The Role of Prevalence of Capsular Polysaccharide in Pathogenicity of *Staphylococcus aureus* Isolated from Mastitic Cows and Buffloes

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Summary

The results showed that a total of 86 *Staphylococcus aureus* strains were recovered from bacteriological examination of 237 Mastitic milk samples collected from cows and buffaloes with an incidence of 36.38%. All *Staph.aureus* strains cultured in serum soft agar revealed that 71 out of 86isolated strains showed diffuse form (encapsulated) and 15 strains were compact form (non capsulated) with an incidence of 82.6% and 17.4% respectively. For pathogenicity tests, the mice group which injected with encapsulated strain was died within 24 hrs while the other group which injected with non capsulated strain was still survived until the end of the experiment (72h).

The extracted capsular polysaccharide prepared from the encapsulated strain of *Staph.aureus* subjected to electrophoresis in SDS gel and stained with Comassie brilliant blue showed a common density band at 58.5kDa and few additional bands at 48.75 and 84 kDa. The immunogenicity of the capsular polysaccharide antigen was determined using Western immunoblot technique.

Introduction

Mastitis can be succinctly defined as an inflammation of the mammary gland. It is developed as a result of complex interaction between host pathogen and environment. Mastitis continues not only as the most common and most costly illnesses in dairy herd, but also constitutes at time a public health hazard (7).

Staph. aureus has emerged as one of the most prevalence organism causing mastitis and once it is established in the mammary gland, it is very difficult to eradicate (**11**). Bacterial capsule is an important virulence determinant for organisms that cause invasive disease; most strains of *Staphylococcus aureus* produce capsular polysaccharide (**19; 9 and 13**). *Staphylococcus aureus* strains are able to produce capsular polysaccharide in vivo under different culture condition.

The enhanced virulence of encapsulated *Staphylococcus aurous* strains is more virulent than that lacking of capsule because these resist phagocytosis in the absence of antibodies and complement **(14)**.

This work was done to study the role of prevalence of capsular weight polysaccharide in pathogenicity of *Staphylococcus aureus*, isolated from mastitic cows and buffaloes by preparation and purification of the capsular polysaccharide, in addition to its pathoginicity to laboratory animal as well as estimation of its molecular and immunogenic potency.

Material and Methods

Samples:

A total of 237 milk samples were collected from clinical Mastitic cases of 70 cows and 20 buffaloes from different farm at El-Giza, and El-Fayoum Governorates.

The samples were collected from each animal under a condition of asepsis with discarding of the first stream of milk **(2)** in sterile screw capped bottles, and then transferred to the laboratory as soon as possible with minimum delay.

Isolation and identification of *Staphylococcus aureus:*

All collected samples were incubated aerobically at 37°C for 24h then centrifuged at 3000 r.p.m for 20 min. A loop full from the sediment was picked up and streaked on surface of blood agar plates then incubated for 24 h at 37°C.

The suspected growing colonies were processed and identified by conventional methods based on colony morphology, Gram stain, tube coagulase, oxidase, and catalase test according to **(15)**

Culture media and procedure: (1)

The isolated *Staphylococcus aureus* strains were grown on trypticase soya broth modified by addition of 0.1 % glucose and 0.5 NaCl and 1 % yeast extract to facilate the production of capsule

Detection of capsule: (19)

Detection of capsule was applied by using of serum soft agar technique. Serum soft agar was prepared from brain heart infusion broth (BHI) by addition of 1 % (vol/vol) normal rabbit serum and 0.15% (wt/vol) agar. All strains were grown in 8 ml brain heart infusion broth and incubated at 37°C for 18 h, and then about 100 µl were transferred in 2 ml volume of brain heart infusion broth and incubated at 37°C for 2 h.

Soft serum agar technique for detection of capsule: (3)

One loop full of a 2h-broth cultures of the strains to be tested was used to inoculate three tubes of serum soft agar. After vigorous mixing, the third tube was found to give a convenient number of colonies. For correct determination of morphology the colony morphology was recorded as diffuse or compact after incubation at 37°C for 24 hour.

Extraction of capsule: (12)

Staphylococcus aureus were grown on trypticase soya agar, the organism were removed by washing the agar plate with PBS ,the bacteria were killed by adding 3 % formalin for 18 h then, washed and suspended in PBS (5 % wet/vol). The bacterial suspension was mixed in a blender for 3 min at 4°C, and then cooled in ice for 3 min, this cycle was repeated four times, and the organism was removed via centrifugation at 4500 xg for 10 min. The supernatants were pooled for used.

Virulence studies: (19)

Two strains of *Staphylococcus aureus* , one is encapsulated and the other was non capsulated were grown on trypticase Soya broth and incubated at 37°C for 18 h in the presence of chloramphenicol (5 µg/ml) the cultures were harvested by centrifugation, washed twice in sterile PBS resuspended in PBS to 2 X 10^7 CFU.

Experimental models: (18)

Two group of Swiss albino mice (8 for each) with an average weigh of 20- 30 gm aged 2month were used for detection of pathogenicity of the isolated strains, six mice from each group were injected intraperitonial with 0.5 ml of prepared culture of the tested strains (encapsulated and non

capsulated) while the other 2 mice were injected with normal saline and left as control the time of lethality was assessed at 24h later.

Antiserum preparation: (4)

New Zealand white rabbit's were immunized with prepared capsule three times a week interval for 4 weeks with increasing amounts (0.1 to 0.4ml). The first immunization was given subcutaneously and each subsequent injection was given intravenously, the animals were bled after the fourth weeks of immunization.

SDS-PAGE and Western immuno-blotting:

Capsular polysaccharide was separated by sodium dodecyl sulphate Polyacrylamide gel electrophoresis according to **(8)**. Capsular polysaccharide separated in 10 % and 5 % (w/v) acryl amide as resolving and stacking gels respectively. After electrophoresis, the gel was subjected to western blotting. The polysaccharide transferred to nitrocellulose sheet and this carried out at a constant voltage of 70 for 1 .5 h. in running buffer containing 25 mM Tris HCL and 192 mM glycine (pH 8.3), after the sheet was placed in sealed plastic box with blocking buffer containing 10% skim milk and incubated at room temperature for 10 min. the nitrocellulose membrane was rinsed in PBS 0.05% Tween 20 (PBST) three times or 10 min. and then incubated with rabbit antiserum diluted 1:500 in blocking buffer containing 2.5% skim milk in PBS, at 25 C or 2 hr. the membrane was washed 3 times with PBST Tween for 10 min. and incubated at 25C or 1 h with peroxidase- conjugated. The membrane was washed with water for 5 min. to stop the reaction

Results

The bacteriological examination of 237 milk samples collected from mastitic cows and buffaloes revealed that 86 *Staphylococcus aureus* strains were isolated with a percentage of 36.38.

Colonial morphology in serum soft agar:

All *Staphylococcus aureus* strains cultured in Serum soft agar revealed the 71 out of 86 showed diffuse form (encapsulated) and 15 strains were compact form (non capsulated) with an incidence of 82.6 % and 17.4 % respectively (Table 1).

Table (1): Animal species, numbers of examined animals, milk samples and morphology of *Staphylococcus aureus*.

colonial morphology on serum soft agar				No. of isolated strains				
Compact		Diffuse						
%	No.	%	No.					
15.6	12	84.4	65	36.67	77	210	70	Cows
33.3	3	66.7	6	33.33	9	27	20	Buffaloes
17.4	15	87.2	71	36.3	86	237	90	Total

Virulence study:

The group of six mice which were injected with encapsulated strain (diffuse form) all member were died within 24 h while the other six mice which injected with non capsulated strain (compact form) lived even after 72 h.

SDS-PAGE and Western immuno-blotting:

The extracted Capsular polysaccharide prepared from the encapsulated strain of *Staphylococcus aurous* electrophoresd in sodium

sulphate polyacrylamide gel stained with Comassie brilliant blue showed a common density band at region of approximately 58.50 kDa and few additional band of 48.75 kDa and 84 kDa. (Table (2) and Photo. 1)

For Western immunoblot the specific immunogenic band of capsular polysaccharide antibody raised against encapsulated strain was blotted at a molecular mass 48 kDa. (Photo.2)

Table (2): SDS-PAGE pattern of capsular polysaccharide of encapsulated *Staphylococcus aureus*.

Amount	Electrophered band molecular (kDa)	Marker molecular weight (kDa)	Lane band
36.05	84	175	1
34.13	58.50	84	2
30.01	48.75	62	3
		47	4
		32.5	5
		16.5	6

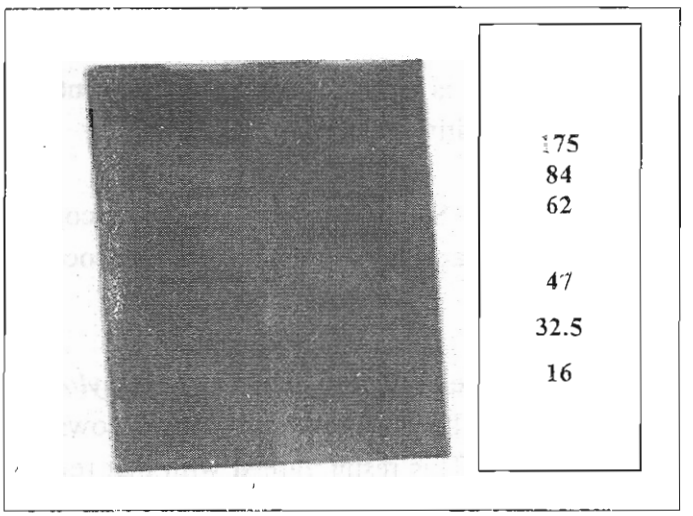

Photo. (1): SDS PAGE of encapsulated Staph. aureus antigen. Lane 1: marker molecular weight (175 to 16.5). Lane 2: electrophoretic encapsulated antigen.

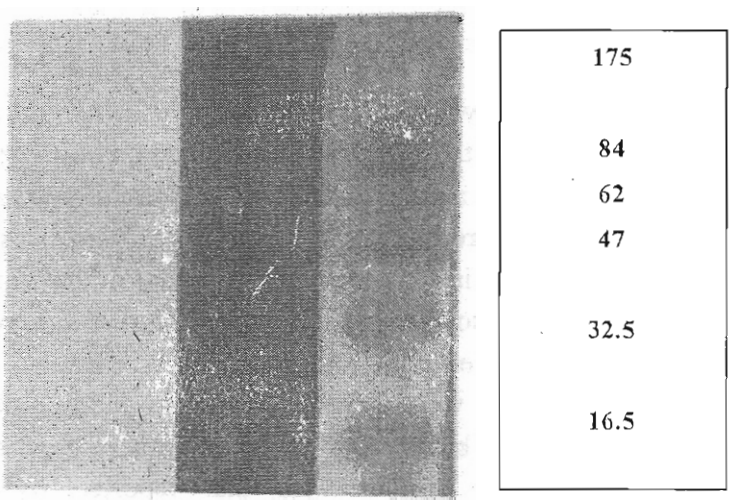

Photo (2): Western Immunoblot of encapsulated *Staph. aureus* antigen

Discussion

Staphylococcus aureus is a common etiological agent of contagious bovine mastitis, it is Gram positive bacterium.

Capsular production by Staphylococcus was first recognized in 1930 by **(5)**. The prevalence of encapsulation among Staphylococcus strains has been appreciated recently.

In the present study, eighty six strains of *Staphylococcus aureus* were isolated from Mastitic milk samples collected from cows and buffaloes with an incidence of 36.3 % .This result agreed with that reported by **(16)**. High rate of isolated *Staphylococcus aureus* may be attributed to hands of worker which considered the main tool in distribution of microorganisms from teat of the animals to the other in addition to the absence of modern hygienic measures applied.

Recently, it has been shown that stock cultures of *Staphylococcus aureus* isolated from bovine mastitis are rarely encapsulated, but it retrieved when strains are cultured in a media facilitated the expression of the capsules as recorded by **(6)** who recorded that the expression of capsule is greatly influenced by the environmental and bacteriological growth condition such as medium used, so in this study the isolated strain grow in modified trpticase soya broth to enhance the production of capsule. In this study, about 71 isolates (82.6 %) were diffuse colonies in serum soft agar after cultivation in trpticase soya broth modified by addition of glucose and NaCl and these results nearly similar with that reported by **(18)**. Nevertheless, our results do not confirm with that obtained by **(17)** who found that 100 % of bovine milk isolates subculture in brain heart infusion broth are compact in brain heart infusion serum soft agar The difference in the geographic origin of isolates could explain this discrepancy. Diffuse

colony morphology in serum soft agar considered a criterion of encapsulation, seems to be a characteristics of most Staphylococcus strains from domestic animal milk **(18)**

In the present study, it is obvious that capsular production of *Staphylococcus aureus* cultivated under a condition of maximum capsular expression was more virulent for mice than non capsulated strains and this may attributed to inter peritoneal injection of encapsulated strains allowed the organism within peritoneal cavity to escape from local defenses and transit more efficiently through the lymphatic to the blood stream of animals, these agreed with that recorded by **(19) and (10)**.

Regarding to the result obtained for SDS-PAGE and Western blot, it was found a common density band appears at a region of approximately 58.50 kDa and few additional bands at 48.75 – 84 kDa this considered as being associated with peptidoglycan and at KDaof 48 for immunogenic band this agreed with that recorded by **(6)**.

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الملخص العربى

الدور الممرض الذى يقوم به غلاف بوليسكرايد فى انتشار الميكروب العنقودى المعزول من لبن الابقار والجاموس المصابة بالتهاب الضرع

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فى هذه الدراسة تم عزل 86 عترةمن الميكروب العنقودى الذهبى بنسبة 36.3% بعد اجراء الفحص البكتريولوحى لعدد237 عينة لبن تم تجميعها من عدد (70بقره و20 جاموسة) وباجراء زرع العترات المعزولة على الاجار الطرى المغذى بالسيرم لمعرفة احتوائها علىالغشاء الخارجى فقد تبين 71عترة بنسبة 82.6% كانت محتوية على الغشاء الخارجى. وباجراء العدوى الصناعية فى االفئران لمعرفة الا ثر الضار الذى يسببه الميكروب المحتوى على الغلاف الخارجى والغير محتوى، فقد تبين ان العترة المحتوية على الغلاف ذات تاثير ضار حيث ماتت الفئران المحقونة به فى فترة الثلاث ايام فى حين أن الفئران المحقونة بالعترة الغير محتوية على الغلاف الخارجى فقد بقية حية حتى بعد مرور فترة التجربة. وقد تم تحضير الغلاف الخارجى للعترة المعزوله المحتوية عليه، وباجراء التحليل الكهربى للغلاف للميكروب العنقودى الذهبى فقد كشف عن وجود أوزان جزيئه مختلفة عند الكيلو دالتون 48.75 – 84 وبتعيين الاجسام المناعية المحقونة فى سيرم الارانب فقد تبين ان الكيلو دالتون 48 مضاد للغلاف الخارجى .