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Detection of Efflux Activity in Macrolide Resistant *Streptococcus pyogenes* Obtained from Patients with Throat Infections

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ABSTRACT

Background: *Streptococcus pyogenes* are bacteria which cause a wide variety of clinical manifestations including pharyngitis. Macrolides are alternative treatment options in cases of penicillin allergy or penicillin treatment failure. Due to a global rise in the emergence of macrolides resistance among these bacteria, the need for easy and rapid identification of isolates over-expressing efflux is paramount. This study detected the efflux-pump mediated resistance in macrolide resistant *Streptococcus pyogenes* using the ethidium-bromide (EtBr)–Agar cartwheel method.

Methods: Overnight cultures of *Streptococcus pyogenes* isolates obtained from patients with throat infection were prepared. Agar plates containing EtBr of different concentrations were prepared. The minimum inhibitory concentration (MIC) of carbonyl cyanide m-chlorophenylhydrazine (CCCP) was determined.

Results: The isolates identified as isolates 190 (Reference strain), 22, 78, and 114 (iMLSB phenotypes) fluoresced at a concentration of 1.0 mg/l of EtBr; isolate 101 did not fluoresce even at a concentration of EtBr as high as 2.0 mg/l. The effect of ½ MIC of CCCP on the MIC of Erythromycin was tested and in the presence of CCCP at a concentration of 4µg/ml, the MIC of erythromycin was decreased by 2 to 4-fold.

Conclusion: The ethidium bromide-agar cartwheel method provided an easy and instrument-free means by which rapid evaluation of antibiotic resistance through the use of efflux pumps in bacterial isolates can be conducted.

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Introduction

Streptococcus pyogenes or group A streptococci (GAS) are aerobic Gram positive bacteria which colonize the throat or skin and cause a wide variety of clinical manifestations ranging from mild to life-threatening invasive streptococcal diseases such as septicemia, streptococcal toxic shock-like syndrome (STSS) and necrotizing fasciitis in infants, children and adults (1). Several reports have shown that there is an increase in the suppurative and non-suppurative complications of GAS infections. The reasons for this resurgence has partly been attributed to changes in the epidemiology and virulence of GAS (2) as well as changes in the susceptibility of the bacteria to commonly used antibiotics (3). Over the years, penicillin has been the antibiotic treatment of choice for streptococcal pharyngitis (4). However, in penicillin-allergic patients or in cases of penicillin failure, another class of macrolides (e.g. erythromycin) has been considered as alternative treatment for streptococcal pharyngitis. Therefore, the emergence, acquisition and spread of resistance to macrolides in *S. pyogenes* and the management of infection constitute an important problem in the family, work or hospital environment.

Macrolide, lincosamides and streptogramins resistance may be due to several distinct mechanisms which include post-transcriptional target site alteration which is caused by rRNA methylases (erm genes), target site mutations, and the acquisition of active efflux genes (mef genes) (5, 6).

Efflux pumps function to limit the intracellular concentration of antimicrobial agents, either by the over-expression of these pumps so as to be able to expel the increasing amount of antibiotics inside the cell, or the efflux pumps accumulate mutations for efficiently extruding the drug (7). Although efflux pumps are known to confer inherent resistance to antimicrobials, they work in synergy with other mechanisms like target site modification to enhance the resistance to a significant level. Therefore, the role that efflux systems play in antibiotic resistance cannot be ignored as inherent resistance may be mostly due to efflux systems (8-10).

Whereas efflux pumps such as mef A, tet A and cml A selectively pump out specific antibiotics such as erythromycin, tetracycline and chloramphenicol respectively, other pumps like nor A, mexAB-opr M, and bmr A are capable of extruding structurally diverse compounds that have distinct mechanisms of action and are therefore called multidrug resistance (MDR) efflux pumps that are capable of expelling antibiotics, dyes, disinfectants, and detergents (11). Active efflux due to mef A genes is the cause of resistance to 14- and 15-membered macrolides, i.e., the M phenotype, but not to the 16-membered macrolides, lincosamides, and streptogramins (12). Due to the rapid emergence and spread of bacterial isolates showing resistance to several classes of antibiotics, methods that can rapidly and efficiently identify isolates whose resistance due to active efflux have been developed, including the ethidium bromide-agar Cartwheel method, which is an easy, instrument-free, agar based method that affords the simultaneous evaluation of many bacterial strains (10). The principle of this method is simple and relies on the ability of the bacteria to expel a fluorescent molecule that is a substrate (commonly used is ethidium bromide) for most efflux pumps.

Materials and Methods

Study Design

A total of 197 throat swab samples were collected from the patients with throat infections that visited the ENT Clinics at the University of Benin Teaching Hospital (UBTH) Benin City, and Stella Obasanjo Hospital, Benin City, Nigeria.

Inclusion and Exclusion Criteria

All consenting patients aged 2 to 65 years who presented with throat infection were recruited into the study. Patients with prior antibiotic therapy or patients with other respiratory tract infections like rhinorrhea or nasal congestion were excluded from the study.

Ethical Approval

Ethical approval was obtained from the Edo State Hospitals Management Board (Ref: A732/T/1) and the University of Benin Teaching Hospital Ethics and Research Committee (Protocol No: ADM/E 22/A/Vol. VII/148200). A signed informed consent was obtained from the subjects, after duly explaining the purpose of the study.

Sample Collection, Processing, Isolation and Identification

Throat swabs were collected from all participants using aseptic technique and these were immediately taken to the Microbiology laboratory. The samples were then inoculated on blood agar containing 5% defibrinated sheep blood and incubated at 37 ºC for 20 hr. The phenotypically distinct colonies that showed β-haemolysis were sub-cultured on fresh blood agar plates to obtain pure bacteria culture. Biochemical characterization and identification of isolates were carried out using standard microbiological techniques such as Gram reaction, catalase test, bacitracin sensitivity test and latex agglutination (HiStrepTM Latex Test kit, Himedia, India) (13).

Antibiotic Susceptibility Test

The isolates were tested for susceptibility to eight antibiotics belonging to seven different classes, namely β-lactams (penicillin G 10 unit and ceftriaxone 30 µg), macrolides (erythromycin 15 µg), lincosamides (clindamycin 2 µg), fluoroquinolones (levofloxacin 5 µg), glycopeptides (vancomycin 30 µg), phenicols (chloramphenicol 30 µg), and oxazolidinone (Linezolid 30 µg) (Oxoid Ltd, Basingstoke, Hampshire, UK). This test was done using the Kirby–Bauer disc diffusion method on Muller–Hinton agar supplemented with 5% sheep blood agar, and the results were interpreted according to the CLSI guidelines while *Streptococcus pneumoniae* ATCC 49619 was used for quality control (14).

Determination of the macrolide-resistant phenotype by the D-zone Test

The macrolide-resistant phenotypes were determined by a double-disc test involving erythromycin (15 µg) and clindamycin (2 µg) discs placed 15–20 mm apart, as described by CLSI. After 16-24 hr of incubation at 37 °C, the absence of a significant zone of inhibition around the two discs was recorded to indicate constitutive resistance (CMLSB phenotype), blunting of the clindamycin zone of inhibition proximal to the erythromycin disc was recorded to indicate inducible resistance (iMLSB phenotype) and susceptibility to clindamycin was indicated with no blunting of the zone of inhibition around the disc and this was suggestive of the M phenotype.

Detection of efflux activity using the Ethidium Bromide – Agar cartwheel method

Ethidium bromide stock solution was prepared in distilled water at a concentration of 50 mg/ml and stored at 4 ºC, protected from light. Bacterial strains were grown in 5 ml of tryptic soy broth (TSB) overnight. The following day the optical density of the bacterial culture was adjusted to 0.5 McFarland Standard. Tryptic soy agar (TSA) plates containing ethidium Bromide concentrations ranging from 0 to 2.0 mg/l were prepared and protected from light. The plates were then divided into 5 sectors by radial lines forming

a cartwheel pattern. The optical density adjusted cultures were swabbed on the EtBr-TSA plates starting from the centre of the plate and spreading towards the edges. Each plate included one reference strain that served as a comparative control for fluorescence analysis. The swabbed EtBr-TSA plates were incubated at 37 °C for 16 hr and examined under a UV trans-illuminator. The minimum concentration of EtBr (MCEtBr) that produced fluorescence of the bacterial mass was recorded and the TSA plates photographed.

Confirmation of efflux activity by determining the MIC of Erythromycin in the presence of efflux inhibitor

The efflux activity was further confirmed by determining the MIC of erythromycin in the presence of efflux inhibitor, carbonyl cyanide m-chlorophenylhydrazine (CCCP). The minimum inhibitory concentration (MIC) of CCCP was determined and the capacity of CCCP to decrease the MIC of erythromycin was assessed. To assure that the bacterial viability was not compromised by the presence of the efflux inhibitor itself, the action of CCCP was employed at a concentration of half of the MIC. All tests were carried out in triplicates and results were expressed as an average of the triplicates.

Data Analysis

All analyses were conducted using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

In this study, a total of 197 samples were collected from the subjects recruited in this study, out of which nineteen (19) samples were positive and the isolates were identified as *S. pyogenes*. The results from antibiotic susceptibility test (Table 1) showed that all the isolates were susceptible to penicillin, ceftriaxone, vancomycin, levofloxacin, linezolid and chloramphenicol. Erythromycin resistance was found in 4 (21.05 %) of the isolates with inducible macrolide, lincosamide and streptogramin B (iMLSB), being the predominant phenotype (75%) followed by the M phenotype (25%). Inducible clindamycin resistance was observed in 3 (15.79%) isolates, as shown in Figure 1.

Phenotypic detection of efflux activity was then carried out on the macrolide resistant isolates using the ethidium bromide-agar (EtBr-Agar) cartwheel method. The fluorescence ability of the isolates is shown in Figure 2. Whereas isolates 190 (Reference strain), 22, 78, and 114 (iMLSB phenotypes) fluoresced at a concentration of 1.0 mg/l of EtBr, isolate 101 (M phenotype) did not fluoresce even at a concentration as high as 2.0 mg/l of EtBr.

The isolates were then evaluated for their susceptibility to erythromycin in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a known bacterial efflux pump inhibitor. First of all, the MIC of the test agents employed in the study were determined (Table 2). The MIC of CCCP on the isolates ranged from 8-16 µg/ml while plates containing erythromycin showed growth even at a concentration of 64 µg/ml. Then, the effect of ½ MIC of CCCP on the MIC of Erythromycin was tested. The concentration of CCCP used (5µg/ml) was well below its MIC; hence, CCCP inhibitory effect on the bacterial isolates used in this study is minimal or null. In the presence of CCCP at 4 µg/ml, the MIC of erythromycin was decreased by 2 to 4-fold. For the reference strain, there was a slight reduction in the MIC of erythromycin in the presence of CCCP which may be due to the antibacterial effect of CCCP.

Table 1. Antibiotic Sensitivity Pattern of Studied GAS Isolates to some commonly used Antibiotics.

Antibiotics (amount/disc)	Susceptibility pattern (%) with respect to diameter of zone of inhibition in mm		
	Sensitive	**Intermediate**	**Resistant**
Penicillin G (10 units)	19 (100%)	-	-
Ceftriaxone (30 µg)	19 (100%)	-	-
Erythromycin (15 µg)	13 (68.42%)	2 (10.53 %)	4 (21.05 %)
Clindamycin (2 µg)	16 (84. 21%)	-	3 (15.79%)
Levofloxacin (5 µg)	18 (94.74%)	1 (5.26%)	-
Vancomycin (30 µg)	19(100%)	-	-
Chloramphenicol (30 µg)	16 (84.21%)	3 (15.79%)	-
Linezolid 30 µg)	19 (100 %)	-	

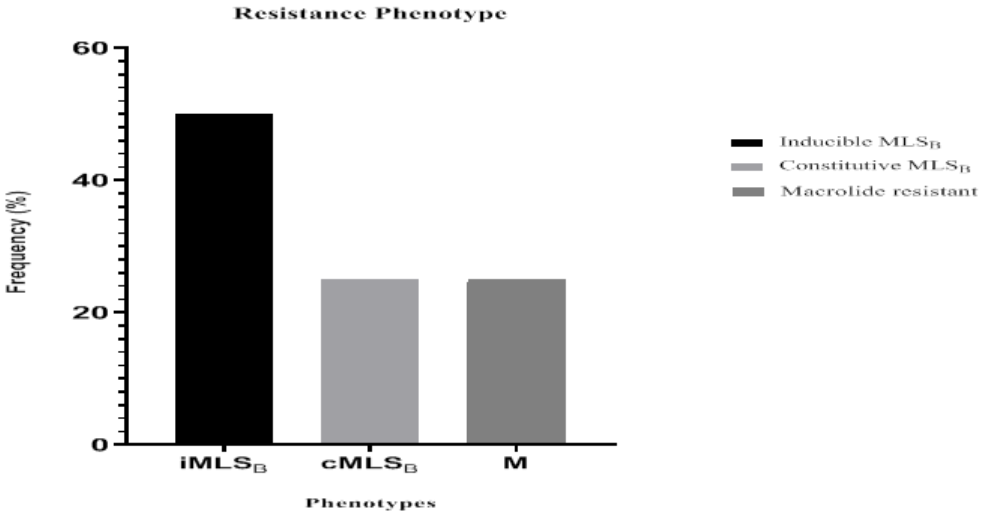

Fig 1. Frequency of macrolide resistance phenotype after conducting D-test among the *Streptococcus pyogenes* isolates .

Table 2. The synergistic effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on the Activity of Erythromycin.

Isolate	ERY Alone	CCCP Alone	ERY + CCCP (Δ)
Reference Strain	0.25 ± 0.00	8.00 ± 0.00	0.125.00 ± 0.00 **(2)**
Isolate 22	8.00 ± 0.00	8.00 ± 0.00	4.00 ± 0.00 **(2)**
Isolate 79	2.00 ± 0.00	8.00 ± 0.00	0.5.00 ± 0.00 **(4)**
Isolate 101	16.00 ± 0.00	16.00±0.00	4.00 ± 0.00 **(4)**
Isolate 114	2.00 ± 0.00	8.00 ± 0.00	0.67 ± 0.29 **(3)**

CCCP = Carbonyl cyanide m-chlorophenylhydrazone, ERY= Erythromycin, MIC= Minimum inhibitory concentration,

(Δ) MIC fold change, i.e. the ratio of the MIC of antibiotic alone to that of antibiotic + CCCP.

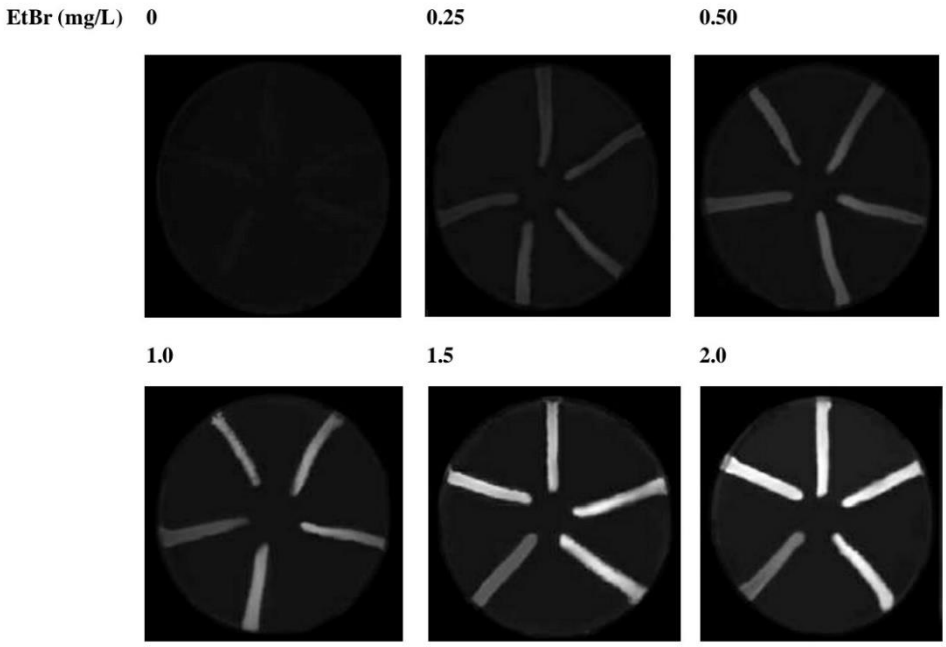

Fig 2. The EtBr-agar cartwheel method applied to macrolide resistant isolates of *Streptococcus pyogenes* in the study population.

Discussion

Pharyngitis is the most common manifestation of *Streptococcus pyogenes* infection. Erythromycin and other macrolides are considered alternative treatments for streptococcal pharyngitis in patients with penicillin allergy or in cases of penicillin treatment failure. Therefore, the emergence and spread of resistance to macrolides in *S. pyogenes* is an important problem in the management of infections.

Although erythromycin resistance is low in most countries of the world, recently some local resistance has emerged due to its excessive use. However, in this study, resistance to erythromycin was seen in 21.05 % of the GAS isolates. Majority of the erythromycin-resistant isolates (75%) belonged to inducible phenotype (iMLSB) followed by the M phenotype (25%).

Active efflux due to mef (A) genes is the cause of resistance to 14- and 15 membered macrolides, i.e., the M phenotype, but not to the 16 membered macrolides, lincosamides, and streptogramins (12). Considering that inherent resistance is mostly due to efflux, easy and rapid identification of isolates over-expressing efflux is paramount. The phenotypic detection of efflux activity was also carried out on the macrolide resistant isolates using the Ethidium Bromide-Agar (EtBr-Agar) cartwheel method, a simple, method that uses agar plates containing increasing amounts of Ethidium Bromide for the detection of efflux activity in bacterial cells (15). The principle of using EtBr – agar cartwheel method is that it uses ethidium bromide as a substrate that allows the detection of an over-expressed efflux pump in comparison to the intrinsic efflux activities of a reference strain. The amount of ethidium bromide that was required

to produce fluorescence in strains expressing efflux pumps was significantly higher than that which produces fluorescence of the reference strain. In this study, a range of fluorescence was detected depending on the ability of the isolates to expel ethidium bromide from the cell. Whereas isolates 190 (Reference strain), 22, 78, and 114 (iMLSB phenotypes) fluoresced at a concentration of 1.0 mg/l of ethidium bromide, isolate 101 (M phenotype) did not fluoresce even with a concentration of ethidium bromide as high as 2.0 mg/l.

This implies that isolate 101 has a high efflux activity compared to the other isolates, and this further confirms the result of the D-zone test which indicated that isolate 101 has an M phenotype. Although the ethidium bromide -agar cart-wheel method is generally used for the identification of over-expressed efflux pumps contributing to multi-drug resistance (MDR) phenotype, the isolates in this study showed specific-resistant phenotypes (macrolide resistance in particular). This is probably the reason why they fluoresced even at low concentrations of ethidium bromide. Multi-drug resistance is usually the result of over-expressed efflux pumps that expel different class antibiotics before they reach their intended targets. Most classes of antibiotics are predisposed to resistance by efflux, with the exception of the glycopeptides family (9).

The results from the EtBr-agar cartwheel method were also assayed for, by observing the effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP), which is used for in-vitro testing of the expression of bacterial efflux pumps, on the activity of erythromycin so as to confirm the activity of efflux system in the erythromycin resistant strains. This confirmation was essential to exclude factors that could have affected fluorescence, such as a decrease in cell permeability of Ethidium Bromide (15). In this study, significant inhibitory activity was achieved by erythromycin in combination with CCCP in comparison to erythromycin alone. This may be due to the activity of CCCP as a protonophore that reversibly attaches to protons and transports them across the13t membrane, thereby resulting in the depolarization of the cell membrane, reduced production of ATP by ATP synthase, and expunging of the electrochemical concentration gradient (16-18).

Conclusion

Studies have revealed that *Streptococcus pyogenes* has remained very susceptible to antibiotics in vitro particularly β-lactamase except for macrolides, lincosmides, and streptogramin B (MLSB) antibiotics. It is pertinent to note that antibiotics resistance has become a serious public health concern and the role that efflux systems play in this resistance cannot be ignored. Although efflux pumps are known to confer inherent resistance to bacteria, they work in synergy with other mechanisms like target site mutation to enhance the resistance to a significant level. Therefore, the ethidium bromide-agar cartwheel method provides an easy and instrument-free means by which efflux pump activity can be evaluated for the rapid identification of bacterial isolates expressing active efflux phenotype.

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Ethics approval and consent to participate

Not needed.

Conflict of interest

Rachel Okojie and Promise Aloysius declare that they have no conflict of interest.

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