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Expression of six possible virulence factors of *C. pseudotuberculosis* in murine macrophage

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ABSTRACT. *Corynebacterium pseudotuberculosis* is a gram-positive bacillus that causes caseous lymphadenitis in goats and sheep. Among its virulence factors are the pld proteins (Phospholipase D), cpp (Serine protease CP40), nanH (Neuraminidase H), sodC (Superoxide Dismutase C), spaC (Adesina SpaC) and pknG (Protein Kinase G). **Objective:** this study aimed to verify the transcription of these genes in macrophages of two lineage of mice infected in vitro with a virulent strain of *C. pseudotuberculosis* (CP1002), through the qRT-PCR technique. In parallel, the expression of pld, cpp, nanH, sodC and spaC genes in these same cells was also studied. **Method:** the total RNA from macrophages of C57BL/6 and Balb/c mice was extracted, and the complementary DNA was synthesized to the extracted RNA. The detection of messenger RNA from PknG, Pld, CP40, NanH, SodC and SpaC was obtained by quantitative PCR. **Results:** the six genes in infected mouse macrophages of Balb/c and C57BL/6 lineages were expressed in vitro in the three-time intervals (1, 12 and 24 hours after infection). The transcription intensity of pknG, pld, cpp, nanH, sodC and spaC genes one hour after infection appears to be greater than in the subsequent intervals, both in Balb/c macrophages and in C57BL/6 macrophages, although in the first cited all the genes show a supposedly accentuated transcript intensity in this interval. The pknG gene appears to have a more intense transcription magnitude than the others in both types of animals. **Conclusion:** the pknG, pld, cpp, nanH, sodC and spaC genes are transcribed into Balb/c and C57BL/6 macrophages, with apparently more pronounced expression in the first hour after infection in both types of cells. All genes appear to exhibit more intense transcription in Balb/c macrophages.

Keywords: *Corynebacterium pseudotuberculosis*, virulence factor, mouse

Avaliação da expressão de seis possíveis fatores de virulência de C. pseudotuberculosis ***em macrófagos murinos***

RESUMO. *Corynebacterium pseudotuberculosis* é uma bacilo Gram-positivo causador da linfadenite caseosa em caprinos e ovinos. Dentre os seus possíveis fatores de virulência estão as proteínas *pld* (Fosfolipase D), *cpp* (Serina-protease CP40), *nanH* (Neuraminidase H), *sodC* (Superóxido Dismutase C), *spaC* (Adesina SpaC) e pknG (Proteína cinase G). **Objetivo:** este estudo buscou verificar, com a técnica qRT-PCR, se existe transcrição destes genes em macrófagos de duas linhagens de camundongos infectados *in vitro* com uma cepa viruleta *C. pseudotuberculosis* (CP1002). Paralelamente,

também foi estudada a expressão dos genes *pld*, *cpp*, *nanH*, *sodC* e *spaC* nestas mesmas células. **Método:** extraiu-se o RNA total de macrófagos de camundongos C57BL/6 e Balb/c e sintetizou-se o DNA complementar ao RNA extraído. A detecção de RNA mensageiro de PknG, Pld, CP40, NanH, SodC e SpaC foi realizada por PCR quantitativa. **Resultados:** houve expressão dos seis genes nos macrófagos de camundongos das linhagens Balb/c e C57BL/6 infectados *in vitro*, nos três intervalos de tempo examinados (1, 12 e 24 horas após a infecção). A intensidade de transcrição dos genes *pknG*, *pld*, *cpp*, *nanH*, *sodC* e *spaC* uma hora após a infecção aparenta ser maior do que nos intervalos posteriores, tanto em macrófagos de Balb/c como em macrófagos de C57BL/6, embora nos primeiros todos os genes mostrem uma intensidade de transcrição supostamente mais acentuada neste intervalo. O gene *pknG* aparenta uma magnitude de transcrição mais intensa do que os demais, em ambos os tipos de animais. **Conclusão:** os genes *pknG*, *pld*, *cpp*, *nanH*, *sodC* e *spaC* são transcritos em macrófagos de Balb/c e de C57BL/6, expressando-se aparentemente de modo mais acentuado na primeira hora após a infecção em ambos os tipos de células. Todos os genes parecem exibir transcrição mais intensa em macrófagos dos animais da linhagem Balb/c.

Palavras chave: *Corynebacterium pseudotuberculosis*, fator de virulência, camundongo

Evaluación de la expresión de seis posibles factores de virulencia de C. pseudotuberculosis en macrófagos murinos

ABSTRACT. *Corynebacterium pseudotuberculosis* es un bacilo grampositivo que causa la Linfadenitis Caseosa en caprinos y ovinos. Entre sus posibles factores de virulencia se encuentra las proteínas *pld* (Fosfolipase D), *cpp* (Serina-protease CP40), *nanH* (Neuraminidase H), *sodC* (Superóxido Dismutase C), *spaC* (Adesina SpaC) e *pknG* (Proteína cinase G). **Objetivo:** este estudio tuvo como objetivo verificar si hay transcripción de estos genes en macrófagos de dos linajes de ratones infectados *in vitro* con una cepa virulenta de *C. pseudotuberculosis* (CP1002) con la técnica qRT-PCR. Paralelamente, también se estudió la expresión de los genes *pld*, *cpp*, *nanH*, *sodC* y *spaC* en estas mismas células. **Método:** se extrajo el RNA total de macrófagos de ratones C57BL/6 y se Balb/c y sintetizó el ADN complementario al ARN extraído. La detección de RNA mensajero de *PknG*, *Pld*, *CP40*, *NanH*, *SodC* y *SpaC* fue realizada por PCR cuantitativa. **Resultados:** hubo expresión de los seis genes en los macrófagos *infectados in vitro* de los ratones de los linajes Balb/c y C57BL/6, en los tres intervalos de tiempo examinados (1, 12 y 24 horas después de la infección). La intensidad de transcripción de los genes *pknG*, *pld*, *cpp*, *nanH*, *sodC* y *spaC* una hora después de la infección parece ser mayor que en los intervalos posteriores, tanto en macrófagos de Balb/c como en macrófagos de C57BL/6, aunque en los primeros mencionados todos los genes muestran una intensidad de transcripción supuestamente más acentuada en este intervalo. El gen *pknG* aparenta una magnitud de transcripción más intensa que los demás, en ambos tipos de animales. **Conclusión:** los genes *pknG*, *pld*, *cpp*, *nanH*, *sodC* y *spaC* se transcriben en macrófagos de Balb/c y C57BL/6, expresándose aparentemente de manera más acentuada a la primera hora después de la infección en ambos tipos de células. Todos los genes parecen exhibir transcripción más intensa en macrófagos de los animales del linaje Balb/c.

Palabras clave: *Corynebacterium pseudotuberculosis*, factor de virulencia, ratón

Introduction

Corynebacterium pseudotuberculosis is the etiologic agent of caseous lymphadenitis, a zoonosis of global occurrence (Benham 1962; Merchant & Packer 1975). The disease affects several domestic animals and shows a high prevalence in Brazil, especially in the goat and sheep herds of the northeastern semi-arid region, with values varying from 9.2% to 72.2% (Meyer et al., 2002).

Caseous lymphadenitis is a disease characterized by the appearance of granulomas that progress to caseous necrosis in superficial and internal lymph nodes, as in some viscera, such as

lungs and liver (Ayers 1977; Arsenault et al., 2003; Al-Gaabary et al., 2009). The economic losses are due to the devaluation of the animals' skin (because of the suppuration of granulomas), condemnation of the carcasses, decrease of the weight and deficiencies in the production of milk and wool (Burrell 1978; Brown et al., 1987; Paton et al., 1988).

The control of caseous lymphadenitis is mainly based on animal vaccination, which in this case is a poorly consolidated procedure, and adoption of complementary measures such as incorporation of new units in the herds, early detection, segregation, treatment or disposal of infected animals, and special care in the routine handling. (Guimarães et al., 2011; Windsor 2011; Kumar et al., 2013). The immunogenic components of the most effective commercial vaccines are attenuated live cells of *C. pseudotuberculosis*, which may be eventually mixed with innocuous forms (toxoids) of phospholipase D, the main virulence factor of *C. pseudotuberculosis* (Carne 1940; Batey 1986; Al-Gaabary et al., 2009).

Additional virulence factors were suggested after the pioneer sequencing of genome of *Corynebacterium pseudotuberculosis* FRC41 by Trost et al., (2010). Analysis of the chromosome of this strain allowed the identification of several genes most likely involved in virulence, such as cpp (CP40), nanH (neuraminidase H), rpfA and rpfB (resuscitation-promoting factors A and B), nor (nitric oxide reductase), dtsR2 (acetyl-CoA carboxylase, beta subunit, involved in mycolic acid biosynthesis) and spaC (SpaC protein, adhesin component). The sodC gene is not listed in the potential virulence determinants of the strain sequenced by Trost et al., (2010), but superoxide dismutase (SodC) is considered a virulence factor by several researchers (Sanjay et al., 2010; Suo et al., 2012; Tonello & Zornetta 2012).

Phospholipase D is an enzyme secreted by *C. pseudotuberculosis* and cpp and nanH genes encode proteins that can also be secreted (Walker et al., 1994; Kim et al., 2010).

Thus, the purpose of this study was to verify if pknG gene, widely conserved in representatives of Actinomycetales (such as *Mycobacterium* and *Corynebacterium*), as well as pld, cpp, nanH, sodC and spaC genes are transcribed into infected murine macrophages in vitro by a virulent strain (CP1002) of *Corynebacterium pseudotuberculosis*.

Material and methods

Cultivation Of Murine Macrophages

C57BL/6 and Balb/c mice were inoculated intraperitoneally with 2mL of sodium thioglycolate (*Brewer's thioglycolate* - SIGMA, USA) and four days later submitted to euthanasia. Peritoneal washes were performed with 10 mL of PBS with 5 mM EDTA (pH 7.4) for recovery of mononuclear cells. These were centrifuged at 500xg for 10 minutes at room temperature and resuspended in 3mL of PBS. The cells were counted by flow cytometry, using the TrueCount (BD Biosciences, USA) system, and placed in DMEM medium with 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Subsequently, the cells were transferred to 24-well plates at a density of $2x10^5$ macrophages/well. After one hour, the wells were washed with PBS and the DMEM medium was added without antibiotics and containing *C. pseudotuberculosis* in an infection ratio of 10 (M.O.I. = 10, multiplicity of infection). One hour later, the wells were washed three times with PBS and added with complete DMEM medium containing antibiotics and incubated for 1 hour, 12 hours or 24 hours at 37 °C, 5% CO_2. Finally, the cells were collected, washed and subjected to RNA extraction with RNAesy Mini Kit (Quiagen).

Corynebacterium Pseudotuberculosis

A virulent *Corynebacterium pseudotuberculosis* strain called CP1002, maintained in exponential growth in BHI medium with 0.1% Tween was used for infection of macrophages.

Rna extraction and Cdna production

RNA extraction was performed using the RNeasy Protect Mini Kit (Quiagen), following the manufacturer´s instructions. The production of DNA complementary to RNA was performed with 3µg/µL of random hexamers (Invitrogen) and 200 U/µL of the enzyme Superscript III Reverse Transcriptase (Invitrogen). Briefly, the steps were as follows: 3 ul of eluate from sample extraction were added to 5.7 µl of ultrapure water, 4 µL of dNTPs and 0.80 µL of random hexamers. This mixture was placed in a thermocycler set at 95º C for 5 minutes, for separation of the RNA strands present in the solutions. In a second tube 4 µl *First Strand Synthesis Buffer*, 1 µl Dithiothreitol, 1 µl RNAse Inhibitor and 0.5 µl Superscript III RT

were mixed. Next, 6.5 µL of this solution was added to the tubes containing the first mix (13.5 µL) after they were removed from the cycler and held in cooler at -4° C for 3 minutes. The extension of the hexamers was done as follows: 10 minutes at 25° C, 30 minutes at 50° C, 15 minutes at 53° C and 10 minutes at 55° C. Reverse transcriptase was then inactivated by incubating the tubes at 70° C for 15 minutes. The cDNA produced was maintained at -80° C until the time of qPCR for the genes studied in this work.

Relative Quantification of Mrna

An exponentially growing liquid culture (BHI with 0.1% Tween) of a virulent *Corynebacterium pseudotuberculosis* strain was homogenized and centrifuged at 6,000g for 5 minutes at 4º C, discarding the supernatant. The pellet formed was preserved in a freezer at -80° C and the nucleic acids were then extracted with the commercial RNeasy Protect Mini Kit (Quiagen). 40 µl of eluate were obtained and an aliquot of this material was used for cDNA production, according to the procedure explained above. Six serial dilutions were prepared from this cDNA using ultrapure water, ranging from 10^{-1} to 10^{-6}. The original solution and the six dilutions were preserved at -80° C until the quantitative reactions were performed. For each of the above-mentioned dilutions, six qPCRs were run using primers for the six genes studied (see Frame 1) plus one pair of primers for the 16S ribosomal RNA gene, used as a normalization control. To each of the 6 tubes containing undiluted cDNA was assigned a fictitious number of 10,000,000 (ten million) molecules of the respective mRNA, corresponding 1,000,000 molecules to the tube with dilution 10^{-1}, 100,000 molecules to the tube with dilution 10^{-2}, 10,000 molecules to the 10^{-3} dilution tube, and so on, up to the number of 10 molecules in the tube containing $^{10-6}$ dilution. After the runs of qPCR, the linear regression analysis was applied to generate a calibration curve for each gene, correlating the logarithm of the number of molecules in ordinates and the Ct (cycle threshold) value in abscissa. In this way, the angular and linear coefficients were obtained for each of the seven straight lines.

These lines were used for the relative quantifications of DNA (and, by extension, mRNA) in the other real-time PCR runs as follows: for each of the seven genes studied, based on the Ct value found in the respective qPCR, it was calculated a quantification factor (corresponding to the number of mRNA molecules), determined as follows: quantification factor = 10W, where w = [coefficient. linear – (Ct x angular coefficient)]. By dividing the values of the quantification factors of the different genes by the respective values of the normalizing gene, the relative amounts of expression of the former are obtained.

Quantitative Pcr

The detection of messenger RNA from PknG was performed similarly to that used for the other genes of this project. The reactions were performed in a final volume of 25 µL, containing: 7.4 µL of RNAse free water, 12.5 µL of *Power Sybr Green Master Mix* (2X) (Applied Biosystems), 1 µL of Bovine Serum Albumin (1 mg / mL), 0.75 µL of each of the 10 µM solutions of the *primers*, *forward* and *reverse*, 0,1 µL of enzyme *Platinum Taq Polymerase* (5 U/µL) and 2,5 µL of cDNA eluates. The primers used are shown in Frame 1. The pairs of primers were drawn with the "Primer Quest" tool on the page *Integrated DNA Technologies*, IDT, available in http://www.idtdna.com/site. The primers selected for pknG aimed to enlarge a gene segment with 250-300 base pairs, with annealing temperature of 64 °C, to allow its use in the same plate with the other primers chosen for this work. The amplification of the targets was performed in the thermal cycler ABI Prism 7500 (Applied Biosystems), under the following time and temperature conditions: initial denaturation for 10 minutes at 95° C, followed by 45 denaturation cycles for 20 seconds at 94° C and annealing for 60 seconds at 62 °C. The result of each qPCR was evaluated through the analysis of the melting curve.

Primers Used

The primers used in this research are listed in Frame 1.

Data Analysis Procedures

After data normalization, a descriptive analysis was performed to compare the expression of the pknG, cpp, pld, nanH, sodC and spaC genes in mouse macrophages.

Frame 1. Primers used in the experiments.

Gene	Sense	Primers sequence
Cpp	Forward	5'-ACG GTA GGC ATA ACT TCC GCT CTT-3'
	Reverse	5'-TCA GCT TGA CCT GGA GCT TGT CTT-3'
Pld	Forward	5'-TGG AAC CTG GAA TCG GAC TTG TGA-3'
	Reverse	5'-TTA TCC ACC CAC CTC TTG ATG GCT-3'
nanH	Forward	5'-TGG CTA AGG CTA TTG AAG ACG CGA-3'
	Reverse	5'-AAT CGT AGC TCC CTC AGC TGC TTT-3'
rna16S	Forward	5'-AGT AAC TGA CGC TGA GGA GCG AAA-3'
	Reverse	5'-CAG GTA AGG TTC TTC GCG GTT GCA T-3'
pknG	Forward	5'-AGC AGA TAT TAC GCA CCC GGG AAT-3'
	Reverse	5'-TTA CCT GGT CCT CGG CAA CAA TGA-3'
sodC	Forward	5'-TTG TCG CGG CCT TCA TGA ACA ATC-3'
	Reverse	5'-CCA TGG CTT CCA CGT TCA TGA CAA-3'
spaC	Forward	5'-AAG CCG TCA ACC GAC TAC CGT TTA-3'
	Reverse	5'-AAT TCA AAC CGG TAG GAG CCA GGA-3'

Ethical aspects

This work was by the Animal Use Ethics Committee of the São Rafael Hospital, Salvador, Bahia, Brazil, under protocol number 06/2012.

Results

The gene expression values normalized by rRNA16S are summarized in Table 1. There was expression of nanH, cpp, pld, sodC and spaC genes in the macrophages of both mouse lineage after 1, 12 and 24 hours of infection. Considering that the calibration curves for all genes were made with cDNA obtained from the pellet of a liquid culture, the expression of these genes in vitro is evident. The intensity of transcription of these 5 genes one hour after infection appeared to be bigger than in subsequent intervals in Balb/c macrophages. In these same cells, there appeared to be no relevant differences in the transcriptional intensity of these genes after 12 and 24 hours of infection. In the macrophages obtained from the C57BL/6 lineage, the transcription of the cpp, pld, nanH, sodC and spaC genes appeared to be independent of the exposure time of the cells to the pathogen. The magnitude of transcription of the pknG gene was apparently higher than that of the other genes in both types of macrophages and in the three-time intervals considered. Even so, its level of transcription dropped and remained practically the same after 12 and 24 hours of infection.

There appeared to be no significant difference in the magnitude of transcription of the cpp, pld, nanH, sodC and spaC genes after 12 or 24 hours of infection in both Balb/c and C57BL/6 macrophages.

Table 1. Relative quantification factors for the expression of cpp, pld, nanH, sodC, spaC and pknG genes in macrophages of Balb/c and C57BL/6 mice, normalized by rna16S gene.

	cpp	*pld*	*nanH*	*sodC*	*spaC*	*pknG*
1BC1	1450	1788	1223	1278	1468	5961
1BC12	262	346	312	370	448	1921
1BC24	331	466	331	394	629	2087
1CC1	624	895	629	838	783	3584
1CC12	433	576	435	567	620	2327
1CC24	370	515	330	429	417	2423

Label: 1BC = Balb/c; 1CC = C57BL/6; 1, 2, 12 = hours after infection.

Discussion

This study reported the expression of six genes (cpp, pld, nanH, sodC, spaC and pknG) in murine macrophages infected with *Corynebacterium pseudotuberculosis.* Studying the lineage J774 of murine macrophages infected with this bacterium, Stefanska et al., (2010) observed that the bacteria could survive in these cells for more than 48 hours, and that the macrophages were lysed. Although our data do not contemplate this time interval, documentation of the expression of these six genes potentially involved in the virulence of the bacterium, even after 24 hours of infection, confirms the role of the genes in the pathogenesis of caseous lymphadenitis. According to a previous study McKean et al., (2007) the expression of the pdd gene had a small but significant influence in the reduction of murine macrophages survival infected with *Corynebacterium pseudotuberculosis*. In addition, the regulation of the expression of this gene is complex and depends on quorum sensing, that is, it is related to the size of the population. Considering that all cultures of the present study were infected with MOIs of 10, the expression of pld quorum sensing-dependent could explain, at least in part, the small fluctuation of the transcription in later stages of infection in both Balb/c and C57BL/6 cells.

The higher transcription of all genes in the first hour after infection, especially in Balb / c macrophages, suggests that in this range the phagocytes have not yet fully mobilized their innate defense mechanisms. C57BL / 6 macrophages had approximately half of the magnitude of gene expression presented by Balb / c macrophage (43%, 50%, 51%, 65% and 53% for cpp, pld, nanH, sodC and spaC genes, respectively), except for sodC. It is known that C57BL/6 mice are more resistant to *Corynebacterium pseudotuberculosis* and this fact could be the reason for this initial difference in expression. Obviously, these data should be viewed with caution, considering the lack of averages for a valid comparison. A bioinformatic study (Trost et al., 2010) on the genome of *Corynebacterium pseudotuberculosis* FRC41 documented the existence of potential binding sites for GlnX, a transcriptional regulator, upstream of the cpp, nanH and spaC genes. It is possible that this transcription factor controls the simultaneous expression of these genes and, if so, the small variation in the transcription intensity of these genes in the later stages of infection can be justified.

In this work, the expression of the pknG gene was documented both in liquid cultures of *Corynebacterium pseudotuberculosis* (BHI medium with 0.1% Tween) and in culture of murine macrophages experimentally infected with the same bacterium. The intense gene expression in both types of macrophages suggests that the enzyme is very important to ensure the success of the infectious process, but it is not possible to make any illusion about the function of the PknG protein in this species of bacteria.

In all mycobacteria sequenced to date, pknG is the last gene of an operon that still harbors the genes Rv0412c (which encodes a possibly conserved membrane protein) and glnH (which encodes a lipoprotein capable of binding glutamine) (Nguyen et al., 2005). Also, in mycobacteria, the function of the PknG protein seems to modulate the activity of the GarA protein, functional equivalent of the OdhI enzyme of *Corynebacterium glutamicum*, involved in the metabolism of glutamine (Pereira et al., 2011). While the intracytoplasmic function of PknG from pathogenic mycobacteria is still discussed, its role in the pathogenesis of infection by interfering with the maturation of phagosomes containing these bacteria seems to be evident (Walburger et al., 2004).

Conclusion

The cpp, pld, nanH, sodC, spaC and pknG genes of *C. pseudotuberculosis* were transcribed both in BHI liquid medium with 0.1% Tween and in macrophages from Balb/c and C57BL/6 mice. Apparently, the intensity of transcription of these genes after one hour of infection was higher in Balb/c macrophages than in C57BL/6 macrophages. The magnitude of pknG gene expression in both types of macrophages seems to be more pronounced than the expression of the other genes in the three-time intervals considered in this study. The transcription of the pknG gene into cultured bacteria in vitro raises the hypothesis that, just as occurs in *Mycobacterium tuberculosis*, the enzyme plays an important role in the metabolism of glutamine.

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