UNIVERSITI PUTRA MALAYSIA

DETERMINATION SEVERITY OF PNEUMONIA, RESPONSES OF HEAT SHOCK PROTEIN 90 (HSP 90) AND CORTISOL CONCENTRATIONS IN VACCINATED AND NON-VACCINATED PNEUMONIC AND NON-PNEUMOIC GOATS

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A project paper submitted to the

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In partial fulfillment of the requirement for the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Determination Severity of Pneumonia, Responses of Heat Shock Protein 90 (HSP 90) and Cortisol Concentrations in Vaccinated and Non-Vaccinated Pneumonic and Non-Pneumonic Goats", by Ahmad Hafizin Bin Ahmad Tarmizi Tan and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project

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DEDICATION

This project paper is dedicated:

To my parents and family,
A small gift for the unending support and affection

To FPV UPM lecturers and staffs,
Especially to Dr Jesse for the support and advice,
and Mr Jefri as well

To my friends,
Supportive DVM 2018 course mates,
and not to forget Vet Buddies

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LIST OF ABBREVIATIONS

%	Percent
^{o}C	Celsius
pg	Picogram
ml	Millilitre
µl	Microlitre
nm	Nanometre
HRP	Horseradish Peroxidase
O.D	Optical density
RPM	Revolutions per minute
ELISA	Enzyme-Linked Immunosorbent Assay
OAV	Oil Adjuvanted Vaccine
APV	Alum Precipitated Vaccine
HSP 90	Heat Shock Protein 90
ANOVA	Analysis Of Variance
et al.	*et alli* (and others)
UPM	Universiti Putra Malaysia
TPU	Taman Pertanian Universiti

ABSTRAK

Abstrak kertas projek dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Putra Malaysia untuk memenuhi keperluan untuk kursus VPD 4999 - Projek Ilmiah Tahun Akhir

PENILAIAN TAHAP KETERUKAN PNEUMONIA, RESPON PROTEIN KEJUTAN HABA 90 (HSP) DAN KORTISOL DALAM KUMPULAN KAMBING YANG DIVAKSIN DAN YANG TIDAK DIVAKSIN YANG DIJANGKITI DAN TIDAK DIJANGKITI PNEUMONIA

Oleh

Ahmad Hafizin B Ahmad Tarmizi Tan

2018

Penyelia: Associate Prof Dr Faez Firdaus Jesse Abdullah

Penyelia Bersama: Prof. Dr Abdul Aziz Saharee

Pasteurellosis pneumonik adalah penyakit pernafasan yang biasa dihadapi dalam ruminan kecil, sama ada kambing atau bebiri dan agen penyebab umum adalah *Mannheimia haemolytica* dan *Pasteurella multocida*. Program vaksinasi dilihat sebagai cara yang paling kos efektif untuk mengawal penyakit ini. Protein kejutan haba 90 (HSP 90) adalah protein tekanan yang melibatkan banyak fungsi termasuk perlindungan sel daripada tekanan. Oleh itu peningkatan expresi dijangka apabila

haiwan terdedah kepada rangsangan stres. Kortisol adalah glucocorticoid yang juga bertindak balas terhadap rangsangan stres dan peningkatan kepekatan dijangka dapat dilihat dalam keadaan yang tertekan. Terdapat kekurangan maklumat tentang keterukan radang paru-paru dan tindak balas protein kejutan haba 90 (HSP90) dan kepekatan cortisol dalam kambing pneumonia yang divaksin dan tidak divaksin. Oleh itu, kajian ini telah dirancang di mana sebanyak 76 ekor kambing dipilih daripada empat ladang ruminan kecil yang terpilih. Kambing-kambing tersebut dikelompokkan kepada tiga kumpulan iaitu kumpulan vaksin dan tidak divaksin yang tidak menunjukkan tanda-tanda klinikal, kumpulan pneumonik yang divaksin dan kumpulan pneumonik yang tidak divaksin berdasarkan pemeriksaan klinikal. Keparahan radang paru-paru ditentukan berdasarkan pemarkahan auskultasi dan keterukan dikelaskan sebagai ringan, sederhana dan teruk. Sampel darah diambil dari kambing-kambing dan analisis serum kortisol dan protein kejutan haba 90 dibuat menggunakan teknik ELISA. Hasil kajian menunjukkan bahawa kambing pneumonia yang tidak divaksin mempunyai skor auskultasi yang lebih tinggi dan dikelaskan sebagai tanda-tanda radang paru-paru klinikal yang sederhana dan parah manakala kambing pneumonia yang divaksin menunjukkan skor pneumonia ringan. Purata kepekatan HSP 90 untuk kumpulan vaksin dan tidak divaksin yang tidak menunjukkan tanda-tanda klinikal dan kumpulan pneumonik yang divaksin adalah 32.9pg / ml ± 4.21 dan 33.78pg / ml ±5.71 masing-masing dan kira-kira 1.6 kali peningkatan dalam kepekatan yang dilihat dalam kumpulan pneumonik yang tidak divaksin (54.76pg /mlets±13.6). Purata kepekatan kortisol ialah 17.74pg / ml ± 4.43 dan 22.98pg / ml ± 4.71 dalam kumpulan vaksin dan tidak divaksin yang tidak menunjukkan tanda-tanda klinikal dan kumpulan pneumonik vaksin masing-masing

dan hanya 19.67pg / ml ± 3.37 dalam kumpulan pneumonik yang tidak divaksin. Analisis statistik HSP 90 dan kepekatan cortisol menunjukkan tidak terdapat perbezaan yang ketara antara tiga kumpulan. Kesimpulannya, kambing yang dijangkiti penyakit radang paru-paru, kumpulan kambing yang divaksin menunjukkan respon sel hos yang ringan berbanding kumpulan kamping yang tidak divaksin.

Kata kunci: *Vaksin pneumonia, tahap keterukan, auskultasi, HSP 90, kortisol*

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in requirement for the course VPD 4999 – Final Year Project

DETERMINATION SEVERITY OF PNEUMONIA, RESPONSES OF HEAT SHOCK PROTEIN 90 (HSP 90) AND CORTISOL CONCENTRATIONS IN VACCINATED AND NON-VACCINATED PNEUMONIC AND NON-PNEUMOIC GOATS

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2018

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Pneumonic pasteurellosis is a common respiratory disease encountered in small ruminants, either in goats or sheep and the common causative agents are *Mannheimia haemolytica* and *Pasteurella multocida*. Vaccination program is seen to be the most cost-effective way to control the disease. Heat shock protein 90 is a stress protein that involves in many functions including protection of cells from stressors, therefore increase expression is expected when the animal is exposed to stress stimuli. Cortisol on the other hand, is a glucocorticoid that also response towards stress stimuli and therefore increase level is expected to see in stressful conditions. There is paucity of

information on the severity of pneumonia and responses of heat shock protein 90 (HSP90) and cortisol concentrations in vaccinated and non-vaccinated pneumonic goats. Therefore, this study was designed where total of 76 goats were selected in this study from selected four small ruminant farms. The animals were grouped into three groups namely normal vaccinated and non-vaccinated group, vaccinated pneumonic group and non-vaccinated pneumonic group based on clinical examination. Severity of pneumonia was determined based on scoring of the auscultation and the severity was classified as mild, moderate and severe. Blood samples were collected from these goats and the samples were subjected for heat shock protein 90 (HSP90) and serum cortisol analyses using ELISA technique. The result of this study showed that the non-vaccinated pneumonic goats had higher auscultation score and were classified as moderate to severe clinical signs of pneumonia. The vaccinated pneumonic goats revealed mild pneumonia score. Mean concentration of HSP 90 for normal vaccinated and non-vaccinated group and vaccinated pneumonic group were 32.9pg/ml±4.21 and 33.78pg/ml±5.71 respectively and approximately 1.6 time increased in the concentration observed in non-vaccinated pneumonic group (54.76pg/ml±13.6). For the mean cortisol concentration were 17.74pg/ml±4.43, 22.98pg/ml±4.71 in normal vaccinated and non-vaccinated group and vaccinated pneumonic group respectively and only 19.67pg/ml±3.37 in non-vaccinated pneumonic group. Statistical analysis of HSP 90 and cortisol concentration showed there was no significant difference between three groups. In conclusion, the vaccinated group showed mild host cell responses compared to non-vaccinated group in pneumonic goats.

Key words: *Pneumonic Vaccine, severity, auscultation, HSP 90, cortisol*

1.0 INTRODUCTION

Small ruminant farming in Malaysia is a fourth major producer in livestock industry which has seen steady increase of output of livestock product of goats and sheep from 2007 until 2011 and linear trend onwards due to increasing trend of per capita consumption of mutton in Malaysia (Department of Veterinary Services, 2017). However, the small ruminant industry is still far behind compared to poultry, swine and cattle industry, as indicated in self-sufficiency level in Malaysia (Department of Veterinary Services, 2017). The reason can be due to majority of goat farmers are smallholder farmers, thus the production output is relatively small and can be contributed by the farm management (Alina Yusoff, Man, and Mohd Nawi, 2016). Farm husbandry especially in term of management of herd health program, is still lacking among the farmers (Jesse, *et al*, 2015) and therefore development of respiratory diseases is still persist due to presence of multiple stressors.

Pneumonia is one of the notable diseases in small ruminant that has great impact on the animal health thus on the production eventually (Scott, 2017). *Mannheimia hemolytica* is the main causative agent of the respiratory disease in goats, characterized by presence of dyspnea, nasal discharge, pyrexia and non-specific signs such as depression, lethargy and inappetence (Scott, 2017). The severity of the disease under field condition is variable. Therefore, it its recommended to perform vaccination on the goats to reduce the risk of contracting the disease (Jesse, *et al*., 2014). The vaccines should offer cross protection against serotypes A2, A7 and A9 for optimum protection as in the use of recombinant vaccine (Sabri, *et. al*, 2012).

Stress is one of the predisposing factors causing the disease in goats. According to Mohamed and Abdelsalam, (2008), stress is the intrinsic condition causing increase susceptibility to infectious diseases due to break down of immunity and can be measured clinically and biochemically. Useful clinical parameters include elevation of heart rate, respiratory rate and body temperature, whereas cortisol, glucose, urea and free fatty acids are useful biochemical parameters to be measured (Mohamed and Abdelsalam, 2008). Stressful factors like transportation stress, high stocking density in pens, environmental changes and concurrent viral infections increase the susceptibility to the disease (Jesse, *et al*., 2014). On the other hand, the most common encountered stressors according to Mohamed and Abdelsalam, (2008) are high humidity environment with extreme cold or hot temperature, poor ventilation, poor management, transportation and overcrowding in small pens.

Cortisol is one of the parameters used in many experimental studies to evaluate the response of the parameter towards the stress factors as seen studies conducted by Ali *et. al*, (2005) and Greenwood and Shutt, (1992). The previous stated studies, mainly investigate the role of acute stress on the cortisol level, whereas studies on chronic stress is more looked into in this study. Another parameter of interest to study the stress response is heat shock protein 90 (HSP 90). Heat shock proteins play important role in physiological processes (Allison, 2012), and also expressed during exposure to stress. Several studies have been done to study the role of heat shock proteins in response to exposure of stress as studied by Dangi, *et. al*, (2014) and Gaughan, *et. al* 2014.

For the present study, the following hypotheses were proposed:

1. There will be significant clinical pneumonic changes between vaccinated and non-vaccinated goats' farms in relation to pneumonia.
2. There will be significant differences in the levels of cortisol and heat shock protein 90 among pneumonic and non-pneumonic goats from the vaccinated and non-vaccinated goat's farms.

The objectives of the study are:

1. To categorize the severity of pneumonia via auscultation by scoring the sounds obtainable at the lung region.
2. To determine the levels of heat shock protein (HSP) 90 and cortisol among pneumonic goats from vaccinated and non-vaccinated goat's farms.

2.0 LITERATURE REVIEW

2.1 Pneumonic Pasteurellosis

Pasteurellosis is a respiratory disease seen in ruminants including goats which affect all life stages (Zamri, 1991) and can exist in two clinical forms, namely pneumonic and systemic form (Donachie, 2007). Pneumonic pasteurellosis is mainly caused by *Mannheimia hemolytica* and *Pasteurella multocida*, whereas systemic form usually cause septicemia in sheep and is caused by *Bibersteinia trehalosi* (Donachie, 2007; Scott, 2017). However, in goats, *B. trehalosi* does not appear to cause respiratory disease in goats (Matthews, 2016). These agents are the commensals of the upper respiratory tract which will cause an infection when the body defense mechanism is impaired (Scott, 2017). *M. hemolytica* of the serotypes A1, A6, A7, A9 and A11 are the pathogenic serotypes and were reported to cause outbreak in the farms (Mohamed and Abdelsalam, 2008). In United Kingdom, serotypes A1, A2 and A6 are the most commonly isolated serotypes (Matthews, 2016). Other than that, primary respiratory viral infections such as parainfluenza-3 virus, reovirus and adenovirus predispose the animals to secondary pneumonic infection (Scott, 2017). In Malaysia, the disease has the highest occurrence during the rainy seasons and lowest during the dry seasons (Jasni, *et. al*, 1990). High occurrence of the disease during rainy seasons are thought to be due to environmental stress as one of the factors as explained in several literatures including by Mattews (2016), Mohamed and Abdelsalam, (2008) and Kelley, 1980 and lack of vaccination program done on the herd as explained by Jesse, *et al*, 2015.

2.2 Clinical Signs

Clinical presentation in acute case of pneumonic pasteurellosis is seen in 10 to 14 days or earlier days post-infection primarily due to breakdown of immunity primarily due to stress (Mohamed and Abdelsalam, 2008). The lesions develop at the lungs which cause marked respiratory difficulty in animals shown by shallow, rapid breathing accompanied by mucopurulent nasal discharge and coughing. However, in acute cases, the animals are usually found dead without showing any clinical signs (Zamri, 1991). Clinical signs of the chronic case are less obvious and transient (Mohamed and Abdelsalam, 2008) but the animals usually become thinner and on recumbency (Zamri, 1991). Auscultation of the lungs reveal increased respiratory rate (Scott, 2017), with increased breaths sound can be listened especially at the anteroventral aspect of the lungs during the early stage of bronchopneumonia and interstitial pneumonia, and at later stage audible harsh lung sound can be auscultated in the case of uncomplicated interstitial pneumonia (Constable, *et. al*, 2017). As exudation increases, crackles can be listened in a severe case (Jesse, *et al*., 2014). The infected animals are also seen to be pyrexic, lethargic, dull, depressed and anorexic (Zamri, 1991; Scott, 2017) with death is also seen in acute cases and survived animals will become chronically infected (Mohamed and Abdelsalam, 2008).

2.3 Pathogenesis

Stress factors and viral infections are the important predisposing factors causing pneumonic pasteurellosis (Mohamed and Abdelsalam, 2008). Environmental stressors are said to increase susceptibility of the animals to relatively avirulent microbes (Kelley, 1980) whereas primary viral infections predispose the animals to

secondary infection with *Pasteurella* and *Mannheimia* (Scott, 2017). Stress and viral infections will cause breakdown of body defenses especially the pulmonary defense mechanisms and therefore will allow multiplication of the agents and at the same time reduces the clearance of the agents (impairment of mucociliary clearance) (Constable, *et. al*, 2017). In the respiratory tract, the ciliating cells and mucous coating on the trachea, bronchus and bronchioles are compromised which allows the agents to reach the trachea, bronchioles and alveoli via gravitational drainage (Mohamed and Abdelsalam, 2008). Invasion and multiplication of the bacteria into the lung tissue will result to lung damage due to production of endotoxin and cause increased in vascular permeability (Scott, 2017). This cause influx of inflammatory cells primarily neutrophils and further exacerbate when the neutrophils lysed, which will release enzymes to further damage the lung tissue (Scott, 2017). In pneumonic pasteurellosis, leukotoxin is an important virulence factor contributing to the pathogenesis and is present in all serotypes of *M. hemolytica* (Whiteley, *et. al*, 1992). The establishment of *M. hemolytica* A2 infection in naïve goats in a study conducted by Zamri Saad, (1998) was seen within 4-7 days post-infection, which lead to reduced phagocytic activity of alveolar macrophages mainly due to potent leukotoxin that enhance infection and reduce phagocytosis. Characterization of lesion of the lungs seen in pneumonic pasteurellosis usually at the cranioventral distribution in the case bronchopneumonia, whereas in case of septicemia or endotoxemia, diffuse lesion of the lung can be seen as in the case of interstitial pneumonia (Caswell and Williams, 2007).

2.4 Cortisol and its role during stress

Cortisol is a corticosteroid, specifically within the glucocorticoid group, is one of the stress hormones produced by adrenal gland other than catecholamines. Glucocorticoids serve many functions, including gluconeogenesis, lipolysis, block inflammatory response and inhibition of protein synthesis (Klein, 2013). Regulation of the hormone is via hypothalamopituitary axis. In a case of stress, hypothalamus is stimulated to produce corticotropin-releasing hormone which acts on anterior pituitary to produce corticotropin (ACTH) which acts on adrenal cortex to produce cortisol. Cortisol itself acts as an inhibitor onto the hypothalamus and anterior pituitary (Klein, 2013). The hormone is also associated with suppression of immune system when there is an increase in the cortisol level (Randall, 2011).

Studies on stress using cortisol as one of the biochemical parameters have been demonstrated in goats in several different studies. A study done by Ali *et. al*, (2005) displayed elevation of plasma cortisol in stress-induced conditions in sheep and goats during transportation. The author of the study also implied cortisol is an important parameter in stress studies as the hormone shows graduated response depending on the severity of the stressors. Similar observation was also seen by a study conducted by Greenwood and Shutt, (1992) whereby increase of free and total cortisol was seen when transporting the goats.

The previous stated studies however, were done to study the cortisol level on acutely stressed animals and may not reflect the true stress status of the goats in their study. On the other hand, a research was done to study the cortisol level on experimentally induced chronically stressed animals in urine and feces, whereby a

single challenge with corticotropin (ACTH) was made onto rocky mountain bighorn sheep for every other day for 29days, therefore simulating the chronic-like stress response (Miller *et. al,* 1989). The result however was contrary to the hypothesis of the author of the study, in which incremental cortisol level was expected to be observed over the period of the study.

2.5 Heat Shock Protein-90 (HSP90)

Heat shock proteins, also known as stress proteins play important roles in physiological activities and also acts as molecular chaperonage which stabilize unfolded protein during protein synthesis stage under the influence of stressors (Allison, 2012), thus improving the cell survival from degradation (Dangi, *et. al*, 2014). Other functions of heat shock proteins include controlling and signaling cell cycle and protection of cells from stress and apoptosis (Zihai and Pramod, 2003). Different types of heat shock proteins are classified according to molecular weight, and each type according to Zihai and Pramod, (2003), serve specific function in physiological activities. They are mainly expressed when there is exposure to stressors stimulation such as environmental stress and disease state (Morimoto, 1998), but they are also present in unstressed conditions (Morimoto, 1998; Agnew and Colditz, 2008). As mentioned by Morimoto (1998), elevation of heat shock proteins will restrict and reduce level of pathology and cell death. For HSP 90, known functions include HSP 90 acts as a buffer of harmful mutations, controlling cell-cycle and protein folding and regulation (Picard, 2002; Zihai and Pramod, 2003). HSP 90 is also known to have an important complex function in regulating and influencing glucocorticoid receptors activities including maturation of glucocorticoid receptors, which allows binding of

steroids on the stabilized receptors (Grad and Picard, 2007), and therefore lead to increase in expression of HSP 90 during stressful conditions which result in production and binding of steroid hormone on the receptors (Dangi, *et. al*, 2014).

It is mentioned by Belhadj, *et. al* (2015) that both HSP 90 and HSP 70 involve in thermotolerance. A study conducted by Dangi, *et. al*, (2014) on evaluation of several HSP genes, which include HSP 60, HSP 70, HSP 90 and HSP 105/110 on goats undergoing short term heat stress. The result revealed significant elevation of all HSP genes in stimulation of heat stress, therefore affirming the role of HSP in cell protection from stressors. On the other hand, a study conducted by Gaughan, *et. al* 2014 selected HSP 70 as a biochemical indicator to study the effect of chronic heat stress in a growing feedlot cattle. The study concluded HSP 70 is a reliable indicator for chronic stress but not a dependable biomarker to study on one stressor as multiple stressors are also contributing to the level of HSP 70. However, currently there are limited clinical studies on the role HSP especially HSP 90 in response to other stressors aside from heat stress in ruminants particularly sheep and goat.

2.6 Vaccination

Vaccination is a necessary preventive measure in controlling pneumonic pasteurellosis in goats. To date, vaccines against the pathogenic serotypes of *M. hemolytica* were developed mostly developed against A1. However, it was reported that vaccines with serotype A1 is o poorly immunogenic and provides poor cross-protection against other serotypes (Emikpe, *et. al*, 2013). A study was done by Sabri *et. al*, (2008) on the use of recombinant vaccines which to provide protection against serotypes A2, A7 and A9 and were given via intranasal route on goats in Sabah,

Malaysia. The result of the study showed, there was marked reduction of mortality rate after the vaccination within the period of study.

On the other hand, a study was done by Chandrasekaran, *et. al*, (1991) on the effectiveness of locally produced oil-adjuvant vaccine (OAV) developed by Malaysia Veterinary Research Institute (VRI) to the experimentally induced pasteurellosis in sheep. The result showed significant reduction of lung lesions were observed in sheep challenged with *M. hemolytica* alone, but no similar observation was observed when another group of sheep challenged with combined *M. hemolytica and P. multocida* infection.

Currently in Malaysia, alum precipitated vaccine (APV) has been developed by VRI to be used on commercial basis. However, to the author's knowledge, there were no studies conducted on the efficacy of either vaccines (OAV or APV) on the protection level of sheep and goats. In contrast, studies on the immune response of the use of these two types of vaccines were well established on haemorrhagic septicemia in buffaloes caused by *P. multocida*. It is known that OAV acts as a depot when injected intramuscularly into the animals, which will release the antigen slowly over time hence provide longer immune protection (Tizard and Tizard, 2018). On the other hand, APV on haemorrhagic septicemia only provides three to four months protective immunity (De Alwis, 1999) or reduced protective immunity after 6 months according to Myint and Jones (2007). To demonstrate protective response of these two types of vaccines on haemorrhagic septicemia in buffaloes, a study was done by Chandrasekaran, *et. al*, (1994) which showed OAV provide better protection than APV whereby all the buffaloes in the vaccinated group survived 3, 6 and 12 months

post-challenge compared to buffaloes vaccinated with APV with all animals survived at only 3 and 6 months post-challenged. It is therefore, studies on the immune response using different types of vaccines on the protection level of pneumonic goats is imperative to help carrying out this study.

Information on the appropriate time of vaccination from the author's knowledge, was not available for the general herd of animals except recommendation by Scott (2017) for annual booster administration four to six weeks before lambing to breeding ewes.

3.0 METHODOLOGY

3.1 Farm Data Collection

Information on the farm's practices were obtained via observation and interview session with the farm's owner and the workers.

3.2 Study Herd

30 vaccinated goats consisted of 15 vaccinated normal and 15 vaccinated pneumonic goats and 46 non-vaccinated goats consisted of 15 non-vaccinated normal and 31 non-vaccinated pneumonic goats were selected via random sampling in three UPM's foster farms and UPM Taman Pertanian Universiti farm within one-week period.

3.3 Clinical Examination and Grouping

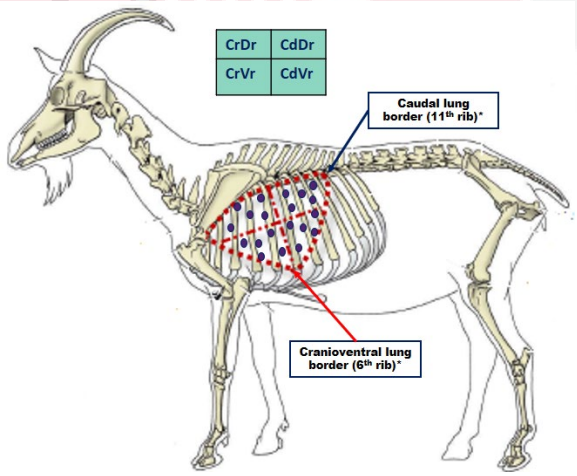

Figure 1 Auscultation method in a goat. Lung field is divided into four quadrants, whereby five auscultation points are determined in each quadrant therefore will yield a total of 40 auscultation points from both lung fields. Image obtained from Dyce, et.al, 2017. Lung borders to ribs based on description by Smith and Sherman, 2009.

Assessment was done on selected goats via observation of the condition of the goats and lung auscultation. Presence of coughing and/or nasal and ocular discharge or any respiratory abnormalities such as dyspnea were noted on the goats examined. Thoracic auscultation was performed on each left and right lung field.The lung field was divided into four quadrants, whereby five auscultation points were determined in each quadrant. During auscultation, the number of points where the presence of crackle or harsh lung sound was counted, which later added from both left and right lung field and divided by 40 points to determine the mean scoring of the animal. Severity of pneumonia is determined according to the scoring which is defined as; <0.325= mild, 0.325< X < 0.65 = moderate, >0.65= severe. From clinical examination, the animals were grouped into 3 groups which consist of normal vaccinated and non-vaccinated group as control group, vaccinated pneumonic group and non-vaccinated pneumonic group as treatment groups.

3.4 Sampling Technique

Blood sample will be collected from the selected goats via jugular venipuncture using 21G vacutainer and whole blood will be stored in red plain tube. Collected blood in the red plain tubes were stored in transport cooler box.

3.5 Serum Extraction and Storage

The collected blood samples were centrifuged at 5000 rpm for 3 minutes to separate the serum from the blood. The procedure was repeated if necessary. The serum was then transferred into 1.5ml Eppendorf tubes. The serum then stored at -20 ^{o}C and -80^{o}C until subjected for serology test (ELISA test).

3.6 ELISA test

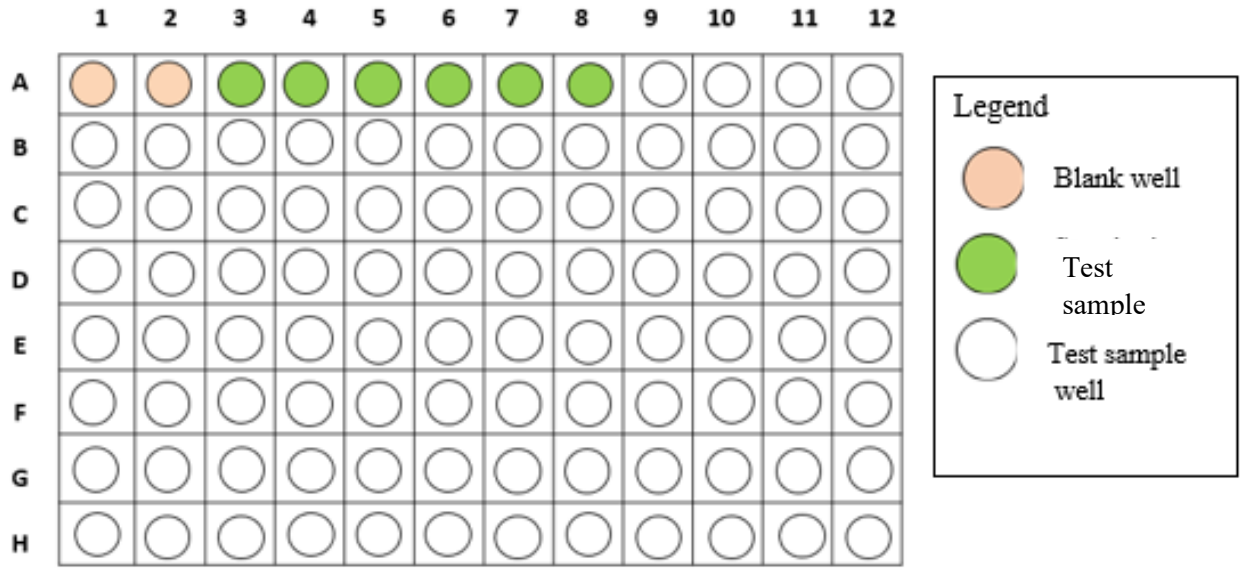

Figure 2: 96 well strip plate (Qayee-Bio company)

The ELISA kit used in this study was manufactured by Qayee-Bio company and specifically used to determine the level of HSP 90 and cortisol in samples via double-antibody sandwich ELISA method.

3.6.1 Washing Liquid Preparation

Washing liquid was prepared beforehand by diluting one part of washing buffer with 19 parts of distilled water.

3.6.2 Procedures

Two blank wells, six standard wells and 76 test sample wells were set on the ELISA strip plate. In the blank wells, the same preparation was applied as in standard and test sample wells, except serum sample and horseradish peroxidase (HRP) were not added in the wells. In the standard wells, the standard was diluted using standard

diluents using the method of serial dilutions, and the concentrations from well A3 to A8 were as follows sequentially; 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml and 0pg/ml. All standard wells were filled with 50µl of standard solution. In test sample wells, 40µl of special diluent and 10µl of serum samples were added to all sample wells. Arrangement of samples were according to four groups (vaccinated normal, vaccinated pneumonic, non-vaccinated normal and non-vaccinated pneumonic). Then, 50µl of horseradish peroxidase (HRP) were added to all wells except the blank wells. The plate was sealed and incubated at 37ºC for 60 minutes. Liquid excess was discarded, and each well was washed five times with prepared washing liquid, with 30 seconds of mixing and shaking for each washing. The washing liquid was discarded, and the plate was tap on the absorbent papers to dry. Then, 50µl of chromogen solution A was added into each well, followed by, 50µl of chromogen solution B. The plate was gently shaken and incubated for 10 minutes at 37ºC away from light. 50µl of stop solution was added into each well to stop the reaction. The plate then was set on standard micro-plate reader, to measure the optical density (OD) at 450 nm wavelength within 15 minutes after stop solution was added. The standard curve linear regression equation was calculated, according to standards' concentration and corresponding OD values.

3.7 Result Interpretation and Data Analysis

Mean pulmonary auscultation was calculated and tabulated in a bar graph. Severity of pneumonia was made based on the mean auscultation of both lung field and the severity is assigned based on the stated scoring.

For heat shock protein 90 and cortisol, standard curve was plotted, and the corresponding concentration of each parameter was determined from the OD values of each sample using CurveExpert version 1.4. Mean concentration for each group for each parameter was calculated and tabulated in a bar chart. Statistical analysis software was used, using SPSS software version 23, to analyze significant level between vaccinated pneumonic group and non-vaccinated pneumonic group.

4.0 RESULTS

4.1 Severity of Pneumonia from Pulmonary Auscultation

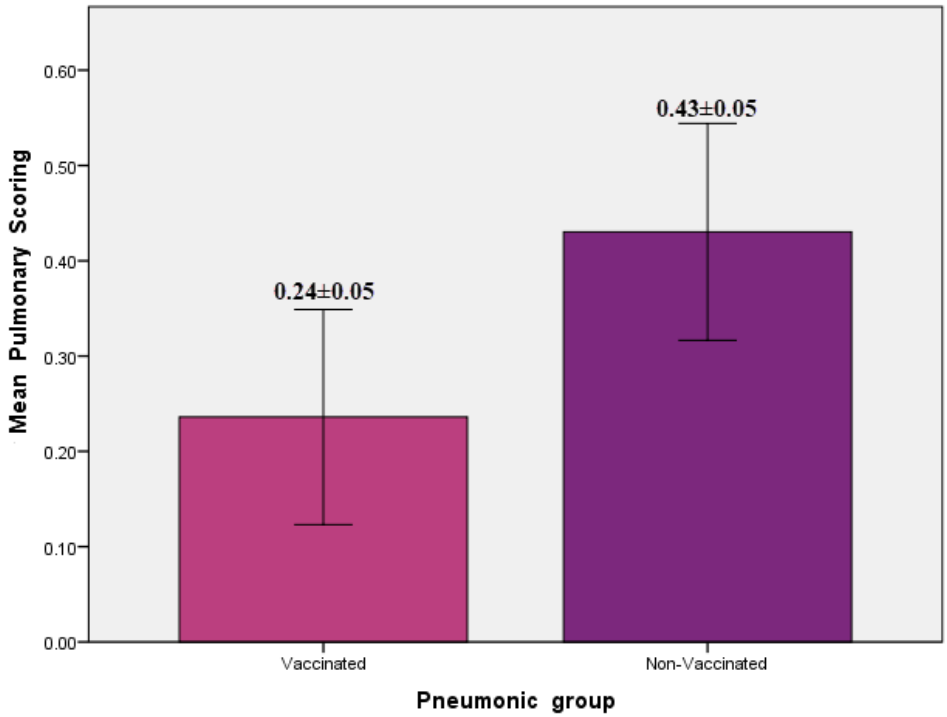

Figure 3:Comparison between severity of pneumonia between vaccinated and non-vaccinated pneumonic goats based on mean from lung auscultation.

Severity of pneumonia is determined according to the mean scoring which is defined as; <0.325= mild, 0.325< X < 0.65 = moderate, >0.65= severe. From the figure 1, vaccinated group falls within mild severity, whereas non-vaccinated group falls within moderate severity.

4.1.2 Mann-Whitney Test

Non-vaccinated pneumonic group showed statistically significant ($p < 0.05$) compared to the vaccinated pneumonic group.

4.2 Heat Shock Protein 90

4.2.1 Mean concentration of HSP 90 between groups

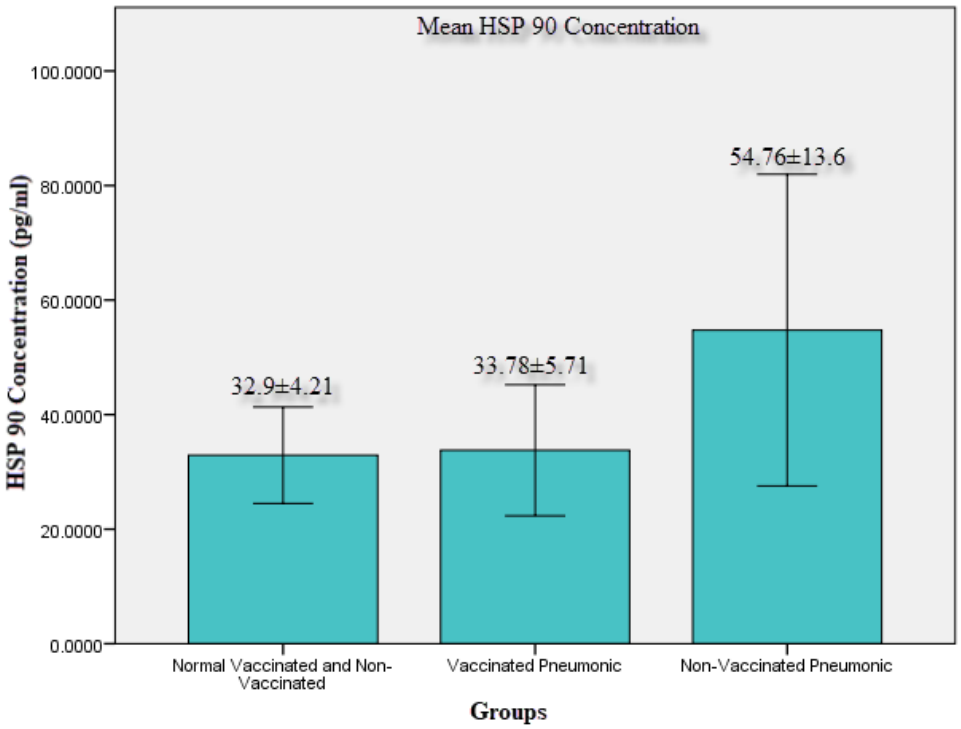

Figure 4: Mean concentration between groups

The above bar chart shows the mean concentration of HSP 90 between three groups. Normal vaccinated and non-vaccinated group and vaccinated pneumonic group appears to have almost the same HSP 90 concentration (32.9±4.21pg/ml and 33.78±5.71pg/ml respectively), whereas non-vaccinated pneumonic group has the highest HSP 90 concentration (54.76±13.6pg/ml) when compared with the other groups.

4.2.2 One-Way ANOVA and Post-Hoc Test

The test statistic showed p >0.05, which means there is no significant difference of the HSP90 concentration. Post-hoc test using Dunnett test showed no significant difference when comparison was made with each group.

4.3 Cortisol

4.3.1 Mean Cortisol Concentration

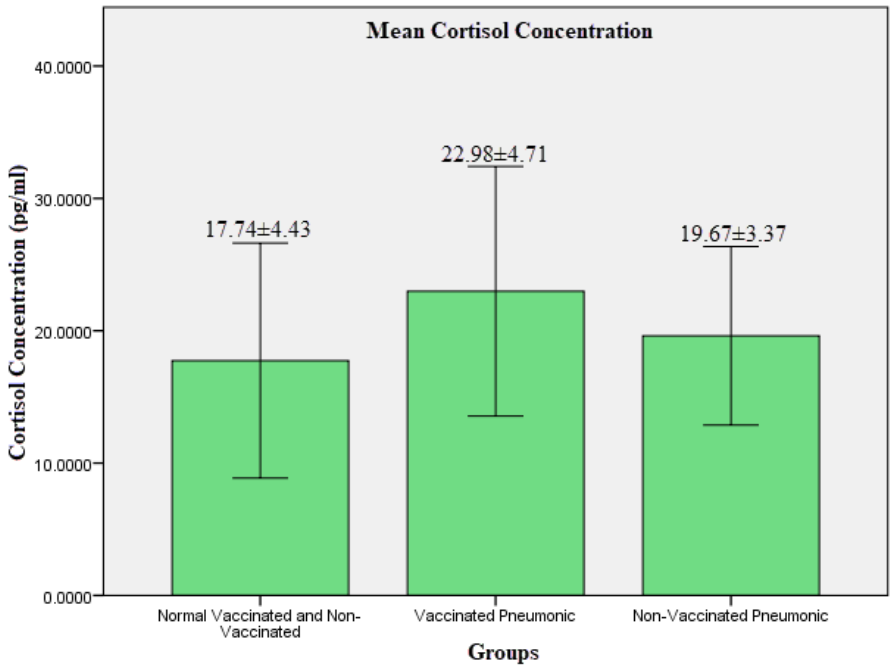

Figure 5: Mean cortisol concentration between groups.

The bar chart in Figure 7 shows the mean concentration of concentration between three groups. All the groups have almost similar cortisol concentration (17.74±4.43pg/ml in normal vaccinated and non-vaccinated group, 22.98±4.71pg/ml in vaccinated pneumonic group and 19.67±3.37pg/ml in non-vaccinated pneumonic group).

4.3.2 One-way ANOVA and Post-Hoc Test

The test statistic showed $p > 0.05$, which means there is no significant difference of the cortisol concentration. Post-hoc test using Dunnett test showed no significant difference when comparison was made with each group.

5.0 DISCUSSION

5.1 Severity of Pneumonia based on Lung Auscultation

In this study, diagnosis of pneumonia was made solely based on clinical examination and pulmonary auscultation, where the mean pulmonary auscultation determined the severity of each goat. It is found that the mean severity based on pulmonary auscultation in vaccinated pneumonic goat is lower and fall under mild severity, whereas non-vaccinated pneumonic goat has higher mean and fall under moderate severity. The severity of pneumonia in this study, can be due to severity on the lesion development on the lung itself, which lead to abnormal sound listened during auscultation as in the case of consolidated part of lung as a result of disease development which result in harsh lung sound (Curtis, et. al, 1986). Difference in severity between the two groups can be explained by protective role of vaccination which provide a degree of protection level on the vaccinated group but none in non-vaccinated group. The statement is in agreement with a study conducted by Chandrasekaran, *et. al*, (1991) whereby the non-vaccinated groups had higher lung lesion score compared with vaccinated groups in experimentally challenged with *P. multocida* and *M. haemolytica* on sheep, therefore supporting the role of vaccination in limiting the disease development and to some extent, limit the severity of the disease in infected vaccinated animals. To the author knowledge, there is no current study conducted where the severity of pneumonia in goats or sheep is assessed by lung auscultation but mainly done via post-mortem on the lungs as seen in study conducted by Chandrasekaran, *et. al*, (1991) and Zamri Saad, (1998). Therefore, to the author

opinion, studying the severity of pneumonia via lung auscultation in tandem with post-mortem would be beneficial for future references.

5.2 Heat Shock Protein 90

The mean concentration of HSP 90 between normal group and vaccinated pneumonic group is similar while highest HSP 90 concentration is seen in non-vaccinated pneumonic group. When comparison is made between vaccinated pneumonic group and non-vaccinated pneumonic group, the statistical analysis showed there was no significant difference between the groups. The reason statistically is the same as explained previously in cortisol parameter. Other than that, the reason of presence of HSP 90 in both groups even in normal group can be explained by few reasons. As explained by Morimoto, 1998, heat shock proteins in general do not only expressed when there is presence of stressors and in the state of disease, but also present in unstressed conditions. Other than that, Gaughan, *et. al* 2014 explained variability of the stress tolerance between the animals result in different reading of HSP expressions, and this explanation is also valid on normal group whereby the group also expose to the same stressors as in other groups. The author also explained that, only initial stress challenge will significantly increase the expression of heat shock protein and no significant changes seen in subsequent expression due to the acclimatization by the animals to the chronic exposure of stress. However, in a study conducted by Dangi, *et. al*, (2014), a short-term heat stress does increase the expression of HSP 90, which support the role of HSP 90 in thermotolerance regulation as mentioned by Belhadj, *et. al* (2015). However, studies are scarce on the role of HSP 90 on other form of environmental stressors especially in chronic stress exposure.

In conclusion, HSP 90 is a reliable parameter to study the effect of heat stress, but more studies should be conducted on the effect of other stressors on the HSP 90 expression especially in chronic exposure.

5.3 Cortisol

The mean cortisol concentration in normal group and vaccinated pneumonic group is almost similar whereas the non-vaccinated pneumonic group has the highest concentration among the other groups. However, the comparison between vaccinated pneumonic group and non-vaccinated pneumonic is not statistically different. Statistically speaking, this is due to the presence of a few outliers which result in the mean of non-vaccinated pneumonic group become higher compare to the other groups, but still does not statistically change the result when comparison is made between vaccinated pneumonic and non-vaccinated pneumonic group. On the other hand, the reason of no significant different between the groups means can be due to the cortisol concentration is not expected to increase in chronically expose stressors mainly due to the environmental stress which agree with the study conducted by (Miller, *et. al,* 1989). The study conducted by Miller, *et. al*, 1989 found that the cortisol concentration in experimentally induced chronic stress in sheep rose temporarily before went back to lower concentration. In contrast with the author's study and by Miller, *et. al*, 1989, studies done on acute stress response on cortisol concentration showed significant changes of the cortisol level after exposure to acute stress as studied by Ali *et. al*, (2005) and Greenwood and Shutt, (1992). Therefore, to the author's opinion, evaluation of cortisol in chronic exposure of stressors may not be a reliable parameter to be studies in naturally exposed herd of animals and is limited by complex

physiological process in experimentally induced chronic stress as mentioned by Miller, *et, al*, 1989.

6.0 CONCLUSION

In summary, non-vaccinated pneumonic group has more severe pneumonia based on pulmonary auscultation scoring compared to the vaccinated pneumonic group. The result validates the role of vaccination that provide immunity protection and therefore reduce the severity of the disease when the animal is infected with the disease. Evaluation of HSP 90 and serum cortisol however, did not show significant changes between vaccinated pneumonic group and non-vaccinated pneumonic group. Therefore, it is concluded that HSP 90 and serum cortisol do not give important consequence and value for interpretation when comparing between the groups in this study.

7.0 RECCOMENDATIONS

Previous studies on experimentally challenge *M. hemolytica* and *P. multocida* on live goats investigated the severity of pneumonic pasteurellosis through clinical signs and post-mortem lesions, but comparison on the severity of the disease mainly by pulmonary auscultation was not made prior to post-mortem. Therefore, by observing the clinical signs and performing pulmonary auscultation, the severity of pneumonia through clinical examination can be studied using the method of scoring like in this study and can be further correlate with the lesions observed during the post-mortem. On the other hand, since evaluation of HSP 90 and cortisol result in no significant difference, the author thinks the main reason is due to one-time sampling study, therefore limiting the value of interpretation. It is suggested that for future studies, the comparison should be made from multiple sampling especially for chronic evaluation of stressors on these parameters. Besides, the author thinks, in a naturally infected herd will result in different stage of disease among the animals in the herd, which will produce variability in the result and prove to be difficult for interpretation. A study with experimentally challenged with the agent and comparison with the control group on the level of concentration of these parameters would produce more convincing results.

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APPENDICES

Appendix 1: Sample result form for goats

Goat ID	Lung auscultation				status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				Status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				Status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				Status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				Status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				Status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected
Goat ID	Lung auscultation				Status
	RCau	RCr	LCr	LCau	Infect.
					Non-infected

Appendix 2: Signalment and auscultation status

VACCINATED FARM

Farm 1

NO	ID	AGE	SEX	AUSCULTATION	STATUS
1	N208	1Y	F	0	NORMAL
2	A17	2Y	F	0	NORMAL
3	102	2Y	F	0	NORMAL
4	417	2Y	F	0	NORMAL
5	R08	2Y	F	0	NORMAL
6	NS201	>2Y	F	0	NORMAL
7	322	2Y	F	0	NORMAL
8	320	2Y	F	0	NORMAL
9	V407	2Y	F	0	NORMAL
10	V007	2Y	F	0	NORMAL
11	R09	2Y	F	0	NORMAL
12	NS105	2Y	F	0	NORMAL
13	206	2Y	F	0	NORMAL
14	12562	3Y	F	MODERATE HARSH	PNEUMONIC
15	605	1Y	F	MILD HARSH	PNEUMONIC
16	617	1Y	M	MILD HARSH	PNEUMONIC
17	601	1Y	M	0	NORMAL
18	603	1Y	M	MILD HARSH	PNEUMONIC
19	R19	2Y	F	MILD HARSH	PNEUMONIC
20	316	2Y	F	0	NORMAL
21	R15	2Y	F	MILD HARSH	PNEUMONIC
22	415	2Y	F	MILD HARSH	PNEUMONIC
23	638	>1Y	M	MILD HARSH	PNEUMONIC
24	624	>1Y	M	MILD HARSH	PNEUMONIC
25	623	>1Y	M	MILD HARSH	PNEUMONIC

Farm 2

NO	ID	AGE	SEX	AUSCULTATION	STATUS
1	686	3Y	F	MILD HARSH	PNEUMONIC
2	2D	3Y	F	MILD HARSH	PNEUMONIC
3	NS9	3Y	F	MILD HARSH	PNEUMONIC
4	A8178	2Y	F	SEVERE HARSH	PNEUMONIC
5	14576	3Y	F	SEVERE HARSH	PNEUMONIC

NON-VACCINATED FARM

Farm 3

NO	ID	AGE	SEX	AUSCULTATION	STATUS
1	8034	2Y	F	MODERATE HARSH	PNEUMONIC
2	2B	2Y	F	MILD HARSH	PNEUMONIC
3	3B	2Y	F	SEVERE HARSH	PNEUMONIC
4	4B	1Y	F	MODERATE HARSH	PNEUMONIC
5	5B	2Y	M	0	NORMAL
6(31)	4207	3Y	F	SEVERE HARSH	PNEUMONIC
7(32)	7B	3Y	F	SEVERE HARSH	PNEUMONIC
8(33)	4206	33Y	F	SEVERE HARSH	PNEUMONIC
9(34)	9B	>2Y	F	SEVERE HARSH	PNEUMONIC
10(35)	10B	3Y	F	MILD HARSH	PNEUMONIC
11	18018	1Y	F	MODERATE HARSH	PNEUMONIC
12	12B	<1Y	F	MILD HARSH	PNEUMONIC
13	13B	>1Y	F	SEVERE HARSH	PNEUMONIC
14	14B	1Y	F	SEVERE HARSH	PNEUMONIC
15	15B	<1Y	F	MILD HARSH	PNEUMONIC
16	8017	1Y	F	MILD HARSH	PNEUMONIC
17	17B	<1Y	F	SEVERE HARSH	PNEUMONIC
18	18B	<1Y	F	MILD HARSH	PNEUMONIC
19	19B	>1Y	F	MILD HARSH	PNEUMONIC
20	KOH4203	3Y	F	MILD HARSH	PNEUMONIC
21	21B	>1Y	F	SEVERE HARSH	PNEUMONIC
22	22B	>1Y	F	SEVERE HARSH	PNEUMONIC
23	23B	1Y	F	SEVERE HARSH	PNEUMONIC
24	24B	2Y	F	0	NORMAL
25	25B	2Y	F	0	NORMAL
26	26B	1Y	F	SEVERE HARSH	PNEUMONIC
27	27B	1Y	F	SEVERE HARSH	PNEUMONIC
28	28B	1Y	F	0	NORMAL
29	29B	<1Y	F	0	NORMAL
30	30B	>1Y	F	SEVERE HARSH	PNEUMONIC

Farm 4

NO	ID	AGE	SEX	AUSCULTAION	STATUS
1	1C	2Y	F	0	NORMAL
2	0144	>3Y	M	0	NORMAL
3	0778	>2Y	F	0	NORMAL
4	9272	>1Y	M	MILD HARSH	PNEUMONIC
5	5C	1Y	F	0	NORMAL
6	6C	2Y	F	SEVERE HARSH	PNEUMONIC
7	7C	2Y	F	SEVERE HARSH	PNEUMONIC
8	8C	>2Y	M	SEVERE HARSH	PNEUMONIC
9	9C	2Y	F	MILD HARSH	PNEUMONIC
10	10C	1Y	F	MODERATE HARSH	PNEUMONIC
11	11C	2Y	F	0	NORMAL
12	12C	2Y	F	0	NORMAL
13	13C	>1Y	F	0	NORMAL
14	14C	1Y	F	0	NORMAL
15	15C	2Y	F	0	NORMAL
16	16C	2Y	F	0	NORMAL

Appendix 3: Mean lung auscultation scoring in vaccinated and non-vaccinated pneumonic goats

Vaccinated Pneumonic

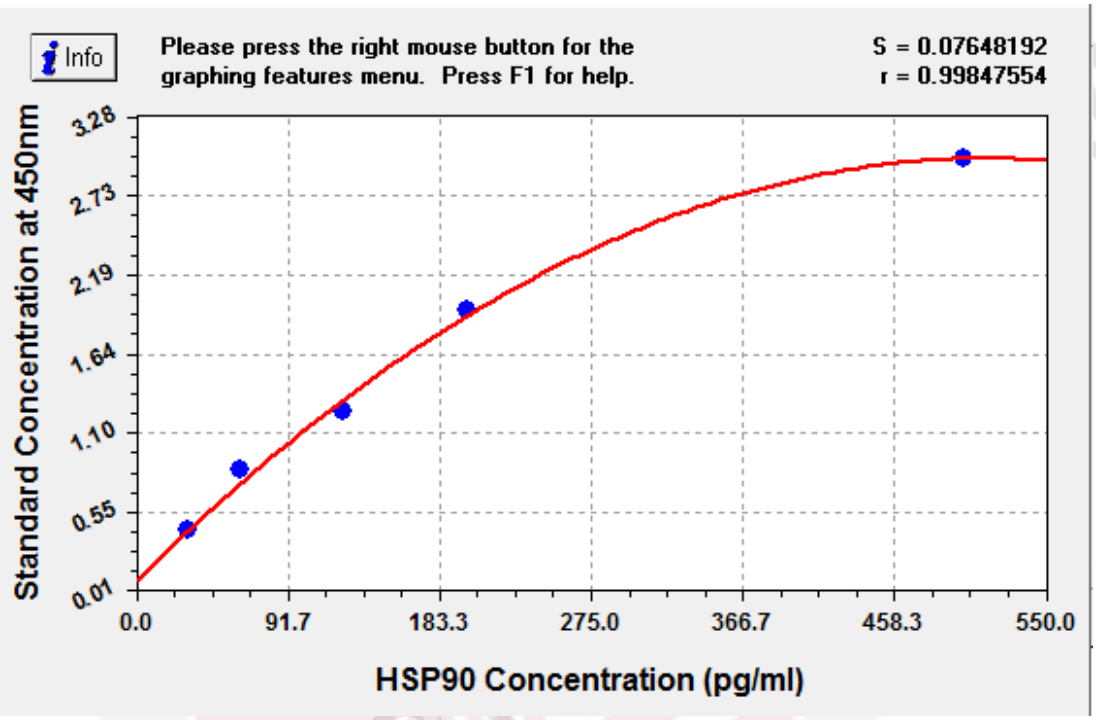
NO	ID	SCORING
1	12562	0.3
2	605	0.13
3	617	0.18
4	603	0.18
5	R19	0.15
6	R15	0.05
7	415	0.15
8	638	0.1
9	624	0.08
10	623	0.13
11	686	0.23
12	2D	0.23
13	NS9	0.13
14	A8178	0.7
15	14576	0.8
	TOTAL	**3.54**
	MEAN	**0.236**

Non-Vaccinated Pneumonic

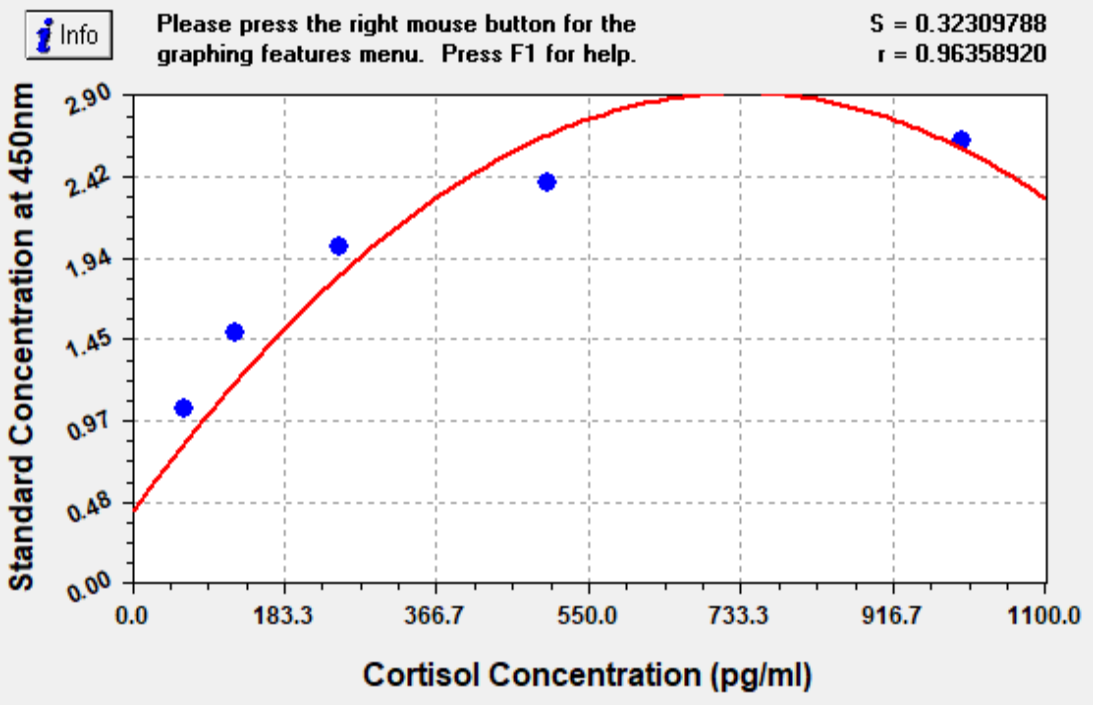
NO	ID	SCORING
1	8034	0.08
2	2B	0.2
3	3B	0.18
4	4B	0.5
5	4207	0.18
6	7B	0.75
7	4206	0.78
8	9B	0.85
9	10B	0.18
10	18018	0.5
11	12B	0.23
12	13B	0.8
13	14B	0.8
14	15B	0.15
15	8017	0.13
16	17B	0.75
17	18B	0.13
18	19B	0.15
19	KOH4203	0.15
20	21B	0.85
21	22B	0.23
22	23B	0.9
23	26B	0.75
24	27B	0.75
25	30B	0.08
26	9272	0.13
27	6C	0.23
28	7C	0.75
29	8C	0.23
30	9C	0.9
31	10C	0.05
	TOTAL	**13.34**
	MEAN	**0.430323**

Appendix 4: Plotted HSP 90 and cortisol standard curve

HSP 90



Cortisol



Appendix 5: Statistical analysis

5.1.1 Test of Normality Between Vaccinated and Non-Vaccinated Pneumonic Groups

Tests of Normality							
GROUP		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
SEVERITY	VACCINATED PNEUMONIC	.315	15	.000	.686	15	.000
	NON-VACCINATED PNEUMONIC	.287	31	.000	.809	31	.000

5.1.2 Mann-Whitney Test

Ranks				
Group		N	Mean Rank	Sum of Ranks
Severity	Vaccinated	15	17.80	267.00
	Non-Vaccinated	31	26.26	814.00
	Total	46		

Test Statistics[a]	
	Severity
Mann-Whitney U	147.000
Wilcoxon W	267.000
Z	-2.012
Asymp. Sig. (2-tailed)	.044
a. Grouping Variable: Group	

5.2 Heat Shock Protein 90 (HSP 90)

5.2.1 One-way ANOVA and Post-Hoc Test

ANOVA

HSP90_concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8546.710	2	4273.355	1.605	.208
Within Groups	194305.380	73	2661.718		
Total	202852.090	75			

Multiple Comparisons

Dependent Variable: HSP90_concentration

Dunnett t (2-sided)[a]

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Vaccinated Pneumonic	Normal Vaccinated and Non-Vaccinated	.8747833	16.3147710	.998	-36.077720	37.827287
Non-Vaccinated Pneumonic	Normal Vaccinated and Non-Vaccinated	21.8603294	13.2130910	.184	-8.066955	51.787614

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

5.3 Cortisol

5.3.1 One-way ANOVA and Post-Hoc Test

ANOVA

Cortisol_concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	274.353	2	137.176	.318	.729
Within Groups	29786.824	69	431.693		
Total	30061.177	71			

Multiple Comparisons

Dependent Variable: Cortisol_concentration

Dunnett t (2-sided)[a]

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Vaccinated Pneumonic	Normal Vaccinated and Non-Vaccinated	5.2355878	6.5703356	.654	-9.671521	20.142697
Non-Vaccinated Pneumonic	Normal Vaccinated and Non-Vaccinated	1.8641727	5.5116607	.924	-10.640961	14.369307

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.