Production of Monoclonal Antibody Specific to Campylobacter jejuni and Its Potential in Diagnosis of Campylobacter Enteritis

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Campylobacter jejuni and Campylobacter coli are important gastrointestinal pathogens causing acute diarrhea with enteritis in humans, particularly in developing countries.¹⁻⁵ They are prevalent in infants under 1 year old⁶⁻⁷ and rank as the third most common cause of acute diarrhea after Rotavirus and enterotoxigenic Escherichia coli.¹⁰,¹¹ In Thailand, a report from the Children Hospital, Bangkok indicated that of 30% bacterial isolation rate, approximately 12% were Campylobacter spp., whereas about 10% and 7% were Shigella spp. and Salmonella spp., respectively.¹² Taylor et al. detected Campylobacter from stool of 105 (18%) of 586 children under 5 years with acute diarrhea in Bangkok.⁴ Of these 105 samples, Campylobacter spp were isolated as the single pathogen in 50 samples (40%). Most Campylobacter strains isolated in developing countries are biochemically and serotypically similar to strains isolated in developed countries and there is little strain variation.⁴,¹³⁻¹⁵ These Campylobacter spp may be both enteroinvasive leading to bloody diarrhea and enterotoxigenic causing watery diarrhea.¹⁶ The various clinical patterns suggest that Campylobacter spp possess more than one virulence factor. In addition, C. jejuni, unlike C. coli, are more often associated with symptomatic infections and with bloody diarrhea.¹⁷,¹⁸

Since Campylobacter enteritis is a considerable world health problem contributing to morbidity in developed countries and to high mortality rates in children in developing countries, it is of clinical importance to develop a specific and rapid diagnostic assay to identify Campylobacter in stool of enteritis patients. The available culture methods for detecting the organism generally are extremely time consuming and costly. Several immunoassay have been developed to detect Campylobacter infection. For epidemiological purposes, immunological reagents using polyclonal antibodies⁹ and monoclonal antibodies (MAbs) against formalin-treated C. jejuni are available for characterization and typing of C. jejuni and

SUMMARY A monoclonal antibody (MAb 3G6) specific for Campylobacter jejuni and Campylobacter coli was produced by immunizing BALB/c mice with a local strain of C. jejuni (28.1). No cross-reactivity was observed with Enterobacteriaceae controls. By immunoprecipitation, MAb 3G6 identified a major protein band of molecular weight 45 kDa and also gave a slight reactivity with 30 and 55 kDa proteins. Using an indirect enzyme-linked immunosorbent assay, MAb 3G6 was able to detect C. jejuni suspended in stool without cross-reactivity to 14 other enteropathogenic bacteria suspended, normal flora in fecal suspension, or fecal debris. In the analysis of fifty clinical specimens, MAb 3G6 detected most positive samples with the exception of one which possessed very low Campylobacter concentration and gave no reactivity to negative samples, demonstrating its high specificity to C. jejuni and C. coli. MAb 3G6 may be suitable as a new tool for the development of diagnostic method for Campylobacter infection.

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C. coli.\textsuperscript{20} However, these methods all require \textit{Campylobacter} colonies isolated from stool culture. An immunological approach for the detection of \textit{C. jejuni} utilizing stool suspension has not been reported.

In this study, MABs that are highly specific for veronal extracted antigen (VE-Ag) of \textit{C. jejuni} have been prepared. These MABs reacted with proteins of 20, 45 and 55 kDa and had the ability to react with intact cells of \textit{Campylobacter}. The preliminary assessment of sensitivity and specificity of the MAB was also performed as a mean of determining its usefulness as an immunodiagnostic marker. Furthermore, clinical specimens obtained from diarrheal patients were analyzed to evaluate the real potential use of the MAB in diagnosis.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

\textit{C. jejuni} isolates 28.1, 29.8, 3.8, 51.8 and 29.9, \textit{C. coli} isolates 19 and 23.8 and other \textit{Campylobacter} isolates were kindly provided by Dr. Udom Leksomboon (The Children Hospital, Bangkok, Thailand), \textit{C. jejuni} and \textit{C. coli} standard strains (ATCC 43502 and 43482, respectively) were obtained from the American Type Culture Collection, Rockville, Maryland. The bacteria were inoculated onto blood agar plate and placed in 37°C incubator with an atmosphere of 89% N\textsubscript{2}, 5-10% CO\textsubscript{2} and 5% O\textsubscript{2}. After 24-48 hours of incubation, bacteria were suspended in brucella broth (Difco Laboratories, Detroit, MI) and spreaded onto columbia agar (Gibco, BRL, Bethesda, MD) supplemented with 10% human blood. After 48 hours the bacteria were stained with Gram stain and examined under light microscope for a seagull-shape bacteria. \textit{E. coli}, \textit{Proteus mirabilis}, \textit{Salmonella choleraeus}, \textit{Salmonella enteritidis}, \textit{Salmonella typhimurium}, \textit{Salmonella paratyphi A}, \textit{Salmonella paratyphi B}, \textit{Salmonella panama}, \textit{Shigella} spp, \textit{Yersinia enterocolitica}, \textit{Vibrio parahaemolyticus}, \textit{Streptococcus pyogenes}, and \textit{Streptococcus agalactiae} were obtained from Dr Suttipant Sarasombat, Department of Immunology, Faculty of Medicine Siriraj Hospital, Bangkok.

**Preparation of antigens**

\textit{C. jejuni} and \textit{C. coli} VE-Ag were prepared as originally described by Barber, Vladoianu and Dimache with some modifications.\textsuperscript{21} In brief, each bacterial isolate was cultured on columbia agar and allowed to incubate for 48 hours in 37°C incubator with 5-10% CO\textsubscript{2}. The bacterial cells were harvested and 3 volumes of aceton were added. The suspension was centrifuged at 10,000 x g, 4°C for 5 minutes and a pellet was washed three times with aceton. Ten grams of aceton-dried cells were suspended in 200 ml of 0.15 M veronal buffer, pH 8.4 and the suspension was allowed to stand with continuous stirring at room temperature overnight. After centrifugation at 12,000 x g at 4°C for 20 minutes the supernatant was removed and dialysed against distilled water at 4°C for 2 days. The dialysed supernatant was filtered through a 0.45 µm Seitz filter (Gibco, BRL) to remove residual bacteria and other cellular debris. Other bacterial antigens were prepared the same way. Protein content was determined by Folin-Ciocalteau method.\textsuperscript{22}

**Immunization of mice**

VE-Ag of \textit{C. jejuni} 28.1 was selected to immunized mice because a good antibody response was obtained with low toxicity. Three six-week-old female BALB/c mice were immunized subcutaneously with 100 µg of antigen emulsified in incomplete Freund’s adjuvant (Difco). On day 14 and 21, 100 µg of the same antigen suspended in incomplete Freund's adjuvant were injected intraperitoneally. Animals were bled and antibodies against VE-Ag were quantitated by indirect enzyme-linked immunosorbent assay (ELISA) before fusion. Five days before fusion, a booster dose of 250 µg of antigen in saline was injected intraperitoneally.

**Fusion Procedure**

Fusion of the splenocytes of a high responding BALB/c mouse (anti-VE-Ag titre, 1/28,000) with the plasmacytoma cell line P3-X63.Ag 8.653 was performed in a ratio of 10:1 in a solution containing 37% (wt/vol) polyethylene glycol (Gibco, BRL) by the method of Margulies \textsuperscript{23} with modifications. Fused cells at a concentration of 1 x 10\textsuperscript{7} cells/ml were dispensed in 96-well tissue culture plate (NUNC, Denmark) which contained a feeder layer of 10\textsuperscript{4} murine peritoneal macrophages. Cells were grown in RPMI 1640 supplemented with 15% fetal calf serum (Flow Lab, Mississauga, Ontario, Canada), 2 mM L-glutamine and 1% hypoxanthine-aminopterin-thymidine (HAT) (Gibco, BRL).

Medium was changed on day 7, substituting HAT with hypoxanthine-thymidine (Sigma Chemical Co, St. Louis, MO). Approximately on day 15, supernatants of wells containing growing clones were tested for MAb directed against VE-Ag. The antibody producing cells were twice recloned by limiting dilution. A clone designated 3G6 was one of the three selected for further study.

**Determination of immunoglobulin class**

Ig isotyping was done by an antibody capture ELISA using isotype and class-specific antisera (ImmuNo Select\textsuperscript{TM} Isotyping System) (Gibco, BRL). Briefly, 50 µl of a monoclonal rat anti-mouse isotype antibody was used to coat the surface of a microtitration plate and acted as the capture antibody. Nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (0.01 M sodium phosphate buffer pH 7.4, 0.14 M NaCl) and 150 µl of hybridoma culture supernatant to be tested was added. After incubation and washing, 150 µl of rat anti-mouse immunoglobulin-alkaline phosphatase

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**References**

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conjugate were added to each well and a color development using alkaline phosphatase substrate p-nitrophenyl phosphate was performed. The intensity of the color is proportional to the amount of antigen in the test sample.

**ELISA procedure**

Screening of hybridoma culture supernatants and detection of *Campylobacter* in clinical specimens were done by indirect ELISA. U-bottom microtitration plates (Immunolon II, Dynatech Industries, Inc., Alexandria, VA) were coated with 100 µl of 10 µg/ml *C. jejuni* VE-Ag or whole-cell suspension (10⁵-10¹⁰ cfu/ml) or 10% fecal suspension in carbonate buffer, pH 9.6, and kept for either 4 hours at 37 °C or 16 hours at 4°C. Any remaining protein binding sites in the plate were saturated by adding 100 µl of 5% milk (Carnation) in PBS. The plates were washed 3 times with PBS containing 0.05% Tween 20 (Sigma) (PBS-T). Culture supernatants (150 µl) from the fusion plates were added. After incubation for 4 hours at 37°C, the plate was washed 3 times with PBS-T. One hundred microliters of goat anti-mouse immunoglobulin horseradish peroxidase conjugate (Dakopatts, Copenhagen, Denmark) diluted 1:2,000 in PBS-T were added. The plate was incubated for 1 hour at 37°C. After washing, 100 µl of 3,3',5,5'-tetramethylbenzidine solution (0.1 mg/ml in 0.1 M sodium acetate-0.1 M citric acid (pH 5.7) with 0.005% H₂O₂) were added to each well and incubated for 20 minutes at room temperature. The color was developed in the dark for 20 minutes at room temperature and the reaction was stopped by adding 25 µl of 4 M H₂SO₄. The optical density was determined on an ELISA reader (Minireader II, Dynatech) at 450 nm. An optical density >25% of the maximum reading of positive control was considered positive.

**SDS-PAGE**

Sodium dodecyl sulfate-10% polyacrylamide gels were prepared as described by Laemmli using 0.75 mm-thick gels. Molecular weight standards (14-205 kDa) (Bio-Rad Laboratories, Richmond, CA) were included in each gel. The gel system consisted of a 10% separating gel and 3% spacer gel. Samples of veronal extracted antigens and immunoprecipitations were dissolved in sample buffer, boiled for 3-5 minutes and then applied to the gel. Electrophoresis was carried out with a constant current of 30 mA.

**Antigen labelling and immunoprecipitation**

*Campylobacter* antigens were radio-labelled with ¹²⁵I (Amersham International Limited, UK) by the chloramine-T technique originally described by Greenwood et al. in brief, pH of antigen suspension was adjusted by adding 1/19 volume of 20x concentration of Tris-HCl pH 8.0. Radioactive NaI was added to the antigens at a ratio of 2 µCi/µg protein. Then chloramine-T was added (0.2 µg/µg protein) and the reaction mixture was incubated at 4°C for 30 seconds. The labelling reaction was stopped by adding excess amounts of sodium metabisulfite (0.2 µg/µg chloramine T). Nal radioactive carrier (approximately 10 times the amount of radioactive NaI used) and 1 µg of BSA/µg protein.

Immunoprecipitation of ¹²⁵I-labelled *C. jejuni* antigens were performed using a technique slightly modified from that described by Ivarie and Jones. To reduce non-specific binding, the ¹²⁵I-labelled antigens were first preadsorbed with a 50% slurry protein-A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). After centrifugation at 10,000 x g at 4°C for 1 minute, the supernatant was carefully removed and divided equally into 5-ml snap-cap tubes (Falcon, Becton Dickenson, Lincoln Park, NJ). Then the supernatant containing labelled antigens was allowed to react with concentrated (6X) MAb, pooled irrelevant hybridoma culture supernatants and pooled immune sera from mice immunized with VE-Ags of *C. jejuni*. The reaction was incubated at 37°C for 1 hour and then kept overnight in the refrigerator. Immune complexes were separated from non-reacted antigens by the addition of a precalibrated amount of protein A-Sepharose. After 1 hour incubation at room temperature, the immune complexes were washed once with NETT (0.1 M NaCl, 0.5 M EDTA and 0.1 M Tris-HCl pH 7.4), twice with NETT buffer and twice with 1% BSA in NETT buffer. The Sepharose beads with the attached immune complexes was suspended in SDS-PAGE buffer and analyzed by SDS-PAGE. Autoradiography was performed using X-OMAT XR film (Eastman Kodak Co, Rochester, NY).

**Clinical specimens**

Fecal specimens from patients with acute diarrhea (50 samples) were speciments routinely submitted to the Children Hospital, Bangkok. The specimens were collected in brucella broth containing 0.1 mg/ml of soybean trypsin inhibitor in 50 mM EDTA. Control feces consisted of 5 samples from children (age range 3 months to 2 years). A 10% fecal suspension was made and 100 µl was used to coat U-bottomed microtitre plate (Dynatech) for *Campylobacter* detection by indirect ELISA. A filtration culture method adapted from that of Steele and McDermott was also performed in parallel at the Children Hospital Laboratory, Bangkok since this method is used routinely to diagnose *Campylobacter* infection. In brief, several drops of fecal suspension were applied directly onto a sterile 0.45 µm membrane filter (Millipore Corp, Bedford, MA) placed on colunbia blood agar base without an-tiotics. The filter was removed after 15 minutes and the plate was incubated microaerobically at 42°C for 24 to 48 hours. *Campylobacter* were able to dart through the filter to produce colonies on the agar surface. Subsequently, Gram stain was performed to search for seagull-shaped bacteria. In addition, specimens were inoculated onto culture plates with antibiotic containing media.
RESULTS

Antibody-producing hybridomas were obtained from 2 different fusions. A total of 736 culture supernatants were screened and only 3 hybridomas producing specific antibodies reacting with VE-Ag and intact cells of Campylobacter were detected. One stable hybridoma designated 3G6 which produced high antibody titre reacted specifically with *C. jejuni* and with lower binding capacity to *C. coli* but not with other Enterobacteriaceae used in the initial screening.

Isotyping revealed that MAb 3G6 is of IgG1 class and has a kappa light chain. Using an indirect ELISA, only 0.5 μg of MAb 3G6 could successfully detected intact *Campylobacter* cells. The MAb was highly reactive to *Campylobacters* as with an O.D. of >1.0 for all isolates (suspended in PBS) tested. The specificity of MAb was determined by ELISA using sonicated antigens and intact cells of other several strains of pathogenic enteric bacteria. None of the enteric bacteria tested reacted with MAb 3G6 (O.D. less than 0.1).

In immunoprecipitation analysis, MAb 3G6 was shown to recognize 30, 45 and 55 kDa polypeptide bands as shown in Fig. 1. The MAb reacted strongly with the 45 kDa antigen and showed slight reactivity with the 30 and 55 kDa proteins.

To evaluate whether MAb 3G6 has potential in rapid diagnosis for *Campylobacter* infection, different concentrations of whole intact cells of *C. jejuni* and *C. coli* were suspended in 10% stool suspensions from 3 normal children aged 3 months to 4 years old. Stool suspensions with 10^5–10^10 cfu/ml of *C. jejuni* and *C. coli* gave positive reactions detected by indirect ELISA when compared to stool suspension alone (Fig. 2). However, the mean optical densities of samples containing stool suspension mixed with *Campylobacter* were constantly less than those of samples containing the bacteria suspended in PBS (Fig. 2). In addition, MAb 3G6 could recognize various

Fig. 1. Molecular weight determination of specific antigens bound to anti-*C. jejuni* antibodies. Autoradiographic patterns of 125I-labelled sonicated antigens of *C. jejuni* are shown in lane A. The 125I labelled antigens were immunoprecipitated with different anti-*C. jejuni* antibodies and subsequently separated on SDS-PAGE. Lane B, the labelled antigens reacted with irrelevant hybridoma culture supernatants represents negative control; lane C, the labelled antigens reacted with polyspecific immune sera; lane D, the labelled antigens reacted with MAb 3G6. Numbers on the left indicate the molecular weight standards. Arrows show 30, 45 and 55 kDa protein bands.

Fig. 2. Detection of *C. jejuni* and *C. coli* by ELISA. ELISA results demonstrate mean optical density of reaction between MAb 3G6 and *C. jejuni* suspended in 3 different normal stool suspension (B, C, D) and in PBS (E). The stool suspension without *C. jejuni* was used as negative control (A).
isolates of Campylobacter in 10% stool suspension as exemplified for seven of them in Fig. 3.

To exclude the possibility that MAb 3G6 would react with pathogenic enteric bacteria in stool suspension, stool was suspended with approximately 10^5 cfu/ml of each of 14 pathogenic enteric bacteria tested. Mean optical densities at 450 nm ranged from 0.02 to 0.08 for other enteric bacteria whereas those of Campylobacter were more than 1. Hence, no cross reactivity of MAb 3G6 with 14 other species of enteric bacteria was detected.

A double blinded study was performed to evaluate the potential application of MAb 3G6 in diagnosis of Campylobacter infection. Fifty stool specimens were obtained from the Children Hospital from November to December 1991. C. jejuni and C. coli were isolated using direct culture on antibiotic containing medium in 7 and by filtration method in 4 of 50 specimens. The indirect ELISA results demonstrated that 6 of these 7 samples were positive (Fig. 4). Three stool specimens which had very low Campylobacter concentration (<10^3 cfu/ml) were negative using filtration method. Of these three samples, one failed to be detected by indirect ELISA. All samples negative on direct culture, gave mean optical density readings less than 0.25. In addition, the optical density readings at 450 nm correlated to the number of Campylobacter found in stool.

DISCUSSION

In this study, we immunologically identified a specific polypeptide of 45 kDa of C. jejuni protein. A MAb (3G6) that reacted with 45 kDa band did not react with other 14 strains of enteric bacteria. These isolates were selected for testing because they are known to cause gastroenteritis and might interfere in an immunologic assay for detection of Campylobacter in stool specimens.

The MAb recognized intact cells of Campylobacter. Therefore, the
determinants or epitopes to which MAb binds on the antigen appear to be exposed or partially exposed on the cell surface. Preliminary data suggest that the antigen may be major outer membrane proteins of C. jejuni.28-32 The major outer membrane proteins of different isolates of C. jejuni have reported molecular weight of 41-45 kDa.29,33 Other proteins identified such as protein of relative molecular weight of 30 kDa which contains cross-reactive epitopes with the major outer membrane protein were also identified by Logan and Trust.34 However, reactivity of MAb 3G6 to outer membrane will need to be confirmed.

Potential application of MAb 3G6 in diagnosis of Campylobacter diarrhea was evaluated. For specificity, indirect ELISA results demonstrated that MAb 3G6 gave no cross-reactivity with 14 other species of enteropathogenic bacteria and reacted with all isolates of Campylobacter tested. However, when suspending Campylobacter in stool of normal children, we found that the ability of MAb 3G6 to detect Campylobacter decreased, compared to those suspended in PBS. The decline in optical density readings may be due to the competitive binding of Campylobacter with other fecal flora or stool components to the limited surface area in the U-bottomed well. Although competitive surface saturation might not cause measurable effect for stool specimens from patients age under 12 months due to low concentration of fecal flora and fecal debris, a double antibody sandwich ELISA was performed directly to the antigen of fecal samples. Our results demonstrated that MAb 3G6 has significant diagnostic potential for detection of whole cells of C. jejuni and C. coli. Indirect ELISA failed to detect Campylobacter when its concentration in stool was less than 10^3 cfu/ml. Nevertheless, such low number of bacteria may not be associated with diarrhea, as in developing countries there are multiple pathogens encountered. Furthermore, there is no reported close-association between the number of Campylobacter and disease pattern. There are, however, frequent isolation of Campylobacter from asymptomatic infection. It remains to determine the specificity of MAb 3G6 to various species and isolates of Campylobacter using clinical specimens from patients suffering from bacterial enteritis.

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REFERENCES
MONOCLONAL ANTIBODY TO CAMPYLOBACTER


