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*Full Length Research Paper*

# Virulence factors and antibiotic resistance in *Staphylococcus aureus* and *Clostridium perfringens* from landfill leachate

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**A total of 34 isolates of bacteria, 20 *Staphylococus aureus* and 14 *Clostridium perfringens* from leachate samples, were investigated for virulence factors and antibiotic resistance. Majority (>70%) of the isolates produced enterotoxins. Many of the *S. aureus* isolates tested positive for deoxyribonuclease, haemolysins and slime production. Staphylococcal enterotoxin A (SEA) was the predominant enterotoxin produced by the *S. aureus.* None of the *S. aureus* was resistant to ciprofloxacin, novobiocin and vancomycin. Six isolates were resistant to methicillin and majority of them were resistant to penicillin, ampicillin and bacitracin. Eleven (78.6%) of the *C. perfringens* isolates produced enterotoxin and were also beta haemolytic. Except for one strain each of *C. perfringens* which were resistant to penicillin and ampicillin-sulbaltam respectively, all others were susceptible to the antibiotics tested. The presence of several of the virulence traits investigated and resistance to commonly used antibiotics in many of the *S. aureus* and *C. perfringens* tested raises concern about their dissemination through leachate to the environment. The accumulation of leachate and possible contamination of surface and groundwater sources also points out its potential risk for public health. There is need for some measures to properly manage waste and consequently reduce surface and groundwater contamination through leachate percolation.**

**Key words:** Virulence factor, landfill leachate, antibiotic resistance, *Staphylococcus aureus*, *Clostridium perfringens*, health impact.

## INTRODUCTION

Microbial analysis of landfill leachates revealed that they contain large number of pathogenic and opportunistic bacteria. Many species belonging to genera such as *Enterobacter, Escherichia*, *Klebsiella, Salmonella, Serratia, Proteus, Pseudomonas* and *Staphylococcus* have been reported (Holt et al., 2000; Adeyemi et al., 2007; Flores-Tena et al., 2007). The infiltration and accumulation of such leachate samples at any depth of soil may affect the quality of surrounding groundwater of nearby habitats in relation to the microbiological quality standard of the drinking water (Fatta et al., 1999). In addition, since domestic animals and birds are known to scavenge around the landfills, there could also be the risk of dispersion of pathogenic species by these animals and birds (Rusin et al., 2000). In a preliminary study involving the ecotoxicological assessment and microbial characterization of leachate from municipal solid waste landfill in Ibadan, Nigeria, Oshode et al. (2008) reported the isolation of potentially pathogenic and toxin-producing microorganisms. Since the risk of transmission of these potential pathogens is high because these is no known containment or treatment system for the leachate generated, it is necessary to evaluate the virulence factors and the antibiotic resistance traits of the isolated species. The detection of toxin production is useful in determining the significance of isolates. This is because the presence of virulence factors, particularly enterotoxins, reinforces the role of some of these isolates as effective agents of food-borne illness. Also the presence of antibiotic resistant genes in these organisms

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is of great concern as they could contribute to the spread of antimicrobial resistance in the food chain. Within this context, the purpose of the present study was to investigate the production of some virulence factors and also determine the antibiotic susceptibility of isolates of *S. aureus* and *C. perfringens* isolated from landfill leachate in Ibadan, Nigeria.

## MATERIALS AND METHODS

### Organisms' isolation, treatment and selection

The bacterial strains used were isolated from landfill leachate in Ibadan, Nigeria (Oshode et al., 2008). *S. aureus* strains were maintained at 4°C on tryptone soya agar (Oxoid, UK) slants while *C. perfringens* isolates were maintained at 4°C in Cooked Meat Medium (Oxoid, UK). A total of 34 isolates, 20 strains of *S. aureus* and 14 strains of *C. perfringens* were selected for this study.

### Haemolysin production

Haemolytic activity was determined on Columbia Blood Agar Base (Oxoid, UK) containing 5% defribrinated horse blood. Plates were incubated aerobically at 37°C for 24 to 48 h for cultures of *S. aureus*. Cultures of *C. perfringens* were incubated at 37°C in an anaerobic jar using Gas Pak System (Oxoid, UK). Zones of clearing around colonies indicated haemolysin production.

### Determination of DNase production by *S. aureus*

DNase Agar (Oxoid, UK) was used to determine DNase production. *S. aureus* strains were streaked on the agar medium and incubated at 37°C for 24 h. After incubation, the culture plate was flooded with 1N HCl and colonies were observed for clearing of colour and this was considered as positive for DNase activity.

### Determination of slime production by *S. aureus*

Production of slime by the isolates was assayed according to the method described by Citak et al. (2003).

### Enterotoxin analysis

The SET-RPLA and PET-RPLA, kits supplied by Oxoid, UK, were used for the detection of enterotoxins in isolates of *S. aureus* and *C. perfringens* respectively. The procedures for analysis and interpretation of results were according to manufacturer's instructions.

### Antibiotic resistance

The antibiotic susceptibility testing for *S. aureus* isolates was determined using the disk diffusion method (Bauer et al., 1966). The medium used was Mueller-Hinton agar and inoculum was prepared in accordance with NCCLS (2006). The antibiotic discs used were ampicillin (25 µg), penicillinG (10 µg) methicillin (5 µg), bacitracin (10 µg), erythromycin (15 µg), vancomycin (30 µg), ciprofloxacin (5 µg), novobiocin (30 µg), gentamicin (10 µg) and tetracycline (30 µg).

For *C. perfringens,* a loopful of colony suspended in peptone water and prepared to 0.5 MacFarland standard was used as inoculum. Defibrinated sheep blood agar was used as the medium. The minimum inhibitory concentrations (MICs) of 5 antimicrobial agents namely, penicillin G (Sigma), clindamycin (The Upjohn Co. Mich), vancomycin (Eli Lilly & Co, Indianapolis), metronidazole (Sigma) and ampicillin-sulbactam (Pfizer, Conneticut) were determined. The agents were reconstituted as directed by manufacturers. Serial two-fold dilutions were prepared and added to the molten defibrinated sheep blood agar. The test isolates were spot-inoculated on the plates. The plates were incubated in an anaerobic jar with GasPak system (Oxoid, U.K.) at 37°C for 48 h.

The MIC was determined according to the approved standard set forth in NCCLS (1993). Plates inoculated with the organisms but without antibiotics serve as the control. The assays were conducted in duplicate for each organism evaluated.

## RESULTS

The production of haemolysins, DNase, slime, enterotoxins and antibiotic resistance among isolates of *S. aureus* is shown in Table 1. Majority (80%) of the *S. aureus* isolates were beta haemolytic. Alpha haemolysis was not detected. Slime and DNase production were positive in 90% of 20 *S. aureus.* Staphylococcal enterotoxin A was the predominant enterotoxin produced by most of the *S. aureus* isolates (85%). Resistance to penicillin was 85% followed by ampicillin, tetracycline, bacitracin, gentamicin, methicillin and erythromycin in that order. Table 1 shows the virulence traits and antibiotic susceptibility of the *C. perfringens* isolates.

Results of the MICs show that two isolates, one each, were resistant to penicillin and ampicillin-sulbactam respectively. Eleven isolates of *C. perfringens* were β-haemolytic and of these 10 produced enterotoxin.

## DISCUSSION

Our findings show that the isolates have the ability to produce a number of exoenzymes which are known to contribute to virulence (Dinges et al., 2000; Sandel and McKillip, 2004). Slime production has been implicated in bacterial adherence, an important factor for bacterial colonization, which is a necessary step in the pathogenesis of many bacterial infections. Many reports in literature have reported the production of slime from *S. aureus* isolated from dairy products (Aguilar et al., 2001; Citak et al., 2003; Vasudevan et al., 2003). Slime production has also been reported to increase resistance to antibiotics (Gunaydin et al., 1995). Production of enterotoxin was detected in all the isolates of *S. aureus*.

Staphylococcal enterotoxin A (SEA) was detected in 85% isolates and in combination with SED in 20% isolates and with SEC in 10% isolates. Two of the non-haemolytic *S. aureus* produced SEB. Our results show that SEA was the predominant enterotoxin produced by the *S. aureus* isolates. SEA is the most frequent enterotoxin involved in staphylococcal food poisoning

**Table 1.** Production of virulence factors and antibiotic resistance of tested isolates.

| Isolate code | Virulence traits, antibiotic resistance |
|---|---|
| SaAERL 3 | β, SEA, SED, $D^+$, $S^+$, bac, met, pen, amp, gen |
| SaAERL 4 | β, SEA, $D^+$, $S^+$, bac, pen, ery, amp, tet, gen |
| SaAERL 7 | non-haemolytic, SEA, $D^+$, $S^+$, bac, pen, amp, gen |
| SaAERL 9 | β, SEA, SEC, $D^+$, $S^+$, bac, met, pen amp, tet |
| SaAERL 15 | β, SEA, SED, $D^+$, $S^+$, pen, amp, tet |
| SaAERL 17 | β, SEA, $D^+$, $S^+$, bac, pen, amp, gen |
| SaAERL 25 | non-haemolytic, SEB, $D^-$, 5, ery |
| SaAERL 28 | β, SEA, $D^+$, $S^+$, bac, pen, amp, tet |
| SaAERL 31 | β, SEA, $D^+$, $S^+$, bac, pen, amp, tet |
| SaAERL 62 | non-haemolytic, SEB, $D^+$, $S^+$, pen, amp, bac |
| SaAERL 82 | β, SEA, $D^+$, $S^+$, pen, amp, tet, gen |
| SaAERL 110 | β, SEA, $D^+$, $S^+$, pen, amp, bac |
| SaAERL 112 | β, SEA, SEC, $D^+$, $S^+$, met, pen, bac |
| SaOARL 1 | non-haemolytic, SEA, pen, ery, tet |
| SaOARL 16 | β, SEA, SED, $D^+$, $S^+$, met, pen, bac |
| SaOARL 22 | β, SEA, $D^+$, $S^+$, pen, amp, bac |
| SaOARL 41 | β, $D^+$, $S^+$, tet, gen |
| SaOARL 44 | β, SEA, SED, $D^+$, $S^+$, met, bac, tet |
| SaOARL 45 | β, SEA, $D^+$, $S^+$, pen, amp, bac |
| SaOARL 46 | β, SEA, $D^+$, $S^+$, pen, amp |
| CpAERL 1 | β, $ent^+$ |
| CpAERL 8 | β, $ent^+$ , pen |
| CpAERL 12 | β, $ent^-$ |
| CpAERL 42 | β, $ent^+$ |
| CpAERL 61 | non-haemolytic, $ent^+$ |
| CpAERL 74 | β, $ent^+$ |
| CpAERL 75 | β, $ent^+$ |
| CpAERL 91 | non-haemolytic, $ent^-$, amp-sulb |
| CpAERL 102 | β, $ent^+$ |
| CpAERL 112 | non-haemolytic, $ent^-$ |
| CpOARL 6 | β, $ent^+$ |
| CpOARL 8 | β, $ent^+$ |
| CpOARL 19 | β, $ent^+$ |
| CpOARL 30 | β, $ent^+$ |

β: beta haemolysis; SEA: staphylococcal enterotoxin A; $S^+$: slime positive; $S^-$: slime negative; ent+: enterotoxin positive; $Ent^-$: enterotoxin negative; $D^+$: DNase positive $D^-$: DNase negative; Antibiotic resistance is represented by bac, bacitracin; pen, penicillin; ery, erythromycin; met, methicillin; amp, ampicillin; tet, tetracycline; gen, gentamicin; Sa: *Staphylococcus aureus;* Cp: *Clostridium perfringens*.

worldwide (Shimizu et al., 2000; Cha et al., 2006), although other staphylococcal enterotoxins are known to be involved in association with SEA. All the isolates of *S. aureus* tested were susceptible to ciprofloxacin, novobiocin and vancomycin. They were however resistant to penicillin, ampicillin, bacitracin, methicillin, erythromycin, tetracycline and gentamicin at 80, 70, 70, 30, 20, 40 and 30% respectively. The high level resistance to penicillin and ampicillin is not surprising because reports in literature revealed a high prevalence of resistance of *S. aureus* from different sources to these antibiotics (Malinowski et al., 2002; Gandara et al., 2006). The *C. perfringens* tested were susceptible to all the antibiotics tested, with the exception of two strains which were resistant to penicillin and ampicillin-sulbactam respectively. Eleven isolates were enterotoxigenic and were also beta haemolytic. Study findings support our hypothesis that pathogenic species of *S. aureus and C. perfringens* are present in leachate from the landfill. *S. aureus*, for example, is a significant pathogen causing many life threatening infections and gastroenteritis (Scherrer et al., 2004). Community acquired infections

associated with methicillin resistant *S. aureus* (MRSA) are on the increase. Although the number of *S. aureus* resistant to methicillin in this investigation are few, that leachate could contribute to the burden of MRSA infections should not be ignored. The lack of published data documenting the production of virulence factors and presence of antibiotic resistant strains of *S. aureus* and *C. perfringens* from landfill leachates hampers our ability to determine whether these traits abound in other regions of the world or not. Collins and Kennedy (1992) had reported the isolation of *S. aureus* among other organisms from leachates. However, it was not known whether the isolates were enterotoxigenic or not. The presence of antibiotic resistant species in leachate in this study reflects an increase in the burden of resistant strains of bacteria in our environment and calls for more research. Preliminary results (Oshode et al., 2008) which reported the presence of faecal coliforms in large numbers (that is ≥ $10^6$ cfu/ml of leachate) indicated that a significant sanitary risk exists for workers managing the landfill, especially with such organisms which have been implicated in gastroenteritis. These findings also support the potential for their transmission in sufficient concentration through scavenging animals and birds. From the results of the present study, the health hazard associated with the contamination of groundwater and surface water by untreated leachate is stressed by the presence of enterotoxin-producing diarrhea-causing bacteria. Since the proximity of the landfill sites to residential areas is close, there is greater possibility of groundwater and surface water contamination with the consequence risk to resource users and to the environment. These findings therefore call for some measures for the proper management of leachate from the landfill in order to reduce or eliminate groundwater/surface water contamination.

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