Attempt to Detect Bovine Antibody against *Fusobacterium necrophorum* by the Agar Gel Double Diffusion Test

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**Abstract.** In order to detect bovine serum antibody against *Fusobacterium necrophorum*, serological investigation was made on bovine isolates of this species by the agar gel double diffusion test. Precipitin lines were observed between most of the HCl-heat extracts prepared from these strains and four anti-*F. necrophorum* sera. Precipitin was developed in rabbits inoculated experimentally with the organism, in the late stage of infection. Precipitin lines appeared between concentrated antigen prepared from the VPI 2891 strain and 16 of 23 sera from cattle affected with hepatic abscess, and between this antigen and 4 of 88 sera from healthy cattle.

It is well known that *Fusobacterium necrophorum* causes purulent lesions in many kinds of animals, especially hepatic abscess in cattle. During the past decade, an increase of *F. necrophorum* infection has been noticed in fattened dairy steers [4, 12, 15] in several countries. Diagnostic studies have also been made with clinic-chemical tests in the recent years [9]. No attempts, however, have succeeded in providing any definite information on the diagnosis of this infection.

The agar gel double diffusion test has recently been used in studying infections with bacteria [11, 14] and demonstrated to be available for detecting antibodies against these bacteria. On the other hand, there are few reports on similar investigation with *F. necrophorum*. In their previous papers [5, 6], the authors described some biological properties of bovine isolates. This paper deals with serological studies on these isolates of *F. necrophorum* by the agar gel double diffusion test and an attempt to demonstrate bovine serum antibody against this organism.

**Materials and Methods**

Strains: A total of 51 strains of *F. necrophorum* were examined in these studies. Of them, 30 strains were originated from bovine hepatic abscesses and 14 strains from the ruminal contents. Each strain was subcultured with MBHI medium [6] for several years. Of 1 reference and 6 stock cultures, 4 strains (VPI 2891, 410, 130 and 118) were supplied by the Miyazaki University, Miyazaki, 2 strains (N 167 and VPI 6161) by the Bergen University, Bergen, and 1 strain (§ 45) by the Osaka University, Osaka. Before use, the differential properties of each strain were reexamined by referring to the VPI Anaerobe Laboratory Manual [3]. In addition, 4 stock cultures (*Fusobacterium varium* ATCC 8501, *Fusobacterium mortiferum* ATCC 9817, *Bacteroides fragilis* subsp. *fragilis* NCTC 9345, and *Bacteroides fragilis* subsp. *distasonis* ATCC 8503) supplied by the Miyazaki University, Miyazaki, were employed in these studies.

Antigens: Ten ml of MBHI overnight liquid culture of each strain was centrifuged at 8,100×g for 20 min and the supernatant discarded. After washing with saline, the sediment was treated by the HCl-heat method [13] with a slight modification. The bacterial cells were resuspended in 0.4 ml of N/5 HCl. The suspension was boiled for 15 min with frequent shaking, cooled immediately in tap water, and centrifuged at 3,600×g for 15 min. The
supernatant was adjusted to pH 7.2 with addition of 
N/5 NaOH.

Concentrated antigen of the VPI 2891 strain was 
prepared as follows. At first, 500 ml of 2-day liquid 
culture was centrifuged. The sediment was washed, 
resuspended in 2 ml of N/5 HCl, boiled, cooled and 
centrifuged in the same manner as mentioned above. 
The supernatant was then transferred to a cello-
phane tubing (Visking Co., New York) and concen-
trated to a 1.0–1.5 ml volume with polyethylene gly-
col 6000 (Wako Chemical Co., Ltd., Tokyo).

Immune sera: Four strains, VPI 2891, An31-2, 
An45-1 and An18-11, showing the typical biological 
properties mentioned in the VPI Manual [3] were 
employed to immunize male albino rabbits weigh-
ing 2 kg. Of them, the An31-2 and An45-1 strains 
had been isolated from bovine hepatic abscesses, 
and the An18-11 strain from the bovine ruminal 
content. Each strain was cultured in 50 ml of MBHI 
liquid medium for 2 days and centrifuged at 8,100×g 
for 20 min. The sediment was washed with saline, 
suspended in a solution which was composed of 
9 ml of saline and 1 ml of 5% phenol solution, and 
allowed to stand overnight at room temperature. 
It was then centrifuged, washed twice, and resus-
pended in such amount of saline as to make 1/5 of 
the original volume. Rabbits were injected intra-
muscularly with 1 ml of a mixture of cell suspen-
sion and incomplete adjuvant (Iatron Co., Tokyo) 
eight times at intervals of 4 days. They were bled 
and sera were harvested from them 1 week after the 
last injection.

Agglutination test: The agglutination reaction 
was conducted by the conventional tube agglutina-
tion method. Agglutinin titers were determined by 
making twofold serial dilutions of the four antisera 
which had previously been diluted 1:25 with sterile 
saline. An equal volume of the homologous viable 
cell suspension (adjusted to the opacity of McFar-
land No. 3 nephrometer standard) was added to 
every tube, including a saline control. All the tubes 
were shaken, incubated in a water bath at 56°C for 
2 hr, and refrigerated overnight at 5°C. Agglutinin 
titer was defined as the reciprocal of the highest 
dilution of antiseraum giving a discernible agglu-
tination.

Agar gel double diffusion test. The procedure 
described by Mansi (1958) was employed in this test. 
Noble agar (Difco, Detroit, Mich.) in saline was 
routinely used. The results were read at room tem-
perature 2 days later.

Experimental infection: Two male rabbits weigh-
ing 2 kg were intraperitoneally inoculated with 8× 
10^6 viable cells of the VPI 2891 strain. After inocu-
lation, sera were obtained from both of them at 
one-week intervals over a 12-week period and ex-
amined for the appearance of antibody against F. 
necrophorum by the agar gel double diffusion test 
and the tube agglutination test. In the former test, 
concentrated homologous antigen was employed.

Bovine sera: A total of 111 sera collected from 
cattle with or without hepatic abscesses were investi-
gated for the existence of precipitin. Concentrated 
antigen prepared from the VPI 2891 strain was 
mainly employed in this investigation. Those sera 
had been collected at the Ube Abattoir in Ube-shi, 
Yamaguchi Prefecture, and the Yahata Meat Center 
in Kitakyushu-shi, Fukuoka Prefecture, Japan, in 
1973 and 1974, and stored at −20°C. F. necrophorum 
was detected from all the hepatic lesions examined. 
These lesions were described in detail in the previ-
ous report [7].

Results

Agglutinin titers of antisera: Before use, 
it the titers of the four antisera were estimated 
by the tube agglutination test. As shown in 
Table 1, the homologous agglutinin titers of 
these antisera against F. necrophorum 
ranged from 1:1,600 to 1:3,200. The titers of 
heterologous reaction among these antisera 
were lower in most tubes, ranging from 1:25 
to 1:800.

Agar gel double diffusion test of strains: 
Table 2 indicates the results of the agar gel 
double diffusion test with respect to the 
reference strain, bovine isolates and stock 
cultures of the genera Fusobacterium and 
Bacteroides. In the homologous and hetero-
logous reaction between the reference strain 
and three bovine isolates and their antisera, 
clear precipitin lines were always observed.

<table>
<thead>
<tr>
<th>Antigen and bovine isolates</th>
<th>VPI 2891</th>
<th>An31-2</th>
<th>An45-1</th>
<th>An18-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPI2891</td>
<td>1600*</td>
<td>&lt;25</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>An31-2</td>
<td>200</td>
<td>3200</td>
<td>800</td>
<td>25</td>
</tr>
<tr>
<td>An45-1</td>
<td>800</td>
<td>400</td>
<td>1600</td>
<td>25</td>
</tr>
<tr>
<td>An18-11</td>
<td>25</td>
<td>&lt;25</td>
<td>50</td>
<td>1600</td>
</tr>
</tbody>
</table>

Remarks.
* Reciprocal of agglutinin titer.
Table 2. Agar gel double diffusion test of reference strain, bovine isolates and stock cultures

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>VPI 2891</th>
<th>An31-2</th>
<th>An45-1</th>
<th>An18-11</th>
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<tbody>
<tr>
<td>Reference strain:</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>F. necrophorum VPI 2891</td>
<td>+**</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bovine isolate:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. necrophorum An31-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F. necrophorum An45-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F. necrophorum An18-11</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Stock culture:</td>
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</tr>
<tr>
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<td>+</td>
</tr>
<tr>
<td>F. necrophorum 410</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>w</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F. necrophorum 130</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F. necrophorum 118</td>
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<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>B. fragilis subsp. distasonis</td>
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<td>-</td>
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</tr>
<tr>
<td>ATCC 8503</td>
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<td>-</td>
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</tr>
</tbody>
</table>

Remarks.
* HCl-heat extract from 10 ml of broth culture.
** +, positive; -, negative; w, weak reaction.

They were presented also in the two stock cultures of F. necrophorum, but not in the other cultures of the genera Fusobacterium and Bacteroides. On the other hand, a precipitin line was also found in most of the antigens prepared from the bovine isolates (Table 3 and Fig. 1). In some strains, one or more additional faint lines were formed between these antigens and antisera. A total of 7 antigens lacked lines against two sera which had been prepared against strains An31-2 and An18-11, respectively.

Experimental infection: No apparent symptoms were found in the inoculated rabbits in the early stage of infection. Loss in body weight began in one rabbit at 6 weeks and in the other at 7 weeks of infection. In the former rabbit, a single precipitin line was observed at 7 weeks and an additional line at 9 weeks of infection. In the latter rabbit, a line was formed at 8 weeks and another line at 10 weeks. Serum agglutinin titers could be estimated at 2 weeks. They reached a maximum, or 1:128 and 1:256, in these rabbits at 6 and 7 weeks, respectively. These titers were maintained up to 12 weeks of infection.

Agar gel double diffusion test on bovine serum: No precipitin lines were observed between antigens prepared from 10 ml of broth culture and sera from cattle with or without hepatic abscesses. When the con-

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>VPI 2891</th>
<th>An31-2</th>
<th>An45-1</th>
<th>An18-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine isolates</td>
<td>44/44**</td>
<td>34/44</td>
<td>44/44</td>
<td>41/44</td>
</tr>
</tbody>
</table>

Remarks.
* Prepared by the same method as antigen in Table 2.
** No. of positive antigens/No. of antigens examined.
centrated antigen was employed in this test, a single line was demonstrated by 16 of 23 sera from cattle with hepatic lesions, and an additional line by some of these sera. On the other hand, an apparent line was presented by sera from a few healthy cattle. Table 4 shows the results of the agar gel double diffusion test on bovine serum with the concentrated antigen.

**Discussion**

Several reports [2, 10, 16] were published on the serological investigation of *F. necrophorum* from various sources. With respect to the agglutination reaction, they indicated that many cultures tended to exhibit a spontaneous agglutination in several suspensions. The authors have also encountered the same difficulty in dealing with bovine isolates which were tentatively divided into phases A and B by referring to Bergey’s Manual [1]. Attempts were made in vain to eliminate this reaction by applying 0.85% sodium chloride solution at different pH, distilled water, 10% MgCl₂ solution, treatment by heating at 100°C for 60 min, and the procedure of freezing (unpublished data). The existence of several serovars in bovine isolates, however, may be expected from the results of the cross agglutination test with cultures showing a weak or no spontaneous agglutination. Further investigation by agglutination test will be necessary to elucidate it in comparison with isolates of various origins.

Recently, Werner [16] reported that cross reactivity was revealed among five stock cultures of *F. necrophorum* by the agar gel diffusion test with freeze-thawed extracts which had been prepared from the whole cell suspensions, but not by the same test with autoclaved extracts. In this study, lines were observed between most of the HCl-heat extracts of bovine isolates belonging to phases A and B, or three extracts from the reference strain and two stock cultures, and rabbit sera. It is suggested that one of the lines may be referred to as antigen common to many isolates of bovine origin. On the other hand, the presence of several serovars may also be estimated from the results of the cross precipitation test. Further investigation is needed to compare isolates from various sources by the agar gel double diffusion test.

It is well known that the main constituent of HCl-heat antigen derived from beta-hemolytic streptococci is polysaccharides of cell-wall origin. In disc electrophoretic analysis of HCl-heat extracts from *F. necro-
phorum of bovine origin, several bands were observed in the column and stained with amido black solution [8]. One of them was detected from most of the extracts and stained faintly by glycoprotein staining. These results suggest that the extracts may be composed of the polysaccharide-protein complex. It is of great interest to investigate the location of this complex in the bacterial cell.

In rabbits experimentally infected, precipitin appeared in the serum in the late stage of infection, while agglutinin was formed in the early stage. Similar findings were obtained from rabbits inoculated with killed cells. Attempts were made to prove antibody in cattle with hepatic abscess from which F. necrophorum had been detected in pure form or with predominance. The present test with concentrated antigen gave satisfactory results and seemed to be available for practical use. Quite recently, the authors have encountered the frequent occurrence of hepatic abscesses suspected to have been produced by infection with this organism in fattening dairy steers fed concentrates. Serological and other additional findings on these animals will be published elsewhere.

It is necessary to clarify the significance of this antigen in the process of F. necrophorum infection and the development of antibody response in bovine serum. In addition, further improvement is needed to make this antigen more specific and sensitive. The new antigen is of great advantage for a correct diagnosis of bovine hepatic abscess caused by F. necrophorum.

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References

要約

寒天ゲル二重拡散法による牛血漬中の抗 Fusobacterium necrophorum 抗体の検出：鹿江雅光・戸田光敏（山形大学農学部家畜微生物学教室）-- 牛血漬中の抗 Fusobacterium necrophorum 抗体を検出するため、本菌参照菌株、牛由来株ならびに保存菌株について、寒天ゲル二重拡散法による血漬学的検査を実施した。参考菌株および牛由来株に対する抗血漬に対して、塩酸加熱抽出液法により作成したホモおよびヘテロの抗原、他のほとんどの牛由来株ならびに保存2菌株の抗原はそれぞれ沈降線を形成した。F. necrophorum VPI 2891 株を実験的に感染させた家兎においては、感染後期の血漬中に沈降線の出現が認められた。次に、VPI 2891 株の濃厚抗原と、野外牛血漬との沈降反応を試みたところ、肝膿瘻牛血漬28例中16例に沈降線が認められた。一方、非肝膿瘻牛血漬においては88例中4例にそれが観察された。


