Detection of Specific Systemic and Local IgG and IgA Antibodies of Pigs after Infection with *Bordetella bronchiseptica* by ELISA

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ABSTRACT. An enzyme linked immunosorbent assay (ELISA) for detection of IgG and IgA antibodies against *Bordetella bronchiseptica* in serum and nasal secretions of pigs was developed. The ELISA that used formolized phase I organisms of *B. bronchiseptica* as an antigen detected antibodies to a capsular K antigen(s) of the organism. In pigs which had an agglutinin titer of less than 10 and an ELISA value of 0.47, on average, of maternal antibodies to *B. bronchiseptica*, IgG antibody in serum increased 4 weeks on average and IgG and IgA antibodies in nasal secretions rose markedly 1 and 2 weeks, respectively, after appearance of more than 5×10^3 colony forming units per m*l* of the organisms in the nasal cavity. In contrast, IgG antibody response in serum was inhibited strongly and no increase of the antibody was observed in pigs that had high titers (an agglutinin titer of 149 and an ELISA value of 1.49, on average) of the maternal antibody. In the pigs, a typical decrease in the production and marked delay in the time course of the production of IgG and IgA antibodies in the nasal cavity were also observed. Thus, although pigs produced systemic and local antibodies to *B. bronchiseptica*, the antibody response was affected dose responsively by the maternal antibody. However, the effect seemed to be a little milder in local antibody responses than in systemic antibody responses. The role of the local antibodies in eradication of the organism in the nasal cavity was not elucidated.—KEY WORDS: *B. bronchiseptica*, ELISA, IgA antibody, IgG antibody, local immunity.

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Studies of antibody responses of pigs after infection or vaccination with *Bordetella bronchiseptica*, which is one of the causative agents of porcine atrophic rhinitis, have been mainly done by detection of agglutinins to formolized bacteria [6]. However, several studies showed that there is no direct correlation between the agglutinin titers and resistance to infection or colonization of the organisms [1, 13, 17]. Adherence of *B. bronchiseptica* to porcine mucosal epithelial cells, which is the first step of infection [20], is related to the phase and mode of the organisms [9, 20], and it is inhibited by the antibody *in vitro*. So it is considered that a similar mechanism may act in the nasal cavity of pigs [20]. Therefore studies of specific antibodies in the nasal cavity and their antibody classes are required for the understanding of defense mechanisms against *B. bronchiseptica* infection. Only a few studies have been done on mucosal antibodies against *B. bronchiseptica* by detection of agglutinins or an indirect fluorescent antibody test using concentrated nasal washings [2, 5, 17]. Our aims was to develop an ELISA which has high specificity and sensitivity, requires only a small amount of unconcentrated samples, and can identify the antibody classes. In this paper we describe the characteristics of the ELISA and detection of systemic and local immunological responses of pigs to infection with *B. bronchiseptica* by the ELISA.

MATERIALS AND METHODS

ELISA antigen: A commercial antigen for detection of aggulutinins to *B. bronchiseptica* (AR antigen "Hokken", Kitasato Institute, Tokyo) which contains 5×10^{11} formolized phase I organisms of H16 strain of *B. bronchiseptica* per milliliter was used throughout the study unless otherwise noted. In some experiments, formolized or heated (121°C for 15 min) phase I and III organisms of strain A19 [8] were also used.

Serum and mucous fluid from the nasal cavity (nasal fluid) for ELISA: Sera collected from hysterectomy-produced colostrum-deprived (HPCD) pigs and pigs experimentally infected with *B. bronchiseptica* were used. Nasal fluid was collected by a conventional method using a cotton swab. The fluid was extracted from the swab with 1 m*l* of phosphate-buffered saline (PBS) containing 0.25% bovine serum albumen (Fraction V, Sigma Chemical Company, St Louis) and 0.05% Tween 20 (Wako Pure Chemical Industry Ltd., Osaka). The extract was stored at −20°C after centrifugation at 10,000 rpm for 15 min. It was used for experiments without dilution.

Absorption test: For the absorption test, serum diluted 1/25 with PBS containing 0.5% bovine serum albumen and 0.3% Tween 20 (diluent) or nasal fluid was mixed with an equal volume of various ELISA antigens containing 1×10^{11} organisms per m*l* and kept at 37°C for 2 hr and then 4°C overnight. The supernatant of the mixture after centrifugation at 10,000 rpm for 10 min was used as the absorbed sample.

ELISA: The assay was carried out as described previously [18]. The main steps are follows: (1) an ELISA antigen (5×10^9 organisms/m*l*) suspended in 0.05 M carbonate buffer at pH 9.5 and the carbonate buffer alone which was served as a control antigen were dispensed in 100 μl volumes into wells of a microelisa plate and stored overnight at 4°C; (2) 100 μl of the samples to be tested was added to each antigen-coated well and the control well and the plate was incubated at 37°C for 1 hr; (3) 100 μl of

peroxidase-conjugated goat anti-swine IgG (Fc fragment) or peroxydase conjugated goat anti-swine IgA (alpha chain specific) (Bethyl Laboratories, Inc., Montgomery, Tx.) diluted 1/2,000 with diluent was added to each well and the plate was again incubated at 37°C for one hr. After each incubation step the plates were thoroughly washed with diluent and distilled water; (4) 100 μl of an enzyme-substrate (o-phenylendiamine dihydrochloride) solution was added and absorbance at 492 nm was determined. The result of the ELISA for each sample was expressed in terms of the ELISA value that showed the difference in optimal dose values recorded for the antigen-coated well and the control well.

Detection of agglutinins: This test was performed by the conventional method in test tubes using the commercial agglutination antigen diluted 1/50.

Pigs and their infection with B. bronchiseptica: Three litters of conventional pigs which were kept in separate pens were used. The first litter (group 1, 3 pigs) was infected intranasally with 7×10^7 colony-forming units (CFU) of the S1 strain of virulent *B. bronchiseptica* when they were 5 days old. The second and third litters (groups 2 and 3 consisting of 3 and 7 pigs, respectively) were infected with the organisms horizontally from the group 1 pigs. Serum and nasal fluid were collected weekly or biweekly after birth and until the end of the experiments.

Isolation and characterization of bacteria from the nasal cavity: Nasal fluid (0.1 m*l*) was cultured on MacConkey agar containing frazoridon, gentamycin, clindamycin and neomycin (25, 0.5, 2.0 and 4.0 mg/*l*, respectively) for quantitative studies of *B. bronchiseptica*, and on Bordet-Gengou agar (Difco laboratories, Detroit, Mich.) containing 10% defibrinated sheep blood for determination of the phase of the organisms. Identification of the bacteria and their phase was carried out by the agglutination test with rabbit anti-capsular serum prepared as described by Ishikawa and Isayama [8].

RESULTS

Optimum antigen dilution for ELISA: The commercial *B. bronchiseptica* agglutination antigen diluted 1/100 ~ 1/400 and 1/100 gave the highest ELISA values for IgG and IgA antibodies, respectively, against the organism. Therefore, the antigen diluted 1/100 was used in subsequent experiments.

Optimum serum dilution for detection of antibodies: As shown in Fig. 1, sera diluted 1/25 ~ 1/100 and 1/25 ~ 1/50 showed the highest ELISA values for detection of IgG and IgA antibodies to *B. bronchiseptica* respectively. Therefore, serum diluted 1/50 was used in subsequent tests. Sera from HPCD pigs showed no signficant ELISA values at any dilutions.

Serum absorption test: Specificity of the test was determined by the antibody absorption test using formolized *B. bronchiseptica* commercial antigen or its heated samples. As shown in Fig. 2, the ELISA values for IgG and IgA antibodies in serum and in nasal fluid were

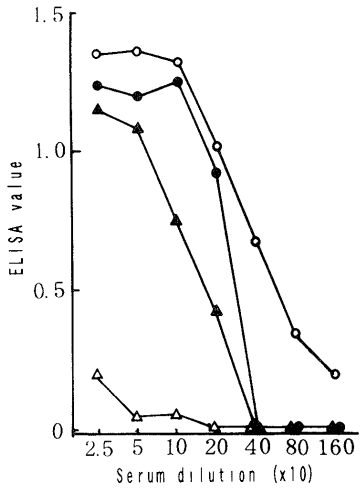

Fig. 1. The optimum serum dilution for detection of acquired (open symbols) and maternal (closed symbols) IgG (○, ●) and IgA (△, ▲) antibodies.

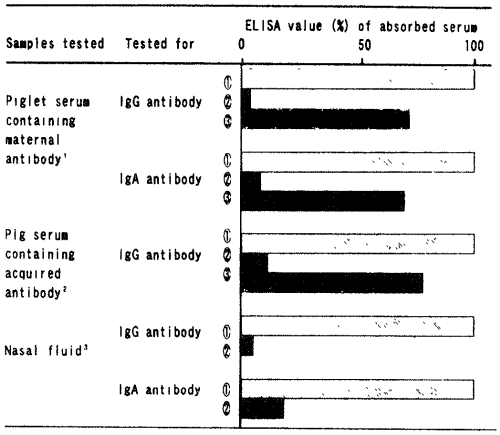

Fig. 2. Antibody absorption test of serum and nasal fluid with formolized *B. bronchiseptica* antigen (②), heated *B. bronchiseptica* antigen (③) and control PBS (①). [1]Serum collected from a 5-day-old piglet of group 3 (see Fig. 6). [2,3]Serum and nasal fluid collected from about a 10-week-old pig of group 1 (see Fig. 6).

reduced to one-tenth of the original titers after absorption with the formolized antigen. In contrast, most of the reactibility of the serum remained after absorption with the heated antigen.

To identify the antibody reacting in the ELISA system, serum collected 9 weeks after infection was absorbed with the commercial antigen (phase I organisms of H16 strain) or phase I or III organisms of the A19 strain of *B. bronchiseptica* that were formolized or autoclaved. As shown in Fig. 3, ELISA values were reduced to one-tenth of the original titers after absorption with formolized antigen of both phase I strains, but not by the heated

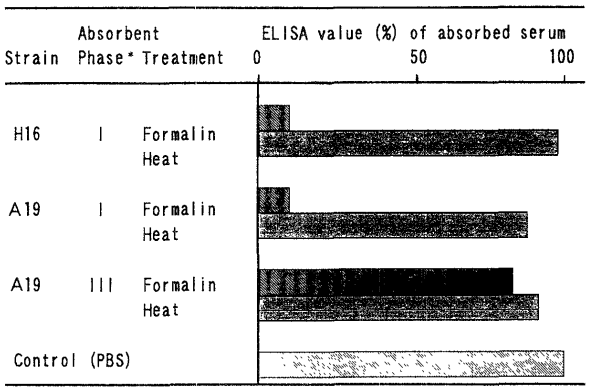

Fig. 3. Serum-IgG antibody absorption test with various antigens. The serum used was from a 10-week-old pig of group 1 (see Fig. 6). *Phase of organisms.

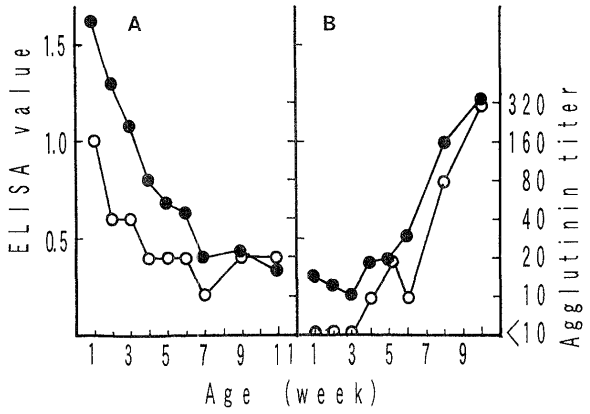

Fig. 4. Sequential antibody titers measured by ELISA for IgG (●) and the agglutinin test (○) in sera of two infected pigs which had high (A) and low (B) titers of maternal antibodies, respectively.

antigen. On the other hand, both formolized and heated antigens of phase III organisms failed to absorb the antibody.

Relationship between ELISA values for IgG antibody and agglutinin titers: Changes in ELISA values and agglutinin titers of two pigs which had different titers of maternal antibodies were investigated weekly after infection with *B. bronchiseptica*. As shown in Fig. 4, the two antibody titers changed in parallel.

Further, the relationship was studied by using 132 serum samples that were collected from 31 pigs before and after infection with *B. bronchiseptica*. As shown in Fig. 5, a high coefficient of correlation (r=0.714) was observed. In this figure, the 7 sera that showed the lowest ELISA values (0–0.07) and agglutinin titer of less than 10 were ones which were collected from HPCD pigs before infection.

Clinical courses of the infection: None of the 13 pigs showed signs of infection during the observation period. In the 3 pigs in group 1, a large number (>5 × 10^3 CFU/m*l*) of *B. bronchiseptica* were continuously found 1

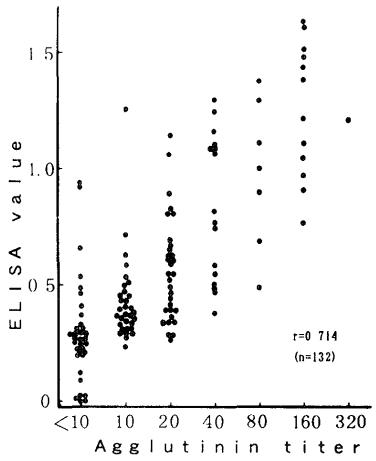

Fig. 5. Correlation between ELISA values of IgG and agglutinin titers.

to 7 weeks after inoculation. However, the number decreased thereafter and only a few organisms were recovered from 2 pigs at 9 weeks. In the 3 and 7 pigs of groups 2 and 3 which were infected spontaneously from group 1 pigs, a similarly large number of *B. bronchiseptica* were detected from 4.0 and 4.7 weeks after birth, on average, respectively. In 8 pigs of the groups, more than 5 × 10^3 CFU/m*l* of *B. bronchiseptica* were detected serially until 10 weeks, but in 2 pigs, the titer decreased to 10 and 100 CFU/m*l*, respectively, at 10 weeks. The organisms isolated throughout the observation period had characteristics of phase I organisms. Atrophy and deformation of the conchae were observed in 2 and 1 pigs, respectively, in group 1, at about 10 weeks after inoculation. No apparent pathological changes were observed in pigs of groups 2 and 3.

Antibody responses of pigs: The antibody responses of each of the 3 groups are shown in Fig. 6 as an average. Antibody titers of serum and nasal fluid of the pigs were first determined 5, 2 and 4 days after birth in groups 1, 2 and 3, respectively. At the time, serum agglutinin titers and IgG ELISA values, on average, were less than 10 and 0.47, respectively, in the 3 group 1 pigs, 107 and 1.14 in the 3 group 2 pigs, and 149 and 1.45 in the 7 group 3 pigs. Apparent IgA antibody was found in only all pigs of group 3 and the ELISA value was 0.96 on average. In nasal fluid, IgG ELISA values were detectable only in group 3 pigs, in which the average was 0.17.

After infection of *B. bronchiseptica*, in group 1 pigs, IgG antibody in serum increased 4 weeks and IgG and IgA antibodies in nasal secretions rose markedly 1 and 2 weeks, respectively, after appearance of a large number of *B. bronchiseptica* in the nasal cavity (aBb). Little increase in IgA antibody in serum was observed in the pigs.

In group 3 pigs maternal IgG antibody in the serum decreased rapidly until they were about 7 weeks old, and the titer remained low thereafter. That is, no apparent rise in the antibody was observed for 6 weeks after aBb. Maternal IgA antibody decreased more rapidly than IgG

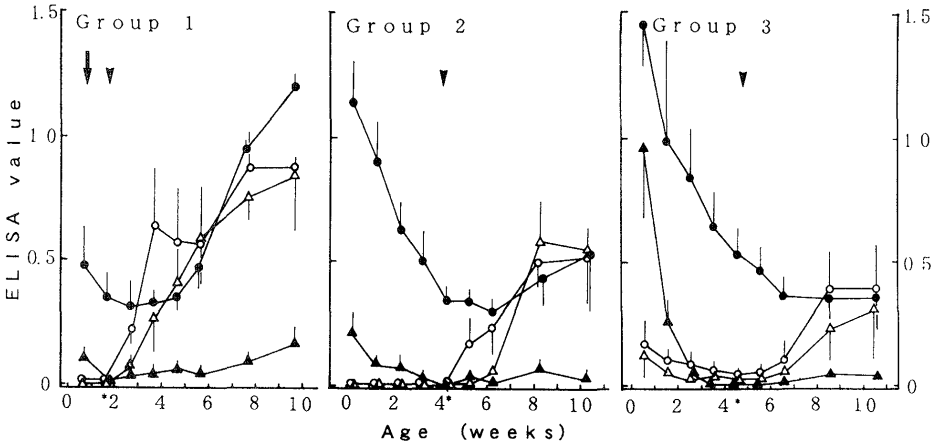

Fig. 6. Antibody responses of three groups of pigs having different levels of maternal antibodies to *B. bronchiseptica*. IgG antibody in serum (●) and nasal fluid (○), and IgA antibody in serum (▲) and nasal fluid (△) are shown. Arrows show the time of inoculation with *B. bronchiseptica*, and both arrowheads and asterisks indicate the time of detection of a large number of *B. bronchiseptica* in the nasal cavity.

antibody and disappeared almost completely by the time the pigs were 3 weeks old. No subsequent development of the antibody in the serum was found during the experimental periods. In contrast, IgG antibody in nasal fluid showed a definite increase at around age 8 weeks, or 4 weeks after aBb, although the titer was apparently lower than that in group 1 pigs. Similarly, a slight IgA response in the nasal cavity was found 4 to 6 weeks after aBb.

Antibody response of group 2 pigs which had relatively high titers of maternal IgG antibody were intermediate between those shown in group 1 and group 3 pigs as shown in Fig. 6.

DISCUSSION

The development of the ELISA using formolized *B. bronchiseptica* makes it possible to investigate the systemic and local IgG and IgA responses of pigs to *B. bronchiseptica* infection as a routine test. The results of the serum absorption test using phase I organisms and their isogenic phase III variants strongly suggested that the antibodies detected by the system were those against the capsular K antigen which is a main constituent of the outer membrane of phase I organisms. It is known that an agglutinin playing a major role in the *B. bronchiseptica* agglutination test is also an antibody against the capsular antigen [8, 15]. The high correlation between the ELISA value and the agglutinin titer may be a result of this fact. Contrarily, it has been shown that there is a very low correlation between the results obtained by an ELISA using a lysate of *B. bronchiseptica* cells and by the agglutinin test [19]. Such disagreement may result from the difference in the antigen used.

A few attempts have been made to measure the antibody to *B. bronchiseptica* in the concentrated washings of the nasal cavity of pigs and dogs by the agglutination test [17] or the indirect fluorescent antibody technique [2]. The use of ELISA for detection and measurement of immunoglobulins in the nasal cavity has the advantages that it requires only a small amount of unconcentrated sample and that it is possible to identify the immunoglobulin class. By this method, as we expected, specific IgA responses were found, and further, a marked increase in IgG antibody was shown. However, it is not known whether the titer of antibody against *B. bronchiseptica* in the nasal cavity reflects the real antibody production in the local tissues, since a large number of the organisms are persistently present on the nasal mucosa and will bind antibodies.

Contamination of the nasal fluid by serum immunoglobulins is the most serious technical problem. However, it seems to be eliminated because of the lack of bleeding at the time of sampling, the difference in constitution of immunoglobulin classes in serum and nasal fluid collected at the same time, and the apparent difference in movement of sequential antibody titers in serum and nasal fluid.

The amount of specific IgG and IgA to *B. bronchiseptica* in nasal fluid during the first few weeks of life in group 2 and 3 pigs that had high titers of IgG and IgA antibodies was very small. This fact shows that transportation of maternal Igs from the serum to the nasal cavity is restricted and seemed to be smaller than that shown previously [3, 14]. Therefore the presence of a high level of Igs in nasal fluid after infection indicates their local synthesis. The distribution of IgG- and IgA-producing cells in the respiratory tract mucosa has been reported [3]. Further, this fact suggests that immunization of sows with *B. bronchiseptica* that expects protection of progenies from colonization of the organism by transmitted maternal antibodies may not act so effectively, as shown previously [17].

Attachment of *B. bronchiseptica* to the mucosal epithelial cells is inhibited *in vitro* by antiserum against formolized phase I organisms but not by antiserum against

heated phase I organisms [20]. In this experiment, *B. bronchiseptica* isolated from the nasal cavity had the characteristics of phase I organisms. Therefore, antibody that is produced in the nasal cavity and that reacts with phase I organisms may play an important role in inhibiting colonization of *B. bronchiseptica* in the nasal cavity and clearing the organism from the cavity. In this experiment, *B. bronchiseptica* almost disappeared at last about 6 weeks after development of local antibodies in the nasal cavity of 2 of the 3 pigs in group 1. In contrast, the organism decreased markedly in number about 2 weeks after development of low titers of local antibodies in 2 of the 7 pigs in groups. Thus it was difficult to find a definite relationship between disappearance of the organisms and development of antibodies in the nasal cavity and, therefore, further experiments are required for determination of the role of antibodies in nasal fluid.

The suppressive influence of maternal antibody on systemic antibody responses has demonstrated by the agglutination test in young pigs infected experimentally with *B. bronchiseptica* or inoculated with *B. bronchiseptica* vaccine [1, 10, 17]. In the present experiment, no IgG antibody increase in serum was observed for more than 6 weeks after aBb in pigs that received large amounts (ELISA values higher than 1.0) of maternal antibodies. The suppression was incomplete in pigs that had small amounts (ELISA values lower than 1.0) of maternal antibodies, and IgG antibody production was observed after a longer lag phase than in pigs that had smaller amounts of maternal antibodies. Similarly, the suppressive effect was observed in local production of IgG and IgA antibodies, but the degree seemed to be a little milder than that observed in systemic antibody production. Thus, the transmitted maternal antibody influenced on amounts of Igs produced and the time course of their production. The mechanisms by which maternal antibodies exert their immunosuppressive effects in infections with various microorganisms [5, 7, 11, 12] are not understood well, although some mechanisms such as diminished T-helper cell activity or enhanced suppressor cell activity have been suggested [7, 16].

REFERENCES

1. Bercovich, Z. and Oosterwoud, R. A. 1977. Vaccination with *Bordetella bronchiseptica* vaccine on a farm with atrophic rhinitis an evaluation of a field experiment. *Tijdschr. Diergeneeskd.* 102: 485–494.
2. Bey, R. F., Shade, F. J., Goodnow, R. A., and Johnson, R. C. 1881. Intranasal vaccination of dogs with live avirulent *Bordetella bronchiseptica*: Correlation of serum agglutination titer and the formation of secretory IgA with protection against experimentally induced infectious tracheobronchitis. *Am. J. Vet. Res.* 42: 1130–1132.
3. Bradley, P. A., Bourne, F. J., and Brown, P. J. 1976. The respiratory tract immune system in the pig. 1. Distribution of immunoglobulin-containing cells in the respiratory tract mucosa. *Vet. Pathol.* 13: 81–89.
4. Brandenburg, A. C. 1978. Bordetella rhinitis in pigs: serum and nasal antibody responses to *Bordetella* bacterins. *Can. J. Comp. Med.* 42: 23–28.
5. De Leeuw, P. W. and Van Oirschot, J. T. 1986. Intranasal vaccination of pigs against Aujeszky's disease: comparison with inactivated vaccines in pigs with low maternal antibody titers. *Res. Vet. Sci.* 39: 34–38.
6. Giles, C. J. and Smith, J. M. 1983. Vaccination of pigs with Bordetella bronchiseptica. *Vet. Bull.* 53: 327–338.
7. Harte, P. G. and Playfair, J. H. L. 1983. Failure of malaria vaccination in mice born to immune mother. II. Induction of specific suppressor cells by maternal IgG. *Clin. Exp. Immunol.* 51: 157–164.
8. Ishikawa, H. and Isayama, Y. 1986. *Bordetella bronchiseptica* phase variation induced by crystal violet. *J. Clin. Microbiol.* 23: 235–239.
9. Ishikawa, H. and Isayama, Y. 1987. Effect of antigenic modulation and phase variation on adherence of *Bordetella bronchiseptica* to porcine nasal epithelial cells. *Am. J. Vet. Res.* 48: 1689–1691.
10. Kemeny, L. J. 1973. Agglutinin response of pigs to intranasal infection of *Bordetella bronchiseptica. Cornell Vet.* 63: 130–137.
11. Kimman, T. G. and Oei-Lie, N. 1992. Role of memory B-cell responses in serum and mucosal fluids of swine for protective immunity against pseudorabies virus. *Am. J. Vet. Res.* 53: 1992–1998.
12. Kimman, T. G., Westenbrink, F., and Straver, P. J. 1989. Priming for local and systemic antibody memory responses to bovine respiratory syncytial virus: effect of amount of virus, virus replication, route of administration, and maternal antibodies. *Vet. Immunol. Immunopathol.* 22: 145–160.
13. Koshimizu, K., Kodama, Y., and Ogata, M. 1973. Studies on the etiology of infectious atrophic rhinitis of swine. VI. Effect of vaccination against nasal establishment of *Bordetella bronchiseptica. Jpn. J. Vet. Sci.* 35: 411–418.
14. Morgan, K. L. and Bourne, F. J. 1981. Immunoglobulin content of the respiratory tract secretions of piglets from birth to 10 weeks old. *Res. Vet. Sci.* 31: 40–42.
15. Ogata, M., Kodama, Y., and Koshimizu, K. 1973. Studies on the etiology of infectious atrophic rhinitis of swine. IV. Agglutination test with formolized antigen for *Bordetella bronchiseptica* infection in pigs. *Jpn. J. Vet. Sci.* 35: 149–155.
16. Reiter, J., Hutchings, P., and Lydyard P. M. 1989. Antibody-mediated modulation of the immune response. *Cell Immunol.* 97: 91–101.
17. Smith, I. M., Giles, C. J., and Baskerville, A. J. 1982. Immunisation of pigs against experimental infection with *Bordetella bronchiseptica. Vet. Rec.* 110: 488–494.
18. Takahashi, K. and Kono, Y. 1985. Development of practical ELISA for detection of antibodies to bovine leukemia virus: A comparison of its sensitivity with that of virus neutralization and agar gel immunodiffusion tests. *Jpn. J. Vet. Sci.* 47: 193–200.
19. Venier, L., Rothschild, M. F., and Warner, C. M. 1984. Measurement of serum antibody in swine vaccinated with *Bordetella bronchiseptica*: comparison of agglutination and enzyme-linked immunosorbent assay methods. *Am. J. Vet. Res.* 45: 2634–2636.
20. Yokomizo, Y. and Shimizu, T. 1979. Adherence of *Bordetella bronchiseptica* to swine nasal epithelial cells and its possible role in virulence. *Res. Vet. Sci.* 27: 15–21.