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Research Article

Granulomatous Natural Swine Pleuroneumonia in Farms of Aguascalientes, Mexico

Alma Lilian Guerrero-Barrera[1]*, Claudia Karina Prieto-Macias[1], Víctor Manuel Loera Muro[1], Juan Manuel Díaz Villaseñor[1], Francisco Javier Avelar-Gonzalez[2], Jairo Mariel Cárdenas[3], Édgar Eduardo Hernández-Cuéllar[1], Francisco Javier Gutiérrez-Cantú[3], and Adriana Cecilia Moreno-Flores[1]

[1]Departamento de Morfologia, Centro de Ciencias Basicas, Universidad Autonoma de Aguascalientes, Mexico

[2]Departamento de Fisiologia y Farmacologia, Centro de Ciencias Basicas, Universidad Autonoma de Aguascalientes, Mexico

[3]Facultad de Estomatologia, Universidad Autonoma de San Luis Potosi, Mexico

***Corresponding Author**: Alma Lilian Guerrero-Barrera, Laboratorio de Biologia Celular y Tisular, Edificio 203, Departamento de Morfologia, Centro de Ciencias Basicas, Universidad Autonoma de Aguascalientes, Av. Universidad, Aguascalientes, Mexico.

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Abstract

Through a random sampling made in 60 pigs slaughtered in San Francisco de los Romo, Aguascalientes, Mexico, lesions characteristic of pleuropneumonia was found in 40 animals (66.6 %). From samples of lung tissue and necrotic lesions fribrin-hemorrhagic, *Actinobacillus pleuropneumoniae* (AP) was isolated and biochemically characterize in 55% (22 pigs) of affected animals. In all cases the presence of AP was confirmed by PCR using gene-specific primer of the Apx toxin IV. Three samples from healthy animals were used as negative control. Sections of 4 μm stained with H-E from injured lungs showed haemorrhagic lesions, thickening of alveolar septa and granulomes associated with bronchi and bronchioles. Through Masson trichromic and Wilder staining (specific for reticular fibers, type III collagen), was showed light to moderate fibrosis in the injured tissue. Using PAS staining was observed disruption of alveolar septa and the presence of the pathogen. Immunolocalization of AP was additionally performed using immunohistochemistry technique with a polyclonal antibody, prepared from a total protein extract of AP biotype 1 strain 4074. Finally, composition of granulomes was determined in these lesions by immunohistochemical technique using specific antibodies. Granulomes observed were not tuberculous and presented macrophages, T cells, activated B cells and epitheloid cells, giant cells rare, but not neutrophils or calcium precipitation.

Keywords: *Actinobacillus pleuropneumoniae; Granulomatous Pleuropneumonia; Granulomes; No Tuberculous Granulomes*

Abbreviations

AP: *Actinobacillus pleuropneumoniae*; PRRS: Porcine Reproductive and Respiratory Syndrome; SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Introduction

Pig meat consumption ranks third in the world, after the beef and chicken [1,2]. The pig meat industry in Mexico occupies the third place and it is economically affected by pig health problems. The main pig health problems are the respiratory diseases, do they cause high mortality and morbidity, decreasing in production and high costs in health care, vaccination and therapeutic treatments. The high mortality and morbidity produced by these diseases also provokes high cost in the introduction of control systems [3].

Swine respiratory diseases are known as Porcine Reproductive and Respiratory Syndrome (PRRS) and are provoked by both bacteria and virus [4]. One of the most devastating bacterial diseases of PRSS is the swine pleuropneumonia [5,6] produced by the Gram-negative bacteria *Actinobacillus pleuropneumoniae* (AP) that belongs to the Pasteurellaceae family.

Swine pleuropneumonia occurs in several stages from acute to chronic [7-10]. The acute phase is characterized by necrotizing haemorrhagic pneumonia, with fibrinous pleuritis associated. The chronic phase is characterized by tissue destruction, active lung inflammation and tissue encapsulation [11-13].

This last pig pleuropneumonia stage results in decreases feed conversion and also produces animals infected without symptoms of disease. Therefore, chronic pleuropneumonia is considered one of the main stages for spread the pathogen. In this stage, the specific and no specific immune response is waiting for being characterized [8,14-16].

In the no specific immune response, the bacterial resistance to phagocytosis favours the persistence of these pathogens for long periods, which induces chronic antigenic stimulation, activation of T cells and macrophages. This causes the formation of tissue encapsulation surrounding microorganisms. In chronic infections such as tuberculosis, this tissue encapsulation corresponds to granulomas [17-19].

Granulomas are constituted by the focal concentration of activated macrophages and large histiocytes or epithelioid cells, with an increased phagocytic capacity and digestion of strange particles [17-19]. These macrophages aggregate form multinucleated giant cells, and sometimes have other cellular components such as lymphocytes and fibroblasts. There are two granuloma types: tuberculous, produced by *Mycobacterium tuberculosis*, or not tuberculous, produced by another infectious agent. The granuloma type depends of the elapsed time from the onset of infection, the infection agent that produces it, and of its specific cell composition. Tuberculous granulomas contain caseous necrosis that has tissue with a cheese-like consistency, but in no tuberculous granulomas this structure is absent. In porcine pleuropneumonia [12], observed in infected lung, encapsulation tissue formation that resembled granulomas.

In *A. pleuropneumoniae* experimental infections, Henning and his team (1998) [15] found the formation of granulomas on the seventh day post-infection and the presence of bacteria in tonsil and Broncho-alveolar lavage. Ohba and collaborators in 2009 [18] found 0.16 % of granulomatous pleuropneumonia in 14818 slaughtered-pigs. In Aguascalientes, Mexico there is no information about granulomatous pleuropneumonia. This study found the presence of no tuberculous granulomas associated with chronic swine pleuropneumonia in animals naturally infected from Aguascalientes farms, Mexico. The bacteria were isolate from lessons and identified by PCR using as probe the gen for Apx IV toxin species specific.

Material and Methods

Isolation of field strains

A random sample of 60 pigs was done in the municipal slaughterhouse of San Francisco de los Romo, Aguascalientes, where pigs are slaughtered both: Aguascalientes state and neighbouring states. In 66.6 % of these pigs (40 animals) lesions characteristic of swine pleuropneumonia caused by *A. pleuropneumoniae* (AP) were detected. From samples of lung tissue with characteristic lesions, AP was isolated in 22 (55 %) of the 40 pigs affected. Three uninjured pig samples were used as negative control. The bacteria isolation was based haemorrhagic and necrotic lesions [20] on blood agar medium (Bioxon), in the presence of β-haemolytic *Staphylococcus aureus* [20,21]. The culture was incubated anaerobically at 37°C for 24h. CAMP positive colonies were seeded in BHI agar (brain-heart infusion, Bioxon) [22], in the presence and absence of V growth factor (NAD, 10 ug/ml) and incubated anaerobically at 37°C. These strains were characterized biochemically with Api20 NE gallery system (bioMerieux). The results were interpreted with Api Lab Plus (bioMerieux) program. The isolates did not require NAD for growth, so were identified as biovar 2 AP.

Strains identity was confirmed by nested PCR, using specific primer as proposed for the Apx toxin gene IV by [23].

PCR assays to confirm the detection of *A. pleuropneumoniae*

PCR reaction mixture was prepared with the following components; 100 ng of DNA from AP, 2.5 µl 10X buffer, 2.5 µl $MgCl_2$ 50 mM, 0.2 U Taq polymerase (Fermentas), 1 µM of each primer; APXIVA Forward (5' TGGCACTGAACGGTGATGAT 3') and APXIVA-Reverse (5' GGC-

CATCGACTCAACAT 3'), 0.5 mM of dNTP's and H_2O for complete 25 µl total. A second reaction mixture was prepared as described previously using 2 µl of the above PCR product in place of the DNA of the strain, and using the oligonucleotides APXIVAN-Forward (5'GGGGACGTAACTCGGTGATT3') and APXIVAN-Reverse (5' GCTCACCAACGTTTGCTCAT 3') for nested PCR. Conditions for the nested PCR were those described by Frey (2003) [23]. The PCR products were analysed by electrophoresis using 1.5 % (w/v) agarose and stained with ethidium bromide (1 µg/ml).

Images of the gels were captured using the Chemi Doc (Bio-Rad) image analyser and the software Quantity One (Bio-Rad).

Preparation of antibodies Against *Actinobacillus pleuropneumoniae* biotype 1 serotype 1 strain 4074

The preparation of specific polyclonal antibodies directed against total proteins of AP 4074, was performed according to Harold and Lane (2014) [24]. First total protein extracts of the bacteria were prepared by the method of Guerrero Barrera and collaborators, 1999 [25]. The extract was separated by 10%, SDS-PAGE, using 50 µg per lane, and was stained with Coomassie blue. Each lane was cut and pulverized with a syringe plunger for insulin, and mixed with complete Freund's adjuvant (Sigma), prior to being injected subcutaneously into a female rabbit weighing 2.5 kg. Before to the first inoculation, a blood sample of 3 ml was taken to obtain preimmunise serum. One week after, a second subcutaneous injection was performed, mixing the total extract with incomplete Freund's (Sigma) adjuvant; then two inoculations were performed with space of a week each, solubilizing the bacteria total extract in aluminium hydroxide. Finally, a last inoculation was carried out intraperitoneally using aluminium hydroxide as adjuvant. In the end of inoculation scheme, total blood collection by cardiac puncture was done. Immune serum was separated, and was adsorbed with a total extract of *Escherichia coli*. Antibodies titer was performed using Western blot method, obtaining a titer of 1:5000.

Tissue processing for staining used in the study

The pig lung samples, from both healthy and infected lung tissue, were fixed in neutral formalin (10%, pH 7.2) and processed for paraffin inclusion technique using a Lab -Tech Histoquinet II, and sections of 4 µm were obtained. Haematoxylin-Eosin staining (H-E), Masson trichrome staining, Wilder and PAS (periodic acid- Schiff) staining were done. All histological stains except PAS, were conducted in accordance with the Manual of Histochemistry of the Armed Forces [26], PAS technique was done in accordance with the Sigma Manual.

Immunohistochemistry

Histological sections (4 µm) were processed by immunohistochemical technique. To detect AP, as primary antibody was used the polyclonal antibody raised against total proteins of strain 4074 AP, biovar 1, serotype 1, as described above. The tissues were subjected to de-waxing, endogenous peroxidase neutralizing with hydrogen peroxide at 3.0 % for 30 min, moisture, antigen-retrieval with 0.01M sodium citrate for 30 min in a pressure cooker, according to the method of Hayat (2002) [27]. These samples were blocked with bovine serum albumin 0.1 % (Sigma) for 1h at room temperature. The first antibody was used in 1: 5000 dilutions, it was incubated at room temperature for 2h, after time, three washes with phosphate buffer solution (1X PBS) were performed, the second antibody, goat anti-rabbit, specifically directed against γ chain, conjugated to peroxidase (Calbiochem) was placed. Second antibody was incubated at room temperature for 2h, after time, three washes with phosphate buffer (1X PBS) were performed. The revelled was done with diaminobenzidine (Sigma). The preparations were counterstained with haematoxylin. The assembly was done with Entellan (Merk). The observations were made in an Optical Microscope Olympus BX 51, and imaging was performed with an Olympus camera PL 642, obtaining representative fields used the Image Pro Plus system (Cybernetics). The same procedure described above was used to detect T lymphocytes, activated B-lymphocytes, neutrophils and epithelioid cells. As primary antibodies, the following monoclonal antibodies were used: for detected anti CD4 T lymphocytes, CBL 127 (Cymbus Biotechnology LTD), anti CD4 (Zymed), anti CD8 (Zymed). To detect activated B cells, monoclonal anti - CD40, CBL 486 (Cymbus Biotechnology LTD) was used. To detect neutrophils, anti- CD15 monoclonal antibody CBL 144 (Cymbus Biotechnology LTD) was used and monoclonal anti- CD-54 (ICAM-1), My13 Clone (Zymed) for detecting epithelioid cells. For macrophages detection, anti-macrophages, LN- Clone 5, isotype IgM (Zymed) antibody was used. As secondary antibody, a goat anti - mouse antibody conjugated to peroxidase (Calbiochem) was used.

Results

Isolation and bacterial strains identification associated with lung lesions

From random sampling of 60 pigs in the municipal slaughterhouse of San Francisco de los Romo, in 40 of these animals (66.7 %) lesions characteristic of swine pleuropneumonia was detected. From the lung tissue samples with characteristic lesions, in 22 of the 40 affected pigs (55%) was isolated and characterized by biochemical methods to *A. pleuropneumoniae* presumably biotype 2, since not need NAD to grow (Figure 1).

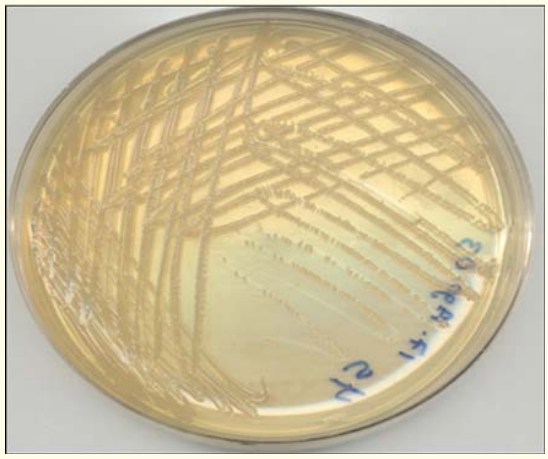

Figure 1: *Fibrino-Hemorrhagic Injury Isolated from Actinobacillus pleuroneumoniae. BHI agar without NAD.*

In all cases, the identity of these strains was confirmed by nested PCR (Figure 2), using a specific Apx toxin IV gene oligonucleotide, reported as a species-specific toxin [28]. Bacteria were not detected in control samples of healthy tissue.

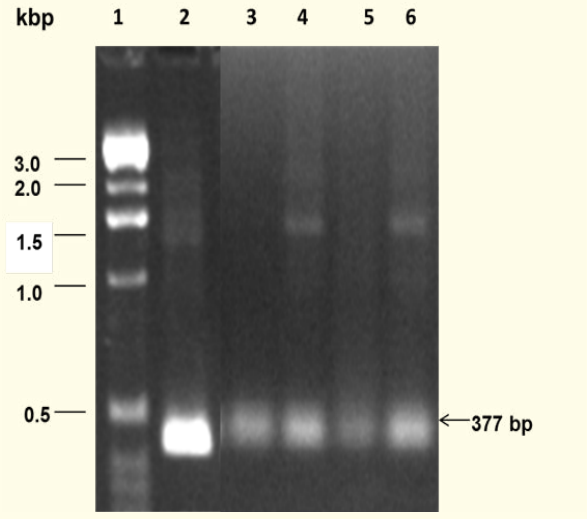

Figure 2: *Corroboration of the Identification of Actinobacillus pleuropneumoniae through Nested PCR using gene Apx IV toxin (Frey, 2003). 1- Molecular size markers. 2- AP biotype 1 serotype 1. 3-6- AP strains isolated from Lesions of Chronic Pleuropneumonia. All Isolates Grew in the Absence of NAD.*

Histological samples injured lungs analysis

From the 22 samples with typical lesions of porcine pleuropneumonia, all showed 100% thickening of alveolar septa (Figure 3). Eighty per cent of the samples showed hemorrhage. Moderate to mild fibrosis associated with the presence of collagen fibers (Figure 4) was detected by Masson staining. Used Wilder staining, septa in type III collagen fibers (Figure 5) were observed. In 36.4% of injured tissues granulomas were observed (Figure 6), also peri bronchiolitis was observed in 27.3 % of samples and chronic pneumonia cases (18 %). Bacteria presence was detected in the alveoli in 73 % of samples by PAS staining and bacteria adhered to the bronchial epithelium and in the lumen of the bronchus in 27.3% of cases (Figure 7).

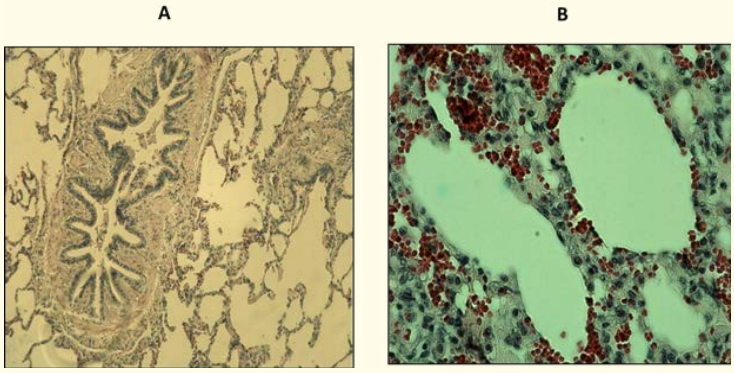

Figure 3: *Lung Lesion Observed by H-E. A) Healthy Lung Staining Hematoxylin and Eosin (Total Magnification 200X). B) Histological Section of a Lung Injury, Staining Hematoxylin and Eosin Approach 400 X, where Thickening of the Alveolar Septa shown and Bleeding.*

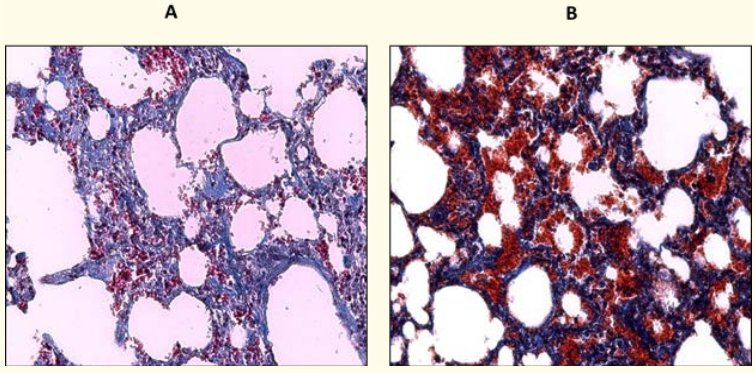

Figure 4: *Masson's Trichrome Stain for Infected Lung Tissue. A) Tissue with Moderate Fibrosis and Moderate Bleeding. B) Tissue with Mild Fibrosis and Heavy Bleed Aspect.*

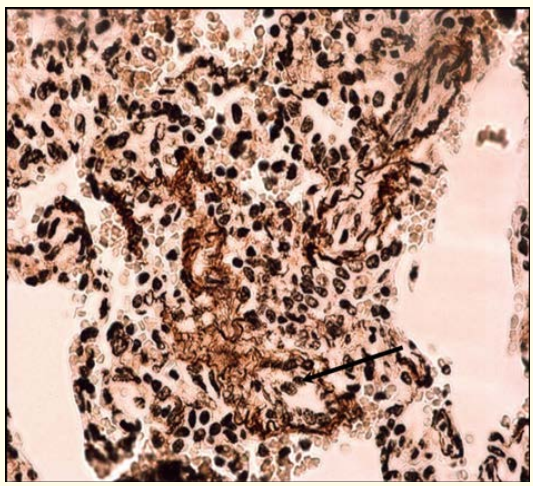

Figure 5: *Lung Injury Stained with Wilder Stain, for Reticular Fibers (400X magnification). Within the Interdental Septum Thickened Reticular Fibers (collagen type III) are Observed, Indicated by the Arrow.*

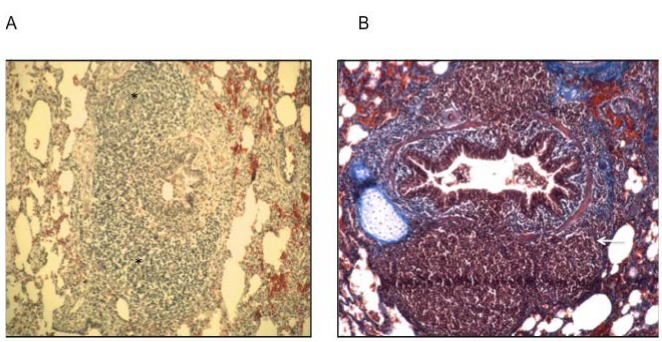

Figure 6: *A) Hematoxylin-eosin stain showing a granuloma associated with bleeding in bronchiole and alveolar septa. B) Masson's trichrome stain showing two granulomas associated with bronchus (100X increase). Asterisks indicate the granulomas and the arrow indicates the presence of collagen fibers around the granuloma.*

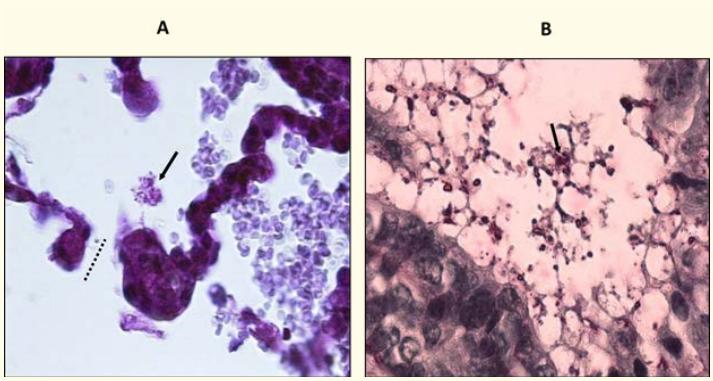

Figure 7: *Lung samples infected with A. pleuropneumoniae biotype 2. PAS staining. A) Bacteria in the alveolar space (arrow) and structures of the interalveolar septum disruption (dotted line). B) Attached bacteria in the bronchial epithelium and in the light of a bronchus (1000X).*

Immunohistochemistry

By immunohistochemical technique, bronchioles and lamina propria of the bronchial mucosa associated bacteria (Figure 8) were detected. No-tuberculous granulomas without precipitation of calcium were detected. In granulomas, CD4 and CD8 T lymphocytes were detected (Figure 9C), activated B-lymphocytes (CD40) were also observed (Figure 9C), epithelioid cells (CD54), few giant cells (Figure 9A), but not neutrophils were detected (CD15) (Figure 9F).

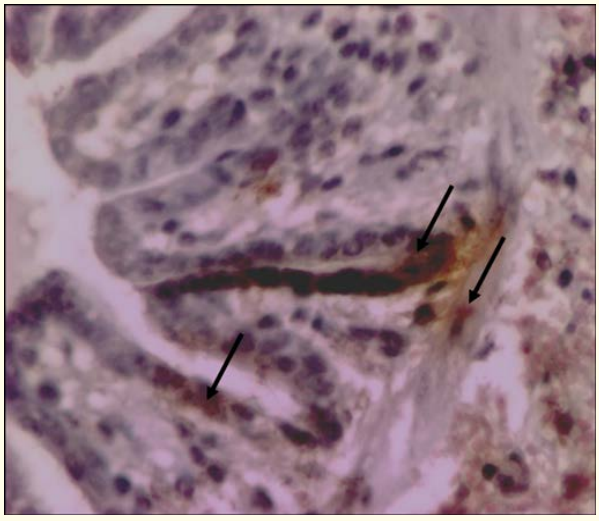

Figure 8: *Actinobacillus pleuropneumoniae immunorecognition in infected lung tissue using a polyclonal antibody directed against total proteins from strain 4074. (400X).*

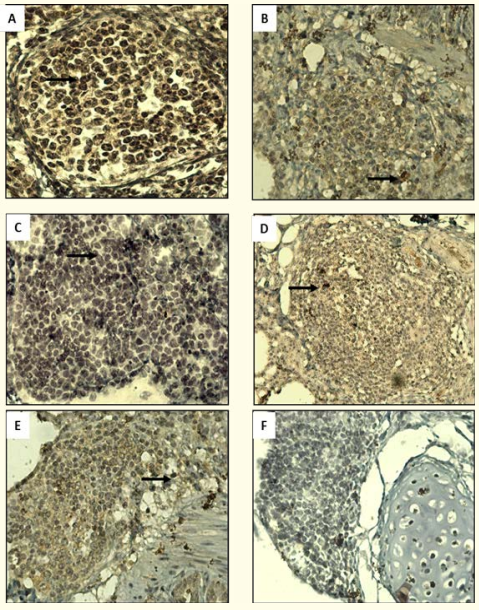

Figure 9: *Composition of granulomas in chronic pleuropneumonia (400X). A) Epithelioid cells recognition with anti- CD54 antibody, the arrow points to a giant multinucleate cell. B) Recognition with anti- macrophages antibodies. C) B lymphocytes recognition with anti-CD40 antibody. D) T lymphocytes recognition with anti CD8 antibody. E) T lymphocytes recognition with anti CD4 antibody. F) Neutrophils recognition with anti CD15 antibody.*

Discussion

Actinobacillus pleuropneumoniae, a member of the family Pasteurellaceae, is the causative agent of porcine pleuropneumonia, it has two biotypes. Biotype 1 has thirteen serotypes NAD dependent. This is the only biotype detected in North America, and 1, 5 and 7are the most common serotypes in the region [29].

Biotype 2 has been reported only in Europe [29,30]. In the present work was achieved, isolate and biochemically characterize AP, that seemed no depend of NAD for growth in 55% of lung samples showed characteristic lesions (Figures 1 and 2). In none case was observed AP dependent of NAD for growing. AP biotype 2, does not require V factor (NAD), is beta- haemolytic, and has been shown to necro-haemorrhagic lesions produced are similar to those caused by biovar 1 [31-35].

In Mexico, only has been reported the presence of AP biotype 1, the most common serotypes 1, 3, 5 and 7, causing hyper acute or acute pleuropneumonia [21]. While other authors in Europe observed the same virulence capacity in both AP biotypes, characterized strains in this study show attenuated virulence. In the host, encapsulation tissue occurs when the pathogenic bacteria live within the host for long time, stimulating the immune system and causing the action of different cell types. The AP long persistence and the encapsulation tissue formation have been associated with of attenuated strains presence, such as the mutants with deletion in anaerobic regulatory HlyX [36,37]. Conversely, hyper acute and acute pleuropneumonia characterized by a haemorrhagic and necrotizing pneumonia, causing a progressive respiratory failure, often fatal [17]. The precise meaning of the presence of biotype 2 AP in central Mexico, and his apparent state of attenuated virulence, must be elucidated in future research, since we have observed in strains present in the environment surrounding the farms, AP that apparently acquire NAD from other sources. Then, it would not necessarily be AP biotype 2, but AP biotype 1 [9].

In this work, using H-E staining [26], negative control samples without lung tissue lesions showed the characteristic architecture, with air spaces delimited by normal morphology and normal septa, not thickened, therefore (Figure 3A). In samples had lesions of alveolar septa, thickening was observed (Figure 3B), but no fibrin deposition in the alveolar spaces, as typically shown in the acute pleuropneumonia [17].

Thickening of the alveolar septa was associated with increased collagen fibers of the connective tissue, resulting in fibrosis (Figure 4). Haemorrhage was also found in the lungs, as shown in subacute, hyper acute and acute pleuropneumonia [17,38]. Have shown that AP has affinity for all types of lung collagen [39], but has a preference for type III collagen (reticular fibers). Figure 5 shows fibrosis caused by the permanence of AP is associated with the presence of reticular fibers. With PAS histochemical staining, bacteria associated with bronchial epithelium in the lumen of bronchioles and inside alveoli were observed.

Retraction structures in alveoli further noted, which are retractable breaks in the walls thereof, similar to the pulmonary emphysema (Figure 7), resulting in a significant decrease in lung capacity being caused in this case by AP [40]. Reticular fibers in lesions of pleuropneumonia presented with a pattern of short and discontinuous fragments (Figure 5). These fibers generally are resistant to attack by many proteases kind, but they can be degraded by several metalloproteinases [41] having as AP, which neutral metalloprotease degrades IgA, IgG, gelatine, haemoglobin and actin [42,43,44].

"*In vitro*", *A. pleuropneumoniae* can adhere to buccal-epithelial cells, tracheal cells and alveolar epithelium, even collagen [39,45-47]. The association between bacteria and epithelial cells could mediate the effective release of exotoxins of bacteria on host cells and cause their destruction, even in the presence of neutralizing antibodies to toxins [48], to thereby achieve underlying the epithelia and spread throughout the bronchial tree extracellular matrix. With immunohistochemical technique was found AP associated in bronchiolar epithelium and the lamina propia, in agreement with the results found with PAS staining (Figures 7 and 8). Both the H-E staining as Masson trichrome staining, granulomas in bronchi and bronchioles associated were detected (Figure 6). However, unlike what was found by other authors [17], detected granulomas were not tuberculous, abundance of collagen fibers around not showed, although a slight staining of

collagen fibers in the periphery (Figure 6 and Figure 9A) was observed. As shown in Figure 5, the decrease in the amount of collagen fibers and shortening reticular fibers in granulomas produced in chronic pleuropneumonia, may be associated with the proteolytic action of these AP virulence factor, as metalloprotease.

Figure 9 shows the recognition of cell types that composed granulomas pleuropneumonia chronic, using various specific antibodies. With anti CD54 antibody the presence of epithelioid activated macrophages was detected, considered gold mark in tuberculous granulomas or typical granulomas, which are in the center of a variety of T cell -mediated injury [14]. Although multinucleated giant cells were scarce, they could also be observed (Figure 9A). Macrophages were detected in the center of the granuloma in most cases, being the predominant population epithelioid cells, which has similarities with the tuberculosis granulomas [14]. These cells are transformed macrophages, with secretory capacity and less phagocytic capacity (53). Activated T and B-lymphocytes were detected, the predominant population was T cells and these cells are from the extravasation of blood flow and converge around the irritation -stimulation infection focus. T cells recognize foreign peptides in the context of major histocompatibility complex class II, presenting them dendritic cells and macrophages (13).

The present study demonstrates the presence of antigen presenter's macrophages in granulomas caused by swine pleuropneumonia. T cells were CD4 and CD8, these cell types are involved in the production of cytokines such as IL2, IFγ, TNF, IL4, IL5, IL 10 and IL 13 and bacterial antigen presentation by dendritic cells in chronic diseases such as tuberculosis [14]. Response T lymphocytes produce cytokines, such as IL-2, that activate other T cells perpetuating the response and INF- γ, which is important for the transformation of macrophages in epithelioid cells and multinucleated giant cells [14]. The presence of neutrophils was also studied, giving a negative result, which is consistent with findings in tuberculosis, where neutrophils are associated with necrosis, but not in the granuloma structure [14]. Neutrophils in porcine pleuropneumonia are associated with necrotic lesions and bleeding in the early stages of the acute phase [49]. However, according to the evidence obtained in this study do not appear to be associated with the chronic phase, neither the formation of granulomas. Granulomas found in this study were different from those tuberculosis granulomas, since deposit calcium salts were not detected, they also not had a lot of giant cells and collagen fibers that delimits are scarce, this coincides with the granulomas present in infections caused by *Mycobacterium avium*, who has loss of cellular organization and disorganized lymphocytes infiltrating [50]. Subsequent studies on porcine pleuropneumonia granulomas may determine the causes of these no caseous granulomas [51].

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