Benedito Rodrigues da Silva Neto
(Organizador)

Pesquisa Científica e Tecnológica em Microbiologia 3

Atena Editora
Ano 2020

Benedito Rodrigues da Silva Neto
(Organizador)

Pesquisa Científica e Tecnológica em Microbiologia 3

Atena Editora
Ano 2020

2020 *by Atena Editora*

Editora Chefe: Profª Drª Antonella Carvalho de Oliveira
Diagramação: Luiza Batista
Edição de Arte: Luiza Batista
Revisão: Os Autores

Atena Editora
Ano 2020

Atena Editora
Ano 2020

Dados Internacionais de Catalogação na Publicação (CIP)
(eDOC BRASIL, Belo Horizonte/MG)

P474 Pesquisa científica e tecnológica em microbiologia 3 [recurso eletrônico] / Organizador Benedito Rodrigues da Silva Neto. – Ponta Grossa, PR: Atena, 2020.

Formato: PDF
Requisitos de sistema: Adobe Acrobat Reader
Modo de acesso: World Wide Web
Inclui bibliografia
ISBN 978-65-5706-143-5
DOI 10.22533/at.ed.435200107

1. Microbiologia – Pesquisa – Brasil. I. Silva Neto, Benedito Rodrigues da.

CDD 579

Elaborado por Maurício Amormino Júnior – CRB6/2422

Atena Editora
Ponta Grossa – Paraná - Brasil
www.atenaeditora.com.br
contato@atenaeditora.com.br

Atena Editora
Ano 2020

APRESENTAÇÃO

Temos o prazer de dar continuidade ao tema de microbiologia inter-relacionado à pesquisa científica e tecnológica iniciado pela editora no ano de 2019. Apresentamos aqui um novo volume deste contexto, denominado “Pesquisa científica e tecnológica em microbiologia, volume 3” contendo trabalhos e pesquisas desenvolvidas em diversos institutos do território nacional contendo análises de processos biológicos embasados em células microbianas ou estudos científicos na fundamentação de atividades microbianas com capacidade de interferir nos processos de saúde/doença.

A microbiologia é um vasto campo que inclui o estudo dos seres vivos microscópicos nos seus mais vaiados aspectos como morfologia, estrutura, fisiologia, reprodução, genética, taxonomia, interação com outros organismos e com o ambiente além de aplicações biotecnológicas. Como uma ciência básica a microbiologia utiliza células microbianas para analisar os processos fundamentais da vida, e como ciência aplicada ela é praticamente a linha de frente de avanços importantes na medicina, agricultura e na indústria.

A microbiologia como ciência iniciou a cerca de 200 anos, entretanto os avanços na área molecular como a descoberta do DNA elevou a um novo nível os estudos desses seres microscópicos, além de abrir novas frentes de pesquisa e estudo. Sabemos na atualidade que os microrganismos são encontrados em praticamente todos os lugares, e a falta de conhecimento que havia antes da invenção do microscópio hoje não é mais um problema no estudo, principalmente das enfermidades relacionadas aos agentes como bactérias, vírus, fungos e protozoários.

Temas ligados à pesquisa e tecnologia microbiana são, deste modo, discutidos aqui com a proposta de fundamentar o conhecimento de acadêmicos, mestres e todos aqueles que de alguma forma se interessam pela saúde em seus aspectos microbiológicos. Portanto a obra propõe uma teoria bem fundamentada nos resultados práticos obtidos em alguns campos da microbiologia, abrindo perspectivas futuras para os demais pesquisadores de outras subáreas da microbiologia.

Assim, desejo a todos uma ótima leitura!

Benedito Rodrigues da Silva Neto

SUMÁRIO

CAPÍTULO 5

Corynebacterium pseudotuberculosis PROTEIN EXTRACT INDUCES IP10 PRODUCTION IN BLOOD SAMPLES OF INDIVIDUALS WITH PULMONARY TUBERCULOSIS

Data de aceite: 01/06/2020

Submission date: 05/03/2020

Rogério Reis Conceição
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/1968670876252647
rogerioreisconceicao@gmail.com

Samanta Queiroz dos Santos
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/9663458541454602

Zunara Victória Santana Batista
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/6616432566735398

Ramon Mendes dos Santos
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/2239694896220013

Silvânia Maria Andrade Cerqueira
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/6382393685853768

Caio Lopes Borges Andrade
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/3027186212966797

Soraya Castro Trindade
Universidade Estadual de Feira de Santana
Salvador-Bahia
http://lattes.cnpq.br/4927186541075656

Fúlvia Soares Campos de Sousa
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/0611165561750495

Lilia Ferreira de Moura-Costa
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/6672994596587713

Marcos Borges Ribeiro
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/7634265862645053

Roberto Meyer
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/9574426480028427

Songelí Menezes Freire
Universidade Federal Bahia
Salvador-Bahia
http://lattes.cnpq.br/8634866589762862

ABSTRACT: *Mycobacterium tuberculosis* and *Corynebacterium pseudotuberculosis* are part

of the CMRN group and share similarities among some virulence/pathogenicity factors. The objective of the present study was to use semi-purified extracts of *C. pseudotuberculosis* strains to evaluate antigenicity in samples of individuals diagnosed with active pulmonary tuberculosis. Voluntary participants with positive tuberculosis (TB) diagnosis and treatment-naïve tuberculosis (n=28), without complaints, no history or symptomatology of TB or *M. tuberculosis* infection (n=28). With heparinized blood, cell stimulation was carried out using mitogen (PWM) and *C. pseudotuberculosis* protein extract (PAT-10, VD57 and FRC-41 strains). For cytokines measurement, BD Cytometric Bead Array (CBA) was used. Semi-purified extracts of all three *C. pseudotuberculosis* strains induced production of cytokines in blood samples from studied groups. In addition, they differentiated studied groups when compared to IFN-γ and IP-10. *C. pseudotuberculosis* strains extracts PAT10, VD57 and FRC41 induced production of IFN-γ, IP-10 in blood samples from individuals infected with *M. tuberculosis*. Under stimuli of extracts used, the detection of IFN-γ and IP-10 differentiated the control group and active TB groups.
KEYWORDS: *Corynebacterium Pseudotuberculosis*; Cytokines; *Mycobacterium Tuberculosis*; Tuberculosis; Antigenicity

1 | INTRODUCTION

Several scientific researches are carried out to identify and obtain reagents that make up laboratory diagnostic kits, and for that, molecules (antigens) of the infectious agents are necessary for evaluation of the host response with *in vivo* or *in vitro* models. Some biomolecules can be shared by different biological agents, such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Corynebacterium pseudotuberculosis*, which can be used in cross-reactive studies, to evaluate immune response and diagnostic tests [1] and it's cost benefit. The diagnosis of *M. tuberculosis* infection should be made clinically and by screening methods. When suggestive, it should be confirmed by laboratory methods, with agent identification, such as in sputum microscopy and molecular tests [2]. In a country with a high incidence rate, such as Brazil, despite the current technological resources used to control tuberculosis, it could be considered necessary to seek innovative strategies regarding diagnostic methods and immunoprophylaxis, aimed at the early diagnosis of TB cases, and its effective treatment, since rapid and safe diagnosis, accompanied by early treatment, are fundamental measures to break the transmission chain and control tuberculosis [3]. On the other hand, the infection by *C. pseudotuberculosis* that compromises superficial and deep lymph nodes has a course of infection that may involve respiratory organs in goats and sheep presents an infectious model similar to that of human tuberculosis [4]. Additionally, due to the continuously discussed similarities between some virulence / pathogenicity factors of these bacterias, the objective of this study was to use semi-purified extracts of *C. pseudotuberculosis* strains to investigate their *in vitro* antigenic

capacity, in the cellular immune response by cytokines release as a model proposed with samples from individuals with active pulmonary tuberculosis.

2 I MATERIALS AND METHODS

2.1 Ethical aspects and Selection of Study Participants

This study was approved by the Research Ethics Committee of the Institute of Health Sciences - CEP-ICS / UFBA (CAAE nº 57662016.8.1001.5662). The study population consisted of 56 volunteers from the Bahia lung disease specialized Hospital and a clinical laboratory in a public University of Bahia through march-june 2017 categorized into two groups. Group 1 (control, n = 28): Individuals without complaints, history or symptoms of pulmonary TB and without M. tuberculosis infection (negative Tuberculin Skin Test / non-reactive TST) and with normal white blood cell count (WBC). Group 2 (active pulmonary TB, n = 28): Individuals with TB diagnosis, as recommended by the Ministry of Health protocols performed at the service unit, (Positive Molecular Rapid Test (GeneXpert®) without resistance to rifampicin, positive smear microscopy and / or culture of BK positive) and with normal WBC. Samples from this group were collected before beginning treatment. Samples of participants who reported contact or consumption of derivatives (meat and / or raw milk) of small ruminants were not included in the analysis avoiding research vies by cross reaction.

2.2 Production of extracts of *Corynebacterium pseudotuberculosis*

Three strains of *C. pseudotuberculosis* were used: strain PAT10, of a pulmonary abscess in a sheep from Argentina Patagonia [5], strain VD57 isolated from a granulomatous lesion in a goat of the municipality of Juazeiro, State of Bahia [6] and strain FRC41, isolated from the inguinal lymph node of a 12-year-old girl with necrotizing lymphadenitis in France [7]. The cultures in Brain Heart Infusion (BHI), the three-phasepartitioning (TPP) technique was performed, according to [8], with minor modifications. Protein dosage was performed using Bio-Rad Protein Assay (Lowry).

2.3 Blood collection

Samples of blood were obtained into heparinized tube to perform the *in vitro* cell stimuli and in EDTA tube for WBC Counting.

2.4 *In vitro* stimulation with *Corynebacterium pseudotuberculosis*

In a sterile 24-well plate for cell culture, 0.5 ml of blood per well was added to each extract at a protein concentration of 25ug / ml, as established in previous standardization.

The pokeweed (PWM) was employed at a concentration of 5ug / ml as a positive control. Pure blood and stimulus samples were incubated at 37°C for 24 hours. The supernatants obtained by centrifugation were immediately stored at -20°C for subsequent cytokine dosing.

2.5 Dosing of cytokines

The cytokines IFN-γ dosage were performed by the Human Th1 / Th2 / Th17 Cytokine Kit (BD®) and IP-10 was dosed by the Human IP-10 CBA Flex Set Kit (BD®), both following the manufacturer's recommendations. Acquisition was predetermined for 3,000 events in FACSCalibur-BD, with Cell Quest Pro Software (BD®). Analysis were done in the FCAP Array ™ software.

2.6 Statistical analysis

For the data normality analysis, the Kolmogorov-Smirnov test was used. T-test was used to analyze cytokine data with parametric (normal) distribution and Mann-Whitney test for nonparametric (abnormal) distribution. Values of $p < 0.05$ were considered statistically significant. Data obtained in the study were analyzed in GraphPadPrism v 5.0 software.

3 I RESULTS

Epidemiological characteristics of the studied population are described in table 1. It is observed that female participants are in a higher proportion than the male participants in the control group, unlike the TB group in which there is an equivalent proportion of both sexes.

Variable		Control (N=28)	TB (N=28)
Sex	Female	20 (71,5%)	14 (50,0%)
	Male	8 (28,5%)	14 (50,0%)
Age	Mean (Interval)	35 (20 – 63)	36 (19 – 55)
Observation of BCG scar	Yes	24	22
	No	4	6
TB contact	Domicile	3	7
	Work	4	8
	Other	1	5
	No Contact	20	8

Table 1 - Clinical-epidemiological characteristics of the study population.

Figures 1 and 2 show the median concentrations of IFN-γ and IP-10 cytokines in the whole blood samples after in vitro stimulation in the two study groups.

In the control group, the median concentrations of IFN-γ without stimulus (S / EST), basal condition, were zero and the value of minimum and maximum of 0.0 - 20.61. There

was statistical significance in the intragroup comparison with the stimuli PAT10, VD57, FRC41 (p <0.0001). In the TB group, the median IFN-γ S / EST concentrations were 4.18 pg / mL and the minimum and maximum values of 0.0-31.88, in the intragroup comparison, significance was obtained in the stimuli VD57 (p = 0.0114) and FRC41 (p = 0.0062). In both groups the medians of the cytokine dosed under all stimuli were larger than the basal. In the intergroup analysis there was significance between S / EST (p = 0.0045), PAT10 (p = 0.0050) and VD57 (p = 0.0007). There is a lower concentration of IFN-γ dosed in the different stimuli of the TB group, in relation to the control group, except in basal condition (Figure 1).

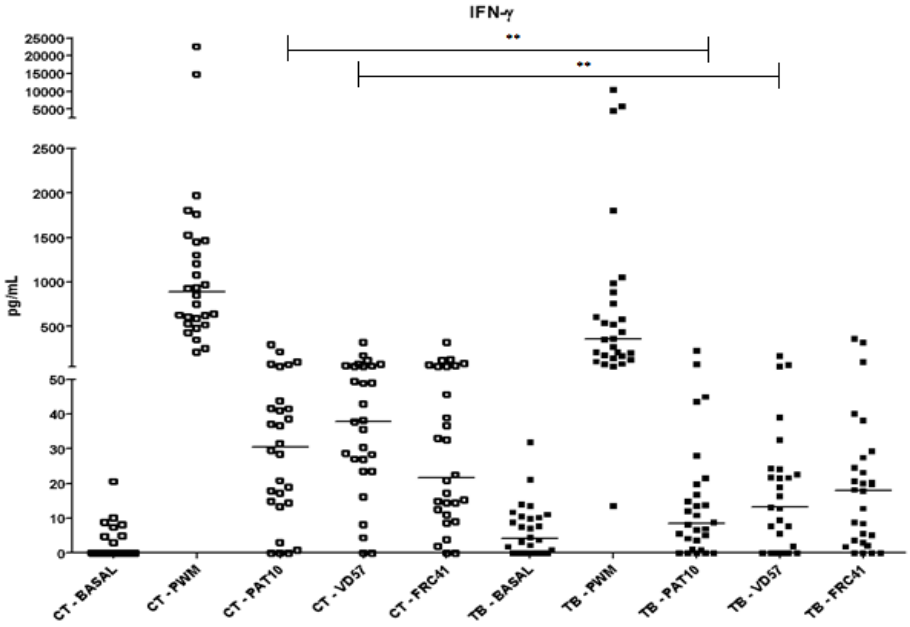

Figure 1 - IFN-γ in peripheral blood stimulated in vitro for 24 hours with extracts antigen secreted by *C. pseudotuberculosis* strains. The median value is expressed in the horizontal line. Being: Control / CT (n = 28) and Tuberculosis / TB (n = 28). PWM: pokeweed; PAT10, VD57, FRC41: extracts of the strains PAT10, VD57 and FRC41 of *C. pseudotuberculosis*, respectively. Pre-determined acquisition of 3000 events with the Cytokine-BD Human Th1 / Th2 / Th17 Kit using Cell Quest Pro-BD® Software. Analysis software: FCAP Array BD®. Statistical analysis: In-group: Basal X CT-PAT10, CT-VD57, CT-FRC41 (all p <0.0001), BASAL X TB-VD57 (p = 0.0114), X TB-FRC41).

The median concentrations and the minimum and maximum values of IP-10 in the control group were 252.2pg / mL and 26.11 - 2806, respectively. The median IP-10 concentrations of basal blood in the control group were lower than the median values under stimulus, PAT10, VD57, FRC41 (p <0.0001). In the TB group, basal median was 877.9pg / mL and minimum and maximum values were 101.6 - 7799. IP-10 concentrations were higher under stimulation with PAT10 (p = 0.0044), VD57 (p <0.0001) and FRC41 (p = 0.0032) than from basal. The IP-10 concentrations of the TB group were higher in relation to the control group. There is a difference of the IP-10 dosed between the CTxTB groups in the conditions BASAL (p <0.0001), PAT10 (p = 0.0470) and FRC41 (p = 0.0044) (Figure 2)

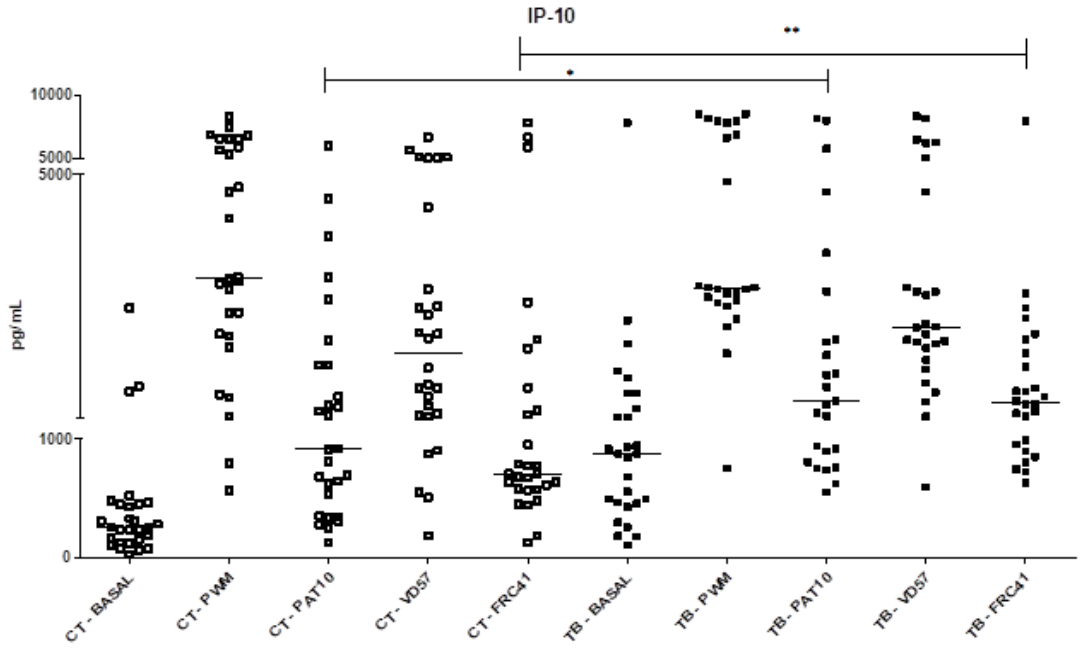

Figure 2 -IP-10 in peripheral blood stimulated for 24 hours with antigenic extract secreted by *C. pseudotuberculosis* strains. The median value is expressed in the horizontal line. Being: Control / CT (n = 28) and Tuberculosis / TB (n = 28). PWM: pokeweed; PAT10, VD57, FRC41: extracts of the strains PAT10, VD57 and FRC41 of *C. pseudotuberculosis*, respectively. Pre-determined acquisition of 3000 events with the BD ™ CBA Human IP-10 Flex kit using Cell Quest Pro-BD® software. Analysis software: FCAP Array BD®. CT-PAT10, CT-VD57, CT-FRC41 ($p < 0.0001$); TB-VD57 ($p < 0.0001$) TB-FRC41 ($p = 0.0032$). The continued bar presents statistical analysis of the difference between groups (CTxTB).

4 I DISCUSSION

The profile of the effective immune response against *M. tuberculosis* is Th1, IFN-γ is the main cytokine involved in this context, but the action of the cytokine alone is not sufficient to promote protection against this bacillus, and thus the contribution of components cell and other cytokines are required to promote effective protection, such as TNF, IL-2 and IL-6 [9]; [10]. Chemokines such as IP-10 also aid the Th1 response profile. On the other hand, the development of Th2 profile involves IL-4 and other cytokines, which act to inhibit the Th1 response against *M. tuberculosis*. IL-10 exerts functions similar to IL-4 [9]; [11]. The antigenic potential of the *C. pseudotuberculosis* semi-purified extracts from strains PAT10, VD57 and FRC41 was demonstrated by the measurement of higher concentrations of cytokines IFN-γ, IP-10, TNF, IL-2, IL-6, IL-10 and IL-17, after stimulation in the study subjects, with the exception of IL-4 in the control group. Increased production of these cytokines when stimulated with *M. tuberculosis* specific antigens was observed in the works of [12] and [13]. Rebouças et al (2011) [14], using *C. pseudotuberculosis* semi-purified antigens in samples of small ruminants with caseous lymphadenitis, showed higher levels of IFN-γ in the group of infected animals after stimulation. PAT10 extract was able to differentiate study groups when IFN-γ concentrations were compared. Although it is found in small amounts, IFN-γ is currently used as a biomarker for TB [15].

Under antigenic stimuli, lower IFN-γ levels were measured in the TB group (Table 1) compared to the control group. This may have occurred because of the short incubation time, already in the presence of mycobacterial antigens, IFN-γ remained at undetectable concentrations before the sixth day of stimulation[16]. In addition, IFN-γ production in response to a specific *M. tuberculosis* antigen appears to decrease in untreated TB individuals [17] which may have occurred in the present study.

Evaluating the cytokine profile in peripheral blood of patients with active TB and healthy contacts, in response to the *M. tuberculosis* 30-Kd antigen, Torres et al. (1998) [18] found lower concentrations of IFN-γ in the TB group. The authors have suggested that IFN-γ plays a protective role in healthy contact individuals. Deenadayalan et al (2010) [19] demonstrated the same results as in this present study.

Similarly to the IFN-γ concentrations, IP-10 concentrations were able to differentiate the study groups, however with higher concentrations in the TB group. Thus, IP-10 may be a useful biomarker to monitor the efficacy of therapy in patients with active TB[20], since this molecule exists in a larger amount of serum which would facilitate its laboratory dosage and clinical interpretation [21]; [22]. Several studies have shown high production of IP-10 in individuals infected with *M. tuberculosis*, allowing the development of updated and simplified test platforms [15].

The VD57 antigen was able to differentiate study groups when compared to the concentrations of IFN-γ. The FRC41 antigen, following the previous comparison, led to the differential production of IP-10 between the groups. The VD57 antigen stimulation allowed the differentiation of the groups when evaluating the concentrations of IFN-γ. The culture with FRC41 antigen culminated in the differential production of IP-10 between the groups.

These extracts have potential for use in an immunodiagnostic kit, although they require complementary studies in order to identify which components of the protein extracts interact with the immune system stimulating the production of the cytokines.

4.1 Acknowledgment

Laboratory of immunology and molecular biology of UFBA and CAPES.

5 I CONFLICT OF INTEREST DISCLOSURE

There are no conflicts of interest.

REFERENCES

1. DORELLA, F. A. et al. ***Antigens of Corynebacterium pseudotuberculosis and prospects for vaccine development.*** Expert Reviews in Vaccine, 8(2):205-213, 2009.

2. FERRI A. O., et al. ***Diagnóstico da tuberculose: uma revisão.*** Revista Liberato. 15(24): 105 – 212, 2014.

3. PEREIRA, A. A. et al. ***Gestão e gerenciamento dos níveis hierárquicos do programa nacional de controle da tuberculose.*** IJHE-Interdisciplinary Journal of Health Education, v. 1, n. 1, 2016.

4. DORELLA, F. A. et al. ***Corynebacterium pseudotuberculosis: microbiology, biochemical properties, pathogenesis and molecular studies of virulence.*** Veterinary research, v. 37, n. 2, p. 201-218, 2006.

5. CERDEIRA, L. T. et al. ***Whole-genome sequence of Corynebacterium pseudotuberculosis PAT10 strain isolated from sheep in Patagonia, Argentina.*** Journal of bacteriology, v. 193, n. 22, p. 6420-6421, 2011.

6. ALMEIDA, S. et al. ***The genome anatomy of Corynebacterium pseudotuberculosis VD57 a highly virulent strain causing Caseous lymphadenitis.*** Standards in genomicsciences, v. 11, n. 1, p. 29, 2016.

7. TROST, E. et al. ***The complete genome sequence of Corynebacterium pseudotuberculosis FRC41 isolated from a 12-year-old girl with necrotizing lymphadenitis reveals insights into gene-regulatory networks contributing to virulence.*** BMC genomics, v. 11, n. 1, p. 728, 2010.

8. PAULE, B. J. A. et al. ***Three-phase partitioning as an efficient method for extraction/concentration of immunoreactive excreted–secreted proteins of Corynebacterium pseudotuberculosis.*** Protein expression and purification, v. 34, n. 2, p. 311-316, 2004.

9. VAN CREVEL, R; OTTENHOFF, T. H. M; VAN DER MEER, J. W. M. ***Innate immunity to Mycobacterium tuberculosis.*** Clinical microbiology reviews, v. 15, n. 2, p. 294-309, 2002.

10. KHANNA, M; SRIVASTAVA, L. M; KUMAR, P. ***Defective interleukin-2 production and interleukin-2 receptor expression in pulmonary tuberculosis.*** The Journal of communicable diseases, v. 35, n. 2, p. 65-70, 2003.

11. ROOK, W.; GRAHAM, A. ***Th2 cytokines in susceptibility to tuberculosis.*** Current molecular medicine, v. 7, n. 3, p. 327-337, 2007.

12. KELLAR, K. L. et al. ***Multiple cytokines are released when blood from patients with tuberculosis is stimulated with Mycobacterium tuberculosis antigens.*** PLoS One, v. 6, n. 11, p. e26545, 2011.

13. YOU, E. et al. ***Evaluation of IL-2, IL-10, IL-17 and IP-10 as potent discriminative markers for active tuberculosis among pulmonary tuberculosis suspects.*** Tuberculosis, v. 99, p. 100-108, 2016.

14. REBOUÇAS, M. F. et al. ***Corynebacterium pseudotuberculosis secreted antigen-induced specific gamma-interferon production by peripheral blood leukocytes: potential diagnostic marker for caseous lymphadenitis in sheep and goats.*** Journal of veterinary diagnostic investigation, v. 23, n. 2, p. 213-220, 2011.

15. AL-ATTIYAH, R.; EL-SHAZLY, A.; MUSTAFA, A. S. ***Comparative Analysis of Spontaneous and Mycobacterial Antigen Induced Secretion of Th1, Th2 and Pro Inflammatory Cytokines by Peripheral Blood Mononuclear Cells of Tuberculosis Patients.*** Scandinavian journal of immunology, v. 75, n. 6, p. 623-632, 2012.

16. RUHWALD, M.; RAVN, P. ***Biomarkers of latent TB infection.*** Expert review of respiratory medicine, v. 3, n. 4, p. 387-401, 2009.

17. SAHIRATMADJA, E. et al. ***Dynamic changes in pro-and anti-inflammatory cytokine profiles and gamma interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment.*** Infection and immunity, v. 75, n. 2, p. 820-829, 2007.

18. TORRES, M. et al. ***Cytokine Profiles for Peripheral Blood Lymphocytes from Patients with Active Pulmonary Tuberculosis and Healthy Household Contacts in Response to the 30-Kilodalton Antigen of Mycobacterium tuberculosis.*** Infection and immunity, v. 66, n. 1, p. 176-180, 1998.

19. Deenadayalan et al (2010)

20. HONG, J. Y. et al. ***Efficacy of IP-10 as a biomarker for monitoring tuberculosis treatment.*** Journal of Infection, v. 68, n. 3, p. 252-258, 2014.

22. RUHWALD, M. et al. ***CXCL10/IP-10 release is induced by incubation of whole blood from tuberculosis patients with ESAT-6, CFP10 and TB7. 7.*** Microbes and infection, v. 9, n. 7, p. 806-812, 2007.

22. WON, E. et al. ***Biomarkers for discrimination between latent tuberculosis infection and active tuberculosis disease.*** Journal of Infection, v. 74, n. 3, p. 28

ÍNDICE REMISSIVO

M

P

R

S

T

V


Atena
Editora
2020