Thermonuclease Seroinhibition Test for Distinguishing *Staphylococcus aureus* from Other Coagulase-Positive Staphylococci

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Since coagulase-positive staphylococci from animals are heterogeneous, another test is necessary to distinguish *Staphylococcus aureus* from them. Staphylococcal thermonucleases appear to be heterogeneous; antisera raised against *S. aureus* isolated from humans inhibit thermonuclease activity as demonstrated by the metachromatic well-agar diffusion method. The serological specificity of the thermonuclease elaborated by *S. aureus* of human origin was demonstrated using three antisera and 407 strains of staphylococci from diverse human and animal sources.

Coagulase and thermonuclease production are regarded as the two main criteria for separating *Staphylococcus aureus* from other staphylococci (1). Both coagulase and thermonuclease tests are convenient and reliable methods for routine identification of *S. aureus* in human infections (2). However, recent studies have shown that coagulase-positive staphylococci from animals are a heterogeneous group (4, 9). Using different physiological tests, Hájek and Maršálek (9) could distinguish six different biotypes: A (human origin), B (poultry and swine), C (bovine), D (hare), E (canine), and F (pigeon). Further chemical and biochemical studies showed that these biotypes could be delineated into two major groups: strains of biotypes A to D form one homogeneous group, and strains of biotypes E and F form another (15). The latter strains have been reclassified recently to a new species, *Staphylococcus intermedius* (8). Additional coagulase-positive strains from poultry, cattle, and pigs form still another homogeneous group and are presently classified as *Staphylococcus epidermidis* biotype 2 (4).

The present study describes a simple procedure for distinguishing *S. aureus* from other coagulase-positive staphylococci. This is based on our own observations, and those of others (14), that thermonucleases produced by *S. aureus* are serologically distinct from the thermonucleases produced by other coagulase-positive staphylococci.

MATERIALS AND METHODS

Cultures. Five strains each of *S. aureus* biotypes A, C, D, E, and F were generously provided by V. Hájek (Palacky University, Olomouc, Czechoslovakia). Six strains of biotype B (5) and two strains of coagulase-positive strains of *S. epidermidis* biotype 2 isolated from poultry (4) were kindly sent by G. Sato (Hokkaido University, Sapporo, Japan). Two other coagulase-positive strains of *S. epidermidis* biotype 2 isolated by Brown et al. from bovine sources (3) were kindly supplied by A. C. Baird-Parker (Unilever, Colworth, U.K.). In addition, 50 isolates of *S. aureus* from cow's milk and 117 coagulase-positive staphylococci from lesions in dogs were studied. All of these staphylococci were subjected to a battery of tests as suggested by Hájek and Maršálek (9) for biotyping *S. aureus*. All of the bovine strains belonged to biotype C, and all but five of the canine strains belonged to biotype E (*S. intermedius*); the five non-E canine strains belonged to biotype A (data not shown). Also included were 150 enterotoxigenic and 55 methicillin-resistant *S. aureus* strains from a previous study (12).

Sera. An antiserum (serum A) prepared against purified thermonuclease from *S. aureus* strain V-8 (7) was kindly donated by C. B. Anfinsen (National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md.). Commercially available sera were also found useful: staphylococcus antitoxin (serum B) from Connaught Laboratories, Toronto, Canada, and anti-alpha hemolysin serum (serum C) from Burroughs Wellcome Corp., Research Triangle Park, N.C. Normal rabbit serum was obtained from Difco Laboratories, Detroit, Mich.

Thermonuclease seroinhibition test. A modification of the metachromatic well-agar diffusion technique (11) was developed to determine the serological inhibition of *S. aureus* thermonuclease in broth. This involved pouring molten toluidine blue O-DNA agar (TDA) medium (10) into petri dishes to a depth of 2

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mm; when gelled, pairs of wells spaced 4 mm apart were cut into the agar. By means of a platinum loop (2 mm in diameter), one well of each pair was filled with serum, and the other well was filled with a brain heart infusion broth culture of staphylococcus grown for 24 h at 37°C and then heated in boiling water for 15 min. Each plate was incubated at 37°C for 1 to 4 h. Inhibition was indicated by the flattening of the bright pink halo of thermonuclease activity at the area near the well containing the serum (Fig. 1). Serial twofold dilutions of the sera were made to determine the highest dilution at which inhibition was still demonstrable.

Serological identification of thermonucleases elaborated by *S. aureus* on agar plates was also feasible. Cultures were streak-inoculated on brain heart infusion agar plates and incubated for 24 h at 37°C. Filter paper disks (Carl Schleicher and Schuell Co., Keene, N.H.; no. 740-E, 8 mm in diameter), soaked with serum, were placed at a 4-mm distance beside a band of staphylococcal growth. The plates were overlaid with 10 ml of molten TDA medium and incubated for 1 to 4 h at 37°C. Inhibition was indicated by the narrowing of the zone of thermonuclease activity near the filter paper disk (Fig. 2).

RESULTS

By the well-agar diffusion method, all strains

FIG. 1. Inhibition of thermonuclease activity is indicated by flattened halos around wells A and C opposite wells containing staphylococcus antitoxin. Thermonuclease from *S. intermedius* culture (well B) is not inhibited by the antitoxin.

FIG. 2. Inhibition is indicated by the narrowing of the zone of thermonuclease activity around *S. aureus* colonies (no. 1 and 3) near the filter paper disk soaked with staphylococcus antitoxin. Thermonucleases elaborated by *Staphylococcus intermedius* colonies (no. 2 and 4) were not inhibited by the antitoxin.

of *S. aureus* of biotypes A to D including the enterotoxigenic and methicillin-resistant strains produced thermonucleases that were uniformly inhibited by sera A, B, and C at dilutions as high as 1:16,384, 1:2,048, and 1:256, respectively. Inhibition of thermonuclease activity was visibly more distinct at lower dilutions of the sera. Strains of biotypes E and F (*S. intermedius*) and the four coagulase-positive *S. epidermidis* strains had thermonucleases that were not at all inhibited by sera A and B; none was inhibited by normal rabbit serum. Only at dilutions of 1:16 and 1:4 of serum C was there inhibition of the thermonucleases of *S. intermedius* and *S. epidermidis* strains, respectively. Sera B and C were inhibitory to staphylococcal enzymes other than thermonuclease, such as lysozyme, protease, and hyaluronidase (data not shown), indicating that the antigen preparations used to raise these antisera were not highly purified.

By the streak-inoculation method, only thermonucleases produced by *S. aureus* showed distinct inhibition by sera A, B, and C at dilutions of 1:5,000, 1:1,000, and 1:100, respectively.

DISCUSSION

Our results indicate that the thermonuclease seroinhibition test is a convenient and reliable means for distinguishing *S. aureus* from other coagulase-positive staphylococci. A highly specific antiserum against *S. aureus* thermonucleases is not necessary; commercially available sera produced against staphylococcal toxins or alpha-hemolysin were adequately specific in their inhibition of *S. aureus* thermonucleases. The test may also be helpful in identifying occasional coagulase-negative strains of *S. aureus* (6, 11).

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