

REGULAR ARTICLE

EXTRACTION & PURIFICATION OF HEMOLYSIN FROM TOXIN PRODUCING MICROORGANISMS AND ITS EFFECT ON FORENSICALLY IMPORTANT BLOOD AND SALIVA SAMPLES

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ABSTRACT

Examination of Blood and saliva samples in forensic investigation is now become crucial part of judiciary as it can be useful for the identification of an individual. Individual can be identified from such biological samples by blood grouping as well as DNA analysis. In the study isolation of microorganisms was done from different surfaces having highest possibility of presence of biological evidence, Gram staining and morphological characterization of microorganisms was done, the extraction of toxin from toxin producing microorganism which was determined by the zone of hemolysis on blood agar media was done by ammonium sulphate and sodium azide method. Purification was done and confirmation of toxin was done by ditch method, TLC & SDS-PAGE. Effect of toxin on blood was examined by Drabkin's method for determining hemolytic unit. Effect of hemolysin on saliva was examined by starch hydrolysis test as well as loss of an antimicrobial Property of saliva. As the toxin isolated is hemolysin toxin means it degrades the blood, but in the study we observed the effect of the hemolysin toxin on saliva as well. As mentioned in results blood samples loses its ABO blood grouping activity as it got affected by toxin. Saliva was also highly affected by toxin isolated, as the saliva loses the amylase activity and antimicrobial activity as well. The study is significant in cases where blood and saliva may found as crucial evidence. As RBCs and surface antigens got degraded by hemolysin blood as evidence may be undetectable by presumptive test and may give false results. Saliva samples also become untraceable during the presumptive test as loses its amylase activity.

Keywords: Hemolysin, toxin, blood, saliva

INTRODUCTION

Hemolysin toxins are mainly of four types, α -hemolysin, β -hemolysin, γ -hemolysin and δ -Hemolysin. α -Hemolysin is the most characterized virulence factor of *S. aureus*. Upon binding to the cell surface, α -hemolysin monomers assemble into a homo-heptamer, forming a pre-pore. The pre-pore subsequently transitions to a mature β -barrel trans-membrane pore (Bhakdi S and Tranum-Jensen J, 1991), thereby leading to the formation of a 14-Å diameter aqueous channel (Song L *et al.*, 1996). This pore allows the transport of molecules smaller than 2 kD (Menestrina, 1986). β -hemolysin does not form pores in the plasma cell membrane but instead is a neutral sphingomyelinase hydrolyzing sphingomyelin, which is a plasma membrane lipid. β -hemolysin's enzymatic activity is required for its hemolytic activity (Huseby *et al.*, 2007; Ira J and Johnston L J, 2008). The mechanism leading to cytotoxicity is still poorly understood. Sphingomyelin is enriched in lipid-ordered membrane microdomains with high content in cholesterol. Sphingomyelinase treatment of synthetic lipid bilayers leads to aggregation of cholesterol-rich microdomains (Ira J and Johnston L J, 2008), suggesting that cell death may result from the modification of host cell plasma membrane fluidity and destabilization of the bilayer structure. Alternatively, cell death might result from the formation of large ceramide-rich signaling platforms. This class of membrane-damaging peptides was identified over 60 years ago with the purification of δ -hemolysin (Wiseman, 1975). Three different mechanisms as reviewed by Verdon *et al.* (2009) have been proposed to explain its hemolytic activity. Briefly, δ -hemolysin could (i) bind to the cell surface and aggregate to form trans-membrane pores; (ii) bind to the cell surface and affect the membrane curvature, thereby destabilizing the plasma membrane; or (iii) at high concentration, act as a detergent to solubilize the membrane. γ -Hemolysin and, to a lesser extent, LukED are hemolytic to rabbit erythrocytes (Morinaga N, Kaihou Y, Noda M, 2003).

Interest in rapid and less invasive diagnostic tests has grown exponentially in the past decade, which has led to extensive research on saliva as a biological fluid for clinical diagnosis (Sun F and Reichenberger E J, 2014). Saliva has some advantages compared to blood and urine, two of the most used diagnostic fluids in laboratory setting. Saliva collection is easy and non-invasive requiring relatively simple instructions for collection and it possesses lower protein content, less complexity and varying composition than serum (Nunes *et al.*, 2011). The main functions of saliva include protection and integrity maintenance of oral mucosal health through lubrication, buffering action, antibacterial and antiviral activity, and food digestion (Humphrey S P, Williamson R T, 2001). Recent proteomic studies have identified and characterized more than 1000

salivary proteins and peptides. Most of these are commonly found in plasma and some are solely produced and secreted by salivary glands having no correlation with blood levels (Denny *et al.*, 2008). In Forensic examination blood and saliva may be found as important evidence. Blood and saliva found at crime scenes may be present on the victim, suspect, or any surfaces can be considered significant and treated as such when documenting, collecting, and preserving. Traditionally blood at crime scenes has been documented and collected for identification of suspect or victim through Blood grouping and DNA, at a crime laboratory. Saliva can be useful for identification of an individual as DNA analysis can be done as well as can determine the presence of any poisonous material or drugs. As hemolysin toxin producing microorganisms may be present in any habitat and are of diverse range may be present on crime scene. As blood and saliva are biological fluids, there are chances of contaminations of microorganisms. Study is done to determine the effect of hemolysin toxin on blood and saliva samples and the extent of effect. Once the effect of hemolysin on forensically important blood and saliva samples will be known then false results and wrong identification of an individual can be overcome.

MATERIALS AND METHODS

As we know when saliva and blood found as evidence most of the time found in dried form. As it was not possible to collect samples from real crime scene so as here in study the mimic of crime scene was created in laboratory. Blood and saliva samples are the major requirements for the study. Human blood sample and saliva samples were collected from the volunteers of the Department of Life Science, Gujarat University, Ahmedabad with consent. 20ml of ABO blood was collected by vein blood collection method by expert from four volunteer. Saliva was collected by maintaining aseptic condition and collected 5 ml from each volunteer. To create simulated crime scene collected 20 ml blood and saliva samples were poured on different surfaces, for each surface 2ml blood and 2ml saliva was used. Surfaces used here in study are metal, aluminium, glass & wood which may useful as weapon, apart from these ceramic, tile, stone, soil, paper and fabric materials surfaces used from which possible presence of evidence to be found. Then poured saliva and blood were allowed to dry on different surfaces as to provide same condition as real crime scene.

Blood agar media was prepared for isolation of hemolysin producing microorganisms by using 3 gms of HiMedia™ blood agar powder in 100 ml of sterile distilled water. For the isolation of microbes cotton swabs were dipped in sterile double distilled water and the wet cotton swab was rubbed on different surfaces by following the procedure as followed by forensic investigator from

crime scene and collected in sterile plastic bag and was allowed to dry for further preparation of culture suspension. For swab preparation sterile double distilled water used instead of normal saline as sterile water is also suitable for preservation of bacteria pathogenic to plants or humans (Liao C H and Shollenberger L M, 2003). A few instances of much more prolonged survival in water have been recorded but Ballantyne (1930) have been unable to find any record of prolonged survival of bacteria in NaCl solution.

Culture suspension was spread on blood agar plates for isolation. The plates were incubated at 37°C. After growth of desired colony on the blood agar plates; these different zone forming colonies were isolated on the blood agar slants (having same composition of blood agar plates) and on the basis of morphological study and nomenclature of individual colony and an activity was determined on the basis of their characteristics of giving hemolytic zone and comparing the diameter of the zone giving colony and the diameter of zone that surrounds a bacterial colony growing on blood agar, which represents partial or complete discoloration or breakdown of Hb. Sterilized Glucose broth flasks were inoculated with selected highly hemolytic five colonies out of fifty five colonies named as: C-SN-1 (γ -hemolytic), G-SN-1 (α -hemolytic), P-SN-1 (β -hemolytic), CON-2 (β -hemolytic), and CON-3 (α -hemolytic). Inoculation had been done by adding a loopful of each colony to its respected flasks. All five flasks were incubated at 37°C for 48-72 hours in orbital shaker at 120 rpm.

25ml of inoculated Glucose broths from each flask were collected in REMI centrifuged tubes. Each tube was contained 8ml of inoculated Glucose broths. 0.02% of Sodium azide was added were Centrifuged at 10,000 rpm and at 4°C for 20 minutes. 1/20 volume of TrisHCl buffer was added to supernatant. 60% of solid ammonium sulphate was added to the treated supernatants and incubated at 4°C for overnight. Next day, centrifuged at 10,000 rpm for 30 minutes. Precipitates were treated with 25 ml of 50 mM TrisHCl buffer (pH: 8.0), 1 ml of 1mM EDTA and 1 ml of 3mM sodium azide. Dialyzed all the treated precipitates against the dialyzing buffer and centrifuged at 10,000rpm for 10 minutes. Supernatant was collected which contained crude hemolysin. Folin Lowry estimation was done at 750nm to confirm the presence of protein (Lowry et al., 1951).



Figure 1 Precipitations of toxin in centrifugal tubes after treated with ammonium sulfate

For Ammonium sulphate precipitation 6 ml of broth cultures were collected from each five flasks in five centrifuge tubes. 3gm of Solid Ammonium sulphate was added in each tube and centrifuged at 2500-3000rpm for 5 minutes at room temperature. Then, precipitates were collected as shown in fig.1 and treated with 10 ml Tris HCL buffer (pH-8.0). Then, precipitates were incubated at 4°C for 24 hours.

For Blood agar ditch method wells were prepared on blood agar plates having 6mm diameter. Then serial dilutions for P-SN-1, C-SN-1, and CON-2, CON-3, G-SN-1 were prepared such as, 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} were added. The plates were incubated at 37°C for 48 hours and examined for zone of hemolysin toxin.

TLC was performed using: 20ml of methanol:water solvent system (70:30) And Butanol:acetic acid:water solvent system (8:2:2). Ninhydrin solution was used as spraying reagent.

SDS PAGE was performed by preparing 12% separating gel and 5% stacking gel. 20 μ l of the samples was loaded in sample well. Electrophoresis was done at 200V & 110A. After sufficient run gel was stained overnight and destained next day and observed for protein bands.

Hemolysis assay had been done on the bases of checking hemoglobin concentration released from the lysed RBCs. Released hemoglobin can be measured by Drabkin's method (Drabkin 1949). Spectroscopic analysis was done at 540nm and Hemolytic unit was measured.

To determine the effect of toxin on saliva Starch hydrolysis test was performed. Saliva was mixed with different concentrations of toxins as shown in table 1. For standard 300 μ l saliva was taken into the microfuge tubes. 1000 μ l 0.5% starch solution was added to the microfuge tubes. 0 min reading was taken as control by testing it with iodine solution in pitted tiles. Color change was observed at every five minutes interval.

Antibiotic activity loss of saliva was measured by preparing 5 N-agar plates using HiMedia N-agar powder Suspension was made from the PA-14 colony on which saliva was giving antimicrobial activity and was spread onto each of the 5 plates. Four wells were ditched on each of the plates and werelabeled as 1,2,3,4. Different concentration of the mixture containing saliva and toxin which were incubated at different time were added to the wells as follows:

Table 1 Concentration of Saliva and toxin mixture with respect to time

Well 1 (Toxin/saliva)	Well 2 (Toxin/saliva)	Well 3 (Toxin/saliva)	Well 4 (Toxin/saliva)	Incubation period(mins)
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	10
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	40
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	60
1/1 ml	0.5/1 ml	0.5/1.5 ml	0.2/1.5 ml	75

RESULTS

Sodium azide method has been performed for the extraction of pure hemolysin toxin from the hemolytic microorganisms. Toxin was obtained in a form of precipitation.

TLC plates which gives positive amino acid bands under the U.V illuminations which have different Rf values from each other which separates hemolysin toxin such as P-SN-1 and CON-2 β -hemolysin toxin on the basis of its relative affinity towards stationary and mobile phase.

Table 2TLC results for the toxins show following results:

Distance travelled by Solvent	Culture Extract	Rf Value (cm)
7.0 cm	P-SN-1	3.5
	CON-2	3.6
	CON-3	3.7

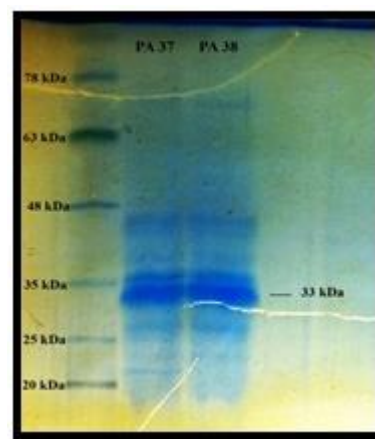


Figure 2 SDS-PAGE analysis for the extracted toxins. Lane: 1 Protein marker, Lane:2 Hemolysin toxin with molecular weight 33 kDa.

As we can see in the figure 2, the bands of the toxin that are toxin 3 and toxin 5 obtained from the different strains in the wells of the SDS-PAGE gel, which are clearly visible after destaining process. And comparing the sample bands with protein ladder gives the bands at 33 kDa size. These bands confirm the presence of the protein that is our crude toxin protein as it shows some light bands above the original band. SDS PAGE analysis confirms the toxin is hemolysin toxin.

As we can see table: 3 Hemoglobin concentrations is increasing with respect to time interval of 1 hr. So it clearly confirms the hemolytic activity of crude protein collected and gives hemolytic unit 1.

Table 3 Hemoglobin concentrations after reacting with toxin

Tubes	Absorbance at 540nm	Hb. Conc.(mg)
Blank	0.000	00.00
Standard	0.009	11.19
C-SN-1	0.042	24.86
G-SN-1	0.025	31.08
P-SN-1	0.020	52.22
CON-2	0.043	53.46
CON-3	0.034	42.27

Starch hydrolysis of saliva

The salivary amylase enzyme present in saliva digests the starch. On adding starch solution to the saliva as control, it gives color change at the interval of every five minutes as shown in in Fig. 3(A). But on adding starch solution to the mixture of saliva and toxin as sample, the toxin starts reacting with saliva, it degrades the salivary amylase and therefore when it was reacted with iodine solution it gives violet color at the interval of every five minutes. No color change was observed with respect to time interval of five minutes up to 5 hours as shown in Fig. 3(B). Which clearly indicates that Salivary amylase lose its activity after reacting with toxin.

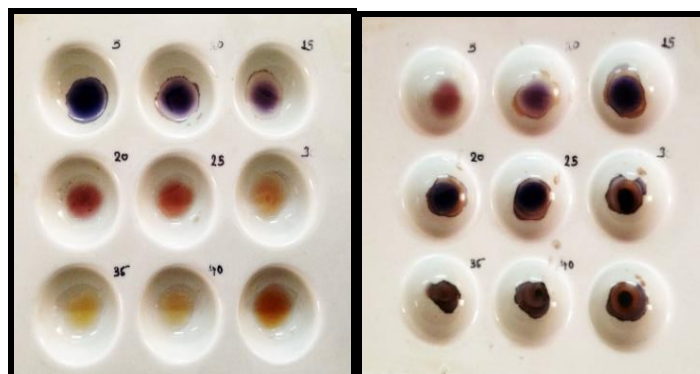


Figure 3 Salivary amylase activity of Control untreated saliva with starch (A) and Sample saliva mixed with toxin and treated with starch solution (B)

Loss of antibiotic activity of saliva

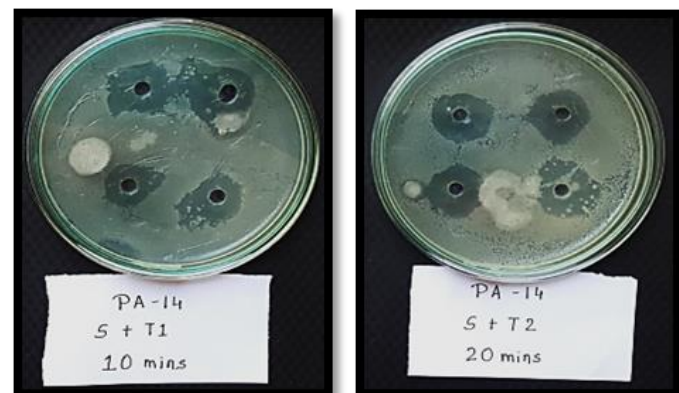


Figure 4 Loss of antibiotic activity of saliva reacting with toxin checked on N- agr media plated with bacterial strain PA14 isolated from biological samples.

As shown in figure 4, when saliva was mixed with toxin and reacted with strain PA-14 on which saliva was giving antimicrobial effect and giving clear zone, after the incubation of 10 minutes, the lysozyme enzyme present in the saliva do not get digested at all by the activity of toxin, therefore strain PA-14 shows the antimicrobial activity against the toxin after 10, 20 and 40mins of incubation. After 60mins of incubation saliva started losing its antimicrobial activity on same strain PA-14 as uneven zones can be seen. , after the incubation of 75 minutes, the lysozyme enzyme present in saliva get digested by the activity of toxin and therefore it does not act on the strain PA-14.

DISCUSSION

As mentioned above **Bhakdi S and Tranum-Jensen J (1991)** stated that α -hemolysin is pore forming toxin secreted by bacteria which degrade the blood cells by binding on cell surface. **Huseby et al. (2007)** and **Ira J and Johnston L J (2008)** have reported that β - hemolysin is not pore forming but degrades blood cells. None of the study have been reported the effect of hemolysin toxins on saliva samples. The study is unique in a way that it specifies the effect of hemolysin toxin for forensically important blood and saliva samples and how it may interfere during forensic investigation and identification procedure of biological samples.

CONCLUSION

Forensic investigation of blood and saliva has now become a significant part of any crime investigation. Blood and Saliva samples may be very useful evidence to identify an individual. Most of the time these samples found at the crime scene in very small quantity through its potency is very high. If such evidence may be affected by any circumstances then it is difficult to identify individual or person involved in crime. As the blood and saliva are biological fluid and there are chances that it may get affected by any external factors such as environmental conditions or any microbial contamination. The present study was carried out on how toxin producing microorganism can affect the blood and saliva samples. As the mimic of the real crime scene was created, toxin-producing microorganisms

were isolated from surfaces from which saliva may be found from the real crime scene. As the toxin isolated is hemolysin toxin means it degrades the blood, but in the study, we observed the effect of the hemolysin toxin on saliva as well. As mentioned in results blood sample lose its ABO blood grouping activity as it got affected by the toxin. Saliva was also highly affected by toxin isolated, as the saliva loses the amylase activity and antimicrobial activity as well. The study is significant in cases where blood and saliva may found as crucial evidence. As the stains found on the crime scene, looked like saliva, a forensic scientist performs primary identification by amylase activity of starch digestion to recognize that the stain is of saliva. But as per the study, if saliva is affected by the toxin it cannot be recognized by the primary identification method.

ABBREVIATIONS: Not Applicable

Competing interests: The authors declare that they have no competing interests.

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