Mechanism of Plasma Clotting by *Erysipelothrix rhusiopathiae*

TOSHIO TAKAHASHI,*1 ISAMU TAKAHASHI,2 YUTAKA TAMURA,1 TAKUO SAWADA,2 TAKAHARU YOSHIDA,2 SHOKO SUZUKI,1 AND MASATAKE MURAMATSU1

National Veterinary Assay Laboratory, Kokubunji, Tokyo 185,1 and Department of Veterinary Microbiology, Nippon Veterinary and Zootecchnical College, Musashino, Tokyo 180,2 Japan

Received 18 May 1990/Accepted 20 July 1990

Experiments were performed to determine the mechanism by which *Erysipelothrix rhusiopathiae* clots plasma. Detection of plasma-clotting activity in four strains of *E. rhusiopathiae* was carried out by mixing a 24-h broth culture of a tested bacterial strain with rabbit plasma (tube coagulation test). Sodium citrate, sodium oxalate, EDTA, and heparin were used as anticoagulants in preparing the rabbit plasma. *E. rhusiopathiae* strains clotted solely citrated plasma in 18 to 24 h. A known coagulase-positive strain of *Staphylococcus aureus* clotted all of the plasma preparations within 1 h. Various constituents of the organisms, such as cell-free culture filtrates, sonicated extracts, and Formalin-killed bacteria, were also checked for their ability to clot citrated plasma. No constituents of any strain of *E. rhusiopathiae* clotted the plasma. Only culture filtrates of *S. aureus* clotted the plasma under these conditions. The spectrophotometric assay demonstrated that two plasma-clotting strains of *E. rhusiopathiae* consumed the citrate in the plasma just before clotting. Of 301 veterinary clinical isolates of *E. rhusiopathiae*, 267 (88.7%) were positive in the tube coagulation test. On the basis of these results, it was concluded that plasma clotting by *E. rhusiopathiae* was due not to extracellular factors such as staphylocoagulase but to consumption of the citrate in the plasma.

*Erysipelothrix rhusiopathiae* is the causative agent of swine erysipelas, which occurs in acute and chronic forms and causes losses in pig production. The coagulation of plasma caused by the majority of pathogenic strains of *Staphylococcus aureus* is a well-known phenomenon (2). Staphylocouagulase, either free or bound, is a protein produced by *S. aureus*. It is generally accepted as a typical feature of the organisms, enabling their differentiation from other similar and related bacteria by the coagulation test. Likewise, the coagulation of plasma by *E. rhusiopathiae* has been noted by Chang and Wu (M. H. Chang and S. F. Wu, Letter, Can. Vet. J. 21: 335, 1980) and by Tesh and Wood (14). Earlier reports of coagulase activity in *E. rhusiopathiae* were published by Nikolov (8, 9). They showed that the phenomenon was based on the existence of coagulase activity in *E. rhusiopathiae*. However, it has not yet been experimentally established whether the plasma clotting is actually caused by the production of a coagulase similar to that produced by *S. aureus*.

The purpose of the present investigation was to determine the mechanism by which *E. rhusiopathiae* clots plasma. Moreover, the difference in the ability of various sources or serotypes of *E. rhusiopathiae* isolates from pigs affected with erysipelas to clot plasma was studied.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. rhusiopathiae* 484, N-10, 487, and 549 (clinical isolates) were selected for the study of the mechanism of plasma clotting. *S. aureus* ATCC 6538P and *Staphylococcus epidermidis* ATCC 12228 were used as coagulase-positive and coagulase-negative controls, respectively.

A total of 301 strains of *E. rhusiopathiae*, including the type strain (ATCC 19414), belonging to various serotypes were investigated for the ability to clot citrated rabbit plasma. *E. rhusiopathiae* ATCC 19414*β* (originating from the spleen of a pig with endocarditis) (serotype 2) was obtained from the American Type Culture Collection, Rockville, Md. The remaining strains were isolated from cases of swine erysipelas in five meat inspection offices and seven livestock hygiene service centers in Japan from 1980 to 1983 (11–13). Infections associated with the isolates included arthritis (148 cases), lymphadenitis (65 cases), endocarditis (30 cases), urticaria (15 cases), and septicemia (42 cases).

**Preparation of plasma.** Fresh citrated rabbit plasma was prepared by placing 20 ml of blood obtained by cardiac puncture into a sterile bottle containing 5 ml of a 5% solution of sodium citrate. Plasma treated with sodium oxalate was prepared in the same manner as plasma treated with sodium citrate. EDTA-treated plasma contained 0.01 ml of a 10% solution of EDTA per ml of whole blood. Heparinized plasma was obtained from blood mixed with 100 U of heparin (Wako Chemical Industries, Ltd., Osaka, Japan) per ml. The plasma from at least two different rabbits was pooled.

**Plasma-clotting test.** Cultures to be tested for their ability to clot plasma were grown in beef infusion (BI) broth (pH 7.6, prepared in our laboratory). BI broth was composed of beef extract liquid (heat extracted from 500 g of beef with 1 liter of water) containing 1% (wt/vol) peptone (Mikuni Chemical Industries, Ltd., Tokyo, Japan) and 0.5% (wt/vol) NaCl. The plasma-clotting test was performed by adding 0.5 ml of a 24-h broth culture of the organism to 0.5 ml of plasma. After gentle mixing, the glass tubes (10 by 75 mm) were incubated at 37°C in a water bath and observed at various times for clot formation. Results were considered positive when firm clot formation was apparent.

**Preparation of constituents from the organisms.** Various constituents were prepared by the following procedure. *E. rhusiopathiae* 484 and *S. aureus* ATCC 6538P were cultured in BI broth at 37°C for 24 h. The bacteria were sedimented by centrifugation at 12,000 × *g* for 20 min, and supernatants were passed through a membrane filter with a pore size of 0.22 µm (culture filtrates). A portion of the cell-free supernatants was concentrated 10-fold by ultrafiltration with a
The sedimented bacterial cells were washed two times with phosphate-buffered saline (pH 7.2) and suspended in the initial volume of phosphate-buffered saline. The cell suspensions were divided into two samples. One sample was inactivated by the addition of 0.5% Formalin at 37°C overnight (Formalin-killed bacteria). The other sample was sonicated with a model UR-200P ultrasonic disrupter (Tomoy Seiko Corp., Ltd., Tokyo, Japan) at 20 KH for 5 min and then centrifuged at 12,000 × g for 20 min. The supernatant fraction was passed through a membrane filter with a pore size of 0.22 μm (sonicated extracts).

These constituents of the organisms described above were checked for clotting ability with citrated rabbit plasma in the same manner as that used for the broth culture. Clotting was eventually recorded after incubation at 37°C for 48 h. Controls for each test included unconinoculated BI broth or phosphate-buffered saline plus plasma (negative control) and a broth culture of each tested strain plus plasma (positive control).

**Determination of citrate and glucose concentrations in the plasma-clotting reaction system.** A UV method of enzymatic analysis with the F-kit (Boehringer Mannheim Yamanouchi K.K., Tokyo, Japan) was used to determine the concentrations of citrate and glucose in a mixture of a 10⁻² dilution of a culture of plasma-clotting strains or non-plasma-clotting strains of *E. rhusiopathiae* and citrated plasma at 0, 6, 12, 18, 24, and 48 h after incubation.

Citrate concentrations were determined as follows (6). Citrate is converted to oxaloacetate and acetate in the reaction catalyzed by the enzyme citrate lyase. In the presence of the enzymes malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate and its decarboxylation product, pyruvate, are reduced to L-malate and L-lactate, respectively, by NADH. The amount of NADH oxidized in these reactions is stoichiometric with the amount of citrate. NADH is determined by measuring its absorbance at 340 nm. Citrate concentrations as low as 0.02 mg/ml could be demonstrated by this method.

Glucose concentrations were determined as follows. d-Glucose is phosphorylated to glucose 6-phosphate in the presence of the enzyme hexokinase and ATP, with the simultaneous formation of ADP. In the presence of the enzyme glucose 6-phosphate dehydrogenase, glucose 6-phosphate is oxidized by NADP to gluconate 6-phosphate, with the formation of NADPH. The amount of NADPH formed in the reaction is stoichiometric with the amount of d-glucose. The increase in NADPH is determined by measuring its absorbance at 340 nm. Glucose concentrations as low as 0.03 mg/ml could be demonstrated by this method.

At the same time, portions (0.1 ml) of serial 10-fold dilutions of a mixture of culture and plasma were poured onto petri plates and mixed with BI agar. After 48 h of cultivation at 37°C, colonies in the agar were counted to determine the number of CFU.

**RESULTS**

**Effect of anticoagulants on plasma clotting.** The effect of anticoagulants on plasma clotting by two selected strains (484 and N-10) of *E. rhusiopathiae*, *S. aureus* ATCC 6538P, and *S. epidermidis* ATCC 12228 was examined. *E. rhusiopathiae* strains clotted solely citrated plasma in 18 to 24 h after incubation, whereas a coagulase-positive strain of *S. aureus* clotted all of the plasma preparations, prepared by use of sodium citrate, sodium oxalate, EDTA, and heparin, within 1 h. A coagulase-negative strain of *S. epidermidis* did not clot any plasma preparation. *E. rhusiopathiae* also clotted the citrated plasma obtained from mice and swine.

**Effect of various constituents of the organisms on plasma clotting.** The clotting ability of culture filtrates, sonicated extracts, and Formalin-killed bacteria was investigated. No constituents of *E. rhusiopathiae* 484 clotted citrated rabbit plasma, except for the whole bacterial culture used as a positive control for clotting. Only culture filtrates of *S. aureus* ATCC 6538P clotted the plasma in 3 h under these conditions.

**Concentrations of citrate and glucose in a mixture of cultures and plasma during incubation.** Changes in citrate and glucose concentrations and in the number of viable bacteria in a mixture of cultures of *E. rhusiopathiae* strains and citrated rabbit plasma during incubation are shown in Fig. 1. Plasma-clotting strains of *E. rhusiopathiae* 484 and N-10 suddenly exhausted the citrate in the plasma after 18 h (just before clotting). In contrast, the initial concentrations of citrate added to the plasma were unchanged in the reactions with non-plasma-clotting strains 487 and 549.

The glucose concentration suddenly decreased to 0.11 mg/ml after 18 h (just before clotting) in the reaction with plasma-clotting strain N-10, correlating with the citrate concentration at each time during incubation. Non-plasma-clotting strain 549 also exhausted the glucose in the plasma after 24 to 48 h.

The number of viable bacteria of the plasma-clotting strains in the mixture reached maximum values (approximately 10⁹ CFU/ml) more quickly than did that of the non-plasma-clotting strains.

**Clotting ability of 301 *E. rhusiopathiae* strains.** The relationship between the sources or serotypes of 301 *E. rhusiopathiae* strains from pigs with swine erysipelas and their ability to clot citrated rabbit plasma is shown in Table 1. *E. rhusiopathiae* ATCC 19414 clotted citrated plasma. Of 301 strains, 267 (88.7%) were positive in the plasma-clotting test. No distinct differences between the strains were found for clotting time, which was always 18 to 24 h. All of the strains that were negative in the clotting test were derived from chronic cases (arthritis, lymphadenitis, and endocarditis) of swine erysipelas, and 82.3% of the strains that were negative belonged to serotype 1a. Of 192 strains belonging to the most common serotype, serotype 2, 187 (97.4%) were positive in the plasma-clotting test.

**DISCUSSION**

The relatively long clotting time and the influence of anticoagulants and bacterial constituents on plasma clotting by *E. rhusiopathiae* indicate the presence of clotting mechanisms different from those of *S. aureus*. It is well known that plasma clotting by *S. aureus* is due to free or bound staphylocoagulase present in cultures (5, 7). Staphylocoagulase reacts specifically with prothrombin in a stoichiometric process. Both reactants possess no enzymatic activity, but their interaction results in the formation of a stable complex, called staphylobrinomen, that possesses specific proteolytic activity. It converts fibrinogen, a soluble protein, into insoluble fibrin in a manner similar to that of physiologically formed thrombin (10).

On the basis of the present results, we presumed that the citrate was utilized or exhausted by *E. rhusiopathiae*, thereby releasing calcium, which was then made available so that normal physiological clotting could occur. To verify this
hypothesis, we used a UV method of enzymatic analysis to determine the concentrations of citrate and glucose in a mixture of cultures and plasma during incubation. Plasma-clotting strains of *E. rhusiopathiae* exhausted the citrate in the plasma after 18 h, whereas the initial concentrations of citrate were virtually unchanged by plasma-clotting strains of *E. rhusiopathiae* after 48 h, despite maximum bacterial growth. In the reaction with a plasma-clotting strain, plasma glucose was suddenly exhausted after 18 h. The present results indicate that the clotting of plasma is caused by citrate utilization or degradation by late-log-phase *E. rhusiopathiae*. Further investigations will be required to clarify the catabolite basis for citrate utilization or degradation. This type of clotting process has been documented for *Serratia marcescens*, *Escherichia coli*, *Pseudomonas aeruginosa*, and group D streptococci (1, 3, 4, 15). These organisms do not produce a true coagulase enzyme but are able to break down the anticoagulant citrate and thus initiate the normal clotting pathways. Tesh and Wood (14) reported that *E. rhusiopathiae* did not use citrate as a carbon source and thus would be unable to cause coagulation by this procedure. Our data, however, show that plasma clotting by *E. rhusiopathiae* is due to the exhaustion of citrate during bacterial growth, in contrast to staphylocoagulase-induced clotting.

A total of 301 *E. rhusiopathiae* strains belonging to various serotypes and isolated from pigs with erysipelas were investigated for their ability to clot citrated rabbit plasma. Of 301 strains examined, 267 (88.7%) were positive in the plasma-clotting test. All of the non-plasma-clotting strains were derived from chronic cases of erysipelas, and most of them belonged to serotype 1a. We previously reported that most of these serotype 1a strains were weakly virulent for mice and swine (12). The virulence factor of *E. rhusiopathiae* is not clearly understood. Therefore, further investigations are required to determine the role of plasma-clotting activity in the pathogenicity associated with *E. rhusiopathiae*.

In general, *E. rhusiopathiae* is easily confused with other gram-positive bacilli, especially *Listeria monocytogenes* or *Corynebacterium* species, isolated from clinical specimens when identification is based on colony morphology and Gram staining. *E. rhusiopathiae* ATCC 19414F could clot citrated rabbit plasma. This clotting ability may be useful in differentiating *E. rhusiopathiae* from other gram-positive bacilli, which do not have this ability (14).

**LITERATURE CITED**