Bacteria Isolated from a Neonatal Intensive Care Unit at an Egyptian University Hospital: Antibiotic Susceptibility and Virulence Factors

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Abstract

Background: The prevalence of bacteria in a neonatal intensive care unit represents a major health problem. We aimed to study the bacteria isolated from infected neonates and bacterial colonization on the environmental surfaces, identify their susceptibility pattern, detect their virulence factors and describe the effect of cefotaxime on the surface adherence properties for the most resistant bacteria.

Methods: Isolates were collected from infected neonates and environmental surfaces, identified and antibiotic susceptibility was determined and were screened for virulence factors and biofilm formation.

Results: A total of 100 clinical specimens and 100 swabs from environmental surfaces were collected. Forty four clinical specimens showed positive bacterial culture, 34 (77.3%) from blood and 10 (22.7%) from ETT aspirate. Fifty one swab cultures from environmental surfaces showed positive bacterial culture. Coagulase-Negative Staphylococci (CoNS) were more abundant (42.7%) followed by S. aureus (21.3%), Pseudomonas spp.(10.7%). K. pneumoniae and S. aureus isolated from ETT aspirate were most resistant to cefotaxime. Adhesion force was visualized as gradual increase in crystal violet color of ETT specimens and bacterial aggregates in scanning electron microscopy.

Conclusions: S. aureus showed more adherence on the surface of ETT specimen than K. pneumoniae. Treatment of ETT specimens with Minimal Inhibitory Concentrations (MIC) prevents the adhesion of the pathogenic K. pneumonia and S. aureus.

Keywords: NICU; Biofilm; Infection control; Colonization; Cefotaxime; Adhesion

Introduction

Healthcare associated infection is any infection that was not present, or in its incubation period at the time of admission [1]. Blood Stream Infections (BSI) are the most frequent neonatal infection in the NICU, followed by respiratory and Urinary Tract Infections (UTIs). They are most commonly caused by S. aureus, Coagulase-negative Staphylococci (CoNS), Klebsiella spp. and E. coli [2]. Hospital outbreaks of multidrug-resistant Klebsiella species, especially those in neonatal wards, are often caused by Extended Spectrum B-Lactamase (ESBL) producers [3] which pose a serious threat to the effective use of β-lactam antibiotics and cephalosporins [4]. The formation of biofilm on endotracheal tubes works as a microbial reservoir, being a potential source of contamination for the lungs of the intubated patients.

Objectives


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and 30 from ETT aspirate. A total of 100 swab from environmental surfaces were collected, 45 from incubators, 20 from ventilators, 10 from faucet, 10 from oxygen mask and 15 from suction machine. For environmental samples, selected sites were chosen, after a period of observation, because there was a maximum traffic in these areas and thus yield for colonization of bacteria was most likely [5]. All samples were collected, processed and identified using standard microbiological methods. The bacterial isolates were phenotypically identified via colonial characteristics, Gram stain and conventional biochemical tests and confirmed by the API 20E Identification System (bioMerieux, France) [6].

Antibiotic susceptibility of the bacterial isolates was performed by the modified Kirby-Bauer disc diffusion method according to the standard procedures of the Clinical and Laboratory Standards Institute (CLSI) 2013 [7]. Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, and Pseudomonas aeruginosa ATCC 27853 used as the quality control strains (Microbiologics, USA) was used as the quality control strain. MDR was defined according to the guidelines of the European Society of Clinical Microbiology and Infectious Diseases [8]. The MIC of cefotaxime (the selected antibiotic) against the most resistant bacterial isolates was determined [9].

Virulence factors produced by bacteria were tested as follows: Biofilm formation was detected by Congo Red Agar (CRA) as previously described [10]. Agar well diffusion assay was used for as saying the degrading enzymes “lecithinase, hemolysin and protease”. Hydrolysis of lecithin was detected on egg yolk agar (TSA supplemented with 10% egg yolk emulsion). Haemolytic activity was determined on blood agar (TSA supplemented with 10% defibrinated sheep blood). Hydrolysis of protease was detected on Casein agar (TSA supplemented with 10% casein or skim milk). The plates were incubated at 37°C for 24 hours [11].

Different concentration of tested antibiotic cefotaxime including MIC, ½ MIC and ¼ MIC (62.5, 31.25 and 15.625 µg/ml) respectively were prepared and tested against S. aureus. For Klebsiella spp (156.25, 78.125 and 39.06 µg/ml) were the tested MIC, ½ MIC and ¼ MIC respectively.

Effect of MIC, ½ MIC and ¼ MIC of Cefotaxime on enzymatic activities and biofilm formation of S. aureus were performed using agar diffusion and Congo Red Agar method (CRA) respectively [10].

Effect of cefotaxime on adherence of K. Pneumoniae and S. aureus were performed by modified tube method and Scanning Electron Microscopy (SEM); a qualitative method for adherence detection [12]. ETT specimens were placed in MIC, ½ MIC and ¼ MIC of cefotaxime suspension with the tested organisms in test tubes. Tubes with ETT specimens were incubated at 37°C for 24 hr. After incubation, tubes were decanted and ETT specimens washed with phosphate buffer saline (pH 7.3) and dried. ETT specimens were then stained with crystal violet (0.1%). Excess stain was washed with distilled water. ETT specimens were dried. The scoring for this method was done according to the results of the control strains. Biofilm formation was considered positive when a visible thick film stained with crystal violet was seen on the surface of ETT specimens. The effect of MIC, ½ MIC and ¼ MIC of cefotaxime on adherence of K. Pneumoniae and S. aureus was visualized by scanning electron microscopy SEM.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Blood Staph aureus n=19</th>
<th>Blood Strept pneumoniae n=4</th>
<th>ETT aspirate Staph aureus n=4</th>
<th>CoNS n=2</th>
<th>Enviromental surfaces CoNS n=32</th>
<th>Staph aureus n=16</th>
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<tbody>
<tr>
<td>Penicillins</td>
<td></td>
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<td>Penicillin (10 units)</td>
<td>7(36.8)</td>
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<td>Cephamycins</td>
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<tr>
<td>Cefoxitin (30 ug)</td>
<td>10(52.6)</td>
<td>-</td>
<td>9(47.4)</td>
<td>1(25)</td>
<td>3(75)</td>
<td>12(37.5)</td>
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<tr>
<td>Glycopeptides</td>
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<tr>
<td>Teicoplanin (30 ug)</td>
<td>9(47.4)</td>
<td>-</td>
<td>10(52.6)</td>
<td>2(50)</td>
<td>2(50)</td>
<td>20(62.5)</td>
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<tr>
<td>Cephalosporins</td>
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<tr>
<td>Cefepime (30 ug)</td>
<td>16(84.2)</td>
<td>-</td>
<td>3(15.8)</td>
<td>3(75)</td>
<td>1(25)</td>
<td>20(62.5)</td>
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<tr>
<td>Antibiotic sensitivity pattern of Gram positive isolates in the NICU.</td>
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was used to investigate the formation of biofilms on the interior luminal surface of the ETT. The SEM samples were processed using standard techniques. Briefly, the ETT specimens were fixed for at least three hours with 2.5% glutaraldehyde and washed twice in sterile Phosphate Buffered Saline (PBS). Dehydration was performed in graded concentrations of ethanol-30, 50, 70, 90, and 100%. Each sample was gold coated using a gold sputter and viewed with JEOL JSM 6510 IV SEM [13].

Results

A total of 100 clinical specimens from neonates and 100 swabs from environmental surfaces were collected. 44 cultures from clinical specimens grew microbes, 34 (77.3%) from blood and 10 (22.7%) from ETT aspirate. Of the 44 positive cultures from patients, 21 (47.7%) positive cultures had monobacterial infection and 23 (52.3%) had mixed bacterial infection, 15 mixed bacterial isolate from blood and 8 from ETT. Also 51 swab cultures from environmental surfaces grew microbes. Of the 51 positive cultures from environmental surfaces, 27 (52.9%) had monobacterial isolates and 24 (47.1%) had mixed bacterial isolates. Forty (53.3%) from incubators, 17 (22.7%) from ventilators, 10 (13.3%) from faucet, 2 (2.7%) from oxygen mask and 6 (8%) from suction machine. After morphological and biochemical characterization of the isolated bacteria, it was found that, Klebsiella spp. were the most common organism followed by S. aureus of both blood cultures and ETT aspirate cultures. Out of 75 isolates from different environmental surfaces Coagulase-Negative Staphylococci (CoNS) were more abundant (42.7%) followed by S. aureus (21.3%), Pseudomonas spp. (10.7%), E. coli (17.3%) and Klebsiella spp. (8%). Sixty seven bacterial isolates from patients were distributed as 29 Gram positive bacterial isolates (46.8%) and 38 Gram negative...
different environmental surfaces 48 isolates were Gram positive

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Klebsiella spp. n=21</th>
<th>Pseudomonas n=4</th>
<th>E. coli n=3</th>
<th>Klebsiella spp. n=6</th>
<th>Pseudomonas n=3</th>
<th>E. coli n=2</th>
<th>Klebsiella spp. n=7</th>
<th>Pseudomonas n=2</th>
<th>E. coli n=3</th>
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<tr>
<td></td>
<td>S (%)</td>
<td>R (%)</td>
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<td>R (%)</td>
<td>S (%)</td>
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<td>S (%)</td>
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<tr>
<td>Ampicillin / cloxacillin (20/100 ug)</td>
<td>10 (42.9)</td>
<td>12 (57.1)</td>
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<td>Cefazolin (100/100 ug)</td>
<td>3 (14.3)</td>
<td>21 (95.7)</td>
<td>2 (83.3)</td>
<td>1 (16.7)</td>
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<td>Ceftriaxone (100 ug)</td>
<td>10 (47.6)</td>
<td>11 (52.4)</td>
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<tr>
<td>Ciprofloxacin (5 ug)</td>
<td>15 (71.4)</td>
<td>17 (28.6)</td>
<td>1 (50)</td>
<td>2 (50)</td>
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<td>Latamoxef (300 ug)</td>
<td>10 (47.6)</td>
<td>11 (52.4)</td>
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<tr>
<td>Metronidazole (300 mg)</td>
<td>15 (71.4)</td>
<td>17 (28.6)</td>
<td>1 (50)</td>
<td>2 (50)</td>
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<tr>
<td>Nitrofurantoin (100 mg)</td>
<td>10 (47.6)</td>
<td>11 (52.4)</td>
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<tr>
<td>Ofloxacin (5 ug)</td>
<td>15 (71.4)</td>
<td>17 (28.6)</td>
<td>1 (50)</td>
<td>2 (50)</td>
<td>-</td>
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<tr>
<td>Tetracycline (250 ug)</td>
<td>2 (9.5)</td>
<td>21 (90.5)</td>
<td>2 (81)</td>
<td>4 (19)</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Tobramycin (30 ug)</td>
<td>8 (38.1)</td>
<td>19 (61.9)</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Trimeprazine / Sulphamethoxazole (1.25/23.75 ug)</td>
<td>10 (47.6)</td>
<td>11 (52.4)</td>
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</table>

**Table 2: Antibiotic susceptibility pattern of isolated bacteria.**

bacterial isolates (56.7%). On the other hand, out of 75 isolates from different environmental surfaces 48 isolates were Gram positive (64%) and 27 isolates were Gram negative bacteria (36%).

All Gram positive bacterial isolates collected from patients and environmental surfaces in the NICU were more sensitive to Ciprofloxacin (Table 1) and all Gram negative bacterial isolates collected from patients and environmental surfaces were more sensitive to imipenem (Table 2). Klebsiella spp. and S. aureus isolated from ETT aspirate had high MDR rate (83.3%) and (50%) respectively. Out of the tested antibiotics cefotaxime was selected for further investigation as all bacterial isolates from ETT aspirate had 100% resistant to it.
Table 1 MDR rate for isolates collected from blood was calculated as follows: in *S. aureus*, it was 47.4% (9/19), in Strept pneumonia, it was zero, however in all Gram positive isolates, it was 82.6% (9/23).

MDR rate for isolates collected from ETT aspirate was calculated as follow: in *S. aureus*, it was 75% (3/4), in CoNS, it was zero, however in all Gram positive isolates, it was 50% (3/6).

MDR rate for isolates collected from environmental surfaces was calculated as follows: in CoNS, it was 37.5% (12/32), in *S. aureus*, it was 12.5% (2/16), however in all Gram positive isolates, it was 29.2% (14/48).

MDR rate for isolates collected from blood was calculated as follows: in *Klebsiella* spp., it was 57.1% (11/21), in *Pseudomonas* and *E. coli*, it was zero, however in all Gram negative isolates, it was 39.3% (11/28).

MDR rate for isolates collected from ETT aspirate was calculated as follows: in *Klebsiella* spp., it was 83.3% (5/6), in *Pseudomonas* and *E. coli*, it was zero, however in all Gram negative isolates, it was 50% (5/10). MDR rate for isolates collected from environmental surfaces was calculated as follows: in *E. coli*, it was 46.2 (6/13), in *Klebsiella* spp., it was 50% (3/6), in *Pseudomonas*, it was 25% (2/8), however in all Gram negative isolates, it was 39.3% (11/28).

In this experiment the Minimal Inhibitory Concentrations (MIC) and the Minimal Bactericidal Concentrations (MBC) of Cefotaxime against the selected strains were determined. The MIC of CTX against *K. Pneumoniae* and *S. aureus* were 156.25 and 62.5 µg/ml respectively. While the Minimum Bactericidal Concentrations (MBC) of CTX against *K. Pneumoniae* and *S. aureus* were 312.5 and 125 µg/ml respectively.

The capability of tested bacteria to produce its virulence factors after exposure to MIC, ½ MIC and ¼ MIC of cefotaxime (156.25, 78.125, and 39.06µg/ml) respectively for *K. Pneumoniae* and (62.5, 31.25, and 15.625µg/ml) respectively for *S. aureus* were examined. The results revealed that 62.5µg/ml cefotaxime (MIC) was sufficient to depress lecitinase, haemolysin and protease activity for *S. aureus* and cefotaxime at ½ MIC and ¼ MIC enhanced these virulence factors. Also the results revealed that 156.25 µg/ml cefotaxime (MIC) was sufficient to depress biofilm formation for *K. Pneumoniae* while 62.5µg/ml cefotaxime (MIC) was sufficient to depress biofilm formation for *S. aureus*. Also cefotaxime at ½ MIC and ¼ MIC enhanced biofilm formation for *K. Pneumoniae* and *S. aureus*.

The effect of MIC, ½ MIC and ¼ MIC of cefotaxime on adhesion visualized by modified tube method and scanning electron microscopy. The results showed that *S. aureus* showed more adherence on the surface of ETT than *K. Pneumoniae*. Also, there was a strong adherence for *K. Pneumoniae* at ¼ MIC (39.06 µg/ml), moderate adherence at ½ MIC (78.125 µg/ml) and no adherence at MIC (156.25 µg/ml) were observed. Also strong adherence for *S. aureus* at ¾ MIC (15.625 µg/ml), moderate adherence at ½ MIC (31.25 µg/ml) and no adherence at MIC (62.5 µg/ml) were observed and is shown in Figure 1,2,3,8,4.

**Discussion**

Out of 67 isolates from neonates 29 isolates were Gram positive (46.8%) and 38 isolates were Gram negative bacteria (56.7%) and *Klebsiella* spp. were the most common followed by *S. aureus* of both blood cultures and ETT aspirate cultures. These results were highly similar to [14] study who reported that Gram negative bacteria were isolated with a rate of 78% (most commonly *K. Pneumoniae*) and Gram positive bacteria were isolated with a rate of 22% (most commonly *S. aureus*) in two NICU’s in Georgia during a period of one year. Also the present results were in agreement with the results of studies done by [15-17] who found that *Klebsiella* was the most common organism causing NI. The organisms isolated from tracheal cultures were 23% *S. aureus*, 23% *Klebsiella* spp., 38.4% Enterobacter spp. and 38.4% *Pseudomonas* [18]. In developing countries Gram-negative rods are major pathogens of NIs in NICUs. Gram-negative rods were isolated from at least 60% of positive blood cultures in developing regions of the world. *K. Pneumoniae* is the major pathogen, responsible for 16%-28% of blood-culture-confirmed sepsis in different regions of the world. Africa and South Asia also have high rates of *S. aureus* infections [19]. Out of 75 isolates from different environmental surfaces 48 isolates were Gram positive (64%) and 27 isolates were Gram negative bacteria (36%). After preliminary identification of the isolated bacteria, Coagulase-Negative Staphylococci (CoNS) were more abundant (47.2%) followed by *S. aureus* (21.3%), *Pseudomonas* spp. (10.7%), *E. coli* (17.3%) and *Klebsiella* spp. (8%). Present results were in agreement with the results of studies done by [7] that Coagulase negative Staphylococcus being the predominant organism (44%) followed by Bacillus spp. (20%), *E. coli* (12.5%) and *Klebsiella* spp. (8.5%).

All Gram positive bacterial isolates collected from patients and environmental surfaces in the NICU were more sensitive to Ciprofloxacin and all Gram negative bacterial isolates collected from patients and environmental surfaces were more sensitive to imipenem. *Klebsiella* spp. and *S. aureus* isolated from ETT aspirate had high MDR rate (83.3%) and (50%) respectively. These results were in agreement with the results of studies done by [20] that imipenem proved to have a broad spectrum and high activity against most tested multiresistant Gram positive and Gram negative bacterial isolates. Resistance against third generation cephalosporins develops more rapidly in the presence of combination of penicillin and an aminoglycoside [21]. Also *K. Pneumoniae* has been found capable to resist many antibiotics especially third generation cephalosporins like cefotaxime, ceftriaxone and cefazidime [22].

The results revealed that *S. aureus* showed positive reactions for the tested virulence factors, while *K. Pneumoniae* was negative for these virulence factors. Also *K. Pneumoniae* and *S. aureus* were biofilm producer. The present results are in accordance with data reported in other studies by [23,24]. They reported that most of isolated *S. aureus* strains were found to produce extracellular enzymes: lecitinase, protease and hemolysins.

About 40% of *K. Pneumoniae* isolated not only from urine, but also from sputum, blood and wound swabs, were able to produce biofilm [25], as well as 63% of *K. Pneumoniae* isolates from urine samples of catheterized patients suffering from UTIs were positive for an in vitro biofilm production [26]. Also a high rate of *K. Pneumoniae* strains isolated from Endotracheal Tubes (ETT) of patients affected by Ventilator-Associated Pneumonia (VAP) were reported to be able to form an in vitro biofilm [27]. Antibiotics at sub-MIC levels interfere with bacterial biofilm virulence expression depending on the type and concentration of antibiotic used [28]. *K. Pneumoniae* was highly resistant to Cefotaxime (MIC 516 mg/L) and in the presence of cefotaxime at sub-MIC concentrations, the biofilm formation enhanced [29]. The gradient in crystal violet color on ETT specimens
indicate that *K. Pneumoniae* and *S. aureus* had the ability to adhere to ETT with different degree and cephotaxime at ½ MIC and ¼ MIC enhanced adhesion to ETT.

The electron-micrographs also depicted that Gram positive *S. aureus* showed more adherence on the surface ETT specimen than Gram negative *Klebsiella* spp. and there was a strong adherence for both organisms at ½ MIC followed by ¼ MIC and no adherence at MIC were observed. Scanning Electron Microscopy (SEM) studies have found that bacteria can colonize Endotracheal Tubes (ETTs) within 24 hours of implantation. The ETT provides an ideal biological niche for bacterial adhesion, and biofilms can form on both the inner luminal and outer surface [30].

In the study of electron micrographs, biofilm positive MRSA isolates were embedded in the matrix material and the quantity of matrix material increased after addition of sub-inhibitory dose of oxacillin in the media [31]. Scanning Electron Microscopy (SEM) studies have showed staphylococci arranged in a matrix on propylene surfaces and it co-related with the results of the conventional methods for biofilm analysis [32].

**Conclusion**

In the present study, Gram negative bacterial isolates collected from blood and ETT aspirate were more common than Gram positive bacterial isolates and *Klebsiella* spp. and *S. aureus* are the most common isolated organisms of blood and ETT aspirate cultures. CoNS, *Klebsiella* spp. and *E. coli* are the most common isolated organisms from environmental surfaces. All isolated organisms were most sensitive to imipenem and most resistant to cefotaxime. *S. aureus* and *Klebsiella* spp. isolated from ETT aspirate culture were the most resistant to cefotaxime representing (100%) resistance. *S. aureus* showed more adherence on the surface of ETT specimen than *K. Pneumoniae*. Treatment of ETT specimens with Minimal Inhibitory Concentrations (MIC) prevents the adhesion of the pathogenic *K. aureus* and *S. aureus*.

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