Antibody to *equi* Factor(s) in the Diagnosis of *Corynebacterium equi* Pneumonia of Foals

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ABSTRACT

Antibody to *equi* factor(s) in cases of *Corynebacterium equi* pneumonia in foals was detected using *C. pseudotuberculosis* exotoxin sensitized calf red blood cells. The test was standardized using antitoxin produced in rabbits by injection of *equi* factor(s). All sera from ten foals with culture-diagnosed *C. equi* pneumonia had antibodies to *equi* factor(s) (titre range 8-256, mean 74.0) and nine sera from 11 foals with suspected *C. equi* pneumonia also showed antibodies (titre range 4-512, mean 136.4). Two of five pneumonia foals with transtracheal aspirate cultures not yielding *C. equi* had such antibodies. Fifty-eight of 59 control horse sera had no antibodies; the one positive serum came from a foal on a farm where *C. equi* pneumonia was endemic. By contrast only five of 15 foals with experimentally-induced *C. equi* pneumonia had antibodies to *equi* factor(s), probably because the acute nature of the disease produced did not mimic the chronic course of the natural disease. Antibody to *equi* factor(s) can be used in the diagnosis of naturally-occurring corynebacterial pneumonia in foals.

Key words: *Corynebacterium equi, equi* factor(s) antibody, foal pneumonia, diagnosis.

RÉSUMÉ

L'utilisation d'hématies de veaux sensibilisées à l'exotoxine de *Corynebacterium pseudotuberculosis* a permis de détecter des anticorps contre le ou les facteurs *equi*, chez des poulains atteints de pneumonie due à *Corynebacterium equi.* La standardisation de l'épreuve s'effectua à l'aide d'une antitoxine produite chez le lapin, par l'injection du ou des facteurs *equi*. Le sérum de chacun des dix poulains atteints d'une pneumonie de laquelle la bactériologie permit d'isoler *C. equi* recelait un titre d'anticorps contre le ou les facteurs *equi* qui variait de 1:8 à 1:256 et dont la moyenne se situait à 1:74. Le sérum de neuf des 11 poulains chez lesquels on soupçonnait une pneumonie à *C. equi* possédait aussi de tels anticorps dont le titre variait de 1:4 à 1:512 et affichait une moyenne de 1:136,4. Par ailleurs, deux des cinq poulains qui souffraient de pneumonie et dont la culture de l'exsudat bronchique s'avéra négative, possédaient aussi de tels anticorps. Le sérum de 58 des 59 témoins ne contenait toutefois pas de ces anticorps; le seul qui en recelait provenait d'un poulain issu d'une ferme où la pneumonie à *C. equi* existait à l'état enzootique. Par contraste, seulement cinq des 15 poulains soumis à une infection pulmonaire expérimentale par *C. equi* possédaient des anticorps contre le ou les facteurs *equi*, probablement parce que le caractère aigu de cette pneumonie différait du caractère chronique de la maladie naturelle. Les anticorps précités peuvent par conséquent servir au diagnostic de la pneumonie naturelle du poulain due à *C. equi.*

Mots clés: *Corynebacterium equi*, anticorps contre le ou les facteurs *equi*, pneumonie du poulain, diagnostic.

INTRODUCTION

Corynebacterium equi is an important cause of pneumonia in foals (1). The infection can be diagnosed in the live animal by bacteriological culture of transtracheally aspirated bronchial material (2), by detection of precipitating serum antibodies in the serum (3) and in young foals by demonstration of *C. equi* sensitized lymphocytes (4). Culture of aspirated material does not always yield *C. equi* in known cases (2). Lymphocyte blastogenesis has the drawback of being a somewhat cumbersome procedure. There is a need for a rapid and simple laboratory method of diagnosing *C. equi* pneumonia.

Corynebacterium equi produces soluble factors which interact with the phospholipase D of *Corynebacterium pseudotuberculosis* to give complete hemolysis of mammalian erythrocytes (5). There are at least two activities having hemolysis-enhancing effect. In horses detection of antibody to *C. pseudotuberculosis* toxin, using prevention of synergistic hemolysis with *equi* factor(s), has been used to diagnose *C. pseudotuberculosis* infections (6). It occurred to us that detection of antibody to *equi* factor(s) in pneumonic foals might be a simple diagnostic test for *C. equi* pneumonia.

MATERIALS AND METHODS

PRODUCTION OF *EQUI* FACTOR

A modification of the method of Knight (6) was used. *Corynebacterium equi* ATCC 33701 was the source of *equi* factor(s). Two loopfuls of a 48-hour plate culture of *C. equi* were inoculated into 250 mL of brain heart infusion broth (Difco, Detroit, Michigan) containing 0.1% v/v Tween 80 (Fisher Scientific). The baffle-flasks

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were shaken at 150 rpm at 37°C for three days before refrigeration at 4°C for one to three days. The broth was then centrifuged at 13,800 x g for 30 min at 4°C and merthiolate added (1:10,000 w/v) to the supernatant. During all subsequent steps the preparation containing *equi* factor(s) was kept at 4°C.

A saturated solution of ammonium sulfate was prepared by dissolving 760 g in 1 L of distilled water, autoclaved and left to stand at room temperature for several days. The solution was then added slowly to culture supernatant at 4°C to give a 55% (v/v) saturated solution. The solution was stirred throughout this procedure. Following refrigeration at 4°C for one to four days the precipitate was recovered by centrifugation at 37,000 x g for 30 min at 4°C, resuspended in 40 mL phosphate buffered saline (PBS), pH 7.0, and dialyzed against distilled water at 4°C for one to three days using 12,000 MW tubing. The dialyzed solution was treated again in the same way. Sodium chloride (9.0 mg/mL) was added to the dialyzed solution, which was filtered through a 0.22 μm filter. The solution was lyophilized and stored at -70°C.

PRODUCTION OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* SENSITIZER

Corynebacterium pseudotuberculosis strain 15 (C.A. Muckle, University of Guelph) was used. Two loopfuls of a 48-hour plate culture were inoculated into 250 mL of brain heart infusion broth with 0.1% (v/v) Tween 80 in a 1 L baffle flask. Cultures were shaken at 150 rpm at 37°C for 18-24 h, then kept at 4°C for one to three days. The supernatant was removed after centrifugation at 13,800 x g for 30 min at 4°C and filtered through a 0.22 μm filter. Merthiolate was added as a preservative at 1:10,000 (w/v) and the supernatant lyophilized.

SYNERGISTIC HEMOLYSIS INHIBITION TEST

Sensitization of red blood cells with *C. pseudotuberculosis* toxin: Steamed trypticase soy agar (Difco) with 0.1% (w/v) sodium azide was dispensed in aliquots of 33.6 mL, autoclaved and kept at 50°C. The volume lost by autoclaving was corrected with sterile distilled water. Weighed lyophilized *C. pseudotuberculosis* supernatant was diluted with PBS to various concentrations and 1 mL added to each tube of agar base. Bovine red blood cells (RBC) washed three times in normal saline were added to each tube to give a final concentration of 1% (v/v). The sensitized RBC agar mixture was poured into 150 x 15 mm Petri plates to give a thickness of 4 mm. Plates were left at room temperature overnight, then refrigerated.

Standardization of test reagents: The lyophilized preparation containing *equi* factor(s) was diluted to various concentrations in PBS. Sterile absorbent discs of 12.7 mm diameter (Schleicher and Schnell Co., Keene, New Hampshire) were touched to the surface of the liquid for five seconds, rotated 180°C and touched for a further five seconds, and then placed on sensitized RBC agar plates. After incubation under conditions described in the Results, the size of the hemolytic zone was measured at right angles to the widest area of hemolysis, across the centre of the disc.

The optimal test conditions were examined by varying the concentration of *C. pseudotuberculosis* sensitizer, the thickness of the agar, the concentration of RBC, as well as the length of refrigeration before use of sensitized plates and the length of incubation after inoculation.

Hemolysis inhibition test: Serial twofold dilutions of serum starting at 1:2 dilution were made in 0.5 mL of PBS, pH 7.2. A 0.1 mL aliquot of each dilution was transferred to wells in a Perspex tray. Equal volumes of two units of *equi* factor(s), as defined in the Results, were added to each well. The trays were covered with parafilm and shaken at room temperature at 60 rpm for 60 min and 12.7 mm discs were then used to absorb the reagents as described. The discs were placed on *C. pseudotuberculosis* sensitized RBC agar plates and incubated at 37°C for three days. The concentration of *C. pseudotuberculosis* sensitizer was standardized as described in the Results. A positive control disc of a saline dilution containing one unit of *equi* factor(s) and a negative control disc containing one unit of *equi* factor(s) with one unit of antibody were included on each plate. Antibody for use in controls was prepared in rabbits by intravenous inoculation of progressive quantities of lyophilized crude *equi* factor(s).

SERUM SAMPLES

Sera from 44 horses admitted to the Ontario Veterinary College Hospital for problems other than respiratory disease were obtained. Sera from 15 foals obtained before experimental *C. equi* infection were also used. Sera from ten foals diagnosed with *C. equi* pneumonia (by lung or transtracheal aspiration culture) and from 16 foals with suspected *C. equi* pneumonia were examined. The majority of the suspected cases came from a farm where *C. equi* pneumonia was endemic. In five "pneumonic" foals pathogens other than *C. equi* were isolated from transtracheal aspirates. Most of the sera had been stored at -70°C for up to two years. Sera from 15 foals taken following experimentally-induced *C. equi* pneumonia were obtained from Dr. R.J. Martens, Department of Large Animal Medicine and Surgery, College of Veterinary Medicine, Texas A & M University. All sera were heated at 56°C for 60 min prior to testing.

RESULTS

STANDARDIZATION OF TEST REAGENTS

Preliminary experiments, not reported fully here, established that the thickness of agar (1-5 mm) did not appreciably alter the sizes of the hemolytic zones. The concentration of *C. pseudotuberculosis* sensitizer had a marked effect on size of the hemolytic zone caused by the *equi* factor(s), but high concentrations (10.0 g lyophilate/L) resulted in complete hemolysis of RBC, in the absence of *equi* factor(s), within a few days of preparation. Complete hemolysis, caused by the sensitizer alone, occurred more quickly with thin plates and low concentrations of RBC. A concentration of 1% (v/v) RBC and agar thickness of 4 mm gave optimal results in the presence of 0.1 g *C. pseudotuberculosis* lyophilate/L. The longer that plates containing sensitizer alone were refrigerated, the wider was the subsequent zone size, after reaction with *equi* factor(s), but this effect was neg-

ligible for plates kept up to two weeks after preparation. All plates were thus used within two weeks of pouring.

The most important variable affecting width of the synergistic hemolytic zone was the length of incubation of discs containing *equi* factor(s). Three days of incubation at 37°C increased the sensitivity of the test about 25-fold compared to one day of incubation. After three days, plates tended to show total hemolysis.

Once the optimal test conditions were determined a unit of *equi* factor(s) was defined as the highest dilution of lyophilate which would, after incubation at 37°C for three days, produce a zone of complete hemolysis extending 1-3 mm beyond a saturated filter paper disc placed on 4 mm thick agar plates containing 1% blood sensitized with *C. pseudotuberculosis* exotoxin. A unit of antibody was the highest dilution of rabbit antiserum which failed to produce a zone of hemolysis around a disc soaked with one unit of *equi* factor(s), after three days of incubation. The amount of *C. pseudotuberculosis* sensitizer required was the lowest concentration, which after three days of incubation at 37°C with a disc saturated with one unit of *equi* factor(s), showed a 1-3 mm zone of hemolysis.

INHIBITIONS OF SYNERGISTIC HEMOLYSIS BY HORSE SERA

Titres were recorded as the highest serum dilution which inhibited hemolysis. Some sera (43%) from both control and pneumonic horses showed hazy hemolytic activity (mean 2.6) up to a titre of 8. This hemolysis was hard to distinguish from synergistic hemolysis and titres were recorded as zero. Some of the sera from pneumonic foals showed hazy hemolysis at lower dilutions followed by inhibition for several more dilutions; titres for these foals were the highest dilution of inhibition. Results are shown in Table I. One of 59 sera from control horses showed a titre of 16. This foal was from a farm where *C. equi* was endemic, although there was no history of pneumonia in the animal.

All foals with culture positive *C. equi* pneumonia but only five of 15 foals with experimental *C. equi* pneumonia had antibodies.

DISCUSSION

Corynebacterium equi produces two enzymes which cause synergistic hemolysis when combined with the sphingomyelin-specific phospholipase D of *C. pseudotuberculosis*; these enzymes are a cholesterol oxidase and a ceramide phosphate-active phospholipase C (5). Of the two enzymes the cholesterol oxidase is probably more important (5). We did not distinguish these two activities in our test and consequently refer to *equi* factor(s) rather than *equi* factor. This study shows that *equi* factor(s) are produced *in vivo* and, as Linder and Bernheimer (5) suggested, may be significant in the pathogenesis of disease caused by *C. equi*. All *C. equi* produce *equi* factor(s) (7).

An important finding was that incubation of *C. pseudotuberculosis* exotoxin-sensitized RBC for three days greatly increased the sensitivity of the test for *equi* factor(s) hemolysis. Antibody to *equi* factor(s) was detected in sera from all the foals with confirmed *C. equi* pneumonia and in nine of 11 foals with suspected *C. equi* pneumonia. The two *equi* factor(s) negative foals in the 11 suspected cases both had acute pneumonia. Antibody was detected in only one of 59 control sera. The one positive control serum was from a foal from a farm where *C. equi* was endemic. Two of five pneumonic foals with bacteria other than *C. equi* isolated from transtracheal aspirates (other foal pneumonias, Table I) had antibody to *equi* factor(s). The failure of transtracheal aspiration to diagnose some cases of corynebacterial pneumonia in foals has been reported (2) and is also suggested by our findings in these two cases. The failure to detect *equi* factor(s) antibody in ten of 15 foals with experimentally-induced *C. equi* pneumonia was puzzling in view of the presence of antibody in diagnosed and suspected cases of *C. equi* pneumonia. This difference from naturally affected animals might be accounted for by the nature of the experimental challenge (by aerosol or umbilical vein), which is likely to have given a more acute infection than the chronic course of the naturally-occurring disease.

The results of this study suggest that detection of antibody to *equi* factor(s) can be used in the diagnosis of naturally-occurring *C. equi* pneumonia. We have not defined the relationship between the level of antibody and the severity of the disease, nor between the onset of antibody production and the stage of the disease. The findings in the experimentally-infected foals suggest that little or no antibody would be detected in the early stages of *C. equi* pneumonia using this test and results from the two negative foals with acute pneumonia in the "suspected" group tend to bear this out. The development of a rapid method of detecting antibody to *equi* factor(s) more sensitive than the technique described here might be a rewarding approach to the diagnosis of *C. equi* pneumonia. The test would be improved with pure preparations of each of the *equi* factor(s) alone or in various combinations.

The presence of hazy hemolysis in about half the sera at dilutions of up to 1:8 reduces the value of the test for animals with low antibody titres. In a collection of sera from horses with *C. equi* pneumonia received after four weeks in the postal system, such hemolysis was more marked. Results for these animals were not included but suggest that such hemolysis may be an aging phenomenon or a result of bacterial contamination of the sera.

Note added in proof:

Since this article was written, Dr. Boris Skalka of the Veterinary School,

Table I. *Equi* Factor(s) Serum Antibody in Foals Diagnosed With or Suspected of *Corynebacterium equi* Pneumonia, with Pneumonia of Other Apparent Origin, or in Normal Horse Sera

	Antibody titre to *equi* factor(s)									
Source	0	4	8	16	32	64	128	256	512	Total
Diagnosed cases			2		2	4	1	1		10
Suspected cases	2	1	1	1		1	2	1	2	11
Other foal pneumonias	3		2							5
Experimental foals	10						2	2	1	15
Control horses	58			1						59

Brno, Czechoslovakia has sent us as yet unpublished data which show that up to 66% of 125 healthy horses of different ages on farms with a history of *C. equi* pneumonia in foals had antibodies to *equi* factors. Antibody was produced in rabbits to *equi* factors and two lines of precipitation identified in gel diffusion tests. Antigenic heterogeneity was not apparent when *equi* factors from 37 strains of *C. equi* were tested against hyperimmune serum. Preliminary descriptions of this work have appeared in Skalka B, Svastova A. Veterinarstvi 1984; 34: 206-208.

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