Inducible clindamycin resistance and $erm$ genes in *Staphylococcus aureus* in school children in Kathmandu, Nepal

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**Aim:** Resistance to methicillin and Macrolide–Lincosamide and Streptogramins B and their association with $erm$ genes in *Staphylococcus aureus* are unknown in Nepal. **Materials & methods:** Nonduplicate nasal swabs from 160 school children were collected from April to September 2018 and processed using standard microbiological procedures. **Results:** Out of 160 samples, 64 (40%) were *S. aureus* in which 17 (26.6%) were methicillin-resistant *Staphylococcus aureus* (MRSA). D-test identified 15 (23.4%) as inducible clindamycin-resistant, which were more prevalent in MRSA (76.4%) than methicillin-sensitive *S. aureus* (MSSA; 4.2%). 18.7% of isolates harbored the $ermC$ gene followed by $ermA$ (15.6%) and $ermB$ (3.1%), and were more in MRSA than MSSA. **Conclusion:** To prevent treatment failure by inducible resistance, D-test must be performed on erythromycin-resistant and/or clindamycin-sensitive isolates.

**Lay abstract:** With the increased prevalence of methicillin-resistant *S. aureus* in hospital- and community-acquired infections, there has been an upsurge in resistance toward Macrolide–Lincosamide–Streptogramin type B antibiotics. This has rendered therapy difficult, thereby increasing morbidity, length of hospital-stay and cost of treatment. Therefore, the correct identification and reporting of *S. aureus* isolates and their susceptibility patterns, more specifically, toward methicillin, clindamycin and erythromycin is very crucial.
MLS\textsubscript{B} resistance for prescribing appropriate therapy in infected patients [13]. If the patient is prescribed with clindamycin without the proper identification of MLS\textsubscript{B} resistance, it can lead to treatment failure [14].

We found multiple studies reporting variable rates of inducible clindamycin resistance in different places [2,15–22]. Moreover, prevalence rates of different \textit{erm} genes are still understudied in Nepal. Therefore, the aim of this study is to give an exact picture of what the prevalence of these genes are in the Nepal community. This will provide a proper direction for future researchers as well as prevent health professional from prescribing antibiotics relating to this condition.

Materials & methods

Materials

All the microbiological media and antibiotic discs were purchased from HiMedia Pvt. Ltd., Co., Mumbai, India. Other chemicals were purchased as primer (integrated DNA technology), Master mix (Takara Bio Inc., Nojihigashi, Japan), DNA Ladder (GeneDirex, Inc., MD, USA), Good view nucleic acid stain- HGV II (SBS Genetech Co., Ltd, Beijing, China), agarose (GeneDireX, Inc.).

Sample collection

After the approval from Nepal Health Research Council, National Ethical Guidelines for Health Research in Nepal (reg. no. 195/2018), a prospective cross-sectional study was conducted over 6 months of period to isolate \textit{S. aureus} from nasal samples collected from students of two different schools of Kathmandu, namely Kirtipur Secondary and Mangal Secondary School. The informed consent was taken from guardian of students before sample collection. Only those participants (school children) who were not taking any medications were included in the study. Nasal swabs were collected by inserting a sterile moistened cotton swabs (HiMedia) into each nostril and transferred to the laboratory keeping in transport media. The laboratory tests were conducted from April–September 2018 in the Microbiology lab of Center for Health and Disease Studies, Nepal. A total of nonduplicate 160 samples were analyzed in the study.

Bacterial isolation & identification

The specimens collected were inoculated in mannitol salt agar (MSA), blood agar (BA) and incubated at 37°C aerobically for 24 h. Beta hemolytic colonies on blood agar and typical mannitol fermenting colonies in MSA were observed. Pin-point-sized colonies on blood agar with diameter of 2–3 mm were indicative of \textit{S. aureus}. Gram stain, catalase, oxidase, O–F and coagulase (free and bound) test were performed for further identification using a standard microbiological techniques [23].

Antibiotic susceptibility testing

Antimicrobial susceptibility was studied by the Kirby–Bauer disk diffusion method on a Mueller–Hinton agar plate (12-cm diameter), following Clinical Laboratory Standard Institute (CLSI) guidelines [1,19]. The tested antimicrobial agents were: penicillin (10 U), cefoxitin (30 \textmu g), gentamicin (10 \textmu g), erythromycin (15 \textmu g), clindamycin (2 \textmu g) and ciprofloxacin (5 \textmu g). Cefoxitin (30 \textmu g) was used for the detection of methicillin resistance. Erythromycin (15 \textmu g) and clindamycin (2 \textmu g) discs placed 15 mm apart were used for detection of inducible clindamycin resistance as per recommended CLSI guidelines [23,24].

Detection of methicillin resistance

With cefoxitin disks, isolates with zone of inhibition $\geq 22$ mm in diameter were considered methicillin resistance and those with $\leq 21$ mm were considered as methicillin susceptible.

Detection of inducible clindamycin resistance

Formation of a flattening shape of the clindamycin inhibition zone $\geq 21$ mm (D shape) around the erythromycin disk has shown in Figure 1, which indicates that erythromycin has induced clindamycin resistance.

ATCC 25923 strains of \textit{S. aureus} were used to perform quality control. A separate in-house strain that showed inducible clindamycin resistance was also used for quality control.

DNA extraction

Genomic DNA was extracted from \textit{S. aureus} isolates from QIAamp DNA Mini Kit, Cat No./ID: 51304 (Qiagen). One to two colonies of isolates were taken with nichrome loop, suspended in nutrient broth and incubated for 24
Inducible clindamycin resistance & erm genes

Research Article

Figure 1. Inducible clindamycin resistant (D-test) positive showing MRSA in MHA media.
CD: Clindamycin; E: Erythromycin; MHA: Muller hinton agar; MRSA: Methicillin-resistant Staphylococcus aureus.

h. The turbidity was checked and DNA was extracted according to the manufacturer’s protocol of QIAamp. The extracted DNA was kept in -20°C until used for PCR.

Amplification of ermA, ermB & ermC genes

DNA amplification was performed using specific primers for detection of erm genes. Primer sequences were designed using Primer-BLAST of NCBI using gene ID 13913675 for ermA and 24247827 for ermC. Primer sequence for ermB was used from research paper [8]. Sequences for primers used for PCR were as follows: ermA/F: 5′-AAGCGGTAACCCTCTGA-3′, ermA/R: 5′-TTCGCAATCCCTTCTCAAC-3′ with amplicon size of 190 bp, ermB/F: 5′-CATTTAACGACGAAACTG-3′, ermB/R: 5′-GAACATCTGTGGTATGGG-3′ with amplicon size of 142 bp, ermC/F: 5′-AATCGTGAACGAGGTTTG-3′ with amplicon size of 299 bp. Each reaction was carried out in final volume of 25 μl with Mastermix (12 μl), forward primer (1 μl), reverse primer (1 μl), DNA template (4 μl) and 7 μl nuclease-free water. PCR amplifications were adjusted according to conditions described in previous studies with some modification [12]. Amplification conditions were as follows: initial denaturation (94°C/4 min for ermA, 95°C/2 min for ermB, 95°C/2 min for ermC), denaturation (94°C/30 s for ermA, 95°C/30 s for ermB, 95°C/30 s for ermC), various annealing temperatures (55°C/30 s for ermA, 50.2°C/30 s for ermB, 52.4°C/30 s for ermC) and 72°C/30 s and final extension at 72°C/5 min and hold at 4°C for infinity. PCR products were analyzed by separating on 1.8% agarose gel electrophoresis, stained with nucleic acid stain solution and finally visualized in gel documentation system [12]. A reaction containing all materials and nuclease-free water except template DNA was used as negative control. A native isolate harboring ermA, ermB and ermC gene was used as a positive control for erm genes. 100 bp ladder from GeneDireX, Inc. was used to identify the size of amplified products.

Statistical analyses

Microsoft Excel 2016 was used to keep the record of the data. The data were analyzed using SPSS version 25 for Windows. Pearson Chi-square test was used to find the association between the two variables. p-value of less than 0.05 was considered statistically significant.

Results

Isolation of S. aureus & antibiotic susceptibility pattern

In this study, out of 160 samples processed, 64 samples showed positive culture growth for S. aureus in which 17 (26.5%) were MRSA. The antimicrobial sensitivity tests among MRSA showed that all isolates were resistance to penicillin (ten units) and highly sensitive (88.2%) to gentamicin. The results of antibiotic susceptibility testing for other antibiotics are shown in Table 1.
Table 1. Result of antibiogram test for methicillin-resistance *Staphylococcus aureus* isolates.

| Antibiotics used | Sensitive (%) | Intermediate (%) | Resistant (%) |
|------------------|---------------|------------------|---------------|
| Gentamycin (10 μg) | 16 (88.2) | – | 1 (5.8) |
| Erythromycin (15 μg) | 4 (23.5) | 3 (17.6) | 10 (58.8) |
| Clindamycin (2 μg) | 12 (70.5) | 2 (11.7) | 3 (17.6) |
| Penicillin (ten units) | 0 | 0 | 17 (100) |
| Ciprofloxacin (5 μg) | 6 (35.2) | 1 (5.8) | 10 (58.8) |

Table 2. Distribution of inducible macrolide–lincosamide–streptogramin type B among methicillin-resistance *Staphylococcus aureus*, methicillin-sensitive *S. aureus* and total isolates.

| Property | MRSA (n = 17) | MSSA (n = 47) | Total isolates (n = 64) |
|----------|---------------|---------------|-------------------------|
| Inducible clindamycin resistance (iMLSₐ) | 13 (76.4%) | 2 (4.2%) | 15 (23.4%) |

iMLSₐ: Inducible macrolide–lincosamide–streptogramin type B; MRSA: Methicillin-resistance *Staphylococcus aureus*; MSSA: Methicillin-resistance *S. aureus*.

Table 3. Result of *erm* genes in isolates according to sensitivity to methicillin.

| Genotype | PCR result | Sensitivity to methicillin |
|----------|------------|---------------------------|
|          | MRSA (%)   | MSSA (%)                  |
| *ermA*   | Positive   | 58.8                      | 4.2                        |
|          | Negative   | 41.2                      | 95.8                       |
| *ermB* [30] | Positive  | 11.7                      | 0                          |
|          | Negative   | 88.3                      | 0                          |
| *ermC*   | Positive   | 70.5                      | 0                          |
|          | Negative   | 29.5                      | 0                          |

MRSA: Methicillin-resistance *Staphylococcus aureus*; MSSA: Methicillin-resistance *S. aureus*.

Inducible clindamycin resistance

Inducible clindamycin resistance was seen among 15 (23.4%) of the total isolates. The distribution of iMLSₐ among MRSA, MSSA and total isolates showed the higher rate of inducible clindamycin resistance in MRSA compared with MSSA (p ≤ 0.05) as shown in Table 2.

Association of *erm* genes in MRSA & MSSA

As shown in Table 3 and Figures 2–4, the electrophoresis results of PCR amplicon showed that 15.6% were *ermA* positive, 3.1% were *ermB* positive and 18.7% were *ermC* positive in which three isolates had both *ermA* and *ermC* genes. In this study, the MRSA isolates harbored the *erm* genes in which *ermB* and *ermC* genes were only present in MRSA, whereas few MSSA also had *ermA* genes.

Discussion

*S. aureus* infection is one of the major causes of infection, mainly in low- and middle-income countries and the rate of emergence of antibiotic resistance is quite alarming [25].

We found a significant number of *S. aureus* from school children, which also show resistance to methicillin, erythromycin and clindamycin. It is very important to correctly identify and report *S. aureus* isolates, especially in clinical and diagnostic settings, including whether the isolates are truly susceptible to clindamycin when they are resistant to erythromycin. A simple D-test can be performed in the laboratory, so that inducible clindamycin-resistant isolates can be excluded for clindamycin therapy [26]. Prevalence of *S. aureus* in a community is multifactorial in nature, depending on the geographical location, socioeconomic status, patient age, species of bacteria, inconsistent use of erythromycin and source of the strain (community or nosocomial) [7,27].

The results of our study have shown that the prevalence of inducible clindamycin resistance was 23.4% among all the isolates. As shown in Table 4, several studies conducted in different parts of the world are inconsistent with the prevalence of iMLSₐ and have previously reported higher variability in prevalence, which ranged from 3.3 to 43% [4,11,13,15,16,21,25,28–33]. Also, the prevalence of iMLSₐ resistance in MRSA was 76.4%, which is much higher than the prevalence rate previously reported by different studies, which were 12.3–35.9% [4,10,25,29,30,34,35]. This
Figure 2. Electrophoresis result of *ermA* gene. Lane 1: DNA marker, Lane 2: negative control; Lane 3: positive control; Lane 4-8: samples and 190 bp for *ermA*.

Figure 3. Electrophoresis result of *ermC* gene. Lane 1: DNA marker, Lane 2: positive control; Lane 3-7: samples and 299 bp for *ermC*. 
Figure 4. Frequency of *erm* genes in *S. aureus* isolates.

Table 4. Comparison of frequencies of *ermA*, *ermB* and *ermC* genes in different studies.

| Study (year)          | Inducible resistance isolates | iMLSB (%) | Ref. |
|-----------------------|-------------------------------|-----------|------|
|                       | *ermA* (%)                   | *ermB* (%)| *ermC* (%) |
| Lina et al. (1999)    | 63.2                         | 0.7       | 25   | 30.2 | [24]  |
| Hamilton-Miller et al. (2000) | – | – | – | 43 | [31]  |
| Fiebelkorn et al. (2003) | – | – | – | 25.2 | [25]  |
| Vivian et al. (2010)  | 29.6                         | 17.1      | 0.66 | 3.3  | [23]  |
| Moosavian et al. (2014) | 41.1 | Nonprevalent | 17.7 | 32.3 | [12]  |
| Aydeniz et al. (2015) | 81.9                         | 0.9       | 10.8 | 27.47 | [26]  |
| Havaei et al. (2016)  | 11.11                        | 22.22     | 44.44 | 4.18 | [22]  |
| Fasihi et al. (2016)  | 11                           | 3.5       | 20.5 | 12.5 | [11]  |
| Adhikari et al. (2017) | – | – | – | 11.48 | [17]  |
| Khashei et al. (2018) | –                           | –         | –  | 8.6  | [9]   |
| Our study             | 66.67                        | 13.33     | 73.38 | 23.4 | [30]  |

indicates an alarming increase in antibiotic resistance. Although iMLSB resistance in MRSA was higher, it was within the range (4–68%) when compared with data of previously reported studies among MSSA [4,10,25,34,35].

Results from our PCR study showed that the prevalence of *ermA*, *ermB* and *ermC* genes were: 15.62, 3.12 and 18.75%, respectively, among inducible clindamycin-resistant isolates. Prevalence of *ermA* gene varied among different studies conducted at different geographic location, which ranged from 11 to 81.9% [11,15,16,28,29,31]. Our study was within this range obtained from those previously conducted. Our study also showed the prevalence of *ermC* gene to be within the range when compared with the rates previously reported by several studies [11,15,16,28,31], which ranged from 0.66 to 44.44%. Due to lack of the pertinent literature in different *erm* genes in Nepal, our study aimed to determine the prevalence of *ermA*, *ermB* and *ermC* genes from *S. aureus* isolates and inducible clindamycin-resistant MRSA isolates. The prevalence of *ermA*, *ermB* [36] and *ermC* genes among iMLSB isolates were 66.67, 13.33 and 73.38%, respectively. The comparison of the prevalence rates of *ermA*, *ermB* and *ermC* genes in different studies are given in Table 2.
Limitations
Regarding the limitations of our study, it was conducted in two different schools of Kirtipur, Kathmandu, Nepal and so while there may be a chance of us extrapolating our findings and applying it across Kathmandu valley, this may not be representative of the whole country. However, Kathmandu is the most populated city in Nepal. Hence, our findings could well represent the whole country. Due to lack of resources and funding, we were not able to perform genotyping or Staphylococcal cassette chromosome mec (SSCmec) typing of *S. aureus*. Furthermore, MIC of antibiotics used was not determined.

Conclusion & recommendations
To conclude, this study identified the significant presence of MRSA in nasal swabs from school children, which clearly emphasizes the importance of sanitation. Furthermore, there is an increase in inducible clindamycin resistance that directly impacts the treatment of the cases. Therefore, this highlights the importance of performing D-test to identify these isolates in detail, which ought to be followed by laboratory in routine identification of *S. aureus*.

Future perspective
As there is presence of significant numbers of *S. aureus* in Nepalese school children as well as resistance to several antibiotics, more research should be done in this area to identify the pathogens, their virulence factors and antibiotic-resistance patterns in order to mitigate the misuse of antibiotics. Furthermore, characterization of gene sequences and typing of strain will give more insight into the genes involved in resistance development.

Summary points
- Methicillin-resistant *Staphylococcus aureus* and inducible clindamycin resistance are emerging as a public health threat, which could lead to increased morbidity and mortality if proper diagnosis and treatment are not done.
- There is a significant association of ermA, ermB, ermC genes and inducible clindamycin resistance, which can be used for rapid diagnosis and treatment.
- Inducible clindamycin resistance was found to be more among methicillin-resistance *S. aureus*.
- Every diagnostic and microbiology laboratories should perform D-test to identify inducible clindamycin resistance if they encounter *S. aureus* isolates sensitive to clindamycin but resistant to erythromycin.

Author contributions
R Timsina, B Timalsina and A Singh conceived and designed the study. B Timalsina designed the primers and PCR assay. R Timsina and U Shrestha processed the samples and performed microbiological tests. R Timsina, U Shrestha and B Timalsina performed molecular biology tests. B Timalsina and A Singh supervised the work and manuscript. All the authors made substantial contributions to editing and writing the manuscript. All the authors read and approved the final manuscript.

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Ethical conduct of research
A written informed consent from parents/guardians of all participants (all aged below sixteen years) was taken prior to the study. The ethical permission was sought from the Ethical Board of Nepal Health Research Council (NHRC), Ram Shah Path, Kathmandu, Nepal (reg no. 195/2018).

Availability of data & materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
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