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Effect of sub-inhibitory dose of cefotaxime on multidrug resistant *Staphylococcus haemolyticus* isolates

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Abstract

Critical care of neonates involves substantial usage of antibiotics and exposure to multi-drug resistant (MDR) nosocomial pathogens. These pathogens are often exposed to sub-MIC doses of antibiotics which might result in a range of physiological effects. There-fore, to understand the outcome of sub-inhibitory dosage of antibiotics on Staphylococcus populations, nasal swab specimens were collected from 34 neonates admitted to the Sick Newborn Care Unit between 2017-2018, a total of 41 non-repetitive isolates were included in this study. Staphylococcus haemolyticus was the prevalent species (58.54%) with high non-susceptibility to cefotaxime (CTX) (79.16%), gentamicin (87.50%), and meropenem (54.17%). Biofilm forming abilities of *S. haemolyticus* isolates in the presence of sub-optimal CTX (30µg/mL), the predominantly prescribed *β*-lactam antibiotic, were then determined by crystal violet assays and extracellular DNA (eDNA) quantitation. CTX was found to significantly enhance biofilm production among the non-susceptible isolates ($p\text{-value}_{Wilcoxin}$ test- 0.000008) with increase in eDNA levels ($p\text{-value}_{Wilcoxin}$ test- 0.000004). Additionally, no changes in non-susceptibility were observed among populations of two MDR isolates, JNM56C1 and JNM60C2 after >500 generations of growth in the absence of antibiotic selection in vitro. These findings demonstrate that sub-MIC concentration of CTX induces biofilm formation and short-term non-exposure to antibiotics does not alter non-susceptibility among *S. haemolyticus* isolates.

Keywords: Cefotaxime**,** *S. haemolyticus*, neonates, sub-MIC, biofilms, short-term evolution

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1. Introduction

Across the world, approximately 2.4 million children lose lives in the first month of birth each year and India contributes majorly to it. India has a neonate mortality rate of 21.7% [1] and 20-41% of preterm neonates admitted to tertiary care hospitals succumb to sepsis [2] often ascribed to multidrug resistant (MDR) bacteria [3]. Among the causal pathogens, members of the *Enterobacteriaceae* and *Moraxellaceae* families dominate, inspite of that, Coagulase-negative staphylococci (CoNS) have been found to contribute significantly [4, 5].

When bacterial populations are exposed to antibiotics, resistance often emerges quickly due to mutations or horizontal gene transfer [6]. Additionally, CoNS are also known for causing device-related infections by forming biofilms [7]. Biofilms, in general, have been reported to have considerably higher resistance to antimicrobial agents as compared to planktonic cells [8] and several studies have found that sub-optimal doses of antibiotics can induce biofilm development in a wide range of species [9,10,11].

The Sick Newborn Care Units (SNCU) are known for considerable use of antibiotics especially third generation cephalosporins, aminoglycosides and penems to a certain extent [12]. This is one of the contributing factors resulting in selection of resistant nosocomial bacteria [13]. In addition, empiric use of the same antibiotics against non-susceptible pathogens among neonates requiring longer duration of stay due to prematurity, might play a vital role in enhancing biofilm-formation. Further, it is also unclear as to how many generations would it take after discontinuation of an antibiotic for a resistant bacterial population to become susceptible again [14].

To address each of these questions, we focused on colonization. Nasally colonizing *Staphylococcus spp.* among the admitted neonates were characterized to identify the predominant nosocomial multidrug resistant species. The next set of experiments were carried out to understand if sub-optimal dosage of the most prescribed group of antibiotics,

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namely, cefotaxime, a third-generation cephalosporin, results in enhanced biofilm formation in the laboratory. Finally, short-term 15-day evolution experiments were carried out to evaluate if discontinuation of the antibiotic could result in loss of non-susceptibility.

2. Results

2.1. Species identification and susceptibility profiling

Among the 34 neonates, the average number of days spent in the SNCU was 7.1. Respiratory distress after birth was the most common clinical symptom (n=17; 50%) in the cohort and only 3 preterm along with 4 term born neonates had higher than normal levels of C-Reactive Protein (Supplementary table 1). To characterize the distribution of *Staphylococcus* spp. colonizing the nares, nasal swabs were collected and spread on MSA upon enrichment. A total of 41 *Staphylococcus* isolates were characterized (MZ636452-MZ636490) from 34 neonates. *Staphylococcus haemolyticus* was found to be the prevalent species (n=24, 58.54%) followed by *Staphylococcus aureus* (n=7, 17.07%) and *Staphylococcus epidermidis* (n=6, 14.63%) (Figure 1A, Supplementary table 2).

Among the 4 antibiotics tested, maximum number of *S. haemolyticus* isolates were non-susceptible (Intermediate and Resistant) to GEN (n=21, 87.50%; MIC: 6- >256 µg/mL), however, 19 (79.16%) were non-susceptible to CTX with MIC values of > 256 µg/mL followed by MRP (n= 13, 54.17%; MIC: 6-32 µg/mL). Only a total of 7 isolates (29.17%) were non-susceptible to AK. Out of 5 CTX sensitive isolates all were susceptible to AK and MRP and 3 were susceptible to GEN (Figure 1B, Supplementary table 2). All the other *Staphylococcus* spp. isolates (n=17) were susceptible to AK and MRP, 5 were non-susceptible to GEN and 3 to CTX. 13 (54.17%) of *S. haemolyticus* isolates harbored both the *mecA* and *blaZ* genes and another 8 (33.33%) had only the *mecA* gene. Two CTX susceptible

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isolates with MIC of 8 µg/mL (JNM17C1 and JNM51C1) were also found to harbor the resistance genes (Figure 1C).

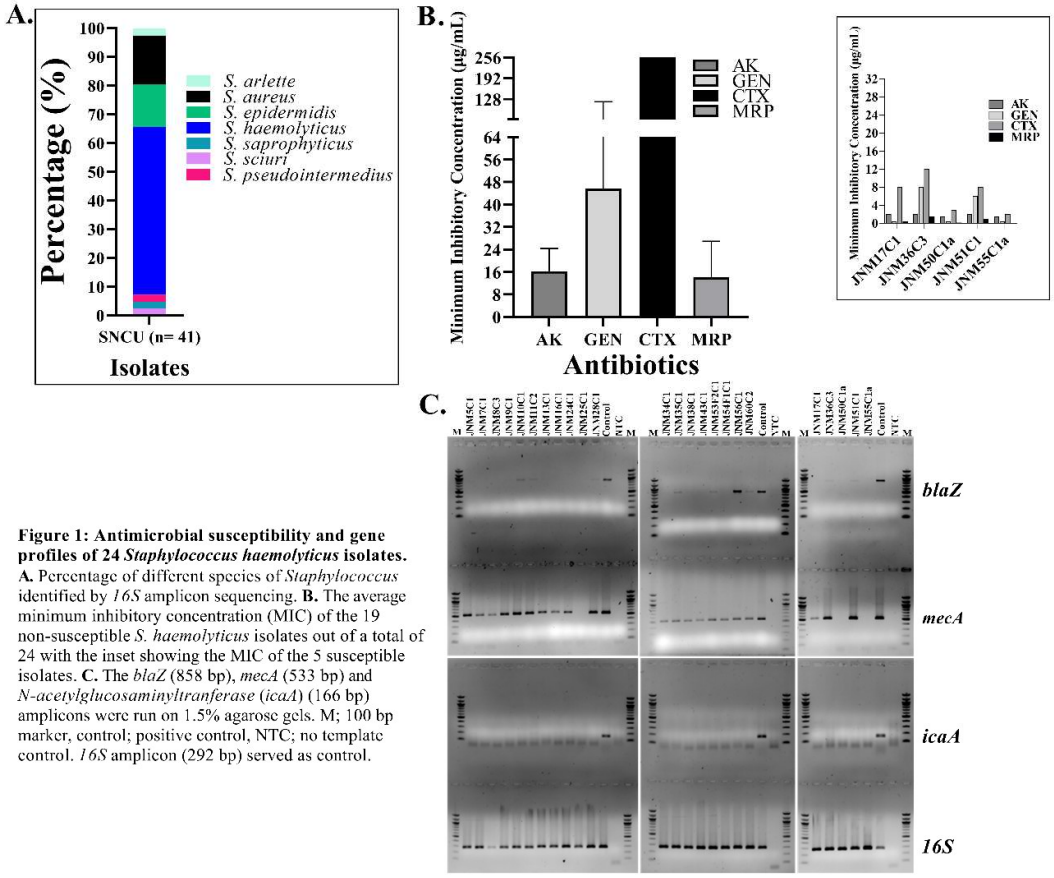

Figure 1: Antimicrobial susceptibility and gene profiles of 24 *Staphylococcus haemolyticus* isolates. **A.** Percentage of different species of *Staphylococcus* identified by *16S* amplicon sequencing. **B.** The average minimum inhibitory concentration (MIC) of the 19 non-susceptible *S. haemolyticus* isolates out of a total of 24 with the inset showing the MIC of the 5 susceptible isolates. **C.** The *blaZ* (858 bp), *mecA* (533 bp) and *N-acetylglucosaminyltranferase* (*icaA*) (166 bp) amplicons were run on 1.5% agarose gels. M; 100 bp marker, control; positive control, NTC; no template control. *16S* amplicon (292 bp) served as control.

2.2. Biofilm enhancement and eDNA release among *S. haemolyticus* isolates

All except for one *S. haemolyticus* isolate (JNM50C1a), grown in TSB_{glu} and TSB_{NaCl} were identified to be biofilm producers after 24 hours. The growth media strongly influenced biofilm formation and a total of 14 out of 24 (58.33%) isolates produced biofilms in TSB_{glu} whereas, 22 (91.67%) isolates formed biofilms in TSB_{NaCl}. In the presence of sub-inhibitory concentration of CTX (30µg/mL), biofilm production was enhanced significantly ($p\text{-value}_{Wilcoxin\ test}$- 0.000008) among all the non-susceptible isolates compared to the controls. By contrast, there was reduction in median optical density values among the susceptible isolates presumably due to cell death (Figure 2A, Supplementary table 3).

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Given that eDNA release has been implicated in *S. haemolyticus* biofilm formation, whether increased biofilm formation also resulted in an increase in quantities of eDNA in the

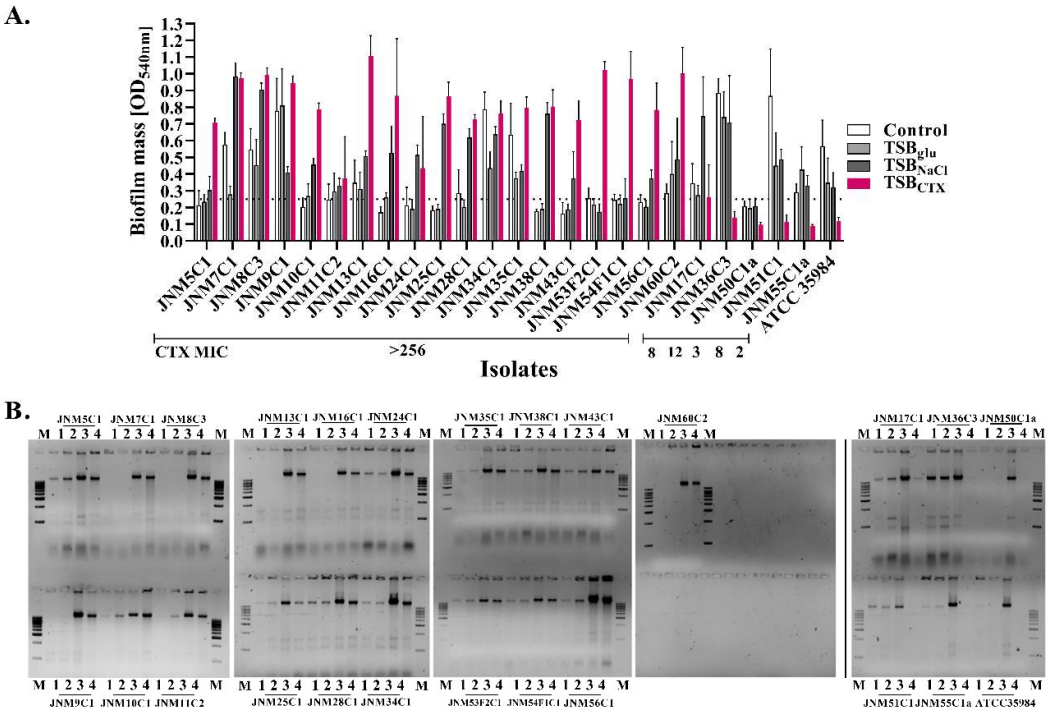

Figure 2: Cefotaxime (CTX), a commonly used *β*-lactam antibiotic enhances biofilm formation among non-susceptible *S. haemolyticus* isolates *in vitro*. A. Semi-quantitative determination of 24 hour biofilm formation under known inducible conditions [tryptic soy broth with 1% glucose (TSB_{glu}) and 3% sodium chloride (TSB_{NaCl})] and 30μg/mL CTX (TSB_{CTX}) using crystal violet assays. The dotted line denotes the cut-off optical density value for biofilm formation (0.25). The biofilm-forming ATCC 35984 *S. epidermidis* strain served as a control. B. Biofilm supernatants run on 0.8 % agarose gels to determine the presence of extracellular DNA (eDNA). M; 1000 bp marker.

biofilms was next evaluated. Biofilm forming sessile cells were harvested and removed by centrifugation and the cell-free nucleic acid in the supernatant was harvested and resolved on 0.8% agarose gels (Figure 2B). Significant increase in eDNA levels were observed in the TSB_{NaCl} ($p\text{-value}_{Wilcoxin\ test}$- 0.000004) and TSB_{CTX} ($p\text{-value}_{Wilcoxin\ test}$- 0.000004) treated groups (Supplementary table 4).

2.3. Whole genome sequencing and resistome mapping of ancestral populations

To generate the resistome profiles of two MDR *S. haemolyticus* ancestral populations (JNM56C1 and JNM60C2), both non-susceptible to CTX, GEN and MRP, paired-end whole genome sequencing was carried out. Iterative *de novo* and reference guided assembly (NC_007168) resulted in alignment of >93% of error-corrected reads. JNM56C1 was

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determined to be a multi locus sequence type (ST) 38 with a chromosome length of 25,54,979 bp (CP063753) at an average sequencing depth of 310. A total of 6 antimicrobial resistance genes were annotated namely, *AAC(6')-Ie-APH(2'')-Ia*, *blaZ*, *dfrG*, *mecA*, *msrA* and *mphC*. Similarly, JNM60C2, a ST-1 isolate, had a chromosome sequence length of 25,11,057 (CP065356) which was ascertained at an average depth of 421. The antimicrobial genes identified were *APH(3')-IIIa*, *AAC(6')-Ie-APH(2'')-Ia*, *blaZ*, *dfrG*, *mecA* and *SAT-4*.The polysaccharide intercellular adhesin (PIA) operon was confirmed to be absent from both the genomes. None of the isolates were found to harbor any plasmids.

2.4. No change in susceptibility in the absence of antibiotic selection

It has been shown in *S. aureus* strains using *in vitro* curing assays that harboring SCCmec imposes a fitness cost [22]. However, if a similar cost is attached with non-susceptibility in *S. haemolyticus*, a decay of non-susceptibility in the absence of antibiotic selection needs to be tested. To understand this, JNM56C1 and JNM60C2, two completely sequenced multidrug resistant *S. haemolyticus* populations with biofilm forming ability (Figure 3A) and known generation time of 30 and 40 minutes respectively (Supplementary table 5) were serially passaged in triplicate for >500 generations (Supplementary table 6) in the absence of antibiotics (Figure 3B). Susceptibility to both *β*-lactam (CTX, MRP) and aminoglycoside (AK, GEN) antibiotics were then compared among the 6 evolved clones and the 2 ancestral populations along with candidate resistance gene amplification. There were no differences in CTX and MRP susceptibility or MIC values among each set of ancestral and evolved populations. Both AK and GEN susceptibilities remained unchanged, however, with 0.25-fold decrease in AK MIC values in 2 of the 6 evolved populations (Figure 3C, Supplementary table 6). In all evolved populations, resistance genes *blaZ*, *mecA* and *AAC(6')*-

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APH(2') were retained albeit with differences in band intensities from the ancestral populations (Figure 3D).

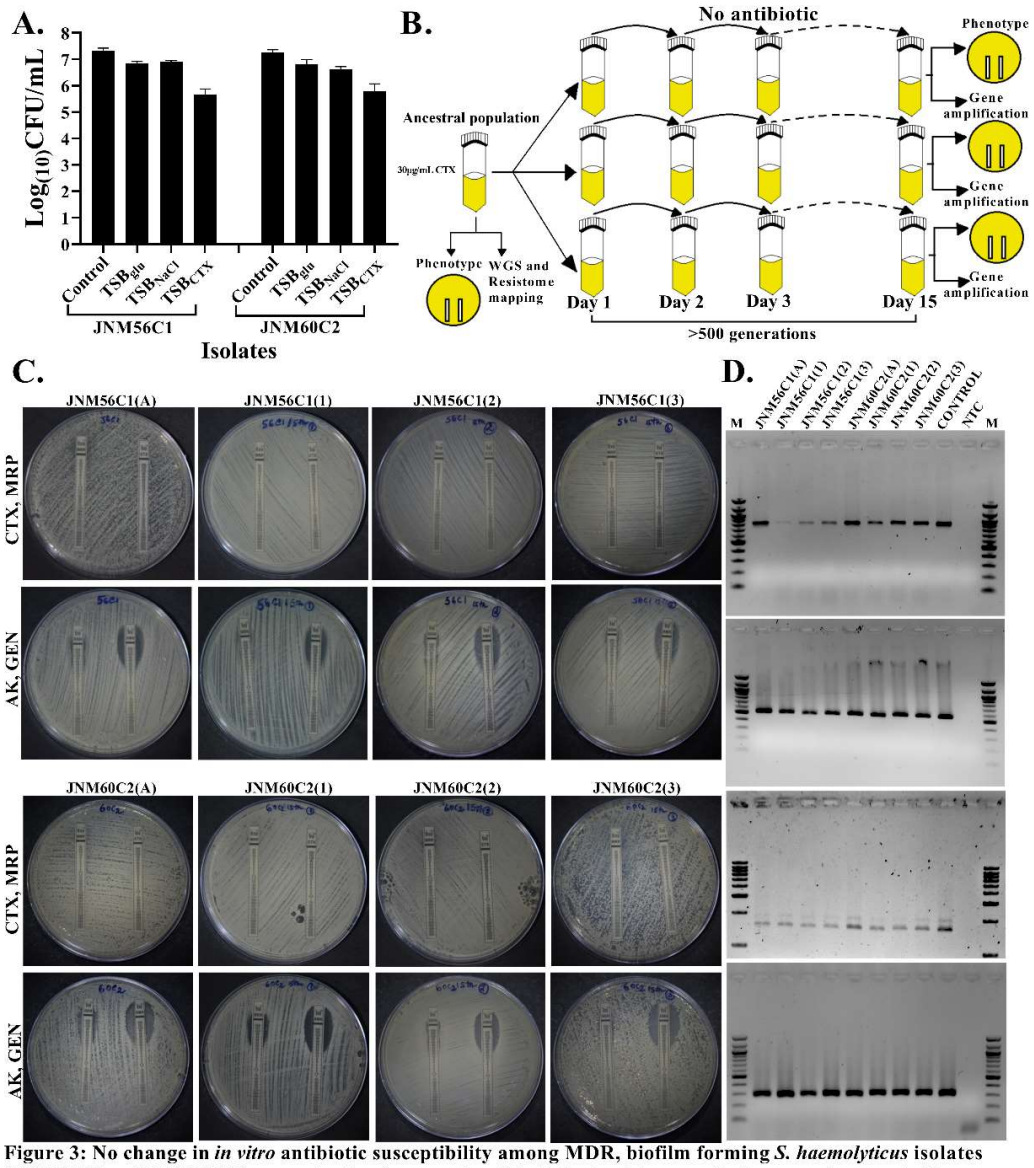

Figure 3: No change in *in vitro* antibiotic susceptibility among MDR, biofilm forming *S. haemolyticus* isolates (JNM56C1 and JNM60C2) grown in the absence of antibiotic in short-term evolution experiments. A. The presence of living sessile cells is confirmed by $\log_{(10)}$CFU counts in both the isolates in the presence of 30µg/mL CTX. The error bars represent standard deviation across three technical replicates. B. Study design of the evolution experiment. C. Comparison of susceptibility to β-lactam and aminoglycoside antibiotics among ancestral and evolved populations using E-tests. D.The *blaZ* (858 bp, panel 1), *mecA* (533 bp, panel 2) and *AAC(6')-APII(2')* (1658 bp, panel 3) and *16S* (292 bp, panel 4) genes were amplified from the ancestral and evolved populations and amplicons were run on 0.8-1.5% agarose gels. M; 100/1000 bp marker, control; postive control, NTC; no template control.

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3. Discussion

Increase in the emergence of MDR pathogens is the major cause of mortality in neonatal sepsis due to the ineffectiveness of first line of antibiotics recommended by the World Health Organization [23]. Neonates admitted to the SNCU, or Newborn Intensive Care Units (NICU) are prone to anticipatory broad spectrum antibiotic therapies [24], possess an immature immune system [25] and have been observed to be colonized by pathogens [26, 27] which make them extremely susceptible to infections. Hence, this study was undertaken to understand the role of antibiotic use and disuse on multi-resistant *Staphylococcus* spp. that frequently colonize neonate nares in an SNCU with high usage of *β*-lactam antibiotics. MDR *S. haemolyticus*, an emerging pathogen known for multi-resistance [28, 29] was observed to be the most frequent colonizer with 79.16% non-susceptibility to CTX and >256 µg/mL MIC value in each case.

To understand if an *in vitro* equivalent of the therapeutic dose of CTX (30 µg/mL) [18] could affect the physiology of high MIC resistant colonizers, we chose to delineate biofilm formation which is known to accentuate antibiotic resistance and colonization. *S. haemolyticus* lacking the PIA operon has been shown to form biofilms in vitro under different growth conditions [17,30,31,32] and the same were used to identify the biofilm-formers. A total of 23 out of 24 isolates were identified to be biofilm formers inspite of the absence of PIA. However, we found TSB_{Nacl} to be a better media for *in vitro* production compared to TSB_{glu}, in contrast to the previous studies [17, 31]. Escalation of biofilm formation in the presence of insufficient antibiotics has been observed in a variety of species through a variety of mechanisms [9,10,11], inspite of that, not much work has been done to understand biofilm development in *S. haemolyticus* and there exists only a single report where nosocomial clones were found to show enhanced biofilm formation on glass and polystyrene surfaces in the presence of ¼ MIC of three antibiotics namely, oxacillin, vancomycin, and linezolid

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[32].Therefore, this study is one of the first showing increase in biofilm formation and eDNA release among MDR, nosocomial *S. haemolyticus* isolates in the presence of sub-inhibitory concentration of CTX.

Evolved antibiotic resistance is a costly affair for many species of bacteria and often result in decreased competitive fitness in the absence of selection pressure [33]. Nevertheless, studies have also exhibited that often bacteria can tackle such trade-offs by way of mutations that compensate the changes [34] and in a long-term evolution experiment comprising of an ancestral streptomycin resistant *Escherichia coli* population by Lamrabet et al, it was clearly demonstrated that after 50, 000 generations of growth in the absence of antibiotic there was no change in susceptibility [35]. Similarly, in our short-term evolution experiments (>500 generations) we observed no changes in the MIC values of CTX and MRP in the evolved clones as compared to both the ancestral populations and minor changes in 1 clone per population in case AK and GEN. However, we believe that a better approach would have been whole genome sequencing of the evolved clones instead of a candidate gene approach to gauge the loss of resistance genes if any in these experiments.

4. Materials and Methods

4.1. Sample collection and isolation of bacterial colonies

Nasal swab samples were collected from 34 neonates (Term born= 7, moderately preterm= 11 and, very or extremely preterm=16) admitted after birth to the SNCU of College of Medicine & JNM Hospital, Kalyani between 2017-2018 (Supplementary table 1). This study was approved by the Hospital and Institutional Ethical Committees. Consenting mothers signed a consent form prior to sample collection. Specimens were grown in Muller Hinton broth (MHB) (Himedia labs, Mumbai, India) and plated on Mannitol salt agar (MSA) (Himedia labs), a selective agar and grown aerobically at 37°C. Single colonies were

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collected and subcultured. Two colonies per sample were isolated from MSA plates (Himedia labs) except for one specimen (JNM8) and two neonates who were followed up on day 4 and 3 respectively (JNM53 and JNM54). The colonies were subcultured in MHB (Himedia labs) and 20% DMSO (Himedia labs) stocks were maintained at −80°C for further analyses.

4.2. Bacterial DNA isolation and species identification

Genomic DNA was isolated from 2 subcultured isolates per specimen using the QIAamp DNA extraction mini kit (Qiagen, Hilden, Germany). Amplification and Sanger sequencing of *16SrRNA*gene were carried out using the S-D-Bact-0008-c-S-20/ S-D-Bact-1391-a-A-17 primer pairs [15] (Eurofins Scientific, Bengaluru, India) to identify the species. One colony per subject was included in the study unless different species were identified.

4.3. Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration (MIC) values were determined using MIC strips (Himedia Labs). The antibiotics tested were cefotaxime (CTX), aminoglycosides; amikacin (AK) and gentamicin (GEN) and the carbapenem; meropenem (MRP). Experiments were carried out according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16].

4.4. Quantification of biofilms

The biofilm forming ability of *Staphylococcus* isolates was determined by a modified crystal violet assay method as described previously [17]. Briefly, 96well polystyrene, flat-bottom microtiter plates were filled with 180µL of tryptic soy broth (TSB) (Himedia labs) and 20µL bacterial cells grown to a Macfarland score of 0.5 in brain heart infusion broth (BHI) (Himedia labs) were added and incubated at 37 °C for 24 hours (hrs) statically. Biofilm forming capacities of all isolates were determined in TSB, TSB with 1% glucose (TSB_{glu}),

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TSB with 3% NaCl (TSB_{NaCl}), and TSB with 30µg/mL CTX (TSB_{CTX}) which is an in vitro equivalent of the therapeutic dosage [18]. After 24 hrs, planktonic cells were removed, adherent cells were fixed with 99% methanol (Finar chemicals, Ahmedabad, India) for 10 mins, and plates were washed once with 1× PBS (Sigma-Aldrich,St. Louis,United States) and air-dried for 10 mins. Modified crystal violet assays were performed, and the absorbance (OD) was recorded at 540 nm. The assays were performed with 6 replicates for each condition in 2 parallel runs. The isolates with an OD of ≥0.25 were considered biofilm positive. *S. epidermidis* ATCC 35984 was used as a positive control. The presence of living sessile cells was determined by colony-forming units (CFU) for selected isolates in the presence of TSB_{glu}, TSB_{NaCl} and TSB_{CTX} in triplicate.

4.5. Extracellular DNA (eDNA) quantification

Extracellular DNA was extracted as previously described by Kaplan et al. with modification [9]. Biofilms were grown in triplicate in TSB, TSB_{glu}, TSB_{NaCl} and TSB_{CTX} in 24 well polystyrene microtiter plates, in a total volume of 1 mL per well. After 24 hrs of growth, the liquid was carefully removed, the plates were washed once with 1XPBS (Sigma-Aldrich, United States) and 50µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) (Sigma-Aldrich, United States) was added to each well. The biofilm-forming cells were scraped off the bottom surface of the wells and were transferred to 1.5mL microcentrifuge tubes. The tubes were centrifuged at 13,000 rpm for 25s and 8µl of each of the supernatants were resolved on 0.8% agarose gels. Densitometric analyses of eDNA were carried out using Image Lab software version 6.0.1(Bio-Rad Laboratories, Hercules, United States).

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4.6. Genomic DNA isolation and whole-genome sequencing

Two isolates (JNM56C1 and JNM60C2) were subcultured in the presence of 30µg/mL CTX. Total DNA from both were purified using the QIAamp DNA extraction mini kit (Qiagen, Germany) and were subjected to paired end whole genome sequencing (2 ×300bp) on an Illumina HiSeq2500 platform (Illumina, San Diego, USA). Both de novo and reference guided assembly was carried out using Velvet and Bowtie2 respectively [19, 20] to build genomes as described previously [21].

4.7. Short-term evolution experiment

The isolates grown in the presence of 30µg/mL CTX formed the ancestral population for each. Generation time for both were calculated from growth curves. The two populations were serially passaged for >500 generations in triplicate for 15 days in fresh Luria-Bertani (LB) media (Himedia labs) in the absence of antibiotic selection at a dilution of 1:100. The ancestral and evolved populations were tested for antibiotic susceptibility for *β-lactam* (CTX, MRP) and aminoglycoside (AK, GEN) antibiotics by Etests.

4.8 Amplification of genes

The presence of *β-lactamase* genes *mecA* and *blaZ*, aminoglycoside resistance gene *AAC(6')-APH(2')*, and N-acetylglucosaminyltransferase *icaA* gene were detected by using PCR amplification. The positive controls used were a laboratory isolate for the *β-lactamase* and aminoglycoside resistance gene and *S. epidermidis* ATCC 35984 for *icaA* gene amplification. A 292 basepair (bp) region of the *16S* gene was amplified as an internal control. Supplementary table 7 lists all the primers used in this study.

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4.9. Statistical analyses

Normality across datasets was evaluated using the Kolmogorov-Smirnov test. To identify significant differences among experimental conditions tested, the Wilcoxon test was performed using GraphPad Prism version 9.1.2 (GraphPad Software, La Jolla, California, USA). A p-value of<0.05 was considered to be statistically significant.

5. Conclusions

To summarize, this study highlights the high nasal carriage rates of MDR *S. haemolyticus* isolates among neonates admitted to the SNCU, demonstrates that these isolates have a tendency towards enhanced biofilm formation upon exposure to insufficient quantities of CTX and further reveals no change in susceptibility to both commonly used *β-lactams* and aminoglycosides when grown in the absence of selection pressure in the short-term. All these findings further reinforce the idea that a definite means of reducing antibiotic resistance is antibiotic stewardship.

Author Contributions: MC ran all the laboratory experiments, analyzed the data and wrote the first draft of the manuscript. TB ran the initial screening experiments and standardized the biofilm assays. MB was the clinician who was involved in specimen collection. BB conceptualized all the experiments, carried out all the next generation sequencing analyses, collated all the data and wrote and reviewed the final draft.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the College of Medicine & Jawaharlal Nehru Memorial Hospital and National Institute of Biomedical Genomics Ethics Committees. The hospital ethical committee reference number is F-24/Pr/COMJNMH/IEC/16/536.

Informed Consent Statement: Informed consent was obtained from all the parents of the subjects involved in the study.

Data Availability Statement: The annotated genome sequences (CP063753, CP065356) and the *16S* amplicon sequences (MZ636452-MZ636490) are available at the GenBank database.

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Conflicts of Interest: None

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