ABSTRACT: This study identified some virulence factors in staphylococci isolated from the faecal samples of children in Ile-Ife, Nigeria and also determined the relationships in the expressions of these virulent factors in the *S. aureus* and coagulase negative staphylococci (CoNS) isolates. Production of coagulase, haemolysin, protease, β-lactamase and formation of capsule and biofilm as well as haemaglutination characteristics were investigated on 180 staphylococci isolates using standard methods. The results showed that expression of virulence factors varied in isolates. The rate of expression was higher in *S. aureus* than in CoNS although this difference was not statistically significant (p>0.05). Strains of CoNS that expressed up to four virulence factors were isolated. Haemolysin was the virulence factor produced by most of the isolates while protease production was the least encountered. The results revealed that the staphylococci strains isolated from faecal samples of children expressed virulence factors and might have an important role in the pathogenesis of infections. It is also shown that those CoNS species isolated from the faecal samples of children could not be ignored or classified as mere contaminants.

Key words: *Staphylococcus aureus*, coagulase-negative staphylococci, virulence factors, faecal, children.

INTRODUCTION

As early as 1963, Williams, in a comprehensive review, had pointed out that perineal and rectal carriages of staphylococci were potential sources of both endogenous and exogenous staphylococcal infections. Recently, Leszczyski *et al.* (2006) described a case of a healthcare worker whose gastrointestinal tract was colonized by methicillin resistant *S. aureus* (MRSA) with subsequent urinary tract infection caused by the same pathogen.

The pathogenic potential of disease inducing staphylococci is associated with a number of biochemical functions which may be considered as virulence factors (Fedtke *et al.*, 2004; Campbell *et al.*, 2006). Although virulence factors have been associated mainly with *S. aureus*, the coagulase negative staphylococci isolated from clinical specimens have been reported to also express these virulence factors (Otto, 2004; Cunha *et al.*, 2006).

Of all the known virulence factors of staphylococci, production of enterotoxin had been widely investigated in faecal staphylococci (Cunha and Calsolari, 2007). Studies reporting investigation into the occurrence of other virulence factors in faecal staphylococci were not found in literature. Yet this information is important in order to evaluate the actual role of staphylococci colonising the gastrointestinal tract of children on the pathogenesis of staphylococci infections in children.

The aim of this study was to examine the expression of some potential virulence factors such as coagulase, haemolysin, capsule, haemaglutination, protease and biofilm formation in intestinal isolates of staphylococci.

MATERIALS AND METHODS

Sample Collection and Isolation

Staphylococci isolates were from faecal samples obtained from children under 3 years of age from Ile-Ife, in Osun State of Nigeria and the adjoining villages. The children, who totalled 293, were among those attending immunisation clinics and day care centres in
Phenotypic and Molecular Identification of Staphylococci strains by the nuc gene

Isolates were identified by colonial characteristics on blood agar and mannitol salt agar, by Gram stain reactions, and by biochemical tests including catalase test, modified oxidase test, alkaline phosphatase test, and slide and tube coagulase test. Further species identifications were done by the fermentation of trehalose, xylose and mannitol, growth in thioglycollate and slide and tube coagulase test. Further species test, modified oxidase test, alkaline phosphatase test, reactions, and by biochemical tests including catalase test for protease production.

Determination of some virulence factors
Test for protease production
The protease test medium was prepared by adding 2% Bacteriologic gelatin (Oxoid, England) and 1% Bacteriologic agar (Oxoid, England) to Nutrient broth (Oxoid, England). All the staphylococcal strains were inoculated on the medium for 24 to 72 h at 37°C. Any clear zones around bacterial colonies indicated protease production.

Test for Haemolysin production
Blood agar plates were employed for this test. Blood agar was prepared by adding 7% of sterile human blood aseptically to sterile nutrient agar which had been cooled to 45°C and mixed thoroughly. Blood agar plates were prepared by pouring a thin layer of nutrient agar into clean sterile Petri dishes and allowed to set. Then, about 10ml of molten blood agar was poured on this nutrient agar layer to give a second layer. All plates were then left to set. To test for the production of haemolysin, the plates were streaked with loopfuls from 18 h bacterial cultures and incubated at 35°C for 24 h. Clear zones around bacterial colonies indicated haemolysin production. The type and extent of haemolysis were also noted.

Test for Exopolysaccharide formation (Capsule Test)
To distinguish between encapsulated and non-encapsulated strains, the combined positive and negative staining method of Okeke and Lamikanra (1996) was employed. The isolates appeared red against a blue background, while the presence of capsule was observed as a clear zone around each cell.

Haemaglutulation test
Preparation of Erythrocyte suspension
Five ml of blood was drawn aseptically from human (type A) blood in a blood bag obtained from Obafemi Awolowo University Teaching Hospital Complex hematology unit into sterile screw covered test tubes. The blood was centrifuged in a swingy bucket centrifuge at 4000g for 15 minutes at room temperature. The plasma and buffy coat of leukocytes were removed by gentle aspiration with a Pasteur pipette. The cells were suspended in 5ml of phosphate buffered saline (PBS) (pH 7.4) and the centrifugation procedure repeated to wash the cells. The cells were washed four times. One percent red blood cells (RBC) in PBS was prepared by weighing 250mg of RBC on a mettler-balance, put aseptically in 25ml of sterile PBS in a sterile 50ml bottle and mixed gently for uniform suspension of RBC to be formed.

The Test Procedure
Haemaglutination test was performed in a manner similar to the method described by Rupp and Archer (1992). The test was performed in U-shaped 96-well microtiter plates. An 0.5 Mc Farland inoculum of each of the organisms was prepared in 5ml PBS in test tubes.
From the tubes 50µl of each suspension were transferred into each of the microtitre plates well. Then, 50µl of the 1% human erythrocyte suspension in PBS was added to each well. After shaking, the plates were incubated at room temperature for 2 hours and the haemaglutination was recorded as positive or negative.

Assay for β-Lactamase production
All the staphylococci were screened for the production of β-lactamase by the cell – suspension iodometric method as described in Adeleke and Olaitan (2003). The cell population in every cell-suspension prepared in phosphate buffered penicillin G was estimated at 10^9 cells per ml on Mcfarland turbidity standards.

Test for Biofilm Formation
Quantitative determination was carried out by the tissue culture plate or microplate method as described by Mathur et al., (2006) using microplate of 96 flat-bottom wells. Adherent staphylococcal cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a microplate reader (DNM – 9602 Microplate Reader, Perlong New Technology, England) at wavelength of 630nm (OD_{630nm}). These OD values were considered as an index of bacteria adhering to surfaces and forming biofilms. The experiment was done in triplicate and the data were averaged and standard deviation was calculated. The results were reported after subtracting the reading for a blank (TSB plus glucose, without bacterial cells). A 3-grade scale as stated by Christensen et al., (1985) was used to evaluate the strains biofilm producing ability: no or weak: OD<0.120; moderate: 0.120<OD<0.240; high: OD>0.240.

Statistical Analysis
Frequencies were obtained and percentages were calculated for study variables. Significant differences between S. aureus and CoNS were determined by the χ² test or the Fisher exact test. All hypotheses were considered significant if p < 0.05.

RESULTS
The distribution of the virulence factors among the S. aureus and CoNS isolates and the significance is given in Table 1. The difference in the expression of all the virulence factors between S. aureus and CoNS isolates was not statistically significant.

The six different virulence factors investigated occurred both singly and in different combinations. For S. aureus, a total of 14 isolates were found to demonstrate the presence of one virulence factor each, 12 demonstrated two, 9 carried three while 2 carried four different markers. Only one of the S. aureus isolates were found to carry the five different factors together. Both α and β-haemolysis were detected in both S. aureus and CoNS strains with α-haemolysis associated more with S. aureus.

Five S. epidermidis, two S. capitis and one strain of S. haemolyticus were also found to express four virulence factors each while the remaining strains of these organisms expressed lower number of virulence factors.

Table 1
Distribution of virulence factors of S. aureus and CoNS strains

<table>
<thead>
<tr>
<th>Virulence factors</th>
<th>Positive</th>
<th>Negative</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
<td>CoNS</td>
<td>S. aureus</td>
</tr>
<tr>
<td></td>
<td>No (%)</td>
<td>No (%)</td>
<td>No (%)</td>
</tr>
<tr>
<td>Haemolysin</td>
<td>23 (56.1)</td>
<td>77 (51.7)</td>
<td>18 (43.9)</td>
</tr>
<tr>
<td>Protease</td>
<td>3 (7.3)</td>
<td>20 (13.4)</td>
<td>38 (92.7)</td>
</tr>
<tr>
<td>Capsule</td>
<td>18 (43.9)</td>
<td>52 (34.9)</td>
<td>23 (56.1)</td>
</tr>
<tr>
<td>Haemaglutination</td>
<td>16 (39.1)</td>
<td>49 (32.9)</td>
<td>25 (60.9)</td>
</tr>
<tr>
<td>β-lactamase</td>
<td>9 (22.0)</td>
<td>18 (12.1)</td>
<td>32 (78.0)</td>
</tr>
<tr>
<td>Biofilm</td>
<td>15 (36.6)</td>
<td>49 (32.9)</td>
<td>26 (63.4)</td>
</tr>
</tbody>
</table>

*Significant at p < 0.05
Table 2
Multiplicity of virulence factors in CoNS isolates

<table>
<thead>
<tr>
<th>Species (No)</th>
<th>Virulence factors, No of strains positive</th>
<th>Haemolysis</th>
<th>Protease</th>
<th>Encapsulation</th>
<th>Haemaglutination</th>
<th>β-lactamase</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis (45)</td>
<td></td>
<td>27</td>
<td>6</td>
<td>13</td>
<td>10</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>S. haemolyticus (26)</td>
<td></td>
<td>16</td>
<td>3</td>
<td>10</td>
<td>8</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>S. capitis (24)</td>
<td></td>
<td>13</td>
<td>2</td>
<td>10</td>
<td>11</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>S. xylosus (11)</td>
<td></td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>S. warneri (8)</td>
<td></td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>S. saprophyticus (8)</td>
<td></td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>S. hominis (6)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>S. schleiferi (5)</td>
<td></td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. lugdunensis (3)</td>
<td></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S. capitis sub</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ureolyticus (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

None of the S. xylosus isolates demonstrated the production of β-lactamase but other virulence factors were expressed. Of the isolates that showed the expression of more than one virulence factors in S. xylosus three produced three virulence factors while five produce two factors. Also, of the strains with single virulence factors expressed, 2 produced haemaglutination and the remaining one was positive for biofilm formation.

Co-expression of virulence factors was also demonstrated in S. saprophyticus strains as one isolate of this organism expressed 3 virulence factors; capsule, haemaglutination and biofilms, 4 carried two factors while one produced only capsule. Two of the isolates did not express any virulence factor. For the S. hominis isolates, apart from the strain that showed the production of β-lactamase, none of the others demonstrated the production of any other virulence factor.

Only 5 S. schleiferi were analysed. One of the isolates was able to express up to 3 different virulence factors, 2 expressed two virulence factors, 1 expressed one factor while the remaining one did not express any virulence factor. This shows high variations in the virulence of this organism.

Three each of S. lugdunensis and S. capitis sub ureolyticus strain were analysed. Two of the three S. lugdunensis isolates expressed three virulence markers, while the remaining one expressed two. None of the three S. capitis sub ureolyticus strains expressed virulence factors.

The results of the determination of the occurrence of virulence factors in CoNS isolates are shown in Table 2. The results showed that expression of virulence factors varied in isolates.

DISCUSSION

Some of the factors that have been regarded as criteria for pathogenicity in staphylococci in the clinical laboratory are coagulase activity, haemolysis, capsule, haemaglutination, protease and slime formation (Otto, 2004). Other biochemical activities considered to contribute to the virulence of pathogenic staphylococci are determinants for inherent or acquired resistance to antimicrobial agents (Campbell et al., 2006). Such activities include the production of β-lactamase by these organisms. These factors were used to characterize the staphylococci isolates obtained in the course of this study.

Capsule formation, for example, is involved with the protection of the microorganisms from phagocytosis. As a result of the decreased phagocytosis, encapsulated strains of S. aureus are usually associated with increased virulence. Therefore it is an important virulence factor for these organisms. Some workers have reported the presence of encapsulated S. aureus strains in the nares (Lamikanra et al., 1985). In this study, the rate of capsule formation positivity was 43.9% for S. aureus and 34.9% for CoNS. This result is comparable to the finding of Turkyilmaz and Kaya (2005) on the incidence of capsule formation in both S. aureus and CoNS isolates from clinical samples. The workers also demonstrated a higher rate of encapsulation in S. aureus than CoNS. The carriage rate is however higher than that reported
by Lamikanra et al. (1985) who reported that out of 309 healthy children in this same community, 11.3% were nasal carriers of encapsulated S. aureus. This difference in carriage rate may have been because different sites of the human body were investigated in the two studies. This view is supported by the observation of Pohllmann-dietze et al. (2000) who observed that encapsulated and unencapsulated S. aureus cells may occupy different niches in the host by virtue of their different adherence capacities. Non-encapsulated strains of S. aureus are already a cause for concern in childhood infections, therefore the probable dispersal of more virulent encapsulated strain calls for increased vigilance. In vitro studies have shown that staphylococcal proteases can cleave and degrade a number of important host proteins including the heavy chains of all human immunoglobulin classes, plasma proteinase inhibitor and elastin (Rice et al., 2001; Karlsson and Arvidson, 2002) indicating that they are important virulence factors. In this study proteases were the virulence factors least produced by the isolates. This might signify that protease production may not be important to these organisms in their metabolic and physiologic processes. Next to protease in term of expression by isolates in this study was the production of β-lactamase. β-lactamase enzymes are responsible for the inactivation of β-lactam antibiotics making them ineffective and thereby enhancing the survival of pathogens (Adeleke and Odelola, 2000). Both S. aureus and CoNS strains tested in this study were found to produce this enzyme signifying it as an important virulence factor in the tested strains. Testing for biofilm formation is another useful marker of the pathogenicity of staphylococci. This is because biofilm colonization by staphylococci facilitates infections that are often difficult to treat and therefore engender high morbidity and mortality (Sauer et al., 2007; Weigel et al., 2007). Many workers have reported that bacteria growing in a biofilm can be up to 1,500 times more resistant to germicides than the same bacteria growing in liquid culture (Weigel et al., 2007).

Thirty-six percent of S. aureus isolates and 32.9% of CoNS were positive for biofilm formation indicating that expression of this property is comparable in these organisms. Other workers have demonstrated a higher rate of biofilm formation in staphylococci using strains from other clinical samples. For example, Mathur et al. (2006) demonstrated biofilm formation in 53.8% of clinical strains of staphylococci. This possibly suggests decreased ability of staphylococci isolates from faeces (compared to other human specimens) to form biofilm. Damage to host cells is in part mediated by staphylococcal haemolysins, which contribute importantly to virulence in S. aureus. Haemolysin production was the virulence factor found to be exhibited most frequently by the isolates in this study. From the results, 56.1% of S. aureus and 51.7% of CoNS strains produced haemolysin. Turkyilmaz and Kaya (2006) had earlier found a comparable rate of 58.9% in S. aureus while the rate for CoNS (28.9%) was comparatively lower. The results of this study showed that haemaglutination rates were 39.1% and 32.9% for S. aureus and CoNS respectively. In another study, the haemaglutination rate was reported as 33% for CoNS (Rupp and Archer, 1992) showing a similarity with this result. However, in the clinical isolates of Turkyilmaz and Kaya (2006) haemaglutination rate was higher (46.7% for coagulase positive staphylococci and 41.1% for CoNS). The capacity of staphylococcal strains to cause haemaglutination is due to various adhesins such as the fibronectins. Therefore haemaglutination can be regarded as a measure of adhesion of pathogens to the host which is the first step in colonization. The expression of these arrays of virulence factors in these organisms shows their pathogenicity potential and confirms the reports of infections due to staphylococci colonizing the gastrointestinal tract (Leszczyski et al., 2006; Bhalla et al., 2007). It is interesting to note that the only isolate that expressed up to five virulence factors was a S. aureus strain. This result is in agreement with the documented high pathogenicity of S. aureus and an increasing association of this pathogen with many infections, both minor and severe. The high rate of expression of virulence factors in various species of CoNS is also noteworthy. CoNS strains that showed positiveness to up to four virulence characters were isolated in this study. These results corroborate the increased recognition of CoNS as important pathogens rather than ordinary culture contaminants.

In conclusion, the results of the present study revealed that these staphylococci strains isolated from faecal samples of children expressed virulence factors and might have an important role in the pathogenesis of staphylococci infections in children. The results also suggest that CoNS isolates associated with the gut have a similar potential for causing disease as the S. aureus isolates obtained from the gastrointestinal tract of children within the study environment.

Acknowledgment
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REFERENCES


