Differentiation of Toxigenic from Nontoxigenic Isolates of Pasteurella multocida by PCR

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A PCR assay was developed for the differentiation of toxigenic Pasteurella multocida subsp. multocida strains, the major etiologic agent for progressive atrophic rhinitis in pigs, from nontoxigenic strains. The PCR targeted a toxA gene encoding a 143-kDa dermonecrotic toxin that is considered to be the central etiologic factor in progressive atrophic rhinitis. toxA fragments were amplified from toxigenic P. multocida isolates but not from nontoxigenic isolates or other bacteria isolated from pigs. The sensitivity of the reaction was as low as 10 pg of chromosomal DNA from a toxigenic strain. The results obtained by PCR of the DNAs of 187 field isolates of P. multocida were consistent with those obtained by the guinea pig skin test and Western blot (immunoblot) analysis. Restriction fragment analysis of the PCR-amplified fragments from 67 field isolates and comparison of the DNA sequences of fragments from capsular serotype A and D strains suggest that the PCR-amplified region, which is considered to encode the major immunodominant determinants of the toxin, would be the same among P. multocida strains. The PCR that we describe should be useful for the diagnosis and the etiologic survey of progressive atrophic rhinitis.

Atrophic rhinitis (AR) is a serious, highly contagious disease of swine characterized by conchal atrophy, facial distortion, sneezing, nasal hemorrhage, and impaired growth (5). In the United States and Japan, Bordetella bronchiseptica was thought to be the principal cause of AR (28), while in Germany and The Netherlands, Pasteurella multocida was considered to be the important primary pathogen (7). After years of controversy, the disease is now classified as a nonprogressive form, which is caused by B. bronchiseptica, and a progressive form (PAR), which is caused by toxigenic P. multocida alone or in combination with other agents (e.g., B. bronchiseptica) (6). The occurrence of PAR caused by P. multocida has not been well accepted in Japan.

A definite diagnosis of PAR cannot be based solely on clinical and pathomorphologic observations, but requires detection of toxigenic P. multocida with nasal and/or tonsillar swabs. Nontoxigenic P. multocida can concurrently infect the nasal cavities and tonsillar surfaces of pigs. The etiologic importance of the toxin (9, 22) necessitates classification of the isolates as toxigenic or nontoxicotic for the diagnosis of PAR.

The tests used to distinguish toxigenic from nontoxigenic strains are classified into the following three groups: (i) biological assays that rely on the activities of the toxin, including a mouse lethality test (20), a guinea pig skin test (4, 20), and cell culture assays (16, 21); (ii) immunological assays that rely on the antigenicity of the toxin, including an enzyme-linked immunosorbent assay (8), a colony blot assay (12), and Western blot (immunoblot) analysis (10); and (iii) a colony hybridization test that detects the toxin gene (11). Among these tests, the DNA assay has several advantages as a diagnostic tool because of the stability of DNA samples and the specificity of the DNA sequence.

In this report we describe a PCR (23) for use in the detection of the toxin gene, which may offer a more sensitive, rapid, and simplified means of identifying toxigenic P. multocida strains. Additionally, the similarities of the toxin genes were examined by restriction fragment analysis and DNA sequencing with PCR-amplified DNA.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. A toxigenic P. multocida subsp. multocida strain, NCTC 12178 (capsular serotype D), was kindly provided by K. Kume (Research Center for Veterinary Science of The Kitasato Institute, Saitama, Japan). Actinobacillus equuli and Actinobacillus suis were obtained from the Japan Collection of Microorganisms (Saitama, Japan). Actinobacillus pleuropneumoniae 4074 (serotype 1), CCMS870 (serotype 2), K17 (serotype 5a), and WF83 (serotype 7) were described previously (14). Aeromonas sp., B. bronchiseptica, Enterobacter sp., Escherichia coli, Haemophilus parasuis, Pseudomonas aeruginosa, Bacillus subtilis, Erysiphe rhiaphis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus suis type 2, and Mycoplasma hypophenmona were isolated from pigs at the Nippon Institute for Biological Science. A total of 187 field isolates of P. multocida were obtained from nasal swabs (152 isolates) and lungs (35 isolates) of pigs from 31 herds. They were identified as P. multocida subsp. multocida with a nonenterobacteriaceae identification kit (API 20NE, API System S.A., Vercieu, France). Capsular serotype A and D P. multocida strains were identified by hyaluronidase sensitivity (3) and passive hemagglutination (2) tests, respectively. E. coli XL-1 Blue endA1 hisD17 supE44 thi-1 recA1 gyrA96 relA1 del[proABlac] [F' proAB lacF' ZdeI5, Tn10( TC') ] was used for transformation.

P. multocida strains were grown in heart infusion medium supplemented with 5% chicken serum. E. coli was grown in Luria-Bertani (LB) medium (13), and for transformation, the LB medium was supplemented with 50 μg of ampicillin per ml. Plasmid pPM35 (11), which contains the 3' half of the toxA gene, was kindly provided by E. M. Kamp (Department of Bacteriology, Central Veterinary Institute, Lelystad, The Neth-
was used P. multocida 32, oligonucleotide 2, 5'-synthesized Plasmid erlands). Plasmid pBluescript II (Stratagene, La Jolla, Calif.) was used for gene cloning.

Oligonucleotides. Four oligonucleotide primers were custom synthesized (Nissilek Co., Ltd., Tokyo, Japan); they had the following sequences: oligonucleotide 1, 5'-TACTCAAT-TAGAAAAAGCCGTTTATCC-3'; oligonucleotide 2, 5'-TCCAGATAATTGTGCATTTTATCAATT-3'; oligonucleotide 3, 5'-TTTACAGACCTTGCACAGGGAA-3'; oligonucleotide 4, 5'-TCTACTACAGTTGCTGTTA TTTTTAAAT-3'. Oligonucleotides 1 and 3 correspond to the sense strand at positions 1983 to 2012 and 2943 to 2972 of the tox4 gene, respectively (see Fig. 1A and B). Oligonucleotides 2 and 4 correspond to the antisense strand at positions 2282 to 2253 and 3212 to 3183, respectively.

Preparation of samples for PCR analysis. Genomic DNA of P. multocida NCTC 12178 was prepared by the method of Smith et al. (27). For preparation of DNA samples from the field isolates of P. multocida and other bacteria isolated from pigs, colonies of the test bacteria grown overnight on agar plates were picked up with a sterilized probe, suspended in 50 µl of sterilized distilled water, and incubated for 5 min at 100°C. Then, the sample was quickly cooled on ice and centrifuged at 18,000 x g for 1 min. Ten microliters of the resultant supernatant was subjected to PCR.

PCR amplification. Amplification of DNA was performed in a total volume of 50 µl. The reaction mixture consisted of 1 µM (each) the two oligonucleotides, 200 µM (each) four deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Toyobo, Tokyo, Japan), 1 x reaction buffer (Toyobo), and sample DNA. The reaction mixture was covered with a drop of mineral oil and was subjected to 25 PCR cycles of 1 min at 92°C, 1 min at 55°C, and 2 min at 72°C in a thermal programmer (model B-641; Kurabo, Osaka, Japan). Ten microliters of the amplification product was separated on a 0.7% agarose gel, and the DNA fragment was visualized by UV fluorescence after staining with ethidium bromide.

Southern blot hybridization. A 1.5-kb HindIII-HindIII fragment and a 0.4-kb HindIII-EcoRI fragment containing a part of the tox4 gene were used as probes to detect the PCR-amplified products. The former fragment was used to detect the products with the combination of primers 1 and 2 and primers 1 and 4, and the latter fragment was used to detect the products with primers 3 and 4. The relevant fragments were excised from pPMF3.5 (11) by enzyme digestion, purified by agarose gel electrophoresis, and labeled with biotin-14-dATP by nick translation (Bionick labeling system; GIBCO BRL, Gaithersburg, Md.).

PCR-amplified DNA was electrophoresed through a 0.7% agarose gel and was transferred to a nitrocellulose filter with a vacuum transfer apparatus (model BC-600; Biocraft, Tokyo, Japan). The blots were hybridized in 50% formamide-5 x SSC (1 x SSC is 0.15 M NaCl and 0.015 M sodium citrate [pH 7.0])-5 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone)-0.1% sodium dodecyl sulfate (SDS)-100 µg of sheared salmon sperm DNA per ml for 14 h at 37°C. Then, the filters were washed twice with 2 x SSC-0.1% SDS for 15 min at room temperature and were finally washed with 0.1 x SSC-0.1% SDS for 30 min at 56°C. The hybridized probe was detected with avidin-conjugated alkaline phosphatase, with 4-methoxy-4-(3-phosphatephenyl) spir (1,2-dioxetane-3,2'- adamantane) used as the substrate (Photo Gene Detection kit; GIBCO BRL). The molecular size markers were bacteriophage λ DNA digested with HindIII plus pBR322 DNA digested with PstI fragments of the following sizes: 23,130, 9,416, 4,361, 2,485, 2,322, 2,027, 1,465, 1,050, 921, and 564 bp.

For detection of the molecular size markers on the blots, the molecular size markers were labeled with biotin-14-dATP by nick translation.

Guinea pig skin test. The Guinea pig skin test was performed by a modification of the method described by Rutter (20). Colonies of test bacteria grown overnight on agar plates were suspended in phosphate-buffered saline (PBS). The suspension was diluted with PBS to a concentration of approximately 10¹⁰ CFU/ml and was then frozen and thawed seven times. The sample was centrifuged at 18,000 x g for 5 min, and the resultant supernatant was filtered through a 0.2-µm-pore-size membrane filter. Two-tenths of a milliliter of the filtrate was injected intradermally into guinea pigs (Japan SLC, Hamamatsu, Japan). The swelling and dermonecrosis of the injection sites were recorded after 24 and 48 h. When the diameter of the affected area reached 5 mm or greater, the test was regarded as positive. When only reddening appeared at the injection site, the test was regarded as doubtful.

Western blot analysis. P. multocida cells were harvested from 0.5 ml of a 14-h broth culture by centrifugation at 18,000 x g for 1 min, resuspended in 0.1 ml of 1 x SDS gel-loading buffer (2 x buffer is 100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), and incubated at 100°C for 3 min. Ten microliters of the sample was loaded onto each lane, and the sample was electrophoresed in a 5.4% stacking gel and a 10% separating gel and was electrophoretically (model BE-310; Biocraft, Tokyo, Japan) transferred to a nitrocellulose filter. The toxin protein was probed with rabbit anti-toxin serum and was visualized with peroxidase-labeled protein A and a substrate solution containing 4-chloro-1-naphthol and hydrogen peroxide.

Restriction fragment analysis. Each of the PCR-amplified products was digested with the restriction enzymes AccI, AluI, and Rsal. The resulting DNA fragments were separated on a 6% polyacrylamide gel and visualized by UV fluorescence after being stained with ethidium bromide. The molecular size markers were pBR322 digested with HapII fragments of the following sizes: 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9 bp.

DNA sequencing and analysis. The sequences of both DNA strands were determined by the dyeoxy chain-termination method (25) with a nonradioisotopic DNA sequencing kit (Sequencing high; Toyobo, Osaka, Japan) as described previously (15). DNA sequence data were analyzed by Genetics (Software Development, Tokyo, Japan).

RESULTS

Sensitivity and specificity of PCR amplification. We focused on a 1.5-kb HindIII-HindIII region (Fig. 1A and B) in the tox4 gene for target sequences of PCR, because (i) this fragment has been shown to hybridize specifically with toxigenic P. multocida strains (11) and (ii) major immunological determinants seem to be encoded in this fragment (18). We then selected four oligonucleotides located around the 1.5-kb HindIII-HindIII fragment of the tox4 gene (Fig. 1A and B) and performed PCR with the following three combinations of the four primers: primers 1 and 4, primers 1 and 2, and primers 3 and 4.

The sensitivities of the PCR assay with the three combinations of primers were determined with chromosomal DNA from a toxigenic P. multocida strain, NCTC 12178, ranging in amount from 100 ng to 1 µg. An amplified fragment of 1.2 kb with primers 1 and 4 (expected size, 1,230 bp; Fig. 2A and B), a fragment of 0.3 kb with primers 1 and 2 (expected size, 300...
FIG. 1. Structure and nucleotide sequence of the PCR-amplified region of the toxA gene. (A) Schematic presentation of the restriction map of the toxA gene and the positions of the primer sets. The numbered arrowheads represent the primers. E, EcoRI; H, HindIII; X, XbaI. (B) Nucleotide and amino acid sequences are a part of the toxA gene between the first HindIII site from the 5'-terminal end and the EcoRI site at the 3'-proximal end. This region contains all of the primers and the target sequences for PCR amplification. The first and second rows show the nucleotide and amino acid sequences, respectively, of the toxA gene from a serotype D strain of P. multocida, as published by Buya et al. (1). Roughly the same sequence was shown by Petersen (17); Petersen had two different nucleotides at positions 2323 and 2324, which are shown by asterisks. The shaded nucleotide sequences indicate the positions of the primers. The underlined nucleotide sequence shows the sequenced region of a serotype A strain. The sites for HindIII and EcoRI are shown above the first row. The numbers above the first row indicate nucleotide positions from the first nucleotide of a coding frame of the gene.
bp; Fig. 2C and D), and a fragment of 0.3 kb with primers 3 and 4 (expected size, 270 bp) were detected from 10 pg of chromosomal DNA. These products were hybridized with toxA probes under stringent conditions. They specifically hybridized with the toxA probe (Fig. 2B and D), indicating the specificity of the amplification. For further confirmation, we sequenced about 300 nucleotides from both the 5' and the 3' ends of the amplified products. The sequencing demonstrated that they contained toxA-specific sequences.

A number of pathogenic and nonpathogenic organisms isolated from pigs were tested with each of the three combinations of the oligonucleotide primers. A. equuli, A. suis, A. pleuropneumoniae, Aeromonas sp., B. bronchiseptica, Enterobacter sp., E. coli, H. parasuis, P. aeruginosa, B. subtilis, E. rhusiopathiae, S. aureus, S. epidermidis, S. suis, and M. hypophun- moniae were all negative by PCR with any of the combinations of these primers. These results indicate that any combination of the primers can be used in the PCR for detecting toxigenic P. multocida strains. Therefore, we chose the combination of primers 1 and 4 because the amplified product with this combination was clearly detected by agarose gel electrophoresis and was of a suitable size for the subsequent restriction fragment analysis.

**Relationship of PCR with other in vivo and in vitro tests for detection of toxin-producing strains.** To see the relationship between the PCR and other tests, 187 field isolates of P. multocida subsp. multocida were examined by the guinea pig skin test, Western blot analysis, and PCR. As shown in Table 1, the results of the three tests were in agreement except for two samples that showed a doubtful reaction by the guinea pig skin test. These two samples were negative by PCR and Western blot analysis and were judged to be nontoxigenic P. multocida. Final results showed that 67 of 187 (35.8%) isolates were interpreted to be toxigenic and the remaining 120 (64.2%) were nontoxigenic by the three tests. Among these 187 isolates 114 (60.9%) strains were capsular serotype A and 73 (39.1%) strains were capsular serotype D. Twenty-five of 114 (21.9%) serotype A isolates and 42 of 73 (57.5%) serotype D isolates were characterized as toxigenic. These results indicate a strong relationship between the PCR for detecting the toxin gene and the other in vivo and in vitro tests that detect the toxin protein.

**Sequence similarity of the PCR-amplified region of the toxin gene among the field isolates.** The PCR-amplified region of the toxin gene seems to contain important immunogenic determinants of the toxin (18). To determine whether there were sequence diversities in this region of the toxin gene between capsular serotypes A and D or among isolates from pigs of
different herds, the DNA segments amplified with primers 1 and 4 from the 67 toxigenic isolates were digested with restriction enzymes AccI, AluI, and RsaI. The restriction patterns were identical, irrespective of the capsular serotypes or the origins of isolates, with any of the restriction enzymes used. For example, the AluI restriction patterns of 17 toxigenic P. multocida strains are shown in Fig. 3.

For closer investigation, the amplified DNA fragment from a strain of capsular serotype A was sequenced. The DNA sequence was identical to that of a serotype D strain described by Buyts et al. (1), and only two nucleotides (at positions 2323 and 2324; Fig. 1B) were different from the sequence published by Petersen (17). This indicates that the PCR-amplified region of the toxA gene is very similar between strains of the two capsular serotypes.

DISCUSSION

Toxigenic P. multocida is recognized as a causative agent of PAR in pigs. However, in Japan, the etiologic differentiation of PAR from nonprogressive AR caused by B. bronchiseptica has not been well accepted. This is due to the inability to distinguish the two diseases by clinical and pathomorphologic observations. Sawata et al. (26) indicated that toxigenic P. multocida is not a primary causative agent of AR in Japan on the basis of field surveys of samples tested by the guinea pig skin test. In contrast, Sakano et al. (24) reported an outbreak of PAR in the Chichibu district of Japan and described the etiologic importance of toxigenic P. multocida. To clarify the prevalence and etiologic significance of toxigenic P. multocida in Japan, a large-scale field survey of the distribution of the organism is needed. The PCR assay described here was developed to address these circumstances.

The advantages of the PCR compared with existing tests include: (i) high sensitivity and specificity, (ii) simplicity of the procedure without the need for cell culture, laboratory animals, or radioisotope facilities, (iii) rapidity of the test, enabling results to be obtained in 4 h, (iv) capacity to handle over 100 samples per day (a thermal cycler for a 96-well microtiter plate is now available), and (v) safety as a result of the avoidance of handling live bacteria (only DNA is handled). Most of these advantages of PCR were based on the detection of a specific DNA sequence that is highly stable and specific.

In comparison with the assay of DNA by the colony hybridization test, the PCR assay has several advantages as a diagnostic tool. PCR offers results faster, is easier to perform, and does not require radioisotopes, a major obstacle in using the hybridization test in diagnostic laboratories. Furthermore, the sensitivity of the PCR assay is about 1,000 times greater than that of the colony hybridization test with a nonradioisotope system (13a).

Another notable advantage of the PCR is that the toxin gene from isolates can be further analyzed by restriction fragment analysis or more completely by DNA sequencing. In the present study, we analyzed the amplified fragment, which seems to encode the major immunogenic determinants of the toxin, from 67 toxigenic P. multocida isolates. Restriction fragment analysis of the PCR-amplified fragment from isolates and the DNA sequence comparison of capsular serotype A and D strains suggest that the sequence of this region would be the same irrespective of the origins of the isolates or their capsular serotypes. This also suggests that the antigenicity of the toxin produced by P. multocida would be very similar. This interpretation is in agreement with previous reports describing the similar antigenicities of the toxin among P. multocida strains (8, 19). Recent research also showed that the purified toxin from serotype A strains as well as the toxin from serotype D strains can induce atrophy of the nasal turbinate bones in pigs (13a).

In the present study, PCR was applied to bacterial colonies on the assumption that the PCR may be applicable for the detection of primary isolates in clinical material grown on selective agar medium. If the PCR could be used directly on nasal or tonsillar swabs of infected animals, it would provide even more rapid results and convenience. In a subsequent study, we are applying this PCR assay for the detection of toxigenic P. multocida strains directly from nasal and tonsillar swabs of experimentally and naturally infected pigs. Preliminary results show that improved detection rates are achieved by direct PCR assay of broth cultures of nasal and tonsillar
swabs. On the basis of the fundamental data presented here, further investigation is needed to evaluate the applicability of the PCR assay for the diagnosis of PAR and for etiologic surveys.

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REFERENCES


FIG. 3. Restriction fragment analysis of the PCR-amplified toxA fragment of P. multocida isolates. Lanes: 1 and 19, molecular size markers; 2, a toxigenic P. multocida strain, NCTC 12179; 3 to 10, toxigenic capsular serotype D isolates from pigs in herds JK, KG, KH, DS, SH, HK, HM, and HH, respectively; 11 to 18, toxigenic capsular serotype A isolates from pigs in herds JY, KC, KT, MN, MY, MM, MS, and MU, respectively. Numbers on the left indicate sizes (in base pairs).