# EXTRACELLULAR BACTERICIDAL FUNCTIONS OF PORCINE NEUTROPHILS

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# ABSTRACT

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Neutrophils are one of the main effector cells of innate immunity and were shown to kill bacteria by phagocytosis more than 100 years ago. Neutrophils are also capable of antimicrobial activity by producing extracellular structures named neutrophil extracellular traps (NETs). This thesis is an investigation of porcine neutrophils and their ability to produce NETs, as well as the antimicrobial ability of secretions from activated porcine neutrophils in combating a variety of common porcine pathogens. Porcine neutrophils were found to produce NET-like structures, and secretions from activated neutrophils were found to possess variable bactericidal activity against common pathogens of swine. Antimicrobial proteins dependent on elastase activity were shown to be partially responsible for the bactericidal activities of activated neutrophils. Several antimicrobial proteins and peptides were identified via proteomic techniques. This work allows for better understanding of innate immunity in swine, and identification of potential targets for addressing porcine health.

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## Declaration of Work Performed

All work reported in this thesis was performed by me, with the following exception:

Paul Taylor, of the Advanced Protein Technology Centre at the Hospital for Sick Children performed the trypsin digests of the neutrophil secretions as part of their Mass Spectrometry service.

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# List of abbreviations

| | |
|---|---|
| ACD | acid-citrate-dextrose |
| ANOVA | analysis of variance |
| BALF | bronchoalveolar lavage fluid |
| BHI | brain heart infusion |
| CFU | colony-forming units |
| CRAMP | cathelicidin-related antimicrobial peptide |
| CMK | chloro- methyl ketone |
| DTT | dithiothrietol |
| DNA | deoxyribonucleic acid |
| FWHM | full-width half-maximum |
| HBSS | Hank's Buffered Salt Solution |
| HDP | host defence peptides |
| $H_2O_2$ | hydrogen peroxide |
| HOCl | hypochlorite |
| IL-8 | interleukin-8 |
| LPS | lipopolysaccharide |
| LRP | leukocyte-rich plasma |
| NAD | nicotanimide adenine dinucleotide |
| NADPH | nicotanimide adenine dinucleotide phosphate |
| NET | neutrophil extracellular traps |
| pBD-1 | porcine beta defensin-1 |
| PBS | phosphate buffered saline |
| PF | proline-arginine-rich prophenin |
| PG | protegrin |
| PMA | phorbol myristate acetate |
| PMAP | porcine myeloid antimicrobial peptide |
| PMN | polymorphonuclear |
| PR-39 | proline-arginine-rich 39-amino-acid peptide |
| ROS | reactive oxygen species |
| RPMI | Roswell Park Memorial Institute-1640 medium |
| SEM | scanning electron microscopy |
| SLE | systemic lupus erythematosus |
| TEM | transmission electron microscopy |
| TFA | trifluoroacetic |
| TLR | Toll-like receptors |

# Chapter 1. Literature Review

## Introduction

Neutrophils are one of the principal effector cells of the immune system. These cells are the first leukocytes recruited to use their potent antimicrobial defence mechanisms in sites of microbial infection and tissue invasion. Neutrophils develop in the bone marrow, and mature cells are exported to the blood stream where they circulate approximately six hours until death (1). In individuals with good health, circulating neutrophils die and are cleared from the blood without participating in an inflammatory response. In times of inflammation and disease, however, the major function of these cells is to destroy invading microbial pathogens (1). The presence of pathogens is detected by macrophages and other sentinel cells, which signal to neutrophils. The neutrophils are rapidly recruited from the circulation to the site of infection and they employ a variety of approaches to control an infection (2).

One of the chief purposes of the innate immune system is to limit pathogens to the infection site and prevent systemic infection (2). Considering this, neutrophils play a major role in microbial control (2).The name neutrophil is derived from the cell's neutral staining properties with haematoxylin and eosin microscopic stains. Polymorphonuclear (PMN) cells or granulocytes are other names given to neutrophils which reflect their cytological appearance. Neutrophils have a multilobed nucleus which may facilitate

neutrophil emigration through narrow junctions between endothelial cells (1). The other identifiable feature of neutrophils is the presence of many granules in the cytoplasm. Many of these granules contain potent antimicrobial molecules used for killing and digestion of microbes (1). These antimicrobial molecules are highly effective but show poor specificity, and as a result, they may be dangerous to host cells in addition to pathogens (1).

The present chapter reviews the current knowledge of neutrophil extracellular traps (NETs), a recently discovered extracellular mechanism of microbial killing involving DNA and cytoplasmic antimicrobial peptides, including cathelicidins. This particular family of antimicrobial peptides is present in porcine neutrophils, and is thought to be a major mechanism of NET-dependant killing of bacteria. NETs appear to promote the effectiveness of the neutrophils by limiting the diffusion of neutrophil secretions, thus preventing damage to host cells via granular contents, as well as potentially enhancing the effectiveness of synergy among antimicrobial peptides by increasing their concentration in the area of infection (2).

Data on NETs and cathelicidins are somewhat limited. NETs are a relatively new concept and have not yet been described in swine. The structure of porcine cathelicidins are well characterized.. However, cathelicidin research is mainly focused on treating infections less common to pigs.

## Phagocytosis and Intracellular Killing

In 1901, Metchnikoff described phagocytosis, a process where microorganisms are enveloped by the phagocyte plasma membrane and engulfed by the cell. The vacuole containing the ingested microbe fuses with neutrophil lysosomal granules to form a phagolysosome (3). The contents of the phagosome are thus subjected to several potent antimicrobial molecules such as antimicrobial peptides, reactive oxygen species (ROS) and hydrolytic enzymes which kill and degrade the ingested microbe. This method of microbial killing prevents potentially harmful release of these antimicrobial molecules into the surrounding tissues (4).

## Mechanisms of Cell Death

Both oxygen-dependent and independent mechanisms are used by neutrophils to kill pathogens. Oxygen-independent methods include releasing antimicrobial proteins and peptides such as BPI, defensins, and cathelicidins into the phagosome (5). These cationic antimicrobial effector molecules bind the membrane of bacteria, perhaps by electrostatic interactions, and disrupt the integrity of the bacterial cell membrane leading to the death of the microbe.

Oxygen-dependent killing occurs through the NADPH oxidase and myeloperoxidase to create superoxide anion and eventually hydrogen peroxide ($H_2O_2$) and hypochlorite (HOCl), which are very potent antimicrobial free radicals. Neutrophils that are actively phagocytosing experience a burst of oxygen consumption (6). This increase in oxygen use is due to a NADPH complex that forms at the phagosomal

membrane. Electrons are transferred from the cytoplasmic NADPH to oxygen on the interior of the phagosome to produce the superoxides and other free radicals (7, 8). These free radicals kill microbes (and damage host cells) by oxidizing proteins and DNA (9). People with mutations in genes that encode the NADPH oxidase complex experience life-threatening infections due to their inability to kill infectious microorganisms (10).

When phagocytosis and microbial digestion are complete, neutrophils undergo apoptosis which is mediated by a family of caspase enzymes (11, 12). Following apoptosis, neutrophil apoptotic bodies are quickly removed by macrophages which contribute to the resolution of inflammation (13).

**Neutrophil Extracellular Traps (NETs)**

In addition to the above mechanisms, a novel microbial killing mechanism associated with neutrophils has been observed. Brinkmann *et al*. noticed that activated neutrophils produced extracellular filaments and webs formed of nuclear components and proteins including elastase (4). The threads that compose NETs are approximately 15 nm in diameter, a characteristic thought to be derived from their unfolded chromatin components**.** Evidence for DNA as a structural component can be seen as NETs are disassembled by DNases, but not by proteases (4). The presence of DNA in NETs has also been demonstrated through the use of DNA intercalating dyes. Histones are another component of the NET structure, confirmed by NETs reacting against histone antibodies (4). Research involving scanning electron microscopy has found that the NET threads also have globular components which are about 30-50 nm in diameter (4). Multiple NET threads may be wound up to make a 100 nm diameter cable, and several cables form

complex web-like structures (4). Experiments suggest that NETs have a flexible structure, which surrounds the cell from which they originate (4).

Scanning electron microscopy (SEM) is commonly used by investigators to observe NETs. For example. using SEM, Brinkman *et al.* found that naïve neutrophils were generally round with slight folding of the membrane, but cells that were stimulated with an agonist such as phorbolmyristate acetate (PMA) or interleukin-8 (IL-8), flattened out and showed membrane protrusions (4). These activated cells produced extracellular net-like structures, that were very fragile and required careful handling in order to preserve and observe them (4). Transmission electron microscopy (TEM) has also been used to demonstrate that NETs are not surrounded by membranes (4).

When neutrophils are activated by pathogens, cytokines (i.e. IL-8) or protein kinase C-activators (PMA), they begin to generate NETs (14). The production of NETs is dependent on signalling pathways that involve Toll-like receptors (TLRs) (14, 15). More NETs are produced via activation by intact bacterial pathogens versus activation by individual TLR agonists, suggesting that multiple signalling pathways are probably involved in NET formation (14, 15).

Fuchs *et al.* describe NET formation as an active process (14) When stimulated, flattened neutrophils develop into more motile, phagocytic cells with maximum oxidative burst activity occuring in the first hour of stimulation (14) Studies using time lapse photography reveal no nuclear changes in the first hour. After the first hour, the

chromatin remodels into euchromatin (transcriptionally active) and heterochromatin (transcriptionally inactive) parts. The nucleus also begins to lose its lobular appearance at this point (14). Approximately 120 minutes after activation, the nuclear membrane separating the chromatin from the cytoplasm "dissolves", allowing the chromatin to mix with other components of the cell. Simultaneously, the granules within the neutrophils are observed to dissolve. After 180 minutes of activation, the mixture of chromatin and granule contents are released into the extracellular environment (14). In these studies, the neutrophils were activated by PMA, *Staphylococcus aureus,* or *Candida albicans,* and this time line and process may reflect NET formation via direct interaction with a microbe or chemical activators. In other studies, however, platelets were activated via TLR4 using bacterial lipopolysaccharide (LPS) and activated platelets bound to neutrophils, activating them to produce NETs within minutes. This suggests a different, indirect method of neutrophil stimulation mediated by activation of other cell types via Toll-like receptors (16)

The process of NET generation appears to be a novel form of cell death (14). Cell death occurs at the moment that NETs are released from the cell (14). Early literature appears uncertain whether NETs were produced from live or dead cells, whether the production of NETs caused cell death, and whether this death was apoptotic, necrotic or something else (4). Recent studies have demonstrated with use of video microscopy techniques which monitored both cell viability and NET formation that neutrophils are alive until the moment that NETs are released (14). For example, Fuchs *et al.* demonstrated that neutrophils activated to make NETs did not have any DNA

fragmentation, and had intact cell membranes until the NETs were secreted (14). A recent review has termed this NET-associated form of cell death "NETosis" (17). The authors propose that NETosis is a unique cell death process because it does not involve caspases, the DNA is not fragmented, and the cell death is not due to a direct insult to the cell membrane such as the complement membrane attack complex (17).

Breakdown of the nuclear membrane occurs in mitosis and meiosis. At this point, it not understood whether the processes involved in NET formation are unique or are similar to those used in cell division (4). The molecular mechanism of NET formation is unknown, but several lines of evidence indicate a role for reactive oxygen species (ROS) in the process. For example, $H_2O_2$ induces NET formation, while catalase treatment (which degrades $H_2O_2$) abolishes NET formation. Also, neutrophils from patients with chronic granulomatous disease, who cannot generate ROS, do not generate NETs. NET formation is just beginning to be studied and more investigations are required to shed light on NET structure, formation, and function.

NETs bind bacteria, but NET-mediated killing is not dependent on phagocytosis. Rather, intact DNA, histones, and other proteins in the NETs are necessary for NET-dependent killing. NETs have been demonstrated to bind to Gram-positive bacteria such as *Staphylococcus aureus* (4) , *Streptococcus pneumoniae* (18, 19) and group A *Streptococcus* (20). Gram-negative bacteria such as *Shigella flexneri* (4) and *Salmonella typhimurium* (4) have also been shown to trigger NET formation. Fungi such as *Candida albicans* (21) can also elicit a NET response from neutrophils. There is a lack of research regarding NET responses to viruses and parasites. The presence of bacterial capsule has

been shown to reduce NET binding ability to *Streptococcus pneumonia*, and this may represent a method used by bacteria to prevent their entrapment in NETs (19).

Neutrophils that have been activated to produce NETs have been shown to kill bacteria even if their ability to phagocytose has been impaired (22). This antimicrobial activity may also be prevented by treating neutrophils with DNases (22). When neutrophils incapable of phagocytosis due to treatment with cytochalasin D are activated by PMA, IL-8, or bacterial components, they are still able to kill microorganisms, confirming the antimicrobial properties of NETs (4). If the NETs are dissolved by treating the same neutrophils with DNAses, antimicrobial activities are eliminated (22).

Histones have been shown to be efficient antimicrobials through the work of James Hirsch in the 1950s. Histones are DNA-bound proteins which are also seen in NETs. It is suggested that histones play a large role in the antimicrobial activities of NETs. Studies reveal that many bacteria are particularly sensitive to histone 2A (4, 19, 20). Histones, however, were not seen to affect the killing of eukaryotes, suggesting that perhaps NETs may also possess some form of antifungal agent.

In addition to histones, there are other proteins, such as protegrins, elastase, defensins and cathelicidins that are associated with NETs (23). It is likely that these proteins are the major effectors of killing. Studies completed by Brooks *et al.* show that at least 20-40 proteins are released from neutrophils stimulated by PMA (24). One protein, ficolin β, is secreted and it is known to possess bacterial binding functions. The

role of ficolin β is unknown, but it is possible that ficolin β has functions associated with NETs (24).

It is hypothesized that high concentrations of antimicrobial agents are contained within NETs. At this point, the mechanism of pathogen entrapment is unknown, but some studies suggest that there is an electrostatic attraction between the negatively-charged pathogen surface and cationic components of NETs. Since there is a known neutrophil-elastase component of NETs, it is possible that elastase-dependant host-defence peptides may play a role in antimicrobial activities.

Since NETs are primarily comprised of chromatin, NETs can be degraded by DNases (4). Studies have shown that some Gram-positive bacteria express DNases or have DNases bound to their membrane. A recent study of group A *Streptococcus* reported that isogenic mutants with no DNase activity were less pathogenic than wild type counterparts (25). Further, Buchanan *et al.* demonstrated that *S. pneumoniae* strains which express DNases are more likely to escape NETs and are also more pathogenic than those isogenic mutants which showed a clear decrease in DNAse activity (20).

The investigator used strains of bacteria that had inactivated DNases. They found 100 times fewer DNase-deficient microbes than the wildtype in the skin of patients with necrotising fasciitis (20). When DNase expression was introduced into the deficient strains, there was a significant increase in lesion size (20). However, as DNase was inhibited by G-actin, the sizes of the lesions were significantly reduced. Both lesions

were examined for neutrophil populations, and the lesions induced by DNase-positive and -negative isolates had similar numbers of recruited leukocytes, but NETs could only be observed in the DNase- negative group (20). Beiter *et al.* produced similar results with *S. pneumoniae* (18). In an *in vitro* study, lungs infected with strains of the bacteria that were capable of producing DNase were found to be far more prolific and pathogenic than strains that did not produce DNase (18). NETs appear to have important roles *in vivo*, and bacteria capable of degrading NETs are more pathogenic, but further work is required to determine the significance of NET formation for host defence.

In a related study, Clark *et al.* showed that NETs may also play a role in sepsis, as activated platelets stimulate neutrophils into producing NETs within minutes of activation under flow conditions (16). This study also suggested that in cases of bacterial infection, NETs may be a more efficient mechanism than phagocytosis for removing bacteria from the bloodstream (16). From the conclusions of this article, it is reasonable to hypothesize that inflammatory conditions involving activated platelets, such as endotoxemia, could induce NET formation. Both platelets and neutrophils are abundant in blood, and it is possible that platelet activation in the lumen of blood vessels could lead to intravascular NET formation.

The production of NETs may not be entirely helpful . Some studies have suggested that failure to dispose properly of NET components may predispose the host to auto-immune diseases such as systemic lupus erythematosus (SLE), which is characterized by auto-antibodies that recognize host DNA and other nuclear molecules

(26). It is also possible that excessive NET formation may contribute to tissue injury. For example, the lungs of patients with cystic fibrosis contain extracellular chromatin, potentially due to an excess of NET production (4).

Additionally, some reports suggest that NETs may not represent the best way to counter bacterial invasion. A study using a murine model has demonstrated that when paired with DNA, cathelicidin-related antimicrobial peptide (CRAMP) had decreased antimicrobial ability with respect to *Staphylococcus aureus.* These data suggest that neutrophils may be able to detect particular bacterial strains and then choose which defence mechanism would be best suited, either phagocytosis or NET formation (27).

Additional work needs to be done to understand how and why neutrophils make NETs, and whether this is a conserved characteristic of all neutrophils. At this point it is not known if all neutrophils are capable of producing NETs. Also, upon infection, neutrophils begin engulfing bacteria by phagocytosis (4). It is not until much later that cells begin to produce NETs. At this point it is not clear why a cell may or may not produce NETs. Further studies are required to establish what factors determine the neutrophil's method of cell killing and which method is more efficient.

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NETs are produced by neutrophils stimulated with Gram-positive and Gram-negative bacteria, fungi, PMA and platelets. It is not known if either viruses or parasites bind to NETs. Additional studies to discover the receptors and mechanisms involved in

the stimulation of neutrophils and the formation of NETs need to be completed. Using this new information it may then be possible to alter NET production by pharmacological means.

The antimicrobial properties of NETs have been demonstrated by several investigators *in vitro*, but the presence or absence of NETs *in vivo* has only received preliminary support. Further studies are required to explore NETs *in vivo*, and determine in what tissues they form during bacterial infections. There has been some suggestion that NETs may have a role in lung tissues during respiratory infection (24). Swine may be ideal candidates for the study of NETs in lung tissues as lung inflammation is prevalent among swine populations due to common bacterial infections.

**Porcine Host Defence Peptides**

Host defence peptides are an important component of innate immunity (28, 29) in both the animal and plant world. In some animals, particularly invertebrate species, these innate immunity peptides provide a first line of defence against bacteria and fungi. In higher order species, however, "host defence peptides" serve antimicrobial and sometimes also immunoregulatory functions (30-32). Although there are many diverse sequences and functions to the peptides, they are generally classed into two families: cathelicidins and defensins (33-37). Defensins are a family of cysteine-rich antimicrobial peptides which are grouped into 3 subcategories: α-defensins, β-defensins, and θ-defensins (38, 39). Defensins are not found in abundance in porcine neutrophils, with only β-defensins having been observed in epithelial cells of respiratory and

gastrointestinal tracts (40-42), so cathelicidins will be the antimicrobial peptides in focus for this research project.

Porcine host defence peptides (HDPs) are a group of small, mostly cationic peptides. They are usually 12-100 amino acids in length and possess an amphipathic configuration (43). Activities associated with HDPs and specifically cathelicidins are lysis of membranes and stereospecific receptor binding (28, 29, 34, 44). This section of the review highlights progresses and discoveries related to cathelicidin research.

Cathelicidins are abundant in epithelial and myeloid tissues. Specifically, they are found in the peroxidase-negative (secondary, specific) granules of neutrophils (45). Members of the cathelicidin family share a common N-terminal proregion, cathelin, a 12 kDa protein found in porcine leukocytes, which gives the cathelicidin family its name (46). The cathelin portion of these peptides is followed by a highly variable C-terminal domain which contains the active microbicidal region of the peptide. Cathelicidins are stored as inactive propeptides and are only activated following cleavage of the conserved region by elastase (43). There are three major subgroups of the cathelicidin family: proline-rich peptides, cysteine-stabilized cathelicidins, and porcine myeloid antimicrobial peptides, each categorized based on their structural components.

Swine possess neutrophils that are rich in cathelicidins, making them ideal candidates for the study of these antimicrobial peptides (47). Pigs have the most varied collection of cathelicidins of any species studied (47). Within each subset of

cathelicidins, pigs also possess a high level of molecular diversity along with multiple isoforms of the peptides (36, 43, 48) Many of the cathelicidins discovered in swine were purified from peripheral leukocytes, or cDNA was located in myeloid tissues (48). There are 11 porcine cathelicidins: proline-arginine-rich prophenin-1 (PF-1), and PF-2, proline-arginine-rich 39-amino-acid peptide (PR-39), cysteine-rich protegrin 1 (PG-1) to PG-5, as well as porcine myeloid antimicrobial peptide (PMAP)-23, PMAP-36, and PMAP-37 (47). All associated genes in swine are contained in chromosome 13 (49). Porcine cathelicidins can be subgrouped into the three categories previously mentioned based on primary amino acid structure. The three groups include a proline-rich group (PR-39, PF-1, PF-2), a disulfide-rich group (PG-1-5), and a highly arginine-histidine-containing PMAP group (49).

Linear proline-rich cathelicidins

PR-39 was originally described in intestinal tissue, and soon after discovered in porcine neutrophils (50, 51). This peptide has a high concentration of proline residues (~50%), as well as large amounts of arginine or phenylalanine depending on the specific cathelicidin (36). The high amount of proline found in the structure helps to prevent degradation by serine proteases, elastases or other proteolytic proteins (36).

The subgroup of cathelicidins that includes PR-39, and includes prophenin-1, and prophenin-2 has been found to have activity against Gram-negative bacteria and mediates bacterial killing without membrane lysis, although they can translocate through biological membranes (47). They generally function by means of stereospecific interaction with pathogen targets. In studies using amino acid substitution, Blecha et al.

demonstrated that the minimum length required to mediate antimicrobial activity in PR-39 is 15 to 26 amino-acids (47).

Cysteine-stabilized cathelicidins

Protegrins are the most studied of all porcine cathelicidins. There are five known protegrins (PG1-5) that have a structure of 16-18 amino-acid residues, and have been shown to possess broad antimicrobial activity (37). Protegrins are primarily found in leukocytes and possess both a common cathelin domain as well as a β-hairpin that makes them similar to defensins (52, 53). Because of this similarity PGs are sometimes called mini-defensins (54). Research has determined that PGs interact with themselves or other molecules in the extracellular milieu to disrupt the membrane of microbes (55).

PMAP Cathelicidins

Three PMAP cathelicidins have been reported in the literature (PMAP-23, PMAP-36, and PMAP-37). The cDNA precursors of these cathelicidins have been located in bone marrow of swine and their names correspond to the associated residue length (31, 49). These peptides have been shown to function as broad-spectrum-antimicrobial agents, but they are not as similar to the other subgroups of cathelicidins (31, 43). Bacterial killing by PMAPs is accomplished by disruption of the pathogen membrane, and mutations in the peptide structure greatly reduces their antimicrobial activity (56, 57).

Antimicrobial Activities of Cathelicidins

Porcine antimicrobial peptides can destroy a variety of microbes (47), including both Gram-negative and Gram-positive bacteria. For example, PR-39 and PR-26 have both been shown to mediate bacterial killing of five of seven Gram-negative and two of seven Gram-positive bacteria (48, 58-61). Some bacterial species, however, do show resistance to antimicrobial activities of cathelicidins (58-61). Certain serovars or isolates of bacteria may also be more susceptible or resistant to the same cathelicidins (58-61). PF peptides appear to be more effective in managing infections of Gram-negative bacteria (51, 62), whereas PMAPs appear to be equally effective in killing both Gram-positive and Gram-negative bacteria, with exceptions. PMAPs have also been shown to be successful in killing some fungi and nematodes, although cathelicidins need to be at much higher concentrations to exhibit this activity (63-65). Protegrins, like PMAPs, possess antimicrobial activity against both Gram-positive and Gram-negative bacteria, again with some species being able to evade this action (47).

The capacity of cathelicidins as an antiviral agent has also been a topic of study. In pigs, only PG-1 has been reported in the literature to possess anti-HIV ability (66). Both lentiviral vectors and retroviral vectors were used in the study, and it was observed that both vectors were inhibited to a similar degree via PG-1 activity (66). Since the vectors used do not share any viral proteins the authors suggest that it is likely that the target for PG-1 and other cathelicidins is cell-related. It is possible that the lipid membrane is a target as it is used by viruses during budding (66).

*In vivo* studies involving cathelicidins in swine have also been conducted. Research identified elevated levels of PR-39 and PG in swine serum following challenge with *Salmonella* (67). In another study, pigs challenged with *Actinobacillus pleuropneumoniae* in an aerosol infection model showed an increase in PR-39 and PF in bronchoalveolar lavage fluid (BALF), but not it serum (68). *In vivo*, neutrophils are commonly exposed to sera, which contains elastase-inhibiting components that might limit elastase-induced activation of cathelicidins. However, Shi and Ganz noted that even in experiments where the neutrophils were activated in porcine serum, there was maturation of the protegrins being studied (23). These investigators suggested that the elastase inhibitors present in the serum can be overcome, likely by the products of the respiratory burst (23). In another exploration of cathelicidin function, Cole *et. al.* blocked the elastase-induced activation of protegrins in a porcine skin wound model (54). Those wounds that were elastase-inhibited showed a reduction in antimicrobial activity compared to those with normal elastase function (54). Furthermore, when the wounds containing the elastase inhibitor were supplied with already-mature protegrins, antimicrobial function was restored (54).

Synergistic activities of antimicrobial peptides

In a study conducted by the Shi and Ganz research group, significant synergy was noted between the activity of a porcine defensin (pBD-1) and cathelicidins (PR-39 and PG-3) (69). When either the defensin peptide or the cathelicidin were incubated with *E. coli* or *S. typhimurium* alone, they were found to be ineffective in mediating bacterial killing; the combination was found to possess elevated antimicrobial abilities (69).

Nonmicrobicidal activities

Recent research has determined that antimicrobial peptides may also be able to affect other biological responses. PR-39 is the most widely studied cathelicidin in its role as a molecular signal (16, 36, 37). PR-39 has been used as a study subject as it mediates its bacterial killing via cellular targets that obstruct protein and DNA synthesis. Since these cathelicidins do not disrupt the cell membrane in killing the targets, it was hypothesized and since shown that PR-39 can also affect other biological processes via similar cellular targets without killing the cell (16, 36, 37). For example, syndecans are a group of proteins found in the extracellular matrix of cells. They play a role in cell growth, cell to cell interactions and cell movement. These proteins have been shown to be upregulated in the presence of PR-39 in wound fluid of swine (70). Furthermore, this elevation of syndecans and other heparan sulphate proteoglycans by PR-39 has been demonstrated to be responsible for wound healing (70). PR-39 can assist in the regulation of vascular cell-cell interaction, and can also inhibit the invasion and metastasis of cancer cells (71-73). Descriptions and explorations of cathelicidins have been mainly focused on their antimicrobial activity, but it is clear that cathelicidins also possess some ability to contribute to other biological functions. Although these abilities may not be related to pathogen elimination, they are related to host defence and recovery from challenge.

## Conclusions

As NETs have been observed in humans, rabbits, horses, mice, cows, and fish, it appears that NET formation by neutrophils is a conserved property of neutrophils (4, 18,

19, 74, 75). One goal in the present series of studies is to investigate the production of NETs by porcine neutrophils.

Porcine host defence peptides are a group of 11 cathelicidins and 13 β-defensins which are involved in the innate immune response (47). In swine, cathelicidin-derived peptides have been given much attention due to their relative abundance in swine neutrophils. Although many studies have been performed examining individual cathelicidins, it would be better to have more studies involving several cathelicidins working in combination, as host-defence mechanisms are often complex. Cathelicidins show potent abilities as antimicrobials and also show potential as antifungal and antiparasitic agents. Several research groups have suggested that perhaps these natural antibiotic compounds could be standardized and used in therapeutic applications (47).

## Objectives

In swine populations, opportunistic bacterial infections are common and economically important. It has been established that porcine neutrophils contribute to the innate immune response and can kill bacteria and limit opportunistic infections. By understanding the mechanisms of extracellular neutrophil killing in more detail, we may improve innate immunity in pigs to opportunistic bacterial infection.

Although NETs have been observed in several species, swine have not yet been studied for the presence, or structure of NETs, or the mechanism by which neutrophil secretions kill bacteria. The proposed research hopes to address this gap by:

1) Establishing whether porcine neutrophils generate neutrophil extracellular traps,
2) Identifying what proteins are responsible for the extracellular antibacterial activity in neutrophils, and
3) Elucidating what bacteria relevant to swine are killed by the identified proteins/peptides released by activated neutrophils

# Chapter 2. Materials and Methods

## Chemicals and Reagents

All chemicals were obtained from Fisher Scientific (Ottawa, Ontario, Canada), except for the Hank's Buffered Salt Solution, PMA, chloro-methyl ketone, and Roswell Park Memorial Institue-1640 medium (RPMI) which were obtained from Sigma (Oakville, Ontario, Canada). Brain heart infusion broth was obtained from Difco (Mississauga, Ontario, Canada). Protein concentrations were obtained using the Bio-Rad DC protein assay kit (Bio-Rad, Mississauga, Ontario, Canada). Carbon mounts were obtained from Ted Pella (Redding, California, USA)

## Isolation of Porcine Blood Neutrophils

The use of animals in this study was approved by the Animal Care Committee of the University of Guelph. Blood was obtained from healthy (~20 kg) Yorkshire-cross weaned pigs from the Arkell swine research station by retro-orbital bleeding with acid-citrate-dextrose (ACD) as anticoagulant. The blood was processed within one hour of collection.

Porcine neutrophils were isolated by dextran sedimentation of erythrocytes, hypotonic lysis, and density-gradient centrifugation of leukocytes as described (76). Each 40 ml sample of whole blood was mixed with 6 ml ACD upon collection and divided equally into two 50 ml conical centrifuge tubes, and 23 ml of 5% dextran in PBS was

added. The tubes were incubated for 20 minutes at room temperature to allow erythrocytes to sediment, then the leukocyte-rich-plasma (LRP) supernatant was aspirated and placed into new 50 ml centrifuge tubes. The LRP was resuspended with RPMI-1640 medium (RPMI), and centrifuged at 228 x g for 20 minutes at 21°C. The plasma was then removed and the pellet was gently smeared against the tube. Erythrocytes were lysed with the addition of 20 ml of 0.2% NaCl solution to each tube, followed by gentle mixing for 30 seconds. Isotonicity was restored with the addition of 20 ml of 1.6% NaCl solution at room temperature. Samples were then centrifuged at 228 x g for 20 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 10 ml RPMI per tube. Each tube of the cell suspension was divided into two and each half was overlaid onto a 4 ml of 80% isotonic Percoll cushion (GE Healthcare, Sweden). The mononuclear cells and the neutrophils were separated by centrifugation at room temperature for 25 minutes at 300 x g. The cells were washed twice with RPMI and resuspended to 1 x $10^7$ cells/ml in PBS with 5% bovine serum albumin. Cells were counted with a hemocytometer and viability ascertained with Trypan blue dye exclusion (viability was routinely >95%). The purity of each cell preparation was greater than 95% as determined by cell counts of Wright's-stained cytospin preparations.

**Activation of Porcine neutrophils**

Neutrophils were activated and the secretions were collected as previously described (77). Briefly, freshly isolated neutrophils were resuspended to 5 x $10^6$ cells/ml in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Some aliquots of neutrophils were activated with 100 ng/mL PMA for 30 minutes at 37°C, then the preparations were

centrifuged for 1 minute at 13,000 x g, and the supernatants of the PMA-activated neutrophils were harvested. Control neutrophils were not treated with PMA and were similarly centrifuged prior to the collection of supernatents. Neutrophil secretions were used immediately or stored at -70°C. For experiments using elastase-inhibited neutrophils, a similar protocol was employed, but the neutrophils were first incubated with chloro-methyl ketone (CMK) (1mM) for 30 minutes at 37°C, prior to the addition of PMA.

**Total Protein Determination**

Total protein concentrations in the neutrophil cell secretions were determined using a BioRad DC protein assay kit. Bovine serum albumin was used as the standard. Absorbance was read with the Ceres UV900 HDi microplate reader (Bio-Tek, Burlington, VT, USA) and accompanying software.

**Scanning Electron Microscopy**

Unstimulated and PMA-stimulated neutrophils from a single pig prepared as methods described above. The neutrophils were first applied to a polished carbon mount inside a Petri dish, and incubated for 30 minutes at 37°C. All samples were then fixed with 50 μl 5% glutaraldehyde and incubated for 230 minutes at 37°C. Carbon discs were then moved to 2.5% glutaraldehyde in covered containers for storage. After the primary fixation, the cells were rinsed with Hank's Buffered Salt Solution (HBSS) three times, and then post-fixed in osmium tetroxide for one hour and washed three times again with HBSS. Samples were dehydrated with a graded ethanol series, and sputter-coated with

gold-palladium metal (4). Samples were kept in a desiccator to prevent moisture absorption, until they were examined with the scanning electron microscope. All samples were viewed with a Hitachi S-570 scanning electron microscope (Mississauga, Ontario, Canada)

**Bacterial Killing Assay**

Eight separate pigs were used in this experiment with the secretions of each pig being used in each treatment group and four replicates of each neutrophil isolation per treatment group. Neutrophil and bacterial killing assays were conducted on different days and each neutrophil isolation was also conducted on separate dates.

Representative clinical isolates of *Actinobacillus suis*, *Escherichia coli* K12, *Streptococcus suis*, and *Pasteurella multocida* were obtained from the Animal Health laboratory at the University of Guelph and grown in brain heart infusion (BHI) broth. Cultures were grown overnight with aeration at 37°C and were used to inoculate fresh broth the following day. These cultures were grown in similar conditions until a measurement of the optical density indicated that there were $10^8$ log phase colony forming units (CFU)/ml. The optical density was measured by the Perkin Elmer Lambda Bio UV/Vis Spectrometer at a wavelength of 600 nm. All of the bacterial cultures were diluted to $10^6$ CFU/ml with PBS and then 30 μl of this preparation was incubated with 100 μl of cell-free secretions of activated or non-activated neutrophils for 30 minutes at 37°C with occasional gentle mixing. After the incubation, 1:100 and 1:1000 dilutions were prepared by adding PBS to the medium. MacConkey agar plates were used for the

*E. coli*, blood agar for the *A. suis* and *S. suis*, and. The plates were allowed to dry for 30 minutes at room temperature and then moved to the incubator at 37°C overnight. CFUs were manually counted the following day.

For evaluating the effect of elastase on bactericidal activity, *A. suis* and *E. coli* K12 were prepared as above, then incubated with cell-free supernatants of non-activated, PMA-activated, or CMK-treated and PMA-activated neutrophils. After incubation, the bacterial preparationswere diluted and plated as previously described and CFUs were manually counted the following day. *S. suis* and *P. multocida* were not similarly tested because of the minimal effect of PMA-activated supernatants on these bacteria.

**Mass Spectrometry**

Neutrophil secretions were trypsin-digested and desalted, as previously described in preparation for analysis by mass spectrometry (78). After total protein concentrations had been assessed as described above, 10 μl from each preparation of neutrophil secretions (non-activated, PMA-activated, and CMK-treated PMA-activated) was taken and dithiothrietol （DTT) was added to a final concentration of 2%. Following this step, 2 μl of trypsin working solution was added to the solution and incubated at 37°C for 2 hours. After the incubation period, 4 μl of 1% trifluoroacetic acid (TFA) was added to the solution.

For desalting, a ZipTip C18 pipette tip (Millipore Corp., Bedford, MA, USA), was equilibrated by aspirating and discarding 10 μl of a 50:50 solution of acetonitrile and

water. The ZipTip was then aspirated with the equilibration solution (0.1% TFA in $H_2O$), followed by aspiration of the sample 15 times to bind the sample proteins to the matrix, and subsequently washed with 10 µl of 0.1% TFA in $H_20$. The washed peptides were eluted from the ZipTip with 50:50 acetonitrile/ $H_2O$ with 0.1% TFA and stored at -70°C prior to analysis by mass spectrometry.

Prior to mass spectrometry, the cell-free secretions of neutrophils were suspended in 0.1% formic acid. Mass spectrometry was performed on a linear ion-trap/orbitrap hybrid instrument (Thermo-Fisher Scientific, San Jose CA) using a split-free nano-LC system (EASY nLC, Proxeon Biosciences, Odense Denmark) at the Advanced Protein Technology Centre, Hospital for Sick Children, University of Toronto. The peptides were concentrated using an on-line C18 trapping column and separated using a 75 µm ID column packed with Magic C-18 resin (Michrom Biosciences) with a gradient of 0 to 40% acetonitrile over 100 minutes. MS data was acquired at 60,000 full-width half-maximum (fwhm) resolution in the Orbitap and MS/MS data was acquired in the linear ion-trap in a data-dependant fashion. The raw data files were compared with a porcine database constructed from the nrdb from NCBI using the Sequest (Thermo-Fisher Scientific, San Jose CA) and X! Tandem (Beavis Informatics, Winnipeg, AB) search engines. The oxidation of methionine and the deamidation of asparagine and glutamine were considered as partial modifications. Parent ion accuracy was 5 ppm and fragment ion accuracy was set at 0.5 Da. The search engine results were analyzed using the Protein Prophet and Peptide Prophet algorithms contained within the Scaffold platform (Proteome Software, Portland, OR).

## Statistical Analysis

Data for bacterial killing assays were analyzed using a one-way or two-way analysis of variance (ANOVA). Given that there is inherent variation between neutrophil isolation dates, data were analyzed where possible with a one-way ANOVA to assess significant difference. Factors included in the ANOVA analysis were date and treatment group. For one-way ANOVA analysis, a Tukey's multiple comparison test was used for post-test analysis when significant differences existed. For two-way ANOVA analysis, Bonferroni post-tests were used if differences were seen to be significant. Data were analyzed with Prism 4 (GraphPad Software, San Diego, California, USA), and are expressed as mean ± SEM.

# Chapter 3. Results

## Scanning Electron Microscopy

To determine whether activated porcine neutrophils are capable of producing NETs, duplicate preparations of purified naïve porcine neutrophils were untreated or exposed to PMA, fixed, and prepared for scanning electron microscopy. NET-like structures were rarely observed (less than 5% of observed cells) in the preparations of unstimulated neutrophils. However, NETs were observed after incubation with PMA for 60 minutes. The observed unstimulated cells were round and there were no visible fibre-like structures (Figure 1). In activated neutrophil populations, however, the cells flattened and had membrane protrusions, and extracellular fibers (resembling NETs) were observed (Figure 2).

## Bactericidal Activity of Neutrophil Secretions

The antimicrobial activity of the secretions released by activated porcine neutrophils was tested against *E. coli, A. suis, S. suis,* and *P. multocida* (Table 1).

The secretions from activated porcine neutrophils were the most effective at killing *E. coli*. Supernatants collected from activated neutrophils at total protein concentrations of 1-1.5mg/ml significantly reduced the numbers of colony forming units of *E. coli*, when compared to supernatants collected from non-activated neutrophils (90.9 ± 4.0% reduction in bacterial numbers [mean ± SEM for the 8 replicate experiments], $p<0.001$,

two-way ANOVA) (Figure 3). This effect was significant for seven out of eight replicate experiments, performed on different days using neutrophils from different pigs.

The same concentration of activated supernatants also significantly reduced colony-forming units of *S. suis* (29.9 ± 12.9% reduction in bacterial numbers, $p<0.05$), and this effect was significant in 4 of the 8 replicate experiments. When similar concentrations of the activated supernatants were used with *A. suis* and *P. multocida,* however, there were only one and two replicates respectively out of eight that demonstrated a significant reduction in colony-forming units (*A. suis*: 35.6 ± 10.2% reduction in bacterial numbers, $p<0.05$; *P. multocida*: 13.2 ± 6.5% reduction in bacterial numbers, $p<0.05$)(Figure 3).

**Effect of Elastase Inhibition on Bacterial Killing**

To explore how much of the bactericidal activity of neutrophil supernatants was due to elastase-dependant peptides and proteins, such as cathelicidins, porcine neutrophils were incubated with the elastase inhibitor CMK, prior to activation with PMA. Secretions of the activated porcine neutrophils were similarly effective at killing *E. coli* as in the previous experiment (83.6 ± 5.1%, $p<0.01$)(Figure 4). Treatment of neutrophils with CMK prior to activation with PMA resulted in a significant decrease in killing ability of the resultant supernatants compared to that of PMA-treated non-CMK-treated neutrophils (28.3 ± 7.8% decrease in killing activity, $p<0.001$, one-way ANOVA)(Figure 4). Secretions of CMK- and PMA-treated neutrophils had significantly greater killing activity compared to that of untreated neutrophils (45.0 ± 5.2% greater bactericidal activity, $p<0.01$, one-way ANOVA)(Figure 4).

The antibacterial activity was also significantly diminished when secretions from CMK-treated neutrophils were incubated with *S. suis*, compared to that from non-CMK-treated neutrophils (43 ± 4.6% reduction in bactericidal activity, $p<0.05$, one-way ANOVA)(Figure 4). This effect was significant in two of the three replicates. There was also a significant difference found between the secretions of untreated neutrophils compared to secretions of CMK- and PMA-treated neutrophils, in two of the three replicates (5% 10.1 SEM, $p<0.01$, one-way ANOVA)(Figure 4). Neither PMA (100ng/mL) nor CMK (1mM) had any detectable effect on bacterial growth (data not shown).

**Mass Spectrometry**

To determine what proteins and peptides were present in neutrophil secretions, samples of activated and non-activated neutrophil supernatants were analyzed by tandem mass spectrometry. In total, there were 60 proteins and peptides were identified in the supernatants of activated and non-activated neutrophils (Table 2). The cathelicidin PMAP-36 was identified as being more abundant by a ratio of 1.65:1 in the secretions of activated compared to non-activated neutrophils (Figure 5). Similarly, lactoferrin was only identified as being present in the activated neutrophil supernatants (Figure 6). No other proteins known to have antimicrobial function were found to be differentially abundant in the non-activated vs. activated neutrophils. Mass spectrometric analysis of the supernatants of CMK-treated neutrophils was not possible because the chlorine group of the CMK permanently binds to the peptides, not allowing precise and accurate analysis via mass spectrometry.

**Table 1.** Effect of supernatants of non-activated and PMA-activated porcine neutrophil on survival of *E.coli* K12, *A. suis*, *S. suis*, and *P, multocida* bacteria. Replicates were incubated in medium with non-activated (PMA-) and PMA-activated (PMA+) porcine neutrophil supernatants, and the CFUs were counted the following day. This table indicates the mean percent killed as well as the standard error of the mean in addition to the total range of bactericidal abilities between the eight biological replicates.

| Bacteria | Mean Percent Killed (± SEM) | Range |
|---|---|---|
| *E. coli* | 90.9% (±4.1%) | 65.1%-99.9% |
| *S. suis* | 29.9% (±12.9%) | 15.2%-54.3% |
| *A. suis* | 35.6% (±10.2%) | 17.9%-67.3% |
| *P. multocida* | 13.2% (±6.5%) | 4.1%-42.5% |

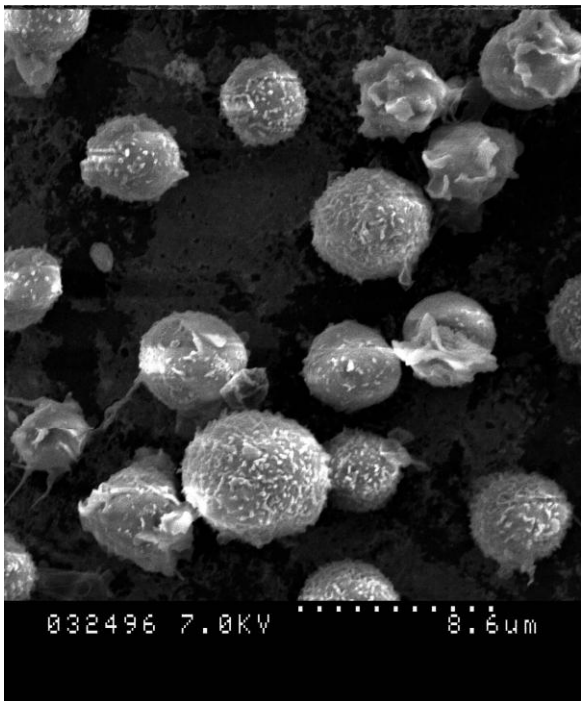


**Figure 1.** Scanning electron micrograph of non-PMA-treated porcine neutrophils. Non–activated porcine neutrophils have a round structure, and no NET-like structures are observed.

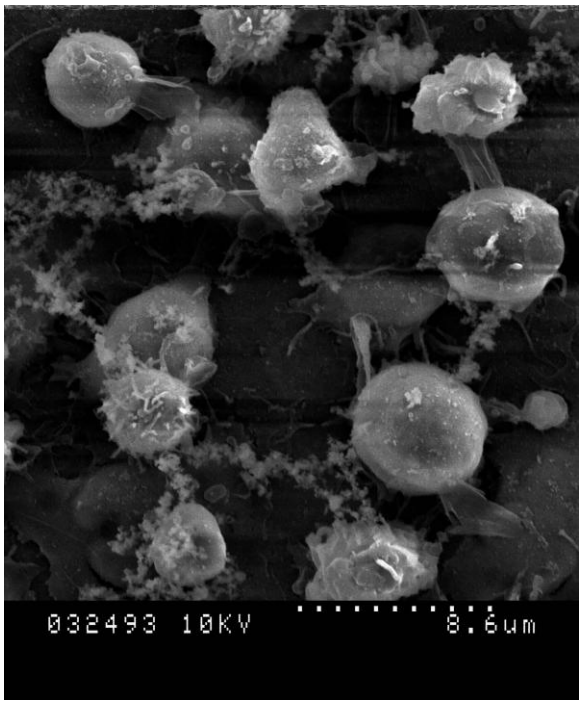


**Figure 2.** Scanning electron micrograph of porcine neutrophils treated with 100ng/mL PMA. Activated porcine neutrophil are surrounded by web-like structures morphologically consistent with NETs.

**Figure 3.** Effect of supernatants of non-activated and PMA-activated porcine neutrophil on survival of *E.coli* K12, *A. suis*, . *S. suis*, and *P, multocida* bacteria. Replicates were incubated in medium with non-activated (PMA-) and PMA-activated (PMA+) porcine neutrophil supernatants, and the CFUs were counted the following day. The graph indicates the results observed in the eight experimental replicates performed on different days using neutrophils of different pigs. Overall, the mean reduction in colony forming units were as follows, 90 ± 4.0% for *E. coli*, 29 ± 12.9% for *S. suis*, 36 ± 10.2% for *A. suis* and 13 ± 6.5% for *P. multocida*.

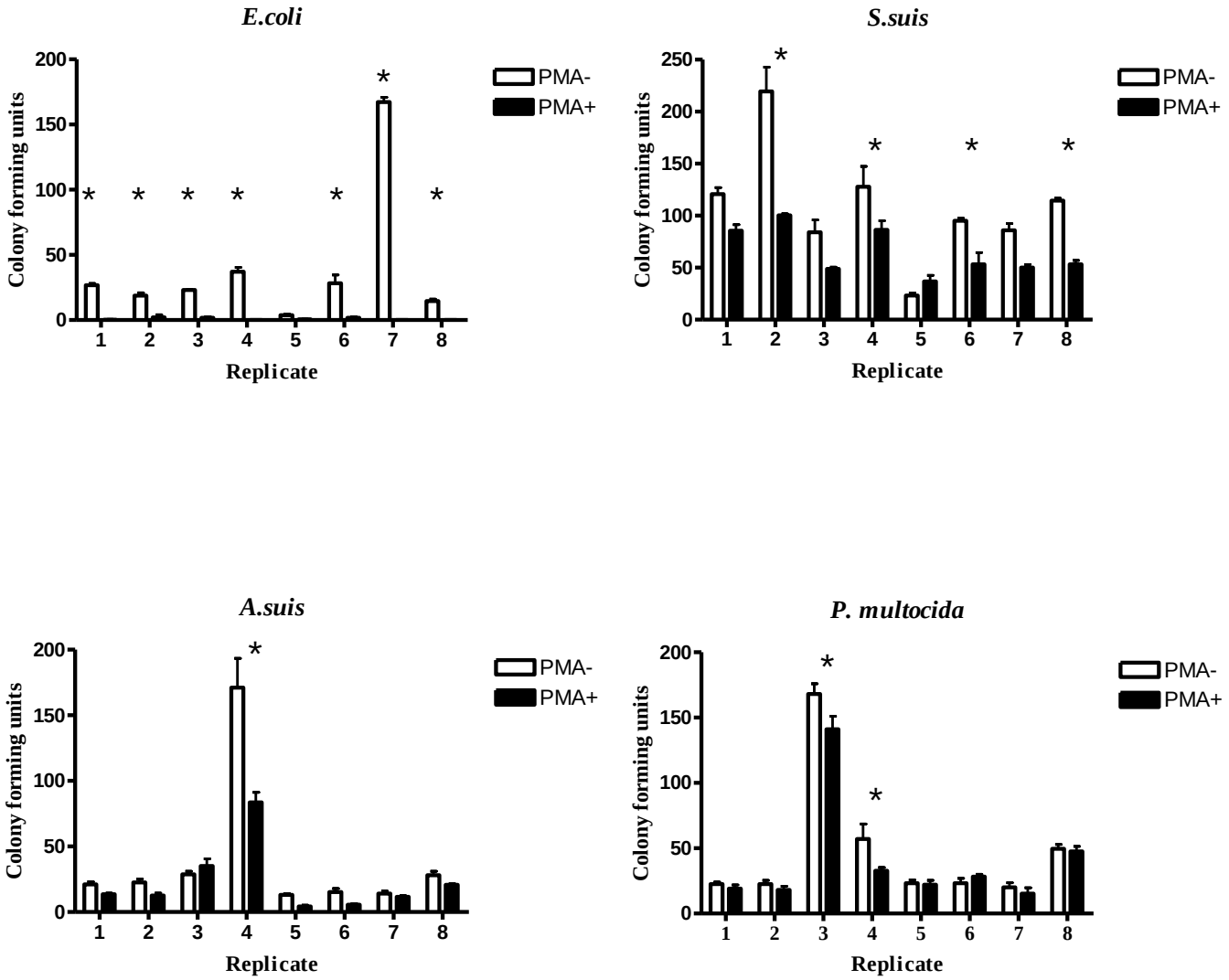

**Figure 4.** Effect of the elastase inhibitor CMK on the bactericidal activity of secretions from activated porcine neutrophils. *E. coli* K12 and *S. suis* bacteria were incubated in medium with supernatants of non-activated neutrophils (PMA-), PMA-activated neutrophils (PMA+), or neutrophils treated with CMK prior to activation with PMA (CMK+/PMA+). Colony forming units were counted the following day. Each graph indicates the results of treatments on one of the three replicates of each bacterium. a, b, c indicate columns with significant differences.

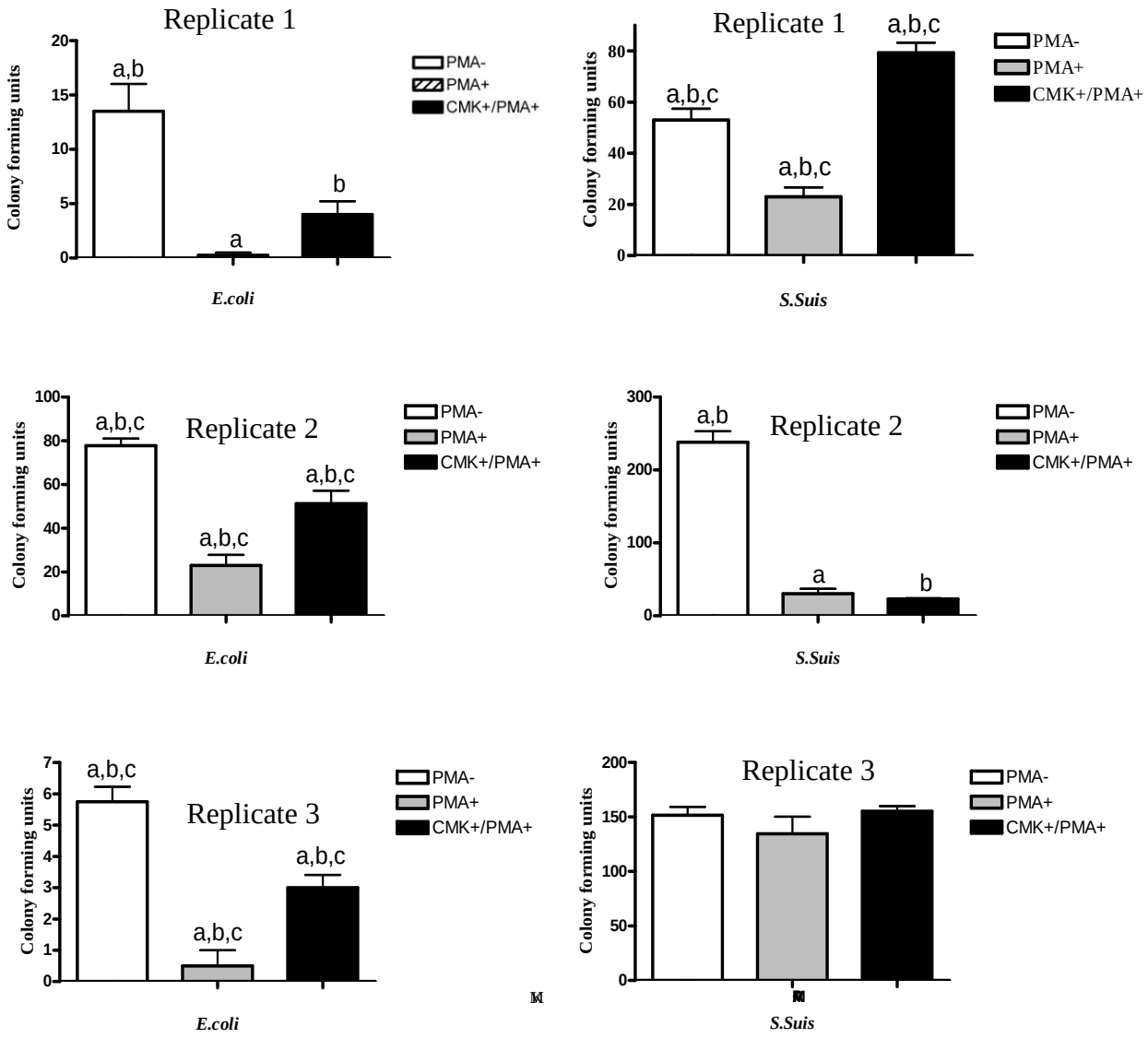

**Table 2.** Identification of proteins in secretions of non-activated and activated porcine neutrophils, as analyzed with tandem mass spectrometry
a: number of matched peptides, % coverage
b: Theoretical mass

| Protein no. | Protein name | Accession no. (NCBI) | MS result in non-activated neutrophils[a] | MS result in activated neutrophils[a] | Th. Mass[b] (kDa) |
|---|---|---|---|---|---|
| 1 | Actin, alpha 1 | gi\|4501881 | 8,22 | 11,29 | 42 |
| 2 | Albumin | gi\|52353352 | 7,8 | 5,8 | 70 |
| 3 | Hemoglobin alpha chain | gi\|122465 | 5,58 | 6,52 | 15 |
| 4 | Chain B, Structure Determination Of Aquomet Porcine Hemoglobin | gi\|809283 | 5,68 | 11,32 | 16 |
| 5 | Fibrillin 1 | gi\|48976131 | 2,2 | 2,1 | 313 |
| 6 | Antibacterial peptide PMAP-36 | gi\|194097491 | 2,10 | 3,25 | 19 |
| 7 | Heat shock 105kDa/110kDa protein 1 | gi\|148225750 | 2,4 | | 97 |
| 8 | Tropomyosin 4 isoform 2 | gi\|4507651 | 2,6 | 2,6 | 29 |
| 9 | PREDICTED: exportin 7 | gi\|194041424 | 2,3 | | 124 |
| 10 | Beta-actin | gi\|20068082 | 2,27 | | 19 |
| 11 | Histone cluster 1, H2bn | gi\|4504261 | 2,13 | | 14 |
| 12 | PREDICTED: similar to thyroglobulin | gi\|194035548 | 2,3 | | 281 |
| 13 | NADH dehydrogenase subunit 5 | gi\|5835873 | 2,9 | | 69 |
| 14 | PREDICTED: similar to myosin VC | gi\|194034708 | 2,1 | | 282 |
| 15 | PREDICTED: similar to Gliomedin | gi\|194034725 | 2,6 | | 59 |
| 16 | PREDICTED: similar to titin | gi\|194043958 | 2,1 | | 603 |
| 17 | PREDICTED: Nance-Horan syndrome | gi\|194044806 | 2,4 | | 160 |
| 18 | Thymosin, beta 4, X chromosome | gi\|10946578 | 2,30 | | 5 |
| 19 | Histone cluster 1, H4a | gi\|4504301 | 2,19 | | 11 |

Table 2 (continued).

| | | | | | |
|---|---|---|---|---|---|
| 20 | Pol1 | gi\|19387582 | 2,1 | | 753 |
| 21 | Protocadherin-11 | gi\|75071591 | 1,2 | | 123 |
| 22 | H3 histone, family 3A | gi\|4504279 | 1,5 | | 15 |
| 23 | Tegument protein/v-FGAM-synthase | gi\|27452770 | 1,2 | | 154 |
| 24 | PREDICTED: dual specificity phosphatase 27 | gi\|194036813 | 1,2 | 1,1 | 129 |
| 25 | Janus kinase 1 | gi\|47523036 | 1,1 | | 132 |
| 26 | PREDICTED: similar to phosphoprotein associated with glycosphingolipidmicrodomains 1 | gi\|194037128 | 1,6 | | 47 |
| 27 | PREDICTED: similar to myotubularin related protein 10 | gi\|194034536 | 1,3 | | 88 |
| 28 | BRCA1 protein | gi\|7839258 | 1,9 | | 25 |
| 29 | PREDICTED: similar to nebulin, partial | gi\|194043732 | 1,0.5 | | 510 |
| 30 | PREDICTED: MYST histoneacetyltransferase (monocytic leukemia) 4 | gi\|194042830 | 1,1 | | 230 |
| 31 | 97R | gi\|8650497 | 1,36 | | 10 |
| 32 | RAB1A, member RAS oncogene family | gi\|72535190 | 1,6 | | 23 |
| 33 | PREDICTED: similar to tau tubulinkinase 1 | gi\|194039323 | 1,1 | | 145 |
| 34 | Recombinase activating protein | gi\|178057333 | 1,3 | | 119 |
| 35 | PREDICTED: similar to katanin p60 subunit A-like 1, isoform 2 | gi\|194040499 | 1,2 | | 55 |
| 36 | PREDICTED: similar to fermitin family homolog 2 | gi\|194034464 | 1,2 | | 67 |
| 37 | PREDICTED: similar to A-kinase anchor protein 6 | gi\|194038792 | 1,1 | | 258 |
| 38 | Cell division cycle 20 homolog | gi\|178056456 | 1,3 | | 55 |
| 39 | PREDICTED: ubiquitin specific peptidase 40 | gi\|194043696 | 1,1 | | 258 |

**Table 2 (continued).**

| | | | | | |
|---|---|---|---|---|---|
| 40 | PREDICTED: similar to FLJ44048 protein | gi\|194043989 | 1,0.5 | | 437 |
| 41 | Xinactin-binding repeat containing 1 | gi\|221139748 | 1,2 | | 199 |
| 42 | PREDICTED: similar to Coiled-coil domain-containing protein 18 | gi\|194035740 | 1,2 | 2,5 | 123 |
| 43 | Clathrin heavy chain 1 | gi\|4758012 | | 1,1 | 192 |
| 44 | PREDICTED: GTP binding protein overexpressed in skeletal muscle | gi\|194037040 | | 1,4 | 34 |
| 45 | Lactotransferrin | gi\|47523782 | | 1,2 | 76 |
| 46 | PREDICTED: similar to telomerase-associated protein 1 | gi\|194038982 | | 2,1 | 288 |
| 47 | PREDICTED: retinoic acid receptor, alpha | gi\|194037340 | | 2,5 | 120 |
| 48 | PREDICTED: similar to Catenin alpha-3 | gi\|194042732 | | 2,4 | 105 |
| 49 | Small calcium-binding mitochondrial carrier 3 | gi\|186886354 | | 1,7 | 52 |
| 50 | Secretogranin-1 | gi\|25453269 | | 1,2 | 74 |
| 51 | PREDICTED: similar to neurofibromatosis 2 isoform 2 | gi\|194043192 | | 2,7 | 70 |
| 52 | PREDICTED: similar to Calmodulin CG8472-PA | gi\|194038236 | | 2,11 | 19 |
| 53 | PREDICTED: coagulation factor XIII, A1 polypeptide | gi\|194037961 | | 3,5 | 83 |
| 54 | Optineurin | gi\|47522752 | | 2,9 | 66 |
| 55 | PREDICTED: similar to Astrotactin-2 | gi\|194033926 | | 2,8 | 41 |
| 56 | PREDICTED: FERM domain containing 6 | gi\|194034446 | | 2,6 | 71 |
| 57 | PREDICTED: similar to discoidin domain receptor family | gi\|194036850 | | 2,5 | 97 |
| 58 | Moesin | gi\|57527987 | | 2,3 | 68 |
| 59 | PREDICTED: similar to DNA excision repair protein ERCC-6 | gi\|194042362 | | 1,1 | 161 |
| 60 | Retinol binding protein 7 | gi\|223634471 | | 1,17 | 15 |

Figure 5.

Figure 6.

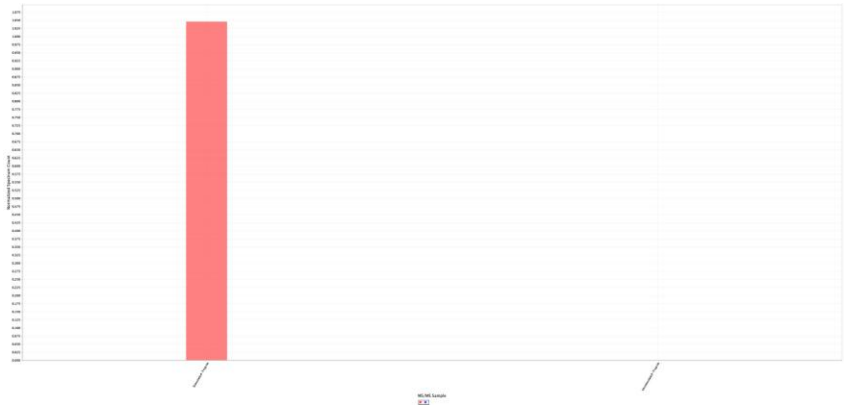

Figure 5. Quantification of antibacterial peptide PMAP-36 in porcine neutrophil secretions analyzed by LC MS/MS. PMAP-36, a peptide known for its antimicrobial properties, was identified at 1.65 times greater concentration in the activated porcine neutrophil secretions versus non-activated porcine neutrophil secretions.

**Figure 6.** Quantification of lactotransferrin in porcine neutrophil secretions analyzed by MS/MS. Lactotransferrin, a protein known for its antimicrobial properties, was identified solely in the activated porcine neutrophil secretions.

# Chapter 4. Discussion

## Scanning Electron Microscopy

Neutrophils are an important effector cell in the innate immune response. In addition to killing pathogens via phagocytosis, neutrophils can also eliminate bacteria and fungi by trapping them in NETs (4). This study characterized the presence of NETs in porcine neutrophils and examined the bactericidal activity of the secretions of stimulated porcine neutrophils against common porcine bacterial pathogens.

Neutrophils isolated from the peripheral blood of swine released NETs when stimulated with PMA. Thread-like structures were observed which were in contact with the stimulated neutrophils, consistent with having been secreted from these cells. These structures were often seen in a web-like pattern. These visual findings were consistent with previous descriptions of NETs in other species (4). Such structures were seen in a lesser amount or not at all in unstimulated neutrophils. The findings are consistent with previous accounts of NETs, suggesting that porcine neutrophils are capable of producing NETs as a potential means of extracellular antibacterial activity.

To confirm whether the observed structures can be truly classified as NETs, immunofluorescence of the NET-like structures would need to be preformed to examine whether DNA and granular proteins are components of the complex. The investigations performed did not include an examination of whether the NETs were capable of binding the bacterial pathogens used in this study, although the literature has demonstrated that

NETs are effective at binding both Gram-positive and Gram-negative bacteria, and have a strong component of granular proteins, from neutrophil granules, including cathelicidins (4).

**Bactericidal Activity of Neutrophil Secretions**

The antimicrobial activity of individual proteins and peptides in neutrophils is complicated to assess, because proteins and peptides coexist and do not act independently in cases of bacterial infection. By choosing pigs as the species of interest, some of the complexity is eliminated, as swine are known to have few or no defensin proteins present in peripheral blood neutrophils. Some porcine cathelicidins have been tested individually for their ability to kill some strains of bacteria (58). However, the literature is lacking a comprehensive study of the combined effect of multiple secreted host defence peptides against the important pathogens that strike porcine populations. Thus, this work incorporates a killing assay using the unfractioned secretions released from both PMA-activated neutrophils and non-activated neutrophils against some important porcine bacterial pathogens.

The data collected suggest that secretions of PMA-activated neutrophils are capable of killing the Gram-negative bacterium *E. coli* K12. The secretions from activated porcine neutrophils resulted in 97% killing of *E. coli* K12. This was a consistent finding, with 87.5% of replicates showing a significant reduction of colony forming units. Although *E. coli* K12 is not commonly pathogenic to pigs, it was used to as a standard as it has been previously demonstrated to be susceptible to porcine

cathelicidins. All secretions were first tested against *E. coli* K12 to assess whether the secretions from activated neutrophils possessed antibacterial properties and could be used to test against other bacterial species. When identical concentrations of secretions from activated neutrophils were incubated with other, more important porcine pathogens; the results were more variable and less significant. Against other Gram-negative species, such as *A. suis* and *P. multocida*, the mean percentage of cells killed was 41% and 16%, respectively. Further, significant reductions were only observed in one and two out of eight trials, respectively, for these two bacteria. The proteins and peptides from activated neutrophil secretions were more effective, however, at killing the Gram-positive bacterium *S. suis*. There was 43% percent killing of *S. suis*, on average among all replicates. In four of the eight replicate experiments, there was a significant reduction of cell numbers observed between the effect of stimulated neutrophil secretions and non-stimulated neutrophil secretions.

A variety of reasons may account for the observed variability in cell killing between bacteria and between replicates. Firstly, to attack pathogens, cathelicidins and other host defence peptides need to have access to the cell membrane, making it necessary for them to pass through the cell envelope. As a result, the thickness, the permeability, and other physical properties of the cell envelope are important to the relative susceptibility to antimicrobial peptides. In terms of peptides whose mechanism of action is to disrupt the cell membrane, the peptides must be able to bind the membrane and integrate themselves into the membrane. Gram-positive bacteria lack an outer membrane and have a thick cell wall comprised of teichoic acid polymers and

peptidoglycan (79). The charge that is associated with these outer wall components is thought to support binding of AMPs and promote bacterial susceptibility. Some bacteria are capable of manipulating the amino acid composition of the teichoic acid, and can become more resistant to AMPs in this manner (79). Gram-negative bacteria are also capable of using similar molecular strategies to modify the charge of the cell membrane, thus promoting resistance to antimicrobial peptides (79). In the present study, neutrophil secretions had a smaller effect against the pathogenic bacteria evaluated in this study compared to the comparatively non-pathogenic *E. coli* K12, and it is possible that this represents an evolutionary adaptation of the pathogenic bacteria to evade the host innate immune response.

Additional reasons for the varied results may also include the nature of the experiment itself. The advantage of using one purified cathelicidin in a bacterial killing assay is that it is possible to determine the precise concentration necessary to damage or kill the bacteria, although this may not be directly relevant to the complex mixture of antimicrobial peptides and proteins present *in vivo*. It was the goal of this study to examine all secreted proteins and peptides acting together against pathogens. Although total protein concentrations were noted, the concentration of a particular protein or peptide was not evaluated. For this reason, it is possible that some of the reduced killing ability seen against some of the bacterial species might indicate that the proteins/peptides responsible for killing a particular bacterium may not be in the optimal bactericidal concentration.

Finally, it is to be expected that there would be some variation between pigs in terms of neutrophil function and the ability of the secretions of stimulated neutrophils to combat different bacteria. This was minimized by sampling from swine that were housed in identical conditions and in a small time period.

**Effect of Elastase Inhibition on Bacterial Killing**

Treating porcine neutrophils with CMK, a known elastase inhibitor, significantly reduced the killing effect of the secretions of activated neutrophils. These secretions induced a significant reduction in the numbers of surviving *E. coli* K12 in all replicates, but this effect was significantly abrogated when neutrophils were first treated with the elastase inhibitor CMK. The number of bacterial CFUs remaining after treatment with supernatants of CMK- and PMA-treated neutrophils was significantly lower than those treated with secretions from non-activated neutrophils, suggesting that CMK-mediated inhibition of elastase only partially abrogated the bactericidal activity of PMA-activated neutrophils.

When the same secretions were incubated with *S. suis*, two of the three replicates had a similar pattern, with secretions of CMK- and PMA-treated neutrophils having a significantly lower killing effect than those from PMA-activated neutrophils.

Bacteria that were incubated with CMK or PMA alone did not demonstrate any notable killing effect (results not shown). Therefore, the CMK-induced reduction of bactericidal activity is attributed to the effect on neutrophil secretions, and not a direct bactericidal effect of the CMK. A second interpretation is that CM might reduce PMA-

induced secretion of neutrophil granules. Although this possibility cannot be excluded, we know of no evidence to support this possibility. A third interpretation is that this effect simply reflects a direct effect of elastase on bacterial survival. Neutrophil elastase has observable but modest bactericidal abilities (80), and these effects could possibly be synergized with other granular antimicrobial proteins found in neutrophils. The fourth and most likely explanation is that elastase is able to cleave antimicrobial proteins and peptides into their active forms (49). In porcine neutrophils, this elastase-activation is most importantly associated with cathelicidins, antimicrobial peptides which contain a common cathelin domain and an active antimicrobial domain (49). When the two domains are cleaved by elastase, the active cathelicidin possesses active antimicrobial properties (49). Based on the observed findings it is likely that these elastase-activated cathelicidins are responsible for a portion of the antibacterial activity of the secretions of activated neutrophils.

Treatment with CMK did not result in a total loss of killing function, suggesting that cathelicidins and other elastase-dependant antimicrobial peptides are not completely responsible for the bactericidal effect of the secretions of activated neutrophils. One possible explanation is that the elastase inhibition was incomplete, although this is considered unlikely because the concentration of CMK and other experimental parameters were similar to those previously published in an experimental system using porcine neutrophils (23). Thus, it is considered more likely that other host defence molecules are also important in extracellular antimicrobial activities of the neutrophil.

**Mass Spectrometry**

Mass spectrometric analysis was performed to explore what proteins and peptides could be positively identified in samples of non-activated neutrophils, activated neutrophils, and CMK-treated activated neutrophils. It was not possible to examine the secretions of neutrophils that were pre-treated with the elastase inhibitor, as the chlorine component of the CMK bound to the proteins and prevented accurate assessment with LC-MS.

Most of the 60 identified proteins and peptides found have no documented antimicrobial activity. The mass spectrometric method identified proteins that are known to be secreted; proteins commonly found in the cytoplasm, membrane, and nucleus; proteins and peptides from primary and secondary granules, and proteins whose expression is induced by stress. Albumin was found in both sets of samples, but since samples were washed in medium containing albumin it was likely acquired during this processing technique.

There were two peptides and proteins that have known host-defence properties, that were found to be either present solely in activated neutrophils or increased in activated neutrophil samples. Porcine lactoferrin was identified only in the activated neutrophil samples. Lactoferrin is a globular multi-functional protein with known antibacterial properties (81). Lactoferrin is found in secondary neutrophil granules, suggesting that the activation method induced degranulation of neutrophils. Lactoferrin is not an elastase-dependant protein, meaning that it could potentially be responsible for the antimicrobial activity noted in CMK-treated samples (81). The other host defence

peptide, found in 1.6 times higher concentration in activated vs. non-activated neutrophil samples, was PMAP-36. This protein is a cathelicidin that is located in the primary granules, consistent with its secretion during neutrophil activation (31). Thus, PMAP-36 is a likely contributor to the elastase-dependent extracellular antimicrobial activity of the secretions of activated porcine neutrophils.

Other cathelicidins, such as PR-39 and prophenins, were expected to be identified but were not observed, suggesting a lack of sensitivity of the analytical technique. Further experiments should involve removal of higher molecular weight proteins by means of size discrimination, and concentration of the sample to facilitate identification of the smaller remaining peptides and proteins. It is possible that more cathelicidin peptides may be identified this way.

**General Discussion**

Several observations in this study indicate that secretions collected from activated neutrophils possess antimicrobial properties, and elastase-dependant cathelicidins are responsible for a portion of this antimicrobial activity. Nevertheless, it should be noted that these studies were conducted in an *in vitro* setting, which may differ significantly in its environmental factors than an *in vivo* setting. The differences found between *in vitro* and *in vivo* locations may contribute to a difference in antimicrobial effect.

Additionally, there is still a need to perform some complementary studies to quantify the inhibition of elastase by the CMK treatment. These experiments would either involve the measurement of elastase activity before and after inhibition using a

colorimetric assay, or by restoring elastase activity to inhibited samples via supplementation with human elastase.

Although several bacterial species were used to conduct these experiments and evaluate the extracellular antimicrobial activities of activated neutrophils, there was only one clinical isolate used per bacterial strain, so it is not known whether similar results would be seen with additional isolates. Further studies using other pathogenic clinical isolates should be done to confirm whether the observations in this study are generally true for multiple isolates of each pathogen. Furthermore, the chemical neutrophil activator PMA was the only method of neutrophil activation used in these studies. Although this is a highly controlled manner of cell activation, antimicrobial cell secretions collected from neutrophils activated by alternative methods, such as lipopolysaccahrides (LPS) or intact bacteria, should be examined.

Bacterial infections in swine are responsible for significant economic losses worldwide. The present findings indicate that porcine neutrophils are capable of killing bacteria using a mechanism never before described in swine. We have been able to observe NETs produced by porcine neutrophils following stimulation with PMA. Just as NETs have been described as being present and a potentially important antimicrobial mechanism in many mammals and fish (4, 82, 83), they may also play a central role in combating common porcine pathogens. Previous research indicates that NETs may serve as an attachment point for granular proteins of neutrophils and not membrane-associated proteins (4). This may indicate that in swine NETs provide a place for antimicrobial

peptides secreted from neutrophil granules, such as cathelicidins, to bind and concentrate their bactericidal activity.

Secretions collected from activated neutrophils were very effective at killing *E. coli* K12, and this result was partially elastase-dependant indicating that cathelicidins were likely responsible for a portion of the killing activity. However, *E. coli* K12 is not an important swine pathogen, and although the findings are important, the results may be less applicable to control of swine diseases. Secretions collected from activated neutrophils were less successful at eliminating *A. suis, S. suis,* or *P. multocida*. Previous accounts using synthetic cathelicidins of particular concentrations resulted in elimination of *P. multocida* (84), but there is no literature documenting the effect of porcine cathelicidins on *A. suis* or *S. suis*. These findings, using the complete secretion of activated neutrophils rather than single proteins, illustrate the complexity of studying antimicrobial peptides in vivo. The results also imply that some bacteria are likely capable of evading both NETs and cathelicidins, suggesting that there are other mechanisms of antibacterial activity that need to be identified and evaluated.

Mature cathelicidins have been generally shown to possess a broad spectrum of antimicrobial activity (31). The findings of this study confirm that cathelicidins have bactericidal activity, in the context of other neutrophil secretions. It appears, however, that cathelicidins do not represent the whole story when it comes to the bactericidal properties of porcine neutrophils. Additional research should be conducted to further characterize the role of porcine cathelicidins and other host defence proteins in vivo.

A goal of this research programme would be to identify a genetic basis for variation in the production and activity of antimicrobial peptides, which may lead to a breeding program to produce healthier swine. Ensuring an optimal concentration and function of antimicrobial peptides in swine could lead to fewer cases of bacterial illness, and a reduced need for antibiotics, resulting in reduced economic losses in swine production. Further research should also be conducted to assess the ability of common porcine pathogens to evade antimicrobial peptides, as increasing the concentration or efficiency of porcine antimicrobial peptides via a breeding programme may not produce satisfactory results if bacteria are resistant to these mechanisms.

In summary, the findings show for the first time that porcine neutrophils are capable of making NETs. The secretions collected from activated neutrophils are effective at killing *E. coli* and *S. suis* but less effective at killing *A. suis* and *P. multocida*. This killing ability was elastase-sensitive, something never before documented in pigs. Future efforts should be made to better understand these complex mechanisms of antimicrobial activity in vivo and their impact on swine health.

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# Appendix 1

Mean CFUs counted for *E. coli* K12, *S. suis*, *A. suis*, and *P. multocida* bacteria when incubated with secretions of non-activated or PMA-activated porcine neutrophils. Eight pigs were sampled, and the neutrophils collected from each pig were incubated with each of the four bacteria. After incubation with cell secretions bacteria were plated and CFUs were counted the following day.

| Bacteria and treatment group | Replicate number | | | | | | | | Mean number of CFUs | Mean Percent Killed ± SEM |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Streptococcus suis*** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | |
| **PMA+** | 86.25 | 53.25 | 48.75 | 53.25 | 50 | 101 | 96 | 36.75 | 65.65625 | |
| **PMA-** | 127.75 | 114.5 | 84 | 95 | 86 | 109 | 281 | 23.25 | 115.0625 | 29.9%(±12.9) |
| ***Pasteurella Multocida*** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | |
| **PMA+** | 32.75 | 47.5 | 141 | 28.25 | 15.25 | 19 | 18 | 22 | 40.46875 | |
| **PMA-** | 57 | 49.5 | 168 | 23.25 | 20 | 22.5 | 22.5 | 23.25 | 48.25 | 13.2%(± 6.5) |
| ***Actinobaccillus suis*** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | |
| **PMA+** | 83.5 | 20.75 | 35 | 5.5 | 11.5 | 13.5 | 12.5 | 4.25 | 23.3125 | |
| **PMA-** | 171 | 28 | 28.75 | 15.25 | 14 | 21 | 22.5 | 13 | 39.1875 | 36.6%(±10.2) |
| ***Escheria coli K12*** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | |
| **PMA+** | 0 | 0 | 1.75 | 1.75 | 0 | 0.25 | 2 | 7 | 1.59375 | |
| **PMA-** | 37 | 14.5 | 23 | 28.25 | 167.25 | 26.75 | 18.75 | 56 | 46.4375 | 90.9%(±4.1) |

# Appendix 2

CFUs counted for *E. coli* K12, and *S. suis* bacteria when incubated with secretions of non-activated,PMA-activated, or CMK-treated, PMA-activated porcine neutrophils. Three pigs were sampled, and the neutrophils collected from each pig were incubated with each bacterium. Bacterial incubations were then plated in quadruplicate and CFUs were counted the following day.

| **Bacteria and treatment** | **Replicate** | | | **Mean CFUs** |
|---|---|---|---|---|
| ***Escheria coli K12*** | 1 | 2 | 3 | |
| **PMA-** | 13.5 | 77.8 | 5.8 | 32.4 |
| **PMA+** | 0.3 | 23.0 | 0.5 | 7.9 |
| **CMK+/PMA+** | 4.0 | 51.3 | 3.0 | 19.4 |

| **Bacteria and treatment** | **Replicate** | | | **Mean CFUs** |
|---|---|---|---|---|
| ***Streptococcus suis*** | 1 | 2 | 3 | |
| **PMA-** | 37 | 112 | 171.8 | 106.9 |
| **PMA+** | 62.3 | 20.8 | 129.5 | 70.9 |
| **CMK+/PMA+** | 39.5 | 5 | 170 | 71.5 |