JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1983, p. 1098–1101
0095-1137/83/111098-04$02.00/0

Vol. 18, No. 5

Differentiation of Staphylococci on the Basis of Nuclease Properties

ROAR GUDDING

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Received 25 April 1983/Accepted 9 August 1983

The quantity, thermostability, and serological pattern of nucleases produced by different staphylococci were studied. Staphylococcal strains were isolated from nine different species of animals or from humans. *Staphylococcus aureus*, *Staphylococcus intermedius*, and *Staphylococcus hyicus* subsp. *hyicus* were vigorous producers of nuclease, whereas the coagulase-negative staphylococci, except *S. hyicus* subsp. *hyicus*, produced significantly less nuclease. The nucleases of all strains were found to be thermostable. *S. aureus*, *S. intermedius*, and *S. hyicus* subsp. *hyicus* could be distinguished from each other and from coagulase-negative staphylococci on the basis of inhibition of nuclease activity by specific antibodies.

The coagulase test and the production of a heat-stable nuclease are considered to be the main criteria for distinguishing *Staphylococcus aureus* from other staphylococci (1, 3, 13). These two characters are reliable for differentiating *S. aureus* of human origin because *S. aureus* is the only coagulase-positive species associated with diseases in humans and because coagulase-negative staphylococci of human origin appear to produce a heat-labile nuclease (11, 13).

The coagulase and the heat-stable nuclease tests alone may not be reliable for identification of staphylococci isolated from animals. Both *S. aureus* and *Staphylococcus intermedius* and some strains of *Staphylococcus hyicus* subsp. *hyicus* coagulate rabbit plasma (3). Furthermore, all three species produce a heat-stable nuclease (4, 10), and the validity of the thermostability of the nuclease of coagulase-negative staphylococci isolated from animals has been questioned (9, 11).

Recent studies have recommended different schemes for the identification of staphylococci based on characteristics such as pigmentation, production of extracellular enzymes, serological properties, and biochemical reactions like the acetoin test and acid production from certain carbohydrates (1, 12, 14, 16, 17).

The aim of the present study was to examine the production, thermostability, and serological pattern of the nucleases of staphylococci isolated from various host species and to assess the suitability of these properties for use in the routine identification of staphylococci.

MATERIALS AND METHODS

Bacterial cultures. A total of 64 staphylococcal strains isolated from different host species were studied (Table 1). Seven human *S. aureus* strains were also included. The following reference strains were used in the study: *S. aureus* ATCC 10832 (Wood 46), ATCC 12598 (Cowan), ATCC 27543, ATCC 29740 (Newbould 305), *S. hyicus* subsp. *hyicus* ATCC 11249, *S. sciuri* subsp. *sciuri* ATCC 29059, and *S. sciuri* subsp. *lentus* ATCC 29070. The strains were maintained on blood agar containing 5% bovine blood.

Identification tests. The strains were classified as belonging to the genus *Staphylococcus* based on the ability to grow in the presence of erythromycin, the sensitivity to lysostaphin, and resistance to lysozyme (1). The organisms were classified according to species by the coagulase test, the nuclease test, production of acetoin and phosphatase, acid production from glucose, sucrose, trehalose, maltose (aerobically), and mannitol (aerobically and anaerobically), and novobiocin susceptibility (1).

Nuclease production. The enzymes used for antibody production and in enzymoserological examinations were produced by growing the microorganisms on a dialysis membrane covering heart infusion agar (19). For testing of nuclease activity and thermostability, the enzymes were produced by overnight growth in heart infusion broth followed by removal of the cells by centrifugation.

Demonstration of nuclease. Toluidine blue DNA Agar (TDA) (15) was used in the agar diffusion tests. The enzyme solutions were deposited in 7-mm-wide wells punched in the 2-mm-thick agar. The plates were incubated at 37°C for 18 h. Nuclease activity was quantitated by measuring the diameter of the pink zones around wells with diluted and undiluted samples.

Thermostability of nucleases. Aliquots (0.5 ml) of the

TABLE 1. Species, origin and source of staphylococcal strains

Bacterial species	Animal species (no. of strains)	Source[a]
Staphylococcus aureus	Cow (14), human (7), dog (3), sheep (2), pig (1), horse (1), poultry (1)	A, B, C, D
Staphylococcus intermedius	Dog (8), pigeon (1)	D, E
Staphylococcus hyicus subsp. *hyicus*	Pig (7), cow (4), poultry (2)	A, F
Coagulase-negative staphylococci	Cow (8), dog (2), pig (1), goat (1), opossum (1)	A, D, F

[a] Sources: A, National Animal Disease Center, Ames, Iowa; B, D. W. Powers, Ames, Iowa; C, M. Bergdoll, Madison, Wis.; D, R. Griffith, Ames, Iowa; E, J. W. Pankey, Homer, La.; F, L. A. Devriese, Gent, Belgium.

crude enzyme solutions were transferred into small glass ampoules and heated to temperatures ranging from 50 to 100°C. The temperature intervals were 5°C, and the heating time was 2 min. The stability at 100°C was also tested by heating the samples from 5 to 120 min. The enzyme activity before and after heating was determined by the agar diffusion method using TDA.

Antisera. Antibodies against staphylococcal nucleases were produced in rabbits according to a procedure used for producing streptococcal antinucleases (6). The nucleases from the following strains were used for antibody production: two bovine, two human, one ovine, and one porcine strain of *S. aureus*, one canine, and one pigeon strain of *S. intermedius*, two bovine and one porcine strain of *S. hyicus* subsp. *hyicus*. The reference strains of *S. aureus* and *S. hyicus* subsp. *hyicus* were used for antibody production.

Enzymoserological differentiation. The inhibitory effect by the antisera on the nuclease activity was examined using a crosswise inhibition test (18). Antisera in filter paper strips were allowed to diffuse into the TDA for 3 h. After removal of these strips, filter paper strips saturated with each nuclease were left to diffuse perpendicularly to the antisera. The effect of antiserum on nuclease activity was also demonstrated by incubating equal volumes of enzyme and antiserum at 37°C for 30 min and determining nuclease activity in the TDA (neutralization test).

RESULTS

Based on the key characters described by Baird-Parker (1) and others (4, 10), the staphylococcal strains were divided into four groups (Table 1). The nuclease producing, coagulase-negative staphylococci, except strains being identified as *S. hyicus* subsp. *hyicus*, were placed in one group including one to three strains of the following species: *S. hyicus* subsp. *chromogenes*, *S. sciuri* subsp. *lentus*, *S. sciuri* subsp. *sciuri* and *S. xylosus*. These staphylococcal species were designated coagulase-negative staphylococci. Among the strains of *S. aureus* were three nonhemolytic strains (one human, one bovine, and one canine); two other strains (one canine and one ovine) did not produce acid from mannitol under aerobic or anaerobic conditions. Most *S. intermedius* strains coagulated rabbit plasma more slowly and weakly than did the *S. aureus* strains. Of the 13 strains of *S. hyicus* subsp. *hyicus*, 5 were coagulase-positive strains.

The nuclease production by all strains of *S. aureus*, *S. intermedius*, and *S. hyicus* subsp. *hyicus* was markedly greater than that of the strains in the group of coagulase-negative staphylococci (Table 2).

The thermostability values of the crude nucleases expressed as the average *D*-value at 100°C (time to effect a 10-fold decrease in enzyme activity) were found to be 133 min (*S. aureus*), 115 min (*S. hyicus* subsp. *hyicus*), and 38 min (*S. intermedius*). Owing to their low enzyme production, *D*-values were not determined for the nucleases produced by the coagulase-negative staphylococci. When heated at temperatures from 50 to 100°C for 2 min, the nucleases of all the tested staphylococci appeared to be heat stable because the activity was either not reduced or only moderately reduced.

The activity of nucleases produced by all staphylococcal strains was tested in the presence of antibodies against *S. aureus*, *S. intermedius*, and *S. hyicus* subsp. *hyicus*. The results of the enzymoserological tests are summarized in Table 3. The results obtained by the crosswise inhibition test and the neutralization test were consistent. There were no differences in the serological properties of nucleases of *S. aureus* and *S. hyicus* subsp. *hyicus* isolated from different species, respectively. Antibodies produced against the nuclease from *S. intermedius* isolated from pigeon inhibited the nuclease activity of

TABLE 2. Nuclease production by staphylococcal strains[a]

Species or group	No.	Nuclease activity zone diam (including well) in mm	
		Avg	Range
S. aureus	29	28.7	23–33
S. intermedius	9	25.0	22–29
S. hyicus subsp. *hyicus*	13	28.7	26–30
Coagulase-negative staphylococci	13	11.5	9–14

[a] Examined in supernatants from cultures grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) incubated for 18 h at 37°C.

TABLE 3. Enzymoserological analyses of nucleases of staphylococcal strains

Nucleases produced by:	Inhibition of nucleases in the presence of antibodies against nucleases of:		
	S. aureus[a]	*S. intermedius*[b]	*S. hyicus* subsp. *hyicus*[c]
S. aureus	+	−, +[d]	−
S. intermedius	−	+	−
S. hyicus subsp. *hyicus*	−	−	+
Coagulase-negative staphylococci	−	−	−

[a] Strains (no.) were isolated from human (2), cow (2), sheep (1), and pig (1) sources.

[b] One strain was isolated from a dog source, unless otherwise noted

[c] Strains (no.) were isolated from cow (2) and pig (1) sources.

[d] One strain was isolated from a pigeon.

the *S. aureus* strains. The inhibition was moderate and less than that of *S. intermedius* strains. The *S. aureus* antisera had no inhibitory effect on *S. intermedius* nuclease. With the exception of the pigeon strain of *S. intermedius*, no antigenic cross-reactivity was demonstrated when nucleases produced by *S. aureus*, *S. intermedius*, and *S. hyicus* subsp. *hyicus* were tested with antibodies against these three species.

In the neutralization test, a slight nuclease activity could be demonstrated even in samples with nucleases and homologous antisera. Consequently, correct interpretation of the test could be done only when a well without antiserum was included for comparison.

DISCUSSION

There are certain presumptions for the use of enzymes and their antibodies as a tool in taxonomy of microorganisms. Ideally, the enzyme should be excreted vigorously by all strains of the organism and there should be a specific and simple test for detecting the enzyme. In general, the nucleases of *S. aureus*, *S. intermedius*, and *S. hyicus* subsp. *hyicus* meet these requirements. The serological difference between the nucleases of these three species provided a basis for a rapid and accurate system for the differentiation of these microorganisms. However, according to the results of the present study, a canine strain of *S. intermedius* should be chosen for antibody production. The results of the serological tests also provide additional evidence for separating *S. intermedius* from *S. aureus*.

Other staphylococcal enzymes have been used in enzymoserological examinations and have given consistent and similar results. The protease of *S. aureus* was serologically different from that of *S. epidermidis* biotype 2 (*S. hyicus* subsp. *hyicus*) and other coagulase-negative staphylococci (2). Antisera against the catalase of *S. aureus* exhibited identical reactions in an immunodiffusion test with extracts from different *S. aureus* strains, but revealed only partial identity with crude extracts of *S. intermedius* and *S. hyicus* subsp. *hyicus* strains (13). In a neutralization test, antibodies against the sphingomyelinase of *S. aureus* were found to inhibit, but not completely neutralize the sphingomyelinase of *S. intermedius* (unpublished data). A similar cross-reaction was demonstrated when *S. intermedius* sphingomyelinase was tested against *S. aureus* antibodies.

The nuclease activity of rabbit sera might represent a possible source of error in the neutralization test. However, owing to the low pH for optimum activity (8), these normally occurring enzymes do not interfere when the pH of TDA is adjusted to 9.0.

All strains of *S. aureus*, *S. intermedius*, and *S. hyicus* subsp. *hyicus* were strong producers of nuclease in contrast to the coagulase-negative staphylococci. However, some poultry strains of *S. aureus* have been reported to have low nuclease production (5).

Present data on the heat stability of *S. aureus* nuclease agree with previous studies (6). In this work, the nuclease of *S. intermedius* was found to be less resistant to heat than the nucleases of *S. hyicus* subsp. *hyicus* and *S. aureus*. Nuclease activity was demonstrated after the nucleases of all the strains, including those of coagulase-negative staphylococci, were heated to 100°C for 2 min. Consequently, in the differentiation of staphylococcal strains isolated from animals, heat stability of the nuclease should not be used as a main characteristic and the term *S. aureus* thermonuclease should be avoided, as it is misleading.

Certain modifications of the neutralization test were attempted (unpublished data). Application of 1 drop of antiserum into wells in TDA and preincubation at 4°C for 2 h, followed by addition of a sample of enzyme solution or broth culture, gave identical results to those for which antiserum and nuclease solution were preincubated. Furthermore, as production of antisera may be an obstacle to some laboratories and no antinucleases are commercially available, antisera originally produced against *S. aureus* hemolysins were tested and found to contain antibodies against the nuclease.

In conclusion, the results of the present study confirmed that the coagulase test and the assessment of thermostability of the nucleases are not suitable as the only criteria for identifying *S. aureus* isolated from animals. However, reliable

differentiation of veterinary isolates of coagulase-positive staphylococci could be performed by testing the serological properties rather than the thermostability of the nuclease.

ACKNOWLEDGMENT

R.G. is a visiting scientist from the National Veterinary Institute, Oslo, Norway, with a fellowship from the Fogarty International Center of the National Institutes of Health (IFO5 TW02927-01A1).

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