Research Article [Haq *et al.*, 7(2): Feb, 2016:4911-4917]
CODEN (USA): IJPLCP ISSN: 0976-7126

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES
(Int. J. of Pharm. Life Sci.)

Antimicrobial and Wound Healing Properties of Human Saliva

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Abstract

To observe antiviral activity of human saliva against Avian Influenza strain H9N2, and comparison of antimicrobial activity against Candida albicans, Staphylococcus aureus in healthy and diabetic human individuals, and saliva's wound healing effects on wounded rabbits.Study was conducted in the Institute of Biochemistry and Biotechnology, and Department of Microbiology at University of Veterinary and Animal Sciences, Lahore. Total of 64 human saliva samples were collected from both sexes. Total of 8 male and female samples (4 diabetic and 4 non-diabetic) were taken from each age range consisting of: 15-25, 25-35, 35-45, 45-55. Antiviral activity of saliva from non-diabetic individuals was observed against Avian Influenza Virus Pk-UDL/01/08 H9N2. Antimicrobial activity of diabetic and non-diabetic samples against Candida albicans and Staphylococcus aureus was observed through agar well and disk diffusion methods. Wound healing of healthy saliva was tested against 30 fully grown adult male rabbits of which 24 rabbits were experimental (saliva applied on induced wound), 3 acted as negative control to check natural wound healing, and 3 were positive control (wound healing medicine was applied).The study showed that antimicrobial activity of human saliva against C. albicans, Staph. aureus and AIV H9N2 was dependent on age of individual with age range 25-35 giving optimum results. Wound healing ability also followed similar trend. Antimicrobial and wound healing ability decreased with increase in age while no antimicrobial activity was shown in saliva of diabetic individuals.Healthy human saliva possesses natural antimicrobial and wound healing abilities.

Key-Words: Human saliva, Avian Influenza H9N2, Candida albicans, Staphylococcus aureus, wound healing, diabetes.

Introduction

Human saliva contains a mixture of peptides, proteins and many miscellaneous substances. It not only maintains the health of the mouth cavity but also provides protection against many pathogens (Verma *et al.*, 2013). This protection has been attributed to the presence of certain antimicrobial peptides such as histatins (White *et al.*, 2009). Histatins have been found to have both naturally occurring antifungal as well as antibacterial properties (Verma *et al.*, 2013). These proteins are also known to play quite an important role regarding natural wound healing in various organisms (*Jia et al.*, 2012).

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To observe the antimicrobial properties of human saliva the present study selected Avian Influenza Virus H9N2, *Candia albicans*, and *Staphylococcus aureus* on the basis of the risk they pose to the human population, while the wound healing abilities of saliva were observed against wounded rabbits. It is worth noting that this is the first study of its kind pertaining to the samples collected from the population of Punjab, Lahore.

Influenza is a common respiratory viral infection of humans and avian species (Pushko *et al.*, 2005). A very common and well-studied virus is the avian influenza virus (AIV). One of the subtypes of AIV is H9N2 which is low pathogenic (LP) and is still persisting in avian species (Sarwar *et al.* 2013). It causes avian influenza in poultry flocks and is thus the main reason of morbidity and mortality in epidemics. In recent years, H9N2 virus has attained a great importance as its infection has zoonotic potential and has been

transmitted to mammalian species, including humans (Lu, 2014).

Staphylococcus aureus is a common cause of skin infections, respiratory infections and food poisoning. It also shows resistance to commercially available antibiotics. A common fungal infection to be diagnosed in humans is Candidiasis. This disease is due to an overgrowth of *Candida albicans* (*C. albicans*). Though the said diploid fungus is part of the normal human gut flora, it poses a threat to individuals whose immune system is compromised (Lal *et al*., 1992). The number of infected patients has increased dramatically (Rodloff *et al*., 2011). Patients suffering from diabetes mellitus (DM) have been reported to be more vulnerable to fungal infections caused by *Candida* species (Gunther *et al*., 2014). Diabetic cases in Pakistan have been projected to be more than 13.9 million by the year 2030 (Whiting *et al*. 2011).

Healing of oral wounds is faster compared to healing in other parts of body and also gives rise to less scaring. Saliva is the major factors which involves in this phenomenon, which in several ways promotes oral wound healing. Functioning and survival of inflammatory cells improves due to presence of saliva because it creates a humid environment, and inflammatory cells are essential for wound healing (Brand and Veerman, 2013).

The current study was designed to observe the natural antimicrobial ability of human saliva and to compare that ability with regards to an individual's age, gender and presence of immunocomprimising ailment such as Diabetis Mellitus. Understanding the natural healing properties of human saliva can help create a new approach in the field of medicine.

Material and Methods

The current study was conducted in the Institute of Biochemistry and Biotechnology, and Department of Microbiology at University of Veterinary and Animal Sciences, Lahore. A total of 64 human saliva samples were collected from both sexes: A total of 8 male and female samples (4 diabetic and 4 non-diabetic) were taken from each age range consisting of: 15-25, 25-35, 35-45, 45-55. Due to the study's wide approach, it was divided into three separate experiments, with each division dealing with various properties of human saliva.

Antimicrobial susceptibility of human saliva

A total of 64 collected human saliva samples were processed for this study. The antimicrobial activity of the selected saliva samples was observed through both agar well diffusion and agar disk diffusion methods. Muller-Hinton Agar was prepared by weighing 19 grams of MH Agar and dissolving it in 500mL of distilled water in a glass bottle. The bottle was then autoclaved at 121ºC for 15 minutes.

For disk diffusion method, petri plates containing MH Agar were prepared and growth of *Candida albicans* and *Staphylococcus aureus* were individually swabbed onto different plates to create a lawn (Rimek *et al*. 2008). Disks were made from Whatman Filter paper (Cat. No. 1440125) and saliva absorption was optimized by testing different sized disks. Disks of 0.5, 2, and 2.5 inches in diameter were able to absorb 20, 140 and 180µL of saliva respectively. The disks were placed at the centre of the media in the plates.

For agar well diffusion method a thin layer of MH Agar was poured onto petri plates and allowed to cool. After that another layer was poured and allowed to solidify over the existing thin layer. Wells were then created on prepared plates of MH Agar using well borer having diameter of 0.5 inches. The fresh *Candida albicans* and *Staphylococcus aureus* growths were individually streaked onto different plates. After streaking the wells were filled with 80µL of saliva samples using a pipette. The properly labeled plates for both agar well diffusion and disk diffusion methods were incubated at 37°C for 24 hours (Verma *et al*. 2013).

Antiviral susceptibility of human saliva

Healthy human saliva samples were used for this experiment. Genetically characterized isolate of Avian Influenza Virus Pk-UDL/01/08 H9N2 of calculated EID50 106.66 was obtained from University Diagnostic Laboratory (UDL) originally preserved in freeze dried form. The collected saliva samples were treated with Pk-UDL/01/08 H9N2 virus with different ratios and at variant incubation time before inoculation in 9 days old embryonated chicken eggs.

For the present study 3 groups of saliva and virus ratio were designed. In Group A the saliva and H9N2 virus ratio was 1:1 by taking 1 mL of saliva and 1 mL of virus. In Group B the saliva and H9N2 virus ratio was 1:1.5 by taking 1 mL of saliva and 1.5 mL of virus. In Group C the saliva and H9N2 virus ratio was 1:2 by taking 1 mL of saliva and 2 mL of virus. Group D was negative control by adding only saliva. Group E was positive control group having untreated virus.

The incubation time after mixing of saliva and virus was also studied at varying time durations. The pre-inoculation incubation time selected after mixing saliva and virus ratios was 0.5 hour, 1 hour, 1.5 hour and 2 hour. For each studied groups 5 vials of saliva and virus mixture were prepared. Vial 1 was incubated at 37ºC for 0.5 hours and Vials 2, 3 and 4 were incubated at 37ºC for 1hr, 1.5hr and 2hr respectively. Vial 5 was not incubated and as such used for inoculation. In the

saliva and virus mixture 200 µL of antibiotic (containing 100 IU/mL of Ceftiofur hydrochloride) was added to avoid any microbial contamination and kept at incubation temperature of 37ºC for half an hour. In case of only virus and only saliva same concentration of antibiotic was used (Khalili *et al*. 2013).

Embryonated chicken eggs (ECE's) being 9 days old were used for this study. For each of the 5 groups a total of 5 vials were used, and for each vial 3 eggs were used for inoculation. In this way, 15 eggs were used for each group. A total of 0.2 µL of inoculum of each sample was inoculated in embryonated chicken eggs via Chorio Allantoic Sac (CAS) route. After inoculation eggs were incubated at 37ºC for 48 hours (Khalili *et al*. 2013). The Allanto Amniotic fluid (AAF) was then pooled out in a separately labeled sterilized 15 mL falcon tubes (Hitchner *et al*. 1980).

Haemagglutination test of harvested fluid was performed using 1% washed chicken red blood cells. Ninety six well U bottom polystyrene micro titration plates were used for HA test. The HA test was conducted according to the procedure described by Alexander and Chettle with some minor modifications (Alexander and Chettle, 1977).

Wound healing property of human saliva

A total of 24 healthy human saliva samples (3 from each age group) were used for this study. Subjects of the study were 30 fully grown adult male rabbits weighing 2.0 to 3.4 kg and ranging in age from 8 to 16 months. They were acclimatized for two weeks in stainless steel cages and fed commercial diets, vegetables, crushed wheat and corn all over the whole experiment. Out of 30 rabbits 24 rabbits were experimental on which saliva was applied, three were negative control to check natural wound healing, and three were positive control on which wound healing medicine was applied.

Wounds (2cm×2cm) were established on the one side of back of rabbits. Saliva was applied on 24 experimental rabbit's wounds after duration of every two hours for two weeks. Polyfax ointment was applied on three rabbits serving as positive controls. The wounds on last three rabbits served negative control for observance of natural healing. Results were observed after 2, 4, 6, 8, 10, 12 and 14 days of experiment.

Results and Discussion

Antimicrobial susceptibility of human saliva

Well diffusion method was unable to yield any significant results as the quantity of saliva required to effectively act against *Candida albicans* and *Staphylococcus aureus* could not be sufficiently held inside each well made through the well borer. Thus, the saliva of both healthy and diabetic individuals was unable to create zones of inhibition around the formed wells. On the other hand disk diffusion method showed favorable results as the disks used in the experiment were able to absorb the required quantity of saliva, not less than 120µL, to give rise to visible zones of inhibition against both *Candida albicans* and *Staphylococcus aureus* as shown in Figures 1 and 2. A comparative look at the antifungal and antibacterial property of human saliva with respect to age is shown in Figures 3 and 4.

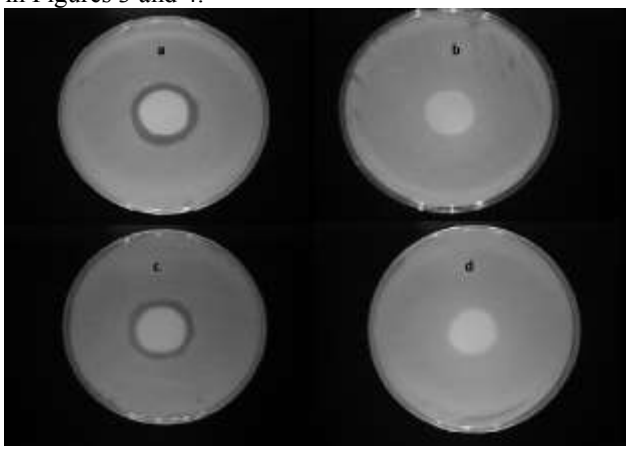

Figure 1: Zone of inhibition by human saliva against *Candida albicans*: a. Healthy male saliva sample age range 25-35. b. Diabetic male saliva sample age range 25-35. c. Healthy male saliva sample age range 35-45. d. Diabetic male saliva sample age range 35-45.

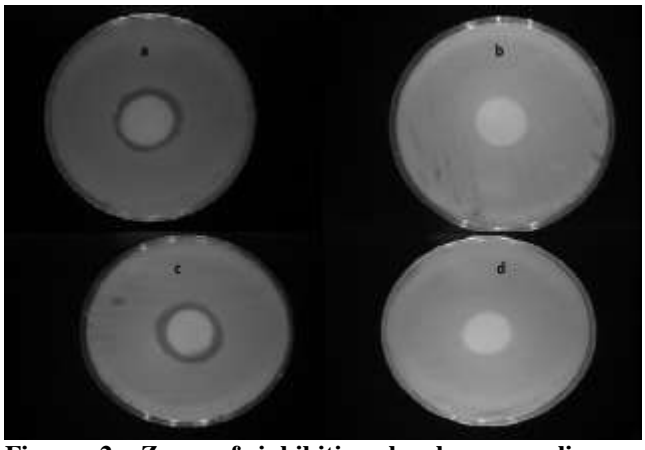

Figure 2: Zone of inhibition by human saliva against *Staphylococcus aureus*: a. Healthy male saliva sample age range 25-35. b. Diabetic male saliva sample age range 25-35. c. Healthy male saliva sample age range 35-45. d. Diabetic male saliva sample age range 35-45.

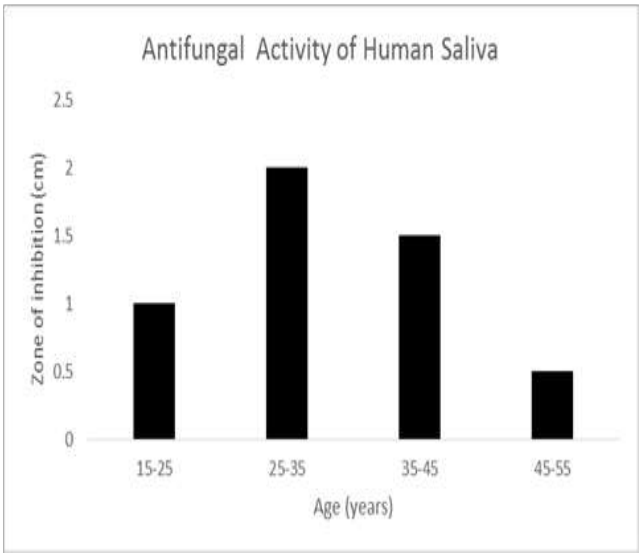

Figure 3: Antifungal activity of human saliva related to age groups.

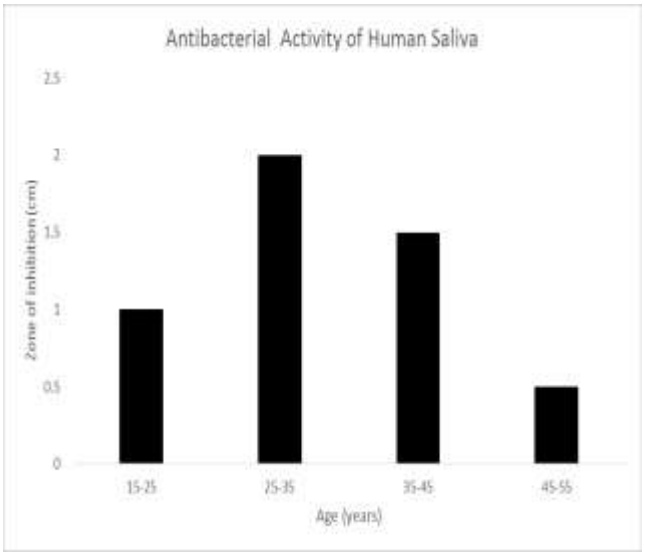

Figure 4: Antibacterial activity if human saliva related to age groups.

Antiviral property of human saliva

The results of antiviral activity of human saliva through performing HA test are shown in Figures 5 and 6.

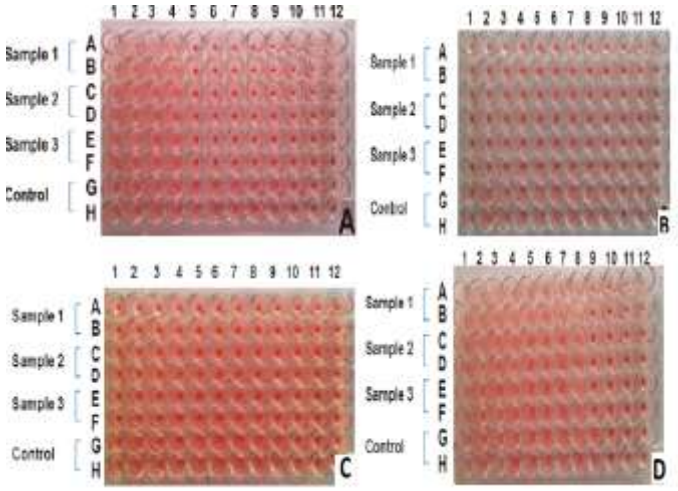

Figure 5: HA titres of H9N2 virus treated with human saliva: A: Male saliva samples age range (15-25). B: Male saliva samples age range (25-35). C: Male saliva samples age range (35-45). D: Male saliva samples age range (45-55).

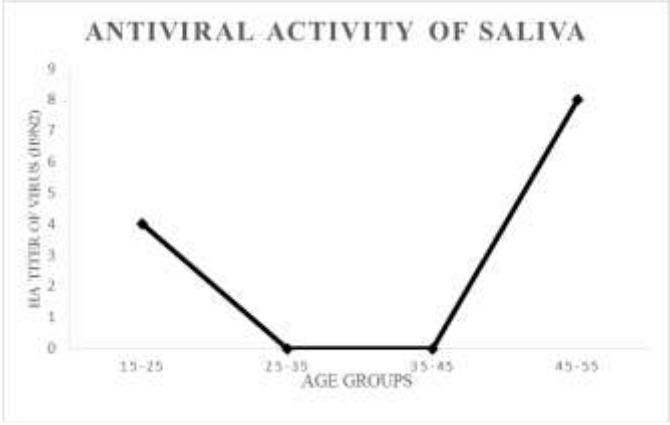

Figure 6: Antiviral activity of saliva related to age groups.

Wound healing property of human saliva

Healing of wounds was observed on 2, 4, 6, 8, 10, 12, and 14 days. The healing speed of wounds on which saliva was applied was higher than the wounds on which wound healing medication (polyfax) was applied, and there was pus formation in the wound of negative control on which natural healing was observed. The comparison of wound healing ability with respect to age of individual is shown in Figures 7, 8, 9, and 10.

Figure 7. Wound healing of experimental group (saliva applied). The figure above depicts healing rate of saliva. Wound is almost healed till the end of last day of experiment. Above shown is the healing result of age group (25-35).

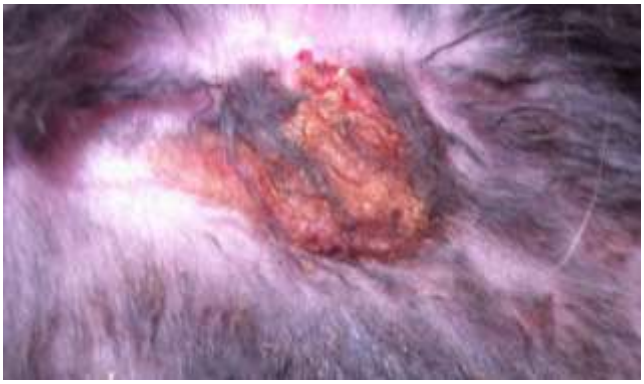

Figure 9. Wound healing in negative control group. The figure above depicts healing rate of negative control group nothing was applied on which to check natural wound healing. Its healing was slowest. There was no healing till the last day of experiment.

Figure 8. Wound healing in positive control (polyfax applied). The figure above depicts healing rate of positive control group on which poly fax was applied.

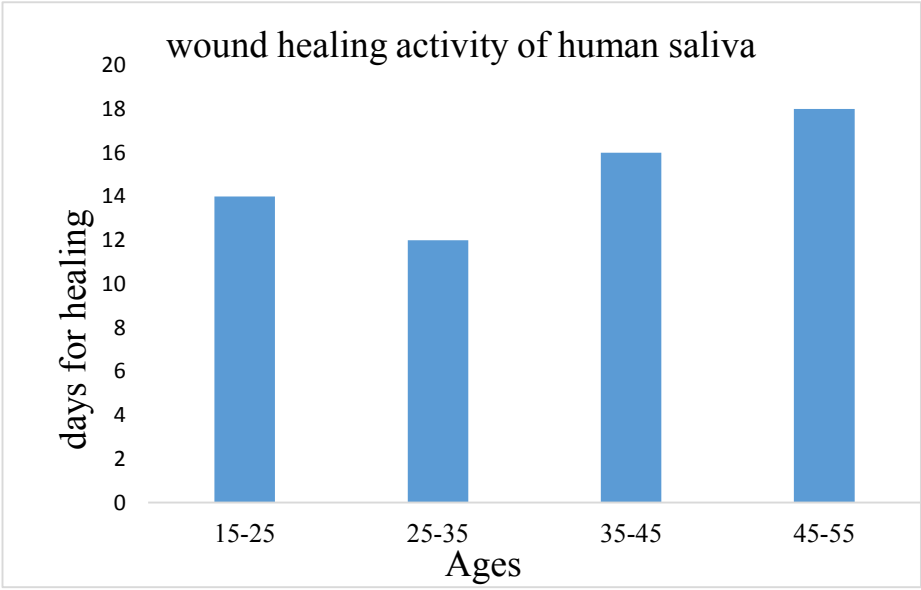

Figure 10: Wound healing activity of human saliva related to age groups.

The results related to antifungal and antibacterial ability showed that in order for human saliva to be effective against fungal and bacterial growths, and in turn create visible zones of inhibition, more than 120µL of each saliva sample needed to be used. The results are in agreement to previous researches with the created zones of inhibition from healthy saliva samples being optimum, 2 centimeters, in diameter belonging from the age range of 25-35. The zones of inhibition gradually decreased in the age ranges of 35-45 and 45-55 indicating that antifungal and antibacterial activity of healthy human saliva decreases with age. Furthermore, the results also showed that diabetic saliva samples didn't create any zones of inhibition. This supports the previous research that immunocompromised individuals have non-existent antimicrobial activity regardless of their age group and gender (Verma *et al*., 2013).

The antiviral results observed through HA tests, in accordance to previous research, showed that saliva requires a minimum of 1 hour incubation period to affect the viral enveloped proteins. However, the mechanism of salivary glycoprotein and how they interfere with virus structure and inhibit its activity is still a question in field of research (White *et al*., 2009). The results of this study also showed that there is no difference amongst the antiviral capacity of saliva amongst male and female individuals. However, antiviral activity, similar to antifungal and antibacterial activities, does depend on age. The reason for this might be due to saliva flow rate variations in children, youngsters and elderly people (Yannan *et al*., 2013).

Wound healing also showed a relation to age. The results showed that wound healing was faster in the group on which saliva was applied compared to the negative group (natural wound healing) as well as the positive group, which contained animals on which wound healing medication had been applied (Jia *et al*., 2012). The results of this study supports the previous research that wound healing was comparatively faster because salivary glands play an important role in the promotion of wound healing (Grossman *et al*., 2004). The results of this study also showed that gender of individuals from any age groups doesn't affect wound healing ability, with both healthy male and female samples showing similar results. This correlates to previous research that the gender of an individual doesn't have a significant effect when it comes to the natural abilities of human saliva (Verma *et al*., 2013).

Conclusion

Based on the results of this present study healthy human saliva possess significant ($p<0.05$) antimicrobial as well as wound healing properties. This innate ability of human saliva, mainly attributed to histatin protein, suggests that salivary proteins can be further used for medicinal purposes.

Acknowledgement

The authors of this study acknowledge the productive help provided by Dr. Maryam Javed (UVAS).

References

1. Alexander, D. and Chettle, N. (1977).Procedures for the haemagglutination and the haemagglutination inhibition tests for avian infectious bronchitis virus, *Avian Pathology*, 6(1):9-1.
2. Brand, H.S. and Veerman, E.C. (2013).Saliva and wound healing, *Chin J Dent Res*, 16(1):7-12.
3. Grossman, L., Li-At, B. and Lipa, B. (2004).Effect of rat salivary glands extracts on proliferation of cultured skin cells- a wound healing model, *Cell and Tissue Banking*, 5:205–212.
4. Gunther, L.S.A., Martins, H.P., Gimenes, F., Pimenta de Abreu, A.L., Consolaro, M.E. and Svidzinski, T.I. (2014).Prevalence of Candida albicans and non-albicans isolates from vaginal secretions: comparative evaluation of colonization, vaginal candidiasis and recurrent vaginal candidiasis in diabetic and non-diabetic women, *Sao Paulo Medical Journal*, 132(2).
5. Hitchner, S., Domermuth, C., Purchase, H. and Williams, J. (1980).Virus propagation in embryonating eggs, *Isolation and Identification of Avian Pathogens*, 312-314.
6. Jia, J., Sun, Y., Yang, H., Wang, X., Liu, L., Zong, L. and Hu, H. (2012).Effects of human saliva on wound healing, *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, 26(5): 563-566.
7. Khalili, I., Ghadimipour, R., Ameghi, A. and Sedigh-Eteghad, S. (2013).Optimization of incubation temperature in embryonated chicken eggs inoculated with H9N2 vaccinal subtype of avian influenza virus, *Veterinary Research Forum*, (4):145-148.
8. Lal, K., Pollock, J.J., Santarpia, R.P. III., Heller, H.M., William, H.K., Fuhrer, J. and Steigbigel, R.T. (1992).Pilot Study Comparing the Salivary Cationic Protein Concentrations in Healthy Adults and AIDS Patients: Correlation with Antifungal Activity, *Journal of Acquired Immune Deficiency Syndrome*, 5: 906-914.
9. Lu, Y. (2014).Potent inhibition of highly pathogenic influenza virus infection using a

peptidomimetic furin inhibitor alone or in combination with conventional antiviral agents, *Philipps-Universität Marburg*, 4: 1-10.
10. Pushko, P., Tumpey, T.M., Bu, F., Knell, J., Robinson, R. and Smith, G. (2005).Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice, *Vaccine*, 23(50): 5751-5759.
11. Sarwar, M., Muhammad, K., Rabbani, M., Younus, M., Sarwar, N., Ali, M. and Ahad, A. (2013).Prevalence of avian influenza viruses in live bird markets of Lahore, *Journal of Animal and Plant Sciences*, 23(2): 388-392.
12. Rimek, D., Fehse, B. and Gopel, P. (2008).Evaluation of Mueller-Hinton-agar as a simple medium for the germ tube production of Candida albicans and Candida dubliniensis, *Mycoses*, 51(3): 205-207.
13. Rodloff, A.C., Koch, D. and Schaumann, R. (2011).Epidemiology and antifungal resistance in invasive candidiasis, *European Journal of Medical Research*, 16: 187-195.
14. Verma, A., Selwal, K.K., Sarsar, V. and Bajwan, M. (2013).Antimicrobial effect of human saliva, *International Journal of Pharmacy and Life Sciences*, 3(3): 230-236. International Standard Serial Number (ISSN): 2249-6793.
15. White, M., Helmerhorst, E., Ligtenberg, A., Karpel, M., Tecle, T., Siqueira, W., Oppenheim, F. and Hartshorn, K. (2009). Multiple components contribute to ability of saliva to inhibit influenza viruses, *Oral Microbiology and Immunology*, 24(1): 18-24.
16. Whiting, D.R., Guariguata, L., Weil, C. and Shaw, J. (2011).IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030, *Diabetes Research and Clinical Practice*, 94(3): 311-321.
17. Yannan, Q., Yaogang, Z., Minzhi, Z., Liuyi, D., Hanjie, Y., Zhuo, C., Wentian, C., Xiurong, W., Hua, Z. and Zheng, Li. (2013).Age- and Sex-Associated Differences in the Glycopatterns of Human Salivary Glycoproteins and Their Roles against Influenza A Virus, *Journal of proteome research*, 12(6): 2742-2754.

How to cite this article
Haq *et al*., (2016). Antimicrobial and Wound Healing Properties of Human Saliva. *Int*. *J*. *Pharm*. *Life Sci*., 7(2):4911-4917.
Source of Support: Nil; Conflict of Interest: None declared

Received: 16.01.16; Revised: 16.02.16; Accepted: 18.02.16