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Short Communication

Distribution of *PLD and FagA*, *B*, *C* and *D* genes in *Corynebacterium pseudotuberculosis* isolates from sheep and goats with caseus lymphadenitis

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Abstract

Caseous lymphadenits (CL) is a chronic and subclinical disease that affects goats and sheep and, consequently, causes economic losses, especially to small producers. The purpose of this study, through use of Polymerase Chain Reaction (PCR), was to verify the presence of virulence genes of phospholipase D (*PLD*), integral membrane protein (*FagA*), iron enterobactin transporter (*FagB*), ATP binding cytoplasmic membrane protein (*FagC*) and iron siderophore binding protein (*FagD*) in 168 isolates of *C. pseudotuberculosis* obtained from cases of caseous lymphadenitis in goats and sheep. *FagA*, *FagB* and *PLD* genes were detected in all 145 strains isolated from abscesses in superficial lymph nodes and in 23 strains isolated from viscera. The *FagC* gene was positive in 167 (99.40%) isolates. The *FagD* gene was detected in 160 (95.23%) isolates. All virulence factors analyzed were found more frequently among isolates collected in the viscera of animals with CL, indicating a multifactorial nature, as well as variations, in the invasive potential of *C. pseudotuberculosis* strains.

Keywords: virulence genes, *C. pseudotuberculosis*, caseous lymphadenits.

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Corynebacterium pseudotuberculosis is a facultative intracellular pathogen that mainly infects sheep and goats, causing the disease called caseous lymphadenitis (CL). In disease manifestations, the main characteristic is abscessing of the lymph nodes (Fontaine and Baird, 2008). Considering the current status of CL prevalence worldwide, including Brazil (Connor *et al.*, 2000), there is a pressing need for more efficient alternatives for disease control that do not only cure sick animals, but also minimize or even prevent the onset of the disease in herds (Çetinkaya *et al.*, 2002; D'Afonseca *et al.*, 2008). The high prevalence of CL in sheep and goats has made studies on ways to detect *C. pseudotuberculosis* virulence factors increasingly important. Although the mechanisms by which the disease is caused remain poorly understood, advances in genomics have allowed the discovery of new genes, especially ones related to *C. pseudotuberculosis* pathogenicity and lifestyle (Quinn *et al.*, 1994; Dorella *et al.*, 2006; Ruiz *et al.*, 2011).

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Hemolysis is an important characteristic of the virulence of *C. pseudotuberculosis* because it has been associated with the uptake of iron, which is a micronutrient important for energy gain and bacterial growth (Jost and Billington, 2004). The gene product for phospholipase D (*PLD*) is regarded as the main virulence factor in *C. pseudotuberculosis* (Hodgson *et al.*, 1999). The *PLD* gene encodes the phospholipase D - *PLD* exotoxin, an enzyme that catalyzes the dissociation of sphingomyelin and increases vascular permeability. This leads to the spread (Hodgson *et al.*, 1994) and survival of *C. pseudotuberculosis* in the cells, and consequently the invasion of the body and transport by phagocytes to regional lymph nodes (Baird and Fontaine, 2007). Although it is not considered to be directly hemolytic, it has been reported as capable of producing synergistic hemolysis (Jost and Billington, 2004).

Other important virulence factors include integral membrane protein (*FagA*), iron enterobactin transporter (*FagB*), ATP binding cytoplasmic membrane protein (*FagC*) and iron siderophore binding protein (*FagD*). These are organized in an operon involved in iron uptake, which provides persistence to *C. pseudotuberculosis* in infections in goats. This operon is located downstream from the *PLD* gene, contributing to the virulence of *C. pseudotuberculosis* (Billington *et al.*, 2002). The finding of seven putative pathogenicity islands containing the classical virulence elements, including genes for iron uptake, fimbrial subunits, insertional elements and secreted toxins, probably mostly acquired through horizontal transfer, contributes to the understanding of how this species causes disease (Ruiz *et al.*, 2011).

The aim of this study was to verify the presence of *PLD*, *FagA*, *FagB*, *FagC* and *FagD* virulence genes in strains of *C. pseudotuberculosis* obtained from caseous lymphadenitis in goats and sheep.

A total of 168 isolates of *C. pseudotuberculosis* were used in this study. The strains were obtained from caseous lymphadenitis samples of goats and sheep originating from farms located in different municipalities of the state of Pernambuco, Brazil: Afranio (n = 2), Floresta (n = 4), Jatobá (n = 2), Petrolândia (n = 4) and Petrolina (n = 38), as well as from animals slaughtered in slaughterhouses located in Petrolina, PE (n = 116) and Juazeiro, BA (n = 2). Of these isolates, 151 were obtained from abscesses in superficial lymph nodes and 17 from abscesses in viscera. Isolates of *C. pseudotuberculosis* were previously identified by means of their morphology and biochemical characteristics (Quinn *et al.*, 1994). The cultures were subcultured in a Tryptone Soy Agar (TSA) medium, and then used for molecular characterization.

The DNA from *C. pseudotuberculosis* isolates was heat extracted in a final volume of 500 uL. Initially the strains were grown in BHI (Brain Heart Infusion) medium for 48 h at 37 °C. Subsequently, 10 colony forming units (CFU) were placed in 500 uL of sterile ultrapure water. Thermal lysis of DNA was done at a temperature of 100 °C for 11 min and cooling at 4 °C for 4 min. After centrifugation at 14,000 *g* for 2 min the supernatant was removed.

PCR was used to check for the presence of *FagA*, *FagB*, *FagC*, *FagD* and *PLD* genes with the use of the specific primers shown in Table 1. The primers for amplification of *FagA*, *FagB*, *FagC*, *FagD* and *PLD* genes were designed with the Primer3 Plus software. The quality of the primers was checked online with the IDT Oligo Analyzer. The primers used for amplification of the *PLD* gene have been described by Hodgson *et al.*, 1990.

The PCR consisted of a final volume of 25 µL containing 4 µL of genomic DNA, 0.4 µM of each primer, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1 mM dNTPs, 2.5 U *Taq* polymerase (Centibiot) and 1 mM of $MgCl_2$ for *PLD* primer, 1.4 mM for *FagA* and 1.3 mM for *FagB*, *FagC* and *FagD* primers. The amplification reaction for the *PLD* gene consisted of the following steps: initial denaturation at 94 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 61 °C for 40 s and extension at 72 °C for 40 s, terminating with a final extension step at 72 °C for 10 min. For the other genes, the difference was in relation to the annealing temperature, which was 58 °C, 55 °C, 60 °C and 61 °C for the *FagA*, *FagB*, *FagC* and *FagD* genes, respectively. The amplification products were then separated in 1.5% agarose gels, stained with ethidium bromide and viewed under ultraviolet light. The specificity of the primers used was confirmed in 1.5% agarose gels, revealing a specific band of the estimated size.

Table 1 - Primers used for the detection of virulence genes.

Primer	Sequence	Product size in base pairs
*PLD*_F	5'ATGAGGGAGAAAGTTGTTTTA3'	924
*PLD*_R	5'TCACCACGGGTTATCCGC3'	
*Fag*_A_F	5'AGCAAGACCAAGAGACATGC3'	245
*Fag*_A_R	5'AGTCTCAGCCCAACGTACAG3'	
*Fag*_B_F	5'GTGAGAAGAACCCCGGTATAAG3'	291
*Fag*_B_R	5'TACCGCACTTATTCTGACACTG3'	
*Fag*_C_F	5'GTTTGGCTATCTCCTTGGTATG3'	173
*Fag*_C_R	5'CGACCTTAGTGTTGACATACCC3	
*Fag*_D_F	5'GAGACTATCGACCAGGCAGA3'	226
*Fag*_D_R	5'ACTTCTTGGGGAGCAGTTCT3'	

PCR is an accurate, efficient and highly sensitive method that can rapidly lead to the identification of a pathogen in material collected directly from lesions, as well as isolates in culture (Pacheco *et al.*, 2007). The use of PCR also permits to detect the presence of virulence genes, thus demonstrating the potential of a disease causing microorganism.

The *FagA*, *FagB* and *PLD* genes were found in all isolates. The *FagC* gene was present in 99.40% of the isolates tested, and the *FagD* gene had a frequency of 95.23%. Frequencies of virulence genes in *C. pseudotuberculosis* isolates according to their origin (viscera or superficial lymph nodes) are shown in Table 2. The combinations of virulence factors observed in 168 isolates of *C.*

Table 2 - Frequencies of virulence genes in *C. pseudotuberculosis* isolates from viscera or superficial lymph nodes.

Gene	Percentage (%) of positive isolates from viscera	Percentage (%) of positive isolates from superficial lymph nodes
*Fag*A	10.11	89.89
*Fag*B	10.11	89.89
*Fag*C	10.18	89.82
*Fag*D	10.62	89.38
PLD	10.11	89.89

Table 3 - Combinations of virulence genes in *C. pseudotuberculosis* isolates.

Gene combinations	Number of positive isolates from superficial lymph nodes	Number of positive isolates from viscera	Total percentage (%)
FagA/*FagB*/*PLD*	1	0	0.60
FagA/*FagB*/*FagC*/*PLD*	7	0	4.17
FagA/*FagB*/*FagC*/*FagD*/*PLD*	143	17	95.23

pseudotuberculosis are shown in Table 3. All samples collected in the viscera were positive for the five genes tested. The *FagD* gene was less frequent in samples collected from slaughtered animals.

In this study, the *PLD* gene was found in all clinical isolates, which is in agreement with other studies that show the spread of the *PLD* gene among *C. pseudotuberculosis* strains (Hodgson *et al.*, 1990; Çetinkaya *et al.*, 2002; Pacheco *et al.*, 2007). All isolates in this study showed a positive reverse CAMP test, indicating expression of *PLD* (data not shown). The mechanisms by which *PLD* is expressed are not well understood, but it can be regulated by a variety of environmental factors that may involve the binding of repressors or activators or structural changes in DNA (McKean *et al.*, 2007). Pacheco *et al.* (2011) compared the proteomes of two strains and demonstrated that *PLD* was expressed only in the virulent isolate. Attenuation of the *PLD* gene reduces the virulence of *C. pseudotuberculosis* isolates and prevents the development of caseous lymphadenitis (McNamara *et al.*, 1994). Knockout isolates for *PLD* may represent new candidates for the production of live attenuated vaccines (Simmons *et al.*, 1998; Meyer *et al.*, 2002).

The *FagA, B, C* and *D* putative genes are related to regulation and uptake of iron by *C. pseudotuberculosis* and are located downstream of the *PLD* gene. These genes are rarely expressed *in vitro*, but when expressed *in vivo,* they increase the virulence of *C. pseudotuberculosis*, which indicates that host factors may be necessary for the expression of these genes (Billington *et al.*, 2002). In the present paper, the *FagA*, *B* and *PLD* genes exhibited a rate of 100% in the isolates tested. In a study by Billington *et al.* (2002), a knockout mutant for the *Fag* gene showed reduced virulence in inoculation in goats. This information is important, as all isolates evaluated in this study were virulent. The *FagC* and *FagD* genes were detected less frequently; but still, this was more than 95%. Interestingly, all isolates negative for the *FagD* gene were obtained from superficial abscesses, suggesting differences in the virulence potential of the clinical isolates and the possibility of participation of *FagD* in the invasive mechanisms expressed by some *C. pseudotuberculosis* strains.

Since treatment of caseous lymphadenitis is difficult, vaccination would be an important alternative (Meyer *et al.*, 2002). The potential of interference of the *PLD* and *Fag* genes in bacterial fitness, and their ability to multiply and survive within the host (Jost and Billington, 2004), place these genes as candidates for production of live vaccines (Hodgson *et al.*, 1999; Billington *et al.*, 2002). Since most studies published so far investigated only a small number of *C. pseudotuberculosis* strains (Billington *et al.*, 2002; Pacheco *et al.*, 2011), the present analysis of a large number of virulent isolates should contribute to mapping the distribution of virulence factors in *C. pseudotuberculosis*.

We could show that genes encoding *PLD* and *FagA*-D virulence factors were present in most of the *C. pseudotuberculosis* strains isolated from animals with CL, indicating a wide distribution in the field and a high pathogenic potential.

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Internet Resources

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