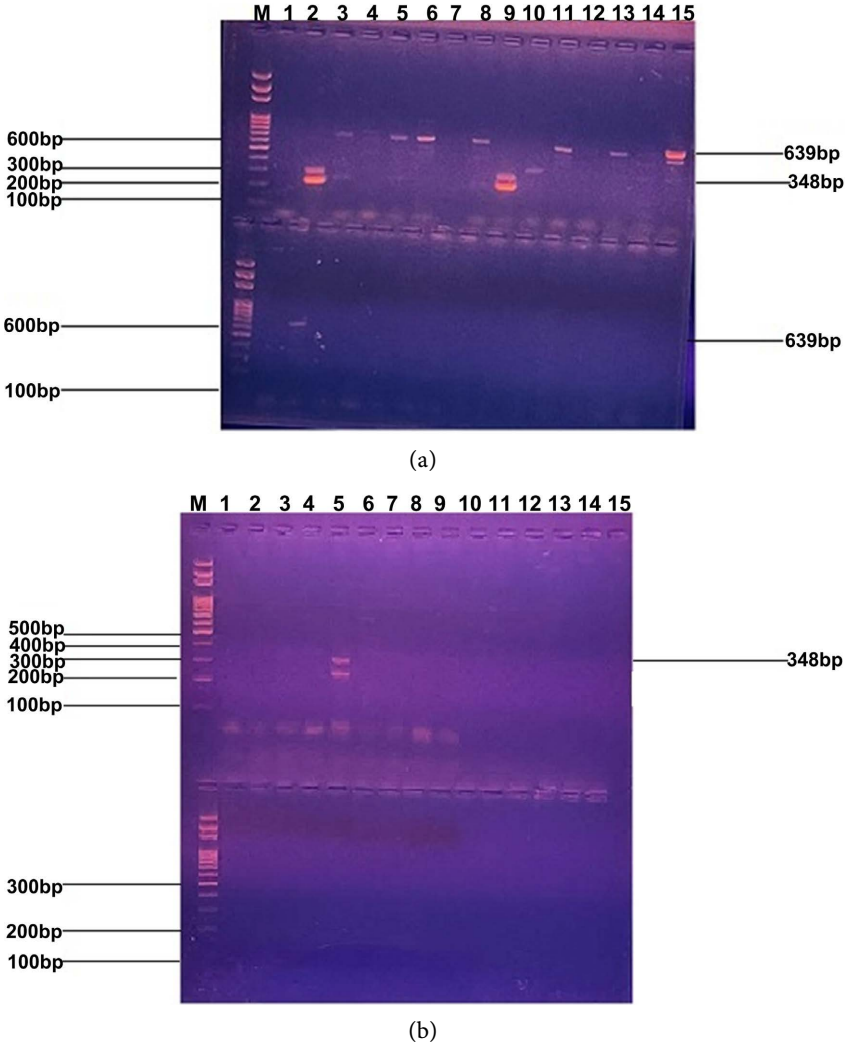

Figure 5. (a) Agarose gel electrophoresis for Multiplex detection of Macrolides Resistance mefA and ermB genes; (b) Agarose gel electrophoresis for Multiplex detection of Macrolides Resistance mefA and ermB genes.

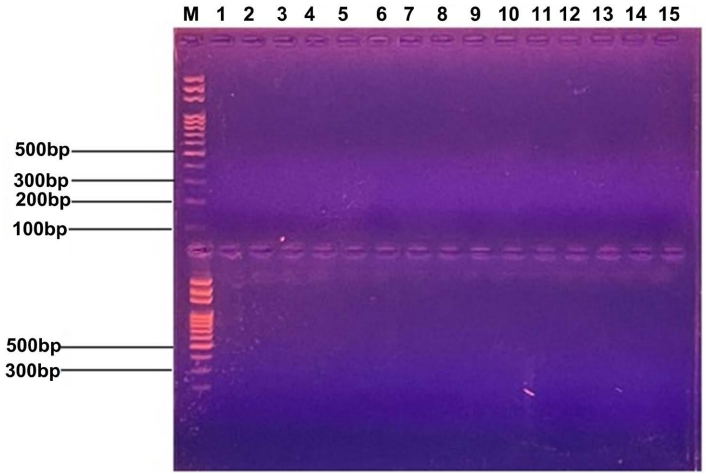

Figure 6. Agarose gel electrophoresis for detection of mphA gene. None of the isolates had mphA gene.

Table 5. Macrolide resistance gene among multi-drug resistance bacterial isolates with the specific primers.

Variable	mefA positive (%)	ermB positive (%)	mphA Positive (%)
Enterococcus faecalis	1 (14.3)	2 (11.1)	0 (0)
Stapylococcus saprophytics	3 (42.9)	3 (11.1)	0 (0)
Staphylococcus lentus	1 (0)	1 (11.1)	0 (0)
Pseudomonas aeruginosa	2 (0)	1 (11.1)	0 (0)
Staphylococcus aureus	3 (28.6)	2 (33.3)	0 (0)
Enterobacter cloacae	1 (14.3)	1 (0)	0 (0)
Klebsiella pneumoniae	0 (0)	2 (11.1)	0 (0)
Staphylococcus xylosus	1 (0)	0 (0)	0 (0)
Serratia marscenses	0 (0)	0 (0)	0 (0)
Enterobacter aerogene	0 (0)	0 (0)	0 (0)
Staphylococcus xylosus	0 (0)	0 (0)	0 (0)
Klebsiella oxytoca	0 (0)	0 (0)	0 (0)
Raoultella ornithinolytica	0 (0)	0 (0)	0 (0)
Serratia ficaria	0 (0)	0 (0)	0 (0)
Staphylococcus eqorum	0 (0)	0 (0)	0 (0)
Total	14 (100)	10 (100)	0 (0)

4. Discussion

Bacterial co-infection with SARS COV 2 was detected in this study. This finding agreed with earlier study which reported bacterial co-infection of 3.2% of all COVID-19 hospitalized patients with 13.5% of those requiring critical care [23]. It also agreed with the reported bacterial co-infection within 48 hours of admission to ICU in 8%, 16.6%, 27.7% respectively [24] [25].

Detection of mefA and ermB genes in this study confirms the earlier report [13]. The possession of chromosomal efflux pumps genes implies that extrusion of the macrolides antibiotic molecules from bacteria to the environment before the drug attains intracellular concentration lethal to the pathogen and this could result in treatment failure with azithromycin. Similarly, possession of ermB gene by isolates in this study could cause methylation of the drug target and consequently, drug target modifications produced by rRNA methylases encoded in erm genes. The macrolides inactivation due to methylation of erm genes could result in failure of palliative treatment with macrolides especially azithromycin used for COVID-19 patients. This could lead to aggravation of disease severity. The absence of mphA in all the isolates in this study could imply that phosphorylases such as those encoded in the mph(A) and mph(B) genes and their effects on macrolides drug target was not a possible explanation for resistance of macrolides. However, the detection of mefA and ermB macrolide genes in multi-drug drug resistant bacterial isolates in this study agreed with previous studies [13] [19] [20] which showed that efflux pump systems encoded by mefA and the methylation encoded by ermB gene could be responsible for resistance to macrolides by some bacterial isolates.

5. Conclusion

Bacteria isolated from this study showed different degrees of susceptibility to Levofloxacin, Amikacin, Ceftazidime, Ciprofloxacin, Cefuroxime, Sulbactam, Ceftriaxone, Gentamycin, Vancomycin, Gentamycin, Clindamycin, Oxacillin and macrolides (Erythromycin and Azithromycin). Bacterial isolates harbouring macrolide-resistant mefA, and ermB genes were detected in this study. None of the isolates had mphA gene. Detection of the mefA and ermB genes which are important genes for resistance to macrolides could have an implication on the failure of therapy with azithromycin or other macrolides antibiotics when used as palliative treatment for COVID-19 patients.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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