Research Article

Phenotypic Determination of Some Virulence Factors in Staphylococci Isolated From Faecal Samples of Children in Ile-Ife, Nigeria

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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, Leszcyski *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 feacal 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

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the community. All specimens were collected with the informed consent of the parents or guardians of the children and the study was approved by the Institutional Review Board of the participating institution.

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 broth and susceptibility to novobiocin as described by Ieven *et al.* (1999). Molecular confirmation of the phenotypic identification of staphylococci was performed by PCR assays for the *nuc* gene using the colony direct method (Tsuchizaki and Kunimoto, 2001). The primers used were nuc-F (5'GCGATTGATGGTGATACGGTT-3') and nuc-R (5'-AGCCAAGCCTTGACGAACTAAAGC-3'). PCR conditions comprised a predenaturation step of 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, followed by final extension for 5 minutes at 72°C.

The samples were electrophoresed for 40min at 90V. A 100-bp DNA Molecular Weight Marker XIV was used as a molecular weight marker in each gel. DNA fragments of 280bp which corresponded to the *nuc* PCR product were visualized for *S. aureus* strains but were absent for all the CoNS strains screened using a UV transilluminator at 300nm after staining with ethidium bromide for 15 minutes and destaining for 30 minutes. *S. aureus* NCTC 6571 was used in all experimental work as control. *S. aureus* (MRSA) ATCC [R] 43300 was used for the polymerase chain reaction analysis as positive control while sterile distilled water was used as negative control.

The staphylococci isolates used in this study included 45 *S. epidermidis*, 41 *S. aureus,* 26 *S. haemolyticus*, 24 *S. capitis*, 11 *S. xylosus*, 8 *S. warneri*, 8 *S. saprophyticus,* 6 *S. hominis,* 5 *S. schleiferi,* 3 *S. lugdunensis* and 3 *S. capitis sub ureolyticus* making a total of 180.

Determination of some virulence factors
Test for protease production
The protease test medium was prepared by adding 2% Bacteriological gelatin (Oxoid, England) and 1% Bacteriological 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.

Haemaglutination 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 heamatology 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 was 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
Heamaglutination 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 heamaglutination 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 bufferd 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 $\chi2$ 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

Virulence factors	Positive		Negative		p-value*
	S. aureus No (%)	CoNS No (%)	*S. aureus* No (%)	CoNS No (%)	
Haemolysin	23 (56.1)	77 (51.7)	18 (43.9)	72 (48.3)	0.533
Protease	3 (7.3)	20 (13.4)	38 (92.7)	129 (86.6)	0.157
Capsule	18 (43.9)	52 (34.9)	23 (56.1)	97 (65.1)	0.192
Haemaglutination	16 (39.1)	49 (32.9)	25 (60.9)	100 (67.1)	0.361
β-lactamase	9 (22.0)	18 (12.1)	32 (78.0)	131 (87.9)	0.063
Biofilm	15 (36.6)	49 (32.9)	26 (63.4)	100 (67.1)	0.583

*Significant at $p < 0.05$

Table 2
Multiplicity of virulence factors in CoNS isolates

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

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, 1expressed 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 positiveness 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 Pohlmann-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-capsulated 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 (Leszcyski *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.

REFERENCES

Adeleke, O. E. and Olaitan, J. O. (2003): The effect of a fixed non-mineral oil on the antibacterial activity of Ampicillin trihydrate against resistant clinical strains of *Staphylococcus aureus. African Journal of Biomedical Research* **6,** 91 – 94.

Bhalla, A., Aron, D. C. and Donskey, C. J. (2007): *Staphylococcus aureus* intestinal colonization is associated with increased frequency of *S. aureus* on skin of hospitalized patients. *BMC Infect Dis* **7,** 105-109.

Campbell, T. L., Henderson, J., Heinrichs, D. E. and Brown, E. D. (2006): The *yjeQ* Gene Is Required for Virulence of *Staphylococcus aureus. Infect Immun* **74**(8), 4918–4921.

Cunha, M. L. R. S. and Calsolari, R. A. O. (2007): Toxigenicity in *Staphylococcus* with emphasis on coagulase negative staphylococci. In; (A. Méndez-Vilas (Ed.). Communicating Current Research and Educational Topics and Trends in Applied Microbiology. *Formatex,* 778-782.

Cunha, M. L. R. S., Rugolo, L. M. S. S. and Lopes, C. A. M. (2006): Study of virulence factors in coagulase-negative staphylococci isolated from newborns. *Mem Inst Oswaldo Cruz Rio de Janeiro* **101**(6), 661-668.

Fedtke, I., Gotz, F. and Peschel, A. (2004): Bacterial evasion of innate host defenses—the *Staphylococcus aureus* lesson. *Int J Med Microbiol* **294,** 189-194.

Ieven, M., Verhoeven, J., Pattyn, S.R. and Goossens, H. (1999): Rapid and economical method for species identification of clinically significant coagulase-negative staphylococci. *J Clin Microbiol* **33,** 1060–1063.

Karlsson, A. and Arvidson, S. (2002): Variation in Extracellular Protease Production among Clinical Isolates of *Staphylococcus aureus* Due to Different Levels of Expression of the Protease Repressor *sarA. Infect Immun* **70**(8), 4239–4246.

Lamikanra, A., Paul, M. D., Akinwole, O. B. P. and Paul, M. O. (1985): Nasal carriage of *Staphylococcus aureus* in a population of healthy Nigerian students. *Journal of Medical Microbiology* **19,** 211-6.

Leszczynsk, P., Weber-Dabrowska, B., Kohutnicka, M., Luczak, M., Gorecki, A. and Gorski, A. (2006): Successful eradication of Methicillin Resistant *Staphylococcus aureus* (MRSA) intestinal carrier status in a Heathcare Worker – Case Report. *Folia Microbiology* **51** (3), 236-238.

Mathur, T., Singhal, S., Khan, S., Upadhyay, D. J., Fatma, T. and Rattan, A. (2006): Detection Of Biofilm Formation Among The Clinical Isolates Of Staphylococci: An Evaluation Of Three Different Screening Methods. *Indian Journal of Medical Microbiology* **24** (1), 25-29.

Okeke, I. N. and Lamikanra, A. (1995): Bacterial capsules: a simple method for demonstration under the light microscope. *British Journal of Biomedical Science* **52**(4), 321-322.

Otto, M. (2004): Virulence factors of the coagulase – negative staphylococci. *Frontier in Bioscience* **9,** 841 - 863.

Po¨Hlmann-Dietze, P., Ulrich, M., Kiser, K. B., Do¨ Ring, G., Lee, J. C., Fournier, J., Botzenhart, K. and Wolz, C. (2000): Adherence of *Staphylococcus aureus* to Endothelial Cells: Influence of Capsular Polysaccharide, Global Regulator *agr*, and Bacterial Growth Phase. *Infect Immun* **68**(9), 4865–4871.

Rice, K., Peralta, R., Bast, D., de Azavedo, J. and McGavin, M. J. (2001): Description of staphylococcus serine protease (*ssp*) operon in *Staphylococcus aureus* and nonpolar inactivation of *sspA*-encoded serine protease. *Infect Immun* **69,** 159–169.

Rupp, M. E. and Archer, G. L. (1992): Hemagglutination and adherence to plastic by *Staphylococcus epidermidis. Infect Immun* **60,** 4322-4327.

Sauer, K., Rickard, A. H. and Davies, D. G. (2007): Biofilms and biocomplexity. *Microbe* **2,** 347-353.

Tsuchizaki, N. and Kunimoto, H. (2001): Rapid microbial gene detection and amplification techniques using colony-direct PCR *Innovations* **17,** 10-11

Turkyilmaz, S. and Kaya, O. (2006): Determination of some virulence factors in *Staphylococcus* spp. isolated from various clinical samples. *Turkish Journal of Veterinary Animal Science* **30,** 127 –132

Weigel, L. M., Donlan, R. M., Shin, D. H., Jensen, B., Clark, N. C., McDougal, L. K., Zhu, W., Musser, K. A., Thompson, J., Kohlerschmidt, D., Dumas, N., Limberger, R, J. and Patel, J. B. (2007): High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. *Antimicrobial Agent and Chemotherapy* **51**(1), 231-238.

Williams, R. E. (1963): Healthy carriage of *Staphylococcus aureus*: its prevalence and importance. *Bacteriol Rev* **27,** 56–71.