BACTERIAL PATHOGENICITY

Characterisation of dermonecrotic toxin-producing strains of *Pasteurella multocida* subsp. *multocida* isolated from man and swine

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Thirty-six isolates, from man or swine, of *Pasteurella multocida* subsp. *multocida* producing ($n = 13$) or not producing ($n = 23$) the dermonecrotic toxin (DNT) were studied by numerical analysis, capsular typing and ribotyping. Toxigenic strains were also characterised by restriction fragment length polymorphism (RFLP) of the *tox*A gene and pulsed-field gel electrophoresis (PFGE). Numerical analysis differentiated the *Pasteurella* species and subspecies, but did not discriminate between toxigenic and non-toxigenic strains. RFLP demonstrated that *tox*A was located in a conserved part of the chromosome of all toxigenic strains. Ribotyping provided evidence of a close association between DNT production and one of the six *Eco*RI ribotypes designated as E2. In contrast, PFGE provided evidence for significant DNA polymorphism amongst the toxigenic strains. Results of phenotypic and genotypic studies suggested that toxigenic strains do not form a clone within the subspecies *multocida*. No difference was found between toxigenic strains of porcine or human origin by biochemical characterisation, capsular serotyping or genomic typing methods.

Introduction

The genus *Pasteurella* includes gram-negative bacteria that are commensal organisms of mucosal surfaces in a wide variety of birds and mammals. The most common species, *P. multocida*, is widespread in wild and domestic animals. DNA–DNA hybridisation studies led to the division of the *multocida* species into three subspecies designated *multocida*, *septica* and *gallicida* [1]. Most strains isolated from swine are *P. multocida* subsp. *multocida* [2] and some of them produce a dermonecrotic toxin (DNT) involved in the pathogenesis of atrophic rhinitis, an economically significant disease. Atrophic rhinitis is characterised by a haemorrhagic discharge, sneezing, torsion of the snout caused by the loss of turbinate bone and weight loss [3, 4]. DNT-producing strains are isolated mainly from swine, but also from dogs, cats and rabbits [5].

In two previous studies, it was suggested that colonisation or infection of the respiratory tract in man by toxigenic or non-toxigenic *P. multocida* subsp.

multocida strains could result from exposure to swine [6, 7]. In animals, DNT induces lesions in liver and kidney [3, 8, 9] and carriage of toxin-producing strains could be harmful to farmers, especially those suffering from chronic pulmonary disease. Human carriage could be of concern also in the control of atrophic rhinitis in swine. Characterisation of *Pasteurella* isolates from man has been done previously [10, 11], but these were not isolates cultured from people after exposure to swine. Recently, *P. multocida* genomic typing methods have been used successfully for epidemiological purposes; for example, to compare strains from wild and domestic animals [12], to study transmission of strains in swine herds [13] and to compare strains isolated from farmers [14]. The objective of the present work was to perform phenotypic and genotypic studies of toxigenic isolates and to investigate the possible transmission of DNT-producing strains from swine to people living in rural areas.

Materials and methods

Bacterial strains

The bacterial strains examined, including reference strains and clinical isolates, are listed in Table 1. A

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Table 1. Characteristics of the *Pasteurella* isolates investigated

Taxon (number of strains)	Source* and isolate designation	Host	Specimen	Capsular type	DNT production	EcoRI ribotype	HindIII ribotype	PFGE pattern
<i>P. multocida</i>	NCTC 10322 ^T	Pig		A	—	E1	H9	
subsp. <i>multocida</i> (36)	NCTC 12177	Pig		A	+	E2	H7	I
	NCTC 12178	Pig		D	+	E2	H8	II
	CIP 5684	Human	CSF	D	—	E1	H1	
	CIP 5724	Human	Wound	D	—	E5	H1	
	CIP 5730	Human	Sinus washing	D	+	E1	H8	III
	PMR 1	Human	Pleural effusion	D	—	E1	H6	
	PMR 9	Human	Sputum	A	—	E2	H10	
	PMR 15	Human	Dog bite	D	—	E3	H11	
	PMR 17	Human	Dog bite	D	—	E4	H8	
	PMR 19	Human	Dog bite	A	—	E4	H9	
	PMR 23	Human	Sputum	A	+	E1	H7	III
	PMR 29	Human	Animal bite	A	—	E6	H11	
	PMR 35	Human	Pleural effusion	A	—	E1	H8	
	PMR 40	Human	Bronchial aspiration	D	—	E1	H8	
	PMR 41	Human	Sputum	A	—	E1	H10	
	PMR 42	Human	Sputum	A	—	E1	H5	
	PMR 43	Human	Sputum	A	+	E2	H11	IV
	PMR 46	Human	Sputum	A	+	E2	H10	V
	PMR 51	Human	Sputum	D	—	E1	H5	
	PMR 78	Human	Cat bite	D	—	E1	H2	
	PMR 79	Human	Bronchial aspiration	A	+	E2	H11	VI
	PMR 85	Human	Pleural effusion	A	+	E1	H8	VII
	PMR 86	Human	Wound	D	—	E1	H12	
	PMR 87	Human	Cat bite	A	—	E1	H6	
	PMR 118	Human	Oropharynx	D	—	E1	H8	
	PMR 125	Human	Oropharynx	A	—	E1	H4	
	PMR 126	Human	Oropharynx	A	—	E1	H8	
	PMR 127	Human	Oropharynx	A	—	E1	H11	
	PMR 130	Human	Oropharynx	D	+	E2	H11	VIII
	SPP 3351	Pig		D	—	E1	H3	
	SPP 6705	Pig		D	—	E1	H5	
	SPP 7294	Pig		D	+	E2	H11	IX
	SPP 10814	Pig		D	+	E2	H2	X
	IEMVT D2	Pig		D	+	E2	H8	III
	IEMVT D706	Pig		D	+	E2	H8	XI
<i>P. multocida</i>	CIP A125 ^T	Human		ND	—	ND	ND	
subsp. <i>septica</i> (1)								
<i>P. multocida</i>	NCTC 10204 ^T	Bovine		ND	—	ND	ND	
subsp. <i>gallicida</i> (1)								
<i>P. canis</i> (1)	NCTC 11621 ^T	Dog		ND	—	ND	ND	
<i>P. dagmatis</i> (1)	NCTC 11617 ^T	Human		ND	—	ND	ND	
<i>P. stomatis</i> (1).	SSI P365	Human		ND	—	ND	ND	

ND, not determined.

*NCTC, National Collection of Type Cultures, London; CCIP, Collection de l'Institut Pasteur, Paris, France; PMR, Collection de *Pasteurella*, Laboratoire de Microbiologie, Faculté de Médecine, Rennes, France; SPP, Station de Pathologie Porcine, Ploufragan, France; IEMVT, CIRAD-EMVT, Maisons-Alfort, France; SSI, Statens Seruminstitut, Copenhagen, Denmark; ^T designates type strain.

stock culture of each isolate was made in Tryptose Broth (Difco) with glycerol 20% v/v and frozen at -80°C . For each experiment, stock cultures were streaked on Trypticase-soy Agar supplemented with horse blood (bioMérieux) 5% v/v. Cultures were incubated at 37°C for 18 h.

Enzyme-linked immunosorbent assay

Differentiation of toxigenic from non-toxigenic isolates was by an enzyme-linked immunosorbent assay (ELISA; Dakopatts) with two monoclonal antibodies, as described previously [15]. Results of the tests were expressed according to Foged *et al.* [16], as the relative absorbance, i.e., the ratio between the absorbance of the test strain and the absorbance of the toxigenic reference strain NCTC 12178, both measured at a wavelength of 492 nm. A relative absorbance of 0.40 was taken as a positive result.

Biochemical characterisation and numerical analysis

All strains were characterised by 78 biochemical tests with three identification kits – API 20E, API ZYM and API 50CH (bioMérieux). API ZYM strips were inoculated with bacterial suspension in distilled water at a concentration equivalent to 4 MacFarland units. API 20E and API 50CH strips were inoculated with bacterial suspension, at 3 MacFarland units, in M63 solution (pH 7.0) supplemented with Bacto Tryptone (Difco) 0.5% w/v and yeast extract 0.02% w/v as described previously (F. Escande, PhD thesis, 1985, Université Paris Sud). API ZYM, API 20E and API 50CH strips were incubated for 4, 48 and 96 h, respectively, at 37°C , and read according to the manufacturer's instructions. Data were recorded in binary code and a numerical analysis was performed with the Taxan program (University of Maryland).

Cluster analysis of the results was performed by the weighted-average-linkage method along with the coefficient of Jacard (S_j).

Serotyping

Capsular type was determined by a passive haemagglutination test with a hyperimmune rabbit serum according to the method of Carter [17].

Synthesis of digoxigenin-labelled probes

The complete sequence of *tox*A, the gene coding for the DNT, was obtained from the EMBL bank (access no. X52478) and analysed with the PCRare® program (Eurogentec) [18] to design two primers Pm1 and Pm2 (Table 2) which were used to amplify a 185-bp product corresponding to the 5' end of a *tox*A-specific probe described by Kamps *et al.* [19]. Polymerase chain reactions (PCR) and nested PCR were performed according to Griffais *et al.* [20]. Five toxigenic strains and five non-toxigenic strains were tested to assess amplification specificity. Incorporation of digoxigenin-labelled dUTP was then performed by nested PCR with an amplified product from the reference strain CIP 5730 and two 20-mer primers, Pm3 and Pm4, corresponding to the 3' flanking sequences of Pm1 and Pm2, respectively (Table 2).

A 1455-bp digoxigenin-labelled probe recognising the 16S rRNA gene was synthesised by a similar nested procedure, with the primers P0mod and PC5 described by Wilson *et al.* [21] (Table 2), 0.1 µg of *Escherichia coli* ATCC 25922 DNA as template, and primers I0 and IC5 (Table 2) internal to the two previous primers.

Slot-blot assay for *tox*A detection

DNA was extracted as described by Boom *et al.* [22], denatured by heating at 95°C, loaded into wells of a slot-blot apparatus (Hoefer Scientific Instruments) and blotted on to a Hybond-N membrane (Amersham). To assess the sensitivity of the probe, 10-fold dilutions of DNA from strain CIP 5730, from 1 µg to 100 pg, were slot-blotted and hybridised according to the manufacturer's recommendations (Boehringer Mannheim).

Ribotyping and *tox*A restriction polymorphism

DNA was extracted by a phenol-chloroform procedure, precipitated by ethanol, dried and resuspended in 10 mM Tris, 0.1 mM EDTA, pH 7.5. DNA solution was digested with *Eco*RI, *Hind*III and *Pvu*II (Boehringer Mannheim) for analysis of *tox*A polymorphism (toxigenic strains), or by *Eco*RI and *Hind*III for ribotyping. Restriction endonuclease digestions were performed for 3 h at 37°C and then DNA fragments were separated by gel electrophoresis in agarose 0.8% w/v, transferred to a Hybond-N nylon membrane (Amersham) and hybridised with DNA probes for 16S rRNA or *tox*A genes.

Pulsed-field gel electrophoresis of total DNA

Restriction endonuclease analysis by pulsed-field gel electrophoresis (PFGE) was performed after digestion of genomic DNA with the infrequently cleaving restriction endonuclease *Apa*I. High mol.-wt DNA embedded in agarose was prepared by the procedure of Allardet-Servent *et al.* [23]. Digests were separated by PFGE in agarose (Appligene) 1% w/v with a Gene Navigator System apparatus (Pharmacia Biotech). A gradient of pulse time of 8–40 s was applied for 40 h at 150 V.

Results

Identification of toxigenic strains

Six *P. multocida* subsp. *multocida* porcine isolates were toxigenic by ELISA: the two reference strains NCTC 12177 and NCTC 12178 and four clinical isolates IEMVT D2, IEMVT D706, SPP 7294 and SPP 10814. As reported previously [7], seven isolates from man were toxigenic: the reference strain CIP 5730 and six clinical isolates, PMR 23, PMR 43, PMR 46, PMR 79, PMR 85 and PMR 130 (Table 1). Twenty-three *P. multocida* subsp. *multocida* isolates and all strains belonging to other taxa, did not produce DNT; in the ELISA test all gave a relative absorbance of <0.10. Capsular types of toxigenic isolates from swine and man were D (five and two isolates, respectively), or A (one and five isolates, respectively) (Table 1).

After PCR with primers Pm1 and Pm2 specific for the

Table 2. Primers used in the synthesis of digoxigenin-labelled probes by PCR

Target gene	Primer	Nucleotide sequence
16S rRNA	P0mod*	AGAGTTTGATCATGG
	PC5*	TACCTTGTTACGACTT
	I0	CTCAGATTGAACGCT
	IC5	GTGGGGTCAGTACTT
<i>tox</i> A (<i>P. multocida</i>)	Pm1	TTTTTCACTGAAGAACAAGCTTTAG
	Pm2	TTTATTTCTCTCTAATCTTCGTAAC
	Pm3	CTCAACGCTTTGAAATAGTA
	Pm4	ACAACATAAGGTTGATTTTC

*From reference [21].

*tox*A gene and electrophoresis, a single band at the expected size of 185 bp was visualised only with the five toxigenic isolates tested. In the slot-blot assay with the *tox*A probe, a marked signal was detected with 10 ng of DNA from *P. multocida* strain CIP 5730, and the probe correctly identified the same 13 toxigenic isolates identified by ELISA. No false-positive result nor background signal was observed with the 23 non-toxigenic isolates.

Numerical analysis

Results of the numerical analysis with 78 biochemical tests are shown in Fig. 1. At a similarity level of 75%, all the *P. multocida* subsp. *multocida* isolates were members of a single cluster. At the similarity level of 80%, there were four distinct areas, A, B, C and D, in the dendrogram. These four areas contained only the 36 isolates of *P. multocida* subsp. *multocida*, distributed as follows: A, 25 isolates; B, 3 isolates; C, 2 isolates; D, 6 isolates. The dendrogram did not clearly reflect the origin of the isolates, but most porcine isolates (except the type strain NCTC 10322) were linked to each other at a similarity level of $\geq 91\%$, and were located in area A. The human isolates were randomly scattered about the four areas. All but one toxigenic isolate were located in the larger area A, and did not form a subphenon of the phenon assigned as *P. multocida* subsp. *multocida*.

Restriction polymorphism

After digestion of DNA by *Hind*III, *Eco*RI or *Pvu*II, the *tox*A probe hybridised with unique restriction fragments of 1.5, 5.2 and 26.0 kb, respectively, in all the toxigenic isolates.

With DNA digested by *Eco*RI, the 16S rRNA probe hybridised with 8–10 restriction fragments (Fig. 2). Six different ribotypes (E1–6) were observed in the 36 isolates, with E1 (20 isolates) and E2 (11 isolates) being the most common (Fig. 2 and Table 1). These two ribotypes differed by a single band of 7.2 kb which was present in E2, but not in E1 (Fig. 2). Ribotype E1 consisted of 3 toxigenic and 17 non-toxigenic isolates. Ribotype E2 consisted of 10 toxigenic and 1 non-toxigenic isolates. Ribotypes E3, E4, E5 and E6 each consisted of one or two non-toxigenic isolates. After *Hind*III restriction, four to eight fragments were visualised and 12 restriction patterns were obtained with the 36 isolates (Fig. 2). There was no difference in the distribution of ribotypes as far as toxin production was concerned: H8, the most common pattern, included five toxigenic and five non-toxigenic isolates.

After *Apa*I digestion and separation of DNA fragments by PFGE, 11 restriction patterns (designated with a roman number I–XI) were found among the 13 toxigenic isolates. The epidemiologically unrelated strains CIP5730, PMR 23 and IEMVT D2 had the same pattern III (Table 1).

Discussion

Cases of pasteurellosis in man are usually categorised as related or unrelated to animal bites or scratches, and most infections unrelated to animal bites are respiratory tract infections. *Pasteurella* spp. are not common inhabitants of the upper respiratory tract of man, but healthy carriage is frequent in pig farmers [6] and toxigenic strains can be isolated from the respiratory tracts of people living or working near swine herds. In contrast, toxigenic strains are rarely if ever isolated from bite infections [7, 24] or bacteraemias (F. Escande, unpublished observations). Toxigenic isolates from swine have been characterised by outer-membrane proteins or lipopolysaccharide electrophoretic patterns [25, 26], and several studies have investigated the specificity of *P. multocida* for different species of animals [2, 11]. However, toxigenic strains from man and swine have not been compared.

In numerical analysis, a similarity level of 75% has valid taxonomic significance for the S_J coefficient and has been suggested as a good criterion for phenetic species [27]. Cutting the dendrogram at the 75% level allowed separation of the different taxa belonging to the genus *Pasteurella*, including the three subspecies of *P. multocida*. Numerical analysis of *Pasteurella* isolates from human infections has been reported previously by Talbot and Sneath [11], with delineation of a homogenous group of *P. multocida* isolates which included isolates from cat bites and 'internal' isolates (i.e., from infections not involving direct inoculation). In the present analysis, most porcine isolates belonged to the same group. In contrast, human isolates were heterogeneous; eight of them were related to the porcine group in the dendrogram at a similarity level of $\geq 91\%$ (Fig. 1). This possibly reflects the fact that man is susceptible to acquisition of infection from several animal species. Moreover, the dendrogram clearly demonstrated that the toxigenic isolates did not form a subphenon of the phenon *P. multocida* subsp. *multocida* and, consequently, biochemical tests could not be used for their identification.

Like biochemical tests, capsular serotyping failed to identify toxigenic isolates: porcine and human isolates were D or A type, which are common types in all animal species. Toxigenic strains of capsular types A or D both occur in swine, but type D strains are more frequently associated with atrophic rhinitis [3]. Type A capsule contains large amounts of hyaluronic acid which confers a 'watery' mucoid phenotype to colonies, as noted previously for isolates from the respiratory tract [28, 29], and confers a greater ability to colonise mucosal surfaces and to resist phagocytosis by polymorphonuclear leucocytes [30].

DNA probes for *tox*A [19] and, more recently, PCR [31] have been described as useful tools for diagnostic purposes. The 135-bp DNA probe specific for *tox*A

Fig. 1. Dendrogram showing relationships among *Pasteurella* spp. isolates based on the S_j coefficient and the weighted-average-linkage analysis.

Fig. 2. Schematic representation and distribution of *Eco*RI (E1–6) and *Hind*III (H1–12) ribotypes among 36 isolates of *P. multocida* subsp. *multocida*.

was tested here in a slot-blot hybridisation assay and compared with a commercially available ELISA. The results were in complete agreement and the slot-blot assays gave a positive reaction with 10 ng of DNA of a toxigenic reference strain. As reported in previous studies, only the DNT-producing isolates possessed the *toxA* gene [19].

Results of digestion by *Hind*III were in agreement with a previous report [19]: two sites were present in the *toxA* gene and, after restriction, the 135-bp *toxA* probe hybridised with a 1.5-kb fragment in all toxigenic strains. Polymorphism of the flanking sequences next to the gene was explored by *Eco*RI (one restriction site in *toxA*) and *Pvu*II (no restriction site in *toxA*). No polymorphism was found with the *toxA* probe: after digestion by either *Eco*RI or *Pvu*II, a single pattern was observed with all toxigenic isolates. It has been reported that after *Sau*IIIa restriction and hybridisation with a *toxA* probe, toxigenic *P. multocida* subsp. *multocida* strains exhibited the same banding pattern [32] and that *toxA* was located on a conserved part of the genome [33]. Flanking sequences of *toxA* have homology with 22 bacteriophages isolated from *P. multocida*, and induction of toxigenic strains by mitomycin C leads to liberation of DNA fragments which hybridise with a *toxA* probe [33]. Consequently, some authors have proposed an exogenous origin of *toxA* [34]. The ribotype E2 differs from the most common ribotype E1 by only one 7.2-kb fragment, and is closely

associated to toxin production. We suggest that the ribotype E2 could be the result of a phage conversion of ribotype E1 strains, by insertion of exogenous, highly conserved DNA into the chromosome. In contrast with *Eco*RI ribotyping, PFGE results provided evidence for significant DNA polymorphism of the toxigenic isolates: 10 *Apa*I macrorestriction patterns were found with the 13 isolates. Analysis of PFGE patterns of *P. multocida* serotype B has been useful to distinguish avirulent strains and virulent strains that cause haemorrhagic septicaemia in cattle, with homogeneity among the Asian haemorrhagic septicaemia strains [35]. The results of the present study do not support the hypothesis that DNT-producing strains form a clone within the subspecies *P. multocida* subsp. *multocida*. Moreover, phenetic characters and genomic typing methods do not discriminate between toxigenic isolates of porcine or human origin and may suggest colonisation of people from a porcine reservoir.

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