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Short communication

Pasteurella multocida isolates associated with ovine pneumonia are toxigenic

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ABSTRACT

The *P. multocida* toxin (PMT), a dermonecrotic protein encoded by the *toxA* gene, is the major virulence factor of capsular type D *P. multocida* strains causing progressive atrophic rhinitis (PAR) in pigs. A high frequency of *P. multocida* isolates harboring the *toxA* gene has been found among ovine pneumonic isolates, although the ability of these isolates to express PMT has never been examined. In this study we have investigated the ability of ovine *toxA* + *P. multocida* isolates ($n = 57$) to express a functional toxin by detection of PMT toxin antigen using an ELISA test and its cytopathic effect in a Vero cell assay. PMT antigen was expressed in the great majority (54/57; 94.7%) of *toxA* + isolates. Moreover, the 100% *toxA* + ovine isolates analyzed produced a cytopathic effect in Vero cells within 24–48 h post-inoculation, identical to that described for porcine toxigenic *P. multocida* isolates. These results show for the first time that, in addition to isolates associated with PAR, isolates of *P. multocida* associated with pneumonia in sheep are also toxigenic. In addition, we found a total agreement (Kappa = 1; C.I. 0.75–1.25) between the detection of the *toxA* gene and the toxigenic capability of *P. multocida* isolates, indicating the PCR detection of *toxA* would be a suitable predictive marker of the toxigenic fitness of *P. multocida*.

1. Introduction

Pasteurella multocida is associated with a number of diseases in domestic and wild animals, including progressive atrophic rhinitis in pigs (PAR) and pneumonia in pigs, cattle and sheep (Harper et al., 2006; Wilson and Ho, 2013). The *P. multocida* toxin (PMT) is the major virulence factor of *P. multocida* responsible for the turbinate bone degeneration manifested during infection in PAR (Lax and Chanter, 1990). It is a dermonecrotic protein (146-kDa) encoded by the *toxA* gene on a lysogenic bacteriophage residing only in the genome of toxicogenic strains (Pullinger et al., 2004). Apart from its well-known role in the pathogenesis of PAR, PMT has several other biological functions. Inoculation of purified PMT can induce liver and kidney damage in addition to atrophy of the nasal turbinate bones in pigs (Lax and Chanter, 1990) and pneumonic lesions in rabbits (Chrisp and Foged, 1991). PMT is also a potent mitogen (Rozengurt et al., 1990) and interacts with several signal transduction pathways to disturb cell growth and differentiation, favoring the evasion of the immune system (Kubatzky et al., 2013). It has been suggested that PMT might dampen the immune response by different toxin-related immune evasion strategies, facilitating the multiplication and survival of *P. multocida* in the host (Kubatzky et al., 2013).

The synthesis of PMT is mainly associated with capsular type D-*toxA* + pig isolates causing PAR, although PMT has been occasionally detected in porcine strains of capsular type A (Djordjevic et al., 1998; Davies et al., 2003). Unexpectedly, a high frequency of *toxA* + *P. multocida* isolates has been found among ovine pneumonic isolates of both capsular types A and D in the last few years (Ewers et al., 2006; Sarangi et al., 2015; Vougidou et al., 2015; Einarsdottir et al., 2016; Shirzad and Tabatabaei, 2016; García-Alvarez et al., 2017), although the ability of these isolates to express PMT has not been investigated. Isolates from sheep and pigs belong to genetically different subpopulations of *P. multocida* (García-Alvarez et al., 2017). This circumstance together with the fact that not all genes are necessarily expressed (Bavananthasivam et al., 2018) led us to investigate the ability of ovine *toxA* + *P. multocida* isolates to express a functional toxin by detection of PMT toxin antigen using an ELISA test and its cytopathic effect in a Vero cell assay.

2. Material and methods

2.1. *P. multocida* strains

This study included 57 pneumonic ovine *P. multocida* isolates harboring the *toxA* gene (*toxA* +): 43 capsular type A, 13 capsular type D and one nontypeable (NT) as determined by multiplex PCR (García-Alvarez et al., 2017). Detailed information about the 57 *toxA* + ovine

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Table 1
Details of the 57 *toxA* + pneumonic ovine *P. multocida* isolates used in this study.

Capsular type	Geographical origin	Year of isolation	No. of isolates	Isolates
A (n = 43)	Badajoz	2008	19	M66, M77, M78, M84, M96, M113, M114, M117, M128, M135, M160, M166, M174, M176, M187, M193, M199, M206, M208
	Cáceres	2008	2	M321, M326
	Cáceres	2009	4	P71, P73, P82, P85
	Madrid	2009	18	P61, P63, P67, P68, P70, P72, P74, P75, P76, P78, P79, P80, P81, P83, P84, P86, P87, P88
D (n = 13)	Badajoz	2008	3	M67, M93, M134
	Cáceres	2008	7	M279, M285, M293, M297, M337, M343, M344
	Madrid	2009	3	P64, P60, P65
NT (n = 1)	Madrid	2009	1	P77

NT, nontypeable as determined by the multiplex PCR described by Townsend et al. (2001).

isolates is shown in Table 1. In addition, three pneumonic *toxA* − isolates of capsular types A (M172), D (P69) and NT (M478), were also included as negative controls in both the ELISA and Vero cell assays. A toxigenic capsular type D *P. multocida* strain (M91996) from porcine atrophic rhinitis kindly supplied by Exopol (Pol. Río Gállego D/8, Zaragoza, Spain) was used as positive control in the Vero cell assays.

2.2. Detection of PMT toxin

PMT toxin was determined in the 57 *toxA* + *P. multocida* isolates using a commercial enzyme immunoassay kit (PMT ELISA Kit, Dako Corporation, Denmark) according to the manufacturer's instructions. Assays were performed from overnight pure cultures of each isolate in Columbia blood agar plates (bioMérieux). According to manufacturer instructions, agar plate growth was harvested with two ml of deionized water and 200 µL of this bacterial suspension were inoculated into two uncoated microwells, covered with sealing tape and incubate overnight at 37 °C for extraction. Bacterial extracts were transferred to microwell coated with monoclonal antibody to PMT in volumes of 50 µL each. After incubation 60 min. at room temperature and washing, conjugate was added. After incubation for 2 h at room temperature and washing, chromogenic substrate was added. The reaction was stopped by the addition of 100 µL of 0.46 mol/L sulfuric acid. Presence of PMT in the extracts of the bacterial cultures was indicated by a yellow color in the microwells. The optical density (O.D) was measured at 490 nm. The final OD-value for each bacterial specimen was the mean of the OD of the two microwells. ELISA assay for each isolate was performed by duplicate in two independent experiments.

2.3. Cytopathic effect of PMT

The in vitro biological effect of PMT of 16 isolates (13 *toxA* + randomly selected isolates and three *toxA* + isolates that were negative on ELISA; Table 2) was assayed in a Vero cell line (Pennings and Storm, 1984). Bacterial extracts were prepared as described previously (Amigot et al., 1998) with a few modifications. Briefly, bacteria were grown on Columbia agar (BioMerieux) and harvested directly from the agar using 4 ml of phosphate-buffered saline, pH 7.4. The suspensions were treated by sonication (three cycles of 50 s each, alternating 3 s on and 2 s off), centrifuged (10 min, 5000 rpm), and filtered through a sterile 0.22-mm pore membrane filter (Millipore). Vero cell monolayers were grown in 24-well tissue culture plates with flat bottoms at 37 °C, 5% CO_2 in a humidified atmosphere, using Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS). Before inoculation, the medium was removed and 200 µl of crude extracts was added to cells in duplicate (Amigot et al., 1998). After 20 min of incubation, 1 ml of DMEN without FCS was added to each well and the plates were incubated at 37 °C, in a humidified atmosphere with 5% CO_2 for a maximum of 5 days. Cell morphology and cytopathic effects were observed daily with an inverted microscope.

Table 2
Pasteurella multocida toxin (PMT) antigen detection by enzyme immunoassay (PMT ELISA) and cytopathic effect detection by Vero cell assay in pneumonic ovine *P. multocida* isolates harboring the *toxA* gene (*tox*A +).

Capsular type	Sequence type (ST)[a]	No. of isolates	PMT detection by ELISA (no. of isolates)	No. of isolates with cytopathic effect on Vero cells/ No. of isolates analyzed[b]
A (n = 43)	ST19	2	1	+ (2/2)
	ST48	1	1	
	ST49	1	1	+ (1/1)
	ST50	10	10	+ (5/5)
	ST54	1	1	
	ST56	4	4	
	ST57	1	1	
	ST58	1	1	
	ST59	1	1	
	ST60	1	1	
	nd	20	19	+ (5/5)
D (n = 13)	ST19	4	4	+ (2/2)
	ST20	1	0	+ (1/1)
	ST50	1	1	
	ST53	1	1	
	ST55	1	1	
	nd	5	5	
NT (n = 1)	ST52	1	1	

NT, nontypeable as determined by the multiplex PCR described by Townsend et al. (2001).
nd, not determined.
[a] Sequence types (STs) were previously determined by García-Alvarez et al. (2017) based on the Multi-host MLST Database (http://pubmlst.org/pmultocida_multihost/).
[b] 16 isolates (13 randomly *toxA* + and PMT producing isolates and three *toxA* + isolates but PMT negative on ELISA test.

2.4. Sequencing of the toxA gene

The *toxA* gene of two isolates representative of the most frequent capsular types and genotypes of ovine *P. multocida* isolates (M297, capsular type A, genotype ST50 and M135, capsular type D and ST19; García-Alvarez et al., 2017) were PCR-amplified sequenced using the primers and conditions described by García (2009). Both isolates gave positive results in both PMT ELISA and Vero cell assays. The amplified products were purified by using a QIAquick PCR purification kit (Qiagen) and sequenced using an automatic DNA sequencer (ABI PRISM 3730; Applied Biosystem). Sequences of both isolates were assembled and compared each other and with the sequence of *toxA* gene of the reference porcine strain NCTC 12178 (accession number X51512) using the FASTA sequence comparison tool (https://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml). Moreover, the *toxA* sequences of isolates M297 and M135 were compared with *toxA* sequences available in the public ENA-database (formely EMBL-database) using the FASTA server (https://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html).

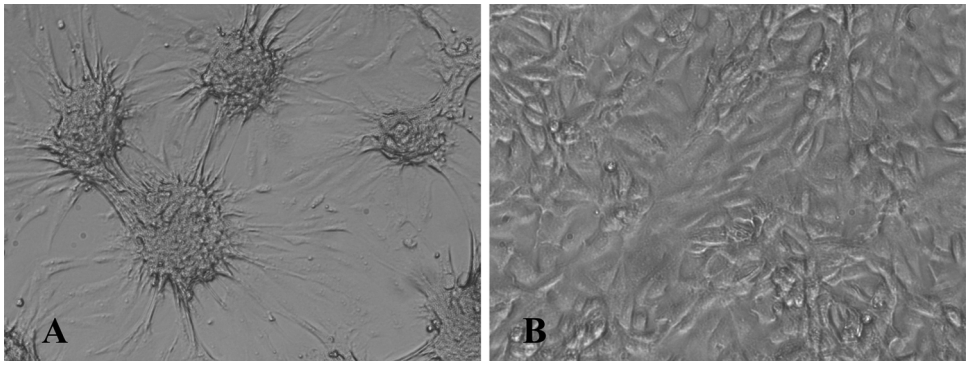

Fig. 1. Effect on Vero cells of PMT toxin produced by culture extracts of *toxA* + (A) and *toxA*- (B, negative control) *P. multocida* ovine isolates after 48 h of incubation at 37 °C and 5% CO_2.

2.5. Statistical analysis

Analysis of the association between PMT antigen expression determined by ELISA test and capsular type of isolates was done calculating the Odds ratio (OR) and the Fisher exact test with a significance level set at $P < 0.05$. Frequency and association measures and their confidence intervals (C.I.) were determined using the Epi InfoTM 7 program of the Centers for Disease Control and Prevention (CDC) (http://wwwn.cdc.gov/). The agreement between *toxA* gene detection by PCR and Cytopathic effect detection on Vero cell assay was estimated by calculating the Kappa coefficient on the WinEpi website (http://www.winepi.net/uk/index.htm). The confidence level was set at 95%. A value *k* equal to 0 indicates that there is no agreement once the chance factor is discounted while a value of kappa equal to 1 indicates a total agreement.

3. Results and discussion

PMT antigen was detected in 54 of the 57 *toxA* + isolates (94.7%, C.I. 85.4–98.9%; Table 2). No significant differences ($P > 0.05$; OR 1.7, CI 0.14–20.5) were observed between isolates of capsular type A (95.3%; 41/43) and D (92.3%; 12/13), indicating that the *toxA* gene is transcribed and expressed in the great majority of the ovine *toxA* + *P. multocida* isolates investigated, regardless of their capsular type. Analysis of the DNA sequences of *toxA* gene of strains M297 and M135 (capsular types A and D, respectively) showed similarity values of 99.9% between them (in 3711 nucleotide overlap) and between 99.6 and 100% with other *toxA* gene sequences available in the public ENA-database. These results indicate that the *toxA* gene is highly conserved in *P. multocida* regardless of their serotype or host origin (Frandsen et al., 1991; Donnio et al., 1999; Einarsdottir et al., 2016). The nearly complete sequences of the *toxA* gene of the ovine strains M135 and M297 have been deposited in the Gene Bank under the accession numbers LS974118 and LR215670, respectively. Comparative analyses of the deduced amino acid sequences of the *toxA* gene of strains M135 and M297 with that from the porcine reference strain NCTC 12,178 revealed a similarity of 99.6% and 99.8%, respectively, suggesting that PMT toxin of the ovine isolates would be biologically functional. This assumption was supported by the fact that culture extracts of the 16 *toxA* + ovine isolates analyzed produced a cytopathic effect in the Vero cells within 24–48 h post-inoculation (Table 2) identical to that described for porcine toxigenic *P. multocida* isolates in Vero cells (Pennings and Storm, 1984) and characterized by the development of knots throughout the monolayer (Fig. 1A). On the other hand, culture extracts of the three *toxA*- isolates did not produce damage to the Vero cells (Fig. 1B). PMT was not detected by the ELISA assay in three of the *toxA* + isolates even though they exhibited an evident cytopathic effect in Vero cells (Table 2). Nevertheless, we found a total agreement ($k = 1$; C.I. 0.75–1.25) between the detection of the *toxA* gene and the toxigenic capability of *P. multocida* isolates determined through cell culture assay. These results indicate that although the ELISA technique has been widely used to detect toxigenic *P. multocida* isolates (Amigot et al., 1998; Hariharan et al., 2000; MacInnes et al., 2008), the PCR detection of the *toxA* gene would be a suitable predictive marker of the toxigenic fitness of *P. multocida*.

Epidemiological data have suggested a relevant role for *toxA* + isolates in ovine pneumonia (Ewers et al., 2006; Sarangi et al., 2015; Vougidou et al., 2015; Einarsdottir et al., 2016; Shirzad and Tabatabaei, 2016; García-Alvarez et al., 2017), but the production of PMT has been mainly associated with capsular type D, and also sporadically with capsular type A, porcine isolates causing PAR (Djordjevic et al., 1998; Davies et al., 2003). The results of this study show for the first time that isolates of *P. multocida* associated with pneumonia in sheep are also toxigenic. PMT toxin is able to induce a persistent inflammatory response (Kubatzky et al., 2013). This persistent inflammatory response could be responsible for the consolidated areas observed in lungs of sheep with subclinical pneumonia caused by *P. multocida*. More than half of the 57 *toxA* + isolates of this study were previously characterized by MLST typing (García-Alvarez et al., 2017) belonging to a relatively large number of STs (Table 2). These data suggest therefore that the toxigenic capacity of sheep *P. multocida* isolates from pneumonia would not be related to particular genetic lineages. These results should be confirmed by the analysis of a large collection of ovine isolates from other geographical origins and genetic backgrounds.

PMT is considered a suitable and effective antigen for vaccination that is able to induce a protective immune response (Foged, 1992; Harper et al., 2006) and is commonly included as a toxoid in many commercial vaccines to control PAR (Zhang et al., 2018). While determining the precise role of PMT in the development of pneumonia in sheep, this study lends some support for the inclusion of this toxin in vaccines for prophylaxis against ovine pneumonic pasteurellosis.

Conflict of interest

The authors declare that they have no conflicts of interest.

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