TOXIN DETECTION IN Pasteurella multocida STRAINS ISOLATED FROM SWINE LUNGS IN THE STATE OF RIO GRANDE DO SUL, BRAZIL

DETECÇÃO DA PRODUÇÃO DE TOXINA EM AMOSTRAS DE Pasteurella multocida ISOLADAS DE PULMÕES DE SUÍNOS NO RIO GRANDE DO SUL

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ABSTRACT
Toxin production and the presence of the toxA gene was assayed in 24 Pasteurella multocida strains isolated from pigs with pneumonia and pleuritis in the State of Rio Grande do Sul, Brazil. Toxicogenicity was assessed through a mouse lethality test and a PCR amplification assay was used to obtain a 300 bp product from the toxA gene. In order to demonstrate that the amplified product corresponded to the toxA gene, the same segment of a reference P. multocida capsular type D toxigenic strain was amplified and treated with the restriction enzyme Taq1, and the restriction fragments corresponded to the expected sizes. Only one out of 24 strains of P. multocida was toxigenic in the mouse lethality test. However, with the use of PCR, nine strains carried the toxA gene. Hypothesis to explain this difference is the suppression of genic expression in vivo or the presence of a defective, non-functional gene.

Key words: Pasteurella multocida, toxin, lung, pigs, PCR.

RESUMO
Foi determinada a produção de toxina e a presença de gene toxA que a codifica, utilizando 24 amostras de Pasteurella multocida isoladas de suínos com pneumonia e pleurite. Para determinar a capacidade toxigênica foi utilizado o teste de letalidade em camundongo. Para a detecção do gene toxA foi utilizada a técnica do PCR, utilizando 2 primers que possibilitaram a amplificação de um produto de PCR de 300 pb. Com a finalidade de comprovar que o produto do PCR amplificado correspondia ao segmento do gene toxA foi feita a clivagem do fragmento da P. multocida tipo capsular D padrão toxigênica com a endonuclease de restrição Taq1, sendo que os fragmentos de clivagem corresponderam aos tamanhos esperados. Apenas uma dentre as 24 amostras de P. multocida foi toxigênica pelo teste biológico em camundongo. Entretanto, com o uso da técnica do PCR, nove amostras foram positivas. São discutidas hipóteses para explicar essa diferença, como possibilidade da ocorrência da supressão da expressão genética in vivo ou a existência de um gene defectivo, não funcional.

Descritores: Pasteurella multocida, toxina, pulmão, suínos, PCR.
INTRODUCTION

_Pasteurella multocida_ (P. multocida) can be present as a commensal in the nasopharynx of apparently healthy animals and as a primary or secondary pathogen in several animal species (Rimler and Rhoades, 1989).

Toxigenic strains of the bacteria have been isolated from humans with tonsillitis, rhinitis, sinusitis, pleuritis, appendicitis, and septicemia, suggesting a potential zoonosis (Nilsen and Frederiksen, 1990, Donnio et al., 1991). Most human isolates belong to capsular types A and D (Carter and Bain, 1960), similar to types isolated from swine (Pijoan, 1992).

In pigs, _P. multocida_ is a normal inhabitant of the nasal cavity and is one of the agents of progressive atrophic rhinitis (De Jong et al., 1980). Toxigenic strains of _P. multocida_ alone or in association with factors such as previous infection by _Bordetella bronchiseptica_ is considered the main etiological agent of the condition (Pijoan, 1992). Toxigenic strains of _P. multocida_ synthesize a 145 kDa toxin codified by the chromosomal gene toxA (Lichtensteiger et al., 1996).

The importance of _P. multocida_ as a primary agent of pneumonia in swine has been thoroughly investigated. According to Pijoan and Fuentes (1987), the microorganism is unable to perform as a primary pathogen, depending on the interaction with other organisms to produce pneumonia (such as adenovirus, Classical Swine Fever virus, Reproductive and Respiratory Syndrome virus, _Mycoplasma hyopneumoniae_ and Aujeszky Disease virus). However, the same authors state that the isolation of _P. multocida_ from swine lungs in slaughterhouses demonstrates the importance of the agent in pneumatic processes, causing great economical losses to the swine industry.

In Brazil, Stepan (1995) examined 5987 pigs and collected 230 lungs with pleuritis and pneumonia in 4 slaughterhouses in the State of Rio Grande do Sul. Bacteriological examination yielded 99 isolates of _P. multocida_ (43%).

The virulence factors of _P. multocida_ strains which cause pneumonia and pleuritis are not well defined. Some authors reported isolation of toxigenic strains from pig lungs (Pijoan et al., 1984; Iwamatsu and Sawada, 1988). However, toxigenicity does not seems important for the experimental reproduction of pneumonia by _P. multocida_ (Baekbo and Nielsen, 1988).

There are several laboratory tests available for discrimination between toxigenic and non-toxigenic strains of _P. multocida_. They include biological assays for toxin activity, such as mouse lethality test (Ahn and Kim, 1994) and dermal necrosis test in guinea pigs (Avella et al., 1993). More recently, molecular tests such as polymerase chain reaction (PCR) (Nagai et al., 1994; Lichtensteiger et al., 1996) have been used.

The present study evaluated toxin production in _P. multocida_ strains isolated from pigs with pneumonia and pleuritis using a mouse lethality test and investigated the presence of the toxA gene by PCR.

MATERIALS AND METHODS

1. Strains

A total of 24 strains of _P. multocida_ were used. Eleven were obtained from pig lungs from four slaughterhouses in the State of Rio Grande do Sul, Brazil, isolated by Stepan (1995) at Laboratório de Patologia Suína do Centro de Pesquisa Veterinária “Desidério Finamor” (CPVDF) (Swine Pathology Laboratory of the Center of Veterinary Research), Eldorado do Sul, identified as PI5, PI23, PI21, PI7, FII14, PIII47, PI8, PIII19, FII9, PIII43, CI12, and 13 strains isolated in the same laboratory from piglets with pneumonia and pleuritis, identified as 76, 805, Ca, Ta, 710, Fr, 1/95, 1265, 1133, 903, 1628, 1363, 1621.

The strains were initially preserved in brain and heart infusion (BHI) media with the addition of 5% horse serum, preserved at −70°C, and later lyophilized. A reference strain of toxigenic _P. multocida_, capsular type D (obtained
from American Type Culture Collection – ATCC N° 12948) and a strain isolated from a pig with atrophic rhinitis (limbriated, capsular type D, obtained from Centro Nacional de Pesquisa de Suínos e Aves – National Poultry and Swine Research Center – EMBRAPA, Concórdia, Santa Catarina, Brasil) were used as controls.

2. Classification

All strains were classified into capsular types A and D. Neutral acriflavine test 1:1000 (Carter and Subronto, 1973) was used to identify D-type strains. Hyaluronidase test was employed to identify A-type strains, using a hyaluronidase producing strain of *Staphylococcus aureus* (Carter and Rundell, 1975).

3. Dermonecrotic toxin detection in mouse

In order to assess the toxigenicity of *P. multocida* strains, the technique described by Ahn and Kim (1994) was modified as follows:

3.1 Strain cultivation

Strains were propagated in TSB (trypticase soy broth) for 36 hours at 37°C. Each bacterial growth was frozen and thawed repeatedly 10 times in liquid nitrogen and centrifuged for 30 minutes at 4000 g. The supernatant was then filtered in a 0.22 mm- membrane.

3.2 Mouse inoculation

21-day-old Webster mice were inoculated intraperitonially with 0.5 ml of the filtrate described above and observed for 10 days. The strains causing death were considered toxigenic.

4. Detection of the Gene toxA by PCR

4.1 Strains

To determine optimal conditions for the amplification of specific DNA fragments of *P. multocida* by PCR, a reference strain of capsular type D was used. All strains were cultivated in BHI for 24 hours at 37°C.

4.2 PCR primers

Two specific primers for *P. multocida* toxA gene were designed, based on Nagai et al. (1994).

4.3 DNA amplification by PCR

The technique used was essentially as described by Sambrook et al. (1989). DNA extracted from reference strains and field isolates was obtained by heating at 100°C of 100 ml of the culture for 5 minutes, followed by cooling at 4°C and centrifugation at 14000 rpm for 2 minutes.

PCR reactions was performed in a 25 ml volume, containing 30 pmol of each primers, 200 mM of each tri-phosphate de-oxy-ribo-nucleotide, 1 unit of Taq DNA polymerase (CENBIOT Enzimas) in 1x reaction buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl, 2,5 mM MgCl₂) and 5 ml of the sample. The following protocol was used in the thermocycler (MJ Research): a denaturation stage at 94°C (5 minutes), followed by an annealing at 55°C (45 seconds) and extension at 72°C (45 seconds) and denaturation at 94°C (45 seconds). The three last cycles were repeated 35 times and the strains were incubated at annealing temperature for 2 minutes, followed by extension at 72°C (5 min).

4.4 Restriction of PCR products with enzymes

To demonstrate that the amplified PCR products corresponded to the toxA gene segment, the PCR amplicons of reference *P. multocida* type D strains were treated with *TaqI* restriction endonuclease (Fig. 2B, lane 4). An aliquot of 10 ml of the amplicon was treated with the enzyme according to the manufacturer’s specification (Pharmacia).
RESULTS

1. Strain classification
   All strains were classified as capsular type A, except for sample 1/95, which belonged to type D.

2. Toxin assay in mouse
   Table 1 shows the results obtained in mouse lethality test. Two strains, the standard *P. multocida* D and 1/95 were considered toxigenic.

3. ToxA gene detection
   Using PM1 and PM2 primers, a 300 bp PCR product was obtained with the D-type *P. multocida* strain (Fig. 2A, lane 12). The restriction fragments corresponded to the expected sizes, according to the analysis of the restriction map presented in Fig. 1.
   In the present study, 9 out of the 24 analyzed strains presented bands with sizes corresponding to the segment of the *toxA* gene observed in the reference strain (lane 1, 2, 7, 8, 15, 17, 19, 22 and 26), besides the fimbriated sample (fimbD), isolated from the nasal turbinate of an animal with atrophic rhinitis (lane 25), (Fig. 2A).

4.5 Analysis of PCR products by electrophoresis in agarose gel
   Aliquots corresponding to 1/5 of PCR reactions or ½ of the restriction reaction were submitted to electrophoresis in 2% agarose gel, stained with ethidium bromide and the DNA bands were visualized under UV light, essentially as described by Sambrook et al. (1989).
**DISCUSSION**

Pneumonia and pleuritis associated to *P. multocida* are very important to pig production and most strains isolated from the lesions belong to capsular type A (Pijoan, 1992).

The ability of *P. multocida* strains to produce toxin is the main factor in the pathogenesis of progressive atrophic rhinitis, frequently associated with capsular type D (Pijoan et al., 1984). However, some toxigenic capsular type A strains have been isolated from pigs with progressive atrophic rhinitis and pneumonia (Pijoan et al., 1984; Ahn e Kim, 1994).

Only one out of 24 strains isolated from pneumonia and pleuritis in our work was classified as capsular type D. All other strains were identified as capsular type A. These results are consistent with other authors (Pijoan et al., 1983, Iwamatsu and Sawada, 1988, Lopez et al., 1988, Awad-Masalmeh et al., 1994), which support the assertion of Rutter (1983) and Pijoan (1992) that *P. multocida* type A strains are more prevalent in pneumatic processes than type D strains. Type A strains are more frequently found in the lung, probably due to the presence of a capsule of hyaluronic acid, which difficults phagocytosis by alveolar macrophages (Pijoan, 1992).
Only one out of twenty four strains was toxigenic in the mouse lethality test, sample 1/95, classified as capsular type D. However, using PCR, 9 strains amplified the segment of the toxA gene. The relationship between P. multocida capsular type, toxin production and pneumonia in pigs is not well defined. Results obtained by diverse authors present differences. Iwamatsu and Sawada (1988), examining 116 strains of P. multocida isolated from pneumatic pig lungs by dermal necrosis assay in guinea pigs, found that only 14.3% of the toxigenic strains belonged to capsular type A. Most (85.7%) of the toxin-producing strains belonged to capsular type D. However, Ahn and Kim (1994), studying 77 strains of type A P. multocida isolated from pig lungs by mouse lethality test, determined that 77% of the strains were toxigenic, and, out of 3 type D strains, 66.3% produced the toxin.

We observed that some strains which yielded PCR products for the toxA gene were negative in the mouse lethality test. However, Lichtensteiger et al. (1996), comparing PCR with other tests, including mouse lethality assay, concluded that PCR was 100% sensitive and specific in the detection of toxigenic P. multocida strains. Amigot et al. (1998), evaluated three techniques for detection of toxigenic P. multocida strains isolated from swine: ELISA test, cytotoxic effect in cat fetal lung cell culture and detection of toxA gene by PCR. He did not observe complete consistency among the 3 tests, as some strains that were negative by ELISA showed weak bands in PCR reaction when in cell culture.

The primers used in our experiment had been previously used in the differentiation of toxigenic and non-toxigenic strains of P. multocida (Nagai et al., 1994). 187 isolates of P. multocida and related bacteria were analyzed in this work, and the PCR method was shown to be sensitive and specific in the detection of toxigenic strains.

Some hypothesis can be raised to explain the fact that some non-toxigenic strains in the mouse test amplified a specific PCR product of the toxA gene. It includes the suppression of the expression of the gene in vitro after subculture. Another possibility would be the presence of a non-functional, defective gene. In this case, the PCR test could be used to detect the mutant or non-functional gene. Lichtensteiger et al. (1996) stresses that a genetic test, such as PCR, could avoid potential problems such as the suppression of the expression of a gene.

Some authors suggest PCR as a fast and safe alternative for toxin detection in P. multocida strains isolated from diseased swine (Nagai et al., 1994, Lichtensteiger et al., 1996). However, this may not always be true, as PCR assay can present false-positive results.

Additional studies, including analysis of new isolates of P. multocida isolated from pneumonia lesions in pigs are suggested, in order to confirm the present results. They may indicate that toxigenicity can be an important factor in strains isolated from this condition in swine.

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