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DETECTION OF DERMONECROTIC TOXIN PRODUCED BY SEROTYPE D STRAINS OF *PASTEURELLA MULTOCIDA*

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This paper presents the results of an investigation of the toxigenic properties of serotype D strains of Pasteurella multocida. A total of 6 strains isolated from nasal swabs of pigs with clinical atrophic rhinitis were examined. Toxigenic properties were determined utilising three tests: mouse mortality assay, skin test on guinea pigs and mouse spleen atrophy assay. The investigated strains of P. multocida showed different ability for toxin production. The toxin prepared by sonication produced dermonecrotic lesions on the skin of guinea pigs. Dermonecrotic toxin (DNT) had lethal toxicity for mice, and also induced marked atrophy of spleens in mice On the basis of these results, serotype D strains P. multocida may be evaluated as toxigenic and the applied assays are suitable for P. multocida toxin detection.

Key words: dermonecrotic toxin, P. multocida serotype D, swine.

INTRODUCTION

Pasteurella multocida is an important bacterial pathogen in domestic and laboratory animals, and its role in human infections is increasingly being recognised (De Jong, 1992; Giles *et al.*, 1980; Schoss *et al.* 1985). In *P. multocida*, five capsular (A, B, D, E, F) and 11 somatic (1-11) serotypes occur in 20 different combinations. Serotypes are often related to host specificity and pathogenicity. Capsular types A and D are usually isolated from pigs(Bechmann and Schoss, 1988; Hoffman *et al.*, 1989). Toxigenic strains belong to type D or type A (Lariviere *et al.*,1992; Pedersen and Elling, 1984). Atrophic rhinitis is a multifactorial disease associated with intensive pig production. A severe form of the disease, usually associated with reduced productivity, is caused by infection with toxigenic strains of *Pasteurella multocida* alone or in combination with *Bordetella bronchiseptica* (De Jong and Borst, 1985; De Jong, 1992; Pedersen and Barford, 1981). *Bordetella bronchiseptica* causes moderate, nonprogressive turbinate atrophy, generally without significant snout changes. In contrast, toxigenic type D and A *P. multocida* provokes severe turbinate atrophy, combined with shortening or twisting of the snout and reduced productivity, known as Progressive Atrophic Rhinitis (PAR; De Jong, 1992). The characteristic lesions of PAR have been reproduced with toxigenic strains of *Pasteurella multocida* and with purified toxin

(De Jong *et al.*, 1980; De Jong, 1992; Giles *et al.*, 1980). The etiological definition of PAR implies that the ultimate diagnosis depends on the demonstration of toxigenic *Pasteurella multocida* in a herd. Thus, the differentiation of toxigenic from nontoxigenic isolates of *P. multocida* is important for diagnosis of the disease and for epidemiological studies on atrophic rhinitis. Since 1980, several diagnostic assays have been developed in order to differentiate toxigenic from nontoxigenic strains of *P. multocida*. The widely used methods were based on biological effects of the *P. multocida* toxin, such as lethal tests in mice, dermonecrotic tests in guinea pigs and atrophic changes in the mouse spleen (Nakai *et al.*, 1984; Sawata *et al.*, 1984). Toxigenicity of isolates can be demonstrated by testing for cytotoxicity in cultured cells , EBL and VERO cells (Pennings and Storm, 1984; Rutter and Luther, 1984). A commercially available ELISA is now widely used to differentiate toxigenic from nontoxigenic isolates of *P. multocida* (Foged *et al.*, 1988). Gene probes based on fragments of the *P. multocida* toxin gene have been described (Kamps *et al.*, 1990). Diagnostic tests, based on detection of DNA fragments have been described (Nagai *et al.*, 1994) and could offer great advantages in detecting toxigenic *P. multocida* in samples where bacterial culture is not possible.

The purpose of the present report was to detect reliably the toxin produced by serotype D strains of *P. multocida*, from nasal swabs of piglets from herds with clinical signs of atrophic rhinitis.

MATERIAL AND METHODS

Pasteurella multocida strains: A total of 6 strains of *P. multocida* was isolated from nasal swabs of pigs with nasal turbinate atrophy, from a herd with clinical atrophic rhinitis.

Strains were isolated on blood agar and Columbia blood agar, which contained bacitracin, gentamicin and clindamycin (Vidić *et al.*, 2000).The isolated strains of *P. multocida* were assigned to capsular, serotype D, according to the method described by Carter and Subronto, (1973).

Preparation of dermonecrotic toxin: *P. multocida* growth on blood agar plates, incubated for 24 hours at 37 °C, was harvested and diluted in phosphate buffered saline to give suspensions containing about 2x 10^{10} cells/ml. The suspensions were sonicated four times for 60 seconds, at an amplitude of 6 kc followed by a 30 min centrifugation at 12000 rpm. The supernatant was filtered through sterile a 0.22 nm membrane filter. The filtrate was used as *P. multocida* dermonecrotic toxin.

a) Lethal test in mice for toxin detection:

For this investigation we used mice weighing ca 15 g. A series of clear filtrates from suspensions (1:2 to 1:64) were injected 0.5 ml i.v. into each animal. We used two animals for each inoculum dilution. The animals were observed for 48 h and the mortality was recorded. Lethal toxicity was expressed as the number of dead mice/ number of inoculated mice. A lethality rate of 100% was taken as the toxicity criterion.

b) Toxin detection by the skin test on guinea pigs.

We prepared dilutions of clear supernatant of suspensions from 1:2 to 1:64 and applied 0.1 ml intracutanously (in.cut.) in depilated abdominal skin of each guinea pig. The reaction was observed 72 hours after injection, and changes in form of necrosis, inflammation and induration were read as positive reactions.

Dermonecrotic titres were expressed as the reciprocal of the highest dilution of toxin with a positive 72 - hour skin reaction.

c) Detection of toxin by the mouse spleen atrophy assay.

For this investigation we used mice surviving on the 7th day as well as those dying between days 5 and 7 after injection with a series of toxin dilutions, from assay a. Surviving mice were killed and their spleens were examined and atrophic or hypertrophical changes recorded.

RESULTS

Table 1 presents the results for toxicity obtained in the mouse lethality test and the dermonecrotic test on guinea pigs. It is evident that the investigated serotype D strains of *P. multocida* showed different levels of toxin production. A lethality rate of 100% was taken as the toxicity criterion. In accordance with this, strains with a toxin titre 1:32 and higher were considered highly-toxic (in our experiment strains S-4 and BP-7). Medium toxic strains were those causing 100% lethality at a toxin titre 1:8 and 1:16 (S-19, SP-39, BP-03). Strains with a titre lower than 1:8 (in our experiment SP-143) were considered weakly toxic. Figure I shows the results of toxin determination via dermonecrotic-tests on guinea pigs. In inoculated animals changes appeared in the form of skin necrosis of diameter I cm, with red borders. Skin necrosis was noticed after only 48 hours. As with mouse lethality test, the tested strains showed different abilities to produce dermonecrotic lesions. Dermonecrotic titres had values from 1: 32 do 1: 4.

Table 1. Detection of *P. multocida* toxins by the mouse lethality test and by the DNT (dermonecrotic test) on guinea pigs

Strains *P.multocida*	Inoculum dilution 1:2	1:4	1:8	1:16	1:32	1:64	assay 100% lethality	DNT titers
S-4	2/2 N	2/2 N	2/2 N	2/2 N	2/2 N	2/2 N.R	1:64	1:32
S-19	2/2 N	2/2 N	2/2 N	2/2 N	1/2 R	0/2 I	1:16	1:16
BP-7	2/2 N	2/2 N	2/2 N	2/2 N	2/2 R	1/2 I	1:32	1:16
BP-03	2/2 N	2/2 N	2/2 N	1/2 R	1/2 R	0/2 -	1:8	1:8
SP-39	2/2 N	2/2 N	2/2 N	2/2 N.R	1/2 R	0/2 I	1:16	1:8
SP-143	2/2 N	2/2 N	1/2 R	1/2 R	0/2 -	0/2 -	1:4	1:4

died/inoculated mice
N - necrosis; R-rubor; I-induration
DNT titers-dermonecrotic titers

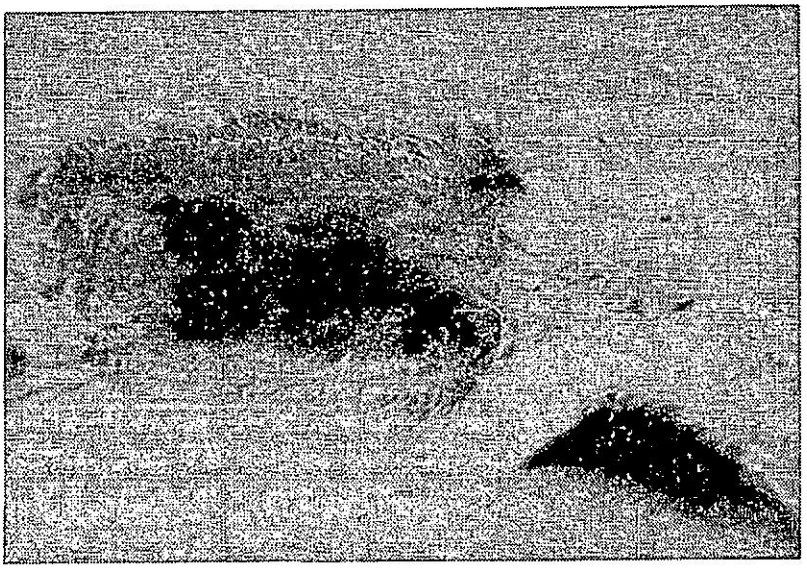

Figure 1. Dermonecrotic changes in guinea pigs after administration of *P. multocida* toxins

Table 2 and Figure 2 show the detection of toxins by observing changes in mouse spleen. Changes on the spleen were registered in mice killed 7 days after inoculation and in those dying after 5 days. Changes appeared as spleen reduction up to approximately 25% of normal weights. Atrophic spleens were sharp-edged, with a light reddish colour and anaemic. Most obvious spleen reduction was observed after inoculation low dilutions of toxin suspension (1:2 and 1:4).

Table 2. Detection of *P. multocida* toxins by the mouse spleen atrophy assay

Strains	Inoculum dilution									
	1 : 4		1 :8		1 : 16		1 : 32		1 : 64	
	LT	WS	LT	WS	LT	WS	LT	WS	LT	WS
S-19	2/2	-	2/2	-	2/2	31.4	1/2	33.8	0/2	30.6
BP-7	2/2	-	2/2	27.6	2/2	30.8	2/2	31.2	1/2	32.6
SP-39	2/2	-	2/2	38.2	2/2	n.t.	1/2	36.3	0/2	42.1
Control	48.7									

LT= lethality - died/sacrificed mice
WS= spleen weight (mg)
n.t.= not tested
- mouse died before 5th day

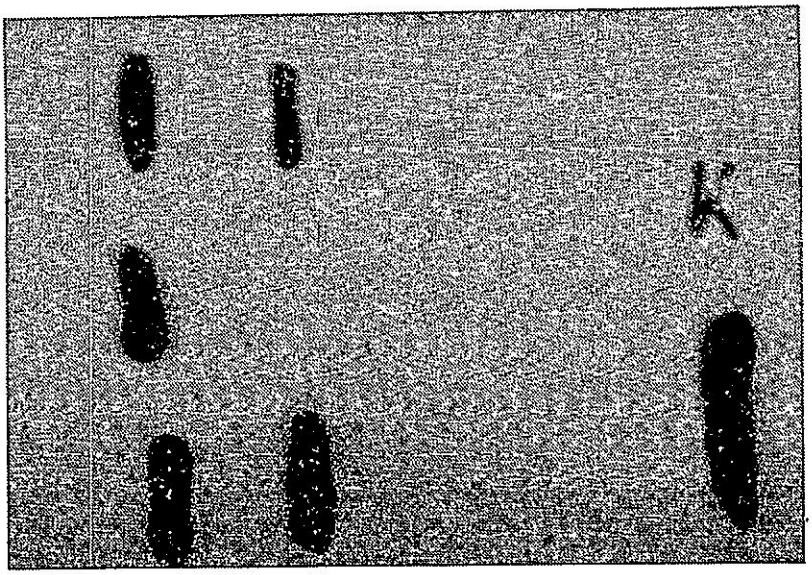

Figure 2. Changes in mouse spleen after application of *P. multocida* toxin

DISCUSSION

The prevalence of infection with toxigenic *P. multocida*, serotype D, is higher in herds exhibiting clinical disease (De Jong, 1992). *P. multocida* can be present in 60-80% of weaned pigs in a herd with clinical atrophic rhinitis in the finishing pigs (Hoffman *et al.*, 1989; Lariviere *et al.*, 1992). Evidence is accumulating to support the view that the severe progressive lesions of atrophic rhinitis of pigs are attributable to infection with toxigenic strains of *P. multocida*. Clinical signs comparable to those seen in field outbreaks of the disease can be reproduced in pigs by experimental infections with toxigenic but not with non-toxigenic strains (De Jong, 1992.; Lariviere *et al.*, 1992). Toxigenic isolates of *P.multocida* can colonise the nasal cavity, elaborate severe toxins and produce progressive lesions of the turbinate bones and snout. There is some evidence that the presence of *B. bronchiseptica* can enhance colonisation by *P. multocida*, particularly the toxigenic type D strains isolated from pigs (Pedersen and Barford, 1981; De Jong, 1992). The cytotoxin of *B. bronchiseptica* is required for optimum growth by toxigenic *P. multocida*. The toxin of *P. multocida* is the main colonisation factor produced by toxigenic strains. The toxin is thermolabile and dermonecrotic and has been named the atrophic rhinitis toxin or PMT - *P. multocida* - toxin (De Jong, 1992; Nakai *et al.*, 1984; Sawata *et al.*,1984). The toxin can produce turbinate atrophy when injected intranasally, and also when given intramuscularly, intraperitoneally, intravenously or intradermally (De Jong, 1992). The toxin enhances osteoclastic resorption and impairs osteoblastic synthesis of the turbinate osseous core and irreversible changes can occur within a few days. The epithelium and the submucosa undergo secondary atrophy and the turbinates may disappear almost completely within 10-14 days (Giles *et al.*,1980;

Hoffman *et al.*, 1989). These lesions can persist until the animal is 90 kg body weight. The turbinate atrophy is not accompanied by an inflammatory reaction. The effect of the *P. multocida* toxin is restricted to the nasal cavity which is supported by the intriguing observation that the turbinate lesions result in shortening and twisting of the snout (De Jong, 1992).

Thus, the differentiation of toxigenic from nontoxigenic strains of *P. multocida* is important in diagnosis and for epidemiological studies on atrophic rhinitis, while assays of the toxin are necessary for its characterisation and for studies of its mode of action. De Jong (1992) observed that pathogenic strains were capable of producing a thermolabile toxin, detected by a skin test in guinea pigs or by a lethality test in mice. Sawata *et al.*, (1984) found dermonecrotic activity in specimens prepared by sonication from *P. multocida*. Furthermore, *P. multocida* DNT exerted degenerative effects on spleens of mice. This toxic phenomenon was similar to lienotoxicity by *B. bronchiseptica* heatlabile exotoxin. Nakai *et al.* (1984) found that toxicity of *P. multocida* DNT was completely inactivated by heating at 70 C for 30 minutes, and was reduced by treatment with trypsin, formaline or glutaraldehyde, indicating that the DNT may be a protein.

De Jong *et al.* (1980) suggested that pathogenicity of *P. multocida* isolated in pigs was closely correlated with the ability to produce DNT. Similar observations have been reported by others (Lariviere *et al.*, 1992: Rutter and Luther, 1984). *Bordetella bronchiseptica* also produced a heat labile exotoxin. The DNT produced by *B. bronchiseptica* killed mice, when given i/p or i/v, produced dermonecrotic lesions and also had degenerative effects on spleens of mice (De Jong, 1992). The DNT produced by *B. bronchiseptica* may be a major virulent factor producing nasal turbinate atrophy in young pigs and in mice (Nakai *et al.*,1984). Differences were not observed in the toxicity between *P. multocida* and *B. bronchiseptica* DNT, confirming the results of De Jong *et al.* (1980). Although biologic and toxic properties of *P. multocida* were similar to those of *B. bronchiseptica* DNT, cross-neutralisation tests between *P. multocida* and *B. bronchiseptica* (Nakai *et al.*, 1984) indicated that DNT from the two bacterial species were serologically distinct.

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DOKAZIVANJE DERMONEKROTIČNOG TOKSINA SOJEVA *PASTEURELLA MULTOCIDA*, SEROTIPA D

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SADRŽAJ

U radu su izneti rezultati ispitivanja toksičnih svojstava bakterije *Pasteurella multocida* serotipa D. Ispitano je 6 sojeva *P. multocida* izolovanih iz brisa nosa svinja sa kliničkim atrofičnim rinitisom. Za dokazivanje toksičnih svojstava primenjena su tri testa: test smrtnosti na miševima, dermonekrotični test na zamorcima i registrovanje atrofičnih promena na slezini miša. Ispitani sojevi *P.multocida* imali su različitu sposobnost stvaranja toksina. Dobijeni toksin stvarao je dermonekrotične promene na koži zamorca, izazivao je smrtnost miševa kao i značajnu atrofiju slezina miševa. Na osnovu dobijenih rezultata sojevi *P.multocida*, serotip D mogu se oceniti kao toksogeni, a primenjeni testovi su podesni za dokazivanje toksina *P.multocida*.