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ORIGINAL ARTICLE

Rate and Mechanism of Erythromycin Resistance in *Streptococcus pyogenes* in Beni-Suef University Hospital

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ABSTRACT

Background*: Group A streptococci (GAS) is a major cause of morbidity and mortality. Erythromycin is an effective macrolide antibiotic for treating GAS infections. However, GAS macrolide resistance has been increased due to either an efflux mechanism (M phenotype), encoded by mef A gene, or by methylation of the ribosomal target resulting in resistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics. Methylase can be expressed either constitutively (cMLS phenotype) or inducibly (iMLS phenotype).* ***Objectives****: The present study aimed to find out frequency of S. pyogenes isolated from patients with upper respiratory tract infections at Beni-Suef University Hospital, determine rate and mechanism of macrolide resistance.* ***Methodology****: The present study was conducted on patients with upper respiratory tract infections attended to otorhinolaryngology clinic, Beni-Suef University Hospital, Egypt, in the period from February to December 2015. Detailed history taking was carried and clinical findings were obtained. Throat or ear swabs were taken and processed by conventional bacteriological methods. S. pyogenes isolates were further tested to determine erythromycin resistance phenotype by D- test, MIC of Erythromycin by tube broth dilution method and for mef A gene by PCR.* ***Results****: Forty two S. pyogenes isolates were identified from (100) swabs taken from either ear or throat specimens (42%), isolates resistance to erythromycin and clindamycin was 83.3% (35) and 31% (13) respectively. The pattern of macrolide resistance was 31% (13/ 42) cMLS phenotype, 52.3% (22/42) M phenotype and no isolate was iMLSB phenotype. Most strains with M phenotype expressed low-level macrolide resistance (MIC 1-4µg/ml), while cMLSB isolates showed a high level of erythromycin resistance (MIC ≥64 µg/ml) (highly significant: p-value 0.0001). The results confirmed a strong correlation between the M phenotype and the mef A gene in GAS (highly significant: p-value =0.001).* ***Conclusion:*** *Incidence of erythromycin resistance was evident among the isolates. To preserve the necessary efficacy, limited use of erythromycin is recommended.*

INTRODUCTION

Streptococcus pyogenes (*S. pyogenes*) is a Gram positive, human specific bacterial pathogen. It is an aerotolerant anaerobic coccus that forms long chains of cells when actively dividing.[1]

Based on the Lancefield classification of serologic typing that depends on surface carbohydrate antigen, *S. pyogenes* is a Group A *Streptococci* (GAS), moreover, serotyping depends also on the expressed surface M protein according to which more than 100 serotypes exist. More recently, this serotyping system has been replaced with the nucleotide sequence of the 5 end of the *emm* gene, producing over 223 serotypes to date.[2]

S. pyogenes has the ability to cause a wide spectrum of diseases and contributes to an immense burden of human illness that contributes in global morbidity and mortality.[3] These diseases can range from mild, non-invasive pharyngitis and impetigo to much more serious invasive diseases such as necrotizing fasciitis and toxic shock syndrome. Furthermore, non-invasive diseases can result in post–infection complications such as acute post–streptococcal glomerulonephritis (APSGN) and acute rheumatic fever (ARF), which may lead to rheumatic heart disease (RHD) and reactive arthritis.[4]

Penicillin has been the drug of choice for the treatment of GAS infections. Even though *S. pyogenes* has remained universally susceptible to β-lactams, in the past 15 years, the rate of penicillin failure has dramatically increased to almost 40% in some regions of the world.[5] Additionally, many patients are allergic to

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penicillin and in those patients macrolides are used as the alternative treatment of choice.[6]

Erythromycin is an effective macrolide antibiotic for treating GAS infections. However, increasing erythromycin resistance in GAS isolates was noted in the 1990s, and in some countries, this resistance peaked in the early 2000s.[7] The fluctuation in GAS macrolide resistance has been associated with changes in macrolide use.[8] So determining erythromycin resistance phenotypes seems to be a useful tool in eradicating GAS infection.[9]

The main known mechanisms of macrolide resistance in *S. pyogenes* are macrolide specific efflux mechanism (M phenotype), encoded by the macrolide efflux protein A (*mef A*) gene, as well as the modification of the ribosomal target by a methylase enzyme. Methylation leads to decreased antibiotic binding and co-resistance to macrolide, lincosamide, and streptogramin B (MLS). Methylase can be expressed either constitutively (cMLS phenotype) or inducibly (iMLS phenotype).[10] Therefore, the objective of this study was to determine the prevalent erythromycin resistance mechanisms of *S.pyogenes* isolated from patients attending outpatient clinic of otorhinolaryngology Beni-Suef University Hospital.

METHODOLOGY

The present study included 100 patients, in the period from February to December 2015. They were enrolled from patients attending at outpatient clinic of otorhinolaryngology, Faculty of Medicine, Beni-Suef University, Egypt.

Detailed history taking was carried out including: history of smoking, family size, chronic disease, skin infection in the previous 14 days and close contact with patient having sore throat in the previous 14 days. Additionally, demographic data (including sex**,** age and residence) and clinical findings were obtained from each patient.

Specimen collection:

Throat swabs were taken from patients with acute follicular tonsillitis and ear swabs were taken from patients with acute otitis media. Samples were transferred and processed immediately in the Medical Microbiology and Immunology Laboratory, Faculty of Medicine, Beni-Suef University.

Identification of *S. pyogenes*:

S. pyogenes yields were identified by Gram stain, colony morphology on 5% sheep blood agar containing medium, incubated for 18-24 hours at 35-37°C with 5% CO_2,[11] catalase test and serologic identification was done by **(Slidex® Strepto Plus A** (bioMérieux® SA).[12]

D- test using Clindamycin and Erythromycin discs (Oxoid, England) was performed in accordance with the CLSI guidelines[13] to determine the 3 phenotypes of erythromycin resistance; M phenotype in which the strain was resistant to erythromycin but sensitive to clindamycin, Constitutive phenotype (cMLSB) in which strain was resistant to both erythromycin and clindamycin & Inducible phenotype (iMLSB) in which the strain was erythromycin resistant and clindamycin inducible i.e blunting of the clindamycin inhibition zone near to the erythromycin disc.[14]

MIC determination:

Resistant isolates to erythromycin were tested by tube broth dilution method to determine the MIC. Erythromycin powder (Sigma Chemical Company) was used to prepare the antimicrobial agent stock solution at concentration of 1280 µg/mL and used after dilution, according to CLSI 2013.[13]

PCR to detect *mef A* gene:

Strains with M phenotype-erythromycin resistance were submitted to further studies to detect *mef A* gene by PCR; according to Rubio-López et al., 2012.[15] DNA was extracted using nucleic acid extraction kit (Vivantis®). The primers used for *mef* A gene amplification were:

- Forward primer (mef-F):
  5`-CAGGGTCATAAAGCCTAAATAG-3` and
- Reverse primer (mef-R):
  5`-GAGGTAAGCTACATAAACTGTG-3`.[15]

Approval of the ethical committee, faculty of medicine, Beni-suef university was obtained and Informed written consent was obtained from all study participants or their guardians.

Statistical analysis:

Qualitative data were presented in the form of frequency distributions with percentages, while quantitative data were presented as means and standard deviation. Cross tabulation test were done in addition to *p*-values for the Chi - square test. *P*-values of <0.05 were considered as statistically significant. Analysis was conducted using Statistical Package for Social Science, (SPSS) version 20 (IBM, Amronk NY).

RESULTS

The current study involved 100 patients, 55% were females, their ages ranged between 1 and 35 (mean 17, 48% were less than 10) years old. Eighty eight % of them were from rural residents, 87% had no chronic disease, 12% had history of skin infection in the previous 2 weeks and 66% had history of contact with someone complaining from sore throat in the previous 2 weeks (table 1).

Furthermore, 64% were diagnosed as acute otitis media, while 36% were diagnosed as cases of pharyngitis and tonsillitis. The collected samples revealed 42 GAS yields (15/36 throat swabs and 27/64 ear swabs *p*-value= 0.692).

Table 1: Results of univariate analysis of potential risk factors for acquisition of GAS & development of erythromycin resistant strains:

Predisposing factor		GAS (Total=42)		Erythromycin susceptibility		
		NO. (%)	P- value	Resistant (total=35) NO. (%)	Sensitive (total=7) NO. (%)	P- value
Age (years)	≥10 11-20 21-30 >30	**21 (50%)** 8 (19.1%) 4 (9.5%) 9 (21.4%)	0.988	17 (48.5%) 8 (22.8%) 4 (11.5%) 6 (17.2%)	4(57%) 0 0 3(43%)	0.32
	Mean 15					
Gender	Male Female	21 (50%) 21 (50%)	0.532	18 (51.5%) 17 (48.5%)	3 (43%) 4 (57%)	**0.**.679
Residence	Urban Rural	2 (4.8%) **40 (95.2%)**	.277	1 (2.8%) 34 (97.2%)	1(14.3%) 6 (85.7%)	0.195
Seasonal	Winter Spring Summer Autumn	13 (31%) 16 (38%) 11 (26%) 2(5%)	**0.02**	12 (34.3%) 14 (40%) 9 (25.7%) 0	1 (14.4%) 2 (28.5%) 2 (28.5%) 2 (28.6%)	**0.025**
Smoking	Smoker Passive Non smoker	8 (19%) 34 (81%) 0	0.858	7 (20%) 28 (80%) 0	1 (14.3%) 6 (85.7%) 0	0.882
Family size (number of persons)	3-6 7-10 11-14	22 (52.4%) 16 (38.1%) 4 (9.5%)	0.841	16 (45.8%) 15 (42.8%) 4 (11.4%)	6 (85.7%) 1 (14.3%) 0	0.07
Chronic disease	Positive Negative	6 (14.3%) 36 (85.7%)	0.710	4 (11.4%) 31 (88.6%)	2 (28.6%) 5 (71. 4%)	0.237
Skin infection in the previous 14 days	Positive Negative	10 (23.8%) 32 (76.2%)	0.009	9 (25.7 %) 26 (74.3%)	1 (14.3%) 6 (85.7%)	0.517
Close contact with patient having sore throat in the previous 14 days	Positive Negative	30 (71.4%) 12 (28.6%)	0.785	27 (77.2%) 8 (22.8%)	3 (42.8%) 4 (57.2%)	0.057

Out of 42 GAS isolates, 35 (83.3%) were resistant to erythromycin (24/ 27 in ear and 11/15 in throat isolates respectively, *p*=0.195) and 13/42 (31%) isolates were resistant to clindamycin with a highly significant *p* value (= 0.0001) (figure 1).

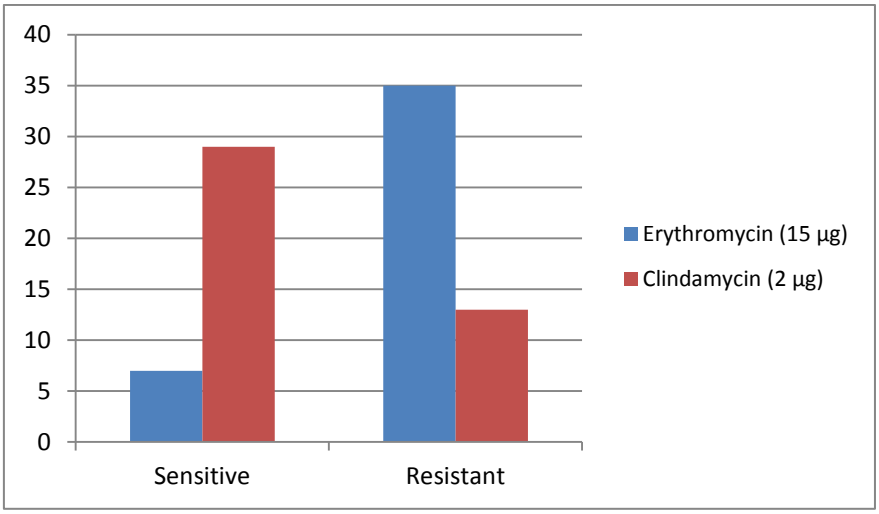

Fig. 1: Susceptibility to Erythromycin and Clindamycin

The D- test showed highly significant M phenotype 22/42 (52.3%) (p =0.000) and cMLSB phenotype 13/42 (31%) (p =0.0001) isolates among the GAS strains tested, while none were with inducible phenotype (iMLSB) (Figure 2).

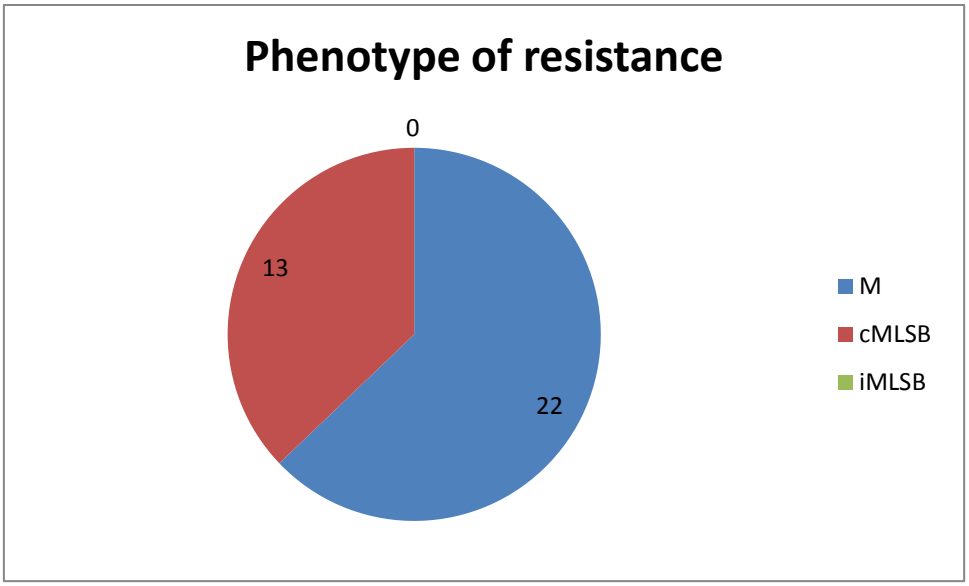

Fig. 2: Phenotype of macrolide resistance

Concerning the predisposing risk factors for acquisition of GAS and development of erythromycin resistant strains there was a significant association only with seasonal exposure (p-value = 0.025) (table 1).

The MIC of most strains of M phenotype was 1-4 µg/ml, while most strains of constitutive phenotype had minimal inhibitory concentration ≥ 64µg/ml with a highly significant p- value (=0.0001) (table 2).

Table 2: The minimal inhibitory concentration (MIC) of erythromycin:

Concentrations µg/ml	No of isolates (total =35)		P- value
	M phenotype (Total =22)	Constitutive phenotype (Total =13)	
1-4	16	0	**0.0001**
8-32	5	3	
≥64	1	10	

The *mef* A gene was detected in all 22 strains of M phenotype (100%) with a highly significant p value of 0.001 (figure 3).

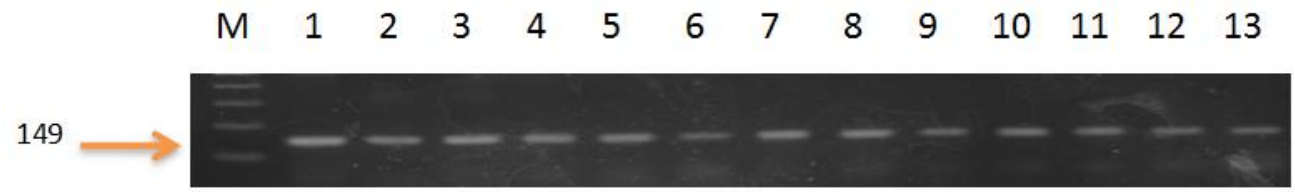

Fig. 3: Agarose gel electrophoresis showed PCR product of *mefA* gene with 149 bp size in DNA extract. Lane M: DNA ladder (100, 200, 300,……etc) Lane 1-13: PCR products of positive samples

DISCUSSION

Streptococcus pyogenes is a main human pathogen that causes over 600 million infections yearly. This genus is capable of colonizing the upper respiratory tract and skin of asymptomatic people; however, it is additionally responsible for a wide range of diseases, including suppurative infections and non-suppurative complications, which may occur either endemically or as outbreaks. [16]

The overall frequency of upper respiratory infections associated with GAS in our study was 42% (41.7% in throat swabs and 42.2% in ear swabs). A similar result was reported from Ethiopia; GAS was isolated in 40.6% of the cases. This similarity in high prevalence of GAS may be due to seasonal and social similarity, where the study in Ethiopia was conducted from February to May; when the carriage and infection rates of GAS reached their maximum.[17]

Unlike the present findings, Wessels results[18] showed that the most common bacterial pathogens in the upper respiratory tract infections were *S. pyogenes* and stated that the percentage of *S. pyogenes* that caused acute tonsillopharyngitis was 23.81%. Moreover, lower percentages were also obtained by Afaf et al., in Egypt; who reported *S. pyogenes* in only 18.5% of the specimens.[19]

In the same context, lower frequencies were also spotted in several studies in Turkey, [20] India, [21] Taiwan[22] and (GAS frequency among URT samples were 11%, 2.8% and 4.1% respectively).

The higher prevalence rate in the present study is probably due to the trends of over prescription of antibiotics in health facilities, variations and differences in methodology, seasons of sample collection and geographical variation of study settings.[23]

The mean age of patients with GAS, in the current investigation, was 15 years old (50% were below 10 years). This was comparable to the work of Fatima et al.[24] who stated that 75% of GAS isolated strains were in 5-10 age group and Esther et al.[25] who reported that the highest frequency of GAS pharyngitis and otitis infection was detected in children aged 1–8 years old. That may be explained by the lack of this population immunity and strain tropism. On the other hand**,** Rijal et al.[26] stated that the frequency of GAS was similar in all age groups of school children.

Out of 42 GAS isolates, 83.3% were resistant to erythromycin and 31% isolates were resistant to clindamycin. Worldwide variations of *S.pyogenes* resistance to erythromycin was observed; it reached erythromycin 21.3% and clindamycin 10.7% in the study done by Ibrahim et al.[27] in Egypt, while in other studies erythromycin resistance was 42% in Poland, 24% in Portugal, 28% in Hong Kong, 21% in Spain and 25% in Italy.[28] Moreover, Ciftci et al.[29] reported resistance to erythromycin and clindamycin as 3.8% and 3.0% respectively.

The D-test, performed in the current investigation, showed highly significant M phenotype 52.3% isolates among the GAS strains tested and cMLSB phenotype 31%, while none were with inducible phenotype (iMLSB). These results agreed with the findings from previous researches, who stated that high erythromycin resistance rates are associated with the M phenotype and no iMLSB pattern was detected.[30]

Pérez et al.[31] stated that among erythromycin-resistant isolates of *S.pyogenes*, a significant increasing trend in the prevalence of MLSB was observed (from 7.0% to 35.5%). Likewise, in Norway and in Bulgaria, iMLSB was the prevalent phenotype in GAS isolates,[32] and a study in France demonstrated predominance of the cMLSB phenotype.[33]

The reasons why a particular phenotype predominates in a specific geographical region are not completely acknowledged; nevertheless, this may be related to differences in the organization of medical care and antimicrobial prescribing practices. Furthermore, the new macrolides; such as clarithromycin and azithromycin; are frequently used in the Egyptian market as preferred treatment for GAS, these long acting macrolides as they ensure a low serum concentration of the antibiotic for a long period of time could select for resistant strains with consequent changes in macrolide resistance rates and phenotypes.[34]

In the current study, among clinical variables only seasonal variations was found to be a predictor for GAS infections. Erythromycin GAS sensitive strains were significantly associated with seasonal exposure and the time of study, which was more common in the period from January to May. This may be explained by the prevalence of *emm4* and *emm22* isolates in this period which showed higher rate of resistance.[35] Unlike the current data, univariate analysis done by Chia-Ying et al.[36] indicated that study period, age, and specimen types were possible factors associated with erythromycin resistant GAS isolates.

In addition, there has been a strong negative association between the age of patients and the occurrence of erythromycin-resistant strains. This seems to be due to the prescription of more antibiotics for children and a higher risk of cross-colonization among children than among adults. [37]

Out of 22 M phenotype strains, 73% expressed low-level of macrolide resistance, while, 77% of cMLSB isolates showed a high level of erythromycin resistance.

These findings are in agreement with the reports recorded in Africa[38] who stated that strains with the cMLSB phenotype have high MICs for erythromycin, while, the M phenotype expressed lower-level of macrolide resistance. Similarly, Farrell et al.[39] stated that *S. pyogenes* M-phenotype have low macrolide MICs 1–

8 µg/mL, cMLSB resistance ranged from 16 to >64 µg/mL, while, inducible MLSB resistance ranged from 1 to 64 µg /mL.

The current data confirmed a strong correlation between the M phenotype and the *mef A* gene in GAS, since M phenotype strains were all positive for *mef A* gene. These results agree with the susceptibility pattern done by Silva et al.[40] who stated that GAS isolates M phenotype confers low to moderate levels of resistance and are generally encoded by the *mef A* gene.

CONCLUSIONS

S. pyogenes has continued to be highly susceptible to antimicrobial agents in vitro since the 1940s, specifically to penicillins, which are usually the first-line of treatment. Macrolide resistance may become a problem, since it has emerged in numerous countries, and as a result, in vitro antimicrobial susceptibility testing should be performed. Such testing will not only allow researchers to distinguish susceptible phenotypes from resistant phenotypes, but also to differentiate between the different resistant phenotypes since they unravel the potential activity of the different macrolide members. Consequently, continuous monitoring of resistance pattern of GAS to macrolides and other alternative drugs is recommended to avoid possible treatment failures. The restricted use of erythromycin is advised to maintain the required efficacy. Furthermore, clindamycin is a good alternative for GAS infections in penicillin allergic children.

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- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES

1. Wintermute EH and Silver PA: Dynamics of the mixed microbial diseases. Genes Dev, 2016; 24:2603–2614.
2. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM and Relman DA: The application of ecological theory toward an understanding of the human microbiome. Science, 2016; 336:1255 – 1262.
3. Jubeh B, Breijyeh Z, and Karaman R: Resistance of Gram-Positive Bacteria to Current Antibacterial Agents and Overcoming Approaches. Molecules, 2020; 25(12): 2888. doi: 10.3390/molecules25122888
4. Barth D, Naicker P, Engel K, Muhamed B, Basera W, Mayosi B, Dale J and Engel M: Molecular Epidemiology of Noninvasive and Invasive Group A Streptococcal Infections in Cape Town. mSphere, 2019; 4(5): e00421-19. doi: 10.1128/mSphere.00421-19
5. Brook I: Penicillin failure in the treatment of streptococcal pharyngo-tonsillitis. Current Infectious Disease Reports, 2013; 15(3):232–235. [PubMed]
6. Jourdan A, Sangha B, Kim E, Nawaz S, Malik V, Vij R and Sekhsaria S: Antibiotic hypersensitivity and adverse reactions: management and implications in clinical practice. Allergy, Asthma & Clinical Immunology, 2020; 16, Article number: 6.
7. Silva-Costa C, Friães A, Ramirez M, and Melo-Cristino J: Differences between macrolide-resistant and -susceptible *S. pyogenes*: importance of clonal properties in addition to antibiotic consumption. Antimicrob.Agents Chemother., 2012; 56:5661–5666.10.
8. Van Heirstraeten L, Coenen S, Lammens C, Hens N, Goossens H and Malhotra-Kumar S: Antimicrobial drug use and macrolide-resistant *S. pyogenes*, Belgium. Emerg. Infect. Dis., 2012; 18:1515–1518. 10.3201/eid1809.120049.
9. Kaplan EL: The resurgence of group A streptococcal infections and their sequelae. Eur J ClinMicrobiol Infect Dis, 2000; 10:55-7.
10. Bozdogan B, Appelbaum P, Ednie N, Grivea I and. Syrogiannopoulos G: Development of macrolide resistance by ribosomal L4 mutation in *S. pyogenes* during miocamycin treatment of an 8-year-old Greek child with tonsillopharyngitis, J ClinMicrobiol. Infect., 2003; 9:966-969.
11. Clinical and Laboratory Standards Institute (CLSI): Performance standards for antimicrobial susceptibility testing, seventeenth informational supplement, 2013; S17; 27 No. 1.
12. Meisal R, Michaelsen TE and Caugant D: *S. pyogenes* isolates causing severe infections: *emm* types, multi locus sequence types, and superantigen profiles. J Clin Microbiol., 2010; 60 (3):872-47.

13. Clinical and Laboratory Standards Institute (CLSI): Disc diffusion supplemental tables Performance standards for antimicrobial susceptibility testing. M100- S23, 2013; (M02-A11).
14. Seppala H, Nissinen, A, Yu Q and Huovinen P: Three different phenotypes of erythromycin-resistance *S. pyogenes* in Finland. J. Antimicrob. Chemother., 2005; 32(6): 885 891.
15. Rubio-López V, Valdezate S, Álvarez D, Villalón P, Medina M and CandSáez Nieto S: Molecular characters, antimicrobial susceptibilities and resistance mechanisms of *S. pyogenes* isolates resistant to erythromycin. BMCMicrobiol., 2012; 12: 215.
16. Lynskey N, Lawrenson R, Sriskandan S: New understandings in Streptococcus pyogenes. Current Opinion in Infectious Diseases, 2011; 24(3):196–202. [PubMed] [Reference list]
17. Hortense Kamga Gonsu, Cynthia Mbimenyuy Bomki, François Djomou, Michel Toukam, Valantine Ngum Ndze, Emilia Enjema Lyonga, Calixte Didier Mbakop and Sinata Koulla-Shiro: A comparative study of the diagnostic methods for Group A streptococcal sore throat in two reference hospitals in Yaounde, Cameroon, The Pan African Medical J., 2015; 20:139.
18. Wessels M: Clinical practice. Streptococcalpharyngitis. N. Engl. J. Med., 2011; 364:648-655.
19. Afaf S, Abdulrahman L, Kholeif A, El-Beltagy Y and Eldesouky A: Bacteriology of tonsil surface and core in children with tonsillitis and incidence of bacteremia during tonsillectomy. Egypt J. Med. Lab. Sci., 2004; 13:1-9.
20. Shulman S, Bisno A and Clegg H: Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America. *Clin Infect Dis*., 2012; 55(10):1279-82.
21. Kumar R, Vohra H, and Chakraborty A: Epidemiology of group A streptococcal pharyngitis & impetigo: a cross-sectional & follow up study in a rural community of northern India. Indian J Med Res., 2009; 130:765–771.
22. Shih C, Lin C and Lu C: Evaluation of a streptococcal pharyngitis score in southern Taiwan. Pediatr Neonatol., 2012; 53: 49-54.
23. Ba-Saddik I, Munibari A and Alhilali A: Prevalence of group A beta-haemolytic streptococcus isolated from children with acute pharyngotonsillitis in Aden, Yemen. Trop Med Int Health., 2014; 19(4):431–439.
24. Fatima F and Shubha D: Prevalence Survey for Assessing Intensity of Group A Beta Hemolytic Streptococci (GABHS) Subclinical Infection Rate in School Children: A Cross Sectional Study Global J of Medical research Diseases, 2013; 13(3).
25. Tamayo E, Montes M and Pérez-Trallero E: *Streptococcus pyogenes emm*-types in northern Spain; population dynamics over a 7-year period. J Clin. Microbiol., 2013; 10.1016-.08.013.
26. Rijal K, Dhakal N, Shah R, Timilsina S, Mahato P, Thapa S and Ghimire P: Antibiotic susceptibility of Group A Streptococcus isolated from throat swab culture of school children in Pokhara, Nepal Med Coll J., 2009; 11(4): 238-240.
27. Ibrahim S, El Sokkary R, Elhewala A, El Anwa M, Awad W, Hamed A and Badawy I: Emerging Resistance to Erythromycin and Penicillin among *S. pyogenes* Isolates in Zagazig, Egypt. Inter J Current Microbiol and Applied science, 2014; Volume 3 Number 10 pp.750- 756.
28. Cantón R, Loza E, Morosini I and Baquer F: Antimicrobial resistance amongst isolates of Streptococcus pyogenes and Staphylococcus aureus in the PROTEKT antimicrobial surveillance programme during 1999–2000. *Journal of Antimicrobial Chemotherapy*, 2002; 50, Suppl. S1, 9–24 (DOI: 10.1093/jac/dkf811).
29. Ciftci E, Dogru U and Guriz H: Antibiotic susceptibility of *S. pyogenes* strains isolated from throat cultures of children with tonsillopharyngitis. J Ankara Med Sch., 2003; 25: 15-20.
30. Villaseñor-Sierra A, Katahira E, Jaramillo-Valdivia A, Barajas-GarcíaMde L, Bryant A and Morfín-Otero R: Phenotypes and genotypes of erythromycin-resistant Streptococcus pyogenes strains isolated from invasive and non-invasive infections from Mexico and the USA during 1999 2010. Int. J. Infect. Dis., 2012; 16(3): 178 181.
31. Pérez-Trallero E, Martín-Herrero J, and Mazón A: Spanish Surveillance Group for Respiratory Pathogens. Antimicrobial resistance among respiratorypathogens in Spain: latest data and changes over 11 years (1996-1997 to 2006- 2007). Antimicrob Agents Chemother., 2010; 54: 2953-9.
32. Jenkins S and Farrell D: Increase in macrolide resistance, United States. Emerg Infect Dis., 2009; 15, 1260-1264.
33. d'Humieres C, Cohen R, Levy C, Bidet P, Thollot F, Wollner A and Bingen E: Decline in macrolide-resistant *S. pyogenes*isolates from French children. Int J Med Microbiol., 2012; 302, 300-303.
34. Amer F, Elbehedy E, and ElAhmady M: Streptococci in an Egyptian urban community: incidence of erythromycin resistance determinants and antibiotic susceptibility profile. Asian Pac. J. Trop. Med., 2008; 1(94): 20 23.

35. Chiang-Ni C, Wu A, Liu C, Chen K, Lin Y, Chuang W, Fang H and Wu J: Emergence of uncommon *emm*types of *S.pyogenes* among adult patients in southern Taiwan. J. Microbiol. Immunol. Infect., 2011; 44:424–429.
36. Chia-Ying Huang, Jui-Fen Lai, I-Wen Huang, Pei-Chen Chen, Hui-Ying Wang, Yih-RuShiau, Ya-Wen Cheng, Li-Yun Hsieh, Shan-Chwen Chang and Tsai-Ling Yang Lauderdale: Epidemiology and Molecular Characterization of Macrolide-Resistant *S. pyogenes* in Taiwan, J Clin Microbiol., 2014; 52(2): 508–516.
37. Klaukka T, Lehtonen R, Nenonen E and Huovinen P: The Finnish Study Group for Antimicrobial Resistance. Outpatient use of erythromycin: link to increased erythromycin resistance in GAS. Clin Infect Dis., 2015; 21:1378–1385.
38. Gagliotti C, Buttazzi R, Di Mario S, Morsillo F and Moro M: A regionwide intervention to promote appropriate antibiotic use in children reversed trends in erythromycin resistance to *S. pyogenes*. Acta Paediatr., 2015; 104:e422–e424.
39. Farrell D, Morrissey I, Bakker S and Felmingham D: Detection of macrolide resistance mechanisms in *S. pyogenes* using a multiplex rapid cycle PCR with microwell-format probe hybridization. J. Antimicrob. Chemother., 2016; 48 (4): 541-544.
40. Silva-Costa C, Friaes A, Ramirez M, and Melo-Cristino J: Macrolide-resistant *S. pyogenes* prevalence and treatment strategies. Expert Rev Anti Infect Ther., 2015; 13 (5):615–28.