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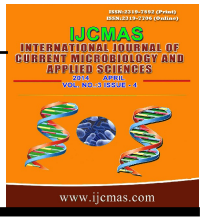

Original Research Article

Detection of slime material in *Staphylococcus aureus* bacteria from ovine mastitis by transmission electron microscope and Congo red agar method

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Keywords

Staphylococcus aureus, slime material, Electron microscope, Congo red stain.

A B S T R A C T

This study was conducted on 17 ovine mastitis *Staphylococcus aureus* isolates to evaluate Slime production by two methods (culturing Congo red agar and quantitative assay using transmission electron microscope). The slime Production of *S.aureus* was recorded in 2 (11.76%) isolates, which produced characteristic black colonies of dry crystalline consistency in Congo red agar, also the result reported 10 (58.8) isolate have red small colonies which considered as (negative result) the other result show 3(17.6) isolate have black smooth colonies and 2 (11.67) isolate have red colonies with dry crystalline consistency which considered as indeterminate result. Examination under electron microscopy the two slime producing *S. aureus* bacterial cells in Congo red agar showed a condensed extracellular and thick layer surrounding the cell wall was observed in >80% of bacterial cells of the Slime producing (SP) colonies.

Introduction

The ability of *Staphylococcus aureus* bacteria to persist in the mammary tissue as a biofilm is one of the possible sources of chronic or persistent infections (Vaseduvan *et al*., 2003). A biofilm is defined as a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donland and Costerton, 2002).

The production of slime material has been produced by in vivo colonization and has been detected in vitro by determining the type of colony morphology produced in Congo red agar (CRA). Bacteria in the biofilm exhibit increased resistance to components of the host's immune system and antimicrobial agents, as compared to their planktonic counterparts; Biofilm-forming ability has been increasingly recognized as an important virulence factor in staphylococci (Oliveira *et al*., 2006). Some investigators reported that slime producing *Staph. aureus* strains had

a higher colonization capacity than its non-slime-producing variants did. Therefore, *Staph. aureus* slime may play a role in the establishment of infection (Ammendolia *et al*., 1999).This specific adhesion pattern is mediated by bacterial cell wall structures containing adhesins, which is a genetically determined feature of bacterial species. Adherence of *Staph. aureus* bacteria to the host cells is mediated by specific cell-surface proteins such as fibronectin, fibrinogen and collagen. The slime is viscous extracapsular layer, weakly immunogenic and of labile structure which is lost or partially lost on in vitro subcultures (Aguilar *et al*., 2001). Slime production was detected also in human and ruminant mastitis strains of *Staphylococcus.* Slime-producing bacteria are considered to have increased ability of colonizing host tissue and better protection from opsonization and phagocytosis (Arslan and Özkardes, 2007). The objectives of this study were to detect slime material in *Staphylococcus aureus* bacteria isolated from mastitic ewes by Electron microscope and Congo red stain.

Materials and Methods

Bacterial isolates – Seventeen *Staph. aureus* isolates, provided by different field in Baghdad area were obtained from culture of ovine milk specimens; Strains were isolated and identified by their colony morphology, hemolysin and coagulase production, Isolates were characterized at the species level by the API Staph system (Biomerieux, France) according to the instructions of the manufacturers. The organism was stored in Trypticase soy broth (TSB), to which 15% sterile glycerol was added, at – 20°C.the method of isolation discussed by (Hammadi and Yousif,2013).

Detection of slime producing bacteria

Slime production of all isolates was evaluated by two different methods, culturing Congo red agar and quantitative assay using transmission electron microscope.

Detection of slime production by Congo red agar methods

Slime production of *Staph. aureus* isolates was evaluated by Congo red agar method (CRA), according to the protocol of Freeman *et al*., (1989). This test was prepared by dissolving the following substance in 1liter of distal water (brain heart infusion broth, 37 g; sucrose 50 g; agar10 g; Congo red agar, 0.8 g). The medium was autoclaved at 121 C° at 15 bound for 15 minutes. Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other medium constituents, then added when the agar had cooled to 55 C. Plates were inoculated and incubated aerobically for 24h at 37 C, followed by storage at room temperature for 48h. A positive result was indicated by black colonies with a dry crystalline consistency.

Quantitative assay using transmission electron microscope.

After an overnight growth on CRA, bacteria were scraped off the plates, placed in 2.5% buffered glutaraldehyde for 90 min, washed twice, centrifuged at 180 x g for 10 min, resuspended in Milloning buffer, and centrifuged again at 180 x g for 10 min to obtain a solid pellet. This pellet was fixed for 30 min in osmium tetroxide and, after a final wash in buffer and dehydration in ethanol, rinsed in propylene oxide and embedded in Epan-Araldite (1:1). Ultrathin (400- to 600-A [40- to 60-

nm]) sections were made, using an LKB-8800 ultra- microtome, and stained with uranyl acetate and lead citrate. Ultrathin sections were studied by transmission electron microscopy (Baselga *et al*., 1993).

Ethics Approval

This study was approved by the ethical and research committee of Veterinary Medicine College/University of Baghdad.

Results and Discussion

Initial characterization of *Staphylococcus aureus* bacteria:- The isolated bacteria from ovine mastitis were identified by conventional methods. All of the 17 strains were showed hemolysis, sharply defined of clear zone in blood agar also found as gram positive, catalase positive cocci and fermented manitol in manitol salt agar. These isolates were rabbit plasma-coagulase positive and they were considered as *S. aureus*. Confirmations of the strains were done using API Staph (Hammadi and Yousif,2013).
Slime production.

Total agreement was observed when the results obtained with the two techniques used (Congo Red Agar and Electron microscopy) were compared.

Congo Red Agar

In our study (17) ovine mastitis *Staphylococcus aureus* isolates examined in CRA test, The slime Production of *S.aureus* was recorded in 2 (11.76%) isolates, which produced characteristic black colonies of dry crystalline consistency, also the result reported 10 (58.8) isolate have red small colonies which considered as (negative result) the other result show 3(17.6) isolate have black smooth colonies and 2 (11.67) isolate have red colonies with dry crystalline consistency which considered as indeterminate result.as in Table (1).

Our results of culthuring of *Staph aureus* on GRA were in agreement with Citak *et al*., (2003); Oliveira *et al*., (2006); Arslan and Özkardes, (2007); Krukowski *et al*., (2008); Jain and Agarwal, (2009) they recorded that CRA were used in detection of the slime production from *S. aureus* isolated from human and animals, this strains produced four different colony phenotypes on CRA: * black colonies of dry consistency and with rough surface; * black colonies with a smooth, round and shiny surface; * red colonies of dry consistency and with rough surface and * red colonies with a smooth surface, round and shiny .

The percentage of positive *Staph. aureus* to CRA appeared in a percentage of 11.7%, this in agreement with Watson, (1989) which reported that in bovine mastitis less than 10% of *Staph .aureus* bacteria produced slime material after in vivo growth and this ratio decreased to when this bacteria growth in vitro because this bacteria lost this exopolysaccharide material after grown in laboratory media.

Citak *et al*., (2003) were reported slime production in 5.1% *S.aureus* isolated from milk samples. Baselga *et al*., (1993) who detected 12% positive strains out of 92 bovine strains tested. Krukowski *et al*., (2008) were reported high percentage (42.37%) of slime producing *S.aureus* mastitis isolates in out of 59 *S.aureus* bacterial isolates. Vasudevan *et al*., (2003) showed that out of 35 *S. aureus* mastitis isolates, 32 strains produced slime material within 24-48 hours.

Table.1 Slime production by *Staphylococcus aureus* in CRA method .

No. of strains tested	Negative result	Indeterminate result		Positive result
	Red-smooth colonies	Black-smooth colonies	Red colonies a dry crystalline consistenc y	Black colonies with a dry crystalline consistency
17	10	3	2	2

Figure.1 Bacterial cells of *S. aureus*, which produces a slime layer (arrowhead), as observed by transmission electron microscopy (magnification, x 80,000).

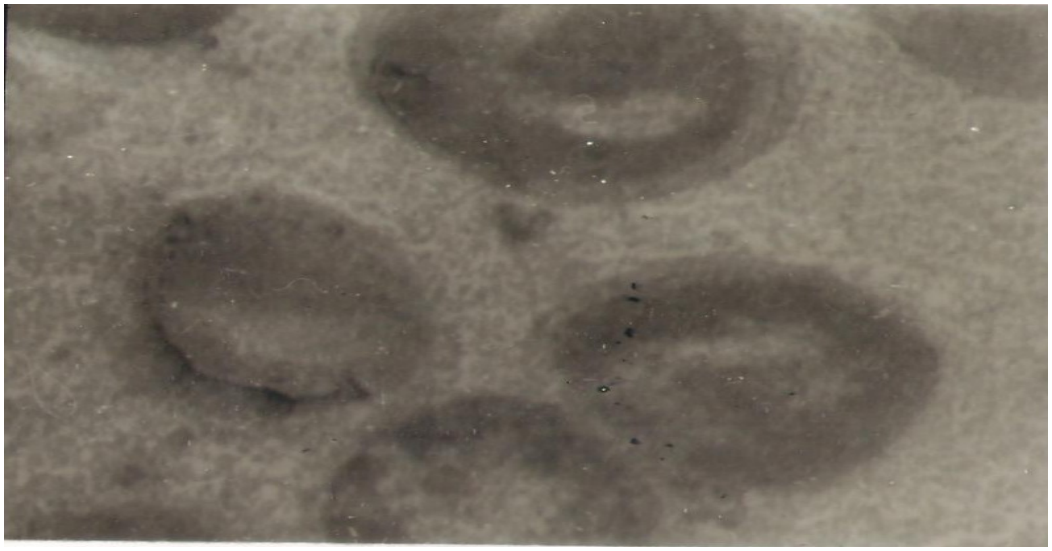
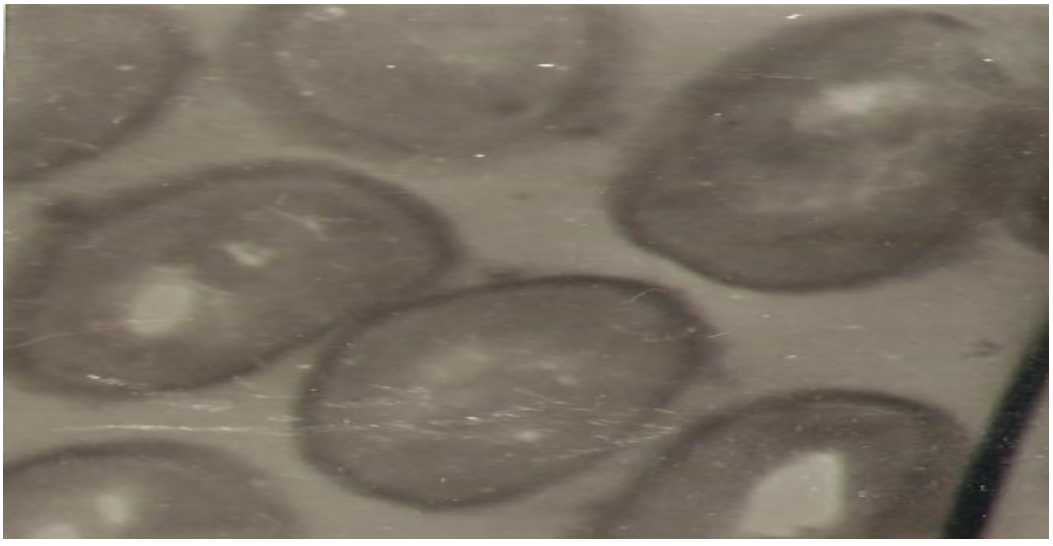


Figure.2 Bacterial cells of *S. aureus*, which didn't produces a slime layer as observed by transmission electron microscopy (magnification, x 60,000).



There are some variations in interpretation of CRA test results between researches were appearing. In which, bright black colonies and black colonies (Citak *et al*., 2003; Oliveira *et al*., 2006; and Jain and Agarwal, 2009) were considered as a positive result. In present result 3 isolates (17.64%) produced black colonies of smooth and shiny surface, which was characterized as indeterminate result. also, the results showed a red colonies with rough surface were described as indeterminate result. But some researcher like Cucarella *et al.,* (2004) describe dry crystalline surface (black or pink rough colony phenotype) as a positive result, without regarding the color.

Knobloch *et al*., (2002) reported that 128 human isolates of *S. aureus* tested in CRA with brain heart infusion (BHI) base produced only red (ranging from pink to orange) colonies, as well as that particular strains formed black colonies on CRA using a TSB base, dry crystalline morphology of these red colonies was observed after 24 hours for the five *S. aureus* strains which were described as slime producing. Such discrepancy when interpreting the results may possibly be due to the fact that the test itself was not originally designed for investigating *Staphylococcus aureus* isolates. Number of studies revealed the ability of *S.aureus* mastitis to produce slime material. The biofilm protect the bacteria from attack by antibacterial or phagocytosis, it inhibits chemotaxis of granulocytes and their opsonic activity, therefore affecting the inflammatory response (Dorocka-Bbkowska and konopka, 2007; fox *et al*., 2005). The bacteria with slime material are 10-1000 fold more resistant to antibiotics than their planktonic form (Gorski and palmer, 2007).

Electron microscope

The present study reported that the two slime producing *S. aureus* bacterial cells (in CRA) when examined under electron microscopy showed a condensed extracellular and thick layer surrounding the cell wall was observed in >80% of bacterial cells of the Slime producing (SP) colonies (Fig. 1). This layer was present in only 5% of cells in the case of their non slime producing (NSP) strain. The differences between SP and NSP strains found in this work in the proportion of exopolysaccharide-surrounded bacterial cells (Fig. 2). this results was in agreement with those found by Watson (1989), who observed that in bovine mastitis, >80% of *S. aureus* bacterial cells produced exopolysaccharide after in vivo growth and that the proportion decreased to <10% when bacteria were grown in regular laboratory media. bacteria able to form opaque colonies in vitro are proposed to be responsible for in vivo colonization, and this bacteria also responsible for infection and virulence (Oliveira *et al*., 2006).

Early detection and management of biofilm-forming staphylococci can be one of the essential steps towards the prevention and management of device-associated nosocomial infections (Nasr et al., 2013).

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