Research Article

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Saliva of different dog breeds as antimicrobial agents against microorganisms isolated from wound infections.

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The study investigated the antimicrobial potential of saliva samples from five breeds of dog namely Alsatian, Belgium shepherd, Doberman, crossbreed of Doberman and Alsatian and Local breeds. The antimicrobial properties of the saliva samples were tested on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* isolated from wounds of patients receiving treatment at the Delta State University, Abraka, Nigeria. The wounds had total bacterial counts that ranged from 1.43×10^6 to 4.7×10^9 CFU/ml. All the breeds` saliva samples contained lysozyme, peroxidase and lactoferrin as antimicrobial constituents. The peroxidase activity was found to be highest at 0.81±0.021U/ml in Alsatian dog and lowest at 0.52±0.11µ/ml in the crossbreed saliva sample. The lactoferrin values ranged from 48 to 75 ng/ml with Belgium shepherd saliva sample having the highest and the local breed saliva sample had the least. The saliva samples were able to inhibit the growth of the isolates. The zones of inhibition of the saliva samples for *S. aureus, P. aeruginosa* and *C. albicans* ranged from 8.53±0.99 to 9.82±1.22mm, 10.77-12.13±1.24mm and 10.62±1.42 to 16.20±1.64mm respectively. The minimum inhibitory concentration of the samples ranged from 6.25 to 50.00%v/v. The saliva samples exhibited potent antimicrobial property against the test organisms even at low concentrations. Sterilized, purified extracts can be formulated and applied in the treatment of wound infections.

Key words: Wound infection, peroxidase, lactoferrin, saliva samples, dog breeds

A wound is a hurt or an injury to living tissues of the body or a break in the continuity of the skin. It ranges from small lacerations to severe wide-spread injuries (Roper, 2002). Studies by Spicer (2002) showed that most wounds result from occurrences such as falls, mishandling of sharp objects, accidents, burns, ulcers. These wounds can become infected from a variety of sources such as the patient's own normal flora, the medical personnel in charge of the patient, the environment or the hospital equipment. Opportunistic pathogens have been known to infect wounds. Signs of wound infections include delayed healing of the wound surface, change in the colour of the wound, granulation tissue, abnormal red skin colour around the wound, increased exudates, increased pain at the wound site, foul odour and sometimes discharge of pus (Brendan and Freedman, 2006). Organisms that may infect wounds include Staphylococcus *aureus*, *S. epidermidis, Streptococcus pyogenes, Diphtheroids, Escherichia coli, Klebsiella* and some fungal species (Spicer, 2002). Treatment of wound infections involving some of these pathogens has become difficult due to the resistance they have developed to the commonly used antibiotics such as penicillins, cephalosporins (Takonelli et al., 2009). Plants are being researched for the purpose of antimicrobials but there is the caution of a need to conserve our natural vegetation. An alternative to plants is being envisaged in saliva. It is a watery substance produced in the mouth of humans and animals. Saliva contains many compounds that are antibacterial and promote healing. It contains enzymes such as lysozyme and peroxidase (Ihalin *et al.*, 2006) which break the chemical bonds in bacterial cell walls. It also has lactoferrin, defensins cystatins (Abiko et al. 2003), protease inhibitior (Ashcrofit et al. 2006) nitrates, lephin,

Ophorphin (Todorovic et al. 2008 and Wisner *et al.* 2006).

In view of the fact that it was believed that wounds of dogs that are constantly licked by dogs healed faster than wounds being treated, this work aims at isolating and identifying microbial isolates from wound samples and determining the antimicrobial potential of saliva from different dog breeds on the isolates..

MATERIALS AND METHODS

Collection of Samples

Wound Sample:

Six samples of wounds were collected from various patients receiving treatments at the Delta State University Health Centre, Abraka Nigeria using sterile swab sticks. The samples were taken from the wounds that were failing to make progress despite an optimal environment for wound healing. The swab sticks were placed into sterile containers and labeled accordingly. They were immediately taken to the laboratory for further analyses.

Saliva Samples

Two samples of Saliva were obtained from each of the four different dog breeds (Alsatian (A), Belguim Shepherd (B), Doberman(C), Cross Breed (Doberman and Alsatian) and Local Breed (E) from Turf Club, Oria, Abraka, Delta State. The samples were labeled appropriately and transported to the laboratory in ice-packs for further analyses.

Isolation and Identification of Organism

This was done using the method of Fawole and Oso (2007). The samples were inoculated onto Nutrient Agar and incubated at 26±2°c for 24 hours. The identification of the different isolates was done using morphological characterization and reaction to biochemical tests (Cheesebrough, 2004).

Test for Lysozyme

This was determined on plates containing each of the dog saliva sample, UV killed cells of *Micrococcus lysodeikticus*. It was sterilized at 121°c for 15mins and about 12ml was poured into sterile plates. Broth cultures, grown overnight at 37°c for 48 hours. Lysozyme production was indicated by a zone of definite clearing surrounding the area of growth.

Test for Lactoferrin

This was done using a modified method of Adebimpe and Lawrence (2003). 100µl of sample was put in a well, covered and incubated at 26°c for one hour. Plate was washed four times and 10µl of lactoferrin detection solution was added to each well. The plate was covered and incubated at room temperature for another 1 hour. 100µl of HRP solution A was added to each well. Plates were covered and incubated at room temperature for 30mins. Plate was washed four times and 100µl of TMB substrate solution added to each well. The plates were developed in the dark at room temperature for 30mins. 100µl of stop solution was added to each well and absorbance measured at 450nm.

Test for Peroxidase

Peroxidase activity was determined using the modified methods of Mansson-Rahembull *et al.* (2004) and Bergemeyer (1974). 2.80ml Buffer (0.1m potassium phosphate, pH 7.0), 0.05ml (0.018m Guaicol) and 0.05ml substrate (0.1ml 30% H_2O_2 diluted with distilled water to 120ml and adjusted to 1cm length path to 0.4 and 0.41 versus distilled water) was pipette into 1cm quartz cell. It was equilibrated at 25°c and monitored at ΔA/ min. Rate of increase in absorbance at 436nm was recorded.

Determination of Minimum Inhibitory Concentration (MIC) of Saliva Samples

Two milliliters of each of the saliva samples was introduced into a sterile test tube. One milliliter was withdrawn and serially diluted into tubes containing peptone water. A standard density of the organisms was made by inoculating three pure colonies of each isolate into 5ml peptone water and incubated at 37°c for 24hours. 0.1ml was taken and used to inoculate each of the serial dilutions prepared. Two other test tubes, one containing only 1ml of saliva and the other containing only peptone water were set up as controls. These were also incubated at 37°c for 24hours. The lowest concentration of the saliva that prevented the growth of the organisms was determined

Antimicrobial Susceptibility Test

This was done by determining the zones of inhibition of each saliva sample using the method of sterile Nutrient Agar at 45°c was poured into sterile Petri-dishes and allowed to gel. 0.1ml of homogenized mixture of the isolates was inoculated onto the media and spread evenly across the surface using a sterile glass spreader. Each of the saliva was used in soaking perforated disks which were placed on different spots on prepared Nutrient Agar plates.

Table 1: Percentage occurrence of each isolate in the wound samples

Organism	Occurrence in each sample					% occurrence
	A	**B**	**C**	**D**	**E**	
S. aureus	**+**	**+**	**+**	**+**	**-**	**80**
P. aeruginosa	**-**	**-**	**+**	**+**	**+**	**60**

+ present, – absent

RESULTS AND DISCUSSION

The bacteria isolated from the wound samples were *Staphylococcus aureus* and *Pseudomonas aeruginosa* (table 1). The percentage occurrence of each isolate in the samples was 80% for *Staphylococcus aureus* and 60% for P. *aeruginosa. S. aureus* being a normal flora of skin might have been introduced into the wound as a result of the breakage in the resistant structure of the skin or might have been introduced into the wound from the hospital environment. Pseudomonas could have been introduced from the environment such as showers, gardens and soil of which the individual might have been exposed to. Pathogenic organisms causing wound infection have been found to vary according to the anatomical site of the wound. Antibiotic resistant organisms such as methicillin resistant *S. aureus* (MRSA) are commonly encountered reflecting the hospital flora (Tacconelin *et al.*, 2009).

The log of the total bacterial counts for the wound samples ranged from1.11$\pm$1.09 to 8.05$\pm$1.95CFU/ml as shown in table 2. The bacterial count was highest in wound simple E and lowest in wound sample B. The total bacterial count of each wound sample showed that all the wound samples were infected. This is in conformity with the study of Bowling et al., (2010) that 950CFU/ml– 16780CFU/ml bacterial load in wound signifies a wound infection. The high microbial count might be attributed to the fact that a suitable environment was provided for the prolific multiplication of the pathogenic organisms on entering the tissue which resulted in an infection.

Table 2: log of Total Bacterial count of the wound samples (CFU/ml)

	Count
Alsatian (A)	1.11$\pm$1.09
Cross breed (B)	8.05$\pm$1.95
Doberman (C)	2.39$\pm$2.31
Belgium Shepherd (D)	7.67$\pm$1.08
Local Dog (E)	1.15$\pm$1.13

All the saliva samples possessed lysozyme, peroxidase and lactoferrin (table 3). Jorma (2002) found that antimicrobial agents such as lysozyme, mucin, and lactoferrin detected in human saliva conferred protein for the whole body. The antimicrobial potentials of the samples are evidenced in the results of the determination of zones of inhibition and the minimum inhibitory concentrations (Tables 4 and 5). All the saliva samples were able to inhibit the growth of the two isolates. The ability of the dog breed saliva samples to inhibit the growth of *S. aureus* increased in the order of Belguim Shepherd › Alsatian› Crossbreed› Doberman while that of *P. aeruginosa* was similar for Belguim Shepherd, Doberman and Crossbreed. The Alsatian dog breed saliva sample had the highest zone of inhibition of 12.13±1.24. Belgium shepherd had the highest amount of lactoferrin while Alsatian had the highest peroxidase activity. This could have contributed to their high antimicrobial activity. There was no growth inhibition in the controls. This indicated that the saliva samples had antimicrobials that inhibited the growth of the organisms. The result is in conformity with that of Famand et al. (2003) that lactoferrin in saliva confers antimicrobial properties against organisms such as *S. aureus, C. albicans, E. coli*, Pseudomonas.

The statistical analysis of the peroxidase activity of the samples using ANOVA showed that there was a significant difference since $P<0.05$.There was no significant difference ($P>0.05$) in the diameters of zones of inhibition.

The minimum inhibitory concentration (MIC) of each saliva sample on the isolates is shown in table 5. Different concentrations of the saliva samples ranging from 6.25% (v/v) - 100 %(v/v) were used. All the saliva samples were able to inhibit the growth of the organisms at one concentration or the other. At concentration where there were growths, the growths were scanty. The minimum inhibitory concentrations on S. aureus were 25%, 50%, 50% 25% and 12.5% for Alsatian, Crossbreed, Doberman, Belgium shepherd and local breeds respectively. The ability of the saliva samples to inhibit the growth of these organisms even at very low concentrations might be attributed to the presence of very potent antimicrobials in the saliva samples.

Table 3: Antimicrobials in each Dog breed Saliva Samples

Sample	Lysozyme	Peroxidase (μ/ml)	Lactoferrin (ng/mL)
Alsatian (A)	+	0.81±0.021	50
Cross breed (B)	+	0.61±0.044	75
Doberman (C)	+	0.61±0.440	55
Belgium Shepherd (D)	+	0.52±0.110	65
Local Dog (E)	+	0.80±0.115	48

One unit of peroxidase is the amount of enzyme which catalyzes the conversion of 1 μmol of hydrogen peroxide per minute at 25C. + Present

Table 4: Zones of Inhibition of each Saliva Sample on the Isolates

	Zones of inhibition (mm)		
	S. aureus	*P. aeruginosa*	*C.albicans*
Alsatian (A)	9.07±1.168	12.13±1.24	14.24±1.32
Belgium Shepherd (D)	8.53±0.9	10.87±0.96	16.20±1.64
Doberman (C)	9.63±1.39	10.77±1.57	12.14±1.22
Cross Breed (B)	10.47±2.17	10.77±1.91	10.62±1.42
Local Dog (E)	4.82±1.22	10.82±0.97	12.21±1.32

Table 5: Minimum Inhibitory Concentration (% v/v)

Concentration	***S. aureus***					***P. aerginosa***					***C. albicans***				
%(v/v)	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	+	+	-	-	-	+	-	-	-	-	+	-	-	-
12.5	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-
6.25	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+

+ Growth inhibition, - No Growth inhibition, A – Alsatian, B - Cross breed, C – Doberman, D - Belgium Shepherd, E – Local Dog.

Lysozyme in saliva samples destroys bacterial cellular membranes (Hamning *et al*., 2010) and limits glucose assimilation by bacterial cells which leads to lowered metabolism, adhesion and aggregation which eventually leads to reduction to growth and hence death (Andres and Fierro. 2010).

Peroxidase identified in the saliva of some animals inhibited the growth of microorganism. It inhibited the uptake and production of acids and prevents the accumulation of hydrogen peroxide (Klimiuk *et al*., 2006). The lactoferrin detected in the saliva samples of the different dogbreeds was found to produce antibacterial activity to human infants. It has been found to bind lipopolysacchandes of bacterial wall and the oxidized iron part of the lactoferrin oxidizes bacteria via formation of peroxides. Membrane permeability is affected and results in cell breakdown (Andres and Fierro, 2010).

REFERENCES

Abiko, Y., Nishimwa, M. and Kaku, T. (2005). Defensins in Saliva and salivary glands. *Medical Electron Microscope*. 36 (4): 247- 252

Andres and Fierro (2010). Antimicrobial Mechanism of Action of Transferrins.

Antimicrob Agents Chemother. 54 (10): 4335- 4442.

Ashcroft, G.S., Lei, K. and Jun, N. (2000). Secretory Leukocyte Proease Inhibitor Mediate Non- redundant functions necessary for wound healing. *Nat. Med.* 6(10): 1147- 1153.

Brendan, H. and Freedman, A. (2006). *Infections BMJ.* 332 (7545): 838- 841.

Cheesebrough, M .(2004). District laboratory practice in tropical countries. Part 2. Cambridge University Press, Uk. 434pp.

Famand, S. and Evans, R.W. (2003). lLactoferrin- A Million functional protein with antimicrobial Properties. *Mol. Immunl.* 40(7): 395- 405.

Fawole, M.O. and Oso, B.A. (2007). *Laboratory Manual of Microbiology* (5th ed.). spectrum Books Ltd, Ibadan. pp 22- 33.

Hanning, C., Spres, B., spitzmiller, B. and Hannning, M. (2010). Efficacy of Enzymatic Mouthrinser for Immunization of protective enzymes. *Arch Oral Bio.* 55(1): 1- 6.

Ihalin, R., Loimaranta, V. and Tenovuo, J. (2006). Origin, Structure and biological activities of peroxidase in human Saliva. *Archives of Biochemistry and Biophysics.* 445 (2): 261- 268.

Jorma, T. (2002). Antimicrobial agents in saliva- Protection for the whole body. *Journal of Dental Research.* 81 (12): 807- 809.

Mansson- Rahem, B. (1986). Specific assays for peroxidases in human saliva. *Arch Oral Bio.* 31(10): 661- 668.

Prescott, M.C., Harley, P.J. and Klein, A.D. (2007). *Microbiology* (7th ed.). WMC Brown Publishers, USA. 1935pp.

Roper, N. (2002). *Pocket Medical Dictionary* (14th ed.). Church hill Livingstone Publishers, London. 38pp.

Spicer, W.J. (2002). *Clinical Bacteriology, Mycology and Parasitology.* Churchhill Livingstone Publishers, London. 537pp.

Tacconelin, E., De Angeli, G., de Waive, C. (2009). Rapid Screening Tests for MRSA at Hospital Admission: *Systemic Review and Meta- analysis Lancet Infect.* 9(9): 546- 554

Todorovic, V., Pesko, P. and Micer, M (2008). Insulin- Like Growth factor-1 in wound healing of rat skin. *Regulatory peptides.* 150(1- 3): 7- 13.

Wisner, A., Dufow, E. and Messaoudi, M. (2006). Human opiorphin. Proceedings of the National academy of Sciences of the United States of America. 103 (4): 17979- 17984.