JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1996, p. 3035–3039 Vol. 34, No. 12
0095-1137/96/$04.00+0

Direct PCR Analysis for Toxigenic *Pasteurella multocida*

CAROL A. LICHTENSTEIGER,[1]* SUSAN M. STEENBERGEN,[1] RUBY M. LEE,[2] DALE D. POLSON,[2] AND ERIC R. VIMR[1,3]

Department of Veterinary Pathobiology, College of Veterinary Medicine,[1] and Department of Microbiology, School of Life Sciences,[3] University of Illinois, Urbana, Illinois 61801, and NOBL Laboratories, Inc., Sioux Center, Iowa 51250[2]

Received 18 April 1996/Returned for modification 24 June 1996/Accepted 23 July 1996

A more rapid, accurate method to detect toxigenic *Pasteurella multocida* is needed for improved clinical diagnosis, farm biosecurity, and epidemiological studies. Toxigenic and nontoxigenic *P. multocida* isolates cannot be differentiated by morphology or standard biochemical reactions. The feasibility of using PCR for accurate, rapid detection of toxigenic *P. multocida* from swabs was investigated. A PCR protocol which results in amplification of an 846-nucleotide segment of the *toxA* gene was developed. The PCR amplification protocol is specific for toxigenic *P. multocida* and can detect fewer than 100 bacteria. There was concordance of PCR results with (i) detection of *toxA* gene with colony blot hybridization, (ii) detection of ToxA protein with colony immunoblot analysis, and (iii) lethal toxicity of sonicate in mice in a test set of 40 swine diagnostic isolates. Results of an enzyme-linked immunosorbent assay for ToxA agreed with the other assays except for a negative reaction in one of the 19 isolates that the other assays identified as toxigenic. In addition to accuracy, as required for a rapid direct specimen assay, toxigenic *P. multocida* was recovered efficiently from inoculated swabs without inhibition of the PCR. The results show that PCR detection of toxigenic *P. multocida* directly from clinical swab specimens should be feasible.

Pasteurella multocida is part of the commensal flora in the upper respiratory tract of pigs. The bacterium induces pneumonia in grower and finisher pigs, usually as a secondary pathogen invading lungs injured by other bacteria or viruses. A subset of *P. multocida* isolates are critical agents in an upper respiratory disease, progressive atrophic rhinitis (4, 6, 7, 11, 12, 17, 36). These isolates synthesize a 145-kDa toxin encoded by the chromosomal *toxA* gene. The ToxA protein is an essential virulence factor for progressive atrophic rhinitis; nontoxigenic *P. multocida* isolates do not cause this disease. Isolates of either common capsule type, A or D, may be toxigenic. The toxin induces turbinate atrophy and poor weight gains in pigs. In experimental animals, the toxin induces skin necrosis in guinea pigs (dermatonecrotic toxin) and is lethal when injected into mice (9). Among swine producers with >200-sow herds, almost half report problems with atrophic rhinitis and about 60% vaccinate against the disease (30). *P. multocida* is commonly transmitted from dam to piglets; segregated, early weaning decreases transmission by this route (1, 15, 40). Clinical signs in an infected herd may wax and wane, hindering accurate diagnosis. Vaccines currently available in the United States are not optimal; their impact on production performance is minimal and they fail to clear herd infection status (12, 15, 27).

Toxigenic *P. multocida* is unintentionally spread to uninfected (clean) herds via the addition of asymptomatic, infected breeding stock, and many aspects of toxigenic *P. multocida* epidemiology and ecology remain unknown, in part, because of the lack of a rapid, sensitive assay to confirm infection. False negative culture results occur when *P. multocida* dies in transport to the laboratory or is overgrown by other bacteria (nasal flora and contaminants) in the culture. Mouse inoculation may improve recovery rate from field specimens (12), but mouse inoculation is not practical or desirable for extensive epidemiological research or for routine diagnostic testing. Toxigenic and nontoxigenic *P. multocida* isolates do not differ on diagnostic biochemical reactions or morphology. Additional testing of laboratory isolates is required to differentiate toxigenic and nontoxigenic strains; both in vitro and in vivo methods have been used (11, 13, 17, 21, 26, 29, 31, 37). However, culture isolation (which fails in some specimens), species identification, and toxin testing of *P. multocida* is time-consuming and costly.

A more rapid, accurate detection assay is needed for sound decisions regarding diagnosis and treatment, to prevent unintentional introduction of infected pigs into clean herds, to support basic studies in ecology and epidemiology of the organism, and to develop more efficacious vaccines. Assays based on PCR are contributing to diagnostic microbiology (2, 3, 16). The conservation of the sequence of the *toxA* gene (8, 22, 32) indicates that assays for the gene, including PCR, are valid for identification of toxigenic isolates. Direct specimen analysis using PCR for toxigenic *P. multocida* should be a more rapid and sensitive assay than the three-step process of bacterial isolation, biochemical identification, and toxigenic testing of isolates. Another upper respiratory pathogen, *Bordetella pertussis*, causes whooping cough in humans and has been detected directly in nasopharyngeal aspirates or swabs by using PCR, with apparently greater sensitivity than culture isolation (5, 18–20, 23–25, 33). Swab selection in such assays is important because components of some swabs have been shown to inhibit PCR (38).

In this communication, we explore the feasibility of PCR for accurate rapid detection of *P. multocida* from swabs. We show that for our reaction conditions, PCR is specific and sensitive for toxigenic *P. multocida*. Sensitivity appears increased over reactions with primers recently reported (29). In addition, as required for a direct specimen assay, we show that toxigenic *P. multocida* is recovered efficiently from inoculated swabs without inhibiting the PCR assay. We conclude that PCR de-

* Corresponding author. Mailing address: Department of Veterinary Pathobiology MC-002, University of Illinois, 2001 South Lincoln Ave., Urbana, IL 61801. Phone: (217) 333-2449. Fax: (217) 244-7421. Electronic mail address: clichten@uiuc.edu.

tection directly from swab specimens should significantly enhance the identification of pigs infected with toxigenic *P. multocida.*

MATERIALS AND METHODS

Preparation of bacteria. *P. multocida* was routinely cultured at 35 to 37°C by using brain heart infusion (BHI) broth or agar. Two swine isolates of *P. multocida*, M33 and M29, were used as assay controls. Our previous characterization assays showed that isolate M33 was toxigenic (caused necrosis of guinea pig skin and was cytotoxic to Vero cells) whereas M29 was not toxigenic (9, 28, 31). Forty other *P. multocida* isolates, numbered 1 to 40, were derived from diagnostic samples (mostly nasal swabs) submitted from conventional pigs. Isolates were identified as capsule type A on the basis of diminished colony size when cells were exposed to hyaluronidase by coculturing with *Staphylococcus aureus* (10). Isolates not affected by hyaluronidase treatment were labelled type D. Viable *P. multocida* (CFU per milliliter) in test aliquots were estimated by using a standard optical density growth curve (wavelength of 600 nm) and were measured by plate counts on BHI agar after overnight incubation. The panel of bacteria used in specificity testing was collected from diagnostic isolates and a veterinary teaching set.

Detection of *toxA* gene. For PCR assays, bacteria were grown to mid- to late log phase in BHI broth, pelleted by centrifugation, and resuspended in water. Template DNA was prepared by heat lysis (50 μl of bacteria incubated in boiling water for 10 min). PCR assays were run with 0.5-μl aliquots of template DNA in a 50-μl reaction volume. Commercial *Taq* polymerase with reaction buffers (Gibco BRL Life Technologies, Grand Island, N.Y.), nucleotides (0.4 mM) (Promega Corp., Madison, Wis.), and commercially synthesized primers (0.2 μM) (Oligos Etc., Wilsonville, Oreg.) were used. The oligonucleotide primers were designed to amplify an 846-mer segment of *toxA* between nucleotides 2096 and 2942 (32); the forward primer was 5′CTTAGATGAGCGACAAGG3′, and the reverse primer was 5′GAATGCCACACCTCTATAG3′. DNA template amplification conditions designed for the primers and short double-stranded DNA amplification were run for 40 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extending at 72°C for 30 s (Thermocycler 9600; Perkin-Elmer Cetus, Foster City, Calif.). The amplicon was routinely resolved and ethidium bromide stained in agarose gels. Fluorescent signals from the stained DNA were captured with computer imaging analysis equipment (Alpha Inotech, San Leandardo, Calif.).

For Southern blot analysis, PCR amplicon resolved in agarose was transferred to a nylon membrane (Micron Separations Inc., Westboro, Mass.) and probed with an oligonucleotide complementary to nucleotides 2670 to 2692 of *toxA* (32) (5′AACCAGGTTGGAGGGCCTTATG3′; Oligos Etc.) by using a standard Southern blot protocol (14). The probe was end labelled with [^{32}P]ATP (ICN Biomedicals, Costa Mesa, Calif.) by using polynucleotide kinase (Gibco BRL) and a Nensorb-20 column (DuPont Company, Wilmington, Del.) following the manufacturers' instructions.

P. multocida isolates for colony blot hybridization were grown overnight in BHI broth in a 96-well microtiter plate. The isolates were spotted onto BHI agar in a grid pattern with metal or wooden rods, and after overnight incubation, the colonies were lifted onto a nylon membrane (Micron Separations Inc.). The bacteria were lysed, hybridized with the ^{32}P-labelled 22-mer probe (see description of Southern blot analysis above), and exposed to radiographic film by using standard protocols (14).

Detection of ToxA protein. Synthesis of ToxA protein by *P. multocida* was tested by two in vitro assays plus a mouse lethality bioassay. One in vitro assay was a slight modification of an established colony immunoblot assay (26). Colonies of bacteria were prepared as for the colony hybridization (as described above) except overnight growth was on blood agar (sheep blood; Remel, Lenexa, Kans.) prior to lifting to nylon membrane. Milk (5%) to block nonspecific protein binding and anti-ToxA monoclonal antibody (1:1,000) were diluted in the published protocol buffers (26). The anti-ToxA antibody was provided by the National Animal Disease Center, Ames, Iowa; source hybridoma 1B2A3 is deposited with the American Type Culture Collection. Bound antibody was detected with peroxidase-conjugated, goat anti-mouse immunoglobulin G antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and enhanced chemiluminescence (Amersham Life Science, Arlington Heights, Ill.).

The second in vitro assay for ToxA protein was an enzyme-linked immunosorbent assay (ELISA). Sonicated filtrate was prepared from overnight growth of *P. multocida* scraped from blood agar plates into phosphate-buffered saline (PBS) containing 6 mM EDTA and 3 mM phenylmethylsulfonyl fluoride. The suspension was sonicated and centrifuged (1,500 × *g* for 30 min). Supernatant was filtered (pore size, 0.22 μm) and used to coat microtiter plates (Immulon 1; Dynatech, Chantilly, Va.) by incubating for 1.5 h in 15 mM sodium carbonate and 35 mM sodium bicarbonate buffer. The coating buffer with 0.05% Tween 20, and 1% gelatin was used for blocking and antibody buffers; the wash buffer was PBS with 0.1% Tween 20. Following blocking for 1.5 h, a 1:200 dilution of the anti-ToxA monoclonal antibody (same antibody as used in the colony immunoblot) was added and incubated for 1 h. Bound antitoxin antibody was detected by sequential incubation with peroxidase-conjugated goat anti-mouse immunoglobulin (diluted 1:10,000) (Sigma) for 60 min and TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories) for 15 min before stopping the reaction with 100 μl of 1 N H_2SO_4. Known positive and negative isolates (based on repeated ELISA and mouse lethality tests) were included as controls on every ELISA plate. Positive reactions were grossly definitively yellow and negative reactions were clear; intermediate reactions did not occur.

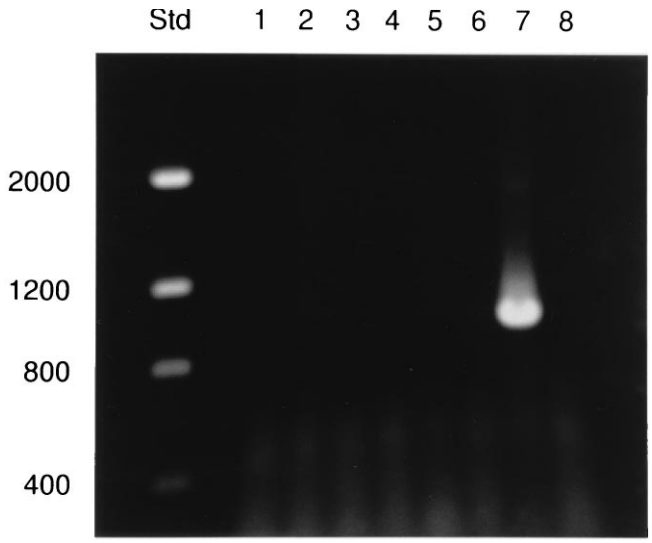

FIG. 1. Specificity of primers for toxigenic *P. multocida*. Agarose gel-resolved, ethidium bromide-stained, PCR product from template DNA of approximately 10^6 CFU of various bacteria and known toxigenic (M33) and nontoxigenic (M29) *P. multocida*. Std, size standards. Lanes: 1, *E. coli*; 2, *Bacillus* sp.; 3, *S. aureus*; 4, *A. hydrophila*; 5, *E. cloacae*; 6, *P. aeruginosa*; 7, *P. multocida* M33; 8, *P. multocida* M29.

The biological activity of *P. multocida* sonicate was tested in adult female BALB/c mice. Aliquots (0.5 ml) of sonicate, prepared as for the ELISA, were injected intraperitoneally in four mice (test mice). Four control mice were inoculated in parallel with heat-inactivated sonicate (70°C, 30 min). Both groups of mice were observed for 48 h. To score the assay, all control mice had to survive while either all or none of the test mice survived. The isolate was scored toxigenic if all the test mice died or was scored nontoxigenic if none of the test mice died. In the rare assay where some control mice died or only one to three test mice died, the assay was repeated.

Handling of swabs. Plastic-shafted, rayon-tipped swabs (Bacti-Swab; Remel) were used without moistening the tip with the built-in transport medium. *P. multocida* recovery was measured in duplicate or triplicate swabs inoculated with an estimated 100 and 500 CFU in 5 μl of water. Bacteria were recovered from swabs by excising the tips into Spin X open-gridded, no-membrane cups (Corning Costar, Charlotte, N.C.) inserted in 1.8-ml capped centrifuge tubes (Evergreen Scientific, Los Angeles, Calif.). A 0.5-ml aliquot of wash water was added, and the tubes were manually inverted a few times. Most of the wash water stayed in the cup until the tubes were centrifuged (13,000 × *g* for 3 min). The CFU of *P. multocida* in the stock suspension and washed from the swabs were determined by plate counts of colonies. After confirming efficiency of bacterial recovery, PCR assays were run on recovered bacteria in parallel with aliquots not added to swabs to confirm that the swabs and the wash procedure did not inhibit the assay reaction.

RESULTS

Validity of PCR for *P. multocida*. To be valid, the PCR assay must be specific for the *toxA* gene of *P. multocida*, applicable to all toxigenic *P. multocida* isolates, and sensitive enough to detect just a few organisms.

The *toxA* primers were tested in the PCR assay with the toxigenic M33 and nontoxigenic M29 *P. multocida* isolates and with various other bacteria that are inhabitants of the swine upper respiratory tract or contaminants of clinical swabs. The expected amplicon was readily detected in the reaction product of *P. multocida* M33 resolved in agarose gel and stained with ethidium bromide (Fig. 1, lane 7). Similar product was not synthesized when *Escherichia coli*, a *Bacillus* sp., *S. aureus*, *Aeromonas hydrophila*, *Enterobacter cloacae*, *Pseudomonas*

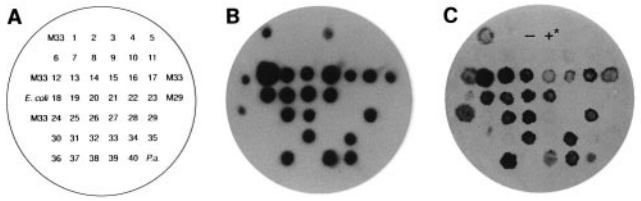

FIG. 2. Colony blot analyses for *toxA* gene and ToxA protein. (A) Grid key; (B) colony blot hybridization for *toxA*; (C) colony immunoblot for ToxA (toxin protein). M33, toxigenic *P. multocida*; M29, nontoxigenic *P. multocida*; P.a., *Pasteurella aerogenes*; *, these two isolates were tested in another experiment; results are indicated over the blot: +, signal; −, no signal.

aeruginosa, or nontoxigenic *P. multocida* M29 provided the template DNA (Fig. 1). In addition, none of the following bacteria gave a detectable product in the PCR assay: *Streptococcus suis*, *Erysipelothrix rhusiopathiae*, *Pasteurella aerogenes*, *Actinobacillus pleuropneumoniae*, and *Bordetella bronchiseptica* (data not shown). The pale indistinct ethidium bromide staining in the lower part of the gel in Fig. 1 apparently is due to DNA amplification from primer dimers, since its intensity, but not the *toxA* PCR product, decreased or disappeared in other experiments when the primer concentration was decreased by 75% (data not shown).

To determine the accuracy of PCR detection of toxigenic isolates, 40 diagnostic swine isolates of *P. multocida* were screened. First, these isolates were characterized by using four other assays to define their toxigenic status. Colony blot hybridization with an internal 22-oligonucleotide *toxA* probe was used to determine which of these isolates encoded the *toxA* gene. The *toxA* probe hybridized to a single *Bam*HI fragment of M33 chromosomal DNA and did not hybridize to M29 chromosomal DNA in Southern blot analysis (data not shown). The probe in colony hybridization detected *toxA* in 19 of the 40 *P. multocida* isolates (Fig. 2A and B). Toxin protein was then assayed in colony immunoblots which were probed with anti-ToxA monoclonal antibody (Fig. 2A and C). Biological toxigenic activity of each isolate was tested in a mouse lethality assay (Table 1). There was concordance of detection of ToxA protein in the immunoblot, *toxA* gene in blot hybridization, and mouse lethality of sonicates. Four of 16 type A and 15 of 24 type D isolates were defined toxigenic by all three assays, and all other isolates were nontoxigenic by all three assays. Bacterial sonicates were also tested for toxin in an ELISA with the same antitoxin antibody as used in the immunoblot (Table 1). ELISA results agreed well with the results of the other three assays except that isolate 39 was negative by the ELISA. The results of PCR analysis are in complete agreement with the colony hybridization, colony immunoblot, and mouse lethality results, indicating good specificity of PCR (Table 1).

Sensitivity of PCR amplicon detection was compared between Southern blot analysis and ethidium bromide staining in agarose gels (Fig. 3). A signal from 150 CFU (Fig. 3A, lane 7) was visible on the ethidium bromide-stained gel under ultraviolet light. A similar signal is visible in lane 7 of Fig. 3B, showing the results of the Southern blot analysis; with direct visual examination, pale signals were seen in the next four lanes of the radiographic film, containing 75, 30, 15, and 3 CFU, respectively (Fig. 3B).

Recovery of *P. multocida* from swabs. To be useful and sensitive for research or diagnostic purposes, there needs to be an efficient means to recover *P. multocida* from nasal and tonsillar swabs. The assay needs to minimize sample handling for efficiency and for avoiding cross contamination of samples. The open-gridded cup readily adapted microcentrifuge tubes for a single tube assay to prepare DNA template. Essentially all inoculated *P. multocida* was recovered from the swabs (Table 2). Two washes with 0.5 ml of water were done, but very few bacteria (1 to 4 CFU) were recovered in the second wash. *P. multocida* recovered from inoculated swabs gave the same PCR signal as did uninoculated *P. multocida* run in parallel PCR assays.

DISCUSSION

The data indicate that our PCR assay is specific and sensitive. Four assays were run to define true positive (toxigenic) and true negative (nontoxigenic) isolates. Except for one apparent false negative ELISA result, there was complete agreement of the toxigenic status of the *P. multocida* isolates based on colony hybridization of *toxA*, colony immunoblot and ELISA for ToxA, and mouse lethality of sonicate. The concordance of PCR results with the defined toxigenic status indicates 100% specificity and sensitivity.

The ELISA, compared with classification status from the

TABLE 1. PCR, ELISA, and mouse lethality toxigenic assay results from diagnostic *P. multocida* isolates

Isolate no.	Capsule type	PCR	ELISA	Mouse lethality assay
1	A	−	−	−
2	A	−	−	−
3	A	+	+	+
4	A	−	−	−
5	A	−	−	−
6	A	−	−	−
7	A	−	−	−
8	A	−	−	−
9	A	−	−	−
10	A	−	−	−
11	A	−	−	−
12	A	+	+	+
13	A	+	+	+
14	D	+	+	+
15	D	+	+	+
16	D	+	+	+
17	D	+	+	+
18	D	+	+	+
19	D	+	+	+
20	A	+	+	+
21	D	+	+	+
22	D	−	−	−
23	D	−	−	−
24	D	−	−	−
25	D	+	+	+
26	D	+	+	+
27	D	−	−	−
28	D	−	−	−
29	D	+	+	+
30	D	−	−	−
31	D	−	−	−
32	D	+	+	+
33	D	−	−	−
34	D	+	+	+
35	A	−	−	−
36	D	−	−	−
37	D	+	+	+
38	A	−	−	−
39	D	+	−[a]	+[a]
40	D	+	+	+

[a] ELISA and mouse lethality assay were repeated with no changes in results; the ELISA result does not match the results from the other assays here or in Fig. 2.

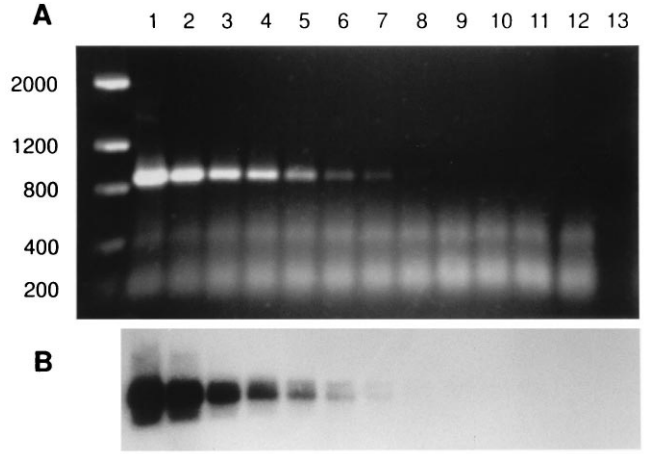

FIG. 3. Sensitivity of PCR assay. *P. multocida* PCR product resolved and ethidium bromide stained in agarose gel (A) and probed for *toxA* with Southern blot analysis using a [^{32}P]ATP-labelled 22-mer internal *toxA* probe (B). Size standards are in the left lane. Lanes 1 to 12 have 3×10^5, 3×10^4, 3×10^3, 1,500, 750, 300, 150, 75, 30, 15, 3, and 0 CFU of *P. multocida* M33 as a template source, respectively; lane 13 is a no template negative control (no bacteria added). A total of 10^6 CFU of nontoxigenic *P. multocida* M29 was added to all PCR assays except for the negative control in lane 13 as a control for sensitivity in the presence of background DNA.

four assays with agreement, had a sensitivity of 94.7% (18 of 19) and a specificity of 100% (21 of 21). Kamps et al. found 2 of 56 isolates that were ELISA negative but positive with a probe for *toxA* gene (21). Those two isolates were not further characterized to differentiate between false assay results or a repressed or cryptic gene not producing toxin polypeptide. Testing for toxin in vitro may be misleading if the gene is repressed under some conditions. A genetic probe such as the PCR assay avoids potential problems of suppressed gene expression. Regulation of toxin expression is unknown; our data suggest that under laboratory conditions all *toxA*-positive isolates express toxin. A PCR-based assay theoretically could give a positive signal from an isolate with a mutated, nonfunctional gene; however, there is no indication of this occurring with our test isolates. Moreover, our colony hybridization and PCR results suggest that these genetic assays are more specific than the colony immunoblot protein assay. The signals were weaker on the colony immunoblot, and the antitoxin antibody cross-reacted with *P. aerogenes* and *E. coli* (Fig. 2). In addition, our evidence indicates that the PCR assay should be adaptable to direct specimen testing without prior isolation of *P. multocida*, which was necessary in the other protocols.

Primers are critical to sensitive PCR results (39); the apparent increased sensitivity of our PCR protocol over that previously reported (about 1,000 CFU, assuming 4.6 fg of DNA per bacterium) (29, 34) is likely due to primer set differences. In addition, the results from the 40 isolates indicate that the reactivity is not restricted by variability of *toxA* gene among isolates or capsule type. In related PCR experiments not shown, using *P. multocida* primers external to *toxA*, we came to the same conclusion as Nagai et al. that *toxA* is in the same location in the chromosome of both capsule type A and D isolates (29).

TABLE 2. Recovery of *P. multocida* from inoculated swabs[a]

Test	*P. multocida* inoculated	*P. multocida* recovered	% Recovered
I	294.5 ± 21.9	299.5 ± 7.8	101.7 ± 2.6
II	129.3 ± 35.3	120.3 ± 19.4	93.0 ± 15.0

[a] Data are CFU ± standard deviations.

In addition to validity, minimal cost and ease of performance are important for both diagnostic and high-volume research assays such as epidemiologic studies. Our PCR protocol is less labor-intensive and avoids hazardous chemicals used in *B. pertussis* protocols in which DNA is extracted or the sample digested with proteinase K prior to PCR. Our open-grid wash cups facilitate efficient bacterial recovery; boiling in the same tubes is all that was needed prior to PCR amplification. Although a single wash recovered essentially all the bacteria from the swabs, we plan to leave two washes in the routine protocol. Some pig swab specimens contain blood, and we have found in pilot studies that one wash does not always effectively lyse the erythrocytes and remove the hemoglobin. Heme of hemoglobin is a potent inhibitor of *Taq* polymerase (35).

Components of some commercial swabs inhibit PCRs (38); inertness cannot be assumed. The selected swabs did not inhibit the polymerase reaction in our studies. Future studies are needed to confirm the absence of, or to validate protocol modifications to avoid, other inhibitors that may be encountered in biological specimens collected from the nose or tonsil. Development of a rapid, valid assay for toxigenic *P. multocida* in nasal and tonsil swab specimens will not only facilitate rapid clinical diagnoses and prompt therapy but will also facilitate epidemiology studies and screening to prevent transmission to clean herds by animal movement.

ACKNOWLEDGMENTS

We thank Jennifer Criley and Jeanette Graves for excellent laboratory assistance.

The project was funded, in part, by the Illinois Department of Agriculture.

ADDENDUM IN PROOF

Kamp et al. recently published the results of a study using a PCR-based assay to detect toxigenic *P. multocida* in swab specimens (E. M. Kamp, G. C. A. M. Bokken, T. M. M. Vermeulen, M. F. de Jong, H. E. C. M. Buys, F. H. Reek, and M. A. Smits, J. Vet. Diagn. Invest. **8:**304–309, 1996).

REFERENCES

1. **Alexander, T. J. L., and D. L. Harris.** 1992. Methods of disease control, p. 808–836. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine, 7th ed. Iowa State University Press, Ames.
2. **Altwegg, M., and J. Verhoef.** 1995. Amplification methods in diagnostic microbiology. Elsevier Science, Amsterdam.
3. **Atlas, R. M., and A. K. Bej.** 1994. Polymerase chain reaction, p. 418–435. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
4. **Baalsrud, K. J.** 1987. The effect of atrophic rhinitis on growth rate. Acta Vet. Scand. **28:**299–304.
5. **Bäckman, A., B. Johansson, and P. Olcén.** 1994. Nested PCR optimized for detection of *Bordetella pertussis* in clinical nasopharyngeal samples. J. Clin. Microbiol. **32:**2544–2548.
6. **Bäckström, L.** 1992. Atrophic rhinitis in swine. Agri-Practice **13:**21–24.
7. **Bäckström, L., D. C. Hoefling, A. C. Morkoc, and R. P. Cowart.** 1985. Effect of atrophic rhinitis on growth rate in Illinois swine herds. J. Am. Vet. Med. Assoc. **187:**712–715.
8. **Buys, W. E. C. M., H. E. Smith, A. M. I. E. Kamps, E. M. Kamp, and M. A. Smits.** 1990. Sequence of the dermonecrotic toxin of *Pasteurella multocida* ssp. *multocida*. Nucleic Acids Res. **18:**2815–2816.
9. **Carter, G. R.** 1990. *Pasteurella* and *Francisella*, p. 129–142. *In* G. R. Carter and J. R. Cole, Jr. (ed.), Diagnostic procedures in veterinary bacteriology and mycology, 5th ed. Academic Press, Inc., New York.
10. **Carter, G. R., and S. W. Rundell.** 1975. Identification of type A strains of

P. multocida using staphylococcal hyaluronidase. Vet. Rec. **96:**343.
11. **Chanter, N., and J. M. Rutter.** 1989. Pasteurellosis in pigs and the determinants of virulence of toxigenic *Pasteurella multocida*, p. 161–195. *In* C. Adlam and J. M. Rutter (ed.), Pasteurella and pasteurellosis, 1st ed. Academic Press, New York.
12. **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, 7th ed. Iowa State University Press, Ames.
13. **de Jong, M. F., H. L. Oei, and G. J. Tetenburg.** 1980. AR-pathogenicity-tests for *Pasteurella multocida* isolates. Int. Pig Vet. Soc. Proc. **1980:**211.
14. **Dillon, J. R., G. S. Bezanson, and K. H. Yeung.** 1985. Basic techniques, p. 1–114. *In* J. R. Dillon, A. Nasim, and E. R. Nestmann (ed.), Recombinant DNA methodology. John Wiley & Sons, New York.
15. **Dritz, S. S., J. L. Nelssen, R. D. Goodband, M. D. Tokach, and M. M. Chengappa.** 1994. Application of segregated early weaning technology in the commercial swine industry. Compend. Contin. Educ. Pract. Vet. **16:**677–685.
16. **Eisenstein, B. I.** 1990. The polymerase chain reaction. A new method of using molecular genetics for medical diagnosis. N. Engl. J. Med. **322:**178–183.
17. **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. Acta Pathol. Microbiol. Immunol. Scand. **100:**1–56.
18. **Grimprel, E., P. Bégué, I. Anjak, F. Betsou, and N. Guiso.** 1993. Comparison of polymerase chain reaction, culture, and Western immunoblot serology for diagnosis of *Bordetella pertussis* infection. J. Clin. Microbiol. **31:**2745–2750.
19. **He, Q., J. Mertsola, H. Soini, M. Skurnik, O. Ruuskanen, and M. K. Viljanen.** 1993. Comparison of polymerase chain reaction with culture and enzyme immunoassay for diagnosis of pertussis. J. Clin. Microbiol. **31:**642–645.
20. **He, Q., J. Mertsola, H. Soini, and M. Vijanen.** 1994. Sensitive and specific polymerase chain reaction assays for detection of *Bordetella pertussis* in nasopharyngeal specimens. J. Pediatr. **124:**421–426.
21. **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.
22. **Lax, A. J., N. Chanter, G. D. Pullinger, T. Higgins, J. M. Staddon, and E. Rozengurt.** 1990. Sequence analysis of the potent mitogenic toxin of *Pasteurella multocida*. FEBS **277:**59–64.
23. **Li, A., D. Jansen, T. M. Finn, S. A. Halperin, A. Kasina, S. P. O'Connor, T. Aoyama, C. R. Manclark, and M. J. Brennan.** 1994. Identification of *Bordetella pertussis* infection by shared-primer PCR. J. Clin. Microbiol. **32:**783–789.
24. **Lichtinghagen, R., R. Diedrich-Glaubitz, and B. von Hörsten.** 1994. Identification of *Bordetella pertussis* in nasopharyngeal swabs using the polymerase chain reaction: evaluation of detection methods. Eur. J. Clin. Chem. Clin. Biochem. **32:**161–167.
25. **Lichtinghagen, R., and R. Glaubitz.** 1995. A competitive polymerase chain reaction assay for reliable identification of *Bordetella pertussis* in nasopharyngeal swabs. Eur. J. Clin. Chem. Clin. Biochem. **33:**87–93.
26. **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.
27. **McGaw, M. B.** 1994. Management techniques for atrophic rhinitis control. Comp. Contin. Educ. Pract. Vet. **16:**1615–1618.
28. **Mendoza, S., A. Ciprian, J. Camacho, and J. A. Montaraz.** 1993. Comparison of three biological assays for the detection of toxigenic properties of *Bordetella bronchiseptica*. Vet. Microbiol. **36:**215–219.
29. **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.
30. **Ott, S. L.** 1994. Influence of herd size on swine vaccination practices. Anim. Health Insight **1994**(Spring/Summer)**:**14–17.
31. **Pennings, A. M. M. A., and P. K. Storm.** 1984. A test in Vero cell monolayers for toxin production by strains of *Pasteurella multocida* isolated from pigs suspected of having atrophic rhinitis. Vet. Microbiol. **9:**503–508.
32. **Petersen, S. K.** 1990. The complete nucleotide sequence of the *Pasteurella multocida* toxin gene and evidence for a transcriptional repressor, TxaR. Mol. Microbiol. **4:**821–830.
33. **Reizenstein, E., B. Johansson, L. Mardin, J. Abens, R. Möllby, and H. O. Hallander.** 1993. Diagnostic evaluation of polymerase chain reaction discriminative for *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica*. Diagn. Microbiol. Infect. Dis. **17:**185–191.
34. **Rolfs, A., I. Schuller, U. Finckh, and I. Weber-Rolfs.** 1992. Biological material amenable to PCR, p. 68–78. *In* A. Rolfs, I. Schuller, U. Finckh, and I. Weber-Rolfs (ed.), PCR: clinical diagnostics and research. Springer-Verlag, New York.
35. **Rolfs, A., I. Schuller, U. Finckh, and I. Weber-Rolfs.** 1992. Substances affecting PCR: inhibition or enhancement, p. 51–60. *In* A. Rolfs, I. Schuller, U. Finckh, and I. Weber-Rolfs (ed.), PCR: clinical diagnostics and research. Springer-Verlag, New York.
36. **Rutter, J. M.** 1985. Atrophic rhinitis in swine. Adv. Vet. Sci. Comp. Med. **29:**239–279.
37. **Rutter, J. M., and P. D. Luther.** 1984. Cell culture assay for toxigenic *Pasteurella multocida* from atrophic rhinitis of pigs. Vet. Rec. **114:**393–396.
38. **Wadowsky, R. M., S. Laus, T. Libert, S. J. States, and G. D. Ehrlich.** 1994. Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum shaft components of a nasopharyngeal swab. J. Clin. Microbiol. **32:**1054–1057.
39. **Wang, H., L. Fliegel, C. E. Cass, A. M. W. Penn, M. Michalak, J. H. Weiner, and B. D. Lemire.** 1994. Primers are decisive for sensitivity of PCR. BioTechniques **17:**82–86.
40. **Wiseman, B., R. Morrison, G. Dial, T. Molitor, B. Freking, M. Bergeland, and C. Pijoan.** 1992. Medicated early weaning: influence of age on growth performance and disease. Proc. Am. Assoc. Swine Pract. **1992:**469–475.