SDS-PAGE and Western blot analysis of somatic and extracellular antigens of Corynebacterium pseudotuberculosis

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Abstract
Different techniques to isolate secreted, cell-surface located and somatic fractions of ***Corynebacterium pseudotuberculosis*** have been studied by SDS-PAGE and immunoblotting, by using a pool of sera from naturally infected goats. The secreted antigens were obtained from culture supernatant of the bacteria grown in a chemically defined medium or in Brain Heart Infusion medium. The surface fraction was obtained by NaCl 1 M treatment and the somatic ones were obtained by using distinct procedures (detergents and ultrasounds). By Coomassie blue staining, we observed 20 bands in the secreted fraction, 35 bands in the surface extract and 40 to 50 bands in the somatic fraction, depending on the extraction procedure. Sixteen immunoreactive proteins were detected among the studied fractions. Bands with molecular weights of 125, 108, 75, 68, 41, 40, 31 and 24 kDa were recognized with higher intensity and all of them were found in the secreted fraction. The use of the chemically defined medium allowed us to evidence the presence of proteins with high molecular masses in the secreted fraction of ***C. pseudotuberculosis,*** which had not been previously described.

Keywords: ***Corynebacterium pseudotuberculosis***. Goat. Antigens. SDS-PAGE. Western blot.

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INTRODUCTION

Corynebacterium pseudotuberculosis is the etiologic agent of various diseases such as caseous lymphadenitis in sheep and goats, ulcerative dermatitis in cows and ulcerative lymphangitis in horses (BATEY, 1986). In addition, isolation of the bacterium from humans directly in contact with infected animals (shepherds, veterinarians, etc) has been reported (BLACKWELL; SMITH; JOYCE, 1974; PEEL et al., 1997). Caseous lymphadenitis is widespread all over the world and is endemic in areas with high concentration of sheep or goat extensive breeding.

C. pseudotuberculosis is a Gram-positive facultative intracellular parasite which is phagocitized by neutrophils and macrophages but is able to multiply inside phagolysossomes, promoting phagocytic cells destruction. Therefore, following the bacterial invasion, there is a formation of pyogranuloma located in superficial or internal lymph nodes, depending on the route of contamination and the stage of the disease. (BATEY, 1986)

The cell wall lipids (CARNE; WICKHAM; KATER, 1956; MUCKLE; GYLES, 1983) and a phospholipase D exotoxin with molecular mass of 31 kDa (LOVELL; ZAKI, 1966) may constitute the two main virulence factors of this microorganism.Walker and others (1994) characterized another secreted antigen, CP40, with molecular mass of 40 kDa, with serine protease activity, which evoked a significant protection rate (82%) in sheep. Many other immunogenic components either secreted or somatic were described by immunoblot, using infected or vaccinated goats or sheep sera (ELLIS et al., 1991a, 1991b; MUCKLE et al., 1992; TER LAAK et al., 1992; BRAITHWAITE et al., 1993; STING; STENG; SPENGLER, 1998). Nevertheless, the results of these studies show some discrepancies regarding the number of the identified antigens, which varies from 2 to 6 in the extracellular fraction and 7 to 11 in the somatic fraction (TABLE 1). Among the antigens present in culture supernatant, Ellis and others (1991a) emphasized proteins of 20, 25, 31,5 and 63 kDa as being very often recognized, while Muckle and others (1992) described two other antigens of 68 and 120 kDa, associated to the cell, as highly immunoreactive,too.

The development of more effective tools for serodiagnostic tests as well as the production of more efficient vaccines for caseous lymphadenitis specifically in goats demand better targeting of immunodominant antigens of ***C. pseudotuberculosis.*** This study aims at giving a further contribution to the description of somatic and extracellular antigens of ***C. pseudotuberculosis*** with a special focus on the secreted fraction which has been obtained through an original approach.

MATERIAL AND METHODS

Bacterial strain, biochemical identification and culture conditions

A wild strain of ***C. pseudotuberculosis***, from the culture collection of the Laboratory of Microbiology of ICS/UFBA, isolated from caseous material from an infected goat in Bahia State, was used in this study. The typifying of this strain was performed by Gram stain, synergistic haemolysis with ***Rhodococcus equi*** (EGEN et al., 1988) and biochemical techniques (API CORYNE kit from BioMérieux SA, Marcy l'Etoile, France). All the protein fractions studied were obtained from the culture in Brain Heart Infusion (BHI) broth, at 37°C for 72 hours, except for the secreted proteins production that were obtained from culture in a synthetic medium, at 37°C for 72 hours.

Preparation of antigens

The secreted fraction was obtained by supernatant culture concentration of C. ***pseudotuberculosis*** grown in BHI medium (37°C, 48 h), or in chemically defined medium (MOURA-COSTA et al., 2002), containing 4,22 g/L Na_2HPO_4- $7H_2O$, 0,14 g/L KH_2PO_4, 0,25 g/L NH_4Cl, 5 mg/Ca_2Cl L, 50 mg/L $MgSO_4$, 12 g/L glucose, 0,1% Tween 80, 2% Minimal Essential Medium (MEM) Aminoacid Solution, 2% MEM Non Essential Aminoacid

Solution, 2% MEM Vitamin Solution (Life Technologies, USA), incubated at 37ºC during 72 hours, under continuous stirring. The bacterial mass was discarded after centrifugation at 3000 x g for 10 minutes and the supernatant was filtered through a 0,22 mm pore size membrane (Millipore, USA). Then, the supernatant was concentrated 50 times by ultrafiltration through a 10 kDa cut-off membrane (Millipore, USA). The concentrated solutions were clarified by centrifugation at 15000 x g, for 30 minutes and stored at –70ºC until use.

The surface extract was obtained by 2 different procedures:

(a) Surface proteins were obtained according to the technique described by Muckle and others (1992). The bacterial mass was washed twice in PBS and then washed twice with 3 volumes of 1M NaCl in 0,1 M PBS pH 7.4. The supernatants were obtained by centrifugation at 12000 g for 2 hours at 4ºC. The pooled supernatants were then clarified by centrifugation at 25000 g for 2 hours at 4ºC and dialyzed for 48 hours against 0,01M PBS, pH 7.4 at 4ºC. The samples were concentrated approximately 20 times by ultrafiltration (10 kDa cut off membrane, Millipore, USA).

(b) Surface proteins were also obtained by extraction with 1 or 2% Sodium Dodecyl Sulphate (SDS) g at 100ºC for 5 minutes, according to the protocol described by Peyret and others, (1993) with ***Corynebacterium glutamicum***.

The somatic extract was also obtained by 2 different procedures:

(a) Extraction by ultrasound: Bacterial mass was washed twice in PBS pH 7.4, centrifuged at 10000 x g for 30 minutes at 4ºC. The pellet was suspended in buffer (2 mL of PBS buffer for each 0.5 mL of bacterial wet mass) and pulse-sonicated (Model 250 Sonifier, Branson Ultrasonics Corporation, USA) at 60% output for 5 minutes (5 times 1 minute, with 1 minute intervals).

(b) Extraction by detergents: Several detergents were tested: sodium dodecyl sulphate (SDS), deoxycholic acid (DOC) and 3-[(3-cholamidopropyl)-dimethylammonio] 1-propanesulfonate (CHAPS). Washed cell pellets were suspended in PBS (1 mL/mg wet mass) with 1% (w/v) detergent and incubated at 60 Cº during 30 or 60 minutes.

Protein measurements

The protein content of the extracts was determined by the Bio-Rad protein assay system, which is detergent compatible at the concentration used in our experiments.

Goat sera and Enzyme-linked immunosorbent assay

The protein fractions immunoreactivity was tested by pooled sera from 10 naturally infected animals confirmed positive by pus culture(positive pool) and 7 animals with no detectable infection (negative pool). Serum from each goat was tested by a highly sensitive and specific (93 and 100% respectively) indirect ELISA using ***C. pseudotuberculosis*** exotoxin (MOURA-COSTA, 2002). All animals from positive pool sera gave high OD values (4 to 10 times ELISA cut off value) and animals from negative pool sera gave much lower OD values than ELISA cut off value.

SDS-PAGE and Immunoblotting analyses

The proteic fractions were separated by one-dimensional polyacrylamide gel electrophoresis under denaturing conditions (LAEMMLI, 1970). We used a discontinuous SDS-PAGE system with a 4% stacking gel and a 10 or 12% running gel. The electrophoresis was carried out in 0,124 M Tris, 0, 96 M Glycine, 0.5% SDS, pH 8.3 migration buffer, for 3 hours at 70-130 V. Each well was loaded with 50 mg of protein. Proteins were visualized by Coomassie Blue R-250 staining or transferred to cellulose acetate membranes (Millipore, USA).

Proteins were transferred to nitrocellulose acetate membranes (Millipore) at 100 V for 1 hour at room temperature as previously described (TOWBIN; STAEHELIN; GORDON, 1979). The transference efficiency

was assessed by Ponceau staining and the membranes were blocked with 5% skimmed milk in 0.05 % PBS-Tween overnight at 4ºC. The membranes were incubated in 1:50 diluted sera in PBS-Tween buffer containing 1 % skimmed milk, for 1 hour at 37ºC. They were then washed 5 times in 0.05% PBS-Tween buffer and incubated for 1 hour with horse-radish peroxidase conjugated rabbit anti-goat immunoglobulins (DAKO A/S, Glostrup, Denmark) diluted 1:100 in PBS-Tween. The membrane strips were washed 5 times in 0.05% PBS-Tween and developed with substrate developer solution (4-Chloro-1-naphtol 0.3% diluted 1:5 in PBS, hydrogen peroxide). The reaction was stopped by a final rinse in distilled water and the strips were dried and photographed.

RESULTS

Secreted antigens

The electrophoretic profiles of the secreted fractions obtained either by the bacterial culture in BHI medium or in synthetic medium (FIGURE 1A) showed a good qualitative homology. However, as expected, the expression rate of some proteins varied according to the culture medium. Therefore, we demonstrated the advantage of a synthetic medium, making the characterisation of a high resolution secreted proteins pattern possible. Separation revealed approximately 20 bands stained by Coomassie blue with molecular mass ranging from 14 to 125 kDa. Proteins with molecular masses 24, 31, 41, 54, 68 and 85 kDa presented higher levels.

The antigenic molecules detected in the secreted fraction displayed molecular masses of 28, 31, 40, 41, 52, 68, 72, 75, 85, 90, 108 and 125 kDa (FIGURE 1B). Only the three bands with molecular masses of 43, 50 and 64 kDa, which were detected in other studied fractions, were not recognized in the secreted fraction (TABLE 3). Finally, no protein from pure and concentrated BHI medium was recognized by naturally infected animal sera (data not shown).

The comparison between the immunoreactive fractions obtained in synthetic medium and in BHI medium did not show any differences regarding the number and characterisation of the main immunodominant bands (FIGURE 1B). Only two proteins (85 and 90 kDa), which appeared to be immunoreactive in the synthetic supernatant, were undetectable in BHI supernatant.

Surface antigens

The 1 M NaCl antigen extraction presented many different protein bands (approximately 30-35 bands), with only partial homology to the protein profile obtained by SDS treatment for 5 min, at 100ºC (FIGURE 2). Based on this result, the SDS treatment at high temperature seemed to be too drastic and probably caused ***C. pseudotuberculosis*** lysis. In the NaCl extract, bands with molecular mass of 24, 43, 50, 53, 72, 75 and 86 kDa were present in highest concentration. The immunoblotting revealed 5 immunodominant bands of 24, 40, 41, 68 and 75 kDa, while higher molecular mass proteins of 90, 108 and 125 kDa were recognized with lower intensity (FIGURE 3).

Somatic antigens

The detergent extraction efficiency was based on the measurement of the amount of protein recovered related to the initial weight of bacterial mass, allowing us to classify the detergents (TABLE 2). The best results were obtained with SDS, followed by DOC and CHAPS. Extending the incubation time from 30 to 60 minutes at 60ºC did not increase the protein recovery with SDS and CHAPS, although a modest increase was obtained with DOC. On the other hand, the extraction conditions at 100ºC during 5 minutes improved the SDS extraction in comparison to the incubation at 60ºC, during 30 or 60 minutes, whereas increasing the SDS concentration from 1 to 2% did not improve the 100ºC, 5 minutes extraction.

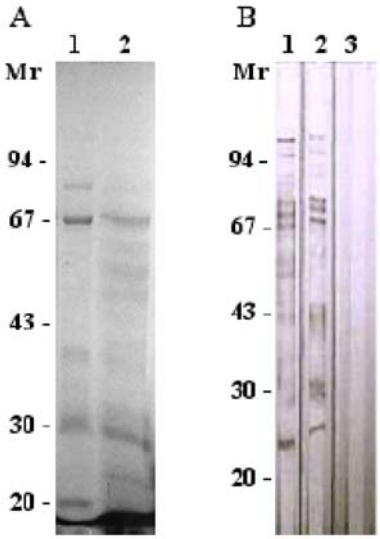

Figure 1A - Coomassie blue stained 10% SDS-PAGE of concentrated supernatant of ***C. pseudotuberculosis*** grown in synthetic medium (1) in BHI medium (2)

Figure 1B - Immunoblot with pooled sera of naturally infected goats

Note: 1. Concentrated supernatant of ***C. pseudotuberculosis*** grown in synthetic medium.
2.Concentrated supernatant of ***C. pseudotuberculosis*** grown in BHI.
3. Concentrated sterilized BHI.

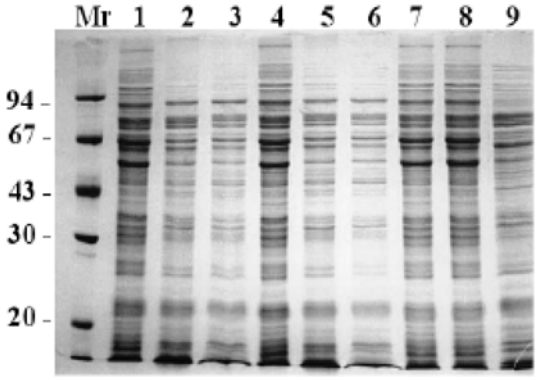

Figure 2 - Coomassie blue stained 12% SDS-PAGE of somatic extracts obtained by different methods and NaCl extract from ***C. pseudotuberculosis***

Note: 1 SDS 1% 60ºC 30 min.
2 DOC 1% 60ºC 30 min.
3 CHAPS 1% 60ºC 30 min.
4 SDS 1% 60ºC 60 min.
5 DOC 1% 60ºC 60 min.
6 CHAPS 1% 60ºC 60 min.
7 SDS 1% 100ºC 5 min.
8 SDS 2% 100ºC 5 min.
9 NaCl 1M.

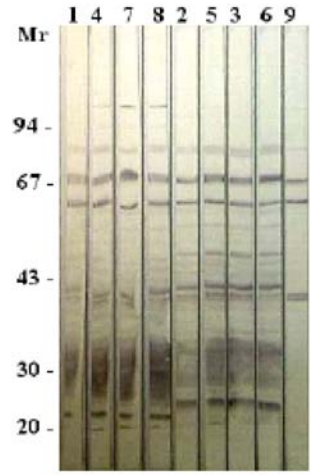

Figure 3 - Immunoblot of somatic extracts obtained by different methods and NaCl extract from ***C. pseudotuberculosis***.

Note: 1 SDS 1% 60ºC 30 min.
2 DOC 1% 60ºC 30 min.
3 CHAPS 1% 60ºC 30 min.
4 SDS 1% 60ºC 60 min.
5 DOC 1% 60ºC 60 min.
6 CHAPS 1% 60ºC 60 min.
7 SDS 1% 100ºC 5 min.
8 SDS 2% 100ºC 5 min.
9 NaCl 1M.

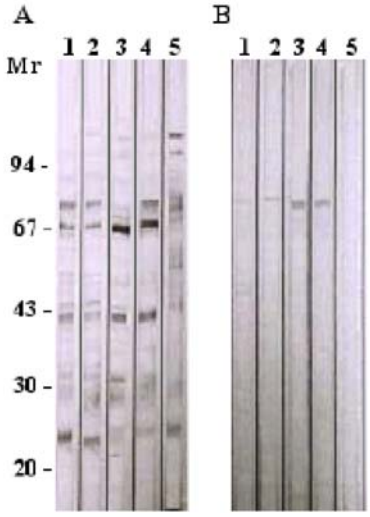

Figure 4 - Immunoblot of different extracts from ***C. pseudotuberculosis.***

Note: ***A*** Seroreativity of pooled sera of naturally infected goats.
B Reactivity of pooled sera of seronegative goats.
1 Somatic extract with DOC (1%, 60º, 60min.)
2 Somatic extract with SDS (1%, 60º,60min.)
3 Somatic extract with ultrasound
4 Surface extract with NaCl 1M
5 Secreted extract.

Table 1 - Secreted and somatic immunogenic structures described by immunoblotting mentioned in several studies

References	Nº of immunogenic bands	Antigens (kDa)
Secreted fraction		
Ter Laak et al. (1992)	6	29; 31; 38; 39; 65; 68
Braithwaite et al. (1993)	4	31,5; 36; 56; 63
Ellis et al. (1991a)	6	20; 25,1; 31,6; 39,8; 63; 68
Walker et al. (1994)	2	31,5; 40
Somatic Fraction		
Ellis et al. (1991b)	10	20; 22; 31,6; 35,5; 39,8; 45,7; 56; 63; 79; 100
Ellis et al. (1991a)	11	12; 25,1; 31,6; 36,3; 39,8; 63; 70; 75; 79,4; 100; 120
Braithwaite et al. (1993)	9	14; 30; 33; 36; 48; 58; 64; 84; 120
Muckle et al. (1992)	7	22; 31,5; 40; 43; 64; 68; 120
Sting et al. (1998)	7	30; 45; 48; 55; 66; 80; 110

Table 2 - ***C. pseudotuberculosis*** somatic proteins extraction recovery according to extraction methodology

Detergents	SDS	DOC	CHAPS	SDS	DOC	CHAPS	SDS	SDS
Concentration (%)	1	1	1	1	1	1	1	2
Incubation (min.)	30	30	30	60	60	60	5	5
Temperature ºC	60	60	60	60	60	60	100	100
Extraction (1)	100	64	53	100	79	56	100	100

(1) Expressed in relative extraction recovery in comparison with SDS treatment

Table 3 - Summary of ***C. pseudotuberculosis*** antigens recognized by sera of naturally infected goats according to the studied fractions

Molecular masses (kDa)	125	108	90	85	75	72	68	64	53	50	43	41	40	31	28	24
Somatic extract by detergentes	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x
Somatic extract by ultrasound	x	x		x		x	x	x	x	x	x	x	x	x	x	
NaCl extract	x	x			x		x	x				x	x		x	
Secreted extract	x	x	x	x	x	x	x		x			x	x	x	x	x

The protein profiles obtained by detergent extractions showed a high qualitative homology between detergents and did not seem to be affected by incubation conditions such as time and temperature (FIGURE 2). However, quantitatively, SDS extraction obtained higher

concentration of proteins with higher molecular masses (>100 kDa). The Coomassie blue staining showed 45 to 50 bands with molecular masses ranging from 14 to 200 kDa. The most intense bands presenting the highest concentration had molecular masses of 14, 28, 31, 33, 53, 64, 68, 75 and 85 kDa.

We observed that the immunogenic somatic fractions varied according to the extraction techniques used. The immunoreactive fractions obtained with the detergent extractions were identical regardless the detergent or incubation conditions applied (FIGURE 3). The sonicated antigen recognition pattern generally followed the extraction pattern obtained by detergents, except in the absence of two immunodominant bands of 24 and 75 kDa (FIGURE 4). We also observed a weakly immunoreactive band of approximately 150 kDa found only in sonicated extract and recognized by the pool of sera from negative animals.

DISCUSSION

In this study, we have compared different techniques to obtain antigenic extracts (secreted, cell-surface and somatic) of ***C. pseudotuberculosis*** and have also described a new method to obtain secreted antigens from the bacterium grown in a synthetic medium. Electrophoretic and western immunoblotting techniques were employed to identify the antigens of ***C. pseudotuberculosis***. We detected 16 immunoreactive bands of 24, 28, 31, 41, 43, 50, 52, 64, 68, 72, 75, 85, 90, 108 and 125 kDa. The 24, 31, 40, 41, 68, 75, 108 and 125 bands were recognized with higher intensity but depending on the fraction studied. ***C. pseudotuberculosis*** secreted proteins SDS-PAGE profile presented here confirms findings from Ellis and others (1991a), who also obtained more than 15 bands from 20 to above 100 kDa by SDS-PAGE, however it diverged regarding the number of proteins recognized by infected animals sera. Beyond the 25, 31, 40, 63 and 68 kDa antigens described by these authors and others (ELLIS et al., 1991b; BRAITHWAITE et al., 1993), we obtained at least 3 more immunodominant proteins of 75, 108 and 125 kDa, all of them present in the cellular fraction. Interestingly, these high molecular masses antigens were detected likewise in ultrafiltrated BHI and would demonstrate that ammonium sulfate precipitation, commonly used for secreted-excreted antigens studies of ***C. pseudotuberculosis*** (ELLIS et al., 1991b; BRAITHWAITE et al., 1993),does not permit a complete immunoreactive proteins pattern. Nethertheless, the study of Ellis and others (1991b) showed that high molecular mass proteins (above 100 kDa) from cellular extract were recognized by serum from culture filtrate vaccinated sheep and would confirm our findings. These authors showed, however, that proteins with low (13-18 kDa) and high (above of 100 kDa) molecular masses were also recognized by serum from animals vaccinated with sterilized BHI medium, whereas, in our case, no BHI medium protein was immunoreactive by immunoblotting when naturally infected animals pooled sera were used.

Although the electrophorectic profile of the NaCl extract is more complex than that obtained by Muckle and others (1992) or Mohan and others (2001), our antigenic recognition results are in agreement with those of Muckle and others (1992) and confirm the importance of the 31, 40 and, especially, the 68 kDa antigens.

The characterization of antigenic proteins from C***. pseudotuberculosis*** somatic fraction solubilized by detergents, proved the higher solubilization capacity of SDS in comparison to the other ionic detergents used. Peculiarly, the higher extraction efficiency was not followed by loss of antigenic recognition in comparison to the extracts obtained with the well-known less denaturing detergents such as DOC and CHAPS (HELENIUS et al., 1979). Moreover, in comparison with the ultrasonic extract, somatic extracts obtained by detergents solubilization presented a higher number of immunoreactive fractions and proved the significance of the extraction methodology used. This may also explain the qualitative variations found in the literature regarding the

immunoreactive bands profile of ***C. pseudotuberculosis***. (MUCKLE et al., 1992; MOHAN et al., 2001)

Finally, the present approach makes clear that the secreted fraction from this bacterium is representative of all humoral response immunodominant antigens. In addition, the technique used to obtain this secreted fraction is simple and made available a purified extract to be used as a tool in future studies involving ***C. pseudotuberculosis*** antigenic fractions. Some previous work developed in our laboratory has already showed that this secreted fraction is very useful for diagnostic purpose like ELISA (MOURA-COSTA, 2002), and is a strong stimulator of cellular immune response in infected goats (REGIS, 2001). Other studies are being carried out to test the ability of this antigen as a component in a protective vaccine.

Análise por SDS-PAGE e Western blot de antígenos somáticos e extracelulares de Corynebacterium pseudotuberculosis

Resumo

Diferentes técnicas para isolar e descrever antígenos das frações secretadas de superfície e somáticas de Corynebacterium pseudotuberculosis foram estudadas por SDS-PAGE e imunoblot, utilizando-se pool de soros de cabras naturalmente infectadas. Os antígenos secretados foram obtidos do sobrenadante de cultura da bactéria cultivada em meio quimicamente definido ou em meio BHI (Brain Heart Infusion). A fração de superfície foi obtida por tratamento com NaCl 1M, e as frações somáticas foram obtidas por vários procedimentos (detergentes e ultra-som). Pela coloração por Coomassie blue, foram detectadas 20 bandas na fração secretada, 35 bandas no extrato de superfície e entre 40 e 50 bandas, a depender do processo de extração, na fração somática. Entre todas as frações estudadas, foram detectadas 16 proteínas imunorreativas. As bandas com pesos moleculares de 125, 108, 75, 68, 41, 40, 31 e 24 kDa foram reconhecidas com maior intensidade e todas elas foram encontradas na fração secretada. A utilização do meio quimicamente definido permitiu revelar, na fração secretada de C. pseudotuberculosis, a presença de proteínas de alto peso molecular que não tinham sido previamente descritas.

Palavras-chave: Corynebacterium pseudotuberculosis***. Cabra. Antígenos. SDS-PAGE. Western blot.***

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Acknowledgements

This work was supported by the Laboratory of Immunology (ICS/UFBA). We thank all the technical staff of the Laboratory of Immunology (ICS/UFBA) and Dr. Silvia Sardi and Dr. Gúbio Soares Campos from Laboratory of Virology (ICS/UFBA) very much indeed for their technical assistance.