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PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF ERYTHROMYCIN RESISTANT GROUP A B HAEMOLYTIC STREPTOCOCCI CAUSING ACUTE TONSILLOPHARYNGITIS IN A TERTIARY CARE HOSPITAL

Microbiology

Elayarani A — Post Graduate Student, Department of Microbiology, Chettinad Hospital & Research Institute, Chettinad Academy of Research and Education

Dr. R. Vasanthi — Professor, Department of Microbiology, Chettinad Hospital & Research Institute, Chettinad Academy of Research and Education

Dr. Priyadarshini Shanmugam* — Professor & Head, Department of Microbiology, Chettinad Hospital & Research Institute, Chettinad Academy of Research and Education *Corresponding Author

ABSTRACT

Introduction: An increased incidence of erythromycin resistance among *Streptococcus pyogenes* was noted by the recent studies worldwide. The present cross-sectional study was carried out in the Department of Microbiology at Chettinad Hospital and Research Institute, Chennai, over a period of one year from November 2016 to March 2018.

Materials and methods: A total of 100 throat swabs were processed by routine culture and sensitivity for the genus and species level identification. The *Streptococcus pyogenes* isolates obtained were considered for phenotypic and genotypic detection of erythromycin resistance by Kirby Bauer disc diffusion test and conventional PCR.

Results: Among the fifty *Streptococcus pyogenes* obtained, 52% of isolates showed resistance to erythromycin by phenotypic method, and only 32% samples showed the presence of *mefA* gene.

Conclusion: Future studies focused on molecular mechanisms involved in erythromycin resistance pattern would help in devising better treatment options.

KEYWORDS

Group A Streptococcus (GAS), MefA gene, Erythromycin

INTRODUCTION

Respiratory Tract infections are the most common infections occurring among human beings accounting for about 10% of morbidity and mortality globally [1,2]. Lower respiratory tract infections are responsible for more hospital admissions than upper respiratory tract infections[3,4] *Streptococcus pyogenes* the most common causative agent of bacterial pharyngitis which is characterized by inflammation of the tonsils and other parts of the throat.About 16,300 deaths occur due to invasive Group A Streptococcal disease annually indicating the burden of infection[5,6]. In children, several factors like genetic variations, multiple exposures and immunity play an important role in causing bacterial pharyngitis [7]. Erythromycin is used as an alternative to penicillin in the treatment of acute tonsillopharyngitis, but resistance by m*efA* gene involved in molecular mechanisms like target site alteration by methylase and active efflux have been recently studied [8,9].

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology, Chettinad Hospital and Research Institute (CHRI), a tertiary care hospital in Chennai. The Institutional Human Ethics Committee approval (71/IHEC/9-16) was obtained on 17th October 2016. It was a cross sectional study conducted over a period of 1 ½ years from November 2016 to March 2018.

Sample size: A total of 100 throat swabs were collected from patients presenting with symptoms of sore throat (acute tonsillopharyngitis), dysphagia and fever.

Inclusion criteria: Patients of all age groups presenting with symptoms of acute tonsillopharyngitis and willing to participate in the study with the informed consent form were considered.

Exclusion criteria: Patients presenting with other chronic upper and lower respiratory tract infections and those not willing to participate in the study.

Sample collection and processing: Two throat swabs were collected from the inflamed areas with the help of a tongue depressor, one swab for the smear and the other for culture. Swabs collected were streaked on the solid media like blood agar, chocolate agar, and the respective plates were incubated at 37˚C in the presence of 5% CO_2aerobically for 24 to 48 hours. Plates were observed the next day for the presence of beta haemolytic colonies on blood agar [Figure1]. Isolates showing Gram positive cocci arranged in short chains, negative slide catalase testand positive PYR test with cherry red colour were confirmed as Genus *Streptococci* and group identification was done by Latex agglutination test (LK06 – HiStrep™ Latex Test Kit).

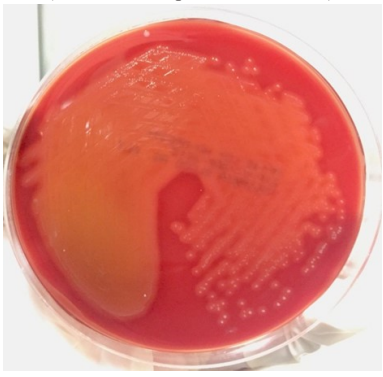

Figure 1: Blood agar plate showing tiny, grey colonies with beta haemolysis.

Antibiotic susceptibility testing was performed by Kirby Bauer disk diffusion method on Muller Hinton agar with 5% sheep blood agar [10]. The test inoculum was standardized with 0.5 McFarland turbidity standards andthe standardized inoculum was lawn cultured on a Mueller Hinton agar with 5% sheep blood agar plate by making an even streak of the swab over the entire surface of the plate in three directions, rotating the plate through an angle of 60˚ after each application. The discs were placed in such a way that they were 15mm away from the edge of the plate and the distance between each disc not less than 25mm. Only 6 discs were placed per petri dish and incubated at 37˚C aerobically overnight. The diameter of the zones was measured and interpreted as "Susceptible(S), Intermediate (I), Resistant(R)" as per CLSI guidelines 2017. The antibiotic discs used were penicillin (10 units), ampicillin (10µg), cefotaxime (30µg), vancomycin (30µg), erythromycin (15µg), azithromycin (15µg), tetracycline (13µg), ofloxacin (5µg), clindamycin (2µg), linezolid (30µg), and bacitracin (0.04 units). For *mefA* gene detection, conventional PCR was carried out with Group A *Streptococcus* isolates which were stored at -20˚C in brain heart infusion broth (BHI) containing 30% glycerol.

DNA Extraction by salting out method was done: About 2-3 isolated colonies from subculture plate were emulsified in BHI broth dispensed in Eppendorf vials and kept in incubator for 18-24 hours. Eppendorf vials were centrifuged for 10 minutes at 5000 rpm and the

supernatant was discarded and 500 µl of lysis buffer was added. To this about 5 µl proteinase K was added. About 10 µl sodium dodecyl sulphate was added and was kept at 55˚C for 2 hours incubation. About 100 µl phenol chloroform and Iso amyl alcohol were added to it. The vials were then centrifuged at 10,000 rpm for 5 minutes; about 100 µl ice cold ethanol was added and then centrifuged at 7,000 rpm for 3 minutes. Then TE buffer [tris (Ie)-2-ethoxyvinyl) borane was added which acts as a storage chemical.

PCR amplification procedure: About1µl of the stored DNA was added to the PCR mixture [Table 1&2] followed by initial denaturation at 96˚C for 1 minute as hot start, followed by 25 cycles of denaturation at 96 ˚C for 10 seconds, annealing at 50˚C for 5 Seconds and extension for 1 minute at 60˚C,the final extension procedure at 60˚C for 4 minutes.

Table -1

PCR Mixture	
Total Volume	25 µl
DNA	1 µl
Forward Primer	3 µl
Reverse Primer	3 µl
Nuclease free Water	3 µl
Master mix	15 µl

Table- 2

Primers used for mefA gene		
Gene Type		**Primers**
Gene for *mefA*	Forward	5'-AGTATCATTAATCACTAGTGC-3'
	Reverse	5'-TTCTTCTGGTACTAAAAGTGG-3'

Gel documentation of DNA fragments: An intercalating dye Ethidium bromide was added to agarose gel and isolation of bands was determined by the examination of the gel under the UV transilluminator. The amplified product of *mef A* gene with the size of 420 bp [Figure 2] was detected using 100bp ladder.

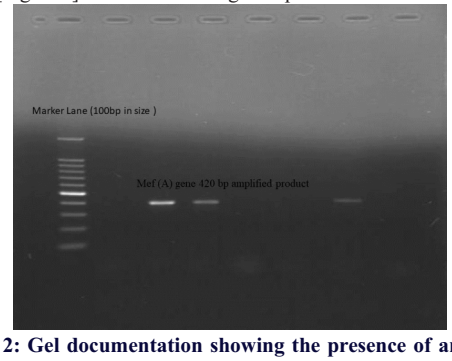

Figure 2: Gel documentation showing the presence of amplified mefA gene at 420bp in samples.

RESULTS

During the study period, about 100 throat swabs were collected and processed, out of which 80 yielded pathogenic bacteria. Among the total 80 isolates obtained, 74% were Gram positive cocci, 6 % were Gram negative bacilli and the rest 20% were normal flora [Figure 3]. Among the 74 isolates of Gram-positive cocci, 50 (67.57%) were *Streptococcus pyogenes*, 17 (22.97%) were other *Streptococci*, 7 (9.46%) were *Staphylococcus aureus* [Figure 4]. Among the 6 Gram negative bacilli obtained, 4 (66.67%) were *Pseudomonas aeruginosa*, 2 (33.33%) were *Acinetobacter spp.* In our study among the 50 isolates belonging to *Group A Streptococcus*, 26 (52%) exhibited phenotypic resistance to erythromycin by disc diffusion method. About 10 (38.46%) of the resistant strains were obtained from the age group between 15 to 25 years, followed by 7 (26.92%) isolates from the age group more than 25 years, and 6 (23.08%) isolates were obtained from below 10 years. In association with the GAS isolates obtained in the different age groups the statistical analysis showed p value of <0.0001 which was found to be more significant in the age group below 10 years [Table 3].

A total of 16 (61.53%) isolates were showing the presence of *mefA* gene out of the 26 GAS isolates exhibiting phenotypic resistance to erythromycin. About 7 (43.75%) isolates showed the presence of *mefA* gene in the age group more than 25 years and 4 (25%) isolates each in the age groups less than10 years and between 10 to 15 years [Table 4].

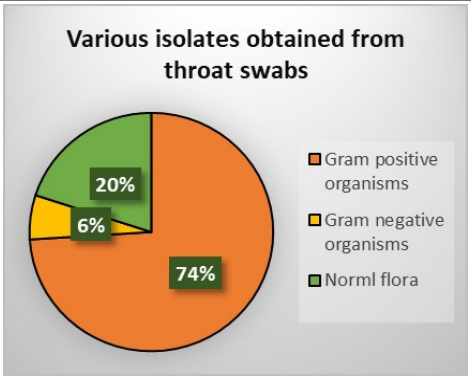

Figure 3: Distribution of the bacterial isolates obtained from throat swabs

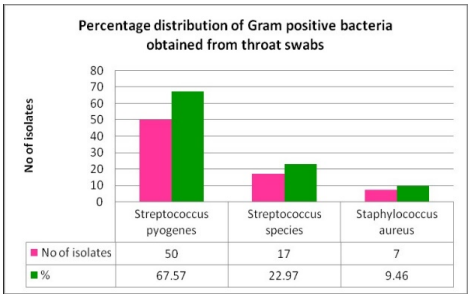

Figure 4: Distribution of Gram-positive bacteria obtained from throat swabs

Table 3

Age wise distribution of Streptococcus pyogenes exhibiting phenotypic resistance to erythromycin.					
Age	Total no of GAS isolates	%	No of GAS isolates showing phenotypic resistance to erythromycin	%	p value
< 10 yrs	26	52	6	23.08	P < 0.0001*
10- 15 yrs	6	12	3	11.54	0.2969
15 - 25 yrs	10	20	10	38.46	1.0000
>25 yrs	8	16	7	26.92	0.7805
Total	50	100	26	100.00	

*P value is fond to more significant it the age group below 10 years

Table 4

Age wise distribution of mefA gene among isolates showing phenotypic resistance to erythromycin.			
Age	No of GAS isolates showing phenotypic resistance to erythromycin	Presence of mef A gene	%
< 10 yrs	6	4	25
10- 15 yrs	3	4	25
15 - 25 yrs	10	1	6.25
>25 yrs	7	7	43.75
Total	26	16	100

DISCUSSION

The incidence of *Streptococcal* infection has gradually increased in India and in few other Asian countries [10,11,12,13,14]. According to the study conducted by Bhardwaj N et al in north Indian population, about 5% isolates of GAS obtained from various clinical samples showed the presence of *mefA* gene [15]. In the present study done, out of 50 (50%) isolates of *Streptococcuspyogenes* obtained from throat swab cultures, 26 (52%) isolates showed phenotypic resistance to erythromycin, out of which only 16 (32%) were positive for *mefA* gene. The frequency of erythromycin resistance is region specific which might be the possible reason for higher resistance level of GAS to erythromycin in our study. Surveillance research from UK and Ireland (2006) confirmed that the level of erythromycin resistance always increases over a period [16]. According to Abraham T et al phenotypic resistance to erythromycin was found in 53% which is a much concern in treating penicillin allergic patients with invasive GAS infection and concluded that

erythromycin usage should be strictly monitored in human as well as livestock to prevent emergence of resistance[17].In our study higher resistance was detected among GAS isolates to cotrimoxazole (100%), followed by erythromycin (52%), which is like the above study.

The resistance of erythromycin had been gradually rising globally since 1955.The macrolide resistance by GAS isolates differs with regions and countries. The highest prevalence was reported in Asia, whereas Lebanon, Turkey and Taiwan had reported the lowest incidence[18,19].The possible mechanism by which GAS exerts erythromycin resistance is by efflux pump because of the presence of *mefA* gene (M phenotype) which was proved by a series studies conducted over a period of time in Argentina covering different cities [20]. Howard et al proposed that combined identification of *mef A* and *erm B* genes (efflux with ribosomal modification) would help to detect the presence of constitutive phenotype (MLS. sub. B) among GAS isolates. Understanding the molecular mechanisms would help to tackle the resistance issue and would aid in future administration of antimicrobials [21]. Lesser rates of resistance have been documented in Serbia with 6.8% and in Romania as 5% [22,23].

Mathur P et alrevealed that there exist several heterogeneities among GAS strains specifically from north and south India estimating almost around 45 different types and subtypes [24]. Several vaccines against GAS isolates acting on the multiple M types provide a wide spectrum of coverage in protection against post streptococcal complications. It has been proved that vaccines prepared with 26 varied M types through a multiple epitope combination give protection against 85% to 90% of M types in the US and Europe [25,26]. Diversity of *emm* genotypes in countries like Nepal, Australia, Brazil and India would not benefit from the above formulation based on 26-valent vaccines due to larger diversity [27].

CONCLUSION

There was no clear association between exotoxin production and disease severity among β-haemolytic *streptococcus* infections. The *mefA gene* distribution in *Streptococcuspyogenes* was more diverse among south Indian population. The possible mechanism by which GAS exerts the erythromycin resistance is by efflux pump because of the presence of *mefA* gene (M phenotype). In conclusion, a high level of antimicrobial resistance was observed amongst β-haemolytic *Streptococcus* and theunderstanding of antibiotic resistance, both by phenotypic and PCR based methods is very important for appropriate treatment choice.

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