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

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Abstract
Caseous lymphadenitis (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 lymphadenitis.

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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).

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 enterobactein 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, insertion 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 μL. 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 μL 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 MgCl₂ 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.

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. pseudotuberculosis isolates from viscera or superficial lymph nodes.

| Gene   | Percentage (%) of positive isolates from viscera | Percentage (%) of positive isolates from superficial lymph nodes |
|--------|--------------------------------------------------|------------------------------------------------------------------|
| FagA   | 10.11                                            | 89.89                                                           |
| FagB   | 10.11                                            | 89.89                                                           |
| FagC   | 10.18                                            | 89.82                                                           |
| FagD   | 10.62                                            | 89.38                                                           |
| PLD    | 10.11                                            | 89.89                                                           |

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

| Primer | Sequence | Product size in base pairs |
|--------|----------|---------------------------|
| PLD_F  | 5’ATGAGGGAGAAGTTTGTATTTA3’ | 924              |
| PLD_R  | 5’TCAACCAGGTTATCGGC3’       |                  |
| Fag_A_F| 5’AGCAAGACCAAGGAGCATGC3’   | 245              |
| Fag_A_R| 5’AGTCTCAGCCCAACCTACAG3’   |                  |
| Fag_B_F| 5’GTGAGAAGAACCCCCGTATAAG3’ | 291              |
| Fag_B_R| 5’TACCGCATTTATCGCAGACTG3’  |                  |
| Fag_C_F| 5’GTGGCTATCTCCTTGTATG3’    | 173              |
| Fag_C_R| 5’CGACCTTATGTTGACATACC3    |                  |
| Fag_D_F| 5’GAGACTATCGACCCAGCAAG3’   | 226              |
| Fag_D_R| 5’ACTTCTTGGGAGCAGTTCT3’    |                  |
Virulence genes in *Corynebacterium*

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 FagD 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
Primer3 Plus software, http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (September 10, 2010).
IDT Oligo Analyzer, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/ (September 10, 2010).

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