Rapid Detection of *Bordetella pertussis* by a Monoclonal Antibody-Based Colony Blot Assay

BJÖRN GUSTAFSSON1,2* AND PER ASKELÖF1

Department of Vaccine Production, National Bacteriological Laboratory, S-105 21 Stockholm,1 and Department of Bacteriology, Karolinska Institutet, S-104 01 Stockholm,2* Sweden

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Monoclonal antibodies to *Bordetella pertussis* filamentous hemagglutinin (FHA) and lipopolysaccharide (LPS) were used in a colony blot enzyme-linked immunosorbent assay designed for rapid detection of *B. pertussis*. Bacterial colonies from Bordet-Gengou agar plates were blotted onto nitrocellulose filter disks, lysed by immersion in chloroform, and reacted with monoclonal antibodies. Following reaction with peroxidase-conjugated rabbit anti-mouse immunoglobulin antisera and 4-chloro-1-naphthol, blue dots representing single colonies appeared on the filters. Blotting of single *B. pertussis* colonies could be performed after incubation for 40 h, i.e., before the colonies were visible by eye on the agar surface. Ten of ten *B. pertussis* strains showed positive blotting reactions with antibodies specific for *B. pertussis* FHA and LPS. Fourteen of fourteen *B. parapertussis* strains reacted with two of the FHA-specific antibodies but not with two of the LPS-specific antibodies. Strains of *B. bronchiseptica* showed a variable reaction pattern. No cross-reactions were observed with strains of *Streptococcus mitis*, *S. pyogenes*, *S. pneumoniae*, *Staphylococcus aureus*, * Branhammera catarrhalis*, or *Klebsiella pneumoniae*. This assay may be useful for identification of *B. pertussis* and *B. parapertussis* in suspected cases of whooping cough.

*Bordetella pertussis*, the causative organism of the disease whooping cough, is a fastidious and slowly growing organism. In the early phase of the disease, the bacteria attach to and multiply on the cilia of epithelial cells of the respiratory tract. Laboratory diagnosis is performed by cultivating the bacteria from nasopharyngeal swabs or washings on selective media, such as Regan-Lowe or Bordet-Gengou medium, for 3 to 7 days (8). However, the frequency of *B. pertussis* infection is considered to be underestimated, partly because of the lack of rapid and reliable laboratory diagnostic techniques (7).

It is important from both diagnostic and epidemiological points of view that accurate and reproducible methods are available for identification of *B. pertussis* infections. The colony blot assay is able to detect bacteria in diminutive amounts and has been used for detection of *Mycoplasma* sp. (5) and *Brucella* sp. (10), as well as hemolysin (4), colonization factor antigen (6), and Shiga-like toxin (12) from *Escherichia coli*.

In this study, we used monoclonal antibodies against *B. pertussis* lipopolysaccharide (LPS) (3) and filamentous hemagglutinin (FHA) (2) in a colony blot assay for detection of *B. pertussis* and *B. parapertussis* colonies growing on selective agar plates. The assay is completed within 6 h, and single colonies are detected on agar plates incubated for 40 h at 37°C. This assay may be useful for detection of *B. pertussis* and *B. parapertussis* in samples from patients.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation.** *B. pertussis* 18323 (phase 1) and 44122/c (phase 4); the clinical isolates *B. pertussis* 636/83, 23/85, 35/84, 60/84, 29/84, 500/85, 40/85, and 210/84 (all in phase 1); *B. parapertussis* ATCC 15237; and the clinical isolates *B. parapertussis* 39/82, 89/82, 229/82, 228/83, 328/83, 670/85, 703/85, 704/85, 751, 762/85, 2/86, P42, and BDI 181 were obtained from the National Bacteriological Laboratory, Stockholm, Sweden. *B. bronchiseptica* B2533/83, B2786/83, 809/86, 755/86, and 754/86 (all clinical isolates) were obtained from E. Olsson, National Veterinary Laboratory, Uppsala, Sweden. *Streptococcus mitis* K122, *S. pyogenes* K124, *S. pneumoniae* CCUG 2839 and CCUG 2497, *Staphylococcus aureus* S209, *Branhammera catarrhalis* 18284, and *Klebsiella pneumoniae* K133 were obtained from the Karolinska Institutet, Stockholm, Sweden. The bacteria were cultivated on Bordet-Gengou agar at 37°C for 24 to 48 h.

The bacteria were suspended in 0.01 M phosphate-buffered saline, pH 7.2. The optical density was adjusted to 0.275 at 525 nm, and 10-fold dilutions were made in phosphate-buffered saline. Samples (0.1 ml each) of a 10−3 dilution of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* suspensions were spread on Bordet-Gengou plates. In some experiments, suspensions of *Streptococcus mitis*, *S. pneumoniae*, *K. pneumoniae*, and *B. catarrhalis* were prepared as described above, and 0.1-ml amounts of a 10−3 dilution of each bacterial suspension were mixed with a *B. pertussis* suspension before being spread onto Bordet-Gengou agar plates.

**Hybridomas.** The production and characterization of mouse monoclonal antibodies to *B. pertussis* FHA (2) and LPS (3) have been described previously. The antibodies were prepared from ascitic fluid obtained by injecting 5 × 106 hybridoma cells intraperitoneally into pristane (Sigma Chemical Co., St. Louis, Mo.).-primed mice (9). The characteristics and specificity of each antibody are described in Table 1.

**ELISA.** Antibody production of hybridomas was measured by an enzyme-linked immunosorbent assay (ELISA) (1) performed in 96-well microtiter trays (Dynatech M 129 A; Flow Laboratories, Irvine, Scotland) as described earlier (2, 3). Briefly, each well was coated with 125 μl of *B. pertussis* FHA (1 μg/ml) or LPS (20 μg/ml) in phosphate-buffered saline and 0.02% NaN3 and left overnight at 22°C. The remaining binding sites were blocked by 1% bovine serum albumin...
TABLE 1. Characteristics of monoclonal antibodies and their ELISA titers

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Immunoglobulin class</th>
<th>Specificity</th>
<th>Titera</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-2-A5</td>
<td>G1</td>
<td>FHA</td>
<td>160,000</td>
<td>2</td>
</tr>
<tr>
<td>16-2-A8</td>
<td>G2a</td>
<td>FHA</td>
<td>320,000</td>
<td>2</td>
</tr>
<tr>
<td>16-2-D9</td>
<td>G1</td>
<td>FHA</td>
<td>640,000</td>
<td>2</td>
</tr>
<tr>
<td>6-4-H6</td>
<td>G3</td>
<td>LPS</td>
<td>64,000</td>
<td>3</td>
</tr>
<tr>
<td>9-1-H5</td>
<td>M</td>
<td>LPS</td>
<td>32,000</td>
<td>3</td>
</tr>
<tr>
<td>9-2-A8</td>
<td>G3</td>
<td>LPS</td>
<td>32,000</td>
<td>3</td>
</tr>
</tbody>
</table>

a Endpoint titer of ascites fluid in ELISA. The endpoint was defined as the highest dilution in a twofold serial dilution still giving an optical density at 492 nm of >0.2 above the background. Wells were coated with B. pertussis FHA and LPS, respectively.

albumin in PBS–0.05% Tween 20, and 100-μl samples of serial dilutions of ascitic fluid were added to the wells. The immune reaction was performed by adding 100 μl of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Copenhagen, Denmark). A 100-μl volume of 3 mM 1,2-phenylenediaminedihydrochloride (Fluka, Buchs, Switzerland) in a substrate buffer consisting of 25.7 ml of 0.2 M Na2HPO4, 24.3 ml of 0.1 M citric acid, and 50 ml of deionized water (pH 5.0) was used as a substrate. Immediately before use, 40 μl of a 30% H2O2 solution was added to the substrate solution. The enzyme reaction proceeded for 15 min at 22°C and was terminated by addition of 50 μl of 1 M H2SO4. The optical density at 492 nm was measured in a Titertek Multiscan (Flow Laboratories) spectrophotometer. An optical density of >0.2 above the background was considered to be a positive result. As a negative control, wells were incubated with ascitic fluid from mice injected with SP2/0-Ag14 myeloma cells (11).

Colony blot ELISA. The bacteria were grown for 24 to 48 h on Bordet-Gengou agar plates. Blotting of colonies was performed by a modification of the method described by Roop et al. (10). Nitrocellulose filters (Schleicher & Schuell, Dassel, Federal Republic of Germany) were cut into disks, placed on the plates, and allowed to adhere to the surface for 10 min at 22°C. The disks were removed, and the adherent bacterial cells were lysed by immersion of each disk for 10 min in a petri dish containing 15 ml of chloroform. The disks were allowed to dry for 15 min and then immersed in a solution consisting of 3% gelatin in 20 mM Tris hydrochloride–0.5 M buffered saline (TBS), pH 7.5, for 45 min at 22°C before incubation with monoclonal antibodies (diluted 1:50) for 2 h at 22°C. Unbound antibodies were removed by washing the disks three times for 10 min each time with TBS–0.05% Tween 20 before incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako) for 1 h. The nitrocellulose disks were washed twice for 10 min each time with TBS-Tween and once for 10 min with TBS and then incubated with a substrate solution containing 60 mg of 4-chloro-1-naphthol (Sigma) dissolved in 100 ml of TBS with 20 ml of methanol. H2O2 (60 μl; 30%) was added immediately before use. The reaction was stopped by rinsing the disks in tap water.

RESULTS

Bacterial colonies formed by B. pertussis, B. parapertussis, and B. bronchiseptica were transferred to a nitrocellulose filter and blotted with monoclonal antibodies specific for B. pertussis FHA and LPS (Table 1).

Inoculating Bordet-Gengou agar plates with diluted preparations of B. pertussis resulted in visible growth of single colonies after incubation for 3 to 4 days. However, a positive blotting reaction was obtained with B. pertussis strains after incubation of the agar plates for only 48 h. Single colonies, not visible by eye, gave rise to distinct blue dots on the nitrocellulose filter (Fig. 1A). Spreading of a single colony with a loop resulted in a positive blotting reaction with bacteria in the primary inoculation after only overnight incubation (Fig. 1B).

Positive blotting reactions were obtained with all of the B. pertussis strains tested, irrespective of whether monoclonal

FIG. 1. Nitrocellulose replicas of cultures of B. pertussis 23/85 blotted with FHA-specific monoclonal antibody 15-2-A5. Agar plates were either inoculated with diluted suspensions of B. pertussis and incubated at 37°C for 48 h (A) or spread with a single colony by loop and incubated overnight (B) before blotting.
antibodies to FHA or LPS were used (Table 2). The strongest color reaction was obtained with monoclonal antibody 15-2-A5. Blotting reactions with *B. parapertussis* and *B. bronchiseptica* strains are given in Table 2.

No cross-reactions were observed with colonies formed by *Streptococcus mitis*, *S. pyogenes*, *S. pneumoniae*, *K. pneumoniae*, *Staphylococcus aureus*, and *Branhamella catarrhalis* (Table 2). Brown dots appeared from colonies formed by streptococci immediately upon transfer of colonies to the nitrocellulose filter. However, these dots were easily distinguished from the small blue dots obtained following the specific immunological reactions obtained with colonies of *B. pertussis*. No blotting reaction of any strain tested was obtained with ascitic fluid from mice injected with SP2/0-Ag14 myeloma cells.

The effects of incubating the agar plates for different periods before blotting them were also studied. Blotting of single colonies of *B. pertussis* 23/85 after 24, 40, or 48 h of incubation with monoclonal antibody 15-2-A5 was performed. Weak but clearly visible staining of single colonies was obtained after incubation of the agar plates for 40 h. The blotting reaction became more distinct with incubation for 48 h, although no colonies were visible on the surface of the agar. No blotting reaction of single colonies was obtained after incubation for 24 h.

**DISCUSSION**

The clinical symptoms of pertussis are often diffuse at the onset of the disease. Rapid methods of diagnosing the infection could eliminate the need for multiple diagnostic tests and inappropriate therapy.

Cultivation of suspected samples on freshly made Bordet-Gengou or Regan-Lowe agar trays is recommended for successful isolation of *B. pertussis*. The trays should be examined daily for 7 days, and serological confirmation should be performed by fluorescent-antibody test (8). This procedure is time consuming and requires access to a fluorescence microscope and personnel skilled in this technique. With the colony blot assay, it is possible to obtain isolation and immunological confirmation of the bacterium within 48 h by using specific monoclonal antibodies and standard laboratory reagents. Differentiation of *B. pertussis* from *B. parapertussis* was possible, since the LPS-specific monoclonal antibodies reacted with *B. pertussis* only, whereas most of the FHA-specific antibodies reacted with both *B. pertussis* and *B. parapertussis*. This may be useful in epidemiological studies.

The assay must be considered sensitive, since each dot represents 1 CFU. Theoretically, a 0.1-ml sample containing 5 to 10 live *B. pertussis* bacteria should be enough to identify a positive sample. However, the clinical isolates used in this study were kept in lyophilized amouplets until used. Fresh nasopharyngeal aspirates should be used for clinical evaluation of this assay.

Monoclonal antibody-based sandwich ELISAs for detection of *B. pertussis* LPS (3) and FHA (2) have been previously developed. The advantage of these assays is that they detect both soluble antigens and whole bacteria and are not dependent on the presence of live bacteria in the sample, whereas the colony blot assay detects only live bacteria. However, LPS- and FHA-specific sandwich ELISAs require antigen concentrations of 160 to 320 (LPS) and 7 to 15 (FHA) ng/ml, which far exceed the antigen concentrations present on 5 to 10 bacteria.

Monoclonal antibodies 15-2-A5, 16-2-A8, and 16-2-D9 have been shown to react with purified FHA in an ELISA
It was also shown that antibodies 15-2-A5 and 16-2-A8 were positive in blotting experiments with sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated FHA, whereas blotting with monoclonal antibody 16-2-D9 showed no band at all. It was suggested that the antigen epitopes responsible for binding of antibody 16-2-D9 may be heat labile and thus denatured during sample preparation (2). In this study, we found that all three antibodies gave rise to dot blots when tested with B. pertussis colonies. Furthermore, FHA-specific monoclonal antibody 16-2-A8 reacted with only three of the B. parapertussis strains in the colony blot assay, whereas the other FHA-specific monoclonal antibodies reacted with all of the B. parapertussis strains. All of this information, taken together, indicates that different epitopes of the FHA-molecule are responsible for binding of each antibody. The different reactivities observed between monoclonal antibody 16-2-A8 and strains of B. parapertussis indicate a heterogeneity in the structure of B. parapertussis FHA that may be used for serological differentiation of this species.

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LITERATURE CITED