FULL PAPER *Bacteriology*

A Possibility of Application of the 105-Kilodaltons Protein of *Brachyspira alvinipulli* Cross-Reacting with Antisera to Five Species in the Genus *Brachyspira* to Diagnosis

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(Received 18 November 2003/Accepted 9 February 2004)

ABSTRACT. The antigenic properties of *Brachyspira (B.) alvinipulli* ATCC 51933 and strain C2 were analyzed and compared with those of *B. hyodysenteriae* ATCC 27164 and ATCC 31212, *B. pilosicoli* ATCC 51139, *B. innocens* ATCC 29796 and *B. aalborgi* NCTC 11492. In gel immunodiffusion tests, a protein in *B. alvinipulli* ATCC 51933 reacted strongly with anti-*B. alvinipulli* ATCC 51933-serum and formed two precipitin lines. Furthermore, by an immunoblotting technique, the 105-kilodaltons (kDa) protein in *B. alvinipulli* ATCC 51933 reacted strongly with each of the antisera to *B. hyodysenteriae, B. pilosicoli, B. innocens* and *B. aalborgi*. Therefore, the 105-kDa protein could be applied to diagnosis of chicken infection by *B. alvinipulli* and *B. pilosicoli*. But the 105-kDa protein reacting with the anti-*B. alvinipulli* ATCC 51933-serum was not confirmed in *B. hyodysenteriae, B. pilosicoli, B. innocens* and *B. aalborgi*. The N-terminal amino acid sequence of the 105-kDa protein isolated from *B. alvinipulli* ATCC 51933 was Met-Lys-Lys-Met-Val-Tyr-Phe-Phe-Gly-Asn. The amino acid alignment of this protein possessed 50% homology with the periplasmic-iron-binding protein BitC in *B. hyodysenteriae*.

KEY WORDS: anti-*B. alvinipulli* ATCC 51933-serum, *B. alvinipulli*, 105-kDa antigen.

J. Vet. Med. Sci. 66(7): 773–778, 2004

Brachyspira (B.) alvinipulli is an anaerobic and weakly beta-hemolytic spirochete [14], and a causal agent of the deterioration of chicks and reduction in egg production [17]. The spirochete colonized the mucosal surface of ceca in one-day-old chicks and 14-month-old hens, and the ceca affected with the spirochetes were dilated and contained pale-yellow watery contents [17]. Lymphocytic typhlitis and proctitis in the infected chicks were observed as histological changes [17].

Many studies on the antigens of *B. hyodysenteriae* have been published [7, 9, 11, 13, 19]. Amongst the reports, a 45-kilodaltons (kDa) polypeptide located in the outer envelope of *B. hyodysenteriae* P18A is more interesting, because it has the ability to produce antibody in gnotobiotic pig serum [13]. A 45-kDa protein in the *B. hyodysenteriae* B204 was related to endotoxin [19]. A 44-kDa periplasmic flagellar sheath protein of *B. hyodysenteriae* was confirmed as a species-specific antigen consisting of glycoprotein, and the protein could produce antibody in adult New Zealand White rabbits [9]. Ochiai *et al.* [11] reported that 22- and 17-kDa proteins present in eight strains of *B. hyodysenteriae* reacted strongly with convalescent pig sera and were intimately related to the antigens responsible for the strong stimulation in pigs [11]. Amongst the protein antigens, the periplasmic flagella have been studied intensively. The hyperimmune pig serum to periplasmic flagella derived from *B. hyodysenteriae* P18A cross-reacted with strains B78, S75/1, B169, P18A, KF9, VS1, MC52/80 and P35/2 of *B. hyodysenteriae*. Convalescent serum of a pig affected with dysentery also reacted strongly with the periplasmic flagella of *B. hyodysenteriae* P18A. These results mentioned above demonstrated that the periplasmic flagella of *B. hyodysenteriae* were major antigens in the spirochete [7], but there are no reports on the immunochemical characteristics of proteins of *B. alvinipulli*. In this study we analyzed the proteins immunochemically and compared the antigens with those of the other *Brachyspira* species.

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MATERIALS AND METHODS

Strains used: *B. alvinipulli* ATCC 51933 and strain C2, *B. hyodysenteriae* ATCC 27164 and ATCC 31212, *B. pilosicoli* ATCC 51139, *B. innocens* ATCC 29796 and *B. aalborgi* NCTC 11492 were used in this study. All strains were grown at 37°C for 72 hr on trypticase soy agar (BBL, U.S.A.) containing 5% (volume/volume) sheep blood under anaerobic conditions with the GasPak System (BBL, U.S.A.). The cells were harvested and washed twice with physiological saline by centrifugation at 15,000 rpm for 10 min at 4°C, and the pellets were stored at –80°C before use.

Hyperimmune serum: Hyperimmune sera were prepared as previously described [1]. The cells in 0.01 M phosphate buffer (pH7.2) were adjusted to approximately 1×10^9 cells ml^{-1}, and were inoculated intravenously into rabbits at intervals of four days. When the antibody titer to the inoculum, reached the maximum titer in agglutination and immunodiffusion tests, the rabbits were bled, and the sera were stored at –20°C.

Gel immunodiffusion test: Gel immunodiffusion test was carried out as previously described [1]. All the strains, except *B. hyodysenteriae* ATCC 31212, were used. Harvested cells were treated with 0.5% sodium dodecylsulfate (SDS) in 0.01 M phosphate buffer (pH7.2) at 45°C for 3 hr,

and then centrifuged at 10,000 rpm for 5 min at 16°C. The supernatants were then placed in the wells, except the center wells, made on 1% agar containing sodium azide at a concentration of 0.1%. The antisera were also placed in the center wells. The reactions between the antigens and the antisera were carried out at room temperature overnight.

SDS-polyacrylamide gel electrophoresis (PAGE): SDS-PAGE with 12.5% (weight/volume) separating gel was carried out as previously described [11]. The harvested spirochete cells were suspended in 50 μl of physiological saline and mixed with 50 μl of dye buffer (0.125 mM Tris, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.2% bromophenol blue). The samples were heated at 100°C for 5 min and centrifuged at 15,000 rpm for 1 min at 16°C. The supernatants were loaded and run through the stacking gel at 14 milliamperes (mA) and separating gel at 19 mA continuously. The gel was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma, U.S.A.).

Immunoblotting technique: Immunoblotting was performed as previously described [10]. All of seven strains and antisera were used. After SDS-PAGE, the proteins were transferred to nitrocellulose membrane (Hybond^{TM}-C, Amersham Farmacia, England) at 80 mA for 1 hr, with a semi-dry blotter (Sartblot II-S, Sartorius AG, Germany). The blotted membranes were immersed in phosphate-buffered saline (PBS) containing 5% skim milk and 0.05% Tween 20 as a blocking buffer overnight at 4°C, and probed with antisera diluted 1:60 with blocking buffer for 1 hr at room temperature. The membranes were washed three times with blocking buffer and re-probed with an alkaline phosphatase-conjugated anti-rabbit IgG serum (Sigma, U.S.A.) for 1 hr at room temperature. The membranes were then washed three times with PBS, and treated with a substrate (0.1% magnesium sulfate, 0.05% Fast Blue BB Salt, 0.05% disodium 1-naphthylphosphate n-hydrate, 59.8 mM boric acid, 45 mM sodium hydroxide).

Preparation of sample for N-terminal amino acid sequencing: The precipitated cells were suspended in physiological saline and mixed with sample buffer (187.5 mM Tris-HCl, 6% SDS, 30% glycerol, 0.03% phenol red, 125 mM Dithiothreitol) and then incubated at 60°C for 1 hr. SDS-PAGE with 7% separating gel was performed at a constant current of 10 mA. The separated proteins were transferred to poly vinilidene difluoride (PVDF) membrane at 80 mA for 1 hr with a semi-dry blotter. The membrane was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma, U.S.A.) for 5 min, and then destained with methanol. After drying the membrane, the band concerning our experiment was cut from the membrane and analyzed with Protein Sequencer Model 490 (ABI, U.S.A.).

Treatment with proteinase K: Treatment with proteinase K (EC3. 4. 21. 14) (Sigma, U.S.A.) was carried out as previously described [18]. The cells were digested with 0.2% proteinase K at 50°C for 2 hr, and analyzed by SDS-PAGE.

Purification of 105-kDa protein in B. alvinipulli ATCC 51933: After SDS-PAGE, the 105-kDa band was cut from the 7.0% (weight/volume) separating gel, and the 105-kDa protein was extracted from the gel-strip by electro-elution. Two volumes of cold acetone was added to the sample dissolved in running buffer (0.1% SDS, 10 mM Tris-HCl), kept at –80°C for 1 hr, and centrifuged at 10,000 rpm for 20 min at 16°C. The precipitate was redissolved in running buffer and analyzed by SDS-PAGE, immunoblotting, and sequenced with a sequencer (490/140C perkinelmer U.S.A.) and PTH analyzer (610A Perkinelmer U.S.A.).

RESULTS

Comparison of the antigenicity of six strains by gel immunodiffusion test: As shown in Fig. 1A, *B. alvinipulli* ATCC 51933 reacted strongly with anti-*B. alvinipulli* ATCC 51933-serum and formed one precipitin line. *B. aalborgi* NCTC 11492 also reacted strongly with the antiserum to *B. alvinipulli* ATCC 51933 and formed one precipitin line. These two lines partially fused into a spur, but anti-*B. aalborgi* NCTC 11492-serum reacted with *B. aalborgi* NCTC 11492 but not *B. alvinipulli* ATCC 51933 (Fig. 1B). On the other hand, *B. alvinipulli* strain C2 did not react with anti-*B. alvinipulli* ATCC 51933-serum (Fig. 1A).

Comparison of the protein profiles of seven strains from five species by SDS-PAGE: One band with a molecular size of 105-kDa was confirmed only in *B. alvinipulli* ATCC 51933 and C2 (Fig. 2).

Comparison of the serological reaction profiles of seven strains from five species by the immunoblotting technique: The protein with a molecular size of 44-kDa in all species reacted strongly with all of the antisera (Fig. 3). Only the 105-kDa protein in *B. alvinipulli* ATCC 51933 (Fig. 3B) and strain C2 (data not shown) reacted strongly with each of the hyperimmune serum to all the species, whereas the antisera to *B. alvinipulli* ATCC 51933 or strain C2 did not react with the 105-kDa protein of the other strains (data not shown) including *B. aalborgi* NCTC 11492 (Fig. 3A).

Proteinase K digestion of B. alvinipulli ATCC 51933: The 105-kDa protein in *B. alvinipulli* ATCC 51933 digested with proteinase K was not detected by SDS-PAGE (Fig. 4).

Alignment of N-terminal amino acid of the 105-kDa protein: The alignment of N-terminal amino acids of the 105-kDa protein was Met-Lys-Lys-Met-Val-Tyr-Phe-Phe-Gly-Asn. The sequencing was performed twice, and the results were reproducible.

Confirmation of protein profiles, serological reaction profiles, and N-terminal amino acid sequence of the purified 105-kDa protein in B. alvinipulli ATCC 51933: The purified 105-kDa protein from *B. alvinipulli* ATCC 51933 produced a single band with a molecular size of 105-kDa by SDS-PAGE (Fig. 5A), and the band reacted well with antisera to seven strains by immunoblotting (Fig. 5B). The N-terminal amino acid alignment of purified 105-kDa protein from *B. alvinipulli* ATCC 51933 was Met-Lys-Lys-Met-Val-Tyr-Phe-Phe-Gly-Asn.

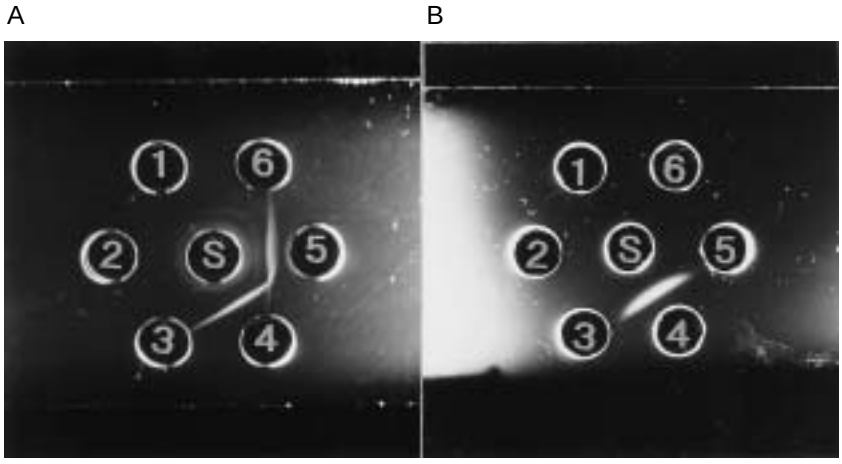

Fig. 1. Reaction between antisera and cells of six strains by gel diffusion. A:anti-*B. alvinipulli* ATCC 51933-serum. B: anti-*B. aalborgi* NCTC 11492-serum. Wells: 1, *B. hyodysenteriae* ATCC 27164; 2, *B. pilosicoli* ATCC 51139; 3, *B. innocens* ATCC 29796; 4, *B. aalborgi* NCTC 11492; 5, *B. alvinipulli* ATCC 51933; 6, *B. alvinipulli* C2; S, antiserum.

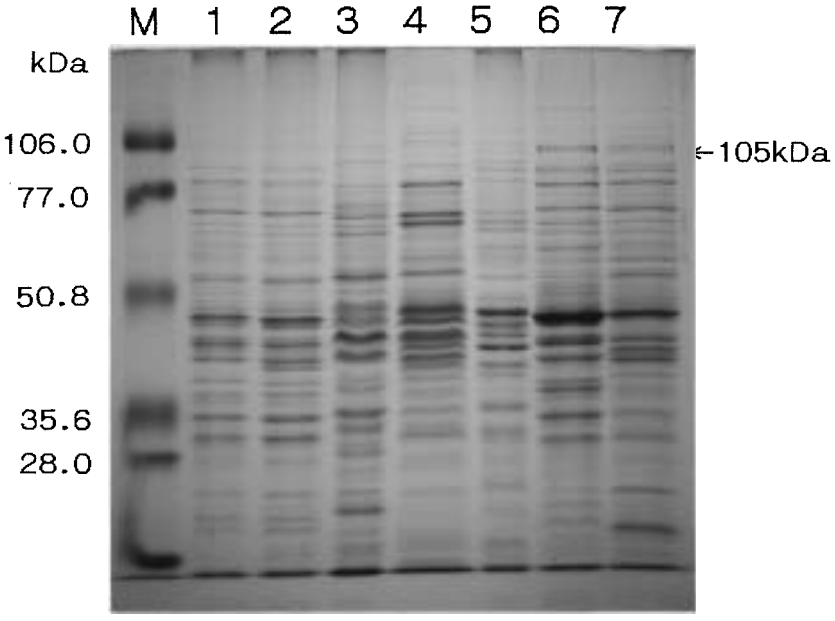

Fig. 2. Comparison of the protein profiles of *B. hyodysenteriae, B. pilosicoli, B. innocens, B. aalborgi* and *B. alvinipulli* by SDS-PAGE. Lanes:1, *B. hyodysenteriae* ATCC 27164; 2, *B. hyodysenteriae* ATCC 31212; 3, *B. pilosicoli* ATCC 51139; 4, *B. innocens* ATCC 29796; 5, *B. aalborgi* NCTC 11492; 6, *B. alvinipulli* ATCC 51933; 7, *B. alvinipulli* C2, M, molecular size markers.

DISCUSSION

In this study, only *B. alvinipulli* ATCC 51933 possessed the 105-kDa antigen consisting of protein and the antigen cross-reacted with antiserum to each of the five species of the genus *Brachyspira*, whereas proteins with a molecular size of about 105-kDa in *B. hyodysenteriae, B. pilosicoli, B. innocens* and *B. aalborgi* did not react with anti-*B. alvinipulli* ATCC 51933-serum. The result indicates that the 105-kDa antigen in *B. alvinipulli* ATCC 51933 may be species-specific, but possess similar epitopes reacting with the antisera to *B. hyodysenteriae, B. pilosicoli, B. innocens* and *B. aalborgi.* It is well-known that periplasmic flagella consist of proteins with molecular sizes of 43.8, 38, 34.8, 32.8, and 29.4-kDa. These proteins were common to eight strains of *B. hyodysenteriae* (B78, S75/1, B169, P18A, KF9, VS1,

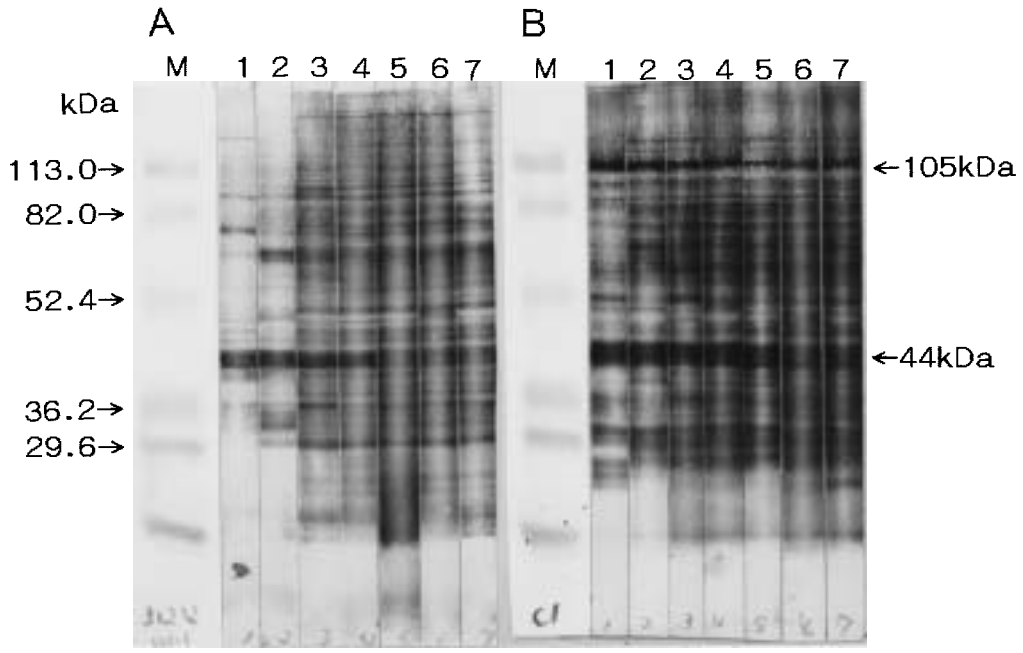

Fig. 3. Comparison of the protein profiles of *B. aalborgi* NCTC 11492 and *B. alvinipulli* ATCC 51933 reacting with seven antisera by immunoblotting. A: *B. aalborgi* NCTC 11492. B: *B. alvinipulli* ATCC 51933. Lanes: 1, anti-*B. hyodysenteriae* ATCC 27164-serum; 2, anti-*B. hyodysenteriae* ATCC 31212-serum; 3, anti-*B. pilosicoli* ATCC 51139-serum; 4, anti-*B. innocens* ATCC 29796-serum; 5, anti-*B. aalborgi* NCTC 11492 -serum; 6, anti-*B. alvinipulli* ATCC 51933-serum; 7, anti-*B. alvinipulli* C2-serum; M, molecular size markers.

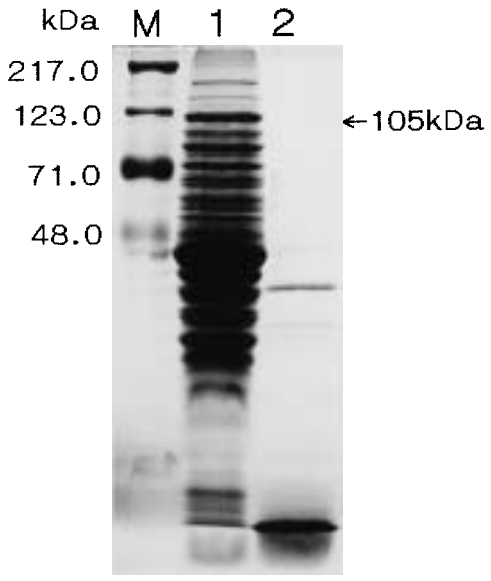

Fig. 4. Comparison of the protein profiles of proteinase K-digested and undigested *B. alvinipulli* ATCC 51933. Lanes: 1, undigested cells; 2, proteinase K-digested cells; M, molecular size markers.

MC52/80 and P35/2) and two non-pathogenic intestinal spirochetes (PWS/A and M1) [7]. Hyperimmune gnotobiotic pig serum to periplasmic flagella of *B. hyodysenteriae* P18A cross-reacted with each of the polypeptides derived from the eight strains of *B. hyodysenteriae* and two non-pathogenic intestinal spirochetes [7]. Convalescent porcine serum also reacted with each of the polypeptides derived from the eight strains of *B. hyodysenteriae* and two non-pathogenic intestinal spirochetes [7]. From the results, the periplasmic flagella may show common antigenicity among species in the genus *Brachyspira* including *B. alvinipulli* ATCC 51933. The 105-kDa protein in *B. alvinipulli* ATCC 51933 is a big molecule, and could not be a subunit protein of periplasmic flagella. Because the 105-kDa antigen in *B. alvinipulli* ATCC 51933 was digestible with proteinase K, it might be a protein, possibly an outer membrane protein. The N-terminal amino acid alignment of 105-kDa protein in *B. alvinipulli* ATCC 51933 was composed of Met-Lys-Lys-Met-Val-Tyr-Phe-Phe-Gly-Asn and the similarity rate of the alignment was 50% of that of periplasmic-iron-binding protein BitC in *B. hyodysenteriae*, which was Met-Lys-Lys-Ile-Val-Leu-Ile-Phe-Thr-Ser [4]. If this is a reasonable homology rate for matching, 105-kDa protein in *B. alvinipulli* ATCC 51933 must be located on the periplasmic membrane.

In the gel immunodiffusion test, the precipitin line between *B. aalborgi* NCTC 11492 and anti-*B. alvinipulli* ATCC 51933-serum made a partial fusion with one line

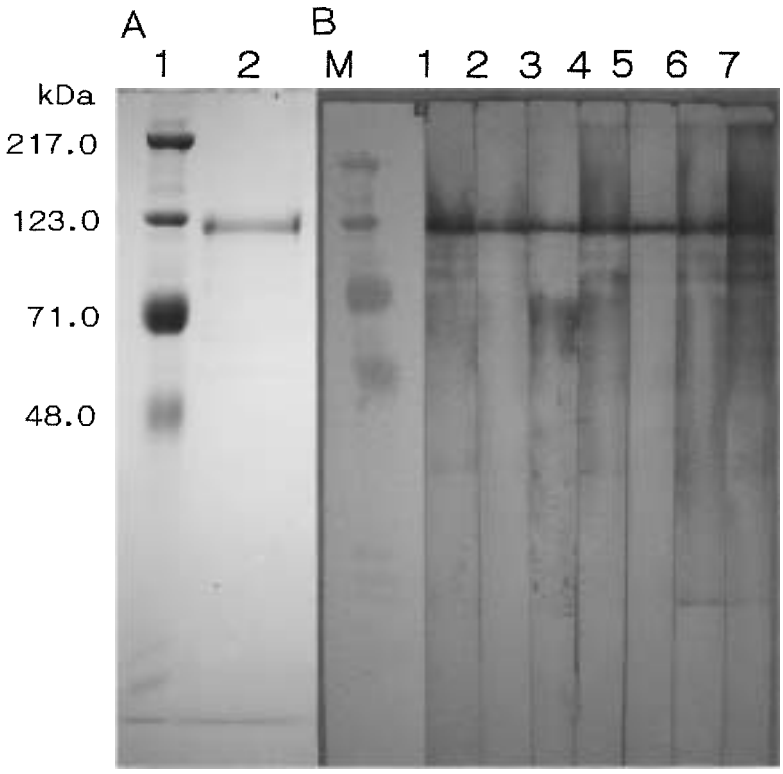

Fig. 5. Profiles of the purified 105-kDa protein in *B. alvinipulli* by SDS-PAGE and immunoblotting. A: SDS-PAGE. Lanes: 1, Molecular size markers (kilodaltons); 2, the purified 105-kDa protein in *B. alvinipulli* ATCC 51933. B: immunoblotting. Lanes: 1, anti-*B. hyodysenteriae* ATCC 27164 serum; 2, anti-*B. hyodysenteriae* ATCC 31212 serum; 3, anti-*B. pilosicoli* ATCC 51139 serum; 4, anti-*B. innocens* ATCC 29796 serum; 5, anti-*B. aalborgi* NCTC 11492 serum; 6, anti-*B. alvinipulli* ATCC 51933 serum; 7, anti-*B. alvinipulli* C2 serum; M, molecular size markers.

revealed by the reaction between *B. alvinipulli* ATCC 51933 and homologous antiserum, and made a spur. Therefore, an antigen in *B. aalborgi* NCTC 11492 may be same as one of antigens in *B. alvinipulli* ATCC 51933. Furthermore, *B. alvinipulli* strain C2 did not react with anti-*B. alvinipulli* ATCC 51933-serum. Antigenicity of *B. alvinipulli* strain C2 to rabbit may be different from that of *B. alvinipulli* ATCC 51933.

On the other hand, the 105-kDa protein in *B. alvinipulli* ATCC 51933 could be applied to the diagnosis of infection by *B. alvinipulli* and *B. pilosicoli*. *B. alvinipulli* is the causal agent of deterioration of chicks and reduction of egg production [17], and economic loss of the egg-production industry. The chicks infected with *B. alvinipulli* ATCC 51933 produced pale-yellow, watery cecal contents and mild lymphocytic typhlitis in 1-day-old chicks and 14-month-old hens [17]. On the other hand, *B. pilosicoli* is another causal agent of the delay of laying, and a highly significant reduction in egg-production in adult chickens [16]. Eggshell quality in the 17-week-old meat breeder females inoculated with *B. pilosicoli* became rough- and soft-shelled [15].

Several serological techniques for the detection of antibodies to *Brachyspira* species have been developed. Kashiwazaki *et al.* [6] reported on the microscopic agglutination test (MAT) for the detection of antibody to *B. hyodysenteriae* in the serum of pigs with experimentally produced disease. Diarra *et al.* [3] also performed the MAT, and identified 18 field isolates as *B. hyodysenteriae.* A slide-agglutination test has been developed to determine the serogroup of isolates of *B. hyodysenteriae* [5]. Moreover, a 30-kDa outer membrane lipoprotein (BmpB) which was specific to *B. hyodysenteriae* has been studied as a candidate for an antigen for diagnosis of swine dysentery [8]. But there are few reports on diagnostic techniques for the detection of antibodies to *B. pilosicoli* or *B. alvinipulli* in infected animals. Although polymerase chain reaction (PCR) with 16S rDNA [12] or 23S rRNA [2] is a useful tool for the identification of *B. pilosicoli*, it cannot detect less than 10^3 spirochete cells in the fecal samples [8]. On the other hand, PCR tests and immunological tests have never been developed for the diagnosis of *B. alvinipulli* [15]. This is the first report on the diagnostic application of 105-kDa protein in *B. alvinipulli* ATCC 51933, and it may be useful for the sero-

logical diagnosis of chick intestinal spirochetosis.

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