JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1998, p. 3342–3346 Vol. 36, No. 11
0095-1137/98/$04.00+0

Two-Color Hybridization Assay for Simultaneous Detection of *Bordetella bronchiseptica* and Toxigenic *Pasteurella multocida* from Swine

KAREN B. REGISTER,[1]* RUBY M. LEE,[2]† AND CINDY THOMSON[3]

Avian and Swine Respiratory Diseases Research Unit, National Animal Disease Center, Agricultural Research Service,[1] U.S. Department of Agriculture, and Boehringer Ingelheim/NOBL Laboratories, Inc.,[3] Ames, Iowa 50010, and Boehringer Ingelheim/NOBL Laboratories, Inc., Sioux Center, Iowa 51250[2]

Received 1 June 1998/Accepted 21 July 1998

***Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* are the etiologic agents of swine atrophic rhinitis. Methods currently used for their identification are time-consuming and suffer from a lack of sensitivity. We describe a colony lift-hybridization assay for detection of *B. bronchiseptica* and toxigenic *P. multocida* that can be performed with a single colony lift derived from a primary isolation plate without the need for pure subcultures of suspect bacteria. Membranes are hybridized simultaneously to probes derived from the *B. bronchiseptica alcA* gene and the *P. multocida toxA* gene. A multicolor development procedure permits sequential detection of bound probes. The assay was tested with 84 primary isolation plates generated from nasal swabs from swine with clinical signs of atrophic rhinitis. Comparison of the results from the colony lift-hybridization assay with those from conventional testing, based on a combination of colony morphology, biochemical reactions, mouse lethality, and enzyme-linked immunosorbent assay, indicated that the colony lift assay has superior sensitivity and comparable specificity. This technique has wide application for diagnostic and experimental studies.**

Atrophic rhinitis (AR) is a costly and widely prevalent respiratory disease of swine. Severe, progressive AR results from concurrent infection with both toxigenic *Pasteurella multocida* and *Bordetella bronchiseptica*. Toxigenic *P. multocida* alone may also cause severe disease, while infection with *B. bronchiseptica* alone generally results in a moderate to mild reversible form. *P. multocida* and *B. bronchiseptica* may also cause bronchopneumonia in swine.

Traditionally, a definitive diagnosis of infection with *P. multocida* or *B. bronchiseptica* is established from clinical signs and isolation of these agents from nasal swabs or biopsy specimens. Suspect colonies are subcultured and subjected to biochemical testing. *P. multocida* isolates must subsequently be tested for toxin production, since nontoxigenic strains are not believed to play a role in AR (3). Conventional identification methods suffer from a lack of sensitivity, since both *B. bronchiseptica* and *P. multocida* are often found in low numbers compared to the numbers of other bacteria found in clinical specimens. Additionally, since *B. bronchiseptica* grows more slowly than most other bacteria commonly found in the upper respiratory tract, its presence may be masked by overgrowth of other organisms.

Previously, we reported on a simple, nonradioactive colony hybridization assay for detection of *B. bronchiseptica* from primary culture plates (19). However, an assay that could detect both toxigenic *P. multocida* and *B. bronchiseptica* would be of great benefit to diagnostic laboratories and would facilitate experimental studies. Here we describe a two-color, nonradioactive colony lift-hybridization assay for simultaneous detection of these pathogens and demonstrate its utility with primary isolation plates derived from clinical samples.

* Corresponding author. Mailing address: Swine Respiratory Diseases Project, USDA/ARS/National Animal Disease Center, P.O. Box 70, 2300 Dayton Rd., Ames, IA 50010. Phone: (515) 239-8275. Fax: (515) 239-8458. E-mail: kregiste@nadc.ars.usda.gov.

† Present address: Biovet USA, Inc., Saint Anthony, MN 55418-2590.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The toxigenic strain *P. multocida* P-4533 (serotype D:3) has been described previously (20). Strain P-1059 (serotype A:3) was determined to be nontoxigenic with a monoclonal antibody specific for the *P. multocida* dermonecrotic toxin in a colony blot assay, as reported previously (13). This assay was also used to determine the toxin-producing abilities of the 60 field strains from swine (provided by Richard B. Rimler, National Animal Disease Center, Agricultural Research Service [ARS], U.S. Department of Agriculture [USDA], Ames, Iowa) included in Table 1. The capsular serogroup of these strains was determined by indirect hemagglutination (20). Somatic serotypes were determined by gel diffusion precipitin tests (7). *B. bronchiseptica* KM22 was obtained from a swine herd with AR and has frequently been used as a virulent challenge strain in vaccine studies (12). Except when indicated otherwise, *P. multocida* was grown on dextrose starch agar and *B. bronchiseptica* was grown on Bordet-Gengou agar with 10% sheep blood at 37°C for 18 to 36 h.

Six swine isolates of *Escherichia coli* and swine isolates of *Salmonella typhimurium*, *Salmonella choleraesuis*, *Salmonella anatum*, *Salmonella heidelberg*, *Salmonella infantis*, *Salmonella montevideo*, and *Salmonella derby* were kindly provided by the Diagnostic Bacteriology section of the National Veterinary Services Laboratories, Animal and Plant Health Inspection Service, USDA.

Probes. A fragment of the *P. multocida toxA* gene consisting of nucleotides 1983 through 3183 was generated by PCR as reported elsewhere (14) and was cloned into the plasmid vector pCRII (Invitrogen, San Diego, Calif.). This plasmid, designated pPMT, was introduced into *E. coli* INVαF′ by electroporation.

The *Bordetella*-specific probe pDLA5, which contains a 4.7-kb fragment that includes the *B. bronchiseptica alcA* gene, has been described (6). A previous study demonstrated the specificity and sensitivity of this probe for identification of *B. bronchiseptica* (19). Commercially obtained plasmid purification columns were used to prepare plasmids pPMT and pDLA5, as instructed by the manufacturer (Qiagen, Chatsworth, Calif.). The insert from pPMT, designated ToxA, and the insert from pDLA5, referred to as AlcA, were released from plasmid vectors by digestion with *Eco*RI. Inserts were gel purified with a GeneClean Spin Kit (BIO 101, Inc., Vista, Calif.) according to the recommendations supplied by the manufacturer. These probes were random prime labeled with either digoxigenin, fluorescein, or biotin by a standard protocol (2).

Colony lifts and hybridization. Nasal swabs obtained from swine with clinical signs of AR were used to streak sheep blood agar plates. Colony lifts were prepared from plates up to 3 weeks old using positively charged nylon membranes (Boehringer Mannheim, Indianapolis, Ind.), according to the manufacturer's recommendations. Nucleic acid was fixed to the membranes by baking at

TABLE 1. *P. multocida* swine isolates tested for toxin production

Capsular and somatic serotype	Geographic origin	No. of strains tested	Expression of toxin[a]	Hybridization with ToxA
A:3	Iowa	7	−	−
A:3	West Virginia	1	−	
A:3	Arkansas	2	−	−
A:3	Michigan	1	−	−
A:3	Wisconsin	1	−	−
A:3	Maylasia	3	−	−
A:3	South America	1	−	−
A:3, 4	Illinois	2	−	−
A:3, 12	Iowa	1	−	−
A:3, 12	Nebraska	3	−	−
A:15	South America	1	−	−
D:2, 12	Missouri	1	−	−
D:3	Iowa	1	−	−
D:3	Michigan	2	−	−
D:3	Wisconsin	1	−	−
D:3, 12	Iowa	1	−	−
D:3, 12	Arkansas	7	−	−
D:3, 12	Belgium	2	−	−
D:7, 12	Belgium	1	−	−
D:12	Iowa	1	−	−
D:12	Brazil	1	−	−
D:3, 11, 12	Iowa	2	−	−
A:3	Nebraska	3	+	+
A:3	Norway	3	+	+
A:3, 4	United Kingdom	1	+	+
D:−	Nebraska	1	+	+
D:3	Iowa	1	+	+
D:3	Wisconsin	2	+	+
D:3	Singapore	2	+	+
D:12	Nebraska	2	+	+
D:12	Iowa	1	+	+
D:12	Arkansas	1	+	+

[a] As determined by colony blot assay with a toxin-specific monoclonal antibody (13).

80°C for 2 h. The membranes were stored in sealed plastic bags at room temperature for up to 2 months prior to hybridization. Cellular debris was removed by incubation of the membranes in 3× standard saline citrate (1× standard saline citrate contains 150 mM NaCl plus 15 mM sodium citrate [pH 7.0])–0.1% (wt/vol) sodium dodecyl sulfate for 1 to 3 h at 68°C, followed by gentle wiping of the membrane surface with a moistened laboratory tissue. Prehybridization and hybridization were carried out at 42°C by a standard protocol (2). Hybridization solutions contained either 15 to 45 ng each of fluorescein-ToxA and digoxigenin-AlcA (for multicolor detection) per ml or 15 ng of ToxA or AlcA alone (for standard colorimetric or chemiluminescent detection) per ml in 5× standard saline citrate–50% formamide–0.02% sodium dodecyl sulfate–*N*-lauroylsarcosine–2% Genius blocking reagent–20 mM sodium maleate. Posthybridization washes were performed as described previously (2).

Detection of probes. The Genius Multicolor Detection kit (Boehringer Mannheim) was used for development of colony lift membranes hybridized simultaneously with a mixture of fluorescein-ToxA and digoxigenin-AlcA. The protocol supplied by the manufacturer was followed. Briefly, after posthybridization washes, probe-target DNA hybrids were fixed to the membranes by exposure to UV light (254 nm) for 3 min. The membranes were incubated in 1% Genius blocking reagent for 30 min, followed by an additional 30-min incubation in a 1:5,000 dilution of anti-fluorescein–alkaline phosphatase (anti-fluorescein–AP) for detection of fluorescein-ToxA. After washing, the membranes were incubated in freshly prepared "red" AP substrate solution (containing naphthol-AS-phosphate and fast red TR). They were allowed to develop, without agitation and with protection from light, for 15 to 45 min. Residual AP was inactivated by incubation of the membranes in 50 mM EDTA (pH 8.0) for 10 min at 85°C. This temperature is expected to melt DNA hybrids; however, the previous UV cross-linking step prevents bound but undeveloped digoxigenin-AlcA from being washed away. A second round of detection identical to the first one was carried out, except that anti-digoxigenin-AP and "blue" substrate solution (containing naphthol-AS-phosphate and fast blue B) were used. Following development of digoxigenin-AlcA, membranes were washed briefly in 10 mM Tris–1 mM EDTA (pH 8.0), allowed to air dry, and stored at room temperature. In one series of experiments "green" substrate reagent (containing naphthol-AS-Gr-phosphate and fast blue B), also included in the Genius Multicolor Detection Kit, was substituted for the red or blue substrate.

In some experiments membranes were hybridized with either ToxA or AlcA alone and were then developed by a standard procedure with anti-digoxigenin-AP or anti-fluorescein-AP, as appropriate (2). Colorimetric detection was carried out by incubation for 5 to 15 min in color substrate solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Chemiluminescent development was accomplished by incubation of membranes in 0.25 mM disodium 3-{4-methooxyspiro[1,2-dioxitane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl} (CSPD) for 5 min. Membranes were then heat sealed in plastic pouches, incubated at 37°C for 15 min, and exposed to Kodak X-OMAT AR film at room temperature.

Conventional identification of *B. bronchiseptica* and *P. multocida*. Modified MacConkey/Bordetella agar plates (MicroBiologics, St. Cloud, Minn.) were streaked with nasal swabs obtained from swine with clinical signs of AR and were incubated at 37°C for 48 h. Nonfermentative colonies were subcultured on sheep blood agar and were subsequently used for biochemical testing. Isolates positive for oxidase, citrate, urea, and growth in 6.5% NaCl, negative for indole, and causing no change or an alkaline reaction on triple sugar iron slants were identified as *B. bronchiseptica* (17).

For detection of *P. multocida*, nasal swabs were immersed in 0.5 ml of phosphate-buffered saline for 5 min. The resulting suspension was injected intraperitoneally into female adult BALB/c mice. Lethality within 48 h was presumptive evidence of the presence of *P. multocida*. Livers from the affected mice were recovered and cultured on sheep blood agar. Following overnight incubation at 37°C, a single colony was picked for an additional subculture, which was used for biochemical testing. Isolates positive for oxidase, indole, and catalase and having the characteristic colony morphology and musty odor were identified as *P. multocida* (16).

P. multocida isolates were further tested for production of the dermonecrotic toxin by a previously described enzyme-linked immunosorbent assay (ELISA) (13). Briefly, sonicated filtrate prepared from pure subcultures was used to coat microtiter plates. After blocking, an antitoxin monoclonal antibody was added, followed by the addition of peroxidase-conjugated anti-mouse immunoglobulin G. TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was used for development. Plates were read on a microplate reader at a wavelength of 450 nm. Samples recorded as positive had mean optical densities of at least 95% of those of the positive control.

RESULTS

Optimal assay parameters. Preliminary experiments were conducted with colony lifts from sheep blood agar plates streaked with a mixture of toxigenic *P. multocida* (strain 4533), nontoxigenic *P. multocida* (strain 1059), and *B. bronchiseptica*. Membranes were subjected to simultaneous hybridization with fluorescein-labeled ToxA and digoxigenin-labeled AlcA and were developed by the multicolor detection procedure. As expected, development of fluorescein-ToxA with the red substrate reagent produced a pink hybridization signal only from the toxigenic isolate of *P. multocida*, and development of digoxigenin-AlcA with the blue substrate reagent produced a purple hybridization signal only from *B. bronchiseptica* (Fig. 1). Hybridization signals were usually visible within 20 min. However, the colors continued to intensify for at least another 15 min. Therefore, 45 min was chosen as the standard development time. Use of both probes, each at a concentration of 30 ng/ml, resulted in strong color development in the absence of background. Increasing the probe concentration further did not increase the background, but neither did it increase the intensities of the signals or reduce the time required for devel-

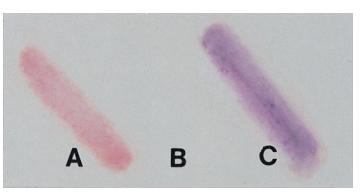

FIG. 1. Colony lift from a plate streaked with toxigenic *P. multocida* (A), nontoxigenic *P. multocida* (B), and *B. bronchiseptica* (C), hybridized with 30 ng each of fluorescein-ToxA and digoxigenin-AlcA per ml, and developed by the multicolor detection procedure.

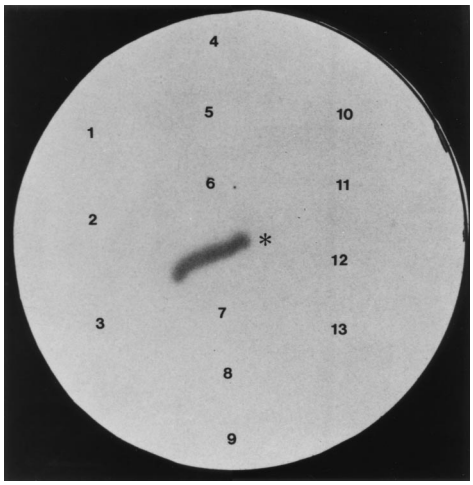

FIG. 2. Colony lift from a plate streaked with swine isolates of *E. coli* (locations 1 to 6) or *S. typhimurium* (location 7), *S. choleraesuis* (location 8), *S. anatum* (location 9), *S. heidelberg* (location 10), *S. infantis* (location 11), *S. montevideo* (location 12), and *S. derby* (location 13); hybridized with digoxigenin-ToxA; and developed with a chemiluminescent substrate. The location of the toxigenic *P. multocida* strain used as a positive control is indicated with an asterisk.

opment. Probes were used at 30 ng/ml each for the remainder of the study. Identical results were obtained when the order of detection was reversed. The color of the hybridization signals from toxigenic *P. multocida* and *B. bronchiseptica* was reversed when the AlcA probe was developed with the red reagent and the ToxA probe was developed with the blue reagent.

Colony lifts from plates up to 3 weeks old performed as well as lifts from fresh plates, except for those which had become excessively contaminated during storage. In the latter case, occasional false-positive signals occurred due to residual bacterial debris that could not be removed. Colony lifts stored at room temperature for up to 2 months performed as well as freshly prepared lifts, although extra care was required to completely remove bacterial debris prior to hybridization.

In addition to the blue and red development reagents, the Genius Multicolor Detection kit contains a third substrate for development that results in deposition of a green pigment on the membrane. Although only two colors are required to perform the assay reported here, the color reagents are not available separately, and discarding the green substrate reagent is not a cost-effective use of the components included in the kit. Therefore, additional membranes were tested by a procedure in which the green substrate was substituted for either the red or the blue substrate. We found that the intensity of color observed with the green substrate reagent is greatly reduced compared to that observed with the red or blue substrates. The degree of reduction is unacceptable, since the positive controls used in blind experiments were sometimes mistakenly identified as negative. Increasing the concentration of the probe chosen for development with the green substrate did not increase the intensity of the color.

Specificity of ToxA. The ToxA probe used in the present study is derived from the central portion of the *toxA* gene. One previous investigation in which a PCR assay for identification of toxigenic *P. multocida* was described suggested that *E. coli* and *S. typhimurium* each contain sequence homologous to this region (8). Other reports failed to confirm this observation (9, 11, 14). To further establish the specificity of the ToxA probe used here, colony lifts of six swine isolates of *E. coli* and single isolates of *S. typhimurium*, *S. choleraesuis*, *S. anatum*, *S. heidelberg*, *S. infantis*, *S. montevideo*, and *S. derby* were hybridized with digoxigenin-ToxA. Negative reactions were obtained with all strains, both from membranes developed by the standard colorimetric procedure and from duplicate membranes developed with the more sensitive chemiluminescent substrate CSPD (Fig. 2). Identical results were observed when ToxA labeled with fluorescein was substituted for digoxigenin-ToxA.

Use of a ToxA-specific probe for identification of toxigenic stains of *P. multocida* further requires that homologous sequence be absent from phenotypically nontoxigenic strains. Available data suggest that this may generally be the case for nontoxigenic *P. multocida* (4, 5, 9, 10, 11, 14, 15). However, these studies included only a few isolates or isolates from a limited geographic region. Furthermore, there is evidence that at least some nontoxigenic strains may harbor silent or incomplete portions of the *toxA* gene (9, 11). Sixty swine isolates, obtained from locations around the world and of diverse capsular and somatic serotypes, were tested for toxin production in a colony blot assay with a toxin-specific monoclonal antibody. Forty-three of the strains were found to be phenotypically nontoxigenic (Table 1). Colony lifts from these strains were negative when hybridized with either digoxigenin-ToxA or fluorescein-ToxA, regardless of whether the membranes were developed by the colorimetric or chemiluminescent procedure. All 17 of the strains identified as toxigenic hybridized to ToxA.

Comparison of multicolor detection with standard identification methods. Nonselective primary isolation plates obtained from nasal swabs of swine with clinical signs of AR were used to compare the sensitivity and specificity of the multicolor detection procedure with those of standard identification methods based on a combination of colony morphology, biochemical testing, mouse lethality, and ELISA. The results for a total of 84 specimens derived from 15 herds are summarized in Table 2. Concordant results were obtained for 78 specimens, 59 of which were negative for both toxigenic *P. multocida* and *B. bronchiseptica*. Of the remaining samples with concordant results, 10 tested positive for toxigenic *P. multocida* and 9 tested positive for *B. bronchiseptica*.

Five plates reported as negative for *B. bronchiseptica* by conventional testing were found to have 10 or fewer AlcA-positive colonies when subjected to the colony lift assay. Identical results were obtained with duplicate colony lifts hybridized to digoxigenin-AlcA and developed by chemiluminescent detection. In all cases, the AlcA-positive colonies were located in an area of the plate containing nearly confluent growth of bacte-

TABLE 2. Results of conventional testing for toxigenic *P. multocida* and *B. bronchiseptica* compared to those of colony lift-hybridization assay

Result by the following assay:					No. of specimens
Conventional testing			Hybridization assay		
P. multocida	Toxigenic *P. multocida*[a]	*B. bronchiseptica*	Toxigenic *P. multocida*	*B. bronchiseptica*	
−	NT[b]	−	−	−	27
+	−	−	−	−	32
+	+	−	+	−	10
−	NT	+	−	+	7
+	−	+	−	+	2
+	−	−	+	−	1
+	−	−	−	+	4
−	NT	−	−	+	1

[a] As determined by ELISA.
[b] NT, not tested; *P. multocida* was not isolated.

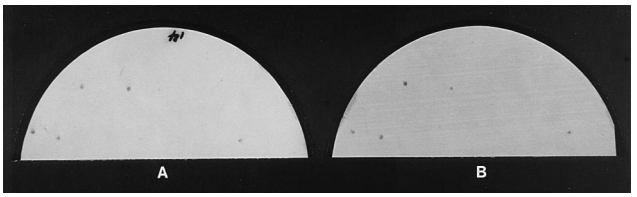

FIG. 3. Colony lifts from a primary isolation plate reported to be negative for toxigenic *P. multocida* by conventional methods. Membranes were hybridized with 30 ng each of fluorescein-ToxA and digoxigenin-AlcA per ml and developed by the multicolor detection procedure (A) or subjected to a colony blot assay with a monoclonal antibody specific for the *P. multocida* dermonecrotic toxin (B).

ria. We were able to isolate from two of these plates a pure culture of *B. bronchiseptica*, identified by biochemical testing and hybridization with AlcA, by restreaking the bacterial growth recovered from the region of the plate that contained AlcA-positive colonies. The age and excessive contamination of the remaining two plates made attempts at isolation of *B. bronchiseptica* impossible.

Five ToxA-positive colonies were detected from one plate reported as containing nontoxigenic *P. multocida* by conventional methods (Fig. 3A). A duplicate colony lift hybridized with digoxigenin-ToxA and developed with a chemiluminescent substrate had an identical pattern of positive colonies. The same five colonies reacted with a monoclonal antibody specific for the *P. multocida* dermonecrotic toxin (Fig. 3B). Due to the age of the original plate, subsequent isolation of toxigenic *P. multocida* was not attempted.

DISCUSSION

A previous report from this laboratory described the use of a *Bordetella*-specific probe in a highly sensitive colony lift-hybridization assay for identification of *B. bronchiseptica* infection in swine (19). This procedure is faster, more objective, and better suited to the analysis of large numbers of samples than standard identification methods based on colony morphology and biochemical testing. Another advantage is the ability to use primary isolation plates, without the need for pure cultures of suspect colonies. However, information regarding the presence of both toxigenic *P. multocida* and *B. bronchiseptica* is required during clinical evaluation of swine herds for AR. Using a commercially available multicolor development kit, we have modified the previously reported colony lift assay such that both toxigenic *P. multocida* and *B. bronchiseptica* can be identified from the same colony lift membrane.

The sensitivity and specificity of the AlcA probe for identification of *B. bronchiseptica* have previously been demonstrated with colony lifts derived from diagnostic samples (19). However, the membranes used in that study were developed with a chemiluminescent substrate, which is approximately 10 times more sensitive than multicolor detection (2). Nevertheless, in the present study all samples reported to contain *B. bronchiseptica* by conventional methods were also positive by the colony lift assay. Additionally, we identified *B. bronchiseptica* from five samples that were reported to be negative on the basis of standard methods. Visual examination did not reveal isolated colonies with a morphology typical of that of *B. bronchiseptica* on the primary isolation plates derived from these samples. This omission is explained by the fact that only a few AlcA-positive colonies were present in an area of nearly confluent growth and illustrates the limitations of conventional methods for identification of *B. bronchiseptica*. We conclude that the colony lift-hybridization assay described here is more sensitive than standard methods since it permits the detection of even a few colonies whose presence and morphology are masked by overgrowth of other bacteria in the sample. Similar findings were reported in a previous study in which 6 of 77 clinical specimens reported to be negative by conventional identification methods were subsequently shown to contain colonies of *B. bronchiseptica* in an area of confluent growth (19).

All samples from which toxigenic *P. multocida* was identified by conventional methods were also positive by the colony lift assay with the ToxA probe, indicating that this technique is at least as sensitive as conventional identification methods. One sample reported as containing nontoxigenic *P. multocida* by standard methods was found to contain a few colonies positive by the colony lift assay with ToxA. As described above, the positive colonies were located in an area of the plate with nearly confluent growth. Identical results were obtained from a repeat colony lift developed with a chemiluminescent substrate. Furthermore, production of the dermonecrotic toxin by the ToxA-positive colonies was demonstrated with an antitoxin monoclonal antibody. The age of the plate and the excessive contamination that occurred during storage prevented us from isolating toxigenic *P. multocida*. However, as was previously shown for *B. bronchiseptica* with the AlcA probe (19), some samples testing negative by conventional methods may contain low numbers of toxigenic *P. multocida* that are identified only in the colony lift assay. Since the tonsil, rather than the turbinate, is the anatomic site colonized most effectively by *P. multocida* (1, 3), low numbers of this organism in nasal swabs are not unexpected. Additionally, it is known that swine may be colonized simultaneously with a mixture of toxigenic and nontoxigenic *P. multocida* (1, 3). Since a single colony was selected for the subculture used for determination of toxin production in this study, false-negative results could occur with samples from swine having mixed infection.

Our results also indicate that the ToxA probe is highly specific. Although the plates used for colony lifts contained large numbers of bacteria other than *P. multocida*, no false-positive results were obtained for the 74 plates reported to be negative for toxigenic *P. multocida* by conventional methods. Previous studies with probes or PCR primers that overlap the sequence of ToxA likewise demonstrated no cross hybridization with approximately 20 other bacterial species, including *E. coli* and *S. typhimurium* (9, 11, 14). In contrast, one additional study did suggest that *E. coli* and *S. typhimurium* may each have sequence homologous to a region that is included in the ToxA probe, since PCR products were obtained from both organisms with primers specific for a portion of this region (8). However, in the study presented here, we found no evidence that ToxA cross hybridizes to *E. coli* or *Salmonella* spp. isolated from swine.

The specificity of ToxA for toxigenic *P. multocida* further depends upon the assumption that nontoxigenic strains lack the *toxA* gene. Most available evidence is consistent with this assumption (4, 5, 9, 10, 11, 14, 15). However, a few reports present conflicting data suggesting that silent or incomplete copies of *toxA* may occasionally be found in nontoxigenic isolates (8, 9, 11). Our investigation did not detect sequence homologous to ToxA in the 38 diagnostic samples demonstrated to contain nontoxigenic *P. multocida* or in 43 nontoxigenic strains isolated from locations around the world. Thus, we conclude that the ToxA probe is highly specific for only toxigenic strains of *P. multocida*.

The assay described in this report includes probes labeled with digoxigenin or fluorescein. Biotinylated probes are also commonly used by many laboratories for nonradioactive de-

tection of nucleic acids and are suggested as another alternative for use with the Genius Multicolor Detection kit. However, we previously found that biotinylated probes are not suitable for identification of toxigenic *P. multocida* since their use results in false-positive signals from nontoxigenic strains (18). It was further demonstrated that the false-positive results are due to residual avidin-binding proteins in the *P. multocida* isolates present on the colony lift membrane. These proteins, which occur in both *P. multocida* and *Pasteurella haemolytica*, bind the streptavidin-AP conjugate used for development of biotinylated probes. Thus, biotinylated probes should not be substituted for digoxigenin- or fluorescein-labeled probes in this assay or in any colony lift procedure performed with samples suspected of containing *P. multocida*.

The multicolor detection kit utilized in the present investigation permits a single colony lift to be used for rapid, sequential detection of ToxA and AlcA. However, the multicolor reagents are considerably more expensive than those required for either conventional colorimetric detection or chemiluminescent detection. In addition, we found the green substrate reagent included in the kit to be unsuitable for the assay, as reported here. Since the multicolor reagents are not available individually, users must discard one-third of the purchased kit. Therefore, some laboratories may wish to consider other options for the development of membranes hybridized with ToxA and AlcA. Either standard colorimetric detection or chemiluminescent detection can be performed with duplicate colony lifts, each hybridized to a separate probe. If only a single colony lift is available, chemiluminescent detection is the only viable alternative. In that case, membranes must be sequentially hybridized with each of the probes and stripped between the two detection procedures. This option has a more extended turnaround time than either multicolor detection or standard colorimetric detection with duplicate membranes, since overnight hybridization with ToxA and AlcA must be performed sequentially rather than simultaneously. The best alternative for development of membranes hybridized to ToxA and AlcA will depend on the priorities of the laboratory performing the assay.

In summary, we report here a colony lift-hybridization assay that includes highly sensitive and specific probes useful for identification of *B. bronchiseptica* and toxigenic *P. multocida* from primary isolation plates. Multicolor detection provides rapid and definitive results from a single colony lift, although other options for development may be preferred depending on the needs of the laboratory conducting the assay. The colony lift assay provides faster, more objective results than conventional identification of these pathogens and should prove useful in both diagnostic and research settings.

ACKNOWLEDGMENTS

We thank Holly Good and Pamala Recker for expert technical assistance.

REFERENCES

1. **Ackermann, M. R., M. C. DeBey, K. B. Register, D. J. Larson, and J. M. Kinyon.** 1994. Tonsil and turbinate colonization by toxigenic and nontoxigenic strains of *Pasteurella multocida* in conventionally raised swine. J. Vet. Diagn. Invest. **6:**375–377.
2. **Boehringer Mannheim Corporation.** 1995. Genius system user's guide for membrane hybridization. Boehringer Mannheim Corporation, Indianapolis, Ind.
3. **De Jong, M. F.** 1992. (Progressive) atrophic rhinitis, p. 414–435. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames.
4. **Foged, N. T.** 1992. *Pasteurella multocida* toxin. The characterisation of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. APMIS **100**(Suppl. 25)**:**1–56.
5. **Frandsen, P. L., N. T. Foged, S. K. Petersen, and A. Bording.** 1991. Characterization of toxin from different strains of *Pasteurella multocida* serotype A and D. J. Vet. Med. B **38:**345–352.
6. **Giardina, P. C., L.-A. Foster, S. I. Toth, B. A. Roe, and D. W. Dyer.** 1995. Identification of *alcA*, a *Bordetella bronchiseptica* gene necessary for alcaligin production. Gene **167:**133–136.
7. **Heddleston, K. L., J. E. Gallagher, and P. A. Rebers.** 1972. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. Avian Dis. **16:**925–936.
8. **Kamp, E. M., G. C. A. M. Bokken, T. M. M. Vermeulen, M. R. de Jong, H. E. C. M. Buys, F. H. Reek, and M. A. Smits.** 1996. A specific and sensitive PCR assay suitable for large-scale detection of toxigenic *Pasteurella multocida* in nasal and tonsillar swabs specimens of pigs. J. Vet. Diagn. Invest. **8:** 304–309.
9. **Kamps, A. M. I. E., W. E. C. M. Buys, E. M. Kamp, and M. A. Smits.** 1990. Specificity of DNA probes for the detection of toxigenic *Pasteurella multocida* subsp. *multocida* strains. J. Clin. Microbiol. **28:**1858–1861.
10. **Kamps, A. M. I. E., E. M. Kamp, and M. A. Smits.** 1990. Cloning and expression of the dermonecrotic toxin gene of *Pasteurella multocida* ssp. *multocida* in *Escherichia coli*. FEMS Microbiol. Lett. **67:**187–190.
11. **Lichtensteiger, C. A., S. M. Steenbergen, R. M. Lee, D. D. Polson, and E. R. Vimr.** 1996. Direct PCR analysis for toxigenic *Pasteurella multocida*. J. Clin. Microbiol. **34:**3035–3039.
12. **Magyar, T.** Personal communication.
13. **Magyar, T., and R. B. Rimler.** 1991. Detection and enumeration of toxin-producing *Pasteurella multocida* with a colony-blot assay. J. Clin. Microbiol. **29:**1328–1332.
14. **Nagai, S., S. Someno, and T. Yagihashi.** 1994. Differentiation of toxigenic from nontoxigenic isolates of *Pasteurella multocida* by PCR. J. Clin. Microbiol. **32:**1004–1010.
15. **Petersen, S. K., and N. T. Foged.** 1989. Cloning and expression of the *Pasteurella multocida* toxin gene, toxA, in *Escherichia coli*. Infect. Immun. **57:** 3907–3913.
16. **Quinn, P. J., M. E. Carter, B. Markey, and G. R. Carter.** 1994. Clinical veterinary microbiology, p. 254–258. Wolfe Publishing, London, United Kingdom.
17. **Quinn, P. J., M. E. Carter, B. Markey, and G. R. Carter.** 1994. Clinical veterinary microbiology, p. 280–283. Wolfe Publishing, London, United Kingdom.
18. **Register, K. B.** 1998. Biotinylated proteins of *Pasteurella multocida* and *Pasteurella haemolytica* cause false-positive reaction with biotinylated probes in colony lift-hybridization assays. Anal. Biochem. **257:**230–233.
19. **Register, K. B., M. R. Ackermann, and D. W. Dyer.** 1995. Nonradioactive colony lift-hybridization assay for detection of *Bordetella bronchiseptica* infection in swine. J. Clin. Microbiol. **33:**2675–2678.
20. **Rimler, R. B., and K. A. Brogden.** 1986. *Pasteurella multocida* isolated from rabbits and swine: serologic types and toxin production. Am. J. Vet. Res. **47:** 730–737.