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Human Seroreactivity to Secreted Molecules of *Corynebacterium pseudotuberculosis*

Ramon Mendes dos Santos[1], Silvânia Maria Andrade Cerqueira[1,2], Caio Lopez Borges Andrade[1], Gabriel Saldanha Müller[1], Vitória Costa de Menezes Santos[1], Hévlyn Ribeiro de Araújo[1], Samanta Queiroz[1], Rogério Reis Conceição[1], Luiz Gustavo Freitas Oliveira[1], Marcos Borges Ribeiro[1*], Silvana Marchioro[1], Lilia Ferreira de Moura-Costa[3], Fúlvia Soares Campos de Sousa[3], Maria da Conceição Aquino de Sá[1], José Tadeu Raynal Rocha Filho[1], Soraya Castro Trindade[1], Eduardo Martins Netto[4], Roberto Meyer[1], Songelí Menezes Freire[1]

[1]Laboratory of Immunology and Molecular Biology of the Health Sciences Institute of the Federal University of Bahia, Bahia, Brazil
[2]Specialized Hospital Otávio Mangabeira, Bahia, Brazil
[3]Microbiology of the Federal University of Bahia, Bahia, Brazil
[4]Laboratory of Infectology of the University Hospital Complex Professor Edgard Santos, Salvador, Bahia, Brazil
Email: *ribeiromb@ufba.br, songelí.mf@gmail.com

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Abstract

Corynebacterium pseudotuberculosis is an infectious agent that occurs in small ruminants causing caseous lymphadenitis, and more rarely in humans causing lymphadenitis and pneumonia. The breeding small ruminants have great economic importance in Brazil. Rural farm workers and veterinary students who acquired this disease suffered from weakening symptoms for weeks, and the identification of the etiological agent was time-consuming and complex. Due to the low prevalence of case records, there is probably no available commercial diagnostic kit for *C. pseudotuberculosis* infection in humans. This study aimed to describe human seroreactivity to secreted antigens from *C. pseudotuberculosis*. Reactivity of serum from farm workers (n = 14), individuals who work with the bacillus at laboratory (n = 8) or individuals without contact (n = 25) was tested with secreted proteins from PAT10 strain of *C. pseudotuberculosis* by Western blotting. Samples of all (100%) farm workers showed reactivity to 31 kDa, 71 kDa and 164 kDa proteins, while laboratory workers showed 87.5%, 62.5 % and 37.5%, and no-contact 20%, 0% and 16%, respectively. All sera recognized the 275 kDa protein. Our data suggest that *C. pseudotuberculosis* secreted proteins are antigenic in humans and the recognition profiles allowed the identification of individuals with and without prior contact with this

bacillus. This is the first paper which describes human reactivity to *C. pseudotuberculosis* in serum samples of workers in Brazil.

Keywords

Human Immunoreactivity, Antigenicity,
Corynebacterium pseudotuberculosis, Secreted Proteins, Western Blot

1. Introduction

Corynebacterium pseudotuberculosis is a pleomorphic, gram-positive, facultative anaerobic and non-motile pathogen that causes pneumonia and lymphadenitis in humans [1] [2]. The first report of human infection by *C. pseudotuberculosis* was a 37-years-old man from Panama in 1966 with no known contact with small ruminants [3]. Since then, new infections have been reported in Australia, the United States, France, New Zealand, Belgium, Spain, Switzerland, China, the United Kingdom and Norway [1] [2] [4]. All of them reported direct or indirect contact with small ruminants or direct contact with the *C. pseudotuberculosis*. The infection is widely described in goats and sheep [5] and other small ruminants [2]. *C. pseudotuberculosis* is endemic in Brazil, including in northeastern states [6]. Human infection, as far as we know, has not yet been reported in Brazil.

Among the main symptoms of this infection in humans are inguinal lymphadenopathy or eosinophilic pneumonia with fever, pain, fatigue, malaise, globus sensation, dysphagia, and dry cough; and lymph node or lung biopsy analyzes from patients showed granulomatous, necrotizing non-specific inflammation without signs of malignancy, non-specific vasculitis, infiltrates of eosinophilic granulocytes and focal necroses [4] [7] [8].

Currently, the *C. pseudotuberculosis* infection diagnosis in humans is not commercially available. In the case reports described in the literature, patients are submitted to several diagnostic tests for various infections and treatments against the disease for several months without knowing the etiologic agent [1] [3] [4]. Besides that, the diagnosis as it has been carried out through invasive methods such as excision or puncture of affected lymph node followed by bacterial culture and biochemical or molecular processes [4], is considered complex. In this paper we describe the human seroreactivity to *C. pseudotuberculosis* and a simple method to identify this infection.

2. Methods

2.1. Ethical Aspects

This project was approved by the Ethics Research Council of the Health Sciences Institute of the Federal University of Bahia. CAAE: 57018116.9.0000.5662.

2.2. Study Population

To meet the criteria for non reactivity inclusion with *Mycobacterium tuberculosis*, the participants of the two groups had a tuberculin skin test (TST) (TST/P-PD—Statens Denmark) and Interferon-γ release assay (IGRA/QTF-TB—Qiagen) negatives. In the study, the following groups were analyzed: Contact Group (CG)—composed of adult individuals who showed or referred a work history with small ruminants on a farm (n = 14) or with *C. pseudotuberculosis* in a microbiology laboratory of the Federal University of Bahia (n = 8); non-contact group (nCG)—composed of individuals who reported no contact with small ruminants or their raw meat and food derivatives, and without a clinical history of an infectious process characteristic of *M. tuberculosis* or *C. pseudotuberculosis* (n = 25).

2.3. Culture Conditions of *Corynebacterium pseudotuberculosis*

The strain PAT10 of *C. pseudotuberculosis* [9] from the bacterial culture collection of the Laboratory of Microbiology Applied to Biotechnology and Immunology (LAMABI) of the Federal University of Bahia was used. For protein secretion, the bacteria were cultured in 500 mL of brain heart infusion (BHI) broth (DIFCO) at 37˚C for 72 h [10].

2.4. Three-Phase Partitioning (TPP) of the Secreted Fraction of *C. pseudotuberculosis* Proteins

Purification and concentration of secreted proteins were performed according to the protocol by Paule *et al.* [10], with few modifications: after centrifugation and collection of the culture supernatant, ammonium sulfate (Synth, Brazil) was added and mixed. The pH was adjusted to 4.0 with HCl solution and then n-butanol was added. This mixture was stirred for 10 minutes and allowed to rest for 1 hour. The interfacial precipitation was collected, centrifuged at 6000 rpm for 10 min, and the interfacial precipitation was collected again and dissolved in a small volume of 20 mM Tris buffer, pH 7.4.

2.5. SDS-PAGE of Secreted Proteins of *C. pseudotuberculosis*

A polyacrylamide gel electrophoresis with a 4% stacking gel and a 12% running gel was carried out in 0.124 M Tris, 0.96 M glycine, and 0.5% SDS, pH 8.3 migration buffer. The *C. pseudotuberculosis* excreted-secreted antigen was added to wells of 6.6 cm by 1 mm and subjected to a current of 15 milliamperes (mA) (Mini-protean—BioRad). The proteins were stained by silver (Amersham Biosciences). The electrophoretic migration pattern of the proteins was analyzed with Gel Analyzer 2010 software.

2.6. Western Blotting with Proteins of *C. pseudotuberculosis*

Secreted proteins were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad) in 25 mM Tris buffer with 192 mM glycine and 20% methanol, with a fixed

voltage at 100 V for one hour. The membrane was stained with Ponceau-S (Vetec, Brazil), cut into strips, washed in sodium phosphate buffer 0.15 M with 0.05% Tween20 (SPB-Tween20) pH 7.2, and blocked with 5% dry skimmed milk in SPB-Tween20, overnight at 4˚C under shaking. The serum of CG and nCG participants was diluted 1:10 in SPB-Tween20 and incubated for 1 h at 37˚C. The membrane strips were washed four times and incubated with horseradish peroxidase-conjugated anti-human IgG (Life technologies) diluted 1:250 for 1 h at 37˚C. After five washes the membrane strips were immersed in a developing solution (8 mL of methanol, 0.024 g of 4-Chloro-*α*-Naphthol (Sigma), 32 mL of SPB and 13.3 mL of hydrogen peroxide 37%) under shaking for about 50 min and rinsed with distilled water. The pattern of the bands was analyzed with Gel Analyzer 2010 software.

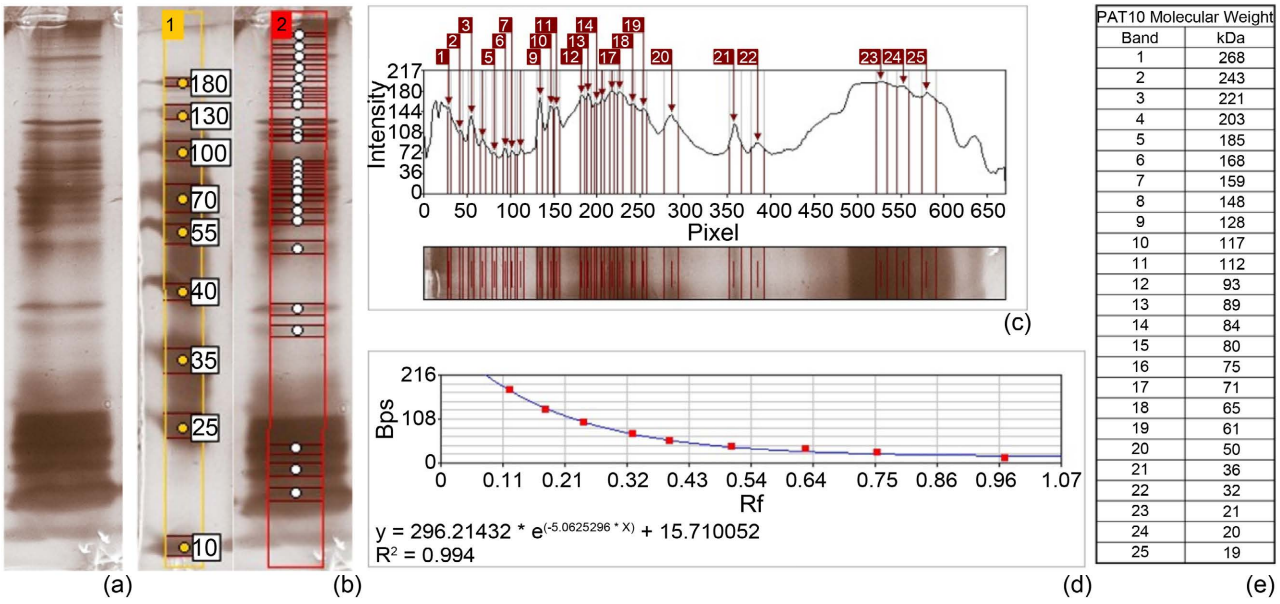
3. Results

3.1. Electrophoretic Profile of Secreted Proteins of *C. pseudotuberculosis* on SDS-PAGE

Secreted proteins of *C. pseudotuberculosis* recovered by TPP revealed twenty-five proteins with a molecular mass ranging from 19 to 268 kDa, on the silver-stained 12% SDS-PAGE gel (Figure 1).

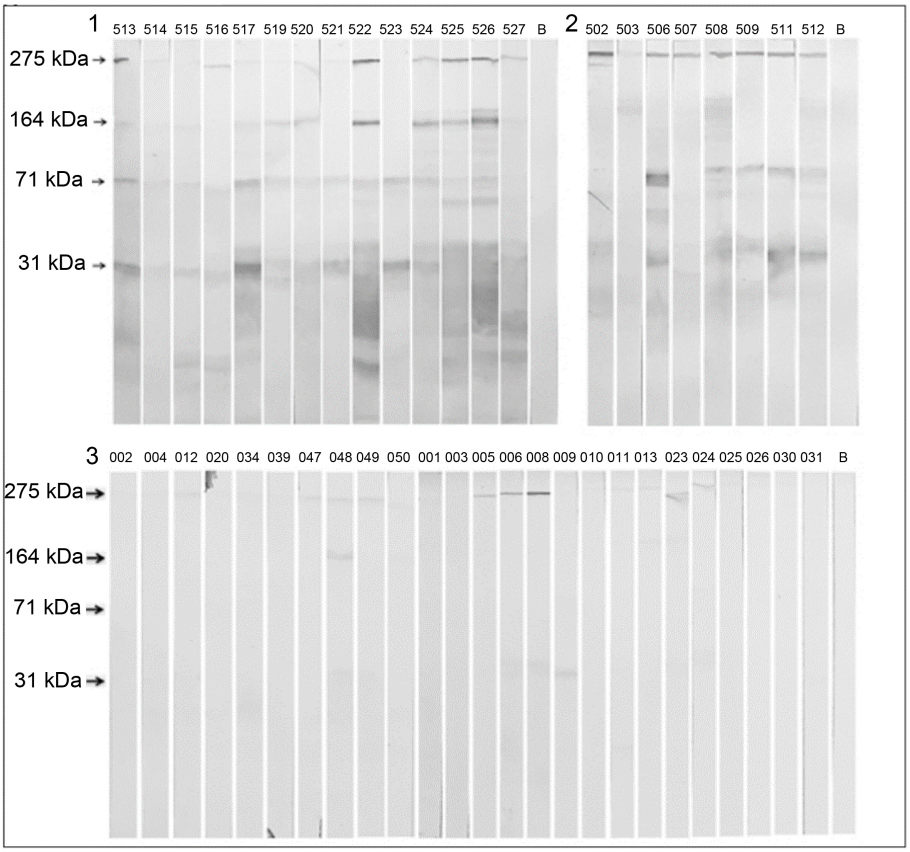
3.2. Reactivity of Human IgG to Secreted Proteins of *C. pseudotuberculosis* by Western Blotting

Serum reactivity of the individuals from the CG to *C. pseudotuberculosis* secreted



PAT10 Molecular Weight	
Band	kDa
1	268
2	243
3	221
4	203
5	185
6	168
7	159
8	148
9	128
10	117
11	112
12	93
13	89
14	84
15	80
16	75
17	71
18	65
19	61
20	50
21	36
22	32
23	21
24	20
25	19

Figure 1. Electrophoretic profile of 12% SDS-PAGE stained with silver salts. *C. pseudotuberculosis* PAT10 strain was grown in BHI medium broth at 37˚C for 72 h and secreted proteins purified by the TPP method. (a): Profile of secreted proteins of *C. pseudotuberculosis*. (b), (c), and (d): Molecular weight standard (Thermo Fisher Scientific ref.: 26616), band identification and analysis by Gel Analyzer 2010 software. (e): Molecular weight of the identified bands. Line band: number of proteins identified in the gel, from top to bottom; Line kDa: MW of correspondent band.



(a)

1: Frequency of reactivity found in immunoblotting with serum from goat and sheep handlers in the municipality of Iaçu/BA; (n = 14).

Bands	Occorrence	Frequency
275 KDa	14	100%
164 kDa	14	100%
71 kDa	14	100%
31 kDa	14	100%

2: Frequency of reactivity found in immunoblotting with serum from individulas working with *C. pseudotuberculosis* in a laboratory of microbiology or animal immunology; (n = 8).

Bands	Occorrence	Frequency
275 KDa	8	100%
164 kDa	3	37.5%
71 kDa	5	62.5%
31 kDa	7	82.5%

3: Frequency of reactivity found in immunoblotting with serum from non-contact individuals with goats and sheep or their raw food derivatives or contact with *C. pseudotuberculosis*; (n = 25).

Bands	Occorrence	Frequency
275 KDa	25	100%
164 kDa	4	16%
71 kDa	0	0%
31 kDa	5	20%

(b)

Figure 2. Western blotting from *C. pseudotuberculosis* PAT10 strain secreted proteins purified by the TPP method with serum from contact group (CG) individuals (a 1 and a 2) and non-contact group (nCG) individuals (a 3). (a 1): Serum IgG reactivity from goat and sheep handlersfrom municipality of Iaçu/BA. The strips from the 14 participants are listed from 513 to 527. (b 1): Frequency of positive reactions found in a 1 (n = 14). (a 2): Serum IgG reactivity from the individuals working with *C. pseudotuberculosis* in microbiology laboratory of the Federal University of Bahia. The strips from the 8 participants are listed from 502 to 512. (b 2): Frequency of positive reactions found in a 2 (n = 8). (a 3): Serum IgG reactivity of individuals of the nCG, who reported no contact with small ruminants or their raw meats and derivatives. The strips from the 26 participants are listed from 002 to 031. The arrows indicate the approximate molecular weight of the proteins appearing in all tested sera (257 kDa), and from the proteins that appeared with low intensity in individuals 08, 13, 48 and 50 (164 kDa) and in the individuals 06, 08, 09, 24, 48 (31 kDa). No reactivity was observed with the protein of approximately 71 kDa in nCG. (b 3): Frequency of reactivity found in a 3 (n = 26). The strip B is the negative control in all panels evaluated.

proteins showed a different profile than the one observed in the nCG by Western blotting (Figure 2(a)). The frequencies of bands with higher occurrences are shown in Figure 2(b).

It was observed in samples from the CG subgroup of farm workers, a higher frequency of approximately 31, 71, 164 and 275 kDa proteins (Figure 2(a)). Among the CG subgroup of laboratory workers samples, it was seen a higher frequency of approximately 31, 71 and 275 kDa proteins and a lower frequency of about 164 kDa protein (Figure 2(a)). On the other hand, in the samples of nCG group, it was observed the presence of 275 kDa protein was constant, with a lower frequency and intensity of 164 and 31 kDa proteins, and no occurrence of 71 kDa protein (Figure 2(a)).

4. Discussion

It was observed that CG participants sera showed reactivity with several proteins of *C. pseudotuberculosis*, highlighting the proteins with 31 kDa, 71 kDa, 164 kDa, and 275 kDa, which presented a frequency of 100% in the individuals that manipulated goats and sheep on the farm. The veterinary community knows the protein of approximately 31 kDa as phospholipase D (PLD), a molecule that gives high virulence and contributes to the pathogenicity of the *C. pseudotuberculosis* infection [11]. The protein with a molecular weight close to 71 kDa is described as neuraminidase H (NanH) or extracellular sialidase and is related to bacterial growth and pathogenicity through the recognition of sialic acid residue in host cells, also found in other species like *C. ulcerans* and *C. diphtheria* [9] [12] [13]. Despite this correlation, further analysis must be done to confirm the protein identity of these bands. References to proteins at 164 kDa and 275 kDa were not found, requiring a biochemical analysis to characterize them.

The protein of approximately 71 kDa was reactive exclusively with the samples of CG individuals, not appearing in nCG ones. A nearby band of about 75 kDa was found by Join-lambert *et al.* [11] when they performed a Western blotting serological reactivity test to diagnose the cause of necrotizing lymphadenitis in a 12-year-old child who had contact with sheep. These findings may indicate that this protein has high immunogenicity and can differentiate, in the serological test, individuals who had and who had no contact with *C. pseudotuberculosis*.

In the last decades, several studies carried out in Brazil evidenced the reactivity of infected goats and sheep against *C. pseudotuberculosis* antigens prepared in the laboratory. Vale *et al.* [14] found reactivity of goat sera with these bacteria proteins between 21 kDa to 92 kDa, including 31.5 kDa and 72 kDa. In another work with naturally infected animals, the 31 kDa and 72 kDa proteins were also found [15]. In Germany, a group of researchers testing *C. pseudotuberculosis* antigens in sera from animals with caseous lymphadenitis identified proteins close to 71 kDa with 70% frequency in sheep and 100% in goats, and proteins close to 31 kDa with a rate of 71% in sheep and 100% in goats [16].

In this work, all individuals of the nCG showed reactivity with protein of 275 kDa. Also were found four sera that showed reactivity with a protein of approximately 164 kDa and, five sera that reacted with a protein of about 31 kDa. These reactivities may result from a cross-reaction of proteins conserved in other bacteria that individuals may have come into contact with, or possible contact with *C. pseudotuberculosis*, unknown to the participant, as occurred in a Panamanian man in 1965 [3].

In the descriptive analysis from the protein profile of the purified extract of *C. pseudotuberculosis* PAT10 strain were found 25 proteins in 12% SDS-PAGE stained with silver salts. Several of these proteins were described in studies of different research groups, with more reference to the proteins between 19 and 94 kDa [10] [14] [15] [16]. These studies were performed using other strains than

the one used in our study, where 11 proteins above 100 kDa were found. The variation in the number of proteins found in other papers may be due to the number of microorganisms collected for bacterial growth, media volume or the staining method used, once silver nitrate staining is 100 times more sensitive than the Coomassie blue used by many researchers [6].

In the current socio-geographical context of infection by *C. pseudotuberculosis* of small ruminants in northeastern Brazil, and the consequent handling by workers that come in contact with these animals, or in the laboratory sector with the manipulation of this pathogenic bacteria, of biological risk NB-2, there is a current demand for infection serological diagnosis in the occupational environment. Its current inexistence is possibly due to the absence of studies aimed at the identification of specific antigenic molecules for human infection. The existence of an immunodiagnostic test, less invasive method if compared to those described in the literature for human [1] [4] [7], may allow epidemiological and occupational studies, besides making the infectology specialty care more efficient. Despite not being a prevalent disease in the general population, the potential risk, although low and preventable, by biosafety measures, for the worker and for the consumers of contaminated meat and derivatives, can have an advance in the diagnosis, with more vision of the one health that should be a goal for everyone.

The professional risk for rural farm workers may reside mainly in the deficient biosafety in the routine activities handling and slaughter infected animals and contaminated products. The use of personal protective equipment (PPE) such as goggles, mask, cap, lab coat, gloves, pants, overalls and boots is essential to prevent contamination by *C. pseudotuberculosis* in livestock activity or working at laboratory with the bacillus. As seen reactivity here in this study population and as referred to in articles dealing with human infection in localities with these diseases in small ruminants. There are also reports of infection by consumers of raw milk and meat from infected animals [2] [17], and boiling or good cooking of these foods must be practiced, preventing the entry of viable bacilli.

5. Conclusions

C. pseudotuberculosis secreted proteins are antigenic for human sera. The electrophoretic profile of the proteins recognized in western blotting by the sera of individuals with *C. pseudotuberculosis* contact or with infected animals differs from the individuals without these contacts.

Further studies should be performed for identification and production of 31 kDa and especially 71 kDa proteins as they are potential indicators of *C. pseudotuberculosis* infection and can be used as a diagnostic tool.

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Ethics Approval Statement

This project was approved by the Ethics Research Council of the Health Sciences Institute of the Federal University of Bahia. CAAE: 57018116.9.0000.5662.

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Potential Conflicts of Interest

There are no conflicts of interest.

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