Detection of Colt Serum Antibody against *Corynebacterium equi* by Agar Gel Diffusion

Muneo NAKAZAWA

Tohoku Branch Laboratory, National Institute of Animal Health, Uminai 31,
Shichinohe-machi, Kamikita-gun, Aomori 039-25

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Abstract. Agar gel diffusion was applied to detect colt serum antibody against *Corynebacterium equi*. Concentrated culture supernates and bacterial sonicates prepared from nine strains of *C. equi* were used as antigens. The presence of precipitating antibodies was demonstrated in the sera of all the affected colts, but not at all in those of the healthy colts examined. Precipitating lines were observed between both antigens and affected colt sera. Precipitating antibodies against concentrated culture supernates appeared only in the 7S globulin fraction. One precipitating line against bacterial sonicates was demonstrated in the 7S globulin fraction and the other one in both 7S and 19S globulin fractions separated with Sephadex G-200 column chromatography.

Corynebacterium equi was first described by Magnusson, who isolated this organism from a foal with purulent pneumonia in Sweden [2]. Infections of *C. equi* in domestic animals have been found in colts [3, 5], cattle [7], sheep [1] and swine [6]. In colts, it is an important pathogen causing abscess formation in lung and intestine, and arthritis [3].

Although attempts were made to detect colt serum antibody against *C. equi* by bacterial agglutination and indirect hemagglutination tests, neither test was entirely satisfactory for diagnostic purposes [4, 8]. Therefore, an improvement of serological method is required for the diagnosis of *C. equi* infection.

In this study, the author attempted to demonstrate colt serum antibody against this organism by agar gel diffusion with the concentrated culture supernate and bacterial sonicate as antigens.

Materials and Methods

Strains: Nine strains of *C. equi* were employed.

Of them, ATCC 6939 was supplied by the courtesy of Dr. R. Yanagawa, of the Hokkaido University, Sapporo, and strains 11DK085, CE220, 24 and 35 by the courtesy of Dr. M. Oka, of the National Institute of Animal Health, Ibaraki. Strains TN-1, 44, 46 and 53 were isolated by the author. Each strains was subcultured onto nutrient agar medium (Eiken) and stored at 4°C until use. The sources and biochemical characteristics of the strains used are described in Tables 1 and 2, respectively.

Preparation of antigens: Each strain was cultured in 2,000 ml of brain heart infusion broth (Difco) at 37°C for 6 days. The organisms grown and culture supernates were separated by centrifugation at 8,000 rpm for 20 min at 4°C. The culture supernate was filtered through 0.45 µm Millipore filter and adjusted to a concentration of 50% by addition with ammonium sulfate. After keeping the mixture at 4°C for 20 hr, the resulting precipitate was collected by centrifugation at 5,000 rpm for 20 min at 4°C, and dissolved in 30 ml of distilled water. The solution was transferred to a Visking cellophane tube and dialyzed against running water for 3 days. The dialyzate was concentrated to a 15 ml volume with polyethylene glycol 6,000 (Wako Chemical Co., Ltd., Tokyo). This antigen was designated CCS.

The organisms washed three times with physiological saline were suspended in 10 ml of saline. The bacterial suspension was sonicated for 20 min in a water-cooled sonic oscillator, Model 200 M

(Kubota Co., Tokyo), at 9 KHz/sec full power. The homogenate was centrifugated at 8,000 rpm for 20 min at 4°C to remove cell debris. The supernate was designated BS.

Colt sera: Sera from four affected colts and from 13 healthy ones were used. Serum TN-0, TN-1, TN-2 and TN-3 were collected 60, 19, 109 and 101 days, respectively, after a clinical pneumonic sign appeared. All the affected colts were examined bacteriologically. *C. equi* was isolated in the order of 10^7 to 10^8 per gram from the suppurative lesions of lung and mesenteric lymph node. No healthy

colts had a past history of infection at a point of time of collecting samples.

Serum fractionation by gel filtration: A 2.7×40 cm column of Sephadex G-200 (Pharmacia Co., Ltd.) was prepared and equilibrated with 0.15 M NaCl in 0.01 M phosphate-buffer solution. Three ml of serum was applied to the column and eluted with the same buffer. The flow rate was approximately 12 ml per hr. Three ml amounts were collected in a fraction collector (Ohtake Works, Tokyo). The protein concentration was examined continually by measuring the optical density at 280 nm by using an autospectrophotometer (Ohtake Works, Tokyo).

Tube agglutination test: Each of the nine strains, cultured on nutrient agar plates at 37°C for 24 hr was harvested, washed, and suspended in physiological saline. Formolized antigen was prepared by the addition of formalin to the suspension to provide a final concentration of 0.3%. On the other hand, heated antigen was prepared by autoclaving at 121°C for 2.5 hr. The autoclaved cells were centrifuged, washed twice, and resuspended in saline. Both antigens were adjusted to the concentration of McFarland opacity tube No. 3. Serial twofold serum dilutions (0.5 ml) were mixed with an equal volume of formolized and autoclaved anti-

Table 1. Source of 9 strains of *C. equi*

Strain	Source
TN-1	Colt: lung abscess
CE220	Colt: lung abscess
11DK085	?
ATCC 6939	Colt: lung abscess
24	Colt: nasal swab
35	Colt: lung abscess
44	Colt: suppurative arthritis
46	Colt: suppurative mesenteric lymphadenitis
53	Colt: lung abscess

Table 2. Biochemical characteristics of 9 strains of *C. equi*

	Strain								
	TN-1	CE220	11D K085	ATCC 6939	24	35	44	46	53
Gram staining	*	*	*	*	*	*	*	*	*
Viscosity	+	+	+	+	+	+	+	+	+
Motility	—	—	—	—	—	—	—	—	—
Oxidase	—	—	—	—	—	—	—	—	—
Catalase	+	+	+	+	+	+	+	+	+
Urease	—	—	—	—	—	—	—	—	—
Tellurite reduction	+	+	+	+	+	+	+	+	+
Nitrate reduction	(+)**	+	—	(+)	+	(+)	+	+	+
Sodium hippurate hydrolysis	+	+	+	+	+	+	+	+	+
Gelatinase	—	—	—	—	—	—	—	—	—
Hemolysin	—	—	—	—	—	—	—	—	—
Fermentation									
glucose	—	—	—	—	—	—	—	—	—
maltose	—	—	—	—	—	—	—	—	—
mannose	—	—	—	—	—	—	—	—	—
rhamnose	—	—	—	—	—	—	—	—	—
sucrose	—	—	—	—	—	—	—	—	—
xylose	—	—	—	—	—	—	—	—	—

* Gram-positive, oval or coccoid forms.

** Weakly positive.

Fig. 1. Agar gel diffusion between colt sera and two antigens prepared from strain TN-1 of *C. equi*. A, CCS; B, BS; 1 to 4, sera from affected colts; 5 and 6, sera from healthy colts.

gens, respectively. The mixtures were well shaken and kept at 37°C for 18 to 20 hr. The titer was expressed as the reciprocal of the highest dilution of test serum showing complete agglutination.

Agar gel diffusion: Gel diffusion plates were prepared on microscopic slides (26×76 mm) using 3 ml of 1.0% molten agar in physiological saline containing 0.1% of sodium azide. After wells 3 mm in diameter were filled with reactants, the slides in a moist chamber were observed at room temperature for 3 days.

Results

Agglutinin titer of colt sera: The seventeen sera collected from affected and healthy colts were examined for bacterial agglutinin titer by using formalized and autoclaved antigens of 9 strains, respectively. Not only healthy but also affected sera showed the lack of detectable agglutinating antibody against both formalized and autoclaved antigens.

Agar gel diffusion on colt sera: Each antigen of CCS and BS prepared from 9 strains was examined against sera from the affected and healthy colts. The presence of precipitating antibodies was demonstrated in all the affected colt sera, but not at all in healthy ones. As shown in Fig. 1, CCS and BS antigens prepared from strain TN-1 formed 1 and 2 precipitating lines against affected colt sera, respectively. No precipi-

Fig. 2. Comparison of antigenicity against affected colt serum between CCS and BS. A, CCS of strain TN-1; B, BS of strain TN-1; 1, serum of affected colt No. TN-2.

tating lines were observed when control concentrated medium was used. On the other hand, at least one common precipitating line against affected colt serum was regularly observed among the antigens prepared from the 9 strains, including ATCC 6939. Comparison of antigenicity between CCS and BS is shown in Fig. 2. These two antigens were prepared from strain TN-1 and formed one identical precipitating line and another specific one against affected colt serum.

Fractionation of precipitating antibody from affected colt serum: For this experiment, serum samples were obtained from

Fig. 3. Agar gel diffusion with two antigens and 7S and 19S fractions from sera of affected and healthy colts. A, CCS of strain TN-1; B, BS of strain TN-1; 1, serum of affected colt No. TN-2; 2, 7S fraction from No. TN-2 serum; 3, 19S fraction from No. TN-2 serum; 4, serum of healthy colt No. 3; 5, 7S fraction from No. 3 serum; 6, 19S fraction from No. 3 serum.

affected colt No. TN-2 and healthy colt No. 3. A slight increase in 7S globulin was observed with serum sample No. TN-2, as compared with serum sample No. 3. Each of the first and second peak was pooled and concentrated to its original serum volume. Each globulin fraction was examined for the presence of precipitating antibody. As shown in Fig. 3, precipitating antibody against CCS antigen was demonstrated only in the 7S fraction. With BS antigen, however, one precipitating line was demonstrated in the 7S fraction and another in both 7S and 19S fractions.

Discussion

Acquired immunity to *C. equi* has been studied by several investigators [4, 8–10]. Recently, Prescott et al. demonstrated the presence of cell-mediated immunity to infection with *C. equi* [9]. On the other hand, with respect to serum antibody, Magnusson, and Cater and Hylton examined it employing bacterial agglutination and indirect hemagglutination tests, but failed to demonstrate any antibody in horses naturally infected with *C. equi* [4, 8]. The author also obtained the same finding; namely, the lack of agglutination of for-

molized and autoclaved antigens by infected colt serum.

In the present study, however, specific precipitating antibodies could be demonstrated in the sera of naturally infected colts by agar gel diffusion with CCS and BS. Precipitating lines were noticed among each CCS and BS antigens prepared from 9 strains and infected colt sera. It is speculated that the precipitating lines observed in this study may be referred to as common antigens of *C. equi*. Further studies are needed to clarify this point.

In this experiment, precipitating antibody against CCS antigen was demonstrable only in the 7S globulin fraction, and that against BS antigen in both 7S and 19S globulin fractions. At least one antigen was common to CCS and BS antigens. It is considered that this common antigen reacts with the 7S globulin fraction, and one antigen specific for BS antigen with both 7S and 19S globulin fractions.

These results indicate that this serological method may be of diagnostic value for detecting natural infection with *C. equi*. However, since the number of samples of infected colt serum used in this study was limited, further investigation should be

carried out with a larger number of samples to elucidate this value. In addition, further improvement is needed to make antigen more sensitive for the detection of precipitating antibody against *C. equi*.

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要 約

寒天ゲル内拡散法による子馬血清中の *Corynebacterium equi* 抗体の検出：中沢宗生（家畜衛生試験場東北支場）——寒天ゲル内拡散法を用いて、*C. equi* に自然感染した子馬の血清抗体を証明した。濃縮した液体培養上清と超音波菌体破壊物の上清を抗原として用いた。沈降線は両抗原とも感染子馬血清4例に対して1本あるいは2本形成されたが、健康子馬血清13例には全く認められなかった。感染子馬血清を Sephadex G-200 で分画すると、濃縮した培養上清に対する沈降線は 7S 分画に、超音波菌体破壊物の上清のそれは 7S 分画だけにあるものと、7S および 19S の両分画にあるものとが認められた。