Detection of Rabbit and Bovine Antibodies to the Outer Membrane of Ruminal *Bacteroides ruminicola* subspecies *ruminicola* by Enzyme-Linked Immunosorbent Assay (ELISA)

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Abstract The enzyme-linked immunosorbent assay (ELISA) was used to detect specific antibodies in rabbit and bovine sera directed to the outer membrane antigen of a ruminal bacteria, *Bacteroides ruminicola* subsp. *ruminicola*. Optimal conditions of ELISA were 10 µg of the outer membrane antigen per well and 1:500 dilution of antiserum from rabbit immunized with organisms of *B. ruminicola* subsp. *ruminicola*. The ELISA recognized antibody against *B. ruminicola* subsp. *ruminicola* subspecies-specifically. Sera from colostrum-deprived calves contained none of specific antibodies, while sera from colostrum-fed calves contained high level of antibodies. The mean titer of sera from 10 day-old calves was significantly ($p < 0.01$) higher than that of 40 day-old calves, but was significantly ($p < 0.01$) lower than that of adult cattle. The mean titer of sera from dairy cows which fed high-roughage diet was significantly ($p < 0.01$) higher than that of feedlot cattle which fed high-concentrate diet. The results suggest that the antibodies against the outer membrane antigen of *B. ruminicola* subsp. *ruminicola* transfer to calves via the colostrum, and that the antibody levels of cows are affected by the way of feed management.

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Key words : *Bacteroides ruminicola*, bovine antibody, ELISA, outer membrane, rumen bacteria

Most of natural antibodies are considered to be produced in responses to continuous low levels of antigenic stimulation of indigenous microflora^{1,2)}. The rumen provides particularly favourable conditions to rumen bacteria as stimulators for natural antibodies production¹⁸⁾. Serological techniques have been used to identify individual strains of rumen bacteria, e.g. *Butyrivibrio*^{3,8)}, *Selenomonas*⁴⁾ and *Bacteroides*^{11,17)}, however, have not been applied to investigate the host immune respon-

ses to rumen bacteria. On the other hand, using the outer membrane or its components such as polysaccharide and lipopolysaccharide (LPS), enzyme-linked immunosorbent assay (ELISA) was performed for detecting antibodies against anaerobic infections in human^{7,12,20)}. Previously we¹³⁾ reported that the antibodies against the outer membrane antigen of ruminal *Bacteroides succinogenes* transferred to calves via the colostrum, and that the titers of cows were affected by the way of feed management.

In the present study, we firstly determined the optimal conditions of ELISA with the outer membrane antigen of *Bacteroides ruminicola* subsp. *ruminicola* and investigated specificity of the methods using immunized rabbit serum, and applied the ELISA to detect specific antibodies in sera from calves and cattle.

Materials and Methods

Bacterial strains: Standard strains of *B. ruminicola* subsp. *ruminicola*, *B. ruminicola* subsp. *brevis*, *B. succinogenes* and *Selenomonas ruminantium* were used. Media and growth conditions of the bacterial strains were described previously¹⁵.

Rabbit immunization: Rabbit antisera to intact bacterial organisms were prepared as described previously¹³. Briefly, the animals were inoculated intravenously 6 times in the first 2 weeks. A booster injection was performed at the fourth week, and the animals were phlebotomized at the fifth week. Each inoculum dose was 5×10^9 live organisms in 0.15 M NaCl.

Preparation of the outer membrane: The outer membrane of *B. ruminicola* subsp. *ruminicola* was prepared by the methods of heat, EDTA treatment, shearing, and differential centrifugation as described previously¹⁵.

ELISA: ELISA was performed as described previously¹³. Briefly, wells of microtiter plate were coated with the outer membrane antigen of *B. ruminicola* subsp. *ruminicola*. After the wells were washed, rabbit antiserum or bovine serum was added. Then, after wash, horseradish peroxidase conjugated anti-rabbit immunoglobulin (Ig G (H + L) goat serum or anti-bovine IgG (H + L) rabbit serum was added as secondary antibody. Substrate was o-phenyldiamine suspended in 0.1 M citrate buffer (pH 4.0) containing 0.3% H₂O₂. Optical density (OD) at 492 nm was read in Immuno Reader (NJ-2000, Nippon Inter Med.). Optimal conditions of ELISA were determined by using various quantities of the antigen (0.25 μ g

to 25 μ g per well), various dilutions of the rabbit antiserum (1 : 50,000 to 1 : 100), a constant dilution of enzyme-conjugated antibody (1 : 2,000), and a constant reaction time (30 min).

Bovine sera: Serum samples were obtained from 24 calves of Japanese Black breed at colostrum-deprived 0 day, and colostrum-fed 10 days and 40 days after birth. Serum samples were also obtained from 50 adult cattle of Holstein-Friesian breed, including 25 dairy cows which fed high-roughage diet and 25 feedlot cattle which fed high-concentrate diet.

Measurement of serum IgG: Concentrations of IgG in sera from calves were measured by single radial immunodiffusion (SRID) kit (ICN ImmunoBiologicals, Israel).

Results

Standardization of ELISA: When antigens ranging from 0.25 μ g to 25 μ g per well were tested (Fig. 1), the curves for two serum dilutions (1 : 500 and 1 : 1,000) were similar. The ELISA values increased under concentrations between 0.25 μ g and 10 μ g of the antigen. At concentrations between 10 μ g and 25 μ g of the antigen, the curve leveled off. When antiserum dilutions between 1 : 50,000 and 1 : 100 were tested at 10 μ g antigen per well

Fig. 1. ELISA values of rabbit antiserum against *B. ruminicola* subsp. *ruminicola* (1 : 500 and 1 : 1,000) in various quantities of the antigen.

Antibody to Ruminant *Bacteroides ruminicola*

(Fig. 2), the ELISA values increased at serum dilutions between 1 : 5,000 and 1 : 500. Therefore, 10 μ g/well of the outer membrane antigen and rabbit antiserum diluted 1 : 500 were chosen for subsequent tests.

Specificity of ELISA to *B. ruminicola* subsp. *ruminicola*: Specificity of ELISA was determined by using 10 μ g per well of the outer membrane antigen of *B. ruminicola* subsp. *ruminicola* and 1 : 500 diluted rabbit antisera raised against some rumen bacterial species (Table 1). The ELISA value of the homologous antiserum was high (1.62), while the ELISA values were negligible level in the heterologous antisera to the other bacterial species such as *B. ruminicola* subsp. *brevis*, *B. succinogenes* and *S. ruminantium*. The value was also negligible in the homologous antiserum absorbed with *B. ruminicola* subsp. *ruminicola*.

Detection of bovine antibodies against the outer membrane of *B. ruminicola* subsp. *rumi-*

nicola by ELISA : Bovine antibodies specific to *B. ruminicola* subsp. *ruminicola* were investigated by the ELISA. At bovine serum dilutions between 1 : 5,000 and 1 : 10, ELISA values showed sigmoid curve at 10 μ g antigen per well.

The ELISA values of sera from calves and adult cattle were estimated under conditions of 10 μ g antigen per well, 1 : 1,000 to 1 : 10 serum dilution, 1 : 1,500 enzyme-conjugated dilution and 30 min reaction (Table 2). The titers of sera from colostrum-deprived calves were not detectable level, while those of sera from colostrum-fed calves were high. Adult cattle showed higher titer than calves. The values of individual animal at 1 : 100 serum dilution were plotted in Fig. 3. The titers of sera from colostrum-fed calves varied from calf to calf, i.e. those from 4 calves of 10 day-old and 19 calves of 40 day-old were not detectable level. The mean titer of sera from 10 day-old calves was significantly ($p < 0.01$) higher than that of 40 day-old calves, but was significantly ($p <$

Fig. 2. ELISA values of various dilutions of rabbit antiserum in a constant outer membrane antigen of *B. ruminicola* subsp. *ruminicola* (10 μ g/well).

Table 1. ELISA values of the outer membrane antigen of *B. ruminicola* subsp. *ruminicola* (10 μ g/well) against the homologous and heterologous rabbit antisera (1 : 500)

Rabbit antisera	ELISA value
anti- <i>B. ruminicola</i> subsp. <i>ruminicola</i>	1.62
anti- <i>B. ruminicola</i> subsp. <i>ruminicola</i> absorbed by the organisms	0.03
anti- <i>B. ruminicola</i> subsp. <i>brevis</i>	0.04
anti- <i>B. succinogenes</i>	0.16
anti- <i>S. ruminantium</i>	0.01

Table 2. ELISA values of calves and cattle, with use of a constant outer membrane antigen of *B. ruminicola* subsp. *ruminicola* (10 μ g/well) and various dilutions of sera

Serum dilution	Calves			Adult cattle	
	0 day-old (n=24)	10 day-old (n=24)	40 day-old (n=24)	Dairy cow (n=25)	Feedlot cattle (n=25)
1 : 10	0	0.69 $\pm$ 0.28	0.32 $\pm$ 0.30	1.20 $\pm$ 0.33	0.85 $\pm$ 0.32
1 : 100	0	0.21 $\pm$ 0.24	0.10 $\pm$ 0.23	0.77 $\pm$ 0.23	0.52 $\pm$ 0.23
1 : 500	0	0.01 $\pm$ 0.15	0.01 $\pm$ 0.14	0.38 $\pm$ 0.15	0.29 $\pm$ 0.15
1 : 1,000	0	0.01 $\pm$ 0.11	0.01 $\pm$ 0.10	0.21 $\pm$ 0.11	0.16 $\pm$ 0.15

ELISA values are expressed as Mean $\pm$ SD.

Fig. 3. ELISA values of calves and adult cattle, with use of the outer membrane antigen of *B. ruminicola* subsp. *ruminicola* (10 µg/well) and bovine sera (1:100).

0.01) lower than that of adult cattle. The mean titer of sera from dairy cows was significantly ($p < 0.01$) higher than that of feedlot cattle and the highest in the experiment.

Total IgG level of calf sera : Concentrations of IgG in sera from colostrum-deprived calves were not detectable level, and those of 10 day-old calves (19.5 ± 4.5 mg/ml, Mean $\pm$ SD) were significantly ($p < 0.05$) higher than those of 40 day-old calves (15.7 ± 5.3 mg/ml). There were positive correlation between the ELISA values (1 : 100 serum dilution) and total IgG level with relatively low coefficient of correlation (0.423).

Discussion

It was demonstrated that the outer membrane of *B. ruminicola* subsp. *ruminicola* possessed subspecies-specific antigen by immunodiffusion, indirect immunofluorescent test and immunoelectron microscopy¹⁶⁾. The present ELISA study also showed no cross-reactivity of the outer membrane of *B.*

ruminicola subsp. *ruminicola* to *B. ruminicola* subsp. *brevis*, *B. succinogenes* and *S. ruminantium*. Thus the outer membrane antigen of *B. ruminicola* subsp. *ruminicola* is considered to be subspecies-specific.

Generally newborn calves acquire passive immunity soon after birth by the intestinal absorption of antibodies present in the dam's colostrum^{6,19)}, and the initial increase in concentration of Igs in serum of the calves decreases during the subsequent 8-16 days by biological degradation⁵⁾. The present observation of increase and subsequent decrease in total IgG concentration in sera from colostrum-fed calves was correspondent to this general agreement. In the present study, specific antibodies against the outer membrane antigen were not detected from colostrum-deprived calves but from colostrum-fed calves. Similar results were obtained in antibodies against the outer membrane antigen of *B. succinogenes*¹³⁾. These results suggest that the antibodies to the outer membrane antigen of

rumen bacteria such as *B. ruminicola* subsp. *ruminicola* and *B. succinogenes* transfer to calves via the colostrum, as was suggested by SHARPE *et al.*¹⁸⁾. The antibodies transferred to calves may influence the settlement of microbial flora in the rumen of newborn calves.

The mean titer of sera from dairy cows was higher than that of feedlot cattle. The relationship between microflora in the rumen and various kinds of feed rations has been published. *B. ruminicola* is the most predominant ammonia-producing bacteria in the rumen of mature cattle²¹⁾, and is the most numerous bacteria in heifers fed a high proportion of hay¹⁰⁾. OGIMOTO *et al.*⁹⁾ demonstrated that the agglutinating titers of antibodies against *B. ruminicola* were higher in grazing cattle than in feedlot cattle. Therefore, the difference of antibody titers between dairy cows and feedlot cattle may reflect the different rumen bacterial populations. The results suggest that the antibody levels against the outer membrane of *B. ruminicola* subsp. *ruminicola* are affected by the way of feed management.

Although the antibodies against rumen bacteria are considered to be natural antibodies^{2,18)}, the mechanism of antibody production and immunological roles in host protection have not been revealed. The ELISA is useful technique to investigate bovine immune responses to rumen bacteria, and it can be applied to detect secretory antibodies as well as serum antibodies of specific Ig classes. It is suggested that the rumen microbial flora may stimulate antigenically host immune system, and play an important role in the development of immune system in calves¹⁴⁾. Further investigations are needed to reveal the relationships between development of immune system and settlement of rumen microbial flora in calves.

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酵素免疫測定法 (ELISA) によるウサギおよびウシ 血清中の抗 *Bacteroides ruminicola* subspecies *ruminicola* 外膜抗体の検出

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酵素免疫測定法 (ELISA) によりルーメン由来 *Bacteroides ruminicola* subsp. *ruminicola* の菌体外膜に対するウサギおよびウシ血清中の特異抗体を検索した。ELISA の反応条件は外膜抗原濃度が 10 µg/well, また, 抗菌体ウサギ血清の希釈倍率は 1:500 とした。 *B. ruminicola* subsp. *ruminicola* の外膜抗原は同菌種の抗菌体血清と特異的に反応し, *B. ruminicola* subsp. *brevis*, *B. succinogenes* および *Selenomonas ruminantium* の抗菌体血清との間に交差反応は認められなかった。ELISA によりウシ血清中の特異抗体を検索した結果, 初乳摂取前の子牛では抗体が全く検出されず, 初乳摂取後には摂取前に比べ高値を示した。10 日齢時の平均抗体価は, 40 日齢時に比べ有意 ($p < 0.01$) な高値を示し, また, 成牛に比べ有意 ($p < 0.01$) な低値を示した。粗飼料を多く給与された乳牛の平均抗体価は, 濃厚飼料を多く給与された肥育牛に比べ有意 ($p < 0.01$) な高値を示した。これらの結果から, 抗 *B. ruminicola* subsp. *ruminicola* 外膜抗体は, 初乳を介して子牛に移行すること, また, 成牛では飼養管理条件によって抗体価に差異があることが示唆された。

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