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Pro-Apoptotic and Anti-Inflammatory Effects of Oral Tilmicosin in a Porcine Model of Pleuropneumonia

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Pro-Apoptotic and Anti-Inflammatory Effects of Oral Tilmicosin in a Porcine Model of Pleuropneumonia" submitted by Erin Melissa Nerland in partial fulfillment of the requirements for the degree of Masters of Science.

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ABSTRACT

The aim of the present project was to assess the effects of oral tilmicosin (Pulmotil^{TM}) in piglets experimentally infected with live *Actinobacillus pleuropneumoniae*. Both chlortetracycline and tilmicosin reduced bacterial load in the lungs of infected animals. In addition, the results demonstrate that when delivered in feed, tilmicosin, but not chlortetracycline, enhances apoptosis in porcine bronchoalveolar leukocytes. This effect was associated with decreased LTB_4 levels in the bronchoalveolar lavages of tilmicosin-treated animals, and a significant reduction of pulmonary lesions. Tilmicosin treatment also inhibited the infection-induced increase in rectal temperatures measured in untreated and chlortetracycline-treated animals. Neutrophil infiltration into the lungs, PGE_2 and IL-1β levels in the bronchoalveolar lavages were not different between any group at any time. In conclusion, the results of this study indicate that tilmicosin administered to piglets in the feed induces apoptosis in neutrophils and reduces inflammation.

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DEDICATION

To my family:

Mom, Dad, Lise, Kev, and Danny

Thank you for all of your support and encouragement in everything that I do.

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ABBREVIATIONS

ABC-1 = ATP Binding Cassette Receptor -1

AIF = Apoptosis Inducing Factor

ANOVA = One Way Analysis of Variace

APAF-1 = Apoptotic Protease Activating Factor-1

ARDS= Adult Respiratory Distress Syndrome

ATP = Adenosine Triphosphate

BAL = Bronchoalveolar Lavage

BRD = Bovine Respiratory Disease

CFU = Colony Forming Units

CO_2 = Carbon Dioxide

CTC = Chlortetraycyline

DED = Death Effector Domain

ddH_2O = Double Distilled Water

DNA = Deoxyribonucleic Acid

ELISA = Enzyme Linked Immunosorbent Assay

FADD = Fas Associated Death Domain

fMLP = formyl Methionine Leucine Phenylalanine

GM-CSF = Granulocyte Monocyte Colony Stimulating Factor

HBSS = Hank's Balanced Salt Solution

HOCl = Hypochlorous Acid

HTAB = Hexa-decyltrimethylammonium Bromide

H_2O_2 = Hydrogen Peroxide

ICAM-1 – Intercellular Adhesion Molecule - 1

Ig = Immunoglobulin

IL = Interleukin

LESARC = Life and Environmental Sciences Animal Research Centre

LPS = Lipopolysaccharide

LTB_4 = Leukotriene B_4

LTC_4 = Leukotriene C_4

MPO = Myeloperoxidase

βNAD = β Nicotinamide Adenine Dinucleotide

NADP = Nicotinamide Adenine Dinucleotide Phosphate

NADPH = Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

PAF = Platelet Activating Factor

PBS = Phosphate Buffered Saline

PDGF = Platelet Derived Growth Factor

PGE_2 = Prostaglandin E_2

PPM = Parts Per Million

RTX = Repeat in Toxin

SEM = Standard Error Measurement

Smac/DIABLO = Second Mitochondrial Activator of Caspases/Direct Inhibitor of Apoptosis Binding Protein

TBP = Transferrin Binding Protein

TGF-β = Transforming Growth Factor - β

TNF = Tumour Necrosis Factor

TNFR = Tumour Necrosis Factor Receptor

TUNEL = Terminal Deoxynucleotide End Labelling

VCAM-1 = Vascular Cell Adhesion Molecule - 1

1.INTRODUCTION

1.1 *ACTINOBACILLUS PLEUROPNEUMONIAE* INFECTION IN PIGS

1.1.1 Epidemiology and Clinical Symptoms

Actinobacillus pleuropneumoniae formerly known as *Haemophilus pleuropneumoniae* was first identified as the cause of lung disease in pigs in 1957 (116). Pleuropneumonia in pigs occurs worldwide and has been increasing in prevalence in the last fifteen to twenty years (110) likely due to crowding, inadequate ventilation, co-mingling of pigs of different age groups, and stress (123). Pleuropneumonia typically affects growing pigs aged two to sixth months and spreads quickly to other pigs in the herd (110). Studies by Paradis *et al.* used a seeder pig exposure model, in which groups of healthy pigs were exposed to a single infected pig. Pigs exposed to the infected animal reached 40% morbidity within 60 hours to 5 days of initial exposure, depending on the inoculum size used to infect the seeder initially(112), (113). Transmission of *A. pleuropneumoniae* occurs via the respiratory route, and intensive rearing of pigs facilitates the spread of the disease (110).

The disease symptoms of pleuropneumonia range from peracute to chronic depending on the number of bacteria that reach the lung, the serotype, and the immune status of the host (30),(121), (135). Up to 24h prior to death, clinical signs may be absent in pigs, while others show signs of severe respiratory distress (110). Symptomatic animals are lethargic, disinclined to move, and anorexic. Fevers of up to 41^0C, laboured

respiration, cyanosis, and bloody, frothy discharge from the snout and mouth are common signs of disease (89), (121). At necropsy, necrotic, hemorrhagic lesions can be found on the dorsocaudal regions of the lung (89).

The peracute form of *A. pleuropneumoniae* is characterized by severe inflammation in which the lungs become dark red in colour and increased vascular permeability results in hemorrhage and edema so severe that fluid and blood ooze from the cut surface of the lung (134). Serosanguinous fluid may fill the thoracic cavity and pleuritis and pericarditis are common (121). Early in infection there is a marked increase of neutrophil infiltration into the lung (89). In the alveoli, macrophages, neutrophils and pulmonary epithelial cells degenerate leading to necrotizing vasculitis and a disrupted blood-lung barrier (89), (136).

As herd immunity among those that survive the disease increases, the acute form of the disease becomes less common, but animals continue to harbour the infection and spread it to others (110).

1.1.2 Virulence Factors

There are 12 different serotypes of *A. pleuropneumoniae*, with serotypes 1, 5 and 7 being the most common ones found in North America (110). Several serotype specific virulence factors such as lipopolysaccharides (LPS), transferrin binding proteins and Apx toxins, among others are involved in the pathogenesis of *A. pleuropneumoniae* (12), (52), (56), (68), (69), (72).

LPS consists of a polysaccharide and a toxic lipid A moiety found on the outer membrane of gram-negative bacteria. Purified *A. pleuropneumoniae* LPS causes tissue damage, however the lesions it induces are not necrotic or hemorrhagic, indicating that

LPS on its own can not induce typical pleuropneumonic lesions but may be a contributing factor to their formation (48). Additionally, LPS appears to be involved in *A. pleuropneumoniae* adhesion to porcine tracheal cells (14), a necessary prerequisite for the induction of disease. Glycosphingolipids, present in the epithelial cells of the respiratory tract are likely the receptors for porcine LPS (3). Abu-Milh *et al.*, demonstrated that whole bacteria, purified LPS, and detoxified LPS of *A. pleuropneumoniae* bind strongly to gangliotriaosylceramide and gangliotetraosylceramide (3).

Iron is essential for respiration in bacteria (91). Several species of *Actinobacillus* have the ability to acquire iron through the binding of host transferrin by receptor proteins. Transferrin is a transport protein that carries iron in the plasma and extracellular fluid to the tissues. The bacteria express these receptors when the iron accessible to them is limited. The two transferrin binding proteins Tbp1 and Tbp2 of *A. pleuropneumoniae* weigh 100kDa and 60 Kda respectively and specifically bind porcine transferrin, but not transferrin from other species thus conferring the species specificity of *A. pleuropneumoniae* (109). The mechanism for iron acquisition involves the Tbp receptors binding and extracting iron from the host transferrin, followed by transport of the iron across the outer membrane of *A. pleuropneumoniae.* Inside the cell iron is bound by a binding protein (124). A study by Baarsch *et al.*, found that, within 4h post-infection, animals inoculated with *A. pleuropneumoniae* had significantly reduced serum iron levels, that remained low for the duration of the 24h study compared to healthy controls (9).

Three different Apx toxins are produced by *A. pleuropneumoniae.* Apx I and Apx II are hemolytic exotoxins, while Apx III is a non-hemolytic exotoxin (51), (72). The

Apx toxins belong to a group of toxins known as repeat in toxins (RTX), which are pore-forming toxins common among pathogenic gram-negative bacteria. The Apx toxins are cytotoxic for porcine alveolar macrophages and neutrophils (12), (33). The Apx toxins play a well defined role in the pathogenesis of pleuropneumonia as they are involved in the evasion of the host's first line of defense. At sublytic doses the Apx toxins impair macrophage phagocytic and chemotatic function, while stimulating oxidative burst in neutrophils and macrophages, thereby releasing damaging oxygen radicals onto the surrounding tissues (38). Knockout mutants of *A. pleuropneumoniae* that are devoid of Apx toxins lose the ability to generate an oxidative burst as measured by chemiluminescence (71). Additionally, the Apx toxins appear to be involved in the formation of lesions as they are cytotoxic for endothelial cells (136). Furthermore, purified Apx toxins instilled endobronchially into pigs, have the ability to induce the characteristic lesions of *A. pleuropneumoniae* (72).

1.1.3 The Role of Neutrophils in the pathogenesis of pleuropneumonia

In acute inflammation, such as is seen in pleuropneumonia, neutrophils play a dual role. Pulmonary clearance of microbes is dependent on the microbicidal activities of neutrophils and resident macrophages (19), (61), (120). Experimental infection with *A. pleuropneumoniae* demonstrates that neutrophil infiltration, measured by neutrophil concentration in bronchoalveolar lavage (BAL), increases significantly within 18 hours of infection (9). The extent to which neutrophils and macrophages can effectively phagocytose *A. pleuropneumoniae* is reduced by the cytotoxic effects of the bacteria on these cells. Furthermore, lesioned areas of the lungs exhibit high numbers of neutrophils,

which are believed to be responsible for the tissue damage associated with pleuropneumonia (88), (89).

1.1.4 Treatment and Control

Due to the severity of the acute disease, treatment is often ineffective. Antibiotic therapy may decrease the rate of mortality, but it is often inadequate in eliminating the bacteria, thus treated animals often still harbour the infection and transmit it to others (110). Tetracycline, spectinomycin, penicillin, sulfathiazole and tiamulin are the antibiotics that have traditionally been used to treat porcine pleuropneumonia (110). In large finishing units, veterinarians recommend treating animals twice daily with injections of antimicrobials over medicating feed and water (110). However, this process is very labour intensive. Prophylaxis with chlortetracycline (CTC), enrofloxacin, or florfenicol given in feed at doses of 1000 parts per million (ppm), 150 ppm and 40 ppm respectively, prevents the development of respiratory disease induced by the intranasal inoculation of 10^6 colony forming units (CFU)/mL *A. pleuropneumoniae* (149). Not surprisingly, serum antibodies to *A. pleuropneumoniae* were detected in all animals, even those pre-treated with the antibiotics (149). With the expansion of finishing units, the incidence of *A. pleuropneumoniae* infections is increasing along with the extensive use of antimicrobials (110).

Due to the large number of virulence factors and the differences in virulence factors between serotypes, there is currently no vaccine that provides complete protection against *A. pleuropneumoniae* (110). The identification and isolation of infected pigs is a critical step in controlling the disease. The variability in clinical symptoms between individually infected animals makes it difficult to identify infected individuals and isolate

them. Serodiagnosis is the only available method to identify those possibly harbouring the infection (110). Veterinarians suggest that any seropositive animals should be considered infected and quarantined (110).

1.2 NEUTROPHILS IN INFLAMMATION

1.2.1 Proliferation and Phagocytic Activity of Neutrophils

The life span of the neutrophil is spent in three different environments: the bone marrow, the blood, and the tissue (10). Proliferation and maturation takes place in the bone marrow. Neutrophils circulate in the blood for an estimated ten hours (10), and those that do not migrate into the tissues are destroyed by the spleen (70). In the event of an infection, neutrophils migrate from the circulatory system into the tissues.

Phagocytosis and degranulation are the two major activities performed by neutrophils at sites of infection. Invading microorganisms become coated with opsonins such as immunoglobulin (Ig) G and complement C3b, which are recognized by an FcγR receptor on the neutrophil (118). In addition to binding the ligand, the FcγR sends signals that are involved in the activation of the cell itself (118). During engulfment, the cell extends pseudopods around the microbe completely enclosing it into a phagosome (118). The phagosome fuses with the lysosome and the granules discharge bactericidal mediators to kill the microbe (118).

1.2.2 Mediators Produced by Neutrophils

The primary bactericidal effects that occur during degranulation are dependant on oxygen (76). Phagocytosis itself stimulates the production of oxygen metabolites through the activation of nicotinamide adenine dinucleotide phosphate

hydrogen (NADPH) oxidase in the lysosomal membrane (75). This active enzyme oxidizes NADPH and reduces oxygen to superoxide anion, which is then converted into hydrogen peroxide (H_2O_2)(76). In and of itself, H_2O_2 is not an effective antimicrobial. The azurophilic granules of neutrophils contain myeloperoxidase (MPO) an enzyme, which reacts with halide such as chloride (Cl^-) to convert the newly produced H_2O_2 into hypochlorous acid (HOCl) (76). A study by Knaapen *et al.*, using circulating neutrophils *in vitro* showed that accumulation of H_2O_2 in neutrophil-conditioned medium is only observed in the presence of an MPO inhibitor (77). MPO, however, is highly abundant in neutrophils and as such MPO concentration is often used as a measure of neutrophil infiltration (8), (73), (95).

HOCl has a number of degradatory effects on numerous tissue types throughout the body. HOCl addition to confluent endothelial monolayers results in a retraction of the cells from each other and induces the formation of large intracellular gaps (145). Physiologically relevant concentrations of HOCl cause extensive protein fragmentation and prevent collagen gelation as measured by the release of acid soluble counts from 3H-collagen, possibly facilitating inflammatory joint destruction (32). This potent oxidant alone does not have the ability to induce DNA single strand breaks in cells, but it may do so in concert with various other molecules such as xenobiotics (82). In the context of the lung, rat lungs perfused with a 50μM solution of HOCl experience edematous injury and a significant increase in wet lung weight within 10 minutes (145). Furthermore, HOCl induces a dose dependent decrease in the surface tension lowering activities of human surfactant (98).

The concentrations of oxygen metabolites produced and released by neutrophils is dependent on the stimulant and its concentration (57). Optimal concentrations of the bacterial product formyl methionine leucine phenylalanine (fMLP) have been shown to induce a brief, but intense release of superoxide anion, yet pretreatment with a lower concentration of fMLP inhibits further response to higher concentrations of fMLP (57).

In addition to oxygen dependent mechanisms, neutrophils produce other enzymes such as elastase and collagenase that are also injurious to tissues. Elastase degrades elastin, extracellular matrix proteins, collagen types I to V, fibrinogen, proteoglycans, and fibronectin (2), (5), (18), (53), (60), (86). Neutrophil elastase also has the ability to increase the production of interleukin (IL)-6, IL-8 and granulocyte monocyte colony stimulating factor (GM-CSF) from epithelial cells (13), (108). Collagenase is stored in the specific granules of the neutrophil in a latent form and cleaves collagens type I and II (76). An *in vivo* rat model of *Pneumocystis carnii* pneumonia is characterized by leaky alveoli and foamy alveolar exudates, and it has been shown that endogenously active collagenase is significantly higher in rats with this pneumonia compared to healthy controls (141).

Other mediators can be released by neutrophils to induce further migration of neutrophils into an area of infection. Leukotriene B_4 (LTB_4), an arachidonic acid metabolite, is a potent chemotactic agent (49) known to induce the recruitment of neutrophils and amplify their numbers in the tissues without enhancing bactericidal mechanisms or oxidative metabolism (111). Leukotrienes are formed in the membrane of the neutrophil after it has been stimulated by bacterial products such as fMLP or leukotoxin (111). *Mannheimia haemolytica* leukotoxin and pneumolysin increase LTB_4

release from bovine and human neutrophils respectively (27), (62). The effects of different toxins on LTB_4 release appears to be species specific as *M. haemolytica* leukotoxin does not affect LTB_4 release from human neutrophils (27).

1.2.3 Innate Immunity, Neutrophil Necrosis and the Perpetuation of Inflammation

Innate immunity is the body's primary line of defense against invading infections. As such, the innate immune system can limit or cure infection before an adaptive immune response is generated. Tissue damage caused by invading pathogens can induce an inflammatory response, characterized by vasodilation, increased capillary permeability, as well as an influx of phagocytic cells (83). The mediators contributing to the inflammatory response may be released from the invading microorganism, cells that have been damaged by the microorganism, or products of white blood cells.

Although neutrophils are well designed to protect the host against microbial pathogens, these leukocytes are a double edge sword as they can also induce tissue damage. During a bacterial infection, neutrophils are exposed to various stimuli, including virulence factors such as the cytotoxic Apx toxins of *A. pleuropneumoniae* (37), (38). These stimuli overwhelm the machinery of the neutrophil leading to organellar swelling, membrane damage and the eventual disintegration of the cell and leakage of the cytotoxic compounds housed within the neutrophil (26), (153). This mode of cell death known as necrosis, results in toxic consequences to the surrounding tissue.

To date, neutrophils have been implicated as a mediator of tissue destruction in a number of diseases such as: ischemia-reperfusion injury, emphysema, rheumatoid arthritis, gout, inflammatory bowel disease, asthma, psoriasis, chronic bronchitis and adult respiratory distress syndrome (ARDS) (17), (92), (97), (115), (146).

The pulmonary parenchyma is delicate and easily injured by the harmful substances produced by neutrophils. Human patients with (ARDS) display severe lung damage in association with increased neutrophil infiltration and abnormal anti-oxidant capacity (115). Furthermore, neutrophil necrosis in an *in vivo* model of endotoxin-induced pulmonary inflammation in mice, is linked to inflammation and tissue damage (97). Damage to the lung parenchyma compromises gas exchange, and as such can have very serious consequences for the host.

1.3 APOPTOSIS

1.3.1 Neutrophil Apoptosis and the Resolution of Inflammation

During acute bacterial pneumonia, neutrophil influx can be so great that the alveolar airspace becomes packed with neutrophils (59), yet under homeostatic conditions there may be no tissue damage associated with this influx. This phenomenon suggests that neutrophils may be removed from an inflammatory site without releasing their toxic compounds onto the tissues (96). The mode of death that the neutrophils undergo and their timely removal may be the pivotal point in the resolution or persistence of inflammation.

Apoptotic cell death is a second mode of cell death, far less damaging to host tissue than necrosis. It is a form of cell death that eliminates unwanted host cells through the activation of a coordinated, programmed series of events. The initial changes in apoptosis begin with the extension of the cytosol into membrane blebs (26), (153). These blebs can be reversibly extruded and reabsorbed (153). Irreversible cytoplasmic condensation occurs next, followed by an increase in cellular density, compaction of

organelles, and nuclear condensation (26), (153). The fibrillar centre of the nucleolus becomes distinct and separate from the surrounding transcription complexes and the nuclear pores vanish (153). At this point, the cell breaks off into a collection of membrane bound apoptotic bodies that still house intact organelles (26), (153) Apoptosis is of great importance because it eliminates the ability of neutrophils to move by chemotaxis, generate oxygen metabolites or degranulate (151), and allows the specific phagocytic elimination of the apoptotic bodies (126).

1.3.2 The Regulation of Apoptosis in Neutrophils

Mechanistically, apoptosis may be triggered via a receptor-mediated pathway, or by intrinsic activation. Death receptors belong to the tumour necrosis factor (TNF) receptor superfamily and include receptors such as Fas (41). Two to six cysteine rich domains located in their extracellular regions characterize the TNFR family of receptors (138). Ligands appear to form trimeric complexes and thus they are thought to promote receptor clustering (11). Clustering leads to apposition of intracellular domains and the transduction of exogenous signals to the cell. Binding of Fas ligand to the Fas receptor induces receptor clustering and recruitment of intracellular adaptor molecules to form the Fas-associated Death Domain (FADD). The aggregation of Fas receptors induces uptake of the adaptor protein FADD/MORT1, to the death domain of Fas (24). One structural component of FADD is the N-terminal death effector domain (DED). FADD DED recruits procaspase 8 (106). The apposition of procaspase 8 to the activated Fas complex causes autolytic cleavage of procaspase 8 to caspase 8, which is released and ultimately leads to the activation of caspase 3 resulting in cleavage of DNA within the cell.

Alternatively, the apoptotic pathway may be initiated at the level of the mitochondria. Loss of signals from neighbouring cells (117), withdrawal of cytokines (28), or activation of p53 from within the cell can stimulate apoptosis intrinsically. The center of control for intrinsically mediated apoptosis is the mitochondria, which function to maintain the redox potential of cell and generate adenosine tri-phosphate (ATP) through oxidative phosphorylation. Situated within the bilayered mitochondrial membrane is the regulatory protein cytochrome-c. The common pathway for intrinsically mediated apoptosis requires the release of cytochrome-c from the mitochondrial membrane into the cytosol. The liberated cytochrome-c interacts with cofactors apoptotic protease activating factor-1 (Apaf-1) and ATP to form an apoptosome, which cleaves procaspase 9 into its active form, caspase 9 (156). Alternatively mitochondria may release second mitochondrial activator of caspases/direct inhibitor of apoptosis binding protein (Smac/DIABLO) to activate caspase 9 (21). Caspase 9 acts on effector caspases such as caspase 3 to initiate the proteolytic cascade leading to programmed cell death. Mitochondria-initiated apoptosis may also occur via the release of apoptosis inducing factor (AIF), which can break down DNA independently of caspases (142).

1.3.3 Clearance of Apoptotic Bodies by Macrophages

As mentioned earlier macrophage phagocytosis of neutrophils is a critical step in the resolution of inflammation (126). An early event in neutrophil apoptosis is the translocation of phosphatidylserine to the outer layer of the lipid bi-layer of the cell membrane. Translocation appears to result from the limited function of translocase, an inward directed pump specific for phosphatidylserine and the concomitant activation of scramblase, which facilitates bi-directional migration across the lipid bilayer (147), (157).

The alteration in activity of translocase and scramblase cause a collapse in membrane asymmetry, which results in the exposure of phosphatidylserine on the cell surface (15). Macrophages possess particular receptors that recognize exposed phosphatidylserine on the surface of apoptotic neutrophils to facilitate phagocytosis (45), (46).

The vitronectin receptor is another receptor found on the surface of macrophages that mediates apoptosis (127). The vitronectin receptor requires the macrophage surface marker CD36 in order to present an adhesive linking molecule, thrombospondin to apoptotic cells.(47), (129). Thrombospondin acts as a bridge between the macrophage and apoptotic cell, although the specific mechanism by which this molecule mediates phagocytosis is still unknown (128). The binding of apoptotic cells to macrophages may also be modulated by opsonic complement fragments on the surface of apoptotic cells that are recognized by the β2 integrin family of receptors (144). Phagocyte receptors such as the LPS receptor CD14, class A and B scavenger receptors and the ATP-binding cassette receptor (ABC-1) may also be involved in the phagocytic uptake of apoptotic cells (129). Other molecules that may be indirectly involved in the recognition of apoptotic cells are the innate immune molecules Surfactant Proteins A and D, which bind to apoptotic neutrophils (58), (131). The expression of these molecules permits macrophages to recognize apoptotic neutrophils and phagocytose them, without the release of pro-inflammatory compounds. Because the neutrophil membrane remains intact the macrophages that engulf the neutrophils do not become active and inflammatory in nature themselves. This allows the dying neutrophils to be disposed of with minimal compromise to the surrounding tissue. It also appears as though the

amount of phosphatidylserine exposed on the membrane's surface is correlated to the efficiency of phagocytosis (43).

A study performed by Fadok *et al.* (1998) (44) established that the phagocytosis of apoptotic neutrophils inhibits the production of pro-inflammatory GM-CSF, IL-1β, IL-8, TNF-α, and leukotriene C_4 (LTC_4) by macrophages. The increased macrophage production of transforming growth factor β (TGF- β), prostaglandin E_2(PGE_2) and platelet activating factor (PAF) is believed to suppress the production of the pro-inflammatory mediators listed above. The mechanisms by which TGF-β, PGE_2, and PAF inhibit pro-inflammatory mediators are still under investigation (44), however it is clear that phagocytic uptake of apoptotic neutrophils aids in the resolution of inflammation as they ingest apoptotic neutrophils before secondary necrosis can occur.

1.4 MACROLIDES

Macrolides are a class of antimicrobials derived from the *Streptomyces* species. Structurally, they are composed of a 14-16 membered lactone ring to which one or more sugars are attached. Macrolides function by reversibly binding to the 50s ribosomal subunit hence inhibiting protein synthesis in bacteria. These bacteriostatic agents are used widely for the treatment of bacterial infection (39), (152). Members of the macrolide class of antibiotics include, but are not limited to such drugs as: erythromycin, azithromycin, clarithromycin, roxithromycin and tilmicosin.

1.4.1 Immunomodulatory Effects of Macrolides in Respiratory Disease

Macrolides are commonly used to treat respiratory tract infections. A number of studies have reported that macrolide antibiotics have anti-inflammatory effects in

addition to their antimicrobial effects. Effective immunomodulation by macrolides has been shown in diseases such as asthma (6), diffuse panbronchiolitis (81), and bacterial infections (40). The anti-inflammatory effects of macrolides have been partially attributed to their accumulation within phagocytes. Although the mechanisms by which macrolides are taken up into leukocytes has not been elucidated, there is evidence that macrolide uptake into neutrophils *in vitro* is associated with calcium channels or a calcium channel-operated mechanism (103). Macrolide concentration within phagocytes can reach concentrations significantly higher than therapeutic concentrations in serum (66) and therefore affect the production of cytokines, hydrogen peroxide, nitric oxide, superoxide and the process of degranulation (79), (84).

Bronchial hyperresponsiveness is a symptom linked to inflammation in asthma patients. Clarithromycin treatment has been shown to decrease eosinophil and eosinophilic cationic protein levels in sputum and blood samples and the ensuing bronchial hyperresponsiveness (4). Roxithromycin has the ability to inhibit the secretion of IL-2, IL-4 and TNF-α by activated human peripheral blood leukocytes (80). In addition, a single 5mg/kg dose of this drug significantly reduces the secretion of IL-3, IL-4, IL-5 and TNF-α (80).

Pseudomonas aeruginosa is believed to play a role in the chronic inflammation that occurs in diffuse panbronchiolitis (81). The advent of erythromycin therapy in the treatment of diffuse panbronchiolitis has increased the survival rate of patients with this disease from 21.9% to over 90% (81). This increased survival rate is seen despite the fact that erythromycin is neither bactericidal nor bacteriostatic for *P. aeruginosa* (81), thus suggesting that erythromycin modulates the inflammatory response in diffuse

panbronchiolitis rather than eliminating the bacteria. Consistent with these findings, erythromycin inhibits *P. aeruginosa* induced IL-8, IL-1β and TNF-α production (81).

1.4.2 Immunomodulatory Effects of Macrolides on Neutrophils

As previously mentioned macrolides accumulate within phagocytes they are carried to their target sites. Macrolides in particular, accumulate within neutrophils. Macrolides have the ability to affect the recruitment of neutrophils to sites of inflammation and to alter their ability to secrete certain mediators.

In vitro studies have shown that erythromycin, roxithromycin, clarithromycin and azithromycin, stimulate the degranulation of neutrophils (1). The macrolides josamycine, spiramycine and oleandomycin do not have this effect (1). Erythromycin, dirithromycin, roxithromycin, clarithromycin and azithromycin also inhibit superoxide production in human neutrophils stimulated with fMLP at physiologic concentrations (140). An *in vivo* murine model system of allergic alveolitis shows that erythromycin reduces neutrophil concentration in BAL fluid 6h post challenge (99). Erythromycin also reduces neutrophil accumulation at the site of challenge in rats that have had LPS injected into the middle ear cavity (42). Studies have shown that the reduction of neutrophil recruitment to sites of inflammation could be attributed to the inhibition of intercellular adhesion molecule –1 (ICAM-1) expression on the endothelial surface (99) or β-2 integrins on the surface of neutrophils (90).

A recent study on bleomycin-induced lung injury in mice (74) demonstrated that pretreatment with 50mg/kg/day of clarithromycin or roxithromycin significantly decreased neutrophil concentrations in bronchoalveolar lavage fluid. Pretreatment with

clarithromycin, roxithromycin or azithromycin also attenuated the increase in wet weight of the lungs, indicating an amelioration of bleomycin-induced edema (74).

A clinical study performed in 1996 by Wenisch *et al.*, sampled neutrophils from patients before and 16h after receiving a single injection of azithromycin (150). Flow cytometric analysis was used to determine leukocyte function and showed that azithromycin treatment decreased the ability of neutrophils to phagocytose *E. coli* and produce reactive oxygen intermediates. Three hours post-injection with clarithromycin, neutrophils also had a decreased ability to phagocytose *E. coli*, but were still able to generate reactive oxygen metabolites, whereas 3h post-injection with roxithromycin neither effect was seen. Additionally, azithromycin has been shown to induce neutrophil apoptosis in circulating human neutrophils, without altering oxidative metabolism or IL-8 production (78). Consistent with these observations, a recent study (31), has shown that 500mg azithromycin taken daily for 3 consecutive days increases neutrophil apoptosis, IL-6 secretion, and vascular cell adhesion molecule 1 (VCAM-1) expression in human peripheral blood neutrophils sampled 28 days after treatment. IL-8 production was found to be reduced 2.5h and 24h post treatment (31).

In light of the importance of the clearance of apoptotic bodies by macrophages, it has recently been shown that treatment of alveolar macrophages with erythromycin or clarithromycin significantly increases phagocytosis of apoptotic neutrophils by increasing the density of the phosphatidylserine receptor on the surface of macrophages (154).

1.5 TILMICOSIN

Tilmicosin is a semisynthetic, 16 membered ring macrolide derived from tylosin and used exclusively in veterinary medicine. It is primarily antibacterial against gram-positive organisms, but also has good activity against gram-negative bacteria such as *Mannheimia* and *Actinobacillus* spp. Clinical studies have shown that treatment with a single injection of tilmicosin (Micotil™) is effective in preventing bovine respiratory tract disease (BRD) (50), (102), (132). Prophylaxis with injectable tilmicosin also reduces *Mannhemia haemolytica* concentrations in nasal secretions, delays the onset of respiratory disease and increases daily weight gain over animals receiving placebo (50), (132). Injectable tilmicosin is also used to treat disease after clinical symptoms appear. It reduces mortality, lowers rectal temperature, improves daily weight gain and decreases *M. haemolytica* in nasal secretions of animals exhibiting clinical signs of disease (102). In addition, intramammary administration of tilmicosin has been shown to be effective in treating *Staphylococcus aureus* infection of the mammary glands in both cattle and sheep (36), (107).

The oral formulation of tilmicosin (Pulmotil™) incorporated into feed at dosages between 200-400ppm successfully controls swine respiratory disease associated with *A. pleuropneumoniae* and *Pasteurella multocida* (101).

1.5.1 Tilmicosin Accumulation Within Leukocytes

The destruction of intracellular bacteria is enhanced by the ability of an antibiotic to be accumulated within phagocytes (64), (93). Macrolides are one of the few antibiotics that can be taken up by phagocytes. Gladue *et al.*, demonstrated that azithromycin administered *in vitro* can reach intracellular concentrations that are 200

times greater than the external concentration and that the drug is delivered to remote sites of infection by phagocytes (54). The efficiency of azithromycin is due, at least in part, to its high affinity for intracellular uptake in phagocytes. As a result the antibiotic is delivered directly to the site of infection and can easily access bacteria as they are phagocytosed (54).

At the 400 ppm dose for tilmicosin, serum concentrations (0.05-0.23 μg/mL tissue) in pigs are below the minimal inhibitory concentration for *A. pleuropneumoniae* (4μg/mL) (125). Scorneaux et al, performed a comprehensive study of tilmicosin accumulation in neutrophils and macrophages in 1998 (133). The study revealed that tilmicosin uptake into neutrophils and macrophages is high (ratio of intracellular concentration versus extracellular concentration (Ci/Ce) = 69 at 4h post exposure and Ci/Ce=16 at 4h, respectively) (133). The only other macrolide found to reach concentrations this high within neutrophils is azithromycin, which has a Ci/Ce of 120 after 3h (54).

The presence of two basic amine groups in the structure of tilmicosin are likely responsible for its localization in the lysosome of the neutrophil, which is an acidic environment (133). Bafilomycin, a proton pump inhibitor, decreases tilmicosin's uptake into neutrophils, suggesting that proton pump activity is required to maintain lysosomal pH (133). Stimulation of neutrophils with LPS and pentoxifylline (a methylxanthine derivative) causes a significant increase in tilmicosin accumulation within phagocytes (133). In the event of tilmicosin removal from the extracellular environment, the release of tilmicosin from within swine neutrophils is quite rapid with 60% loss of the

intracellular concentration within 30 minutes (133). This fast efflux is similar to that seen in mouse macrophages incubated with erythromycin and roxithromycin (20).

1.5.2Tilmicosin-induced Neutrophil Apoptosis

Recent findings have established that tilmicosin increases neutrophil apoptosis during bacterial infections, and thus may have an anti-inflammatory benefit in addition to its bacteriostatic effect (23). Indeed, in a bovine model of pneumonic pasteurellosis, tilmicosin given in its injectable form Micotil^{TM} effectively controls *M. haemolytica* infection, induces neutrophil apoptosis and reduces pulmonary inflammation (23). Subsequent studies using circulating bovine neutrophils show that this effect is observed regardless of the presence or absence of live bacteria, is cell specific, and that other antibiotics, including penicillin, ceftiofur and oxytetracycline, or the corticosteroid dexamethasone, do not have this effect (22). Mechanistically, tilmicosin appears to induce apoptosis independently of increased Fas expression on neutrophils (unpublished data by Lee et al.) and without altering levels of soluble TNF-α (23).

1.6 SUMMARY

A. pleuropneumoniae infection is a highly contagious disease in pigs, which is spread through the respiratory route (110). Crowding, inadequate ventilation and commingling of pigs of different age groups are factors that contribute to the increasing incidence of pleuropneumonia in gang-housed pigs (110). Unfortunately, vaccines and antibiotic therapy have limited success in ridding animals of the infection and animals that survive often become chronically infected, harbour the bacteria and spread it to others (110). Neutrophils are one of the first immune cells that encounter *A.*

pleuropneumoniae in the lung (89). These cells engulf and kill foreign organisms through phagocytosis. In the event of an overwhelming infection, additional neutrophils are recruited to the area via lipid mediators (49), (111). Although these cells are designed to protect the host from infection, stimuli produced by microbes can overwhelm the machinery of the neutrophil leading to organellar swelling, membrane damage and the eventual disintegration of the cell and leakage of harmful compounds housed within the neutrophil (89), (136), (153). In contrast to necrosis, neutrophil apoptosis is a key mechanism for the removal of extravasted neutrophils and contributes to the resolution of inflammation in the lung as well as in other tissues (29), (130). Ultimately, apoptotic cells and their fragments are removed by other cells, such as macrophages (29), (59), (130). In this fashion, apoptotic neutrophils are cleared before they are given the opportunity to cause tissue injury.

Tilmicosin is a macrolide antibiotic used as a subcutaneous formulation to treat respiratory infections in cattle (Micotil™), or as a feed formulation to control bacterial pneumonia in swine (Pulmotil™) (55), (101). The treatment success of tilmicosin has been attributed to its pharmacodynamic concentration in appropriate tissues and low inhibitory concentrations (35), (100), (102). Tilmicosin has a very high affinity for uptake within neutrophils, in which intracellular concentrations 69 times greater than those achievable in the serum have been reported (133). In addition, there is increasing evidence to suggest that tilmicosin also generates anti-inflammatory effects, via mechanisms that have yet to be fully characterized (22), (23), (102).

Previous research in a bovine model suggests that injectable tilmicosin is pro-apoptotic for neutrophils and has other anti-inflammatory effects coupled with its

bacteriostatic effects (22), (23). To date, it has not been established if the pro-apoptotic and anti-inflammatory benefits conferred by tilmicosin are a species-specific phenomenon and/or are related to its mode of delivery.

1.7 RESEARCH OBJECTIVES AND HYPOTHESIS

The overall objective of this study was to investigate the effects of oral tilmicosin on pulmonary inflammation and neutrophil apoptosis in an *in vivo* model of porcine pleuropneumonia. Findings from this study may further explain the immunomodulatory effects of tilmicosin. The specific objectives of this study are:

1. To successfully grow and prepare an inoculum of *A. pleuropneumoniae* that induces severe, acute pneumonia in piglets.
2. To determine if oral tilmicosin (Pulmotil^{TM}) has pro-apoptotic and anti-inflammatory effects in an *in vivo* model of porcine pleuropneumonia induced by *A. pleuropneumoniae*.

It is hypothesized that the macrolide tilmicosin, given orally, will demonstrate anti-inflammatory and pro-apoptotic properties in swine, whereas oral CTC, another commonly used treatment for pleuropneumonia will not show any such properties.

2. METHODS AND MATERIALS

2.1 BACTERIA AND INOCULUM PREPARATION

A. pleuropneumoniae serotype 1 was obtained from Dr. Marcelo Gottschalk (St. Hyacinthe, PQ). This strain is an original, virulent isolate from a pig that died of porcine pleuropneumonia. Inoculum was prepared by growing 10μL aliquots (stored at –70°C) on Brucella agar supplemented with 0.2% βNAD (β nicotinamide adenine dinucleotide) (a blood factor involved in the coagulation cascade) and 5% horse serum. Plates were incubated at 37°C and 5% CO_2 for 24 hours. Fifteen colonies were selected from the plates, suspended in 25mL of Brucella broth supplemented with 0.2% βNAD, 5% horse serum and incubated for 12h at 37°C and 5% CO_2. Bacterial concentrations were estimated using optical density readings (Figure 4) and confirmed by Colony Forming Unit (CFU) enumeration on supplemented Brucella agar. Inoculum was diluted appropriately with sterile, endoxtoxin free saline to obtain the desired concentration for infecting the animals. Diluted inoculum was placed on ice during the inoculation procedure.

2.2 INOCULATION PROCEDURE

Anaesthesia was induced by giving each piglet an intramuscular (IM) injection of ketamine (35mg/kg body weight), followed by general halothane (2-bromo-2-chloro-1, 1,1-trifluoroethane, MTC Pharmaceuticals, Cambridge, ON, Canada). A sterilized, 20 gauge aluminium wire (Canadian Tire, Calgary, AB) was inserted into a 2.7 mm, sterile, all-purpose catheter (Rusch Inc, Oakville Ont.) to give form to the catheter and ease its

guidance into the trachea. The catheter was introduced into the trachea through the mouth using a Miller 4 stainless steel laryngoscope (Jorgensen Laboratories Inc. Loveland Col.), once the catheter was inserted to the bifurcation of the trachea, the wire was pulled out from inside the catheter. Two mL of an *A. pleuropneumoniae* suspension was injected through the catheter with a 10 cc syringe into the lungs and then the catheter was removed. Animals were returned to their rooms and left to recover, under observation.

2.3 BACTERIAL IDENTIFICATION

Experiments were carried out to verify the identity of the bacteria in the inoculum as well as that of bacteria recovered from BAL fluids following intratracheal infections. This was carried out using sugar fermentation profile analysis as well as oxidization and urease capacity (Table 1). Briefly, bacteria to be tested (*A. pleuropneumoniae* from inoculum, bacteria recovered from porcine lungs after experimental infection, and *M. haemolytica*, *P. multocida*, *P. pneumotropica* and *Mycoplasma bovis* for comparison) were grown on either the supplemented Brucella agar described in the previous section (for *A. pleuropneumoniae* growth), or Columbia blood agar (for the other bacteria) for 12h. One bacterial colony of each isolate was diluted in 1mL of sterile buffer, 100 µL of this solution was added to a control base solution with no sugar, a 0.005% w/v glucose solution, a 0.005% w/v maltose solution, a 0.005% w/v sucrose solution, a 0.005% w/v trehalose solution or a 0.02% w/v urea broth.

Table 1: Bacterial identification tests for *A. pleuropneumoniae* compared to other bacterial species. *A. pleuropneumoniae* from the inoculum, as well as the live bacteria retrieved from BAL fluids of experimentally infected animals exhibited identical profiles, characteristic for *A. pleuropneumoniae* (i.e. positive for fermentation of glucose, maltose, and sucrose but negative for trehalose, as well as urease- and oxidase-positive). This profile was distinct from that of the other bacterial species tested.

Bacteria:	*Actinobacillus pleuropneumoniae* isolate	Bacterial Recovery from the porcine lung	*Mannheimia haemolytica*	*Pasteurella multocida*	*Pasteurella pneumotropica*	*Mycoplasma bovis*
Glucose*	+	+	+	+	+	-
Maltose*	+	+	+	-	+	-
Sucrose*	+	+	+	+	+	-
Trehalose*	-	-	-	+	+	-
Urease**	+	+	-	-	+	-
Oxidase***	+ Very weak	+	+	+	+	-

* Glucose, maltose, sucrose and trehalose tests for the ability of the bacteria to ferment these sugars

** The urease test tests for the ability of the bacteria to break down urea

*** The oxidase test tests for the ability of the bacteria to oxidize

in a 96-well enzyme linked immunsorbent assay (ELISA) plate, and incubated for 12 hours at 37° C. In the sugar tests, a change in colour from pink to yellow/orange indicates the bacteria isolate is positive for fermentation of that sugar. A change in colour of the urea from orange to pink/fuschia indicates the bacteria are positive for urease. Additionally, oxidization tests were also carried out. Bacterial colonies on plates were exposed to a sterile swab dipped in a solution containing 10 g / L tetramethyl reagent and 10% v:v of 0.25% ascorbic acid in sterile, distilled water. A dark blue/purple colour develops on the swab within ~5 seconds if the bacteria is positive for oxidation.

2.4 PORCINE MODEL

Weaned, three week old piglets, weighing approximately 6kg were gang housed in a level 2 biohazard rooms at the University of Calgary Animal facilities (LESARC), in a controlled environment (20°C, 40% humidity, 12:12 photoperiods). Animals had access to food and water *ad libitum*. The study was set up in two separate phases: 1) establishment of the model and 2) treatment phase. The purpose in establishing the model was to determine an inoculum size that would induce severe, acute pneumonia, but was not fatal within 24h.

Establishment of the model: To establish the model the following experimental schedule was adhered to:

Day 0:	Piglets arrival
Day 0-Day 7:	Acclimation
Day 0-Day 14:	Sham-treatment feeding
Day 14:	Inoculation

Day 14 + 3h: Experimental Sampling for 3h time point

Day 14 + 24h: Experimental Sampling for 24h time point

Piglets were weight-ranked, and randomly assigned to one of three groups:

1) Controls, inoculated intratracheally with sterile, endotoxin free saline
2) Infected, inoculated intratracheally with 2.82×10^6 or 6.31×10^8 CFU live *A. pleuropneumoniae*, serotype 1 in endotoxin free saline.

For the establishment phase the following parameters were measured: neutrophil infiltration in BAL (% and neutrophils/mL BAL), bacterial recovery, and LTB_4 concentration. During the establishment phase it was determined that the inoculum size that would induce severe, acute pneumonia was 6.31×10^8 CFU live *A. pleuropneumoniae*. Bacterial recovery was measured by spot plating BAL onto selective Brucella agar supplemented with 0.2%βNAD, 5% Horse serum, 2μg/mL crystal violet, 1μg/mL lincomycin and 128μg/mL bacitracin.

Subsequent studies proved that this inoculum concentration was too high as a substantial number of animals died within 24h (25 out of 28 animals). Therefore a lower dose of 1.5×10^7 CFU was given to the animals during the treatment phase to induce a similarly severe pneumonia, which was not lethal within 24h.

Treatment phase: Upon the determination of an appropriate inoculum, the second phase of the study was carried out as follows:

Day 0: Piglets arrival

Day0-Day7: Acclimation

Day 8-Day 14: Treatment feeding period

Day 14: Inoculation

Day 14 + 3h: Experimental sampling for 3h time point.

Day 15: Experimental sampling for 24h time point.

Animals were weight ranked and randomly assigned to 1 of 3 experimental groups and given 2mL of a 7.5 x 10^6 CFU/mL inoculum.

1) Infected, inoculated intratracheally with 1.5 x 10^7 CFU live *A. pleuropneumoniae* serotype 1 in endotoxin free saline. Animals were fed untreated pig starter rations.
2) Infected tilmicosin-treated, inoculated intratracheally with 1.5 x 10^7 CFU live *A. pleuropneumoniae* serotype 1 in endotoxin free saline. Seven days prior to infection, pigs were given food containing tilmicosin *ad libitum* in starter rations (Pulmotil^{TM}, 400 ppm).
3) Infected CTC-treated, inoculated intratracheally with 1.5 x 10^7 CFU live *A. pleuropneumoniae* serotype 1 in endotoxin free saline. 7 days prior to infection pigs were given food containing CTC *ad libitum* in starter rations (1100ppm).

Drug dosage was based on maximal concentrations of tilmicosin or CTC allowed for drug delivery in feed worldwide (63) (Elanco Animal Health, personal communication). Animal handling and all experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Calgary Animal Care Committee. Weight gain was assessed as a measure of feed intake and rectal temperatures were recorded in all experimental groups. Other parameters measured in the second phase of this study were: IL-1β concentrations, bacterial recovery, neutrophil infiltration (% and neutrophils/mL BAL), MPO activity in non-

lesional and lesional tissues, gross pathology of the lung, lesional surface area of the lung, histology of non-lesional and lesional tissues, LTB_4 concentration, apoptosis of BAL leukocytes, PGE_2 concentration, and macrophage phagocytosis of BAL neutrophils, using methods described in the next pages.

2.5 BRONCHOALVEOLAR LAVAGE (BAL)

Three and 24 hours after bacterial challenge, animals were euthanized with an overdose of sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada) and a percutaneous incision was made into the trachea. A 2.7 mm, sterile, all-purpose catheter (Rusch Inc) was introduced into the trachea, extending approximately to the bifurcation of the trachea. Bronchoalveolar lavage samples were collected by 3 sequential washings with 10 mL of pyrogen-free, sterile phosphate buffered saline (PBS). Samples were collected into sterile 50mL Polypropylene centrifuge tubes. Enumeration of the leukocytes was performed using a haemocytometer (Bright-Line improved neubauer, American Optical Corp, Buffalo, NY). A portion of each sample was serially diluted and plated onto *A. pleuropneumoniae* selective media containing, and incubated overnight at 37°C for enumeration of *A. pleuropneumoniae* colony forming units. Clearance of bacteria in CTC and tilmicosin-treated animals was determined by comparing bacterial recovery in BAL samples with the bacterial recovery from infected-untreated animals. BAL fluid was also aliquoted (100μL) and centrifuged, using a cytospin (Shandon Southern Products Ltd, Cheshire, United Kingdom) for 10 min at 20x g onto a microscope slide. Slides were fixed and stained using DiffQuik (Baxter Healthcare Corp, St. Paul, Minn). Neutrophil infiltration and macrophage phagocytic activity were

calculated for each sample as follows. Neutrophil infiltration was defined as the percentage of neutrophils in total leukocytes in the BAL. Cytospins were viewed under light microscopy at 400x magnification as shown in Figure 1. Mean value of percent neutrophils was determined by three cell counts of one hundred cells each. Macrophage phagocytic activity was determined by a phagocytic index defined as the percentage of macrophages engulfing one or more neutrophil. Cytospins were viewed under light microscopy at 1000x magnification as shown in Figure 2. Mean values of percent macrophage phagocytosis of neutrophils were determined by three cell counts of one hundred macrophages each. The remainder of the BAL was centrifuged for 10 min at 10^0C and 250 x g. The supernatant was aliquoted and stored at -70^0C for later analyses. The pellet was suspended in 10 mL 1x Hanks Balanced Salt Solution (HBSS) (Gibco HRL, Life Technologies Inc., Grand Island, NY) for leukocyte purfication.

2.6 LEUKOCYTE PURIFICATION

As porcine BAL neutrophils and macrophages have similar specific gravities (data not shown), experiments were carried out on leukocyte populations instead of purified neutrophils. The resuspended pellet (10 mL) was centrifuged for 10 min at 10°C and 250 x g. The supernatant was discarded and an erythrocyte lysing step was carried out by resuspending the pellet in 1 mL ddH_2O for 30 seconds. 1 mL of 2 x HBSS was added after the 30 seconds to restore tonicity. Only one lysing step was performed to minimize the damage to the leukocyte membrane. The sample was centrifuged again for 10 min at 10 C and 250 x g. The supernatant was discarded and the purified pellet was suspended in 500 μL of HBSS. Purified cells were counted using a haemocytometer and

Figure 1: Photomicrograph of a cytospin preparation of BAL fluid from an infected tilmicosin-treated piglet 3h post-inoculation with 1.5×10^7 CFU of live *A. pleuropneumoniae*. The cells were stained with DiffQuik and viewed under direct light microscopy at 400x magnification. Bar equals 2μm. Black arrows point to neutrophils and white arrows point to macrophages.

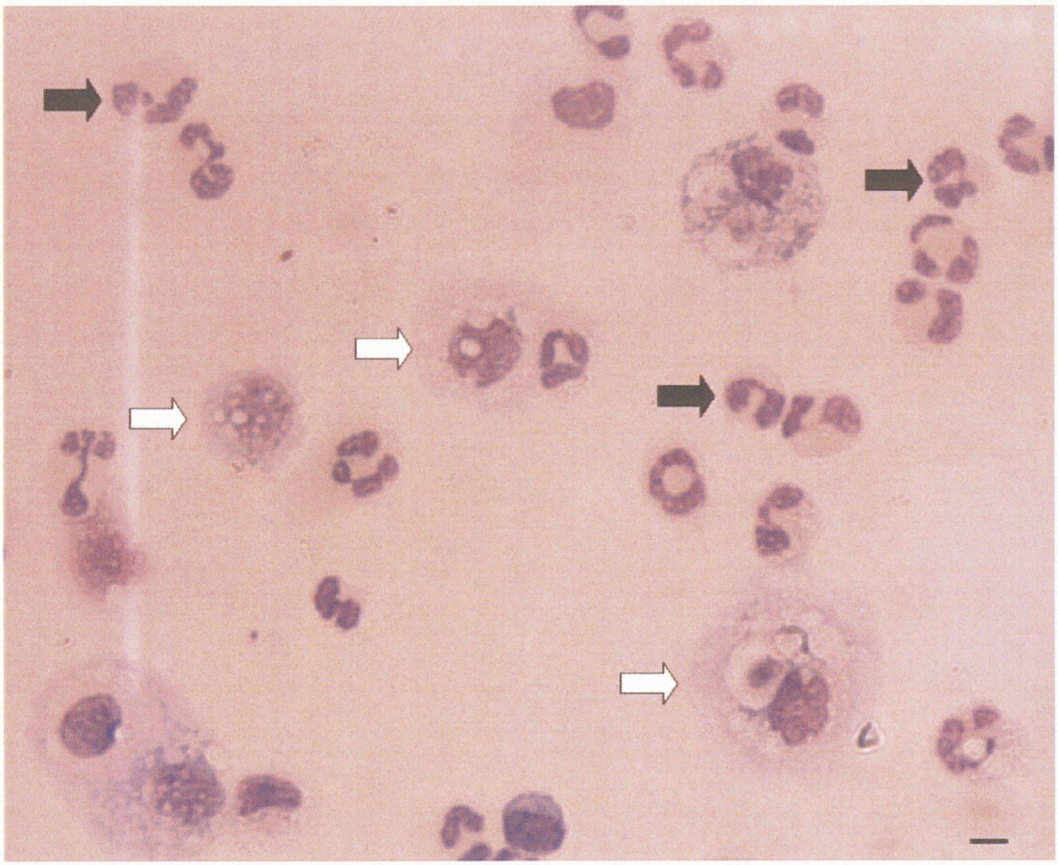
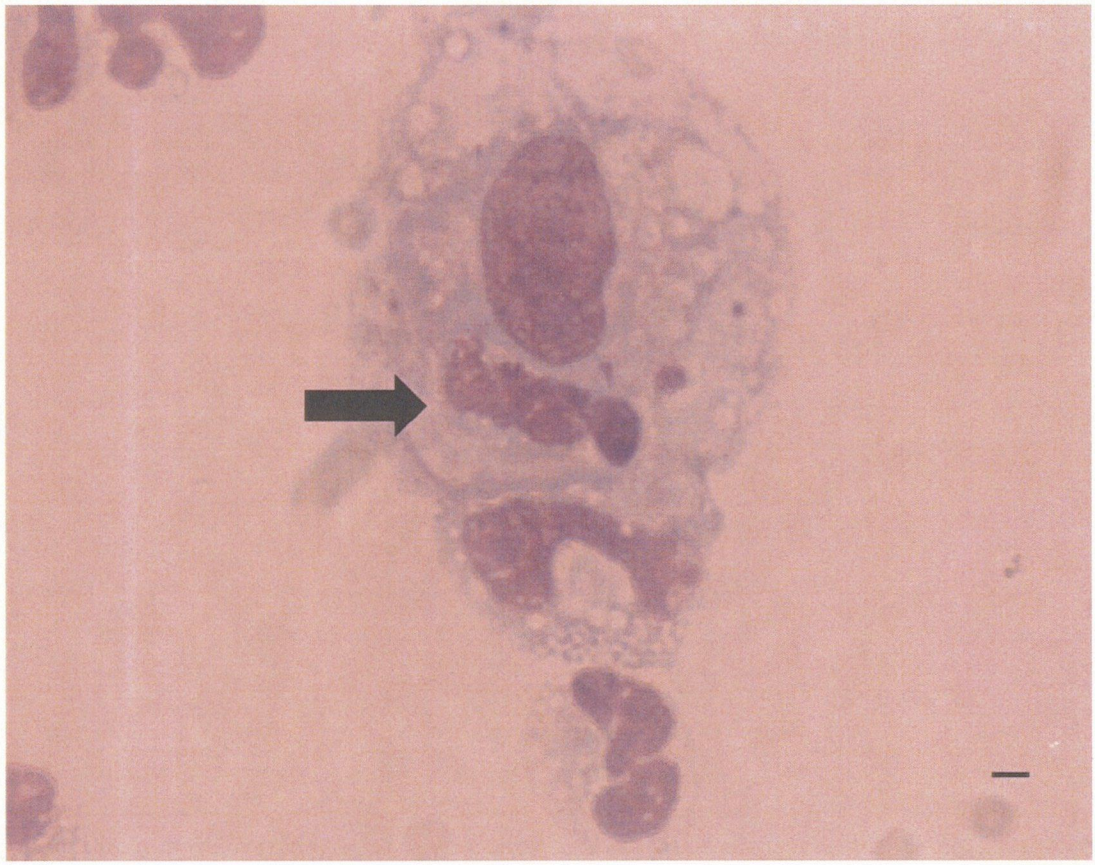


Figure 2: Photomicrograph of a cytospin preparation of BAL fluid from an infected tilmicosin-treated piglet 3h post-inoculation with 1.5 x 10^7 CFU of live *A. pleuropneumoniae*, showing macrophage phagocytosis of apoptotic BAL neutrophils. The cells were stained with DiffQuik and viewed under direct light microscopy at 1000 x magnification. Bar equals 2μm. The black arrow points to a phagocytosed neutrophil.



the population purity was calculated from a cytospin slide. Both BAL and purified leukocytes were exposed to 0.1% trypan blue (Flow Laboratories Inc, McLean, Va.) to assess cell viability. Remaining purified cells were diluted to 10^5 cells/mL in sterile endotoxin free saline and frozen at -70^0C until needed for the cell death ELISA.

2.7 MYELOPEROXIDASE ACTIVITY

Samples of non-lesional and lesional (when lesions were present) lung tissue were snap frozen in liquid nitrogen and transferred to -70^0 C. Within a week of sample collection MPO activity was measured using an MPO assay kit (Cytostore/UTI Calgary, AB) according to manufacturer's instructions. Briefly, tissue homogenates were diluted 1:10 in hexa-decyltrimethylammonium bromide (HTAB) buffer (according to tissue weight) sonicated and spun at maximum speed in a microcentrifuge for 2 minutes. The supernantant was aliquoted into ELISA plate wells to which two substrates, 1% H_2O_2 and O-dianisidine dihydrochloride were added. The enzymatic activity of MPO was measured at 450 nm in an ELISA plate reader.

2.8 GROSS PATHOLOGY AND LESIONAL SURFACE AREA OF THE LUNG

After euthanasia and BAL collection, the lungs and heart were removed from the animal and washed in a water bath. Photographs of the lungs were obtained using a digital camera (Nikon Coolpix 995, Nikon Corp. Tokyo, Japan). Planimetric analysis was used to quantify the lesional surface area of the lung. Briefly, a grid overlay was placed on the photos and the total surface area of lesional tissue was compared to the total

surface area of the entire lung. Lesional surface area was defined as the percentage of total lung with lesions.

2.9 HISTOLOGY

Samples of non-lesional and lesional (when available) lung tissue were fixed in fresh 4% paraformaldehyde for 24 hours at 4^0C. Tissues were dehydrated in ethanol, embedded in paraplast paraffin wax (Oxford Labware, St. Louis MI), sectioned and stained with haematoxylin and eosin. Qualitative observations of non-lesional and lesional alveolar integrity, as well as cellular influxes were recorded with a Leica DMR research microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Photometrics CoolSNAP (Roper Scientific, MA) high performance digital camera.

2.10 INTERLEUKIN-1β (IL-1β) ACTIVITY

IL-1β concentration in BAL supernatants was measured using a sandwich enzyme immunoassay (Quantikine porcine IL-1β kit, R&D Systems, Minneapolis Mn) performed according to manufacturer's instructions. The assay recognizes both recombinant and natural porcine IL-1β. There is no significant cross-reactivity with porcine IL-1α, IL-4, IL-6, IL-8, IL-10, PDGF, TGF-β, TNF-α. Plates were analysed using a THERMOmax microplate reader (Molecular Devices Corp, Menlo Park, Calif.) at 450 nm. The detection limit of the assay is 10 pg/mL.

2.11 LEUKOTRIENE B_4 (LTB_4) ASSAY

LTB_4 concentration in BAL supernatants was measured using a competitive enzyme immunometric assay kit (Leukotriene B_4 enzyme immunoassay kit, Cayman Chemical Co, Ann Arbor, Mich) performed according to the manufacturer's instructions. Specificity for the assay is 100% for LTB_4 and < 0.01% for arachidonic acid, 5(S)-, 12(S)- and 15(S)-hydroxyeicosatetraenoic acid as well as leukotrienes C_4, D_4, E_4 and F_4. Plates were analysed using a THERMOmax microplate reader (Molecular Devices Corp, Menlo Park, Calif.) at 405 nm. The assay has a detection limit of 4 pg/mL.

2.12 PROSTAGLANDIN E_2 (PGE_2) ASSAY

PGE_2 concentration in BAL supernatants was measured using a competitive enzyme immunometric assay kit (Prostaglandin E_2 enzyme immunoassay kit, Cayman Chemical Co, Ann Arbor, Mich) performed according to the manufacturer's instructions. Specificity for the assay is 100% for PGE_2, 43% for PGE_3, 18.7% for PGE_1, 1% for 6-keto $PGF_{1\alpha}$, 0.25% for 8-*iso* $PGF_{2\alpha}$ and <0.01% for arachidonic acid, conjugated linoleic acid, misoprostol, tetranor PGEM, tetranor PGFM, thromboxane B_2, and prostaglandins A_1, A_2, A_3, B_1, B_2, D_2, $F_{1\alpha}$, $F_{2\alpha}$ and $F_{3\alpha}$. Plates were analysed using a THERMOmax microplate reader (Molecular Devices Corp, Menlo Park, Calif.) at 405 nm. The assay has a detection limit of 31pg/mL.

2.13 LEUKOCYTE APOPTOSIS

Leukocyte apoptosis was quantified using an ELISA kit (Cell death detection ELISA kit, Boehringer-Mannheim, Laval, PQ, Canada) according to the

manufacturer's instructions. The kit is a sandwich enzyme immunoassay, which measures histone regions (H1, H2A, H2B, H3 and H4) of mono- and oligonucleosomes that are produced during apoptosis. Photometric development was measure kinetically by reading the plate at 405 nm at 5, 10, 15, and 20 minutes (THERMOmax microplate reader, Molecular Devices Corp, Menlo Park, Calif.). Apoptosis was measured in triplicate in 10^5 leukocytes from each group and expressed as the absorbance ratio for cell lysates from infected CTC and infected tilmicosin-treated animals versus the absorbance for cell lysates from infected untreated animals, which was arbitrarily set at 1. The detection limit for this ELISA is 10^2 apoptotic cells.

2.14 STATISTICS

All data were expressed as means $\pm$ SEM. Student's t-test or one-way analysis of variances (ANOVAs) were used to compare parametric data where appropriate. Mann-Whitney Rank Sum Tests or Kruskal-Wallis multiway factorial analysis of variances were used for non-parametric data. Post-tests were performed if a P value of <0.05 was obtained, using Tukey's test or Dunn's test for multiple comparison of parametric data (ANOVAs) or non-parametric data (Kruskal-Wallis multiway factorial analysis of variances) respectively. Results expressed as percentages underwent an arcsine transformation before statistical analysis and were transformed back to percentages for the expression of mean $\pm$ SEM. $P \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1 INOCULUM PREPARATION

3.1.1 Growth Curve and Calculating the Inoculum Size

A. pleuropneumoniae, serotype 1 proliferated in brucella broth supplemented with 0.2% βNAD and 5% horse serum over the first 15 h, after which their numbers declined. At 12 h, bacteria were still in growth phase (Figure 3). Plating of serial dilutions of inoculum preparations grown for 12 h in broth at 37^0C suggests that a spectrophotometric reading of approximately 0.1 (at 600 nm) will yield approximately 1 x 10^7 CFU/mL live *A. pleuropneumoniae* (Figure 4). Spectrophotometric reading of Brucella medium supplemented with 0.2% βNAD and 5% horse serum alone was used as the blank solution against which subsequent readings containing live bacteria were calculated.

3.2 ESTABLISHING THE MODEL

3.2.1 Results From Sham-treated Control Animals

Data for non- infected, sham-inoculated animals (n=3 animals per group) are presented in table 2, and show baseline control values for all parameters assessed in the infected animals. Inoculation with endotoxin free saline alone does induce mild inflammation as can be seen from the increase in neutrophil infiltration 3h post-infection in sham treated pigs (Table 2). In the BAL obtained from these uninfected animals, no live *A. pleuropneumoniae* could be recovered, and neutrophil infiltration, as well as LTB_4, values were low (Table 2).

Figure 3: Growth of *A. pleuropneumoniae*, serotype 1 in Brucella broth supplemented with 0.2% βNAD and 5% horse serum over 24h incubation at 37° C. Values are mean ± SEM from 5 separate experiments.

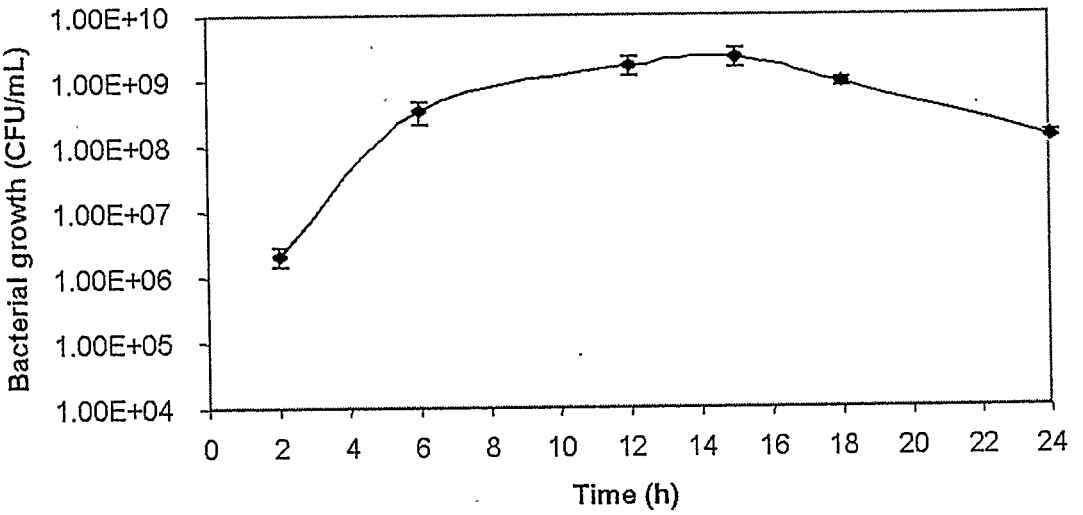

1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Bacterial growth (CFU/mL)
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)

Figure 4: Spectrophotometric readings for dilutions of live *A. pleuropneumoniae*, serotype 1 in Brucella broth supplemented with 0.2% βNAD and 5% horse serum. An approximate reading of 0.1 yields 7 $\log_{10}$ CFU/mL live bacteria, as calculated from serial dilutions plated onto Brucella agar supplemented with 0.2% βNAD and 5% horse serum after 24 h incubation at 37^0C.

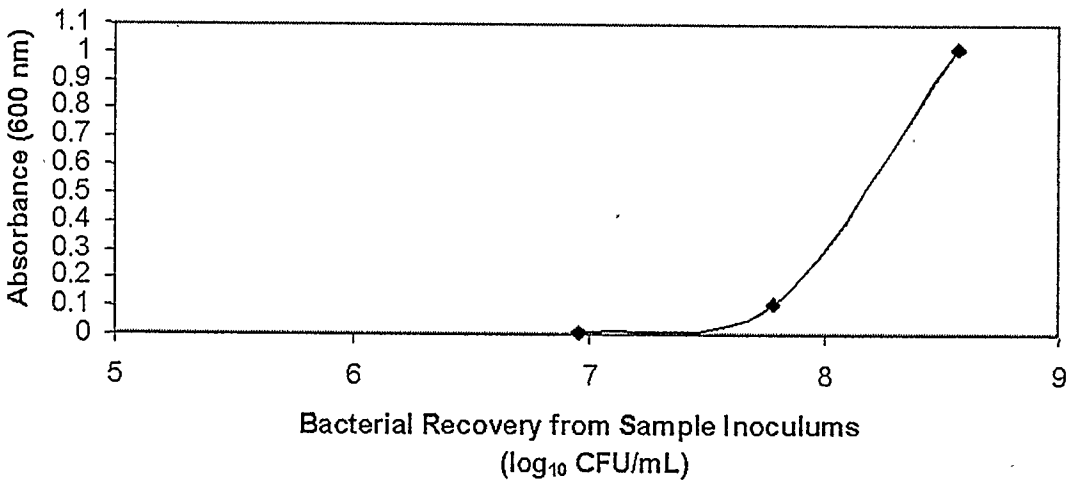

Absorbance (600 nm)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
6
7
8
9
Bacterial Recovery from Sample Inoculums
(log10 CFU/mL)

Table 2: Neutrophil infiltration (% and neutrophils/mL BAL), *A. pleuropneumoniae* recovery (log_{10} CFU/mL), and LTB_4 synthesis in BAL fluids from non-infected control piglets, 3h and 24h post sham-inoculation with sterile PBS.

Values are mean $\pm$ SEM. n=3 per group.

	Neutrophil Infiltration (%)	Neutrophil Infiltration (cells/mL)	Bacterial Recovery (Log_{10} CFU/mL)	LTB_4 (pg/mL)
3h	17 ± 2	$2.58 \pm 3.63 \times 10^6$	0	1.3 ± 0.084
24h	2 ± 1	$1.08 \pm 8.33 \times 10^6$	0	4.87 ± 2.03

3.2.2 Results From Animals Infected with *A. pleuropneumoniae*

In an attempt to establish an appropriate inoculum size for the future antibiotic testing experiments, piglets were infected intratracheally with either 2.82 x 10^6 or 6.31 x 10^8 CFU live *A. pleuropneumoniae.* One of 4 piglets given the high inoculum died prior to the 24 h sampling time, all others survived until sample collection. No significant difference in bacterial recovery was associated with either the inoculum size (2.82 x 10^6 or 6.31 x 10^8 CFU) or the time post-inoculation (3h or 24h) as seen in table 3. Infection was associated with an influx of neutrophils into the bronchoalveolar spaces (Table 4). Values for percent neutrophil infiltration as well as neutrophils per mL of BAL fluid in piglets that received the higher inoculum 24h post-inoculation were 52.75 ± 15.2% and 4.14 ± 2.19x10^6 neutrophils/mL BAL, respectively (Table 4). However, these values are not significantly higher than those found in piglets that received 2.82 x 10^6 CFU at either 3 or 24h post-inoculation or 6.31 x 10^8 CFU at 3h post-inoculation. Similarly, LTB_4 synthesis was elevated in all infected animals (Figure 5). LTB_4 levels at 24 h in the BAL fluids of piglets given the 6.31 x 10^8 CFU inoculum were significantly higher than those measured in all other groups, being 74.44% higher than in animals inoculated with 2.82 x 10^6 CFU 24h post-inoculation and 59.42% greater than animals inoculated with 6.31 x 10^8 CFU at 3h post-inoculation (Figure 5).

3.3 TREATMENT PHASE

A group was defined by the treatment that the animal received as well as the time point for which sampling was done, with the exception of weight gain in which animals

Table 3: *A. pleuropneumoniae* recovery from the BAL fluids of piglets 3h and 24h post-inoculation. Piglets were given either a 2.82×10^6 or 6.31×10^8 CFU bacterial inoculum. Values are mean $\pm$ SEM. n=3-4 per group. No significant difference is observed between any groups.

CFU in inoculum	3h Log_{10} CFU/mL	24h Log_{10} CFU/mL
2.82×10^6	3.02±0.489	5.2±1.14
6.31×10^8	5.31±1.06	6.47±0.66

Table 4: Neutrophil infiltration expressed as a percentage of BAL leukocytes and neutrophils/mL of BAL fluid from animals infected with either 2.82×10^6 or 6.31×10^8 CFU of live *A. pleuropneumoniae*. No difference was found in neutrophil infiltration between either group, 3h or 24h post-inoculation.

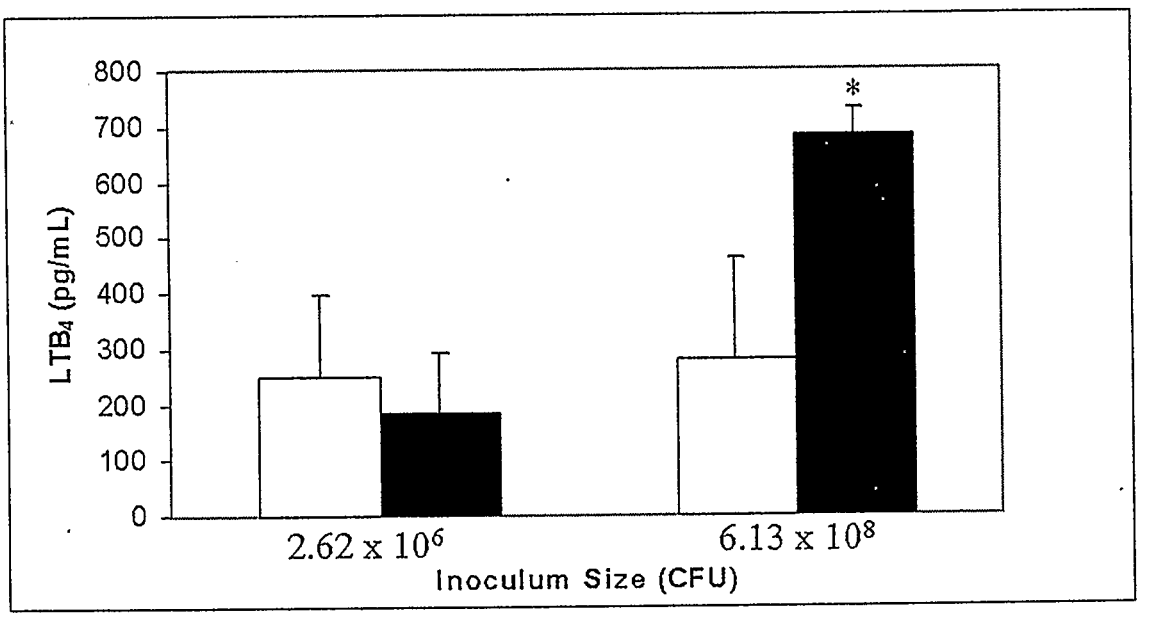
Values are mean $\pm$ SEM. n=3-4 per group.

CFU in inoculum	3h % Neutrophils (Neutrophils/mL)	24h % Neutrophils (Neutrophils/mL)
2.82×10^6	48 ± 22.7 $(9.43 \pm 4.59 \times 10^5)$	31.75 ± 11.9 $(13.6 \pm 4.83 \times 10^5)$
6.31×10^8	39 ± 16.5 $(1.77 \pm 1.48 \times 10^6)$	52.75 ± 15.2 $(4.14 \pm 2.19 \times 10^6)$

Figure 5: LTB_4 levels in the BAL from piglets infected with either 2.82×10^6 or 6.31×10^8 CFU of live *A. pleuropneumoniae*. No difference was found in LTB_4 values at 3h (□) post-inoculation. Twenty-four hours (■) post-inoculation animals given the high inoculum had significantly higher LTB_4 values compared to animals given the low inoculum at 24h post-inoculation.

Values are means ± SEM. n=3-4 per group.

*$P<0.05$ vs. low inoculum animals at 3h.


800
700
600
500
400
300
200
100
0
LTB4 (pg/mL)
*
2.62 x 10^6
6.13 x 10^8
Inoculum Size (CFU)

were grouped by treatment only. One untreated animal died in the study during the inoculation procedure.

3.3.1 Clinical Observations: Weight Gain and Fever

Piglets were weighed on a daily basis beginning on the day of arrival (day 0) until the day of inoculation (day 14). The mean gain for untreated, CTC-treated and tilmicosin-treated animals over the course of 14 days was 3.084 kg, 4.125 kg, and 3.805 kg respectively (Figure 6). All animals weighed significantly more 14 days after arrival, and there was no difference in the weight of animals between the three treatment groups on the day of arrival or after 14 days as shown in figure 6. Twenty-four hours post-inoculation, untreated and CTC-treated animals show significantly increased rectal temperatures compared to their temperatures 15 minutes before inoculation (Figure 7). This increase in rectal temperature is not seen in tilmicosin-treated animals 24h post-inoculation. There was no statistically significant difference in rectal temperatures between experimental groups at the 15 min or 24h time points.

3.3.2 IL-1β Levels in BAL

Concentrations of BAL IL-1β, a potent pyrogen, were measured 3h and 24h post-inoculation. At 3h or 24h post-inoculation, concentrations of BAL IL-1β were not significantly different between any group (Figure 8).

3.3.3 Bacterial Recovery

At 3h post-inoculation both CTC and tilmicosin significantly reduced *A. pleuropneumoniae* colonization in the bronchoalveolar space, by 23.2% and 45.4% respectively, compared with infected, untreated animals as depicted in table 5. At 24h post-inoculation, bacterial numbers were not different between any group (Table 5).

Figure 6: Body weights of piglets experimentally infected with 1.5 x 10^7 CFU of live *A. pleuropneumoniae* on the day of arrival (□) and at time of infection (■) (Day 14). Animals weighed significantly more 14 days after arrival than on the day of arrival. Weights were not significantly different between any group on the day of arrival or at day 14 (time of experimentation).

Values are mean ± SEM. n=10-14 animals per group.

*$P<0.05$ vs. animals from the same group on the day of arrival.

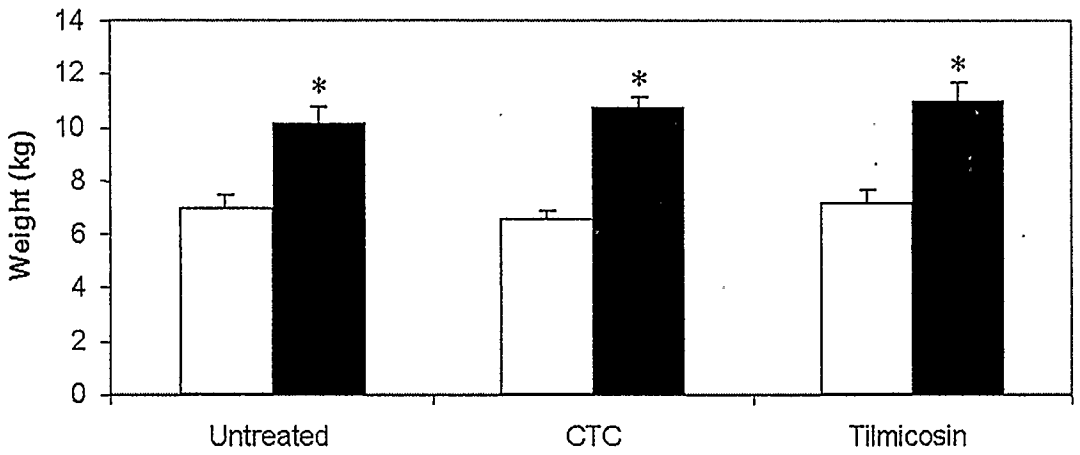

14
12
10
8
6
4
2
0
Weight (kg)
*
*
*
Untreated
CTC
Tilmicosin

Figure 7: Rectal temperatures of piglets experimentally infected with 1.5 x 10^7 CFU of live *A. pleuropneumoniae* 15 minutes prior to infection (□) and 24h (■) post-inoculation. Values are mean ± SEM. n= 4-6 per group.

*$P < 0.05$ vs. untreated animals 15 min. prior to infection, #$P< 0.05$ vs. CTC-treated animals 15 min. prior to infection.

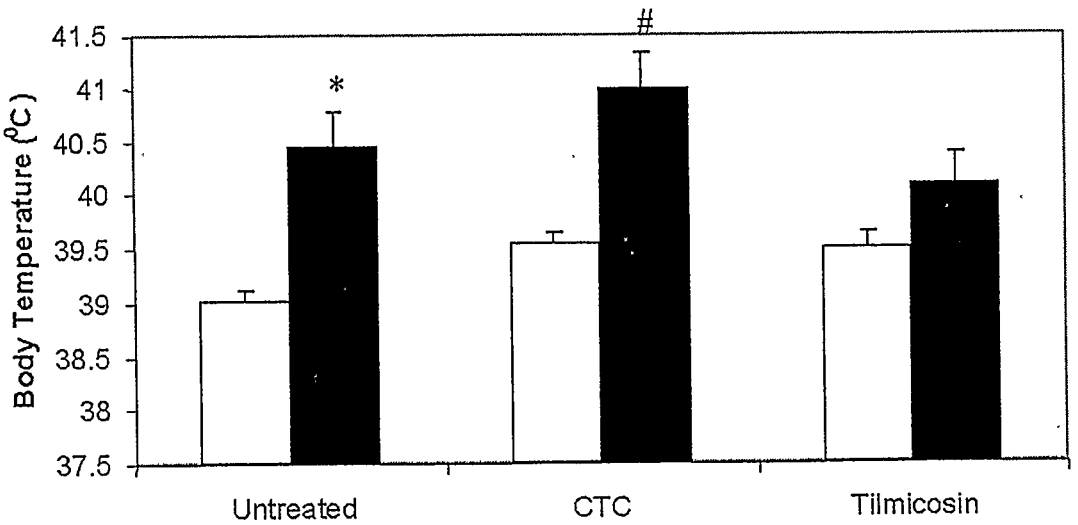

41.5
41
40.5
40
39.5
39
38.5
38
37.5
Body Temperature (°C)
*
#
Untreated
CTC
Tilmicosin

Figure 8: IL-1β concentrations in BAL fluids from piglets experimentally infected with 1.5×10^7 CFU of live *A. pleuropneumoniae*. Values were not significantly different between any group at either 3h (□) or 24h (■) post-inoculation.

Values are mean ± SEM. n= 4-6 per group.

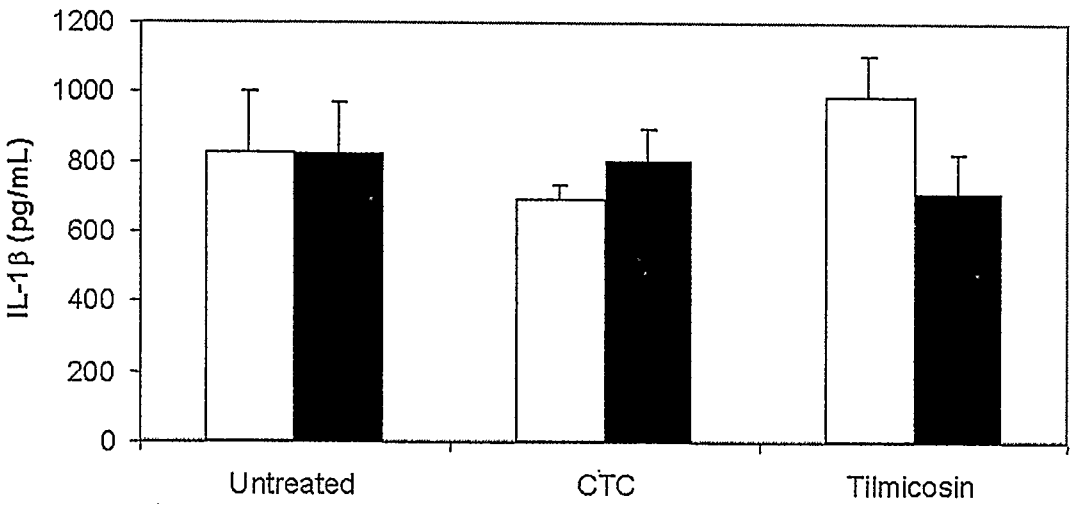

1200
1000
800
600
400
200
0
IL-1β (pg/mL)
Untreated
CTC
Tilmicosin

Table 5: Recovery of live *A. pleuropneumoniae* in BAL fluids of piglets experimentally infected with 1.5 x 10^7 CFU of live *A. pleuropneumoniae*. Both CTC and tilmicosin-treated animals had significantly lower CFU counts versus untreated animals at 3h but not at 24h post-inoculation. Also, tilmicosin-treated animals had significantly lower CFU counts versus CTC-treated animals at 3h post-inoculation.

Values are mean $\pm$ SEM. n=4-6 per group.

*$P < 0.05$ vs. untreated 3h post-inoculation

** $P < 0.01$ vs. untreated 3h post-inoculation

# $P < 0.05$ vs. CTC 3h post-inoculation

	3h Log_{10} CFU/mL	24h Log_{10} CFU/mL
Untreated	5.14 ± 0.22	4.40 ± 0.34
CTC	$3.95 \pm 0.33^{*}$	4.52 ± 0.10
Tilmicosin	$2.81 \pm 0.13^{**\#}$	4.46 ± 0.75

3.3.4 Polymorphonuclear Leukocyte Infiltration and Myeloperoxidase Activity

Significant neutrophilic infiltration was seen in all piglets by 3h post-inoculation (greater than 50% of all leukocytes) (Table 6). Neutrophil infiltration was elevated in all groups, and it was not significantly different between any group at any time. To further characterize infiltration of leukocytes into the lung, MPO activity was measured in non-lesional and lesional tissue. Twenty-four hours post-infection, the MPO activity in non-lesional tissue was significantly less than in lesional tissue in both untreated and CTC treated groups (Figure 9). Statistics could not be applied to the tilmicosin treated group as only one animal out of six had lesion formation 24h post-infection. Three hours post-infection the MPO activity of non-lesional tissue was not significantly different among the three treatment groups (data not shown). At this time no lesional lung tissue had formed.

3.3.5 Pulmonary Lesions

Infection with *A. pleuropneumoniae* induces the formation of necrotic, hemorrhagic lesions in the lung by 24h post-infection (Figure 10). The lesional surface area was significantly reduced in animals treated with tilmicosin (Figures 10 and 11). Only one out of six tilmicosin-treated animals showed signs of pulmonary lesions, while all of the piglets in the untreated and the CTC-treated groups had lesions 24h post-inoculation. Histological analysis of lesional areas indicates that pulmonary lesions are associated with severe neutrophilic infiltration and loss of alveolar structure (Figure 12). No lesions were seen 3h post-infection (data not shown).

Table 6: Neutrophil infiltration expressed as a percentage of BAL leukocytes and neutrophils/mL of BAL fluid in piglets infected with 1.5×10^7 CFU of live *A. pleuropneumoniae*. No statistically significant difference was seen between any group at 3h or 24h post-inoculation.

Values are mean $\pm$ SEM. n= 4-6 per group.

	3h % Neutrophils (Neutrophils/mL)	24h % Neutrophils (Neutrophils/mL)
Untreated	63.25 ± 5.14 (3.85 ± 1.92 x 10^6)	30. 4 ± 6.13 (4.95 ± 1.95 x 10^5)
CTC	53.2 ± 11.8 (5.47 ± 3.27 x 10^6)	52.4 ± 4.77 (1.49 ± 5.31 x 10^5)
Tilmicosin	61.8 ± 13.1 (2.15 ± 7.81 x 10^5)	44.67 ± 12.6 (8.42 ± 4.46 x 10^5)

Figure 9: MPO concentration, used as a marker of neutrophil infiltration, in lesional (■) and non-lesional (□) areas of the lungs of piglets 24 h post-inoculation with 1.5×10^7 CFU of live *A. pleuropneumoniae*. Compared with non-lesional areas, tissues obtained within lesions have high MPO contents. MPO levels were not different between untreated, CTC-treated, or tilmicosin-treated animals. No lesions were seen in the lungs 3h post-inoculation (not shown).

Values are mean ± SEM. n = 4-5 per group (with the exception of tilmicosin / lesional (n=1) where only 1 animal showed any signs of pulmonary lesions).

$^{*}P<0.01$ vs. untreated lesional tissue.

$^{\#}P < 0.01$ vs CTC-treated lesional tissue.

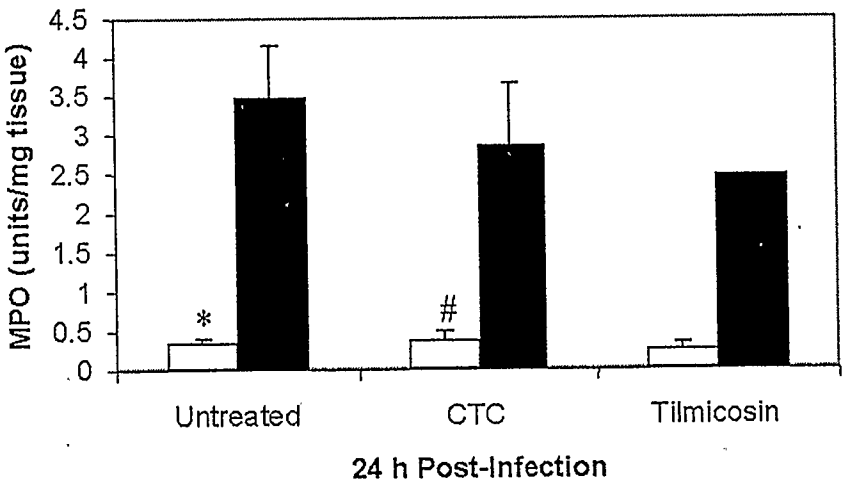
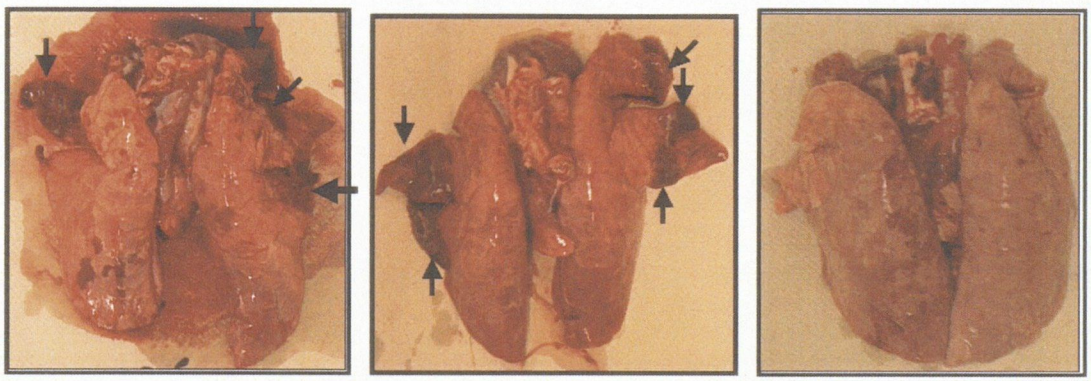

MPO (units/mg tissue)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
#
Untreated
CTC
Tilmicosin
24 h Post-Infection

Figure 10: Representative lung morphology post-mortem in untreated piglets (A), infected CTC-treated piglets (B), and infected tilmicosin-treated piglets (C), 24 h post-inoculation with 1.5 x 10^7 CFU of live *A. pleuropneumoniae*. Black arrows point to the pneumonic lesions seen in untreated and CTC-treated animals. Lesions are almost completely absent from lungs of tilmicosin-treated animals.



A B C

Figure 11: Surface area of pulmonary lesions in piglets 24 h post-inoculation with 1.5 x 10^7 CFU of live *A. pleuropneumoniae.* Mean surface area of lesions in tilmicosin-treated animals was significantly less than in the untreated and CTC-treated animals. Mean lesional surface area in the lungs of CTC-treated piglets was not different from that measured in the lungs of untreated animals. Pulmonary lesions were not seen 3h post-inoculation (data not shown).

Values are mean $\pm$ SEM, n= 4-6 per group.

*$P < 0.001$ vs. untreated, # $P < 0.001$ vs. CTC-treated.

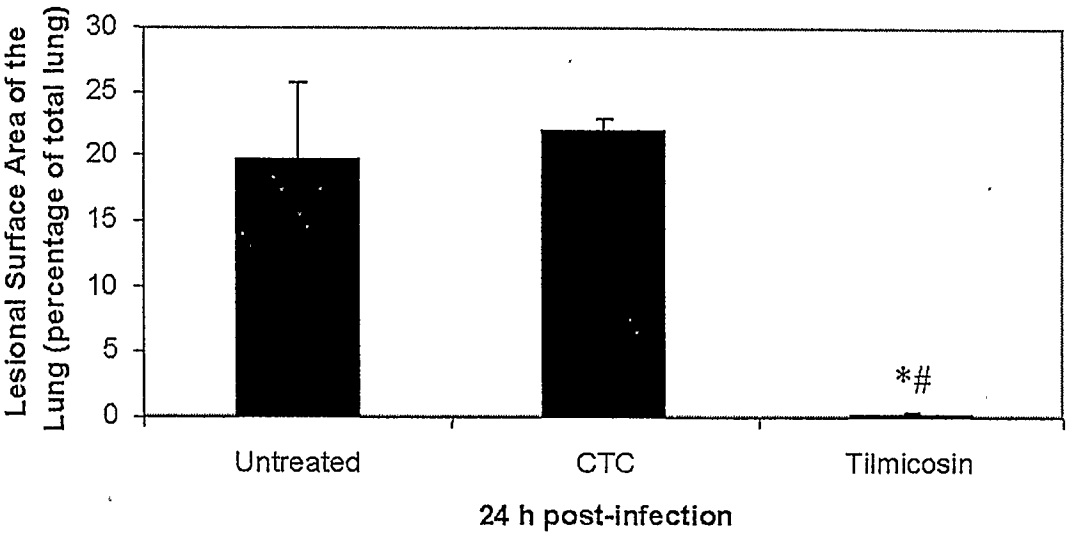
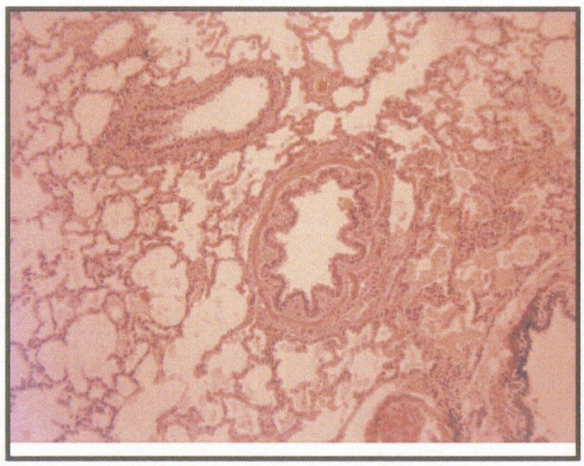
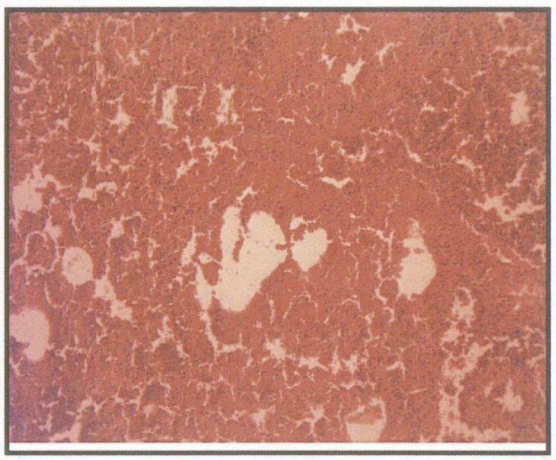

Lesional Surface Area of the Lung (percentage of total lung)
30
25
20
15
10
5
0
*#
Untreated
CTC
Tilmicosin
24 h post-infection

Figure 12: Representative lung histology in pulmonary tissues from piglets 24 h post-inoculation with 1.5 x 10^7 CFU of live *A. pleuropneumoniae*. Sections were obtained from non-lesional areas or from lesional areas of the infected lung. Extensive neutrophilic infiltration can be seen in lesional tissues.

H & E, 100 X original magnification.



Untreated Non-lesional Tissue



Untreated Lesional Tissue

3.3.6 Leukotriene B_4 Synthesis

Concentrations of LTB_4, a potent pro-inflammatory mediator in the BAL, were measured in each group at 3h and 24h post-infection and compared to levels in sham-treated animals. At 3h after infection with *A. pleuropneumoniae,* concentrations of LTB_4 were significantly higher in untreated animals compared to sham treated animals (Figure 13). Infection caused a significant rise in LTB_4 levels in untreated and CTC-treated animals compared to sham treated animals 24h post-inoculation (Figure 13). This increase was inhibited in tilmicosin-treated animals.

3.3.7 Leukocyte Apoptosis

A quantitative ELISA comparing the production of apoptotic nucleosomes by BAL cells was used for this study. As illustrated in figure 14, 3h post-infection BAL leukocytes from tilmicosin-treated piglets had significantly higher levels of apoptosis compared to BAL cells from CTC-treated animals. At 24h post-inoculation, no difference was seen in BAL leukocyte apoptosis among groups (data not shown).

3.3.8 Prostaglandin E_2 Synthesis

Concentrations of BAL PGE_2, a potential anti-inflammatory mediator in the lung, were measure 3h and 24h post-inoculation. At 3h or 24h post-inoculation, concentrations of BAL PGE_2 were not significantly different between any group (Figure 15).

3.3.9 Macrophage phagocytosis of BAL neutrophils

Elimination of apoptotic neutrophils by alveolar macrophages is an important contributor to the resolution of pulmonary inflammation. At 3h post-inoculation, 6 ± 2.828 and 6.4 ± 2.619 % BAL neutrophils were phagocytosed by alveolar macrophages in untreated and CTC-treated animals respectively (Figure 16). In tilmicosin-treated

Figure 13: LTB_4 levels in the BAL from piglets experimentally infected with 1.5×10^7 CFU of live *A. pleuropneumoniae*. Three hours (□) post-inoculation LTB_4 values were significantly higher in untreated animals compared to sham treated. Twenty-four hours post-inoculation (■) BAL LTB_4 values were high in untreated and CTC, but remained at baseline levels in tilmicosin-treated animals compared to sham treated animals. Values are means ± SEM. n=4-6 per group.

$^{\#}P<0.05$ versus sham treated at 3h, $^{*}P<0.05$ vs. sham treated at 24h.

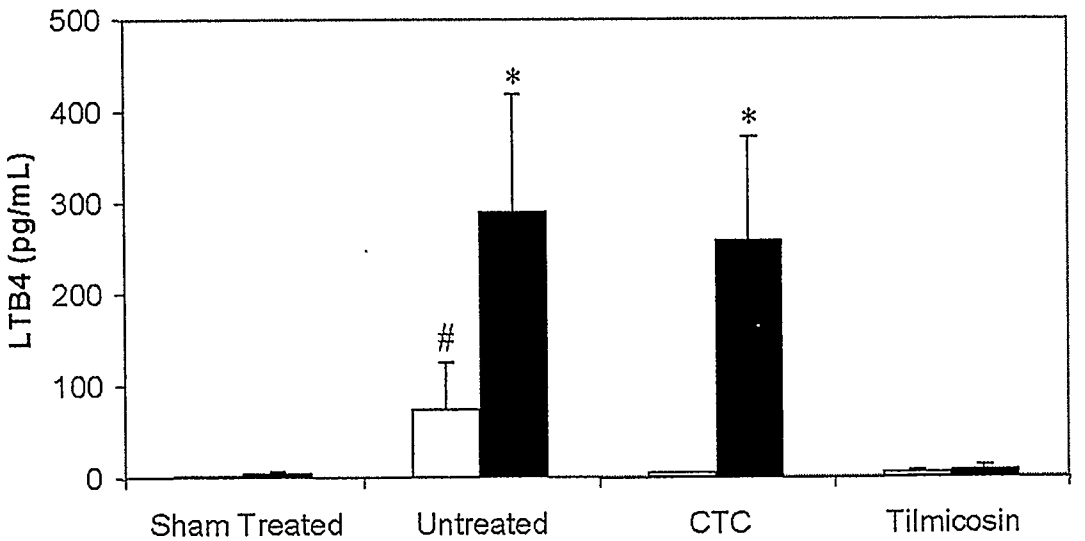

500
400
300
200
100
0
LTB4 (pg/mL)
#
*
*
Sham Treated
Untreated
CTC
Tilmicosin

Figure 14: Apoptosis ratios of BAL leukocytes from CTC-treated (♦) piglets and tilmicosin-treated (■) piglets versus apoptotic values measured in BAL leukocytes from untreated animals (dotted line), set at 1.0, 3h post-inoculation. Ratios were calculated over 20 min of ELISA development. At all time points of ELISA development, ratios seen in tilmicosin-treated animals were significantly higher compared to those of CTC-treated animals, which were not different from values in untreated animals.

Values are mean ± SEM. n=4-6 per group.

* $P < 0.05$ vs. CTC-treated.

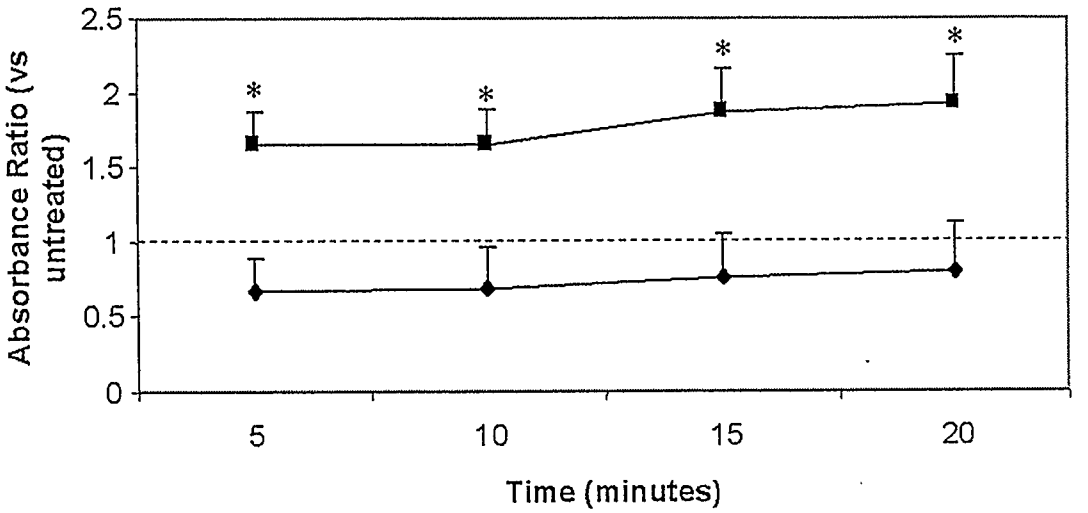

Absorbance Ratio (vs untreated)
2.5
2
1.5
1
0.5
0
*
*
*
*
5
10
15
20
Time (minutes)

Figure 15: Concentrations of PGE_2 in BAL fluids of piglets infected with 1.5×10^7 CFU of live *A. pleuropneumoniae*. Values were not significantly different between any group at either 3h (□) or 24h (■) post-inoculation.

Values are mean ± SEM. n= 4-6 per group.

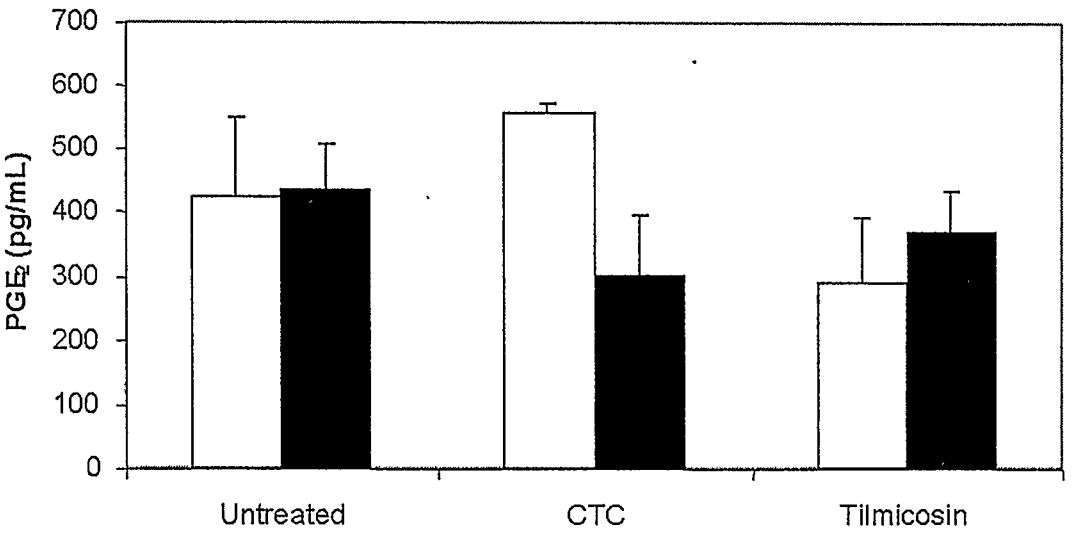
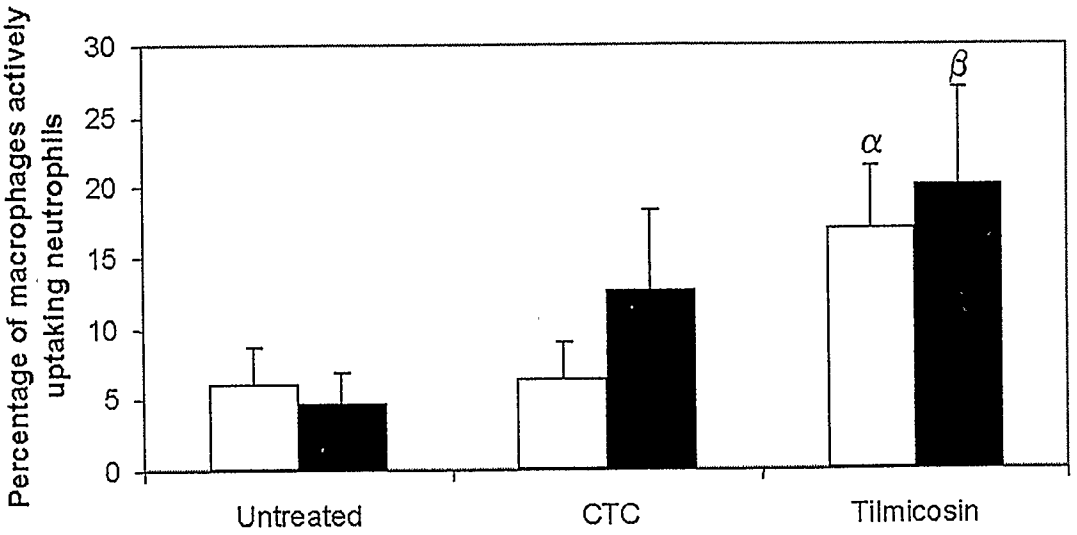

700
600
500
400
300
200
100
0
PGE2 (pg/mL)
Untreated
CTC
Tilmicosin

Figure 16: Percentage of BAL macrophages that phagocytosed at least 1 neutrophil at either (□) 3h post-inoculation or (■) 24h post-inoculation in infected untreated, CTC-treated or tilmicosin-treated animals. Measurements were made using cytospin preparations of BAL and observed under light microscopy. Values failed to reach statistically significant differences between groups at each time point.

Values are means ± SEM. n=4-6 per group.

α P=0.083 vs. untreated and CTC at 3h, βP=0.103 vs. untreated and CTC at 24h.


Percentage of macrophages actively uptaking neutrophils
30
25
20
15
10
5
0
α
β
Untreated
CTC
Tilmicosin

animals this number had reached 17 $\pm$ 4.461%, but this rise failed to reach statistical significance (P=0.083). Similar observations were made 24h post-inoculation as shown in figure 16.

4. DISCUSSION

In an effort to assess the pro-apoptotic and anti-inflammatory potential of an oral formulation of the macrolide tilmicosin, this study assessed the effects of this antibiotic in the porcine lung infected with *A. pleuropneumoniae*. Firstly, the animal model of pleuropneumonia was established to investigate the inflammation associated with this disease. This model helped to establish valuable experimental parameters and an appropriate inoculum size prior to studying the effects of tilmicosin treatment. Experimental infection with *A. pleuropneumoniae* was associated with fever, severe inflammation, elevated LTB_4 in the bronchoalveolar space and caused pulmonary lesions consistent with those found in pigs with porcine pleuropneumonia. Results from the treatment phase of this study indicate that both CTC and tilmicosin reduced bacterial load in the lungs of infected animals (3h post-infection). Tilmicosin treatment, but not CTC also inhibited the infection-induced increase in rectal temperatures measured in untreated and animals. Tilmicosin treatment has significant anti-inflammatory benefits as evidenced by the inhibition of pulmonary lesions and reduced LTB_4 levels in the bronchoalveolar lavages, despite the concurrent presence of live bacteria in the lungs. Furthermore, the results demonstrate that when delivered in feed, this antibiotic, but not CTC, enhances apoptosis in porcine bronchoalveolar leukocytes. . Neutrophil infiltration into the lungs and MPO concentration in non-lesional and lesional tissues was not different among groups. In conclusion, the results of this study indicate that tilmicosin administered to piglets in the feed may have anti-inflammatory benefits in the course of porcine pneumonia via mechanisms, which are associated with the induction of leukocyte apoptosis.

4.1 *A. PLEUROPNEUMONIAE* GROWTH AND INOCULUM PREPARATION

Prior to the initiation of the study, a reliable method of growing and preparing an inoculum needed to be established. *A. pleuropneumoniae* is a fastidious bacterium, meaning that it grows poorly or not at all in conventional media (105). As well, *A. pleuropneumoniae* is dependent on β NAD (a blood factor produced when NADH reduces oxidized vitamin K) for growth, therefore media must be supplemented with β NAD (105). In the framework of the present study, growth of *A. pleuropneumoniae* peaked after 15 hours of incubation. In the interest of generating a virulent inoculum, a time point during log phase was selected. Therefore, *A. pleuropneumoniae* was grown over 12 hours for the preparation of the inoculum.

In order to determine an approximate inoculation size, spectrophotometric readings of inoculum dilutions were obtained and plotted versus CFU counts. For an inoculum preparation, appropriate dilutions were made based on absorbance readings and the final inoculum was read again to ensure accuracy, and CFUs were enumerated from serial dilutions.

4.2 ESTABLISHMENT OF THE MODEL

It is well established that neutrophils play a central role in eliminating bacteria from the lung. Enhanced infiltration and activity of neutrophils in the lungs plays a pivotal role in protection against *A. pleuropneumoniae* infection in piglets (89). Consistent with these findings, experimental infection caused an increase in neutrophil

infiltration in the bronchoalveolar space of infected piglets over sham treated controls. These findings are in agreement with those of Baarsch *et al.*, who previously reported an increase in neutrophil infiltration into the porcine lung 24h post-inoculation with *A. pleuropneumoniae* (9).

The eicosanoid LTB_4, is a potent chemoattractant for neutrophils in the inflamed lung (49), (139). LTB_4 also induces neutrophil degranulation and lysozyme release, which further amplifies the inflammatory response (111), (139). LTB_4 accumulation has been implicated in chronic inflammation of mucosal sites and, as such, is a reliable marker of inflammation (65), (137), (148). Although specific studies regarding LTB_4 accumulation during *A. pleuropneumoniae* infection have not been performed, it is well known that LTB_4 concentrations are increased during bacterial infections. Furthermore, *M. haemolytica*, a bacterial pathogen belonging to the same family as *A. pleuropneumoniae*, increases LTB_4 release from neutrophils (25), (62). In the bovine lung experimentally infected with *M. haemolytica*, severe inflammation is associated with a more than sevenfold increase in BAL LTB_4 concentrations compared with baseline concentrations at 3 hours post-infection (23). Consistent with these observations, the present study describes significantly elevated LTB_4 levels in the lungs of piglets 24h post-infection with *A. pleuropneumoniae*. Based on these elevated LTB_4 levels, 6.31 x 10^8 CFU was determined as an inoculum size that would induce severe, acute pneumonia. Subsequent studies proved that this inoculum concentration was too high as a substantial number of animals died within 24h (25 out of 28 animals). Therefore a lower dose of 1.5 x 10^7 CFU was ultimately given to the animals for the treatment phase of this study.

4.3 TREATMENT PHASE

Average weight gain, as a measure of feed intake, from the day of arrival until the day of infection show that all piglets gained weight throughout the 14 day study period, and that there was no difference in weight gain among piglets from different treatment groups. Thus, all piglets were eating approximately the same amount of food.

Tilmicosin treatment reduced the elevated temperature associated with experimental *A. pleuropneumoniae* infection. Circulating IL-1β is a potent endogenous pyrogen released by leukocytes in response to infectious agents (119). IL-1β can induce fever by entering the hypothalamus, and by activating the brain vasculature to stimulate the release of mediators such as prostaglandins or nitric oxide (119). Erythromycin treatment of patients with diffuse panbronchiolitis decreases both serum levels and bronchoalveolar lavage concentrations of IL-1β (81), (104). In a murine model of chronic respiratory infection caused by *P. aeruginosa* clarithromycin significantly reduces IL-1β concentration in aqueous lung extracts (155). Roxithromycin also inhibits IL-1β increases in mice lungs instilled with LPS (143). In an attempt to correlate the reduction in rectal temperature seen in tilmicosin treated animals 24h post-infection with a reduction in endogenous pyrogen production, IL-1β levels in bronchoalveolar lavage samples were measured. Bronchoalveolar IL-1β was elevated in all groups, regardless of time point or treatment. Similarly, PGE_2 levels remained high in all groups. These finding suggest that the apparent anti-pyrogenic benefit of tilmicosin measured in the present study cannot be explained by reduced IL-1β or PGE_2 in the bronchoalveolar space. It remains possible that other mediators such as IL-6 or TNF-α are modulated by

tilmicosin in this particular model. Regardless, future studies measuring other pyrogenic and/or cytokine and eicosanoid levels in the serum need to be performed to improve an understanding of the mechanisms through which tilmicosin reduces fever.

It is well established that both tilmicosin and CTC given in feed are effective treatments for the control of *A. pleuropneumoniae* infection in swine (16), (67), (100), (101), (114), (149). Results from the present study at 3h post-infection are consistent with these observations. Interestingly, the anti-infective effects of the antibiotic could not be detected 24h post-infection. More research is needed to determine whether this reflects the fact that the animals were too sick to eat after the introduction of *A. pleuropneumoniae* or whether this phenomenon may represent a feature of the pharmacokinetics of these oral antibiotics.

Neutrophil infiltration into the bronchoalveolar space of infected piglets remained elevated in all animals regardless of time point or treatment. Secretion of MPO from the cytosol of neutrophils occurs during phagocytosis or following exposure to an antibody coated surface (75). While MPO may also be contained in small amounts in other cell types, it is a reliable measure of inflammatory cell infiltration. MPO catalyses a reaction between hydrogen peroxide and halide to produce oxidized halogens that are potent anti-microbials with the ability to damage host tissue. To test for the differences in neutrophil infiltration between lesional and non-lesional tissue, an MPO assay was performed on both types of tissue collected from animals twenty-four hours post-infection, the time point at which lesions were present. All groups showed over a 7.5 fold increase in MPO activity in lesional tissue versus non-lesional tissue. The findings also revealed that there was no difference in MPO activity in the non-lesional or lesional tissue between any

groups, suggesting that neither CTC nor tilmicosin-treatment impaired neutrophil influx. It is important to note, that out of six tilmicosin treated animals, only one animal actually had lesion formation whereas in the other two groups 100% of animals infected had lesion formation 24h post-infection. In untreated and CTC-treated animals approximately 20% of the surface area of the lung was covered with lesions, while only 0.17% of the surface area of the lung of tilmicosin-treated animals is lesional, which was significantly less than the values measured in the other two groups ($P<0.001$). Histological micrographs revealed the compromised functionality of "lesional lung tissue". Although, non-lesional sections of the lung were inflamed, the alveolar structures remained largely intact. In contrast, lesional sections showed excessive leukocyte infiltration and the obliteration of functional alveoli. These results indicate that neutrophils become sequestered within lesional areas and may secrete MPO into the surrounding tissue. Furthermore, these observations suggest that tilmicosin, but not CTC, prevents the formation of pulmonary lesions during *A. pleuropneumoniae* infection, and hence more effectively maintains alveolar integrity and lung function. The inhibition of lesion formation and reduction in fever associated with tilmicosin treatment 24h post-infection, may translate into reduced predisposition to future infections, better growth rate and the maintenance of normal respiratory function in these animals.

Results from this study confirmed that elevated LTB_4 levels may be found at sites of inflammation (139). Moreover, the effects of oral tilmicosin in piglets with pleuropneumonia are consistent with those found in bovine pneumonic pasteurellosis. Indeed, 24 hours post-infection, both untreated and CTC-treated animals had a greater than 3.5 fold increase in bronchoalveolar LTB_4 levels compared with the baseline

concentrations detected in sham-treated animals 24 hours post-infection. In contrast, piglets treated with tilmicosin did not experience an increase in BAL LTB_4 concentration 24 hours after inoculation with *A. pleuropneumoniae* compared to sham-treated animals, despite the concurrent presence of bacteria. Recent observations (Lee *et al.* submitted publication) indicate that tilmicosin may directly impair spontaneous LTB_4 release from circulating bovine neutrophils *in vitro*. The mechanisms responsible for the apparent effects of tilmicosin on arachidonic metabolism warrant further investigation.

The microbicidal activity of neutrophils is one of the key mechanisms for pulmonary clearance of infectious agents. Although neutrophil recruitment is fundamentally protective against bacterial infections, the effectiveness of neutrophils in clearing *A. pleuropneumoniae* from the porcine lung is compromised by the cytotoxic activity of the viable bacteria on phagocytes (122). It is well known that toxic oxidants and enzymes released from neutrophils injure pulmonary endothelial cells and pneumocytes (7), (94). The cytotoxic effects of *A. pleuropneumoniae* on neutrophils can be attributed to various factors including hemolysins, a family of RTX (repeat in toxin) proteins similar to the leukotoxin produced by *M. haemolytica* (33), (34). The RTX toxins of *A. pleuropneumoniae* enable this pathogen to evade phagocytic uptake by neutrophils. Moreover, these toxins also have the ability to form pores in the membrane of host cells such as neutrophils, thereby inducing osmotic swelling and oxidative burst typical of necrotic cell death (153). Therefore the infected lungs quickly become infiltrated with necrotic neutrophils, which spill their pro-inflammatory contents onto the tissues (153). In this fashion, self-perpetuating necrosis of neutrophils is clearly a contributing factor to the pathogenesis of porcine pleuropneumonia. Neutrophil necrosis

may in fact, be partially responsible for the formation of the lesions illustrated in the present study, as it has been previously demonstrated that areas of coagulative necrosis are preceded by inflammation characterized by the exudation of neutrophils (89).

In contrast to necrosis, neutrophil apoptosis is a key mechanism for the removal of extravasted neutrophils and contributes to the resolution of inflammation in the lung as well as in other tissues (29), (130). Apoptotic neutrophils do not function as normal neutrophils do. They lose, at least in part, their ability to generate a respiratory burst or degranulate, and thus they do not release toxic oxygen species or enzymes onto the tissue (151). Research has established that macrolides possess the ability to alter neutrophil function and thus may have anti-inflammatory effects. Erythromycin, a 16 ring macrolide, inhibits superoxide production in human neutrophils stimulated with fMLP at physiologic concentrations and reduces neutrophil infiltration into the bronchoalveolar space in a murine model of allergic alveolitis (99), (140). Flow cytometric analysis of neutrophils from patients receiving a single injection of azithromycin demonstrated that azithromycin reduces the ability of neutrophils to phagocytose *E. coli* and produce reactive oxygen intermediates (150). Additionally, azithromycin has been shown to induce neutrophil apoptosis in circulating human neutrophils, without altering IL-8 production (78). Recent observations indicate that clinical concentrations of tilmicosin given sub-cutaneously to cattle induces neutrophil apoptosis, and that this effect exhibits some degree of drug specificity (22). We hypothesized that oral administration of tilmicosin may stimulate leukocyte apoptosis in the *A. pleuropneumoniae* infected lung, thereby attenuating the inflammation associated with this acute bacterial infection. Results from this study indicate that leukocyte population recovered from BAL fluids

obtained 3 hours post-infection from tilmicosin-treated animals had up to 2.5 fold increases in apoptotic activity compared to CTC-treated animals. The rate of apoptosis in the CTC-treated animals was not different from infected, untreated animals.

Ultimately, apoptotic neutrophils and their fragments are removed by other cells, such as macrophages and other "non-professional" phagocytes, including neighbouring epithelial cells (29), (59), (130). This allows for the phagocytic clearance of apoptotic neutrophils before they are given the opportunity to undergo secondary necrosis and cause tissue injury. Early on in neutrophil apoptosis, phosphatidylserine is translocated to the outer leaflet of the cell membrane. Macrophages possess receptors that recognize the exposed phosphatidylserine on the surface of apoptotic neutrophils to facilitate phagocytosis (45), (46). Tilmicosin-induced apoptosis of circulating bovine neutrophils has been shown to be associated with increased phosphatidylserine translocation on the outer leaflet of the neutrophil membrane, as measured by Annexin-V-Fluos staining of neutrophils (22). It was also demonstrated that this phenomenon is drug-specific as treatment with penicillin, ceftiofur, oxytetracycline or the corticosteroid dexamethasone does not increase phosphatidylserine expression (22). It remains to be shown if tilmicosin may also affect the expression of other surface markers of apoptosis, such as the vitronectin receptor, CD36, thrombospondin, CD14, or ABC-1 (47), (58), (127), (129), (131). In the context of this study, while the apparent trend for increased phagocytic uptake of neutrophils by alveolar macrophages in tilmicosin-treated animals would be consistent with the observed pro-apoptotic effects of the antibiotic on neutrophils, values failed to reach statistically significant differences between groups ($P=$

0.083 vs. untreated and CTC 3h post-inoculation and P= 0.103 vs. untreated and CTC at 24h post-inoculation (Fig. 16).

Fadok *et al* have shown that macrophages exposed to apoptotic cells increase their secretion of PGE_2, which may in turn reduce the ability of macrophages to release pro-inflammatory cytokines like IL-1β, when stimulated by LPS (44). Conversely, a study by Lakritz *et al.*, indicates that injectable tilmicosin, given to calves at higher doses than typically used in field practices, reduces LPS-stimulated bovine alveolar macrophage PGE_2 production (85). In the present study, elevated PGE_2 concentrations from porcine BAL measured at both 3 and 24 hours post-infection were not reduced by oral tilmicosin treatment. Tilmicosin's ability to modulate PGE_2 production by macrophages may be dependent on the dosage or route of administration of tilmicosin. Future studies need to investigate the effects of tilmicosin on neutrophil-macrophage interactions and the resulting profiles of pro- and anti-inflammatory mediator release.

4.4 CONCLUSION

In conclusion, the results from this study indicate that oral tilmicosin treatment reduces fever in animals 24h post-inoculation, without altering bronchoalveolar IL-1β and PGE_2 levels. Tilmicosin induces leukocyte apoptosis 3h post-inoculation without altering neutrophil infiltration or tissue MPO activity. Tilmicosin significantly reduces pulmonary accumulation of LTB_4, whereas untreated and CTC-treated animals maintain elevated LTB_4 levels. Tilmicosin also prevents the formation of the necrotic, hemorrhagic lesions characteristic of *A. pleuropneumoniae* infection. Significant inhibition of LTB_4 synthesis, fever reduction and inhibition of lesion formation occurred

only in tilmicosin-treated animals despite the concurrent presence of live bacteria (>2.5 Log_{10} CFU/mL BAL). While this suggests that the anti-inflammatory effects of tilmicosin reflect the pro-apoptotic effects of the drug rather than its anti-bacterial properties this remains a topic for further research. Together, the results demonstrate that oral tilmicosin has pro-apoptotic and anti-inflammatory benefits in a porcine model of pleuropneumonia.

4.5 FUTURE STUDIES

To further qualify and quantify leukocyte apoptosis, *in situ* terminal deoxynucleotide end labeling (TUNEL) will be performed on paraformaldehyde fixed tissue samples, as described by Leite *et al.* and examined with fluorescence microscopy (87). Also, serum levels of IL-1β, and PGE_2, from piglets needs to be measured to determine the mechanism by which tilmicosin modulates fever during acute *A. pleuropneumoniae* infection.

Lastly, the mechanisms by which tilmicosin induces neutrophil apoptosis remain unknown. Previous research has shown that levels of soluble TNF-α and Fas expression on the surface of neutrophils are not altered by tilmicosin (23). This combined with tilmicosin's high affinity for intracellular uptake in phagocytes (133) suggests that tilmicosin induces apoptosis through an intracellular mechanism. Future experiments, involving the incubation of paraformaldehyde fixed cytospins of porcine BAL fluid with anti-active caspase-8 and anti-active caspase-9, will assess if tilmicosin induces neutrophil apoptosis by activating the intrinsic mitochondrial pathway via caspase-9 or the extrinsic pathway via caspase -8.

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