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Specificity of DNA Probes for the Detection of Toxigenic *Pasteurella multocida* subsp. *multocida* Strains

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Five DNA probes directed against different regions of the gene that encodes the dermonecrotic toxin of *Pasteurella multocida* subsp. *multocida* were examined for their ability to identify toxigenic *P. multocida* subsp. *multocida* strains. The specificities of the probes were studied with 96 strains of *P. multocida* subsp. *multocida* and 22 strains of 11 other bacterial species. Results of colony hybridization assays using these probes indicated that two of the five probes have potential diagnostic value.

Atrophic rhinitis, a major respiratory disease in pigs, is characterized by sneezing, nose bleeding, shortening or twisting of the snout, atrophy of the nasal turbinate bones, and, in severe cases, impaired growth. *Pasteurella multocida* subsp. *multocida* strains that produce a dermonecrotic toxin (DNT), which is also called osteolytic toxin, are associated with the severe progressive form of the disease and are therefore considered to be the major etiologic agents. *P. multocida* subsp. *multocida* strains that do not produce the toxin do not cause atrophic rhinitis (4, 10, 13; M. F. de Jong, H. L. Oei, and G. J. Tetenburg, Proc. Congr. Int. Pig Vet. Soc., p. 211, 1980).

P. multocida subsp. *multocida* infection in pigs can be diagnosed by bacteriological examination of nasal or tonsillar swabs (L. A. M. G. van Leengoed and E. M. Kamp, Proc. 4th Int. Symp. Vet. Lab. Diagn., p. 922, 1986). Several tests are used to distinguish toxigenic from nontoxigenic *P. multocida* subsp. *multocida* strains: a mouse lethality test, a guinea pig skin test, cell culture assays, and enzyme-linked immunosorbent assays (2, 4a, 12; de Jong et al., Congr. Int. Pig Vet. Soc.). The discriminative properties of these tests all depend on the detection of the DNT protein or its activity. DNA probes that detect the gene for DNT may offer a new approach for the identification of toxigenic *P. multocida* subsp. *multocida* strains.

The gene encoding the DNT of *P. multocida* subsp. *multocida* was recently isolated (5, 7, 11), and the name *toxA* has been proposed (11). Hybridization studies using a few *P. multocida* subsp. *multocida* strains suggested that nontoxigenic strains do not possess the *toxA* gene (5, 11). We continued and extended these studies, examining the specificities of five DNA probes, each of which represents a different region of the *toxA* gene.

Nucleotide sequence analysis enabled us to map the *toxA* gene (1). A recombinant lambda phage (Fig. 1A) that contained the whole gene was used to generate several subclones. The insert DNA was digested with *Eco*RI-*Xba*I, *Xba*I, or *Xba*I-*Sac*I. Three DNA fragments that covered the *toxA* gene and about 1.8 kilobases of flanking sequences were isolated by agarose gel electrophoresis and electroelution (8). Subsequently, the fragments were subcloned into pKUN19 by standard recombinant DNA techniques (6, 8). From the generated subclones pPmE3.1, pPmF3.5, and pPmH4.20 (Fig. 1B), five different DNA probes were prepared (Fig. 1C). Probe 1, an *Eco*RI-*Xba*I fragment of about 2,000 base pairs (bp), was isolated from subclone pPmH4.20; and probe 2, an *Xba*I fragment of 900 bp, was isolated from subclone pPmE3.1. Probe 3, a *Hin*dIII fragment of 1,502 bp; probe 4, a *Hin*dIII-*Eco*RI fragment of 440 bp; and probe 5, an *Eco*RI-*Sac*I fragment of 719 bp, were isolated from subclone pPmF3.5. Probe 1 covers the 5′ end of the gene, and probe 5 covers the 3′ end.

With these probes several bacterial strains were tested for *toxA*-related sequences by a colony blot hybridization procedure (3). Briefly, strains were grown on blood agar plates in the presence or absence of 0.1% NAD for 16 h at 37°C. Colonies were transferred onto GeneScreen Plus membranes (Du Pont), and the DNA was bound to the membranes according to the recommendations of the manufacturer. Membranes were prehybridized for 1 h in 50% formamide, 1% sodium dodecyl sulfate, 1 M NaCl, 10% dextran sulfate, and 100 μg of sheared heat-denatured salmon sperm DNA per ml. DNA fragments to be used as probes were labeled with [α-^{32}P]dATP (3,000 Ci/mmol) by nick translation (Boehringer GmbH). The specific activity of the probes was approximately 10^8 dpm/μg of DNA. One nanogram of the heat-denatured DNA probe was added to 5 ml of prehybridization solution. After overnight hybridization at 42°C, the membranes were washed extensively in 2× SSC and 1% sodium dodecyl sulfate at 65°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). Finally, the membranes were washed twice under stringent conditions in 0.1× SSC–1% sodium dodecyl sulfate at 65°C for 30 min. For autoradiography, the membranes were exposed to X-ray film (X-Omat AR; Eastman Kodak Co.). Colony blots were hybridized with ^{32}P-labeled chromosomal DNA of *P. multocida* subsp. *multocida* to act as controls.

Except for two, the bacterial strains that we used (Table 1) were isolated in The Netherlands from nasal or tonsillar swabs taken from pigs of various herds. The two exceptions were atypical pasteurella isolates that were isolated from a cattle herd in which fattening bulls and heifers suffered from atrophic rhinitis. These isolates produced a toxin with properties similar to those of the DNT of *P. multocida* subsp. *multocida*. The atypical pasteurella isolates were related to the family *Pasteurellaceae*, but their precise taxonomic position remained unclear (4a). All strains were classified phenotypically by the methods described in Cowan and Steel's manual (1a). All *P. multocida* strains fermented sorbitol but not dulcitol and were therefore classified as *P.*

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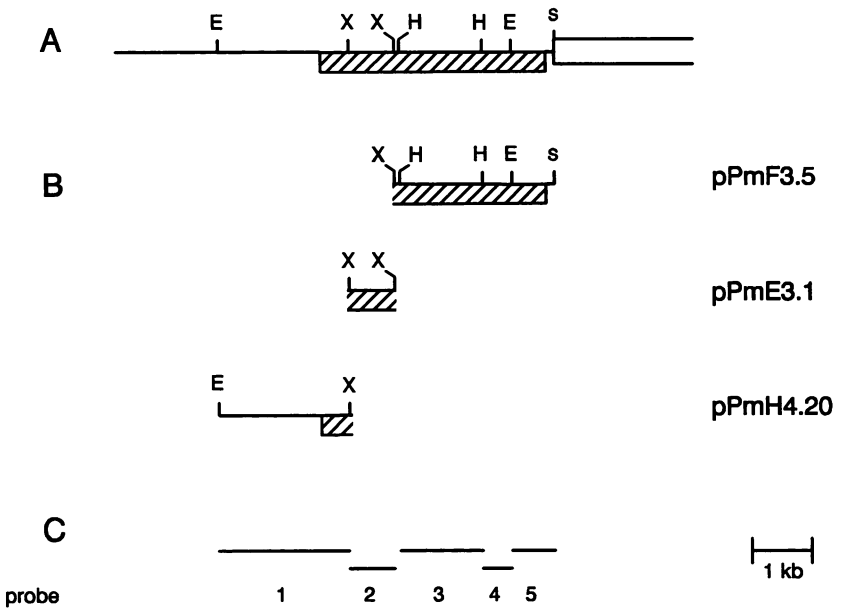

FIG. 1. Strategy for isolation of the five DNA probes. (A) Partial restriction endonuclease maps of the recombinant lambda phage; (B) insert DNA in subclones pPmE3.1, pPmF3.5, and pPmH4.20; (C) locations of the five probes, which together cover the *toxA* gene. Probe 1 covers the 5′ end of the gene; probes 2, 3, and 4 are internal gene fragments; and probe 5 covers the 3′ end of the gene. The solid line represents insert DNA. The open bar indicates vector DNA, and the hatched bar indicates the *toxA* gene. E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; S, *Sac*I.

multocida subsp. *multocida* (9). To distinguish between toxigenic and nontoxigenic strains, culture supernatants were examined for the presence of DNT in a double-antibody sandwich enzyme-linked immunosorbent assay (4a).

The strains were used to examine the specificities of the five DNA probes. As anticipated, all five probes hybridized with the 40 toxigenic *P. multocida* subsp. *multocida* strains and with the two atypical pasteurella isolates (Table 1). Colony hybridization results of probes 2 and 3 are shown in Fig. 2. Probes 1 and 3 seemed to be specific for the toxigenic pasteurella strains, because they did not hybridize with *Actinobacillus pleuropneumoniae*, *A. suis*, *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella typhimurium*, *Aerococcus viridans*, and *Streptococcus suis* type 2 (Table 1, Fig. 2D). Probe 2 hybridized not only with the toxigenic pasteurella strains but also with *E. coli*, *K. pneumoniae*, and *S. typhimurium* (Table 1, Fig. 2C). Probes 4 and 5 also hybridized with these strains, but the hybridization signals were much weaker (Table 1). The hybridization signals of probe 2 with *E. coli*, *K. pneumoniae*, and *S. typhimurium* were highly significant. The basis for this observation is, however, unknown since no significant homologies were found between the DNA sequence of the *toxA* gene and sequences in the EMBL/GenBank data bank (1).

TABLE 1. Hybridization of various bacterial strains with five DNA probes

Bacterium[a] (no. of strains tested)	No. of strains that hybridized with the following DNA probe:				
	1	2	3	4	5
Pasteurella multocida subsp. *multocida*					
Toxigenic (40)	40	40	40	40	40
Nontoxigenic (56)	2	0	1[b]	1[b]	1
Atypical pasteurella (2)	2	2	2	2	2
Actinobacillus pleuropneumoniae (3)	0	0	0	0	0
Actinobacillus suis (1)	0	0	0	0	0
Bordetella bronchiseptica (3)	0	0	0	0	0
Pseudomonas aeruginosa (2)	0	0	0	0	0
Escherichia coli (3)	0	3	0	3[b]	3[b]
Klebsiella pneumoniae (1)	0	1	0	1[b]	1[b]
Proteus vulgaris (1)	0	0	0	0	0
Salmonella typhimurium (2)	0	2	0	2[b]	2[b]
Aerococcus viridans (1)	0	0	0	0	0
Streptococcus suis type 2 (3)	0	0	0	0	0

[a] All bacteria were isolated from pigs, except the two atypical pasteurella strains, which were isolated from cattle and which produced a toxin similar to DNT (4a).

[b] Hybridized weakly.

Of the 56 nontoxigenic *P. multocida* subsp. *multocida* strains tested, 54 did not hybridize with the five probes (Table 1). Strain CVI 15749 hybridized only with probe 1; strain CVI 95723-18 hybridized with probes 1 and 5 and only weakly with probes 3 (Fig. 2B, arrow) and 4. Apparently, most of the nontoxigenic strains (54 of 56) do not possess the *toxA* gene, whereas a few have an incomplete or highly mutated gene. On the basis of these data, we concluded that the *toxA* gene is present in all toxigenic strains of *P. multocida* subsp. *multocida* and that it is absent in almost all nontoxigenic strains. In addition, we expect that when more than one probe is used, nontoxigenic strains with an incomplete *toxA* gene will be distinguished from toxigenic pasteurella strains. Because probes 1 and 3 hybridized specifically with toxigenic pasteurella strains and not with strains of the other bacterial species, these gene sequences have potential diagnostic value. Since probes 1 and 3 also recognized the atypical pasteurella isolates (Table 1, Fig. 2C and D), which produce only a minimum of toxin (4a), the probes

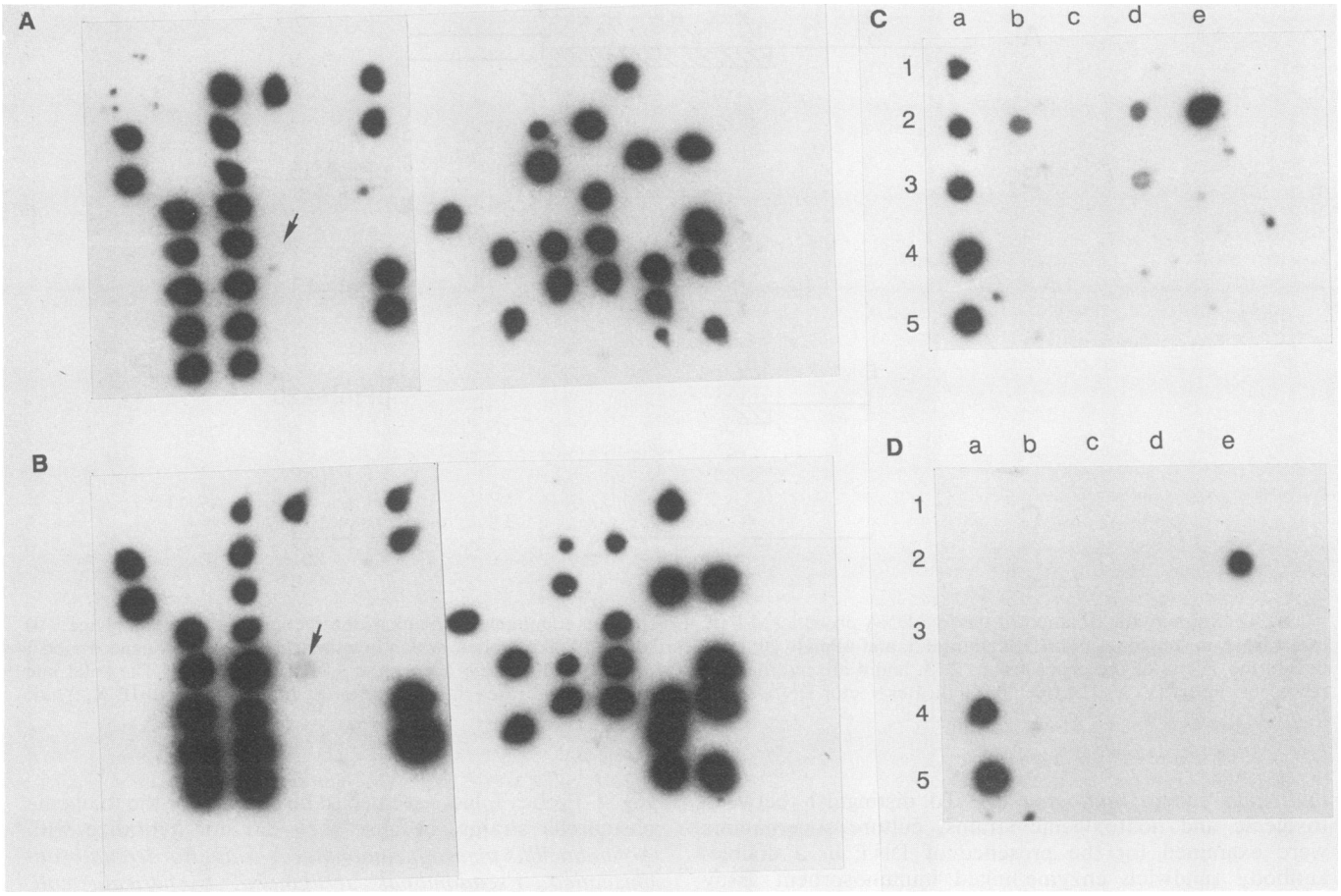

FIG. 2. Autoradiograms of colony hybridization experiments. (A and B) Identical blots of 96 *P. multocida* subsp. *multocida* strains. Note that the 40 toxigenic and 56 nontoxigenic *P. multocida* subsp. *multocida* strains are interspersed on each filter. (C and D) Identical blots of 24 strains of 12 different bacterial species: a1 to 3, *E. coli*; a4 to 5, atypical pasteurella isolates; b1, *A. suis*; b2, *K. pneumoniae*; b3 to 5, *A. pleuropneumoniae*; c1 to 3, *B. bronchiseptica*; c4, *P. aeruginosa*; c5, *A. viridans*; d1, *P. aeruginosa*; d2 to 3, *S. typhimurium*; d4 to 5 and e1, *S. suis* type 2; e2, toxigenic *P. multocida* subsp. *multocida* strain; e3, nontoxigenic *P. multocida* subsp. *multocida* strain; e4, *P. vulgaris*. Colony blots A and C were hybridized with probe 2, and colony blots B and D were hybridized with probe 3. The arrows in panels A and B indicate a colony of a nontoxigenic *P. multocida* subsp. *multocida* strain (CVI 95723-18) that hybridized weakly with probe 3 (B) but not with probe 2 (A).

seem to be even more important. In our current study, we are investigating the diagnostic value of probes 1 and 3 in terms of sensitivity and specificity with nasal and tonsillar swabs. In addition to using the colony hybridization assay, we are now exploring the polymerase chain reaction method with oligonucleotides in the regions of probes 1 and 3. If the polymerase chain reaction method can be used directly on nasal or tosillar swabs of infected animals, it may be even more rapid and sensitive than the colony hybridization assay (14).

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