Monoclonal Antibodies Directed Against the Outer Membrane Protein of *Bordetella avium*

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Bordetella avium is the etiologic agent of coryza and rhinotracheitis in poultry. This respiratory disease is responsible for substantial economic losses in the poultry industry. Monoclonal antibodies (MAbs) were produced against the outer membrane proteins (OMPs) of *B. avium* isolated from diseased chickens. BALB/c mice were immunized with the extracted *B. avium* OMPs. Then the splenocytes from immunized mice and SP2/0 myeloma cells were fused using PEG 4000. Three stable hybridoma clones (designated as 3G₁₀, 4A₃, and 4E₈) were produced via indirect ELISA and three rounds of subcloning. The MAbs were classified as IgG1, and can recognize the 58 kDa OMP band by Western blot assays. No MAb cross-reactivity with chicken *Proteus mirabilis*, *Escherichia coli*, and *Salmonella* was observed. A double antibody sandwich ELISA (DAS-ELISA) was developed using the rabbit polyclonal antibodies as the capture antibody and MAb 4A₃ as the detection antibody. Under the DAS-ELISA, the minimum detectable concentration of *B. avium* was 1×10^4 CFU/mL, and no cross-reactivity occurred with chicken *Proteus mirabilis*, *Escherichia coli*, and *Salmonella*. Results showed that the DAS-ELISA has good sensitivity and specificity. Clinical application showed the DAS-ELISA was more sensitive than the plate agglutination test. This study may be used to develop a quick and specific diagnostic kit, analyze epitopes, and establish systems for typing *B. avium*.

Introduction

BORDETELLOSIS IS AN UPPER RESPIRATORY DISEASE OF poultry caused by *Bordetella avium*, which undergoes both vertical and horizontal transmission. Bordetellosis in poultry is clinically characterized by death of chicken embryos, low hatchability, rapid chick death, and ophthalmia in adult chickens.^(1–4) In Canada, Fillion and colleagues first reported coryza in turkeys caused by *Bordetella*.⁽⁵⁾ Kersters and colleagues subsequently named this bacteria *B. avium* after a systematic research.⁽⁶⁾ In China, Zhu and colleagues isolated *B. avium* for the first time from diseased embryos and chicks.⁽⁷⁾ In recent years, epidemiological investigations have shown that bordetellosis generally exists in different areas in China. The infection rate of this highly contagious disease ranges from 10 to 50% and results in substantial economic loss to the poultry industry.⁽⁸⁾

At present, identification of *B. avium* depends on isolation, agglutination test, agar gel precipitin test, fluorescent antibody technique, indirect ELISA, and other methods.^(9,10) Each technique offers its own advantages and disadvantages, such as complicated operation, time-consuming steps, and poor antibody specificity. Therefore, it is necessary to develop a special diagnostic reagent for the early diagnosis of *B. avium*. Considering monoclonal antibodies (MAbs) have better specificity and sensitivity that identify unique epitopes, they are irreplaceable in the detection and differentiation of sub-

types of pathogenic organisms. Hence, MAbs are broadly applied in the diagnosis and prevention of infectious diseases in animals.^(11–14)

In the last few years, the immunity provided by outer membrane protein (OMP), an important component of cytomembranes, has increasingly attracted the attention of researchers. Tests proved that OMPs stimulate both humoral and cellular immunity in animals. Furthermore, OMPs have displayed cross-reactivity in different bacterial serotypes.^(15,16) Our laboratory has researched various aspects of the OMPs of *B. avium* such as extraction techniques and immunogenicity. Hu and colleagues indicated that *B. avium* OMPs confer immune protection to chicks.⁽¹⁷⁾ Tan and colleagues analyzed B cell epitopes and predicted dominant B cell epitopes.⁽¹⁸⁾ In the present study, our group used *B. avium* OMPs to immunize BALB/c mice and feeder cells for hybridoma production. Then a double antibody sandwich ELISA (DAS-ELISA) was developed using prepared MAbs and rabbit polyclonal antibodies against *B. avium* OMPs. This study provides rapid, specific, and sensitive identification of *B. avium*.

Materials and Methods

Materials

A total of 22 *B. avium* strains were isolated from diseased chickens. Sera from 130 chickens with oculonasal discharge,

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sneezing, dyspnea, and decreased weight gain were collected from a large chicken breeding farm in Liaoning Province. *Proteus mirabilis* from infected chickens, *Escherichia coli*, *Salmonella*, and their OMPs, as well as rabbit polyclonal antibodies against *B. avium* OMPs were all preserved by the Microorganism Research Laboratory of Shandong Agricultural University.

BALB/c mice were purchased from the experimental animal center of Shandong University (Jinan, China). The study protocol was approved by the Animal Care and Use Committee (ACUC) of Shandong Agricultural University. Myeloma cells (SP2/0) were preserved by the Microorganism Research Laboratory of Shandong Agricultural University (Taian, China).

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island, NY). Hypoxanthine, aminopterin, thymidine, and peroxidase-conjugated goat anti-mouse IgG were supplied by Sigma (Beijing, China). A mouse MAb isotyping kit was purchased from Thermo Scientific (Shanghai, China). Polyethylene glycol (PEG 4000) was purchased from Amresco (Beijing, China).

Extraction and purification of OMPs

According to the method described by Wooldridge and colleagues with some modifications, a bacterial strain (GenBank no. HM545299) was selected from preserved *B. avium*.⁽¹⁹⁾ The aforementioned strain has a higher virulence and better cross-reactivity with other isolates of *B. avium*. The strains were cultured in bouillon medium at 37°C for 18 h. The bacteria were collected by centrifugation at 10,000 g for 15 min at 4°C, and then washed three times with chilled phosphate-buffered saline (PBS). The pelleted bacteria were suspended in 10 volumes of Tris-MgCl₂ buffer (Tris-HCl buffer with 10 mmol/L MgCl₂ [pH 7.8]), and subsequently sonicated 10 times (500 W, broken time 60 s, interval 60 s). Cells debris was removed by centrifugation at 10,000 g for 20 min. The supernatant was ultracentrifuged at 100,000 g at 4°C for 1 h. The pellet was resuspended in the same volume of Tris-MgCl₂ buffer with 2% Triton X-100 at room temperature for 30 min and ultracentrifuged at 100,000 g at 4°C for 30 min. The pellet was suspended in PBS and quantitated using the Bradford method.⁽²⁰⁾ Then the OMPs were analyzed by SDS-PAGE. The extracted OMPs were used as the immunized antigens.

The extracted OMPs were concentrated and purified by ultrafiltration and chromatography on Sephadex G-100 gel chromatographic columns. The purified OMPs were quantitated and analyzed by the Bradford method and SDS-PAGE.^(20,21) The purified OMPs were used to select positive hybridomas.

Immunization, cell fusion, and hybridoma selection

Immunity was induced in eight female BALB/c mice by intraperitoneal inoculation with 50 µg of extracted OMPs emulsified in Freund's complete adjuvant. The mice were boosted with the same dose of Freund's incomplete adjuvant after 2 weeks and at 5-week intervals thereafter. A final injection (200 µg) was intravenously given to the mouse without the adjuvant. At 1 week after the second booster, the antibody titers of the immunized mice were determined by indirect ELISA. The mouse that produced the highest ELISA titer was

selected for hybridoma production. When the ELISA titers exceeded 10⁴, the mouse was sacrificed and its splenocytes were prepared for fusion.

The fusions were carried out as described previously.⁽²²⁾ Briefly myeloma cells SP2/0 and splenocytes from immunized mice were fused with myeloma cells using 50% polyethylene glycol 4000 (PEG 4000). The cells were cultured in 96-well plates in DMEM with 10% fetal bovine serum and hypoxanthine-aminopterin-thymidine (HAT). After 12 to 15 days, the supernatants were screened using indirect ELISA established by Yang and colleagues⁽⁹⁾ to detect for the specific antibodies of *B. avium* OMPs. The selected positive hybridomas were subcloned for three rounds using the limiting dilution method.

Production of MAbs

Twelve-week-old female BALB/c mice were injected intraperitoneally with 0.5 mL of pristane. After one week, they were injected intraperitoneally with positive hybridoma (5 × 10⁶ cells). Ascitic fluid was harvested after one week and centrifuged at 10,000 g for 10 min. Then the supernatant oil and precipitates were removed. A large amount of MAb was purified twice with ammonium sulfate precipitation at 50% saturation and stored at -70°C.^(23,24)

The MAB isotypes were determined by testing the hybridoma cell culture fluid using a mouse MAB isotyping kit according to the manufacturer's instructions. The antibody titers of the hybridoma cell culture fluid and ascitic fluid were determined by indirect ELISA.

Western blot analysis was performed by following an established protocol. Briefly, the extracted *B. avium* OMPs resolved by SDS-PAGE were transferred onto a nitrocellulose membrane in transfer buffer. The transfer was performed at a constant current 300 mA for 2 h. Then the *B. avium* OMPs were probed with the hybridoma supernatant of selected MAbs according to the method by Frederick and colleagues.⁽²⁵⁾

Specificity and stability of MAbs

Positive hybridomas were cultured consistently for 3 months and resuscitated three times after cryopreservation. The specificity and stability of the MAbs were determined by indirect ELISA. The reactivities of the antibodies with the OMPs were compared among different bacteria: *B. avium*, chicken *P. mirabilis*, *E. coli*, and *Salmonella*.

Primary applications for MAbs: double antibody sandwich ELISA (DAS-ELISA)

According to the method described by Xiong and colleagues with some modifications,⁽²⁶⁾ an ELISA microplate was coated with rabbit polyclonal antibodies against *B. avium* OMPs in carbonate buffer at 4°C for 12 h, washed with PBST (PBS containing 0.05% Tween-20) and blocked with 5% skim milk in PBST at 37°C for 1 h. The plate was washed three times and blocking solution was added to the test samples by serial dilution followed by incubation at 37°C for 1 h. After washing, diluted MAbs against *B. avium* OMPs and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG were added to the wells and incubated at 37°C. The plate was washed and the substrate solution (sodium phosphate-citrate buffer containing 1 mg/mL tetramethylbenzidine and 0.003% H₂O₂ [pH

5.0)) was added. Finally, the reaction was terminated with 2 M H_2SO_4 and the absorbance at 450 nm was measured.

Standardization and optimization of DAS-ELISA

To develop a DAS-ELISA, rabbit polyclonal antibodies were used as capture antibodies and the MAb 4A₃ selected based on antibody titers was used as detection antibodies. The rabbit polyclonal antibodies were diluted 1:200, 1:400, 1:800, and 1:1600. The MAb was diluted 1:400, 1:800, 1:1600, and 1:3200. A square method was used to determine the optimum diluted concentration. The condition of coating was chosen by incubation at 4°C for 12 h or at 37°C for 4 h. The HRP-labeled goat anti-mouse IgG was diluted by 1:1000, 1:2000, 1:4000, and 1:8000 to detect the best concentration. The time for color development was presumed at 5 min, 10 min, 15 min, and 20 min. The other steps were as mentioned above. A positive well absorbance approaching 1.0 and the highest calculated P/N value (P, absorbance of the positive well; N, absorbance of the negative well) indicate the optimum reaction condition.

Specificity, sensitivity, and repetitiveness of the DAS-ELISA

B. avium, chicken *P. mirabilis*, *E. coli*, and *Salmonella* were used in the DAS-ELISA to determine the cross-reactivity of the antibodies with different bacteria. The lowest detectable value was determined through the serial dilution of *B. avium*. The developed DAS-ELISA was used to detect *B. avium* five times.

Clinical application of the DAS-ELISA

The other 21 *B. avium* strains were detected using the established DAS-ELISA method. Sera of 130 chickens were assayed using a plate agglutination test and the 130 chickens were assayed using the DAS-ELISA. Negative control samples were also assayed. When the calculated P/N value was >2.1, the result was considered positive.

Results

The OMPs of *B. avium*

The extracted *B. avium* OMPs used in our study were analyzed using SDS-PAGE. Briefly, the OMPs consisted of five protein bands with molecular weights of approximately 58, 47, 41, 24, and 16 kDa (Fig. 1), and the OMPs concentration was 640 µg/mL. The extracted OMPs were used as the antigen in the immunization protocol.

The purified *B. avium* OMPs consisted of a strong band with a molecular weight of approximately 58 kDa (Fig. 2). The concentration of the purified OMPs was 156 µg/mL. The purified OMPs were used as the antigen in indirect ELISA as coating during the selection of positive clones.

Immune effect of *B. avium* OMPs on BALB/c mice

Indirect ELISA was used to detect antibody titer in serum samples. Both BALB/c mice exhibited low background antibody before immunization and were acceptable for further immunization. After three booster immunizations, the highest antibody titer in mouse serum sample was 1:10,240 (Table 1).

FIG. 1. SDS-PAGE analysis of OMPs of *B. avium*. M, standard protein molecular weight; lane 1, extracted OMPs of *B. avium*.

Production and characterization of MAbs

The mouse splenocytes were fused with myeloma cells SP2/0. The fusion rate and positivity rate of the wells were 65% and 12%, respectively. Positive clones were selected and subcloned. Three strong positive hybridoma clones (designated as 3G₁₀, 4A₃, and 4E₈) were selected based on their reactivity in indirect ELISA and stability in culture. The clones exhibited exceptional growth characteristics and antibody production.

Antibodies secreted by the three hybridoma cell lines were all found to be IgG1 isotypes. Antibody titers in the hybridoma cell culture fluid were 1:320, 1:640, and 1:640, and the titers in ascitic fluid were 1:64,000, 1:128,000, and 1:128,000, respectively. In Western blot analysis, all MAbs recognized a 58 kDa protein band (Fig. 3). In the specificity test, except for the OMPs of *B. avium*, the MAbs could not react with other OMPs (chicken *P. mirabilis*, *E. coli*, and *Salmonella*). After culturing the hybridomas consistently for 3 months and resuscitating three times after cryopreservation, the antibody titers of the cell culture fluid and ascitic fluids reached approximately 1:320 to 1:640 and 1:64,000 to 1:128,000, respectively.

Establishment and application of DAS-ELISA

The optimal dilutions of the rabbit polyclonal antibodies and the MAbs in DAS-ELISA were determined by

FIG. 2. SDS-PAGE analysis of purified OMPs of *B. avium*. M, standard protein molecular weight; lane 1, purified OMPs of *B. avium*.

checkerboard titration; they were 1:800 and 1:1600, respectively. The coatings were performed at 4°C for 12 h. The time for color development was 15 min. The minimum detectable concentration of *B. avium* under the DAS-ELISA was 1×10^4 CFU/mL. The specificity test did not show cross-reactivity with chicken *P. mirabilis*, *E. coli*, and *Salmonella*. Based on the data of five repeated tests, the results were consistent. Thus, DAS-ELISA was proven a sensitive and reliable tool for *B. avium* detection.

TABLE 1. OD VALUES OF HIGHEST ANTIBODY TITER MOUSE SERUM BY INDIRECT ELISA

Dilution	Before immunization	After immunization
1:640	0.122	2.232
1:1280	0.113	2.013
1:2560	0.104	1.307
1:5120	0.095	0.920
1:10,240	0.088	0.235
1:20,480	0.070	0.113
Negative control	0.077	0.083
Blank control	0.035	0.039

Criteria for positive test results were OD_{positive} (OD for positive well) >0.1 and OD_{positive}/OD_{negative} (OD for negative well) ≥2.1.

FIG. 3. Western blot analysis of specific MAbs against *B. avium* OMPs. M, standard protein molecular weight; lane 1, 3G10; lane 2, 4A3; lane 3, 4E8.

The result of the DAS-ELISA demonstrated that the *B. avium* 21 strains were all positive. The plate agglutination test detected 96 of the 130 chicken serum samples positive. By contrast, the DAS-ELISA indicated that 110 chicken samples were positive, including 96 positive chicken samples tested under plate agglutination (Table 2). The coincidence rate between the DAS-ELISA and the plate agglutination test was 100%. However, the DAS-ELISA was more sensitive than the plate agglutination test.

Discussion

Bordetellosis is a highly contagious upper respiratory tract disease in poultry, characterized by oculonasal discharge, sneezing, dyspnea, and decreased weight gain. *B. avium* is highly transmissible—infected poultry, contaminated litter, and water all can transmit the bacteria. The hatching rate is typically reduced, and hatched poult infected with *B. avium* transmit disease to healthy poults. Under these conditions, the disease is often more severe with increased mortality because of increased stress. A rapid and definitive diagnosis of the disease is the outstanding prerequisite for controlling bordetellosis.⁽²⁷⁾

Diagnosing bordetellosis based on gross lesions and elaborate laboratory procedures for bacterial isolation is time

TABLE 2. DETECTION RESULT OF SERUM SAMPLES BY PLATE AGGLUTINATION TEST AND DAS-ELISA

Detection method	No. of samples	No. of positive samples	Positive rate (%)
Plate agglutination test	130	96	73.8
DAS-ELISA	130	110	84.6

consuming and expensive. Therefore, a rapid and precise laboratory diagnosis requires specific assays. DAS-ELISA is the most common method for detecting antigens. This assay is established by a pattern of polyclonal antibody-antigen-MAb by preparing rabbit polyclonal antibodies and MAbs with high titers; antigens subsequently combine with the distinct epitopes of two antibodies. Thus, DAS-ELISA is highly sensitive and specific for detection.

The most important step for establishing the DAS-ELISA is preparing highly sensitive and specific MAbs. The *B. avium* OMPs include mainly antigenic substances and cross-react with other *B. avium* serotypes. The OMPs are highly conserved and are highly immunogenic, which lays the foundation for preparing specific MAbs. Selecting ideal immunogens is the first step in producing MAbs. The immunogenicity of the antigens, immunizing dose, and the purity of the immunogen influence the success of MAb production. In this study, the extracted OMPs were used as immunogens, and the hybridoma cell lines were selected using purified OMPs. Three of the strongly reacting MAbs were used for further characterization and application in immunoassays.

The DAS-ELISA is established using rabbit polyclonal antibodies and the MAb against *B. avium* OMPs. Our results show that the DAS-ELISA has good specificity, sensitivity, and repetitiveness. The coincidence rate of detecting the other 21 *B. avium* strains isolated in our laboratory was 100%. Concomitantly, this method is more sensitive than the plate agglutination test. Therefore, the assay can be developed as a quick and specific diagnostic kit for controlling and preventing bordetellosis.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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