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Identification of 15- to 17-Kilodalton Antigens Associated with Virulent *Rhodococcus equi*

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Antigens of *Rhodococcus equi* were analyzed by immunoblotting with naturally infected foal sera. Immunoblots of whole-cell antigen preparations of clinical isolates of *R. equi* revealed that major protein bands with molecular masses of 15 to 17 kDa were present in all clinical isolates tested and all isolates virulent for mice. In contrast, the 15- to 17-kDa antigens were not identified by immunoblotting in ATCC 6939, a type strain of *R. equi* that was avirulent for mice. Whole-cell antigens of 102 environmental isolates were investigated by immunoblotting and the mouse pathogenicity test. Twenty-five of these isolates were demonstrated to contain the 15- to 17-kDa antigens by immunoblotting and were virulent for mice. The remaining 77 environmental isolates lacked the 15- to 17-kDa antigens and were avirulent for mice. These data suggest that the diffuse 15- to 17-kDa proteins are virulence-associated antigens with immunogenicity in foals and that they may be useful in marking virulent *R. equi* contamination in the environment of a horse-breeding farm.

Rhodococcus equi is an important bacterial pathogen which causes a serious, often fatal pneumonia and/or enteritis in foals aged 2 to 3 months (1). *R. equi* is a mucoid organism with a distinct lamellar polysaccharide capsule and pili (25, 26) which produces soluble factors (equi factors) which interact with phospholipase D of *Corynebacterium pseudotuberculosis* to cause complete hemolysis of mammalian erythrocytes (2). Moreover, it is thought to be a saprophytic soil inhabitant and to act as an opportunistic pathogen in young foals (1).

Attempts have been made to elucidate the virulence of *R. equi*, and it has long been recognized that variations in the outcome of experimental infections of foals are related to differences in bacterial virulence (7, 11, 22). Several investigators have reported differences in virulence in mouse models between equine lung isolates and soil isolates and have showed that the lethality of *R. equi* is related to the ability of the organisms to resist phagocytosis and intracellular killing by murine macrophages (13, 23). However, little is known of markers and/or factors associated with the virulence of *R. equi*. Capsule and equi factors are thought to be candidate virulence determinants. Serologic studies of *R. equi* indicate that considerable antigenic heterogeneity of the capsular antigen exists among the strains of the organism (14, 15). The relationship between the capsular serotypes and the pathogenicity of the organism is not known, and a recent study has shown that differences between serotypes found on farms appear to reflect geographic rather than virulence differences (14). Equi factors do not seem to play a major role in pathogenesis, since all *R. equi* strains produce equi factors with similar antigenicities (17, 19).

Recently, an equine humoral immune response to *R. equi* was demonstrated by an enzyme-linked immunosorbent assay (ELISA) (6, 8, 20), suggesting that a specific antibody may be more important to disease resistance than has been considered previously (12, 21). Chirino-Trejo and Prescott (5) examined rabbit antisera and foal sera to identify protein antigens in *R. equi* by an immunoblot technique. Some of those antigens seem to be useful in serodiagnostic testing for the infection; however, the importance of these antigens in bacterial virulence remains to be determined. Thus, there is little information regarding antigens associated with the virulence property and their immunogenicity in infected hosts.

The purpose of our study was to determine the immunologic response of foals naturally infected with *R. equi* to the major protein antigens of *R. equi* and, from these data, to identify virulence-associated antigens.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were *R. equi* ATCC 33701 and ATCC 6939. In addition, 116 isolates were used: 2 isolates (strains CE220 and T48) from the lungs of two foals naturally infected with *R. equi* (13, 22); 12 isolates (strains L1 to L12) from lesions associated with pneumonia and enteritis in a foal naturally infected with *R. equi*; and 102 isolates (strains K1 to K102) from soil at a horse-breeding farm at which the infected foal was bred. Soil samples were collected from two sites at the farm. These were soil from within 2 m of the stable in which the infected foal was stalled and soil from the paddock for a mare with a foal at foot. All of these strains, except for strain CE220, were isolated, identified, and serotyped in our laboratory as previously described (24). The isolates were identified as gram-positive pleomorphic bacilli which were nonmotile and non-acid fast. The colonies were nonhemolytic, creamy, mucoid, moist, and glistening on blood agar; after 2 to 3 days of growth, the colonies were mucoid and pink. The isolates produced catalase and urease and reduced nitrate but were oxidase negative and did not produce gelatinase. Glucose, sucrose, lactose, maltose, mannose, xylose, and rhamnose were not fermented. Strain CE220 was obtained from M. Nakazawa, National Institute of Animal Health, Tsukuba, Japan.

Foal sera. Serum samples were collected from five foals naturally infected with *R. equi*. Two of these five infected

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foals (foals 3 and 4) were ones from which isolates T48 and L1 to L12 were obtained. Control serum samples were obtained from healthy foals.

Preparation of antigens. *R. equi* ATCC 33701 was used for the preparation of supernatant antigens. The bacteria were cultured in yeast extract-casein-cystine broth at 37°C for 5 days. The antigens in the culture supernatant were precipitated with 35% saturated ammonium sulfate and then with 55% saturated ammonium sulfate. The precipitates were suspended in 0.01 M phosphate buffer (pH 7.0) and dialyzed against phosphate buffer overnight. The dialysates were designated the 35% precipitate and 55% precipitate antigens, respectively. Whole-cell antigens were prepared by harvesting 48-h growth at 37°C from yeast extract-casein-cystine broth. Samples were solubilized in sodium dodecyl sulfate (SDS) reducing buffer containing 0.0625 M Tris hydrochloride (pH 6.8), 10% (vol/vol) glycerol, 2% SDS, 5% 2-mercaptoethanol, and bromphenol blue and boiled for 10 min. The undissolved material was sedimented by centrifugation at 7,000 × *g* for 3 min. Whole-cell antigen preparations contained approximately 10^7 cells per 10 μl of sample buffer per lane, and precipitate antigen preparations contained approximately 20 μg of protein per 10 μl of sample buffer per lane.

Gel electrophoresis and immunoblot analysis. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the discontinuous method of Laemmli (9) with a vertical slab gel of 12.5% and a stacking gel of 4.5% polyacrylamide. Ten microliters of sample was loaded per lane and electrophoresed at 150 V for 1.5 h. Upon completion of SDS-PAGE, protein-containing bands were determined by staining with 5% Coomassie blue or were transferred to nitrocellulose sheets (Toyo Roshi Inc., Tokyo, Japan) for subsequent immunologic analysis.

The blotted nitrocellulose sheets were incubated in Block Ace (a blocking agent made from milk; Yukijirushi Nyugyo Inc., Tokyo, Japan) for 60 min at 37°C to block any unbound sites on the membranes. The sheets were incubated for 2 h at 37°C with foal serum samples which had been diluted 1:100 in Block Ace. The sheets were washed (three times for 10 min each time) in 0.05% Tween 20 in Tris-buffered saline (pH 7.4). Horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) was diluted 1:1,000 in Block Ace and added to the sheets, and the sheets were incubated for 1 h at 37°C. After the sheets were washed, the substrate diaminobenzoic acid (Bio-Rad Laboratories, Richmond, Calif.) and hydroperoxide were added. Development was stopped by washing the sheets in distilled water.

ELISA. The ELISA was performed by established methods described by Takai et al. (20).

Mouse pathogenicity test. Isolates were grown for 48 h in yeast extract-casein-cystine broth at 37°C. Cultures were diluted 10-fold with sterile saline. Six mice were used for each environmental isolate. Two groups of three mice each for each strain were tested at two inoculum levels (approximately 10^7 cells and 10^6 cells). Twenty- to 23-g ddY mice were each given 0.2 ml of the bacterial suspension intravenously. The mice were observed for 10 days, and deaths were recorded. Strains that killed two or more mice at the concentration of 10^6 cells were considered to be virulent. Two different inocula were used to ensure that the avirulent strain did not kill mice even at the concentration of 10^7 cells. The virulence of clinical isolates was estimated by determining the 50% lethal dose by the probit method on groups of five mice. The inoculum range used was 10^4 to 10^8 cells in 0.2 ml of the bacterial suspension for all of the strains, except for strain ATCC 6939, for which the range was 10^5 to 10^{10} cells. The number of deaths was recorded for 10 days after intravenous inoculation, and the 50% lethal dose was calculated by the method of Reed and Muench (18).

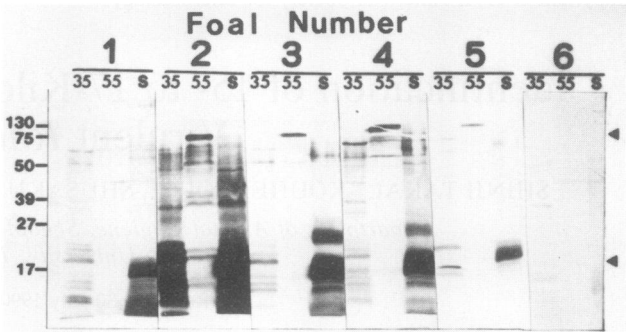

FIG. 1. Immunoblot analysis of culture supernatants and whole-cell preparations of *R. equi* with sera from five naturally infected foals and serum from a healthy foal. Culture supernatants in which protein antigens were precipitated by 35% saturated ammonium sulfate (lanes 35) and 55% saturated ammonium sulfate (lanes 55) and whole-cell preparations (lanes S) of *R. equi* ATCC 33701 were analyzed by immunoblotting. Lanes 1 to 5 correspond to the foals naturally infected with *R. equi*. Lane 6 corresponds to the healthy foal. The numbers on the left indicate molecular masses in kilodaltons. Arrowheads indicate 74-kDa and 15- to 17-kDa proteins.

RESULTS

Reaction of naturally infected-foal sera with *R. equi* antigens in immunoblotting. The immunoblots of the three different preparations of *R. equi* antigens from SDS-PAGE with sera from five naturally infected foals are shown in Fig. 1. Comparison of the immunoblot profiles of the three antigen preparations revealed that infected-foal sera reacted with several components of the *R. equi* antigens. Sera from five infected foals reacted with components of the whole-cell antigen with approximate molecular masses of 15 to 17 kDa and with components of the 55% precipitate antigen with an approximate molecular mass of 74 kDa. Serum from infected foal 2 recognized several components of the 35% precipitate antigen with approximate molecular masses of 10 to 21 kDa. The most intense and consistent reactions occurred with the diffuse proteins with approximate molecular masses of 15 to 17 kDa. Sera from healthy foals showed little or no reaction with the three antigens (some of the results are shown in Fig. 1). Sera from infected foals 1, 2, 3, and 4 had ELISA antibody values (optical density at 492 nm) ranging from 0.6 to 1.7 for *R. equi* antigens. Serum from foal 5 had an ELISA antibody value of 0.3. Sera collected from healthy foals (including foal 6) had ELISA antibody values of <0.1. These findings suggest that the 15- to 17-kDa antigens were produced in vivo by *R. equi* and were distinctly recognized by infected hosts.

Detection of 15- to 17-kDa antigens in clinical isolates from lesions of lungs and lymph nodes of naturally infected foals. To determine whether clinical isolates had 15- to 17-kDa antigens, we tested 14 clinical isolates from lesions of infected foals and 2 control strains for 15- to 17-kDa bands by immunoblotting with the infected-foal serum (from foal 4). This serum was chosen since it showed good reactivity with *R. equi* antigens and was obtained in quantities large enough to pursue this experiment. All of the clinical isolates as well

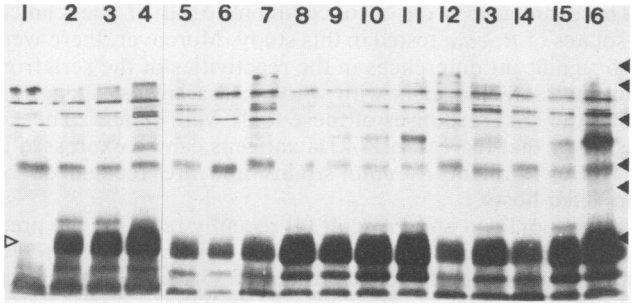

FIG. 2. Immunoblot profiles of 14 clinical isolates and 2 type strains of *R. equi*. Whole-cell preparations were analyzed by immunoblotting with serum from a naturally infected foal (foal 4). Lanes: 1, strain ATCC 6939; 2, strain ATCC 33701; 3, strain CE220; 4, strain T48; 5, strain L1; 6, strain L2; 7, strain L3; 8, strain L4; 9, strain L5; 10, strain L6; 11, strain L7; 12, strain L8; 13, strain L9; 14, strain L10; 15, strain L11; 16, strain L12. The molecular weight markers (M_r) phosphorylase *b* (130,000), bovine serum albumin (75,000), ovalbumin (50,000), carbonic anhydrase (39,000), soybean trypsin inhibitor (27,000), and lysozyme (17,000) are indicated by arrowheads on the right. The arrowhead on the left indicates the 15- to 17-kDa antigens.

as strain ATCC 33701, but not strain ATCC 6939, contained the 15- to 17-kDa antigens. The whole-cell antigens of the 16 strains were compared by SDS-PAGE. No major differences were detected among the 16 strains tested (data not shown). The only minor difference detected was the lack of a faint and diffuse 15- to 17-kDa band for strain ATCC 6939.

Association between mouse pathogenicity and the presence of 15- to 17-kDa antigens in *R. equi*. To clarify the relationship between mouse pathogenicity and the presence of 15- to 17-kDa antigens in clinical isolates of *R. equi*, we examined the mouse pathogenicity of the 14 clinical isolates from lesions of lungs and lymph nodes and of the 2 control strains (Table 1). All mice inoculated with 10^6 cells of the 14 clinical isolates and strain ATCC 33701 died on day 3 or 4. On the other hand, mice inoculated with 10^7 cells of strain ATCC 6939 survived and showed no clinical signs of pathogenicity, such as weight loss, during the experiment.

TABLE 2. Comparison of immunoblot profiles and mouse pathogenicity test results in clinical and environmental isolates of *R. equi*

Isolates (*n*)	15- to 17-kDa antigens[a]	No. of isolates with the following mouse pathogenicity test result[b]:	
		Virulent	Avirulent
Clinical (14)	+	14	0
Environmental (102)[c]	+	25	0
	−	0	77

[a] See Table 1, footnote *a*.
[b] Strains were considered virulent if at least two of three mice injected intravenously with 10^6 CFU died within 10 days.
[c] Soil samples were collected from a horse-breeding farm with endemic *R. equi* infection.

Application of 15- to 17-kDa antigens as a marker for virulence to differentiate virulent and avirulent *R. equi* isolates from soil. One hundred and two soil isolates from the horse-breeding farm at which the infected foal (foal 4) was bred were tested for the presence of 15- to 17-kDa antigens and mouse pathogenicity (Table 2 and Fig. 3). Twenty-five of the 102 (21%) isolates contained 15- to 17-kDa antigens, as determined by immunoblotting (some of the results are shown in Fig. 3). Several bands, except for the 15- to 17-kDa bands, were common to each of eight soil isolates on the immunoblots (Fig. 3). These common bands were also present in the immunoblots of the remaining 94 soil isolates. All mice inoculated at the two inoculum levels (10^7 and 10^6 cells) of these 25 isolates died on day 3 or 4. On the other hand, none of the mice inoculated with 10^7 or 10^6 cells of the remaining 77 isolates (lacking the 15- to 17-kDa antigens) died during the experiment (Table 2), indicating a strong association between the presence of the 15- to 17-kDa antigens and mouse pathogenicity.

TABLE 1. Results of immunoblotting, 50% lethal dose determinations, and serotyping for 14 clinical isolates and 2 type strains of *R. equi*

Strain	Source	15- to 17-kDa antigens[a]	50% Lethal dose	Prescott's serotype	Reference or source
Clinical isolates					
CE220	Lung abscess	+	2.1×10^6	3	13, 14
T48	Lung abscess	+	3.2×10^6	3	22
L1	Lung abscess	+	1.7×10^6	3	This study
L2	Lung abscess	+	1.9×10^6	3	This study
L3	Lung abscess	+	2.5×10^6	3	This study
L4	Lung abscess	+	2.0×10^6	3	This study
L5	Lung abscess	+	2.9×10^6	3	This study
L6	Lung abscess	+	1.7×10^6	3	This study
L7	Lung abscess	+	1.7×10^6	3	This study
L8	Lung abscess	+	1.6×10^6	3	This study
L9	Bronchial lymph nodes	+	1.7×10^6	3	This study
L10	Bronchial lymph nodes	+	1.5×10^6	3	This study
L11	Bronchial lymph nodes	+	1.6×10^6	3	This study
L12	Mesenteric lymph nodes	+	1.5×10^6	3	This study
Type strains					
ATCC 6939	Lung abscess	−	2.0×10^9	1	10, 23
ATCC 33701	Lung abscess	+	1.2×10^6	1	13, 15

[a] + and −, Presence and absence of 15- to 17-kDa antigens, as determined by immunoblotting.

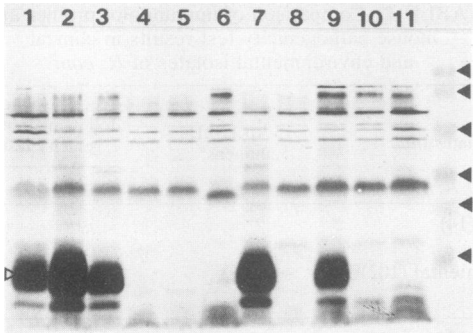

FIG. 3. Immune detection of the 15- to 17-kDa *R. equi* antigens in whole-cell preparations of environmental isolates. Whole-cell preparations of eight isolates were analyzed by immunoblotting with serum from a naturally infected foal (foal 4). Strains ATCC 33701, L6, and L10 were used as positive controls. Lanes: 1, strain ATCC 33701; 2, strain L6; 3, strain L10; 4, strain K1; 5, strain K2; 6, strain K3; 7, strain K4; 8, strain K5; 9, strain K6; 10, strain K7; 11, strain K8. Arrowheads are as in Fig. 2.

Relationship between virulence and capsular serotypes of *R. equi*. To determine the relationship between virulence and serotypes of *R. equi*, we compared the capsular serotypes and virulence of the 14 clinical isolates, the 102 environmental isolates, and the 2 control strains. The two control strains, ATCC 33701 and ATCC 6939, belonged to Prescott's serotype 1, and the 14 clinical isolates from lesions belonged to Prescott's serotype 3, as determined by the agar diffusion test (Table 1). The capsular serotypes of the 102 soil isolates are shown in Table 3. Six of the 25 (24%) virulent strains from soil belonged to Prescott's serotype 3, and the other 19 virulent strains were untypeable. On the other hand, 9 of the 77 (12%) avirulent strains from soil belonged to Prescott's serotype 3, 7 belonged to 1, 4 belonged to 2, 3 belonged to 5, 4 belonged to 6, and 2 belonged to 7. Forty-eight were untypeable. The prevalence of Prescott's serotype 3 was high in the clinical isolates from Aomori prefecture. However, some environmental isolates belonging to serotype 3 were virulent, and others were avirulent.

DISCUSSION

The studies presented here demonstrated that sera from foals with *R. equi* infections recognized epitopes from the 15- to 17-kDa antigens and that these diffuse proteins were present on the clinical isolates as well as on some of the environmental isolates which were virulent for mice.

To identify the proteins associated with infection, we compared the seroreactivities of antigens prepared from culture supernatants and whole cells of *R. equi* ATCC 33701 with sera from the five infected foals by immunoblot analysis. Of the several major antigens identified, 15- to 17-kDa antigens were strongly recognized by the infected-foal sera.

TABLE 3. Serotyping of 102 environmental isolates of *R. equi*

Mouse pathogenicity test result	Total no. of isolates	No. (%) of isolates						
		Of Prescott's serotype:						That were untypeable
		1	2	3	5	6	7	
Virulent	25			6 (24)				19 (76)
Avirulent	77	7 (9)	4 (5)	9 (12)	3 (4)	4 (5)	2 (3)	48 (62)

These antigens were also common to all of the clinical isolates of *R. equi* tested in this study. Moreover, there were no significant differences in the reactivities of the sera from the five infected foals with whole-cell antigens, suggesting that the host responses of these foals were similar. Thus, it is likely that the 15- to 17-kDa antigens can be expressed in vivo by virulent *R. equi* strains and are recognized by infected hosts.

Chirino-Trejo and Prescott (4) recently reported the presence of a prominent and somewhat diffuse protein of 17.5 kDa and a less prominent protein of 15.1 kDa in a clinical isolate from the lungs of an infected foal; these proteins were absent from the type strain, ATCC 6939. These antigens were strongly recognized by rabbit hyperimmune serum; however, the reactivity of infected-foal sera with these antigens was not examined by immunoblotting (5). In the present study, the 15- to 17-kDa bands were always observed to be diffuse, and it was difficult to distinguish them as two separate bands in the immunoblots. However, the 15- to 17-kDa antigens may be the same proteins that were observed by Chirino-Trejo and Prescott (5) with rabbit serum. The difference in the immunoblot profiles of foal and rabbit serologic responses may have resulted from the different routes of antigenic presentation, the different strains used, or the different antibody titers of the sera.

The 74-kDa band found in the 55% precipitate also was recognized strongly by the sera from the five infected foals. This antigen, called equi factor, was prepared by the method of Prescott (16); our result supports the observation that their serologic determination can be used in the diagnosis of naturally occurring *R. equi* infections. The molecular mass of equi factor has been reported to be 74 kDa (2). The 74-kDa band described in this study may represent this equi factor, but further study is needed for confirmation.

Variations in the virulence of isolates of *R. equi* have long been known. Flatla (7) found that *R. equi* strains isolated from soil had no effect when fed to foals but that clinical isolates caused severe lesions. Martens et al. (11) and Takai et al. (22) noted that the laboratory strain isolated originally by Magnusson (10), ATCC 6939, failed to induce lesions in foals following aerosolization or intratracheal inoculation, which readily induced pneumonia with clinical isolates. The virulence of *R. equi* in mice correlates with the clinical disease in horses. Consequently, the mouse model has become the most widely used and accepted method of determining strain virulence (1, 3, 13, 23). In this study, the 15- to 17-kDa antigens were produced by all of the clinical isolates, ATCC 33701, and some of the environmental isolates. All strains containing these antigens were virulent in mice, while avirulent strains, including ATCC 6939, lacked the 15- to 17-kDa molecules, suggesting that these antigens may play an important role in the pathogenesis of *R. equi* infections and/or serve as a marker for virulence.

Inhalation or ingestion appears to be the major route of infection in foals, and the primary source of infection is believed to be the soil (1). In the present study, 25 of the 102 soil isolates were pathogenic for mice, and interestingly, these isolates were obtained from a stud in which *R. equi* infection was endemic. Similar results were reported by Bowles et al. (3). Four of the six strains isolated from the environment failed to proliferate in parenchymal organs and caused minimal lesions, whereas the remaining two strains from the environment (from a stud with epidemic *R. equi* infection) and all of the clinical isolates caused multiple pyogranulomatous lesions in the liver and spleen (3). These studies suggest that soil contamination with virulent *R. equi*

may be related to the prevalence of *R. equi* in foals in horse-breeding farms. It is noteworthy that outbreaks of the disease could sometimes be averted by moving the in-foal mares to areas in which the disease had not occurred (10). The present study clearly reveals that soil is a potential source of the infection and suggests that farms with the potential for endemic infection can be distinguished on the basis of whether virulent *R. equi* has contaminated their environment. Further studies, in both Japan and other countries, are needed to test the prevalence of virulent *R. equi* among environmental isolates collected from areas in which *R. equi* infection is endemic as well as those in which *R. equi* infection has not occurred to confirm this suggestion.

All of the clinical isolates tested in this study belonged to Prescott's serotype 3, as did 6 of 25 (24%) virulent strains and 9 of 77 (12%) avirulent strains isolated from the soil. This result clearly indicates that the appearance of a certain capsular serotype is not associated with virulence. Nakazawa et al. (14) have shown that differences between serotypes found on farms appear to reflect geographic rather than virulence differences. Thus, the prevalence of serotype 3 in the virulent isolates may reflect a geographic difference rather than a high correlation with clinical disease.

In summary, we have identified by immunoblotting a diffuse group of proteins in clinical isolates of *R. equi* that band at the 15- to 17-kDa region of an SDS gel. These proteins have also been found in environmental isolates virulent for mice. These data demonstrate that these *R. equi* proteins are present in epidemiologically important strains, making them potentially useful as virulence markers in antigen detection assays. Further studies are needed to clarify the composition, structure, function, immunology, biosynthesis, and regulation of these proteins.

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