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Bacterial adherence: the role of serum and wound fluid

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Bacteria are known to initiate wound infections and have been found associated with wound infections. The purpose of this study was to investigate the bacterial adherence potential (BAP) wounds. The bacteria used were *Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli* and *Staphylococcus aureus* isolated from infected wounds and assayed for their adherence ability using wound fluid and serum. The BAP were achieved by exposing the pathogens to freshly excised wounds. The adhered bacteria were then eluded and quantified using log (CFU/cm^2) on Mueller Hinton Agar per cm^2 of tissue. The results indicated that wound fluid and serum has a remarkable bacterial adherence potential (BAP) when exposed to freshly injured wounds as when compared to distilled water and no agent.

Key words: Bacteria wound, adherence, wound fluid, serum.

INTRODUCTION

Wound infection is recognized as a prominent route of bacterial infections. Many bacterial agents are known to cause wound infections (Yah et al., 2004a, 2004b). Initial injury to the skin triggers coagulation and an acute inflammatory response followed by cell recruitment, proliferation, and matrix synthesis. Disruption in any of these stages of the healing response can either delay or significantly impair wound healing (Richard et al., 2005; Norbury and Kiewetter, 2007).

According to Mertz et al. (1987), the delay in eukaryotic wound healing is the attachment and invasion of bacteria to the surface of the wounded skin. The attachment involves interactions between components of bacterial cell wall and complementary receptors on the host cell surfaces (Beachey, 1983; Chhatwal et al., 1990). The pili and fimbriae on gram negative bacteria and fimbriae on Gram positive bacteria allow the bacteria to adhere to the host tissues (Mertz et al., 1987; Simpson et al., 1982). Fibronectin on mammalian cell has been reported to be one of the components mediating adherence of staphylococci and streptococci to various cell types of the host (Chhatwal et al., 1990; Mosher, 1984).

The development of these bacterial infections depends on the amount of the initial bacterial inoculum and the systemic and local defense mechanisms (Prescott et al., 2006). An acute wound becomes chronic when it fails to heal properly at the appropriate time (Yah et al., 2004a). To better understand the dissemination and pathogenesis of bacterial wound infection, we attempted to potentiate the adherence of bacteria to wounds using serum and fluid. This is because, newly wounded or incised or burned tissues, when infected show a rapid oozing of fluid and serum from the infected wound site. There is a dearth of accessible information regarding bacterial virulence factors associated with serum and wound fluid as natural mediators of bacterial adherence potential (BAP) to wound surfaces. The purpose of this study was to investigate the BAP of common isolates from wounds in the presence of wound agents.

MATERIALS AND METHODS

Bacterial strains and selection of isolates for analysis

The following four bacterial strains were obtained from wound samples and used for the study: *Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli* and *Staphylococcus aureus*. The swabs of wound samples were obtained randomly from routine specimens from different infected sites of patient's wounds attending University of Benin Teaching Hospital (UBTH). These samples

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Figures 1 and 2. Wounded portion of the animal.

Figure 3. Collection of wound fluid.

were inoculated aerobically on sterile glucose broth, nutrient agar, blood agar, Eosin methylene blue, Mannitol salt agar, MacConkey agar, and chocolate agar at 37^oC for 24 to 48 h. The colonies of each representative isolates were then characterized using standard bacteriological methods (morphologically and biochemically). The isolates were confirmed by gram stain, motility, nitrate reduction, catalase, coagulase, oxidase, citrate test, bile solubility test, pigment formation, hydrogen sulfite test, fruity odor, growth at 42^oC, DNase, hemolysin production and sugar fermentation (Cowan and Steel, 1993). They were further sub-cultured and stored on nutrient agar slants at 4^oC for further analysis. The isolates to be used for the analysis were serially diluted and standardized into 0.5 McFarland turbidity (1×10^5 CFU/ml).

Experimental animals

Ten American Landrace young piglets, 3 - 6 months old, weighing approximately 5 to 6 kilo grams each, were bought from a reputable farm in Benin City (Dewtiz Farm Ltd, Benin, Nigeria) Edo State, Nigeria and quarantined for 10 days in the University of Benin animal house before initiating for the study. The animals were fed on growers marsh feed (Topfeeds Mills Ltd, Sapele, Nigeria) by Johvet Vet Clinic Benin City throughout the course of the experiment. Water was given *ad libitum* and they were housed in Iron steel cages (1 piglet per cage) with 12 h light and dark periods. The animals were certified by Johvet Vet Clinic and performed with approval of the animal care and use committee in Nigeria.

Wounding experiments

The hair was shaved from the back of each animal (from the tho-

racic to the lumbar vertebrae region) using a sterile scalpel blade (Wuxian Metical Blade Factory, Shanghai). The shaved portion was then washed with a non-antibiotic soap (Joy Soap, PZ Nigeria Ltd) using distill running water before wounding. The animals were anesthetized with an intramuscular injection of Ketamine hydrochloride 13 mg/ml (Kamala Overseas, Marine Drive Mumbai) and supported with localized nerve block injection using lidnocaine 2% (Kamala Overseas, Marine Drive Mumbai, India). With the use of an adjustable surgical incision set (Kepro Veterinary, Holland), sterile scalpel blade and measuring ruler, rectangular partial thickness wounds measuring 3 mm by 4 mm and 0.3 mm deep were made in the para-veterbral and thoracic area of the animal. The wounds were separated from one another by 4 cm bands of wounds with the use of adjustable surgical incision set to cut depth of 0.3 mm and 10 strips of skin (3 cm long, 3 cm wide and 0.3 mm deep) were excised from the animals (Figures 1 and 2). The excised skins were then placed in separate plates containing Mueller Hinton Agar (Difco Laboratories, Detroit, Mich USA) with the epidermal side up, while the dermal side was in contact with the agar. Within 2 h of excision, the dermal side of the excised skin was glued to a clean non-greasy microscopic slide using cyanoacrylate glue (Aron Techno Company, Osaka, Japan). A sterile plain tube with inside diameter 10 mm, height 1 cm cut to size, cyanoacrylate glue was attached on the epidermal side. This tube encloses the rectangular wound surface to form a well. The experiment was performed according to the modification of Mertz et al. (1987).

Wound fluid

The wound fluid was collected by the modification of Mertz et al. (1987). To obtain the wound fluid 12 full thickness 15 mm punch biopsies were made on the paraveterbral and thoracic areas on one side of the animals. Benzoin was placed on the normal skin around the biopsy sites and covered with polyurethane occlusive dressing (Tegaderm; 3 M, St. Paul, Minn, USA). The animals were loosely wrapped with bandages and dressed with nylon as shown in Figure 3. After 24 h, the accumulated wound fluid was recovered from beneath occlusive dressing by inserting a 5 ml syringe needle through the dressing into the biopsy site and aspirating the fluid.

The fluid was then placed in sterile eppendorf tubes. The fluid was then centrifuged in a micro-centrifuge (2000 Hambrg 63- Germany) at 12,000 g for 30 s. The pure wound fluid was then aspirated and stored in sterile eppendorf tubes for further analysis.

Serum

The serum was collected both by venipuncture and at the wounded sites into eppendorf tubes and centrifuged in a micro-centrifuge (2000 Hambrg 63- Germany) at 12,000 g for 30 s. The serum was

then aspirated and stored in sterile eppendorf tubes for further analysis.

Wound treatment and adhesion essay

To determine the effect of wound fluid and serum on bacterial adherence, 0.5 ml of either wound fluid or serum and distilled water (control) was added to the wells containing the wound. The fourth well did not contain any of the above agents (NA). The wounded skins were exposed to the above agents for 1 h before challenging with 0.2 ml of bacterial suspension of serially diluted standardized 0.5 McFarland turbidity (1×10^5 CFU/ml) without removing the wound fluid, serum, distilled water and into the well without no agents (NA). The samples were then incubated for another 1 h at 35°C in a shaker 40 rpm (Flash Shaker Stuart, England). The non adhered bacteria were removed by washing the wells with distilled water by vortexing the specimen using an auto-vortex (Stuart Mixer-Great Britain) for 3 to 5 s repeatedly 3 times. The non adhered cells were carefully aspirated.

Quantitation

The adhered bacteria cells to the wounded tissues were determined by removing a 6 mm punch biopsy from the center of the wounds carefully. The biopsy tissue was placed in a sterile mortar containing 10 ml of distilled water. The tissue was then minced with a mortar pestle to facilitate the elution of the bacteria cells from the tissues. The homogenized tissues was serially diluted and inoculated into Oxoid-Mueller-Hinton agar (Difco Laboratories, Detroit, Mich, USA) plates by pour plating method. The agar plates were then incubated for 24 h at 35°C. The colony forming unit (CFU) was then determined and the number of bacteria adhered per area of wounded skin was calculated and the results reported as CFU/cm² area.

Statistical analysis

The wound fluid, serum, distilled and no agent (NA) results were analyzed using the Wilcoxon Rank Sum Test, and repeated measures ANOVA was used for wound area data. The probability value reflects a difference from zero; consistent with $\log_{10} = 0$, P values < 0.05 were considered significant.

RESULTS

In an effort to improve and standardize the factors that contribute to BAP to wounds, four bacterial species commonly isolated from wounds in our community were used for this study. The results reveal that BAP to wounds is widely distributive and not specific to the factors used as shown in Figures 4a to 4d. The adherence concentration of *P. aeruginosa* in wound fluid ranged from 6.26 log (CFU/cm²) to 9.60 log (CFU/cm²) and from 6.23 log (CFU/cm²) to 9.45 log (CFU/cm²) in serum. In distilled water it ranged from 3.23 log (CFU/cm²) to 5.53 log (CFU/cm²) and 3.11 log (CFU/cm²) to 5.32 log (CFU/cm²) as shown in Figure 4a. The results showed a very significant difference between (p<0.01) the adherence capabilities of *P. aeruginosa* in serum and No agent (NA) likewise between wound fluid and No agent (NA) to wounds (p<0.01). The significant differenc-

es varied between serum and distilled water (p<0.01) and between wound fluid and distilled water (p<0.05) as shown in Figure 4a. There was no significant difference (p> 0.05) between distilled water and No agent (NA) likewise serum and wound fluid in the attachment ability of *P. aeruginosa* to wounds (Figure 4a).

The studies performed to determine the adherence aptitude of *S. aureus* to the various agents to wound varied as shown in Figure 4b. The concentration of *S. aureus* in wound fluid and serum ranged from 6.11 log (CFU/cm²) to 9.68 log (CFU/cm²) and 6.04 log (CFU/cm²) to 9.30 log (CFU/cm²) respectively while in distilled water it ranged from 3.32 log (CFU/cm²) to 6.32 log (CFU/cm²) and 3.45 log (CFU/cm²) to 6.04 log (CFU/cm²) in no agent (NA) at all (Figure 4b). The results showed a significant difference between (p<0.05) the adherence abilities of *S. aureus* in serum and no agent (NA) likewise between wound fluid and no agent (NA) to wounds (p<0.05). The same significant difference was also observed between serum and distilled water (p<0.05) and between wound fluid and distilled water (p<0.05) (Figure 4b). There was no significant difference (p> 0.05) between distilled water and no agent (NA) likewise serum and wound fluid in the adhesion ability of *S. aureus* to wound.

The adherence aptitude of *P. mirabilis* to provoke infection to wounds are shown in Figure 4c where the concentration of *P. mirabilis* in wound fluid and serum ranged from 6.01 log (CFU/cm²) to 9.51 log (CFU/cm²) and 5.97 log (CFU/cm²) to 9.34 log (CFU/cm²), respectively. In distilled water it ranged from 3.97 log (CFU/cm²) to 6.36 log (CFU/cm²) and 3.94 log (CFU/cm²) to 6.28 log (CFU/cm²) in no agent (NA) at all (Figure 4c). The results showed a significant difference between (p<0.05) the adherence aptitude of *P. mirabilis* in serum and no agent (NA) likewise between wound fluid and no agent (NA) to wounds (p<0.05). The same significant difference was also observed throughout between serum and distilled water (p<0.05) and between wound fluid and distilled water (p<0.05) (Figure 4c). There was no significant difference (p> 0.05) between the adherence capability of *P. mirabilis* in the presence of distilled water and no agent (NA) likewise between serum and wound fluid to wounds.

The results in Figure 4d show the concentrations of the attachment capacities of *E. coli* to wound. The attachment of *E. coli* in the presence of wound fluid ranged from 5.36 log (CFU/cm²) to 9.91 log (CFU/cm²) and in serum it ranged from 6.28 log (CFU/cm²) to 9.85 log (CFU/cm²). In the presence of distilled water the concentration of *E. coli* ranged from 3.32 log (CFU/cm²) to 5.80 log (CFU/cm²) and 3.25 log (CFU/cm²) to 4.52 log (CFU/cm²) in No agent (NA) at all respectively (Figure 4d). The results also showed a very significant difference between (p<0.01) the adherence abilities of *E. coli* in the presence of serum and no agent (NA), similarly between (p<0.01) wound fluid and no agent (NA) to wounds. The significant difference was also observed between serum
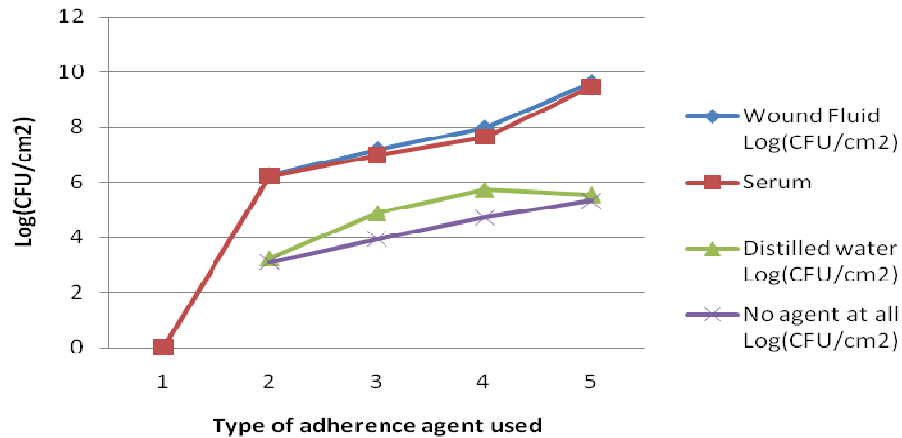
Figure 4a. Effect of wound agents on the adherence ability of *Pseudomonas aeruginosa*.
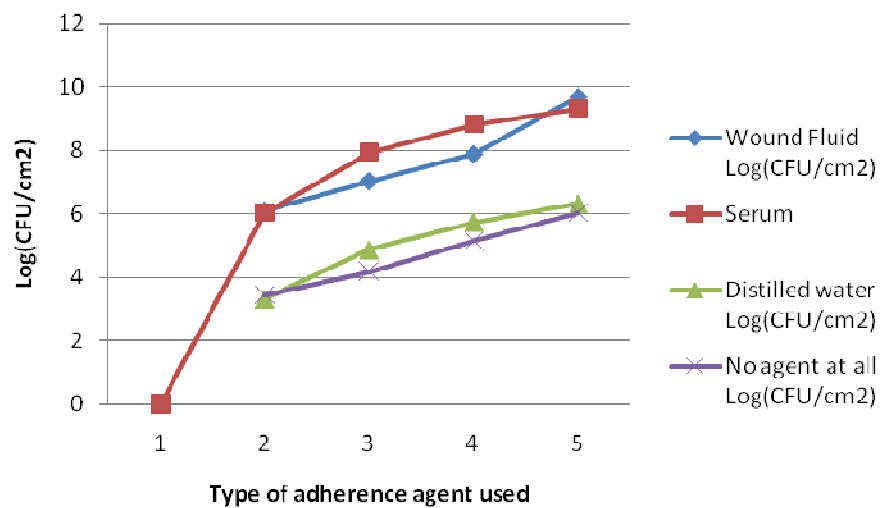

Figure 4b. Effect of wound agents on the adherence ability of *Staphylococcus aureus.*
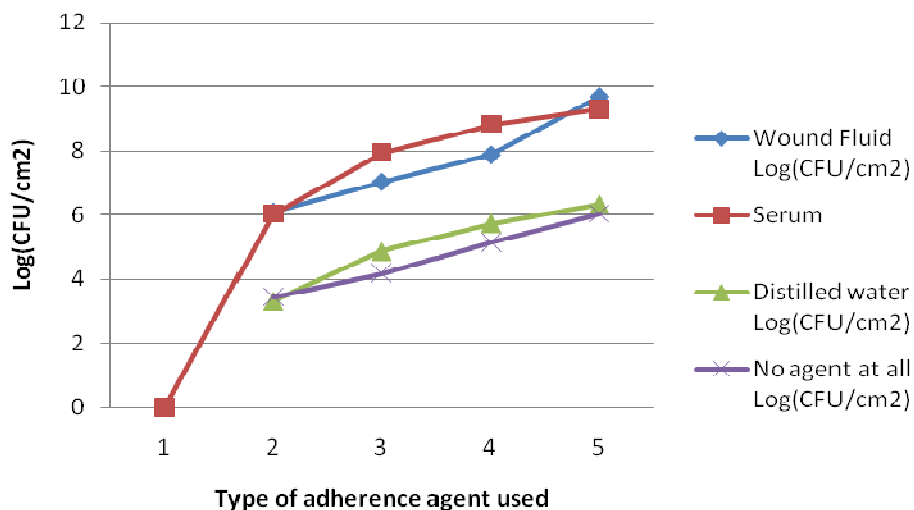

Figure 4c. Effect of wound agents on the adherence ability of *Proteus* species.
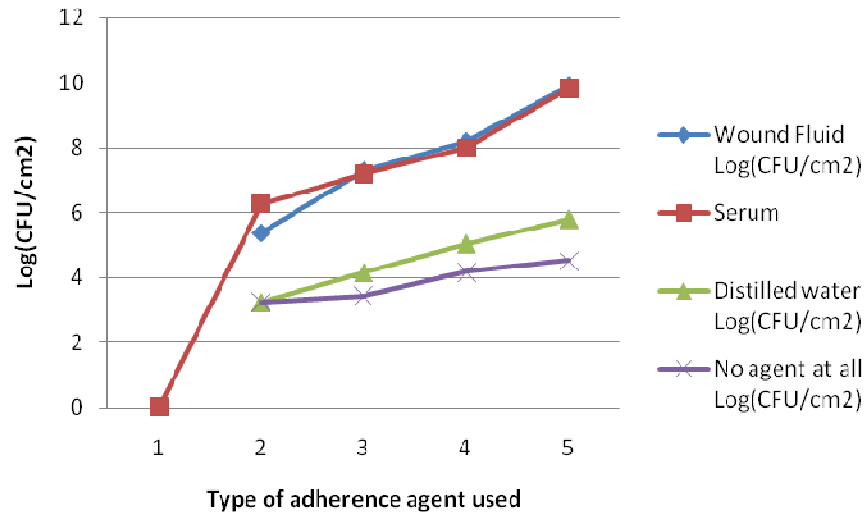
Figure 4d. Effect of wound agents on the adherence ability of *Escherichia coli.*

DISCUSSION

Studies however, have shown that there is a synergy between wound agents and microbial adhesion and penetration into wounds ((Mertz et al., 1987; Chhatwal et al., 1990). These microorganisms express a blind of multiple effects during wound injury resulting into wound infection. These biologic effects are distributed within the bacterial cells and the host wound tissues itself where they may exert similar enzyme-substrate like affinity. In this study we tested the ability of *P. aeruginosa, P. mirabilis, E. coli* and *S. aureus* commonly isolated from wounds to freshly injured wounds in the presence of wound fluid, serum, distilled water and no agent (NA) and tried to correlate their adherence ability with the microbial density. The study however, exposed the detrimental association between wound agents and microorganisms commonly isolated from wounds.

Our results also showed a significant difference between wound fluid and distilled water/no agent (NA) and between serum and distilled water/no agent (NA) in the attachment capability of bacterial isolates to wounds. This, therefore, showed that wound fluid and serum encourage the adherence of bacterial isolates commonly encountered in our community to wounds. As shown in Figure 4a, *P. aeruginosa* showed a very significant difference between (P<0.01) the adherence properties of *P. aeruginosa* in the presence of serum and no agent

and distilled water (p<0.05) and between wound fluid and distilled water (p<0.01) (Figure 4d). There were no significant results (p> 0.05) between *E. coli* potential attachment in the presence of distilled water and no agent (NA) as well as between serum and wound fluid (p> 0.05) to wound.

(NA) likewise between wound fluid and no agent (NA) to wounds. In the presence of *P. aeruginosa* to wounds a significant difference was found to vary between serum and distilled water (P<0.01) and between wound fluid and distilled water (P<0.05). A similar result was also obtained with *E. coli* as shown in Figure 4d. The same trends were found in *Proteus* and *S. aureus* (Figures 4b, 4c). This however, contradicts the earlier reports of Mertz et al. (1987) that wound fluid and serum prevent the attachment of *P. aeruginosa* to wounded skin. Other researchers have shown that serum and wound fluid can cause bacterial cell lysis (Prescott et al., 2006; Bercovici et al., 1975). The lysis ability may occur when the host has sufficiently built up antibodies ready to counteract the mediating process of the bacteria attachment event. In the presence study, by the time of initiating the attachment process for the experiment, has not produced enough antibodies. Secondly, the tissues were excised from the host and placed in Mueller Hinton Agar for the experiment. Thirdly, the course of infection by various pathogens varies within isolates before an infection can be established. The serum, wound fluid, no agent (NA) and distilled water in this study had no bactericidal action. The experimental animals were not inoculated with the pathogens prior to the wounding process.

The results also correlated with those earlier reported by Chhatwal et al. (1990) that increase in bacterial concentration increases the adherence capability of the bacteria to wounds. This therefore, shows that bacterial adherence to debilitated host tissues is dose dependent as revealed by the presence results. This was similar to those earlier reported by Toy et al. (1985) that increasing the concentration of fibronectin caused an increase in the attachment of *S. aureus* to fibrin thrombi. Fibronctin is a glycoprotein found in the blood and in the extracellular matrix connective tissues (Stites et al., 1987).

The results also showed that bacteria adhere to wounds with different affinity (Figures 4a to 4d). The adherence properties of *S. aureus* showed a significant difference between ($P<0.05$) the presence of serum and no agent (NA) likewise between wound fluid and no agent (NA) to wounds. The same significant difference was also observed between serum and distilled water ($P<0.05$) and between wound fluid and distilled water ($P<0.05$). On the other hand there were no statistically significant differences between ($p> 0.05$) the bacteria attachment potential in distilled water and no agent (NA) as well as between serum and wound fluid ($p> 0.05$) to wound. The lack of appreciable differences between wound fluid and serum was due to the fact that the two wound agents do have potential ability to promote bacterial attachment to freshly injured wounds while the distilled water and no agent (NA) do not have enhanced factors necessary to promote BAP to wounds.

Conclusion

The results generally gave a high density of BAP in wound fluid and serum as compared to distilled water and no agent (NA). This predicted the presence of chemical elements in these substances capable of enhancing microbial attachment to freshly injured wounds. With the presence results, the findings therefore, augment the fact that wound fluid, serum and likely other wound agents can act as cofactor influencing the attachment of bacteria to wounds as well as enhancing the infectious process of wound. Also our observations on the BAP in the presence of wound agents showed the need of careful handling of wounds to prevent wound infections and better understanding concerning wound infection.

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