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RESEARCH ARTICLE

MURINE SKIN DAMAGE CAUSED BY *STAPHYLOCOCCUS AUREUS* DNA AND *PROPIONIBACTERIUM ACNES* DNA

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ABSTRACT

One hundred and twenty skin swabs specimens were collected from acne patients. Thirty eight (45.2%) specimens were identified as *Staphylococcus aureus* and twenty seven (32.1%) specimens were identified as *Propionibacterium acnes*. Most of *S. aureus* isolates were susceptible to methicillin and all isolates were susceptible to vancomycin. All *P. acnes* isolates were susceptible to all antibiotics used in this study. DNA was extracted and purified from the most sensitive isolates (*P. acnes* P7 &*S. aureus*S24). The effects of *S. aureus*w hole cells were higher than the effects of *P. acnes* whole cells, while both isolated DNA were able to produce more severe inflammatory changes than the whole bacterial cells. Furthermore, increasing the DNA concentration caused an increase in the intensity of skin inflammation. Moreover, the effects ofhigh GC ratio *P. acnes* DNA developed the highest inflammatory effect than the low GC ratio *S. aureus*DNA.

INTRODUCTION

Staphylococcus aureus is a Gram -positive cocci that occur singly, in pairs, tetrads, short chains, non-motile, pyogenic and regarded as serious opportunistic pathogen responsible for several infections. Most species are facultative anaerobes with low G+C content of DNA ranging 30-39 mol % (Götz *et al.*, 2006).

Propionibacterium acnes is pleomorphic rod, Gram positive, nonmoitle, non-spore former, produces large amount of propionic acid as a major end product of metabolism, typically grows under anaerobe conditions and belongs to the 'high GC' group G+C content of DNA ranging 53-67 %. *P. acnes* is an opportunistic pathogen, causing a range of infections as well as being associated with a number of inflammatory conditions(Holland *et al.*, 2010).

According to the evidence that bacterial DNA and oligodeoxynucleotides elicit an inflammatory response in various organs of the body due to the abundance of unmethylated cytosine phosphate guanosine sequences (CpG motifs) have shown to exert potent immunostimulatory properties (Dalpke *et al.*, 2006; Klinman et al.,1997).

Skin normally harbors bacteria, since these bacterial cells are exposed to degradation and autolysis a matter lead to release its contents and eventually increase skin infection (Mölne *et al.*, 2003).

Upon such fact it is noteworthy to compare between the skin damage resulting from bacterial DNA with low and high GC ratio extracted from *S. aureus* and *P. acnes*, respectively.

MATERIALS AND METHODS

Specimen's collection

One hundred and twenty skin swabs specimens were collected from patients visiting Al-Kindey teaching hospital and MedinatAltib (Medicalcity) teaching hospitals.

Patients (55 males and 65 females), ranging from 13 to 35 year (mean age 23.5 year), with inflammatory acne vulgaris were selected for the study. They had not received antibiotics in the previous 3 weeks, nor havebeen treated for acne before enrolment, and gave informed consent fortaking clinical specimens.

Isolation and identification

After cleaning acne lesions with 70% ethanol, the contents weresqueezed out and pus was collected by aid of two cotton swabs in order toisolate *S.aureus* and *P. acnes*(Basal *et al.*, 2004). Regarding *S.aureus* isolation, First swab was immersed in a tube containing nutrient broth which was usedas transport medium, subsequently, it was incubated for 18-24 hours at 37 ˚C; afterincubation loopfull inoculum was streaked on mannitol salt agar thenincubated aerobically at 37°C for 18-24 hours; the yellow mannitol fermentor colonies were selected then a single colony was inoculated on Blood agar for the activation and detection of bacterial ability to lyses red blood cells (β-hemolysis).Identification was achieved depending on the morphological features on culture medium and biochemical tests according to Forbes *et al.*(2007). API-Staph system was employed to confirm the identification

Concerning *P. acnes* isolation, the second swab was immersed in a tube containing thioglycolate broth, afterward, 0.1 ml inoculum was transferred into another thioglycolate tube. Thereafter, it was incubated for 2-4 days at 37°C. Then

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aloopful inoculum was streaked on *P. acnes* isolating medium and incubated anaerobically using an anaerobic gas-generating system at 37°C for 4-7days. Identification was accomplished by cultural and microscopical properties as well as biochemical tests. Api 20A system was used to confirm the identification.

Antimicrobial susceptibility

Antimicrobial susceptibility test to amoxicillin, ampicillin, ampiclox, cefotaxime, methicillin, oxacillin, penicillin and vancomycin was performed in accordance to Bauer-kir by disc diffusion method using Mueller Hinton medium (Bauer *et al.,* 1966), in case of *P. acnes* the medium supplemented with 5% human blood and 0.1 % of tween 80 to ensure purity and good growth, the inocula were prepared by suspending colonies from 48 hrs culture plates (Shames *et al.,* 2006).

Extracting and purifying of genomic DNA

Genomic DNA from the most sensitive strains was extracted and purified using Wizard® genomic DNA purification kit (Promega, Madison, WI, USA), following manufacturer protocol. The isolated DNA samples were electrophorized (65 V for 1 hr). DNA bands were examined under the UV light (300 nm) transmitted through the gel(Tang and Stratton, 2006).

Histopathological study

Bacterial suspension preparation

Few single pure colonies were taken to a sterile normal saline tube with turbidity adjusted to approximately 1.5×10^8 CFU/ml by comparison to McFarland turbidity standard (tube no. 0.5).

Animals

The guidelines established by the "Guide for the Care and Use of Laboratory Animals" were followed. Eight week old female white mice BALB/C each weight 20 to 25 g were used. They were housed in plastic cages under standard conditions of temperature, light, feed and water. All animals were randomly assigned to groups; A through L (as triplicates).

Injection protocol

Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight)(Kogahara *et al.,* 2009).Thereafter, transcutaneous 10 mm in length wounds were performed on the backs of the mice, 2-3 drops of inoculum material mentioned in table 1, was applied to the wound.

Histological technique

Mice were sacrificed after three days. Injured skin specimens were removed, fixed with 10% formalin for 24 hours at room temperature (20-25ºC), then embedded in paraffin according to standard histologial methods, after fixation tissue was held in 70% alcohol until proceeded and embedded in paraffin using standard techniques. The sections were examined by light microscope under magnification power 10X and 40 X (Humason, 1972).

Table 1 Animal groups included in the present study

Animal group	Description	Inoculum
A	Control group	Sterile saline solution
B	*S. aureus* treated group	live cells of *S. aureus*
C	*P. acnes* treated group	live cells of *P. acnes*
D	DNA control group	sterile TE buffer
E	*S. aureus* DNA group	*S. aureus* DNA (10 µg/µl)
F	*S. aureus* DNA group	*S. aureus* DNA (20 µg/µl)
G	*S. aureus* DNA group	*S. aureus* DNA (30 µg/µl)
H	*S. aureus* DNA group	*S. aureus* DNA (40 µg/µl)
I	*P. acnes* DNA group	*P. acnes* DNA (10 µg/µl)
J	*P. acnes* DNA group	*P. acnes* DNA (20 µg/µl)
K	*P. acnes* DNA group	*P. acnes* DNA (30 µg/µl)
L	*P. acnes* DNA group	*P. acnes* DNA (40 µg/µl)

RESULTS AND DISCUSSION

Thirty eight (45.2%) specimens were identified as *S. aureus*, whereas; twenty seven(32.1%) specimens were identified as *P. acnes*.

Our findings demonstrate that acne is a persistent problem for peopleof all ages especially in teenage years, but clearly women seem to be affected by this medical condition more than men. Females often have a high prevalence to hormone changes during puberty (during pregnancy, within the menstrual cycle and menopause),furthermore certain medicine like birth control pills, make up an oily pomade that contain large amount of oil can be related to the acne development(James, 2005).Although early reports and several individual observations indicate that dietary factors, especially chocolate and meat have an influence other exacerbation of acne. The association could not be proven. However, there is a significant linear dose-dependent relationship between both acne prevalence and severity and the number of cigarettes smoked daily (Schaefer *et al.,* 2001; Jappe, 2003).Although several studies pointed to *Propionibacterium* as the most common agent acne lesions (Thiboutot, 2000; Qa'dan*et al.,* 2005; Prabhu *et al.,* 2010), the present study, obviously, indicates that *S. aureus* has significantly higher isolation percentage in comparison to *P. acne*. These differences could be attributed to the variation in geographical and isolation period which may affect the bacteria involved in acne vulgaris(Ashkenazi *et al.,* 2003).However, *Staphylococcus* is described as one of the main population in the microflora of the follicle; both have been implicated as responsible for stimulation of monocytes and cells PMNs, which leads to production of cytokines and other immunological factors that could explain some of the chronic inflammation acne(Rodriguez-Cavallini and Vargas-Dengo, 2004). Hassanzadeh *et al.* (2008) reported that the same result was noticed;*S. aureus* formed 41% of acne causes; while *P. acne* was responsible for about 33% of them. Furthermore, the other study findings are nearly in agreement with the result accomplished by Toyoda and Morohashi(1998)as it revealed that *S. aureus* was considered as a major infectious agent of acne beside *S. epidermidis*, *P. acne* and *Micrococcus* spp. Alsoour study agrees with the study of Rodriguez-Cavallini and Vargas-Dengo(2004) who stated that *Staphylococcus*, as a single agent or combined with other agents, was isolated in 93% of the cases, *Propionibacterium* in 59 %, although only in 6.5 % of the cases as a single agent.

Antibiotic susceptibility

From the result, we observed various susceptibilities to different antibiotics among isolates. The results are summarized in figure 1 and show that two (0.052%) *S. aureus* isolates were resistant to methicillin, all isolates (100%) were susceptible to vancomycin. Twenty three isolates were resistant to cefotaxime (60.5%), 15 (39.4%) isolates to ampicillin, 29 (76.3%) to penicillin, 8 (21%) to oxacillin, 14 (36.8%) to amoxicillin and 19 (50%) isolates to ampiclox.

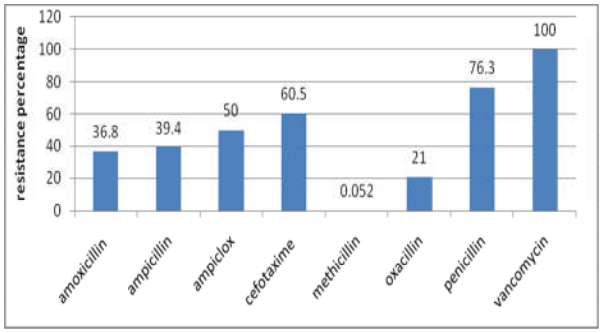


Figure1 Antibiotic susceptibility of *S. aureus* isolated from acne

In the present study, all methicillin resistance*S. aureus* isolates were susceptible to vancomycin this result approach obtained by Steinkraus (2007) and Kaleem *et al.(*2010) who observed similar results.

Regarding *P. acnes*, all isolates were susceptible to all antibiotics used in this study. Gübelin et al.(2006)demonstrated that *P. acnes*is naturally susceptible to various antimicrobial classes including, β-lactams, macrolide, lincosamide, quinolone, tetracycline's and aminoglycoside. Jappe(2003) reported that *P. acnes* is sensitive to awide range of antimicrobials in vitro, but only few antibiotics can reduce the bacterial colonization of the deeper parts of the follicle.

The reason behind using beta lactam antibiotics in the present study is that cytolysis of bacterial cell caused by beta lactam antibiotics will expose DNA to nearby tissue eventually making it more likely to cause skin inflammations.Deasy(2009) stated that due to the popularity of beta-lactam drugs overuse, prescription of drugs, over dosing, *S. aureus* isolates have been able to develop counter-measures to traditional drug therapies and created development problem.

DNA isolation and purification

Concentration and purity of the DNA samples was determined spectroscopically. The optical density ratio of OD260/280 was within the acceptable range; 1.7 - 2.0 i.e. the isolated DNA was pure (Glasel, 1997). In order to confirm this purity both isolated bacterial DNA were submitted for electrophoresis and the result revealed a development of a single band seen by UV transillumination.

In vivo study

Affected skin regions developed familiar external inflammation manifestations. This cardinal signs are redness and swelling. Redness and heat are due to increased blood flow at body core temperature to the inflamed site; swelling is caused by accumulation of fluid (Ruth, 2009).

Histopathological study

The histological sections of control mice skin show that there was a normal structure appearance of epidermis, dermis and hypodermis as shown in figure 2.

Cells *Staphylococcus aureus*whole cells

The histological sections of skin that has been wounded and challenged with 1.5×10^8cfu/ml of *S. aureus* S24 show expansion of dermis, vasodilatation and a mild infiltration of mononuclear cells as shown in figures 3.

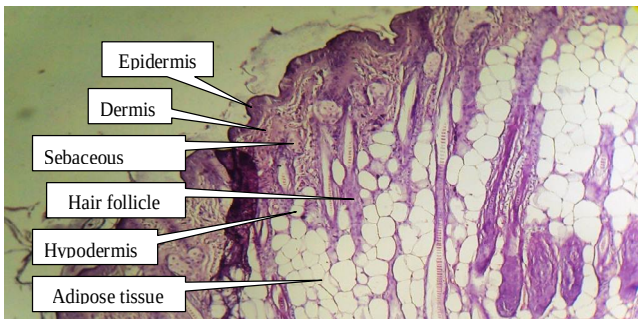


Figure 2 Cross section in normal mouse skin. 10X, H &E.

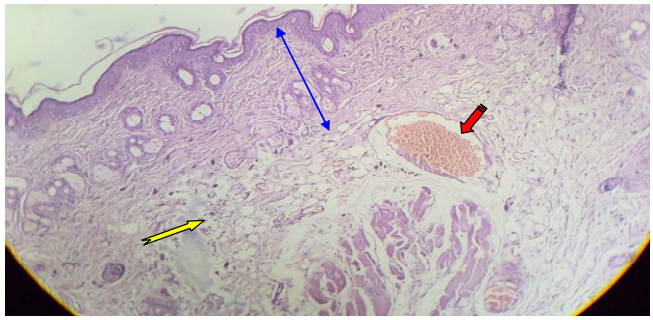


Figure 3 Cross section in mouse skin challenged with 1.5×10^8 cfu/ml of *S. aureus* S24 shows mild infiltration of inflammatory cells ( ), expansion of dermis ( ) and vasodilatation ( ). 100X, H &E.

***Propionibacterium acnes* whole cells**

The histological sections of skin which was infected with 2-3 drops of 1.5×10^8 cfu/ml viable organisms of *P. acnes* P7show that there was mild mononuclear cells infiltration and vasodilatation as shown in figure 4.

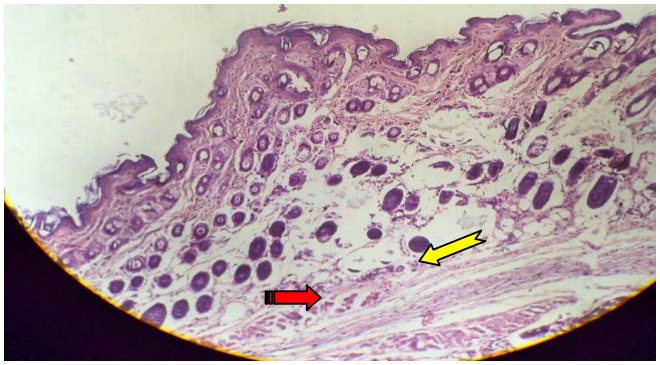


Figure 4 Cross section in mouse skin challenged with 1.5×10^8 cfu/ml of *P. acnes* P7 shows mild infiltration ( ) and vasodilatation ( ). 100X, H &E.

***Staphylococcus aureus* DNA**

The *S. aureus* DNA at concentration of 10 µg/µl didn't induce any of inflammatory changes. While Hemorrhage, infiltration of inflammatory cells, expansion of dermis and hyperplasia of epidermis were the effects of 20 µg/µl of *S. aureus* DNA as shown in figure 5a. In comparison with control group, 30 µg/µl caused infiltration of inflammatory cells and expansion of dermis as it is illustrated in figure 5b.Forty µg/µl were able to cause more severe changes in the experimentally wounds represented by haemorrhage, tissue necrosis, infiltration of inflammatory cells (figure 5c), Odema and vasodilatation (figure 5d).

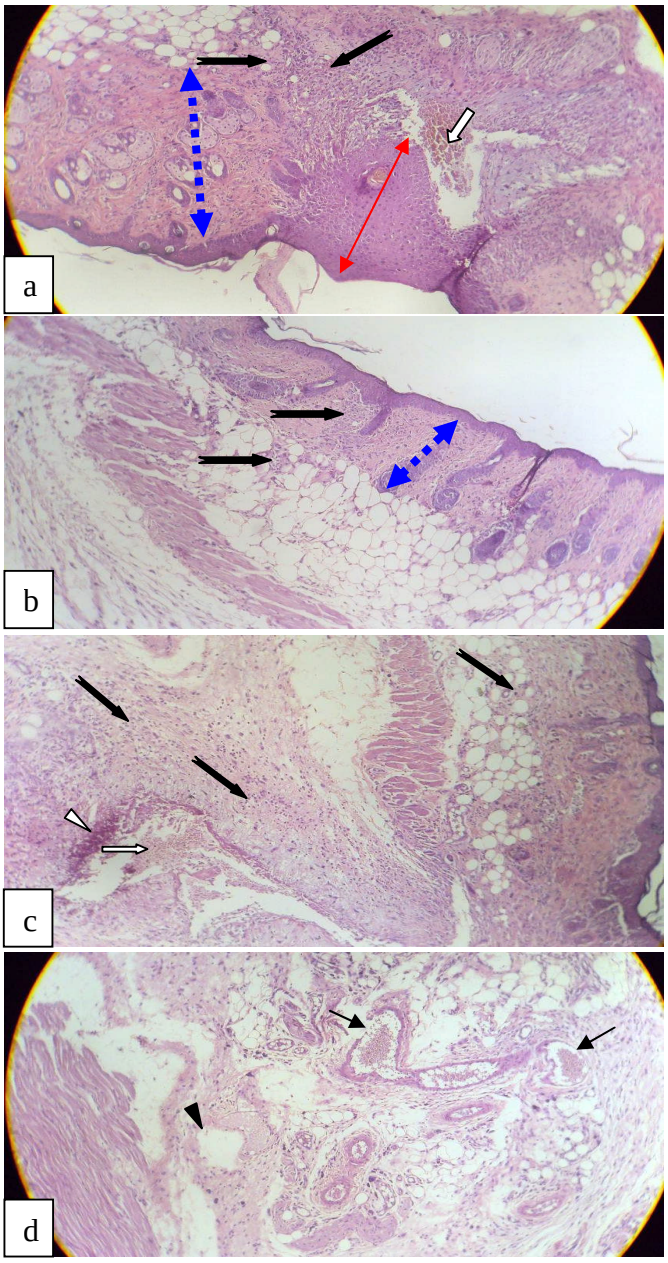
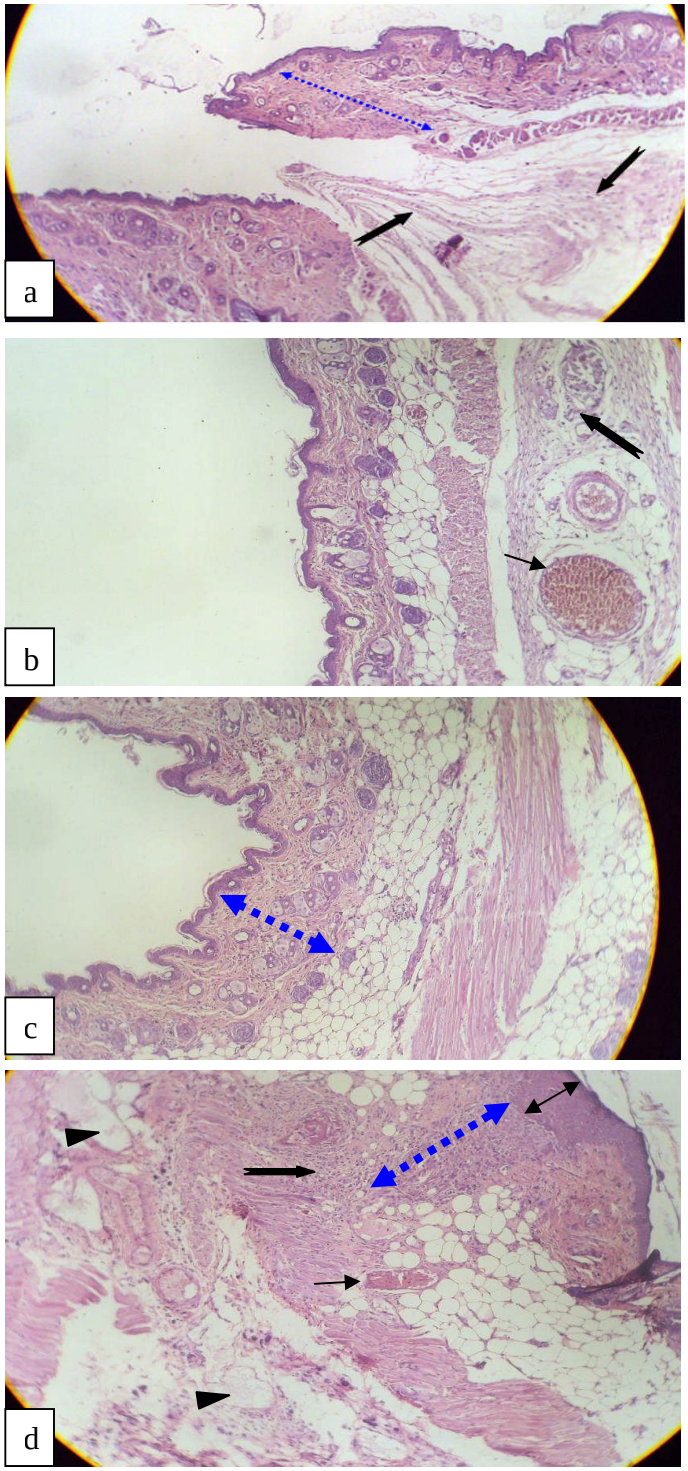


Figure 5 Cross section in mouse skin challenged with 20 µg/µl (a) 30 µg/µl (b) and 40 µg/µl (c,d) of *S. aureus* DNAshows Hemorrhage (white arrow) Infiltration of inflammatory cells (black arrows), Expansion of dermis (dotted arrow) hyperplasia of epidermis (double head solid arrow), tissue necrosis (white triangle)Odema (black triangle) and vasodilatation (thin black arrow) 10X, H &E.

***Propionibacterium acnes* DNA**

Mild Infiltration as well as expansion of demis were the effects of 10 µg/µl of *P. acnes* P7 DNA as shown in figure 6a. While 20 µg/µl of *P. acnes* P7DNA caused Infiltration of inflammatory cells, vasodilatation(figure 6b)and expansion of dermis as shown in figure 6c.Skin inflammation response characterized by expansion of epidermis and dermis, huge infiltration of inflammatory cells, vasodilatation and odema were the effects of *P. acnes* P7 DNA in concentration 30 µg/µl as shown infigure 6d.Forty µg/µlof *P. acnes* DNA P7 triggered Skin inflammation represent by Expansion of epidermis and dermis, infiltration of inflammatory cells (figure 6e) and Vasodilatation and tissue necrosis (figure 6f).



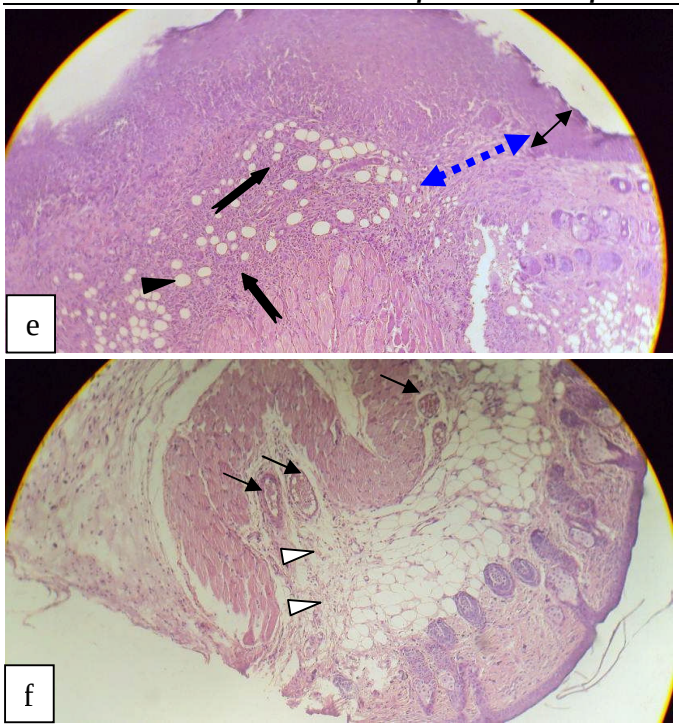


Figure 6 Cross section in mouse skin challenged with10 µg/µl (a)20 µg/µl (b,c) 30 µg/µl (d) and 40 µg/µl (e,f) of *S. aureus* DNAshows Hemorrhage (white arrow) Infiltration of inflammatory cells (black arrows), Expansion of dermis (dotted arrow) hyperplasia of epidermis (double head solid arrow), tissue necrosis (white triangle)Odema (black triangle) and vasodilatation (thin black arrow) 10X, H &E.

When host encounters an injurious agent (microbe or other antigens) the inflammatory process includes recognition of foreign material, vascular dilatation and leakage of fluid and cells into the tissues, attraction of immune cells to the site of injury, eliminate of the foreign organisms or dead material by the immune cells and release of chemical signals to initiate the repair process (Johnson *et al.,* 2002).

Acute inflammation has two major components, vascular changes when alterations in vessel caliber occur resulting in increased blood flow (vasodilation) with structural changes that permit plasma proteins to leave the circulation (increased vascular permeability) and move into the extracellular tissue, these changes are reflected microscopically by numerous dilated small vessels packed with erythrocytes, Hemorrhage is the abnormal bleeding of the blood vessels (Kumar *et al.,* 2007).

In the early phase of inflammation, vasodilation and increased volume of blood flow lead to a rise in intravascular hydrostatic pressure, resulting in movement of fluid from capillaries into the tissues. Fluid accumulation in extravascular spaces is called odema(Goldsby *et al.,* 2003).

The increase in the number of peripheral blood neutrophils is often an indication of acute infection and it is inflammation response (Doan *et al.,* 2008). An unfortunate side effect of leukocytes may be damage to normal host tissues (Reviglio *et al.,* 2009).

The expansion in the epidermis and dermis due to cells infiltration and fluid accumulation in or around the site of injury, while hyperplasia is defined as an increase in tissue cell mass because of an increased number of cells, it following any kind of injury (Wilcock, 2008).

Skin wounding triggers a cascade of inflammatory events that leads to rapid recruitment of phagocytes from the circulation to the site of injury. For instance, prolonged release of proteolytic enzymes, oxygen free radicals, and proinflammatory cytokines owing to excessive leukocyte infiltration (Pierce, 2001;Dovi *et al.,* 2003).

Necrosis is the unprogrammed premature localized death of cells and living tissue. It is caused by external factors, such as infection or trauma (Goldsby *et al.,* 2003).

Histological analysis showed that the effects of *S. aureus* whole cells were more intense than *P. acnes* whole cells that may belong to *S. aureus* processing virulence factors other than DNA which all may contribute in pathogenesis of bacteria (Langley et al., 2003; Mertz and Kinney, 2007).

Moreover, both isolated DNA had more influential than the effect bacterial whole cells, from our result we noted that increasing in concentrations exceed the intensity of skin inflammation, 40 µg/µl were the most influential concentrations with maximun effects.

Histologic examination indicated that the effect of *P. acnes* DNA which have high GC ratio was most influential effects than the impact *S. aureus*DNA with low GC ratio that may be due to the relative abundance of unmethylated cytosine-guanosine(CG)dinucleotides which is attributable to cause the activation of TLR9 and the immunostimulatory effect (Dalpke *et al.,* 2006;Krieg *et al.,* 1995; Neujahr et al.,2001).

In conclusion, *S. aureus*has significant higher isolation percentage in comparison to *P. acnes* from acne.Both isolated bacterial DNA possesses immunostimulatory potential and able to trigger skin inflammation more than bacterial whole cells. DNA of *S. aureus* and *P. acnes* caused skin damage in a dose dependent manner. *Propionibacterium acnes* DNA (high GC ratio) caused more damage in murine skin than *S. aureus* DNA (low GC ratio).

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Conflicts of Interest: Authors declare there are no conflicts of interest.

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