Comparison of Animal Sera for Suitability in Coagulase Testing

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The sera of several animals were examined for suitability in coagulase testing. The assay for coagulase-reacting factor (CRF) activities of the whole sera indicated the following relative concentrations of CRF: human > pig > rabbit > horse > bovine, chicken, and lamb. Human, pig, and rabbit sera had adequate amounts of CRF for coagulase testing. The plasmin activities of the different sera, arranged from the strongest to the weakest, were as follows: rabbit > human > lamb > horse > bovine, chicken, and pig. Fibrinolysis was observed when rabbit, human, lamb, or horse sera were incorporated into coagulase test agars. Pig serum was superior to the other sera for use in the plate test for coagulase since it had adequate amounts of CRF and the plasminogen-plasmin system was not activated by staphylokinase or staphylococcal Müller factor. Heparinized pig plasma was more suitable than citrated pig plasma since citrate interfered with the growth of Staphylococcus aureus, and the use of heparinized plasma prevented false-positive coagulase reactions due to citrate utilization.

Few reports on the suitability of different animal plasmas for coagulase testing have appeared in the literature. Field and Smith (8) compared the plasmas of several animal species for use in the slide test. Taylor and McDiarmid (18) used rabbit, sheep, and ox plasmas in the plate test. Numerous plating media have been prepared with human or rabbit plasmas (6, 7, 10, 15, 17). Duthie and Lorenz (6) reported that cow, sheep, dog, guinea-pig, and mouse plasmas exhibited a relative deficiency in coagulase-reacting factor (CRF), whereas the plasmas of man, monkey, horse, cat, pig, fowl, and rabbit contained substantially more CRF. Meyer (12) reported on the adequacy of the coagulase test by using plasma from different animals.

Rabbit plasma is generally used for routine coagulase testing and is stipulated in the official method for detecting Staphylococcus aureus (21). The use of rabbit plasma is undesirable in the plate test since staphylokinase (SK) and staphylococcal Müller factor (MF) activate the plasminogen-plasmin system. The plasmin (E.C. 3.4.4.14) thus formed causes fibrinolysis and false-negative reactions.

The plate test for coagulase indicates the presence of S. aureus by the formation of opaque zones (fibrin halos) around colonies of coagulase-positive staphylococci. The use of plasminogen-free CRF in a plating medium reduced the occurrence of false reactions (13). Although satisfactory for research purposes, it was recognized that the preparation of CRF by gel-filtration and filter-sterilizing large amounts of bovine fibrinogen for use in a plating medium may be too complex for routine use in many laboratories. The purpose of this study was to compare beef, chicken, horse, human, monkey, pig, and rabbit sera for their suitability in coagulase testing. The criteria used to determine suitability were the presence of adequate amounts of CRF for use in the plate test and the absence of fibrinolysis.

MATERIALS AND METHODS

Cultures. S. aureus ATCC 12600, 13565, 14458, 19095, and 23235 and S. epidermidis 12228 were received directly from the American Type Culture Collection. S. aureus 265-1 was obtained from E. P. Casman of the Food and Drug Administration. All cultures were grown on Staphylococcus medium no. 110 (Difco) slants at 37 C and were stored at 4 C. After growth in Tryptic Soy Broth (Difco) for 24 hr at 37 C, these cultures were used as the inocula for the plating assay of the CRF activities of the different sera.

Plasminogen activators. A partially purified protein preparation from S. aureus 265-1 was used as the source of SK and MF. This material possessed coagulas activity; its preparation was described elsewhere (14).
Serum and plasma. Bovine, chicken, horse, human, lamb, pig, and rabbit sera were obtained from Microbiological Associates, Inc. (Bethesda, Md.) The whole sera and the CRF fractions were used in the plate determination of CRF activity. The whole sera were also used in the qualitative determination of serum plasmin activity.

Rabbit blood was collected in sterile tubes after marginal ear vein puncture. After removal of the blood clot, the sera were cleared of red cells by centrifuging at 1,000 x g for 10 min. Equal volumes of sera from eight rabbits were pooled and then frozen (−15 C) until use. Pig blood was collected at the time of slaughter. The blood was collected in 1-liter jars containing 500 ml of the following anticoagulant buffers: 0.4 M sodium citrate at pH 7.2, 0.3 M sodium citrate plus 1.9% (w/v) NaCl at pH 7.2, 0.2 M sodium citrate at pH 7.2, 10 units/ml heparin (Calbiochem, Los Angeles, Calif.; sodium, B grade; 167 units/ml) per ml in 0.05 M potassium phosphate buffer at pH 7.2, and 20 units/ml heparin per ml in 0.05 M potassium phosphate buffer at pH 7.2. The blood cells were removed by centrifugation at 500 x g for 30 min. This resulted in plasmas, diluted 1:1 in anticoagulant buffer, at final concentrations of 0.2 M sodium citrate, 0.15 M sodium citrate with 0.85% NaCl, 0.1 M sodium citrate, 5 units/ml heparin per ml, and 10 units/ml heparin per ml. The plasmas were filter-sterilized and stored at 4 C.

Pig serum was obtained by collecting the blood in sterile containers. After removal of blood clots, equal volumes of the sera from eight animals were pooled, filter-sterilized, and frozen (−15 C) until use. The rabbit and pig sera were used in the arginine esterase and plasmin assays.

Comparison of CRF activities of different animal sera. Dilutions of each test serum and bovine fibrinogen at a final concentration of 2 mg/ml were incorporated into Coagulase Mannitol Agar (CMGA; BBL). Each plate was inoculated in duplicate with 24-hr Tryptic Soy Broth suspensions of the test cultures. The plates were incubated at 37 C and were examined periodically for the appearance of fibrin halos around the growing colonies.

Plasminogen-free CRF was prepared from each serum tested. Rabbit red blood cells were washed repeatedly in 0.85% NaCl and were then lysed in distilled water. A constant amount of this red hemoglobin preparation was added to each test serum before gel-filtration, and the CRF fractions were collected (13). Dilutions of the CRF fractions of each type of serum were assayed for CRF activity as above.

Qualitative determination of serum plasmin activity. A 0.8% amount (w/v) of Ion Agar no. 2 (Oxoid) was heated and cooled to 50 C. Nonfat dry milk (Galloway-West Company, Fond DuLac, Wis.) and Methiolate (Eli Lilly, Indianapolis, Ind.) were added to give final concentrations of 1% and 0.01%, respectively. A 3-ml amount was dispensed per microscope slide and allowed to harden. Two wells, approximately 4 mm in diameter and 4 mm apart, were cut in the agar. One well on each slide was filled with approximately 0.1 ml of plasminogen activator preparation (concentration, 10 mg/ml) in distilled water. The other well on each slide was filled with one of the different animal sera. The slides were incubated at 35 C in a moist chamber and were examined periodically. Plasmin activity was indicated by zones of clearing (casein hydrolysis) between the adjacent wells where the plasminogen and SK or MF (or both) diffused together and reacted.

Arginine esterase and plasmin activity of rabbit and pig sera. A modification of the manometric technique of Lassen (11) was used to determine the arginine esterase activity of pig and rabbit sera. The substrate used was α-N-benzoyl-L-arginine ethyl ester-hydrochloride (BAE; Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.033 M. At 25 C, the 18 mM NaHCO₃ buffer with 0.85% NaCl gave a pH of 7.4 in the reaction mixture after gassing for 5 min at 780 mm Hg and allowing 5 min for equilibration to be established at approximately 760 mm Hg. Plasminogen activation occurred during the gassing and gas equilibration. A 1.8-ml amount of 10 mg/ml plasminogen activator preparation in the bicarbonate buffer plus 1 ml of rabbit or pig serum were used to demonstrate the arginine esterase activities produced by SK and MF action on the serum plasminogen-plasmin systems. One ml rabbit or pig serum (1 ml) was added to 1.8 ml of the bicarbonate buffer to determine the esterase activity of each serum. A 1.8-ml amount of the plasminogen activator preparation plus 1 ml of the bicarbonate buffer were used to assess the arginine-esterase activity of the staphylococcal enzyme preparation. Carbon dioxide evolution was measured directly with a Gilson Respirometer (Gilson Medical Electronics, Middleton, Wis.).

Rabbit and pig serum protease activities were compared by using α-casein (Sigma Chemical Company, St. Louis, Mo.) as the substrate. The plasminogen activator preparation and α-casein were rehydrated with 0.1 M potassium phosphate buffer at pH 7.4. This buffer was the same as described by Vesterberg et al. (22) for the assay of plasmin activity. After incubating 0.5 ml of 10 mg/ml plasminogen activators with 0.5 ml of rabbit or pig serum for 10 min at 35 C, 0.5 ml of the substrate was added, and the protease determination was made by the method of Bergmeyer (2).

RESULTS AND DISCUSSION

Although the use of plasminogen-free CRF from rabbit serum gave excellent results in the plate test, the preparation of the CRF and filter-sterilizing bovine fibrinogen required too much time for routine use in many laboratories. Since the use of whole plasma would greatly simplify media preparation, a survey was conducted to determine the usefulness of different animal sera in coagulase testing.

Comparison of the CRF activities of different animal sera. The relative amounts of CRF present in the animal sera tested were indicated by the strength of the fibrin halos produced at the higher dilutions of each test serum. The 10-hr reading indicated the following relative concentrations of CRF: human > pig > rabbit > horse > bovine,
| Test serum  | Reaction of serum to various test cultures<sup>a</sup> |
|------------|------------------------------------------------------|
|            | 10% | 6.7% | 5.6% | 4%  | 2.7% | 1.3% | 0.67% | Control |
| Bovine     | 1   | -    | -    | -   | -    | -    | -     | -       |
| Chicken    | -   | -    | -    | -   | -    | -    | -     | -       |
| Horse      | 1 1 1 - 2 1 | 1 1 1 - 2 2 | -    | 1 1 1 - 2 1 | 1 1 1 - 2 1 | -    | -    | -       |
| Human      | Not done | 2 4 F 4 4 2 | 2 2 F 4 4 1 | 4 4 F 4 4 4 | 4 4 F 4 4 4 | 4 4 F 4 4 4 | 4 4 F 4 4 4 | 4 4 F 4 4 4 |
| Lamb       | - 1 - 1 - | -    | -    | -   | -    | -    | -     | -       |
| Pig        | 4 4 4 4 4 4 | 4 4 4 4 4 4 | 4 4 4 4 4 4 | 4 4 4 4 4 4 | 4 4 4 4 4 4 | 4 4 4 4 4 4 | 4 4 4 4 4 4 | 4 4 4 4 4 4 |
| Rabbit     | 0.56% | 0.4% | 0.27% | 0.13% | 0.067% | 0.033% | -     | -       |

<sup>a</sup> Symbols: -, no fibrin halo; 1, weak fibrin halo; 2, moderate fibrin halo; 3, strong fibrin halo; 4, intense fibrin halo; F = fibrinolysis. Boldface percentages indicate per cent serum.
chicken, and lamb (Table 1). These data are similar to that reported by Duthie and Lorenz (6), except they indicated fowl serum had considerable amounts of CRF. The difference in source of the fowl serum is the most likely explanation for the different results.

The plate examination revealed that human, pig, and rabbit sera contain suitable amounts of CRF for coagulase testing. The plates containing pig serum gave the best reactions since the fibrin halos were more opaque than the halos in the other media, and strong reactions were given with up to 10% serum incorporated into the medium. Both human and rabbit sera produced their optimal reactions with serum concentrations of 2.7% or less in the plating media. Media incorporating the horse serum never produced the strong reactions observed with pig, human, or rabbit sera (Table 1).

It is possible that the decrease in reactivity at the higher serum concentrations was due to inhibitors or fibrinolysis. The presence of growth inhibitors is unlikely since the growth of each strain appeared the same on all concentrations of each test serum. S. aureus 13565, 14458, 265-1, and 23235 are strong producers of SK and MF. It is noteworthy that all fibrinolysis observed at the higher serum concentrations was around colonies S. aureus 13565, 265-1, and 23235. S. aureus 14458 did not produce significant fibrinolysis in these experiments. No fibrinolysis was evident around colonies of S. aureus 12600 and 19095 since these strains do not produce plasminogen activators. S. epidermidis 12228 gave negative coagulase reactions on all test media (Table 1).

The comparison of the CRF activities of the plasminogen-free CRF fractions of each serum gave results similar to those obtained with the whole sera, i.e., human, pig, and rabbit fractions produced the fastest reactions and bovine, chicken, and lamb fractions exhibited negligible CRF activity. In contrast to the whole sera experiments, the fastest reactions were observed with media containing the highest concentrations of added CRF. This was due to the elimination of serum inhibitors or to the elimination of fibrinolysis due to the action of plasmin.

Qualitative determination of serum plasmin activity. The plasmin activities of the different animal sera, arranged from the strongest to the weakest, were as follows: rabbit > human > lamb > horse > pig, bovine, and chicken. The pig, bovine, and chicken sera produced no zones of clearing by 48 hr at 35°C. The degree of proteolysis was taken as a measure of the extent of activation of plasminogen by SK and MF; consequently, the plasminogen-plasmin systems of pig, bovine, and chicken sera were not activated by the staphylococcal enzyme preparation. These findings paralleled the fibrin halo stabilities when the whole sera were incorporated into CMA. These data are similar to the species reactivity to SK reported by Clifton and Cannamela (3) and Gerheim and Ferguson (9).

The plating assay of CRF and the qualitative determination of plasmin activity indicated that pig serum was the only test serum to meet the criteria for suitability in coagulase testing, i.e., adequate amounts of CRF and no proteolysis due to activation of the plasminogen-plasmin system. Since rabbit plasma is presently accepted as the substrate of choice for coagulase testing (21), these findings indicated the importance of a comparison of pig and rabbit sera plasmin activities after activation of plasminogen with SK and MF.

Arginine esterase and plasmin activity of rabbit and pig sera. Troll et al. (20) reported that streptokinase-activated plasmin catalyzed the hydrolysis of L-lysine and L-arginine esters. Troll and Sherry (19) found that the arginine esterase activity and some lysine esterase activity were due to plasmin. Troll and Sherry also noted that arginine esters were well suited for the assay of plasmin activity since serum inhibitors had little effect on their hydrolysis.

The BAEE activities of rabbit and pig sera are shown in Fig. 1. The rabbit serum plus SK and MF gave approximately twice the esterase activity of the pig serum plus plasminogen activators. Similarly, rabbit serum produced about twice as much CO₂ evolution as the pig serum. The staphylococcal enzyme preparation contained insignificant amounts of arginine esterase activity. The BAEE hydrolysis observed with rabbit and pig sera without SK and MF indicated that the sera contained esterase activity that was not due to plasmin or that some of the serum plasmin was activated in the test system. The three- to fourfold increase in CO₂ evolution in the presence of the staphylococcal enzymes indicated the staphylococcal preparation activated both rabbit and pig arginine esterase activity.

Since the hydrolysis of the synthetic arginine ester did not explain the difference in fibrin halo stabilities when rabbit and pig sera were incorporated into coagulase test media, a protein substrate was chosen to measure protease activity. The plasmin assay using α-casein as the substrate demonstrated that rabbit serum plus SK and MF was strongly proteolytic, whereas pig serum plus SK and MF had negligible proteolytic activity (Fig. 2). These findings indicated that pig serum plasminogen-plasmin system was not activated by SK or MF; consequently, the stability of fibrin halos on media prepared with pig serum or plasma was due to the absence of plasmin activity.
This plasma. The plasmas in 0.2 M citrate and 0.15 M citrate plus 0.85% NaCl proved to be inhibitory to growth in concentrations which would produce strong fibrin halos. The inhibition of the growth of S. aureus by citrate was reported by Rammell (16). Inhibition of growth or coagulase production was not observed when heparinized plasma was used in coagulase test agars. Preliminary results indicate that the use of the plasma with 10 units of heparin per ml may be more suitable than the use of the plasma containing 5 units of heparin per ml since some fibrin clumping has been observed on plates prepared with 14% pig plasma containing the lower concentration of heparin. The use of heparinized plasma also prevented false-positive reactions by organisms which utilized the citrate and allowed spontaneous clotting to occur.

A gram-positive coccus, which was isolated from a frozen food, produced a false-positive reaction on CMA prepared with pig plasma. This isolate was coagulase-negative by the standard

The difference in plasin activity of rabbit and pig plasmas may prove to be important for application in the tube test for coagulase. The recommended procedure for performing the coagulase test requires periodic examination for clot formation during the test period (21). This is due to the possibility of fibrinolysis when rabbit plasma is used since fibrinolysis may cause false-negative reactions in the tube test. It is expected that future research will demonstrate that this type of false-negative reaction is not a problem when pig plasma is used in coagulase testing. Pig plasma diluted 1:1 in anticoagulant buffers gave slower reactions in the tube test than rabbit plasma. Similar clotting times were obtained when a final concentration of 1 mg of bovine fibrinogen per ml was added to the pig plasma.

Heparinized pig plasma was more suitable than citrated plasma for use in coagulase test agars. It was determined that 14% (v/v) pig plasma (diluted 1:1 in 0.1 M citrate) gave a faster and stronger fibrin halo than higher or lower concentrations of

![Graph showing absorbance at 280 nm vs. percent (w/v) α-casein concentration](image)

**FIG. 1.** Comparison of α-arginine esterase activities of rabbit and pig sera. Esterase activity was determined at 25°C by using 18 mM NaHCO₃ + 0.85% NaCl buffer and 0.033 M BAEE as the substrate. Of the plasminogen activator preparation from S. aureus 265-1, 6 mg/ml was used as the source of staphylokinase (SK) and staphylococcal Müller factor (MF). The extent of proteolysis was determined by measuring the percent absorbance at 280 nm of the trichloroacetic acid-soluble reaction products. Symbols: ○, rabbit serum + SK and MF; □, pig serum + SK and MF.

![Graph showing CO₂ evolution vs. time](image)

**FIG. 2.** Comparison of plasin activities of rabbit and pig sera. The plasmin activities of rabbit and pig sera were determined after activation of S. aureus 265-1 partially purified protein at a final concentration of 5 mg/ml in the activation mixture. The reaction was stopped at 20 min by the addition of 3 ml of 5% trichloroacetic acid. Symbols: ○, rabbit serum + SK and MF; □, pig serum + SK and MF; ●, rabbit serum; ■, pig serum; △, SK and MF.
tube test (21) but was a strong acid producer on CMA. Growth of this organism on CMA produced a zone of opacity around the colony, after 24 hr, which was difficult to distinguish from the fibrin halos around coagulase-positive Staphylococcus colonies. This isolate also produced false-positive reactions on Baird-Parker medium (1) and on TPEY (4).

The false coagulase reaction was eliminated by incorporating pig plasma into media without added carbohydrate, such as Tryptic Soy Agar. Similar reactions were produced on CMA prepared with pig serum and with pig plasma so the effect involved more than action on fibrinogen. It was concluded that acid production in carbohydrate-containing media caused the false reaction. Davis and Davis (5) reported a similar false reaction due to pH effects. Since the coagulase reaction is a distinguishing characteristic of S. aureus, it is recommended that pig plasma be incorporated into a medium free of utilizable sugar to avoid this type of false reaction.

These data indicate that the natural properties of pig serum and plasma may be used to advantage in coagulase testing. The availability of pig blood leads us to believe that the use of pig plasma in coagulase testing will be considerably cheaper than the use of rabbit plasma.

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