Dermonecrotic toxin production by strains of *Pasteurella multocida* isolated from man

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Summary. Ninety-four clinical isolates of *Pasteurella multocida* of human origin were tested for dermonecrotic toxin (DNT) production by three methods: dermonecrotic test in guinea-pigs, Vero cell culture cytotoxicity and ELISA. The strains were isolated from patients living in a rural area with widespread intensive pig breeding. Six strains were found to be toxigenic by the three tests. A major protein band of M_r 145 Kda corresponding to DNT on immunoblots was demonstrated in extracts from these strains. All were isolated from respiratory tract (diseases 5, healthy carriage 1). The difference between isolates from the respiratory tract and isolates from wounds inflicted by pets was statistically significant with regard to DNT production ($p < 0.02$). A possible role of the toxin in pulmonary diseases caused by *P. multocida* has yet to be established.

Introduction

Pasteurella multocida is a common commensal of the oropharyngeal flora of domestic animals including pets. Clinical manifestations due to this micro-organism in man are mainly infections secondary to bites or scratches, and pulmonary infections.¹

There are a few reports of virulence factors produced by this species. Its attachment to animal cell membranes involves fimbriae² and a mannose-sensitive haemagglutinin has been described in strains of porcine origin.³ Some strains possess a capsule with antiphagocytic properties⁴ and recently such activity has been attributed to an outer-membrane protein in strains of avian origin.⁵ Siderophore production has also been demonstrated⁶ and high neuraminidase production seems to correlate with higher virulence of strains,⁷ but the best known virulence factor in animal pasteurellosis is the dermonecrotic toxin (DNT) which causes atrophic rhinitis in pigs.⁸ The activity of this toxin has been demonstrated experimentally in different species including pigs, mice and guinea-pigs. Its production occurs in strains from several hosts—pigs, poultry, calves, dogs and cats.⁹

The purpose of this study was to determine the incidence of DNT producing strains among isolates of *P. multocida* from man, particularly from a rural population associated with swine. We examined the capacity of 41 strains isolated from the respiratory tracts of people living in a rural area to produce this toxin. These strains were compared with 53 isolates from wounds inflicted by dogs or cats to determine

whether isolates from the respiratory tract are distinct with regard to DNT production.

Materials and methods

Bacterial strains

Ninety-four strains of *P. multocida* were isolated from patients in two French hospitals between 1983 and 1989. All were matched by standard methods of identification to the subsp. *multocida*.¹⁰ Forty-one isolates were from the respiratory tract; 20 of these were from sputum or bronchial aspirates from patients living in two rural districts in Brittany and suffering from superinfection of chronic bronchitis or acute pulmonary infection, and 21 were oropharyngeal isolates from healthy people working in piggeries located in one of the two districts.¹¹ Other strains ($n = 53$) were isolated from wounds inflicted by pets. Of the respiratory isolates, 33 were from patients closely associated with pig farming; of the eight non-pig-associated respiratory isolates, three were from cattle breeders and five from patients not living in rural communities. Association with pigs was not determined from wound isolates. All isolates were collected randomly during 1983–1989 and no effort was made to select isolates from pig farmers, except for the 21 oropharyngeal strains from breeders.¹¹

Determination of A and D capsular type was by two non-immunological methods.^{12,13}

Six culture-collection strains were used in all tests: *P. multocida* strains NCTC 12177 and 12178, CIP 5730 and D 706 as positive controls and *P. multocida*

strains CIP 5684 and CIP 5724 as negative controls. The two strains from the National Collection of Type Cultures, London (NCTC) were previously reported to be toxigenic.¹⁴ The three strains from the Collection de l'Institut Pasteur, Paris (CIP) were previously tested by two methods and one of them (CIP 5730) was toxigenic (N. Foged, personal communication). *P. multocida* D 706, a toxigenic strain of porcine origin, was kindly provided by A. Breard, IEMVT, Maisons-Alfort, France.

Bacterial extracts

For dermonecrotic and cytotoxicity tests and immunoblotting, each strain was cultured overnight on blood 5% agar at 37°C. Growth was harvested with swabs, suspended in distilled water and sonicated for 5 min with a Vibracell apparatus (Bioblock, Illkirch, France). After centrifugation for 20 min at 10 000 *g*, supernates were filtered successively through 0.45- μ m and 0.22- μ m membrane filters (Millipore).

For ELISA, bacterial suspensions in distilled water were further incubated overnight at 37°C then centrifuged and filtered through a 0.45- μ m filter and the filtrate was retained.

Dermonecrotic test

Dermonecrotic activity was investigated by injecting intracutaneously 0.1 ml of the extract of each strain into a guinea-pig weighing *c* 200 *g*. Each animal was carefully depilated on both sides before injection. The reaction was observed 48 h after injection and a dermonecrotic lesion > 5 mm in diameter was read as a positive result.

Cell cultures

Vero cell monolayers were grown in 96-well tissue-culture plates with flat bottoms (Falcon, Grenoble, France) in Eagle's Minimum Essential Medium containing fetal calf serum 10%. The plates were inoculated with 50 000 cells in each well. Monolayers were formed within 24–48 h at 37°C in a humidified atmosphere of CO₂ 5% in air.

Cytotoxic test

Each sonicated extract (15 μ l) was diluted 1 in 10 in Eagle's medium with fetal calf serum 2% and 100 μ l of each preparation was added to the cell culture. Cells were examined for cytotoxicity after incubation at 37°C for 48 h by observation with an inverted microscope. When a positive result was obtained, titration and neutralisation were performed with, respectively, two-fold and 10-fold dilutions of each preparation. Titres were expressed as the highest dilution producing alteration in 50% of the cells. The neutralisation assay was performed as follows: 100 μ l of a positive extract diluted 1 in 10 was mixed with

10 μ l of anti-dermonecrotic monoclonal antibody (anti-DNT MAb) P3F51 (Dakopatts Laboratories, Glostrup, Denmark); the mixture was incubated for 30 min at 20°C and tested on Vero cell monolayers.

ELISA

The method of Foged *et al.*¹⁴ was used with 96-well microtitration plates (Falcon) coated with anti-DNT MAb P3F51. After incubation of each bacterial extract, including positive and negative controls, the plates were rinsed twice with phosphate buffered saline, pH 7.0 containing Tween 20 0.05% and bovine serum albumin 1% (PBS-T-SA). Biotinylated anti-DNT MAb P3F37 (Dakopatts Laboratories) was then added. Biotin was recognised by peroxidase-conjugated avidin (Sigma). The colour reaction was developed with *o*-phenylenediamine (Abbot Laboratories, Rungis, France) in citrate-phosphate buffer, pH 5.0. Between the two steps of the procedure, plates were rinsed carefully three times with PBS-T-SA. The colour reaction was finally blocked by 1M H₂SO₄. Measurement of absorbance (*A*) was assessed at 492 nm for each extract with a Titertek Multiscan photometer (Flow Laboratories, Puteaux, France), and relative absorbance *A*/*A*₀ was calculated with *A*₀ = *P. multocida* NCTC 12178 extract absorbance.

Immunoblotting

The proteins in the bacterial extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with polyacrylamide 8% gels.¹⁵ Electrophoresis was performed in a vertical electrophoresis unit model 2001 (LKB, Saint-Quentin-en-Yvelines, France) at 60 mA for 15 min and then at a constant current of 100 mA until migration was complete (about 3 h). Blotting was done as described by Towbin *et al.*¹⁶ Transfer took 2 h at 0.5 mA and 4°C in a model 2005 Transphor LKB unit. A lane with mol. wt markers (Rainbow PMW Markers, Amersham, Les Ulis, France) was cut and the nitrocellulose sheet was then blocked with PBS-T-SA for 16 h at 4°C. Lanes corresponding to each extract were cut and incubated with anti-DNT MAb P3F51 diluted 1 in 100 with PBS-T-SA. After three washes with PBS-T-SA the strips were incubated with peroxidase-labelled rabbit anti-mouse immunoglobulin (Diagnostics Pasteur, Marnes-la-Coquette, France). After three washes with PBS-Tween 20 0.05%, 3,3'-diaminobenzidine tetrahydrochloride (Prolabo, Paris, France) 0.5 mg/ml was added and the reaction was stopped with acetic acid 0.5% solution as soon as the bands became visible.

Statistical analysis

Statistical analysis of the data was done with a corrected χ^2 test.

Results

Dermonecrotic test

Among the 94 clinical isolates of *P. multocida*, necrotic lesions were produced in guinea-pig skin by extracts from only six strains. All the positive controls produced similar necrotic reactions but none of the negative controls did so. The origins of the strains, capsular type and circumstances of their isolation are shown in table I. The patients who were colonised or infected by these strains all lived in a rural environment.

Cytotoxic test

A characteristic alteration of the cellular layer was observed only with extracts of clinical isolates and control strains that gave positive reactions in the dermonecrotic test. After incubation for 48 h the cell layer had shrunk with a clustering of rounded and refringent but always adherent cells. Titres of cytotoxic activity are shown in table I. For all positive strains, the activity of 100 μ l of extract diluted 1 in 10 was neutralised by 10 μ l of anti-DNT MAb P3F51.

ELISA

A and A/Ao values are shown in table II. Extracts from the toxigenic clinical isolates had a relative absorbance (A/Ao) of $\geq 75\%$, whereas extracts from non-toxigenic strains showed an A/Ao value $\leq 12\%$.

Immunoblotting

Immunoblotting of the toxigenic strains disclosed a major band with an apparent M_r of 145 Kda and several minor bands (figure). Similar profiles were obtained with the extracts of the three positive controls, including porcine strain D 706, and of the six toxigenic clinical isolates, but no band was observed with extracts from the two negative controls and the 88 non-dermonecrotic and non-cytotoxic strains.

Statistical analysis

No isolate from bite or scratch infections was toxigenic. There was a statistically significant difference between the results obtained with strains isolated from the respiratory tract and those isolated from wounds ($p < 0.02$).

Discussion

It has been known for some years that certain strains of *P. multocida* produce a toxin. The clinical relevance of toxin production has been assessed in pigs, in which it provokes atrophic rhinitis.⁸ The toxin is an intracellular protein of 143–160 Kda according to different authors^{17–19} and it can be detected by several methods—guinea-pig dermonecrosis,²⁰ cytotoxicity on cultures of embryonic bovine cells (EBL)²¹ or Vero cells,²² ELISA, and immunoblotting with MAbs.²³

In our study, there was complete correlation between

Table I. Sources of toxigenic clinical isolates, capsular type and cytotoxic activity

Strain no.	Capsular type	Professional exposure	Age	Clinical features	Cytotoxic titre (Vero cells)
1	A	Farmer	59	Chronic bronchitis	256
2	A	Farmer	62	Chronic bronchitis	128
3	A	Farmer	67	Chronic bronchitis	64
4	A	Farmer	56	Infectious pneumopathy	64
5	A	Farmer	50	Chronic bronchitis and pleurisy	64
6	D	Pig breeder	38	Healthy carrier	64

Table II. Results of toxin assays on 94 clinical isolates and six control strains of *P. multocida*

Organism	Dermonecrotic test	Cytotoxic test	ELISA	
			A	A/Ao
Toxigenic clinical isolates (n = 6)	+	+	> 0.94	> 75%
Non-toxigenic clinical isolates (n = 88)	—	—	< 0.155	< 12%
Positive controls:				
<i>P. multocida</i> NCTC 12177	+	+	1.348	107%
<i>P. multocida</i> NCTC 12178	+	+	1.262	100%
<i>P. multocida</i> CIP 5730	+	+	1.429	113%
<i>P. multocida</i> D 706	+	+	1.288	102%
Negative controls:				
<i>P. multocida</i> CIP 5684	—	—	0.072	6%
<i>P. multocida</i> CIP 5724	—	—	0.085	7%

Figure. Immunoblot of extracts of three control strains and five clinical isolates. Lane a, strain CIP 5684; b, strain NCTC 12178; c, strain D 706; d, non-toxigenic clinical isolate; e-g, toxigenic clinical isolates.

the results obtained with the three tests employed. The Vero cell line, although less sensitive than the EBL cell line, yielded effective results for screening strains. Our ELISA data were in agreement with those of Foged *et al.*; the relative absorbance was above 39% and below 9% for toxigenic and non-toxigenic strains, respectively, in their study of 615 porcine isolates.¹⁴ Our immunoblotting profiles showed several minor protein bands not noted in their previous report. This discrepancy was probably due to more stringent conditions of preparation of the samples as a similar electrophoretic method and the same MABs were used. The major protein band had a M_r (145 Kda) compatible with those previously reported for the DNT.

Toxin production by *P. multocida* has been reported only for subsp. *multocida* and never for subsp. *gallicida* or *septica*, nor for *P. canis* (formerly dog-type *P. multocida*). The pig is a natural reservoir of these strains, although they can be isolated from other farm animals.⁹ For this study, we collected 94 strains isolated from various sites in human patients. Six strains produced a toxin that was biologically and immunologically identical with that described for strains isolated from pigs. Five of these strains were from the respiratory tracts of patients who had chronic bronchitis with superinfection (three strains) or severe infectious pneumopathy, with or without pleurisy (two strains). The sixth strain was isolated from the oropharynx of a healthy carrier. All but one of these strains had a capsule of type A. In France, capsular types A and D have been isolated equally frequently from human infections.²⁴ In the USA, the A type is predominant in man, especially in respiratory infections,²⁵ whereas atrophic rhinitis in pigs is usually provoked by D type toxigenic strains.²⁶ The absence of toxin production among strains from animal bite wounds does not support a role of the toxin in pasteurellosis secondary to bites or scratches.

Colonisation or infection of the respiratory tract by *P. multocida* occurred mainly in patients who were in frequent contact with piggeries (33 out of 41 isolates).

Contact occurred in the course of their professional activity or as a result of the presence of a piggery near their home. Both hospitals that contributed to the collection of these strains were located in a region that produced 46% of the French pork meat in 1986. The incidence of pleuro-pulmonary infections due to *P. multocida* among farm workers has been emphasised by several authors. In the USA, 37 such cases were described in subjects living in rural areas, of whom 27 were farmers.²⁷ In France, an increase in pleuro-pulmonary pasteurellosis in one of the two districts involved in our study was recorded.²⁸ These authors advanced the hypothesis that there may be a relationship between an increase in the incidence of this unfamiliar disease and the development of intensive pig breeding. Colonisation or infection by toxigenic strains also correlated closely with exposure to pigs.

Although the pathophysiology remains obscure, it is traditionally held that the occurrence of *P. multocida* pulmonary infections in man is linked to the pre-existence of an underlying disorder (chronic bronchitis, bronchiectasis, ORL or pulmonary neoplasia) and to contact with pets, or less frequently to oropharyngeal carriage of the organism.^{27,29} The bacterium then behaves as an opportunist pathogen. Nevertheless, the statistically greater incidence of toxigenic isolates from respiratory sites does not provide conclusive evidence for the DNT as a virulence factor that would favour the onset of an overt infection. Its possible role in respiratory diseases secondary to *P. multocida* colonisation, which represent 60% of human pasteurellosis other than infections from animal bites¹ has yet to be established. However, clinical and experimental data from in-vivo and in-vitro studies are available. The necrotic activity of DNT on liver cells has been demonstrated in the pig and the rat and led to severe dysfunction.^{30,31} For the lung, experimental or clinical evidence is not so well established. In swine, toxigenic *P. multocida* have no primary pathogenic activity in pneumonia,^{32,33} but the proportion of toxigenic strains isolated from the necrotic type of pneumonia is statistically higher and they are able to

extend catarrhal pneumonic lesions caused by a primary pathogen such as *Haemophilus pleuropneumoniae*³³ to a purulent type. Moreover, a synergic effect has been reported between DNT and swine Herpesvirus 1, the Aujeszky's disease pathogen, leading to enhanced lethality *in vivo* and to increased production of virus *in vitro*.³⁴ In EBL cells incubated with DNT, nucleic acid synthesis and adenylate cyclase activity are not altered but there is a very significant decrease of protein synthesis³⁵ and virus appears to

utilise the alterations induced by DNT in cellular metabolism to enhance its replication.³⁴ These facts could indicate a possible interaction of DNT with various pathogenic agents, including viruses and bacteria. Further investigations are necessary to demonstrate that such a mechanism is involved in the pathophysiology of human respiratory pasteurellosis or infections which involve toxigenic strains of *P. multocida*.

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