

Study of Erythromycin Resistance Genes in *Staphylococcus aureus* Isolated from Throat Swab Culture

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Throat swabs were collected to isolate *S. aureus* by using blood agar media. Biochemical tests were performed. Genomic DNA was isolated and PCR was done for erm A, ermB and erm C genes followed by bioinformatics analysis. Isolates were screened on erythromycin disc (erythromycin range 1- 125mg/l). All 47 *S. aureus* isolates found to be resistance to erythromycin by phenotypic methods contained at least 1 were erythromycin resistance gene. That's mean all *S. aureus* isolates which were resistance to erythromycin by disc diffusion method were found as positive by PCR for erm A, B, and C genes.

Keywords: Throat swabs, bacteria, ermA, ermB, ermC.

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μ m and characterized by single cocci, which divide in more than one level to form grape-like clusters. To date, there are 45 species and eight sub-species in the genus *Staphylococcus*, many of which preferentially found the human body (Kloos and Bannerman, 1994), however *Staphylococcus aureus* and *Staphylococcus epidermidis* are the two most characterized and studied strains.

The staphylococci are non-motile, non-spore form facultative anaerobes that grow by aerobic respiration or by fermentation. They grow easily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that differ from white to deep yellow. Most species have a relative complex nutritional requirement, However, in general they require an organic source of nitrogen, supplied

by 5 to 12 essential amino acids, e.g. arginine, valine, and B vitamins, including thiamine and nicotinamide (Kloos and Schleifer, 1986; Wilkinson, 1997).

Members of this genus are catalase-positive and oxidase-negative, differentiates them from the genus Streptococci, which are catalase-negative, and have a different cell wall composition to Staphylococci (Wilkinson, 1997).

S. aureus is both a commensal organism and a pathogen. The anterior nares are the main ecological niche for *S. aureus*. Approximately 20% of individuals are persistently nasally colonized with *S. aureus*, and 30% are intermittently colonized. However, numerous other sites may be colonized, including the axillae, groin, and gastrointestinal tract. Colonization provides a reservoir from which bacteria can be introduced when host defenses are breached, whether by shaving, aspiration, insertion of an indwelling catheter, or surgery. Colonization clearly increases the risk for subsequent infection (Wertheim *et al.*, 2005; Kluytmans *et al.*, 1997).

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Erythromycin is a metabolic product of *Streptomyces erythreus* and consists of a 14-member lactone ring to which are attached two deoxy-sugars, desosamine and cladinose. The macrocyclic lactone ring is the source of the class name, macrolide. Erythromycin, like most macrolides, appears to act by binding in the ribosomal tunnel through which the nascent peptide moves and thus can be considered a peptidyltransferase inhibitor (Schmidt, 1999).

MATERIAL AND METHODS

Sample collection

185 samples were collected from healthy students, aged between 17-28 years, present at

CytoGene Research and development laboratory from January 2016 to May 2016. All specimens collected constitute of both male and female.

Isolation and Identification Bacteria

The throat swab samples were immediately cultured on 5% sheep blood agar media plates. The culture obtained from primary culture on 5% sheep blood agar plates. It was sub-cultured on brain heart infusion to obtain a pure growth. The isolates were identified based upon their culture characteristic, morphology and by using Biochemical methods (Bergey's Manual of Systemic Bacteriology) (Holt *et al.*, 1984).

Antibiotic Sensitivity Test

Antibiotic sensitivity test was done by Disc diffusion (Kirby bauer) method using

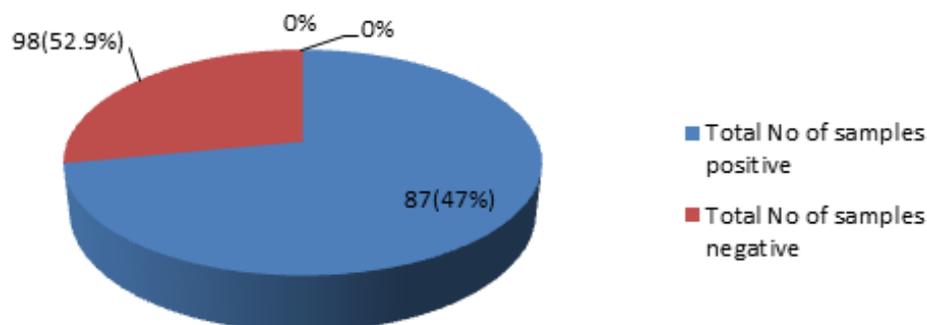


Fig. 1. Distribution of samples showing positive and negative for α-hemolysis on blood agar media

Table 1. Primers of ermA, B, C and 16SrRNA

Primers	Sequences (5' - 3')	Conc (Unit/μl)	T _m Value	Length (bp)
erm A-F	TATCTTATCGTTGAGAAGGGATT	3	57°C	24
erm A-R	CTACACTTGGCTTAGGATGAAA	3	53°C	22
erm B-F	TATCTGATTGTTGAAGAAGGATT	3	57°C	24
erm B-R	GTTTACTCTTGGTTTAGGATGAAA	3	57°C	24
erm C-F	CTTGTTGATCACGATAATTCC	3	53°C	22
erm C-R	ATCTTTTAGCAAACCCGTATTC	3	53°C	22
16S-F	GGAGGAAGGTGGGGATGACG	3	57°C	20
16S-R	ATGGTGTGACGGGCGGTGTG	3	57°C	20

Table 2. Biochemical test results for the isolates from throat swab

Bacteria	Catalase Test	Coagulase Test	Gelatine hydrolysis	Mannitol fermentation Test	MR	Vp	Indole Test	Citrate utilization Test
<i>S.aureus</i>	+	+	+	+	+	+	-	+

* "+" = Positive; "-" = Negative

erythromycin, penicillin, Amoxicillin, clindamycin, tetracycline, ciprofloxacin and trimethoprim against all the isolates

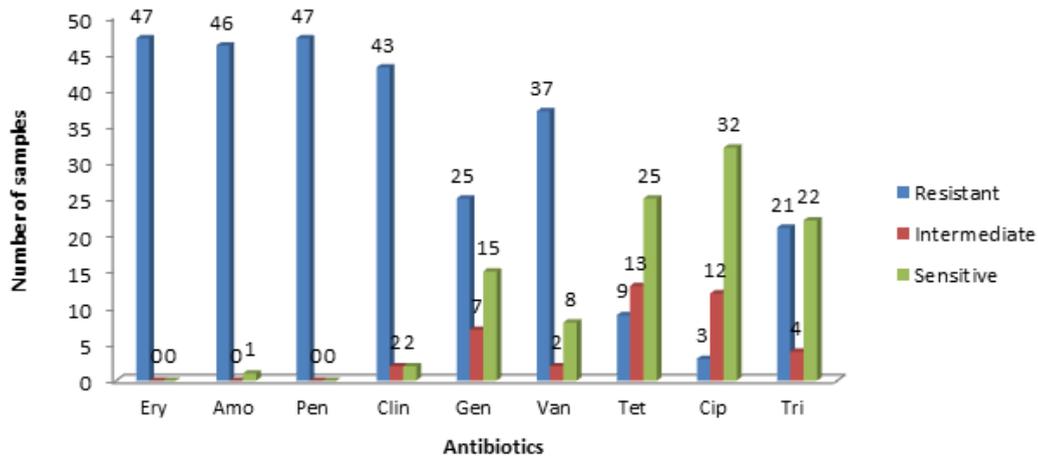
DNA Isolation from bacterium

Isolation of genomic DNA was performed using the Phenol chloroform method. Extraction of bacterial DNA was done through broth bacterial culture. Taken 2 ml culture broth in an Eppendorf tube and centrifuged at 10000 rpm for 10 mins only. There after supernatant was discarded and the pellet was dissolved in T.E. buffer. 10% SDS was added and tubes were kept in water-bath for cell lysis. After 1-2 hours tubes were again centrifuged and the supernatant was collected. Phenol: Chloroform: Isoamyl ratio was added and the samples were

again centrifuged. An upper transparent layer containing DNA was collected and 3 M sodium acetate was added for precipitation of DNA and kept in ice cold conditions. Again double volume of ethanol added in eppendorf tubes and centrifuged. Discard the supernatant and air dry the tubes and add T.E. buffer to the tubes for loading in Agarose gel electrophoresis.

PCR amplification

Template DNA was used in each primer (forward and reverse primer) by adding dNTP and four deoxyriboneucleotide tri phosphate. Taq polymerase was used in each sample. Amplification was carried out in a thermal cycler. Temperature for Initial denaturation at 96p C for 3 minutes followed



*Ery = Erythromycin; Amo = Amoxicillin; Pen = Penicillin; Clin = Clindamycin; Gen = Gentamicin; Van = Vancomycins; Tet = Tetracycline; Cip = Ciprofloxacin and tri = Trimethoprim

Fig. 2. Antibiotic resistance profile of *Staphylococcus aureus*



Fig. 3. Agarose Gel Electrophoresis (a) 0.8% Agarose gel (b) Buffer used- 1X TAE (c) Visualization dye- EtBr, Lane 1,2,3,4,5,6,7,8,9,10,11 Genomic DNA of *S. aureus* isolates compared with Lane 12-1kb DNA Ladder

by 30 cycle, each cycle consisted of denaturation of DNA for 1 second at 95p C, annealing of the primers for 30 seconds at 35p C and elongation 72p C for 7 minutes.

Primer sequences

Analysis by bioinformatics

The comparison of the nucleotide sequences of the unique fragment with the sequences available in the Genbank database was carried out by using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast>). Phylogenetic tree were generated on the basis of sequences of Myelo peroxidase gene sequences using Clustal W 1.8 (Thompson *et al.*, 1994). A method poly genetic tree was constructed using the sequences obtained from the samples.

RESULTS AND DISCUSSION

Isolation and Identification of *S.aureus*:
Current study was performed in 5 months period from January to May in the year 2016. The study was conducted among 185 healthy students of CytoGene Research and Development Laboratory in Lucknow. Spreading of the swab samples were done on blood agar media followed by streaking on Brain heart Infusion agar media. Out of 185 throat swabs samples 87(47%) of samples were positive i.e., β hemolytic colonies and 98(52.9%) were non-hemolytic (no zone around the colonies). In this study, from total throat swab samples 47% of sample showed positive for β hemolysis

like Devapiry *et al.* (2012) work where a similar percentage of β -hemolytic population was obtained on blood agar media. The differences in presence of *S.aureus* (F.Cal=17.552) in relation to different age group was statistically significant.

The isolates were identified based on their morphological and biochemical characteristics. The biochemical tests for identification of isolates and their results are shown in the table (table 2) given below. The characteristic properties of the isolates determined the species as *Staphylococcus aureus*.

All the identified *S.aureus* populations were tested for their antimicrobial susceptibility patterns. The isolates showed high resistance to erythromycin, penicillin, Amoxicillin and

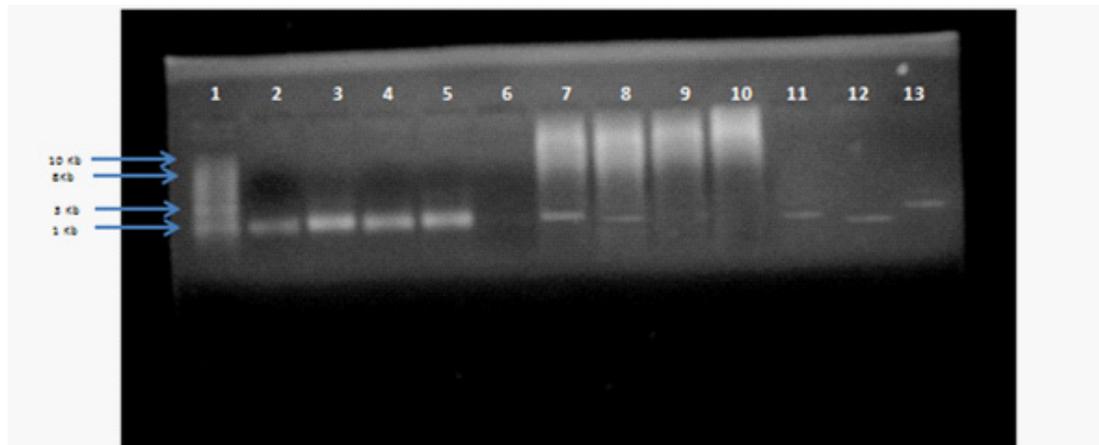


Fig. 4. Agarose gel electrophoresis of PCR product for 16S rRNA amplified gene (size more than 1.4 Kb) compared with (1Kb) DNA ladder

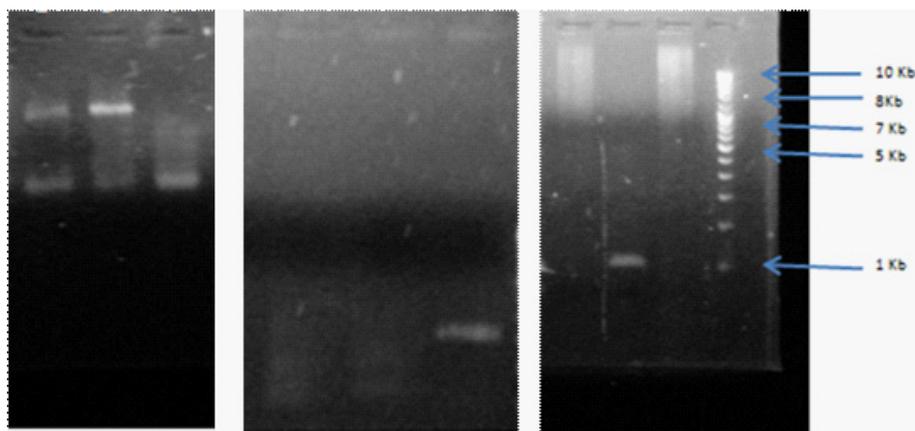


Fig. 5. Agarose gel electrophoresis of PCR amplification for ermA gene, erm B gene, erm C gene (amplified size 737bp) compared with (1Kb) DNA ladder lane 1, 2, 3 shows sample 4, 5, 6 for erm A gene which shows positive results of band. DNA ladder lane 4, 5, 6 shows sample 8,9,10 for erm B in which only lane 10 showed positive results of band. DNA ladder lane 7, 8,9,10 shows sample 1,2,3,4 for erm C in which 7,9,10 only showed positive result of band

clindamycin (100%, 100%, 97.87%, 91, 48%) respectively. Antibiotic resistance rates for gentamicin, vancomycins were moderate (53.19%, 78.72%) respectively, while *S.aureus* isolates showed low resistance against tetracycline, ciprofloxacin and tri methoprime (19.14%, 6.38% and 44.68%) respectively. These results were found similar to earlier studies of Agne *et al.* (2010), Nizami *et al.* (2012) and Kaur and Chate (2015). However this study showed dissimilarity with previous studies including Ahmad and Srikanth (2013) and Adebayo *et al.* (2011).

Primers for internal regions of erm(A), erm(B), erm(C) have been described previously in table (Table 1). Genomic DNA from the resistant isolates which possessed either erm(A), erm(B) or erm(C) were isolated and subjected to PCR analysis with primers specific for erythromycin resistant genes i.e., erm(A), erm(B) and erm(C). The results of isolation of DNA from the bacterial samples are shown below:

Genomic DNA was isolated from the five isolates of *S. aureus* and 16S rRNA region

of DNA was amplified using universal 16S rRNA primers in thermal cycler. All the five isolates of *S.aureus* obtained as genomic DNA were amplified by PCR and characterized by sequencing. The BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis of the sequences was done followed by CLUSTAL OMEGA (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Thus the isolate were confirmed as *S.aureus* as its maximum identity to staphylococcus species was obtained. In this study 16SrRNA detected in 45 (95.74 %) out of 47 *S. aureus* samples.

The result obtained showed the aligned sequences with my query sequence. The sequences with maximum identity were selected to construct the phylogenetic tree. The resistance patterns of *S.aureus* results determined by conventional methods were compared with the results of the (*erm*) genes which observed by PCR technique. The isolates which showed positive result with conventional methods were subjected to PCR-based detection of erythromycin resistance genes i.e., erm(A), erm(B) and erm(C).

Table 3. Distribution of erm genes among S.aureus resistance to erythromycin

Total No of <i>S. aureus</i> isolates	NO &(%) of <i>S.aureus</i>					
	ermA	%	ermB	%	ermC	%
47	29	61.7	7	14.89	11	23.40

P value=7.815

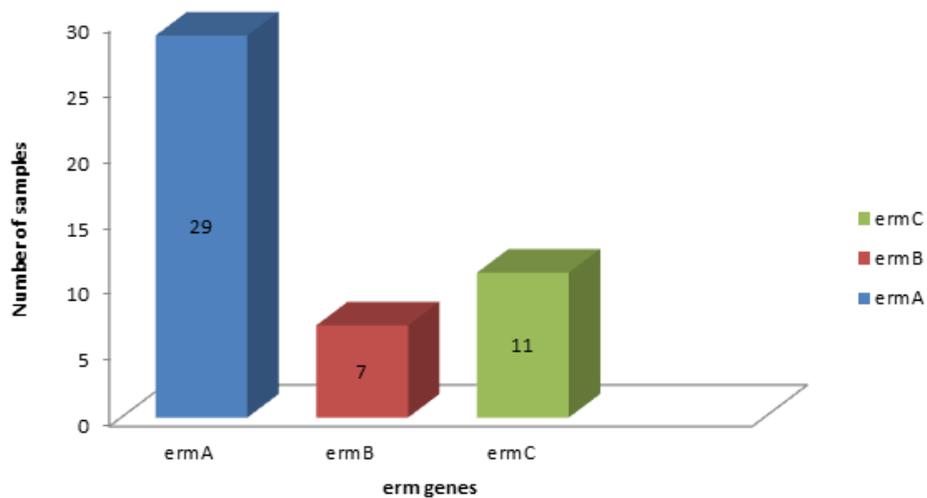


Fig. 6. Distribution of erm genes among S.aureus resistance to erythromycin

Erythromycin resistance in Staphylococci was predominantly mediated by erythromycin resistant methylase encoded by *erm* genes (Zamantaret *et al.*, 2011). Forty seven (47) of *S. aureus* were resistance to erythromycin; these isolates contain any of erythromycin resistance genes tested (*erm* A, B, C). In our study, the most prevalent resistance gene in *S. aureus* was *erm* A (29/47; 61.7%), followed by *erm* C (11/47; 23.40%). Less common were *erm* B which occurring in (7/47; 14.89%) of the erythromycin resistant *S. aureus* isolates tested. The differences in prevalence of *erm* A, B and C ($p=7.815$) in *S. aureus* was statistically significant (Table 4.10, Figure 4.10). In general our observations are in line with the findings of (Lina *et al.*, 1999; Franz *et al.*, 2000). They found that the *erm* A gene was more common in *S. aureus*, similar findings to our results were responsible for erythromycin resistance in 60% among erythromycin resistance *S. aureus* isolated (Saderi *et al.*, 2011). The *ermA* gene was solely responsible for erythromycin resistance until 1971, while *erm* C became dominant between 1984 and 1988 (Westhet *et al.*, 1995). Although *erm* B was present in only a minority of strains, it was formerly found only in animal strains (Eady *et al.*, 1993). In contrast to (Lina *et al.*; 1999; Nicola *et al.*; 1998) we found an association between different *erm* genes, namely *ermA* in combination with *ermC*, in *S. aureus* isolates.

CONCLUSION

All 47 *S. aureus* isolates found to be resistance to erythromycin by phenotypic methods contained at least 1 were erythromycin resistance gene. That's mean all *S. aureus* isolates which were resistance to erythromycin by disc diffusion method were found as positive by PCR for *erm* A, B, and C genes. From the above study it is concluded that the throat has to be considered as an important carriage site for *S. aureus* and should be included when screening for *S. aureus*. The antibiotic resistance grow day by day it is important to use suitable antibiotic after proper laboratory diagnosis such as culture and antibiotic susceptibility tests and treatment should depend on it. The resistance mainly to erythromycin in *S. aureus* was due to the presence of *erm* A, *erm* B and *ermC*.

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