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Nonradioactive Colony Lift-Hybridization Assay for Detection of Bordetella bronchiseptica Infection in Swine

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Current methods for the isolation and identification of Bordetella bronchiseptica from clinical samples are time-consuming and are based, in part, on subjective observations. We describe the use of a Bordetella-specific DNA probe in a nonradioactive colony lift-hybridization assay for the identification of B. bronchiseptica. Eleven of 82 clinical specimens were found to contain B. bronchiseptica by this method, while only 5 of these were reported to contain the organism when the specimens were analyzed by traditional methods. The chromosomal fragment containing a sequence complementary to the probe appeared to be conserved in B. bronchiseptica isolates from swine from a variety of sources. The assay is more rapid than culture and biochemical testing since it can be performed directly on primary culture plates, even when they are heavily contaminated with other bacterial species. Only minimal training is required to accomplish the assay successfully, and the results are easy to interpret.

Bordetella bronchiseptica is a primary etiologic agent of swine atrophic rhinitis and primary bronchopneumonia in piglets. Moderate and severe outbreaks are of considerable economic importance because they are often accompanied by a reduced growth rate and inefficient feed conversion (4, 11).

A definitive diagnosis of B. bronchiseptica infection in swine is established by isolation of the organism from nasal swabs or lung biopsy specimens. Suspect colonies, identified by morphology, are further subjected to a variety of biochemical tests. This method of identification is costly and time-consuming. Excessive contamination of the sample by normal flora is common, resulting in overgrowth of other organisms present. Furthermore, visual recognition of suspect B. bronchiseptica colonies requires a certain level of skill and experience.

Although various selective media have been proposed for use by different investigators, none has been proven to be entirely satisfactory. In some cases, specimens plated on selective media that did not yield B. bronchiseptica were not evaluated by another method (5, 7, 13). This is an important point, since it has been reported that selective media can retard the growth of B. bronchiseptica when the organism is present in low numbers (20). Some media permit the growth of other bacterial species which can cause B. bronchiseptica to display an atypical colony morphology (20). Such colonies are not likely to be singled out for further biochemical testing, particularly by inexperienced personnel. One study recommended the use of blood agar supplemented with cephaloxin (13), although the concentration of antibiotic used was not indicated. This recommendation should be viewed with caution since some studies have reported that B. bronchiseptica is susceptible to broad-spectrum cephalosporins (25).

A faster and more objective method for the identification of B. bronchiseptica infection in swine would clearly be advantageous to clinical veterinary laboratories and would also facilitate experimental studies. Here we describe the use of a nonradioactive colony lift-hybridization assay for the quick and easy identification of this organism from mixed cultures.

MATERIALS AND METHODS

Bacteria and inoculum. A previously described (9) porcine isolate of B. bronchiseptica, MBORD 846, was used to inoculate piglets. Bacteria were grown at 37°C for 18 to 24 h on Bordet-Gengou agar supplemented with 15% defibrinated sheep’s blood. Organisms were scraped from the surface of the plate and were suspended in sterile phosphate-buffered saline (PBS) at an A600 corresponding to approximately 10⁷ CFU/ml.

Biochemical characterization of all organisms was carried out by the Clinical Microbiology Laboratory at the College of Veterinary Medicine, Iowa State University. Gram-negative cocccobacilli that grew on MacConkey agar and that displayed a smooth, domed, hemolytic colony morphology on blood agar were selected for further testing. Bacteria identified as B. bronchiseptica tested positive for oxidase, urease, citrate, and nitrate reduction and tested negative for indole, H₂S production, gelatin hydrolysis, and production of acid from glucose, mannose, inositol, sorbitol, rhamnose, sacrose, melibiose, and arabinose.

Animals and experimental design. For the first phase of the study cesarean-derived, colostrum-deprived (CDD) piglets were housed in isolation units receiving filtered air. They were maintained on a sterile diet of SPF-Lac (Pet Ag, Hampshire, Ill.). At 2 days of age the piglets were infected intranasally with 10⁸ CFU of strain MBORD 846. Control piglets were administered an equal volume of sterile PBS. Immediately prior to inoculation, the nares of each piglet were swabbed, and the swabs were cultured on sheep’s blood agar at 37°C for up to 3 days. At 21 days postinoculation, the piglets were euthanized and tissue was collected from the tonsil, turbinate, trachea, and lung. Following homogenization in sterile PBS, tissue suspensions were diluted and plated in duplicate onto sheep’s blood agar. The plates were incubated at 37°C for approximately 24 h. Some plates were used immediately for colony lifts, while others were stored at 4°C for up to 3 weeks prior to the procedure.

For the second phase of the study primary isolation plates (sheep’s blood agar) from nasal swabs of farm-raised swine suspected of infection with B. bronchiseptica were obtained from the Clinical Microbiology Diagnostic Laboratory at the Veterinary College of Iowa State University.

Nonradioactive colony lift-hybridization assays. Colony lifts were performed directly from primary isolation plates by using positively charged nylon membranes (Boehringer Mannheim), according to the manufacturer’s recommendations. Prehybridization and hybridization were carried out at 42°C as described previously (2) by using a maleic acid-based buffer. The hybridization solution contained 10 ng of digoxigenin-labeled probe per ml in 50% standard saline citrate (SSC; 1% SSC contains 150 mM NaCl plus 15 mM sodium citrate [pH 7.0])-50%
formamide-0.02% sodium dodecyl sulfate (SDS)-0.1% N-lauroylsarcosine-2% blocking reagent-20 mM sodium maleate. Following removal of the membranes from the hybridization solution, they were washed twice for 5 min each at room temperature in 2× SSC containing 0.1% SDS and twice for 15 min each time at 65°C in 0.1% SSC containing 0.1% SDS. Bound probe was detected by using an anti-digoxigenin-alkaline phosphatase antibody conjugate and LumiPhos as detailed previously (2). Membranes were sealed in plastic bags and were incubated at 37°C for 1 h prior to exposure to Kodak XAR5 film. A 5-min exposure was sufficient for detection of hybridized probe. Membranes were stripped for subsequent rounds of hybridization by incubation at 37°C for 30 min in 0.2 N NaOH-0.1% SDS.

**Southern blotting.** The procedure of Woo et al. (24) was used to isolate genomic DNA from some bacterial colonies that displayed positive hybridization signals on colony lift membranes. Aliquots were digested with EcoRI, and the fragments were separated by electrophoresis in 0.6% agarose gels and transferred by capillary action to charged nylon membranes. After baking at 80°C for 2 h, hybridization and detection of bound probes were carried out as detailed above.

**Probes.** A previously characterized plasmid containing a 4.7-kb EcoRI fragment from *B. bronchiseptica* MBORD 846, designated pDLA5 (10), was used as a probe to identify *B. bronchiseptica* from colony lift plates. The EcoRI fragment includes at least three open reading frames essential for the synthesis of the hydroxymate-type siderophore alcaligen (10, 16), which is produced by *B. bronchiseptica* under iron-limiting conditions (9). The probe is not species specific, since it also hybridizes to genomic DNA from *Bordetella pertussis* (8). The parent plasmid, pGEM3Zf+ (Promega Corporation, Madison, Wis.), was used as a negative control probe. Plasmids were digoxigenin labeled by the random priming method as described previously (2).

**RESULTS**

**Identification of *B. bronchiseptica* from infected CDCD piglets.** In the initial phase of the study we used the nonradioactive colony lift-hybridization procedure for the identification of *B. bronchiseptica* cultured from tissues of experimentally infected CDCD piglets. In two separate experiments a total of 15 piglets were randomly assigned to either the experimental or control piglet groups (Table 1). From each piglet we performed a primary evaluation of 82 clinical samples by standard microbiological techniques identified that four of the colonies were *B. bronchiseptica* (denoted by arrows), and all four colonies hybridized with pDLA5. The remaining colonies gave weak signals because of the incomplete removal of cellular debris.

![FIG. 1. Colony lift-hybridization membranes from tissues of CDCD piglets infected with *B. bronchiseptica*. (a) Membrane derived from a plate seeded with homogenized lung. All colonies on the plate were hemolytic and had biochemical properties and morphology consistent with *B. bronchiseptica*. All colonies hybridized with probe pDLA5. (b) Membrane derived from a plate seeded with homogenized tonsil. Many colony types were present. Biochemical testing indicated that all four of the colonies were *B. bronchiseptica* (denoted by arrows), and all four colonies hybridized with pDLA5.](http://jcm.asm.org/Downloadedfrom)

When plates were used for colony lifts and the resulting membranes were probed with pDLA5, a strong signal was obtained from all colonies identified as *B. bronchiseptica* (Fig. 1a). In our first attempts with this assay some weak signals were also detected from other colony types. The membrane in Fig. 1b shows an example of such a result; in this instance the film was overexposed to facilitate photographic reproduction. We later determined that these weak signals were due to incomplete removal of bacterial debris from the membrane following the denaturation and neutralization steps. The manufacturer suggests that gentle pressure and a moistened towel be used for this step. We found that Kimwipes work well, provided that sufficient pressure is applied to completely remove the debris. We were able to apply firm pressure without causing damage to the membrane, as long as the Kimwipe was thoroughly saturated with prehybridization solution. When care was taken to completely remove such material from the filters, only the expected colonies hybridized with pDLA5. None of the colony lifts from plates inoculated with the tissue of animals given PBS exhibited a hybridization signal when pDLA5 was used as a probe. When membranes were stripped and rehybridized with the parent plasmid, pGEM3Zf+, no hybridization was detected from membranes derived from either infected or control piglets (data not shown).

**Identification of *B. bronchiseptica* from clinical samples.** Initial evaluation of 82 clinical samples by standard microbiological techniques identified *B. bronchiseptica* in 5 of them. When the primary isolation plates were tested in the colony lift-hybridization assay, a total of 11 samples were found to contain bacteria that hybridized with probe pDLA5. Five of the hybridization-positive samples were those from which *B. bronchiseptica* was identified by standard methodology. These five plates contained at least a few isolated colonies of *B. bronchiseptica* that could be visually recognized as suspect by personnel with the proper training and experience. The remaining six samples exhibited hybridization signals from areas of the plate that contained confluent growth from a mixture of bacterial types, making it virtually impossible to identify suspect colonies. To determine whether the hybridization signals from these six plates were true positives, bacterial growth from the appropriate areas was restreaked onto additional sheep’s blood agar plates, and the colony lift-hybridization assay was performed. All six plates displayed isolated colonies that had a morphology typical of that of *B. bronchiseptica* and that gave positive hybridization signals with pDLA5. Pure cultures of these colonies were submitted to the Clinical Microbiology Laboratory at Iowa State University for analysis and were identified as *B. bronchiseptica*. All membranes were stripped and reprobed with pGEM3Zf+, and no hybridization to this plasmid was detected.

**Southern blot analysis of clinical isolates.** In order to determine whether the chromosomal fragment containing sequence complementary to pDLA5 was conserved in the 11 clinical isolates, purified DNA from each was digested with EcoRI and was used to prepare a Southern blot. All isolates contained a single 4.7-kb fragment that hybridized with pDLA5 (Fig. 2). This fragment comigrated with the homologous fragment from strain MBORD 846. When the blot was stripped and rehybridized with pGEM3Zf+, no signals were visualized.
Specificity of pDLA5 for *Bordetella* spp. We have previously determined that the gene contained in probe pDLA5 is involved in the synthesis of a hydroxamate-type siderophore produced by *B. bronchiseptica* under iron-limiting conditions (9, 10). Fast-atom bombardment mass spectrometry and proton nuclear magnetic resonance analysis demonstrated that this siderophore is identical to alcaligen, a siderophore synthesized by *Alcaligenes denitrificans* (16). This organism is commonly found in soil and water and, as a result, may also be present in the upper respiratory tracts of pastured swine. For this reason we wished to determine whether pDLA5 would cross-hybridize with DNA from *A. denitrificans*. When pDLA5 was used to probe a colony lift membrane from a pure culture of the type strain of *A. xylosoxydans* subsp. *denitrificans* (ATCC accession number 15173), no hybridization was detected (Fig. 3). In addition, purified DNA from this organism that was digested with EcoRI and used in a Southern blot also failed to bind the probe (Fig. 2, lane 2).

**DISCUSSION**

The method currently used for the identification of *B. bronchiseptica* from experimental samples or clinical specimens, i.e., biochemical testing of colonies that display a characteristic morphology, is costly and time-consuming. Furthermore, the subjective evaluation of colony characteristics as a screening morphology, is costly and time-consuming. Furthermore, the chiseptica from experimental samples or clinical specimens, probe (Fig. 2, lane 2).

![FIG. 2. Southern blot analysis of *B. bronchiseptica* strains and *A. denitrificans*. Chromosomal DNA was digested with EcoRI, electrophoresed, transferred to nylon membranes, and hybridized with probe pDLA5. Lanes: 1, digoxigenin-labeled bacteriophage lambda HindIII marker; 2, *A. denitrificans* ATCC 15173; 3, *B. bronchiseptica* MBORD 846; 4 to 14, clinical isolates of *B. bronchiseptica* identified in the study.](http://jcm.asm.org/)

The colony lift-hybridization assay described in this report provides a number of advantages over currently used methods for the identification of *B. bronchiseptica*. Colony lifts can be done directly from primary isolation plates, without the added time and expense of generating pure cultures for biochemical testing. We were able to detect the presence of single colonies of *B. bronchiseptica* even when they were almost completely overgrown by other organisms. The assay is easy to perform and requires no special equipment other than an incubator with a rotating platform. A large amount of probe can be labeled and then reused many times. We have used the same probe solution repeatedly over the course of a year with no loss of activity. Interpretation of the results is simple and straightforward. The assay appears to have greater sensitivity than the "gold standard" of culture and biochemical testing, since *B. bronchiseptica* was identified from six samples that were initially reported as negative by experienced clinical laboratory personnel. If these samples had been diluted prior to plating, at least some might have been identified as containing *B. bronchiseptica*. However, this is an additional step that laboratories rarely perform.

We found that this assay was virtually 100% specific for *Bordetella* spp., although larger numbers of clinical samples should be tested to confirm this preliminary observation. It should be noted that the probe pDLA5 hybridizes to *B. pertussis* (8) and presumably to *Bordetella parapertussis*, as well as to *B. bronchiseptica*. Since *B. pertussis* and *B. parapertussis* are strictly human pathogens, this cross-reactivity should not present a problem for the identification of *B. bronchiseptica* from veterinary specimens. Recently, two new *Bordetella* species have been described, *Bordetella hinzii* has been isolated from poultry and humans (3, 21), and *Bordetella holmesii* has been isolated from several patients with septicemia (23). Nucleic acid relatedness studies suggest that both of these species are somewhat divergent from *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (21, 23). We have not determined whether *B. hinzii* and *B. holmesii* contain sequence homologous to the pDLA5 probe. However, neither species has been associated with respiratory disease in swine. The remaining species in this genus, *Bordetella avium*, is isolated almost exclusively from avian hosts. It is phenotypically and genotypically distinct from the other members of the genus (1, 6, 22), and we have found that pDLA5 does not hybridize with this organism under the conditions used in the present study (18).

The bacterium *A. denitrificans* is known to be very closely related to *Bordetella* spp. and to *B. bronchiseptica* in particular (6, 12). A recent study has established that the siderophores produced by *A. denitrificans* and *B. bronchiseptica* are identical (16). Since the chromosomal fragment used as a probe in the colony lift-hybridization assay contains genes involved in siderophore synthesis, we suspected that DNA from *A. denitrificans* might also hybridize with it. However, we were unable to show such cross-reactivity in either a colony lift-hybridization assay or a Southern blot analysis. Southern blot analysis of the 11 clinical isolates identified in the present study demonstrated that the *EcoRI* fragment that hybridizes to pDLA5 is indistinguishable in size from that of strain MBORD 846. We have also done Southern blot analysis of a number of other *B. bronchiseptica* isolates from swine from the United States and Hungary. All contain an *EcoRI* fragment with the same mobility as that of MBORD 846 that hybridizes to probe pDLA5 (18). This result is not unexpected since it has been previously reported that isolates of *B. bron-
chiseptica from swine show little clonal diversity (17). Although a more extensive survey of strains would be useful, these results indicate that the probe used in the present study is likely to be highly conserved in B. bronchiseptica isolates from swine from diverse locations and, therefore, suitable as a hybridization target for diagnostic purposes.

Recently, a number of investigators have reported PCR-based diagnostic assays for the direct detection of B. pertussis from clinical specimens (for a review, see reference 15). Some of the amplified sequences are also present in B. burgdorferi (16), B. hinzii (17), and B. bronchiseptica (18) and, therefore, suitable as a hybridization target for these organisms. The colony lift-hybridization assay can be performed by staff with a minimal amount of training. Although workers are also crucial for the success of diagnostic laboratories would be unable to provide the PCR-dedicated rooms and equipment required to avoid contamination. Highly trained workers are also crucial for the success of diagnostic PCR. In contrast, the colony lift-hybridization assay can be performed by staff with a minimal amount of training. Although it is not as rapid as PCR, it reduces the time required for the definitive identification of B. bronchiseptica from roughly 4 days to 2.5 days. We carried out hybridization overnight as a matter of convenience and have not determined the minimum amount of time required for the detection of positive colonies. However, it may be possible to shorten the procedure by decreasing the hybridization time.