Secretion of Alpha-hemolysin by Bovine Mammary Isolates of *Staphylococcus aureus*

K. Kenny, F.D. Bastida and N.L. Norcross

ABSTRACT

A total of 262 strains of *Staphylococcus aureus* isolated from the mammary gland of dairy cows were examined for the production of α-hemolysin. Strains were cultured in a liquid medium of casein hydrolysate and yeast extract in an atmosphere of 7% (v/v) CO_2 in air. The assay consisted of a dot immunoblotting technique employing bacterial culture supernatants and a mouse monoclonal antibody specific for α-hemolysin. Ninety-four percent (247) of 262 strains were positive for α-hemolysin by this method, when cultured in the laboratory. This figure is compared with those obtained in previous studies which typically based their results on the hemolytic patterns of isolates on blood agar plates.

RÉSUMÉ

Nous avons examiné la production d'alpha-hémolysine par 262 souches de *Straphylococcus aureus* isolées de glandes mammaires de vaches laitières. Les souches ont été cultivées dans un milieu liquide contenant de l'hydrolysat de caséine et des extraits de levures en présence d'un mélange air-CO_2 (93-7, v/v). La présence d'alpha-hémolysine a été déterminée par immunobuvardage des surnageants de culture bactérienne à l'aide d'un anticorps monoclonal spécifique. La réaction s'est révélée positive pour 247 (94 %) des 262 souches. Ces résultats sont comparables à ceux obtenus sur les capacités hémolytiques des souches sur gélose au sang.

Bovine mastitis is a major disease of dairy cattle with financial losses reported of over $2 billion per annum (1). *Staphylococcus aureus* is one of the major pathogens of the gland. Some progress in controlling mammary infection by this organism has been achieved through dipping of teats with a disinfectant after milking (2). Therapy of dry cows with antibiotics formulated in a slow release base, attempts to eliminate existing *S. aureus* infections and prevent new ones. The failure of therapy during both the lactating and dry periods to achieve bacteriological cures with this pathogen is common (3). The limited success of current control methods has stimulated efforts to characterize virulence factors of the pathogen in the hope that subsequent immunological intervention may be engineered to protect the host.

Alpha-hemolysin (α-hemolysin) is a 33,000 molecular weight extracellular protein of *S. aureus* (4). This membrane damaging toxin is a basic polypeptide with a pI of 8.5 and possesses lethal, cytotoxic, dermonecrotic and hemolytic properties (5). A comparison of a parent strain of *S. aureus* producing α-hemolysin with mutant progeny, derived by site-directed mutagenesis, which lacked the hemolysin, in the mouse mastitis model provided strong evidence that this protein is a virulence factor (6). Studies in rabbits revealed that infusion of α-hemolysin into the mammary gland caused hemorrhagic necrosis and ischemia, while the presence of antibodies specific for the toxin converted the gangrenous form of the disease to the abscess form (7,8). Specific antibody present in the milk and serum of infected cows suggest that α-hemolysin is secreted *in vivo* during mammary infections (9). Low anti-α-hemolysin titers were found in normal dairy cattle, with higher titers present in dairy cattle with staphylococcal mastitis (10).

Previous studies estimated 20.2 to 100% of bovine mammary isolates produced this hemolysin (11-22). A summary is provided in Table I. Hemolytic patterns on agar plates to which washed erythrocytes from different species had been added and the ability of antitoxin to inhibit such lysis were recorded. The development of hybridoma technology and dot immunoblotting assays may offer more sensitive and more specific methods for examining α-hemolysin production by isolates. We wished to evaluate the usefulness of such methods to delineate α-hemolysin production by isolates of *S. aureus* from the bovine udder.

A total of 262 isolates of *S. aureus* were used. In order to obtain a wide sample of strains, these were cultured from the milk of dairy cows located throughout the state of New York. Bacteria were maintained on tryptic soy agar plates, containing 5% citrated bovine blood. All strains possessed typical colony morphology, displayed gram-positive cocci in stained smears and were catalase positive. These isolates tested positive in the tube

Department of Clinical Sciences, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853.

Reprint requests to Dr. N.L. Norcross.

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TABLE I. The results of previous studies examining α-toxin production by bovine mammary isolates

Study	No. tested	% + ve	Aeration	Methods[a]	Erythrocytes utilized	Ref
1	114	20.2	air	a,b,d,e	cow, horse, sheep, rabbit	11
2	479	26	air	a,b,e	cow	12
3	154	27	air	a	cow, sheep	13
4	149	45	20% CO_2	a,b	sheep, rabbit, human	14
5	92	56.5	30% CO_2	a,b,e	sheep, rabbit	15
6	189	59.3	air	a,b	cow, horse, sheep, rabbit, human	16
7	688	62	air	a	sheep, rabbit, some tested on human	17
8	818	62.6	air	a	sheep, rabbit, some tested on cow	18
9	912	68.4	10% CO_2	a,b,c,d,e	horse, sheep, rabbit	19
10	69	74	air; air + CO_2	a,b,e,f	cow, sheep, rabbit	20
1	114	78	30% CO_2	a,b,d,e	cow, horse, sheep, rabbit	11
11	301	85	CO_2	a,b,c,d	cow, sheep, rabbit	21
12	395	100	20% CO_2	a,b,e,f	sheep, rabbit	22

[a]Methods:
a = Hemolytic patterns on blood agar plates
b = The blood agar plates utilized washed erythrocytes
c = CAMP reaction
d = Potentiation of δ-toxin by β-toxin
e = Inhibition of hemolysis by antitoxins
f = Hemolytic reactions of culture supernatants

coagulase test using rabbit plasma. A colony was transferred from the blood plate to a tube (20 × 130 mm) containing 10 mL of casein hydrolysate, yeast extract medium (23). This tube was incubated for 20 h at 37°C in an atmosphere of 7% CO_2 (v/v) in air, with shaking (150 rpm). The bacteria were removed by centrifugation and the supernatant harvested.

Hybridoma 2C3.D7 was obtained from the fusion of splenocytes from α-toxoid immunized mice with P3X myeloma cells. This hybridoma produces a mouse monoclonal antibody (MAb) of IgG_1 isotype specific for α-hemolysin. Specificity was confirmed in an enzyme-linked immunosorbent assay (ELISA) using α-toxin purified by the method of Cassidy and Harshman as antigen (24). A highly purified toxin preparation, kindly provided by Dr. M. Thelestam, was subjected to gel electrophoresis and proteins were then transferred to nitrocellulose membrane. Hybridoma 2C3.D7 exhibited a strong reaction with a band of about 33,000 daltons, which was the only band observed with this preparation in the silver stained gel. Nitrocellulose paper (BioRad, Richmond, California) was cut into 10 cm squares and through the use of a template, fabricated from plexiglass perforated with 3 mm diameter holes, 1.0 μL of each supernatant was spotted onto the paper. One square accommodated 41 test samples. The paper was placed in a 14 cm diameter plastic petri dish and incubated at 37°C for 2 h. A blocking step was performed by the addition of 30 mL of 5% normal rabbit serum in 0.145 M NaCl, 0.015 M NaH_2PO_4, pH 7.2 (phosphate-buffered saline [PBS]) to each dish which was incubated for 30 min at 37°C with gentle rotation. The paper was washed four times in PBS containing 0.05% Tween 20 (PBST), allowing a 5 min soak between washes.

Culture supernatant of hybridoma 2C3.D7 was diluted 1:10 in PBST and 20 mL added to each petri dish. The paper was incubated for 90 min at 37°C and after five wash cycles in PBST, 20 mL of a 1:4000 dilution of peroxidase-conjugated affinity purified goat antimouse IgG added (Jackson Immunoresearch Laboratories Inc., West Grove, Pennsylvania). The incubation was repeated and the paper washed two times with PBST and three times with PBS. A substrate stock solution of 3 mg/mL 4-chloro-1-naphthol in methanol was diluted with five parts of 0.145 M NaCl, 0.05 M Tris-HCl, pH 7.4 and H_2O_2 was added to a final concentration of 0.01% (v/v). Ten mL of the substrate was added to each petri dish which was then incubated at 37°C for 10 min with rotation. The reaction was stopped by washing in distilled water and reactions scored visually from 0 to 4+. Positive reactions were scored as 2+ or greater.

Three control spots consisting of a standard α-toxin preparation and culture supernatant from strains DU5848 and DU5789 were carried on each nitrocellulose square. DU5848, a bovine isolate, produces α-hemolysin, while DU5789 is an isogenic stain where the α-hemolysin gene has been inactivated by site directed mutagenesis (6). To examine whether the enzyme catalase was interfering with the immunoblot, supernatants from 40 strains which gave 4+ reactions were spotted onto paper and incubated at 37°C for 2 h. The paper was washed once in PBS and exposed to substrate for 15 min. Protein A, a cell wall component of staphylococci which may be present in culture supernatant, can bind to the Fc portion of some immunoglobulin molecules. To reveal whether protein A could be causing false positive reactions, supernatants from these 40 positive strains were spotted onto paper and the assay continued using a mouse monoclonal antibody (clone 8H6.C2) of IgG_1 isotype with specificity for bovine IgG_2. The capacity of the peroxidase-conjugated goat antimouse IgG to recognize rabbit IgG was examined by placing 1 μL volumes of 25%, 12.5% and 6.25% normal rabbit serum in PBS, onto nitrocellulose paper. After incubation and washing the conjugate was added at working dilution and the immunoblot continued as described above.

Of 262 strains examined, 247 (94.3%) produced α-hemolysin (Table II). A weak reaction (1+) was evident with seven strains and although these strains may produce low levels of hemolysin, for the purposes of this

TABLE II. The degree of reaction of culture supernatants in the dot immunoblot

Immunoblot reaction	0	+	++	+++	++++
Number of strains	8	7	72	103	72

study they were classed as negative. The α-hemolysin and DU5848 preparations gave 4+ reactions on each paper, while the DU5789 supernatant gave a response of zero (Fig. 1). No reaction was obtained when supernatant spotted paper was exposed directly to substrate. The use of the mouse MAb specific for bovine IgG_2 instead of the MAb specific for α-hemolysin gave no reaction when the assay was continued in the normal manner. The goat antimouse conjugate displayed no evidence of binding to normal rabbit serum dotted onto the nitrocellulose.

The dot immunoblot technique with a MAb specific for α-hemolysin is sensitive, displaying a 2+ reaction with as little as 0.6 ng of hemolysin (0.6 μg/mL). This concentration of toxin failed to produce lysis when added to an equal volume of 2% washed rabbit erythrocytes. The sensitivity of the α-hemolysin dot immunoblot compares favorably with that described for a system designed to detect bovine serum albumin (25). The reaction is also specific, demonstrating no reaction with the supernatant of a stain lacking a functional α-hemolysin gene yet providing a 4+ reaction with the parent strain.

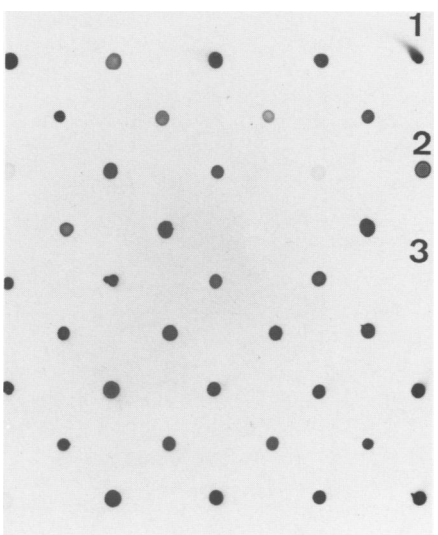

Fig. 1. Immunoblot of culture supernatants for α-hemolysin. Position 1 is a standard α-hemolysin preparation (1.25 ng), position 2 is a positive α-hemolysin-producing strain, position 3 is a non-α-hemolysin-producing strain. The remaining positions are the bovine mammary isolates.

A disadvantage of this method would be that other factors produced by *S. aureus* could give false positive reactions. There was no evidence to suggest catalase interfered with the assay. Protein A did not appear to be of significance, since no reactions were obtained when a mouse MAb of the same isotype, but specific for bovine IgG_2 was employed. Normal rabbit serum was used both to block the nitrocellulose paper and to bind to any protein A which could be present. The affinity purified goat antimouse conjugate displayed no specificity for rabbit IgG and it was decided that the use of 5% normal rabbit serum as a blocking agent was satisfactory. This rabbit serum possessed no neutralizing activity for α-hemolysin.

About 7% of strains were considered negative. Previous studies examining bovine mammary isolates of *S. aureus* revealed that from 20.2 to 100% of strains produced this protein under *in vitro* culture conditions. The figure of 94.3% obtained in the current study suggests that α-hemolysin production is a feature of most bovine mammary isolates. This result probably reflects both the culture conditions and the method used to detect toxin. The use of a yeast hydrolysate medium in an atmosphere of 7% (v/v) CO_2 in air enhances α-hemolysin production and the dot immunoblotting technique used to assay hemolysin production is both sensitive and specific. The yeast hydrolysate medium was not used in any of the previous studies.

Earlier reports detected the protein by examination of the hemolytic patterns of colonies on agar plates to which washed red cells of different species had been added. Strains which secrete δ-hemolysin exhibit zones of complete lysis when streaked on blood plates utilizing washed rabbit and sheep erythrocytes in a manner similar to strains producing α-hemolysin (26). Studies which attribute the ability of culture supernatants to lyse rabbit and sheep red cells after incubation at 37°C to α-hemolysin, failed to consider that the hemolytic pattern used to detect α-hemolysin could also be due to δ-hemolysin of *S. aureus* acting alone or when potentiated by β-hemolysin of this organism (27). The present study has avoided the problems associated with previous studies which used hemolytic patterns as an indicator of α-hemolysin. Antiserum from animals immunized with α-toxoid were used in some studies to inhibit α-hemolysin activity, but such antiserum may contain antibodies which neutralize δ-hemolysin. These factors may partially explain the wide variation in the results of these studies.

There is a degree of evidence suggesting that α-hemolysin is a virulence factor of this pathogen for the mammary gland. A decrease in the severity of staphylococcal mastitis in cows vaccinated with killed bacteria and alpha-toxoid was noted and this amelioration was attributed to neutralizing antibodies (28). Derbyshire found that a cell-toxoid provided a high level of protection to goats from intramammary challenge and only those vaccines with alpha-toxoid exhibited this feature (29). This laboratory is currently examining a parent strain secreting α- and β-hemolysins with a mutant progeny, created by site-directed mutagenesis, which lacks these proteins for ability to infect the bovine udder. While both strains colonized the gland, four of ten cows which received the mutant strain eliminated the bacteria but the parent strain persisted in all ten cows. Clinical signs were severe with the parent strain while mild inflammation was noted with the mutant strain. These results are suggestive that this difference between parent and mutant progeny is due to either or both hemolysins. If α-hemolysin is further characterized as a virulence factor it will be important to determine the extent to which specific antibodies induced by vaccination can interfere with its role.

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