UNIVERSITI PUTRA MALAYSIA

DETECTION OF COMMON PATHOGENS IN RECTAL SAMPLES OF PRE-WEANED PIGLETS WITH DIARRHOEA

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DETECTION

OF COMMON PATHOGENS

IN RECTAL SAMPLES OF PRE-WEANED PIGLETS WITH DIARRHOEA

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Detection of common pathogens in rectal samples of pre-weaned piglets with diarrhoea" by Joey Lai Yee Qing and in our opinions, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD4999 - Final Year Project.

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DEDICATION

This thesis is especially dedicated to:

My family,

My loving parents and brothers,

Who has provided me with much support and encouragement,

My mental support,

Sarah Moy,

My supervisors,

Dr. Michelle Fong Wai Cheng,

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ABBREVIATIONS

%	**Percent**
>	**More than**
µL	**Microliter**
°C	**Degree Celsius**
bp	**Base pairs**
DNA	**Deoxyribonucleic acid**
g	**Gram**
min	**Minutes**
ml	**Milliliter**
PCR	**Polymerase Chain Reaction**
RNase	**Ribonuclease**
sec	**Second**
TAE	**Tris-acetate-ethylenediaminetetraacetic acid**
UK	**United Kingdom**
UPM	**University Putra Malaysia**
USA	**United States of America**
UV	**Ultraviolet**
w/v	**Weight in volume**
spp	**Species**

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

PENGESANAN PATOGEN-PATHOGEN BIASA

DALAM SAMPEL REKTAL ANAK BABI SEBELUM

DICERAIKAN SUSU YANG MENGALAMI CIRIT-BIRIT

Oleh

Joey Lai Yee Qing

2023

Penyelia: Dr. Michelle Fong Wai Cheng

ABSTRAK

Kajian ini memberi tumpuan kepada pengesanan beberapa patogen biasa yang berkaitan dengan cirit-birit pada anak babi sebelum diceraikan susu, termasuk salmonellosis enterik, enteritis klostridial, koksidiosis, dan gastroenteritis koronavirus serta hubungannya dengan pelbagai faktor penternakan. Kajian menganalisis 183 sampel rektal yang dikumpulkan dari anak babi sebelum diceraikan susu dengan cirit-birit di 15 ladang yang merangkumi Perak, Selangor, dan Johor. Sampel-sampel ini dikumpulkan dalam 61 kumpulan berdasarkan litter individu (n=61). Semua sampel rektal telah dikaji untuk pemisahan bakteria *Salmonella* spp dan *Clostridium perfringens*, serta kiraan oosit McMaster yang diubah suai (OPG) untuk *Cystoisospora suis* dan reverse transcriptase Polymerase Chain Reaction (rt-PCR) untuk pengesanan keluarga

Coronaviridae. Secara amnya, 49.2% anak babi yang mengalami cirit-birit sebelum diceraikan susu berasal dari induk babi yang baru, dan 67.34% berasal dari saiz litter lebih daripada 11 ekor anak babi. *Clostridium perfringens* spp dan *Coronaviridae* adalah patogen yang paling biasa dikesan dalam semua sampel cirit-birit, menunjukkan kadar prevalen yang ketara masing-masing 97.96% dan 77.05%, dengan korelasi ($r=0.285$, $p<0.05$) diperhatikan di antara kedua-dua patogen tersebut. Ini diikuti oleh *Cystoisospora suis* (14.75%) dan *Salmonella* spp. (3.28%). 73.77% daripada jumlah sampel adalah dijangkiti secara serentak oleh dua hingga tiga patogen. Terdapat korelasi positif lemah hingga sederhana yang signifikan antara jangkitan serentak dan kehadiran *Clostridium perfringens* ($r=0.386$, $p<0.05$), *Coronaviridae* ($r=0.561$, $p<0.01$), dan masa pengosongan kandang penyusuan ($r=0.282$, $p<0.05$). Masa pengosongan antara 3-5 hari berkait rapat dengan peratusan jangkitan serentak yang lebih rendah ($p<0.05$) berbanding dengan 1 hari dan 7 hari, mencadangkan bahawa tempoh masa ini mungkin berperanan sebagai strategi yang berkesan untuk meminimumkan jangkitan serentak pada anak babi. Kajian lanjut tentang penilaian skor feses, penjajaran patogen tambahan, dan penilaian impak ekonomi disyorkan untuk meningkatkan lagi kedalaman dan aplikabiliti penyelidikan ini.

Kata kunci: Cirit-birit pada anak babi sebelum diceraikan susu, patogen biasa, faktor penternakan, korelasi

An abstract of the project paper presented to Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 - Project.

DETECTION

OF COMMON PATHOGENS

IN RECTAL SAMPLES OF PRE-WEANED PIGLETS WITH DIARRHOEA

By

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2023

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ABSTRACT

Pre-weaned piglet diarrhoea is a significant challenge in the swine industry, causing high pre-wean mortality rate reaching up to 19%, translating into substantial economic losses. This study focuses on detection of several common pathogens associated with diarrhoea in pre-weaned piglets, including enteric salmonellosis, clostridial enteritis, coccidiosis, and coronaviral gastroenteritis and their relationship to various husbandry factors. The study analysed 183 rectal samples collected from pre-weaned piglets with diarrhoea across 15 farms spanning Perak, Selangor, and Johor. These samples were

grouped into 61 pools based on individual litters (n=61). All rectal samples were subjected for bacterial isolation of *Salmonella* spp. and *Clostridium perfringens*, as well as Modified McMaster oocyst count (OPG) for *Cystoisospora suis* and nested reverse transcriptase Polymerase Chain Reaction (rt-PCR) for *Coronaviridae* family detection. In general, 49.2% of pre-weaned diarrheic piglets are from young parity sows, and 67.34% from litter size more than 11 piglets. *Clostridium perfringens* spp and *Coronaviridae* were the most commonly detected pathogens in all diarrhoeic samples, exhibiting striking prevalence rates of 97.96% and 77.05% respectively, with significant correlation ($r=0.285$, $p<0.05$) observed between the two pathogens. This is followed by *Cystoisospora suis* (14.75%) and *Salmonella* spp. (3.28%). 73.77% of total samples were co-infected by two to three pathogens. There were significant weak to moderate positive correlations between co-infection and the presence of *Clostridium perfringens* ($r=0.386$, $p<0.05$), *Coronaviridae* ($r=0.561$, $p<0.01$), and farrowing crate emptying time ($r=0.282$, $p<0.05$). An emptying time of 3-5 days was associated with a significantly lower percentage of co-infections ($p<0.05$) compared to 1 day and 7 days, implying that this timeframe may serve as an effective strategy to minimise co-infections in piglets. Further study of faecal scoring assessment, additional pathogen sequencing and economic impact assessment are recommended to further enhance the depth and applicability of this research.

Keywords: Pre-weaned piglet diarrhoea, common pathogens, husbandry factors, correlation.

CHAPTER 1: INTRODUCTION

Pre-weaned diarrhoea in piglets is one of the most frequent health and economical issues in modern production, which can be associated with high mortality, slow growth rates, and rising medical expenses (Luppi *et al*., 2023). In this study, we aimed to investigate the presence of selected infectious pathogens causing pre-weaned diarrhoea in piglets and correlate it with farm husbandry factors. The selected common pathogens are *Cystoisopora suis*, *Clostridium perfringens*, *Coronaviridae*, and *Salmonella* spp.. *Cystoisospora suis,* formerly known as *Isospora suis* (Barta *et al*., 2005) is a significant parasite, causing swine neonatal coccidiosis that affects intensive pig farming worldwide (Mundt *et al.,* 2005). *Clostridium perfringens* causing clostridial enteritis tends to occur mainly in non-immune piglets less than one week old (Henry Too, 2019). *Coronaviridae* causing viral gastroenteritis are highly contagious intestinal infections and clinically associated with occasional vomiting, profuse diarrhoea, and dehydration in piglets less than 2 weeks of age. According to MSD veterinary manual, pigs of all ages are susceptible to enteric salmonellosis that is caused by *Salmonella* spp.. Besides common pathogens, farm husbandry factors also play a key role in commercial swine production. Piglets are exposed to various stressors such as vaccination, mixing, fighting, temperature fluctuation, changes to the environment, feed, local microbiota, and nursery structure (Faccin *et al*., 2020), these stressors can cause them to be immunosuppressed and vulnerable to diseases. Besides, there are consistently reports of intestinal

inflammation, and disturbed intestinal microbiome composition and functionality as outcomes of poor sanitary housing in pigs (Le Floc'h *et al*., 2009). Chilling also reduces intestinal activity and increases bacterial colonisation (Rhouma *et al.*, 2017). Besides, Gu *et al*. (2010) reported that large temperature differences between the piglet abdomen and the chilly floor surface can increase their susceptibility to diarrhoea. This can be supported by which states the prevalence of diarrhoea was higher in the rainy season than in dry season. Amatucci *et al*. (2022) also reported that sow parity can influence the quality of colostrum and further affect piglets' health.

1.1 Objective and Justification

Pre-weaned diarrhoea increases pre-weaned piglet mortality which brings significant economic impact since it causes profit loss for farmers and increases management cost of farms. Nowadays, pork prices are rising, especially because of the significant short supply since the outbreak of African Swine Fever in the year 2021. Hence, it is important for us to detect and identify the common pathogens causing pre-weaned diarrhoea and find the correlation with husbandry factors to control the occurrence of pre-weaned diarrhoea in farms. It may aid in targeting pathogens and persuade the farmers that applying good farm husbandry practices would avoid economic loss by tackling pre-weaned diarrhoea.

The study was conducted with the following objectives:

1. To detect several common pathogens of pre-weaned diarrhoea.

2. To analyse and correlate the common pathogens of pre-weaned diarrhoea with farm husbandry factors.

1.2 Hypothesis

Null hypothesis 1: There is no detection of common pathogens from rectal samples of pre-weaned piglets with diarrhoea.

Alternative hypothesis 1: There is detection of common pathogens from rectal samples of pre-weaned piglets with diarrhoea.

Null hypothesis 2: There is no correlation between detection of common pathogens and farm husbandry factors.

Alternative hypothesis 2: There is correlation between detection of common pathogens and farm husbandry factors.

CHAPTER 2: LITERATURE REVIEW

2.1 Coccidiosis

Cystoisospora suis, formerly known as *Isospora suis* (Barta *et al*., 2005) is a significant parasite, causing swine neonatal coccidiosis that affects intensive pig farming worldwide (Mundt *et al.,* 2005). It is practically always caused by *Cystoisospora suis* although adult pigs can be infected with *Eimeria spp.* as well (Hinney *et al*., 2021). It causes pre-weaned piglet diarrhoea and reduced weight gain in pre-weaned piglets, usually in the first 3 weeks of life, following ingestion of oocysts that have sporulated in the environment, especially in high temperatures (32-35°C) and humidity conditions and leads to unthriftiness at weaning, considerably impairing animal health and productivity (Feix *et al*., 2020).

2.1.1 Pathogenesis

C. suis develops fast with a prepatency of only five days after oral ingestion of sporulated oocysts (Joachim and Shrestha, 2020). During this endogenous development with several meront generations followed by gamogony, *C. suis* infects and damages the villous epithelium of the jejunum and ileum, leading to atrophy, fusion and necrosis (Harleman and Meyer, 1985; Vítovec and Koudela, 1990; Mundt *et al*., 2006, Mundt *et al.*, 2007). Common clinical signs are non-hemorrhagic diarrhoea of pasty to watery consistency (Sangster *et al.*, 1978; Mundt *et al*., 2006). Enteritis leads to malabsorption

followed by a reduction of weight gain or in severe cases emaciation (Lindsay *et al.,* 1985; Mundt *et al.,* 2006).

2.1.2 Clinical signs

Coccidiosis is a protozoal disease that affects the small intestine and causes scour in suckling piglets. *C. suis* was the most common enteropathogen associated with diarrhoea in piglets from 5 days of age until weaning (Driesen *et al*., 2008). *C. suis* mostly affects suckling piglets age from 5-10 days and has characteristics of high morbidity and low mortality but mortality increases with the presence of secondary bacterial infection (Stuart and Lindsay, 1986). Infected suckling piglets show clinical signs in the second and third week of life (Lindsay *et al*., 1992). Infected piglets show clinical signs such as yellow to grey, frothy to pasty rancid-smelling diarrhoea, perineum stained with faeces, reduction in weight and depressed (Stuart and Lindsay, 1986), while infected older pigs are usually asymptomatic and act as carriers (Shrestha *et al*., 2015). Economic losses in pig farms are mainly due to mortality, impaired performance, retarded growth, and treatment cost (Stuart *et al*.,1980). Additionally, it is believed that coccidiosis makes piglets more vulnerable to secondary bacterial and viral infections, which raise morbidity, mortality, and administrative costs (Chae *et al*., 1998). *C. suis* is more prevalent in piglets raised in confinement, where the warmth and humidity in the farrowing crate favour the sporulation of *C. suis* highly resistant oocyst, while the *Eimeria* species is frequently detected in pigs raised outdoors or at low temperatures (Henry Too, 2019).

2.1.3 Diagnosis

Andrews (2023) reported that the infected piglets' age, lack of response to antibiotic therapy, and the discovery of oocytes in the faeces all aid in the diagnosis. Swine neonatal coccidiosis is suspected in sucking piglets between 7-14 days with clinical signs of diarrhoea that are not responding to the antibiotic treatment. The best, quickest and cost-efficient diagnosis method is detecting the presence of oocyst in faeces using faecal floatation and determining the number of oocyst using modified McMaster technique (Joachim *et al*., 2018). The oocysts are usually present in large numbers and found after clinical signs set in. Oocysts can be detected with saturated sodium chloride or glucose solution and recognized following their size, shape, and sporulation patterns. Staining of impression smears of the mucosa from the jejunum and ileum can help in diagnosing pigs who died before the oocyst shedded (Andrews, 2023). Faecal samples should be collected about 2 to 3 days after the onset of diarrhoea (Henry Too, 2019). Usually many non-pathogenic faecal oocysts are present in normal pigs too (Andrews, 2023), hence it is important to identify those oocysts as *C. suis* (Henry Too, 2019). Post mortem findings such as histologic examination and impression smears of affected jejunum and ileum assist in diagnosis (Andrews, 2023) since they show lesions such as villous atrophy, ulceration and necrosis of the tips of villi (Henry Too, 2019). Endogenous forms of coccidia can be found in the epithelial cells too.

To differentiate *C. suis* and *Eimeria spp*., *C. suis* has a considerably faster development in terms of sporulation. After sporulation, cystoisospora oocysts contain two sporocysts with four sporozoites each while Eimeria oocysts contain four sporocysts with two

sporozoites each. Besides, *C. suis* is almost exclusively found in suckling piglets while *Eimeria spp*. mostly affect younger pigs due to prominent age resistance in animals older than 3 weeks of age, patent infections and clinical disease are rare (Joachim & Schwarz, 2015).

2.1.4 Treatment & control

According to Stuart & Lindsay (1986), usage of anticoccidials on sows have little effect in preventing and controlling porcine neonatal coccidiosis as sows are not the major source of coccidiosis for neonatal piglets. Toltrazuril appears to be effective when given orally to piglets at 4-6 days of age individually for the best result according to Henry Too (2019).

All farrowing pens and areas should have good sanitation and disinfect indoor accommodation with effective disinfectants (Andrews, 2023) such as ammonia compounds, bleaches or live steam. Besides, dry farrowing pens can inhibit sporulation of coccidial oocysts (Henry Too, 2019).

2.2 Clostridial enteritis

Gram-positive, spore-forming bacilli known as clostridia range from being completely anaerobic to moderately oxygen-tolerant (Uzal *et al.*, 2023). Spore-forming ability allows *Clostridium perfringens* to stay in the swine ecosystem; they are ubiquitous Gram-positive anaerobes that can be easily isolated from various

environments (Chan *et al.*, 2012). *Clostridium perfringens* type C is the main enteric clostridia responsible for neonatal diarrhoea in pigs. Its strains produce severe segmental, necro-hemorrhagic enteritis in pigs and are identified by the formation of alpha toxin (CPA) and beta toxin (CPB) (Miclard *et al.*, 2023). Clostridial enteritis is relatively uncommon in South East Asia as compared to other infectious causes of piglet diarrhoea such as *E.coli* and rotavirus (Henry Too, 2019). *C. perfringens* can be found ubiquitously in soil, decaying vegetation, and gastrointestinal (GI) content and faeces of healthy humans and other animals, although it is a very significant pathogen (Uzal *et al.*, 2023). Clostridial enteritis tends to occur mainly in non-immune piglets less than one week old (Henry Too, 2019), they exposed to sow faeces that are colonised by these bacteria, which probably proliferate rapidly in the gut given the unbalanced intestinal microbiota when the *C. perfringens* outgrow the other bacteria (Songer and Uzal, 2005). There will be a decrease in *C. perfringens* count for faecal samples in weanling and grower-finisher pigs since the population of *C. perfringens* decreases in the pig intestine as other bacterial species establish their populations or may be due to the increase use of antimicrobials during the nursery and grower-finisher stages (Chan *et al.*, 2012).

C. perfringens type C are transmissible between piglets and resistant to adverse environmental factors, including UV light, some disinfectants and heat (Songer and Uzal, 2005). It is a primary pathogen, but more common to be secondary to other infectious diseases caused by coccidias, rotavirus, transmissible gastroenteritis (TGE) and porcine epidemic diarrhoea (PED) virus (Henry Too, 2019 ; Songer and Uzal, 2005). However, Chan *et al.* (2012) reported that the number of *C. perfringens* isolates

was higher in pre-weaned piglets compared to pigs in other stages of production. In bacterial cultures, *C. perfringens* type A is similar to other types of this microorganism and can reach 108-109 CFU per gram of contents in pigs with diarrhoea (Chan *et al.*, 2012).

2.2.1 Pathogenesis

C. perfringens type C causes necrosis of enterocytes at the jejunal villi apices which progresses to mucosal necrosis including damage to microvilli, mitochondria and terminal capillaries due to bacterial adhesion (Miclard *et al.*, 2023). CPB brings lethal and necrotizing effects in tissue damage. The course of the disease is usually acute in neonatal piglets, chronic situation can persist for 1 to 2 weeks usually in older pigs, and is recognized by dehydration and persistent diarrhoea without blood (Songer and Uzal, 2005). At necropsy, the most characteristic lesion, severe haemorrhage and possible presence of gas bubbles are observed in the small intestine, varying from red to black colour (Henry Too, 2019). Microscopically, the acute disease in young piglets is characterised by hemorrhagic necrosis of the intestinal wall. It starts in the mucosa but slowly affects all layers of the intestine (Songer and Uzal, 2005).

2.2.2 Clinical signs

Henry Too (2019) described clinical disease can occur from the first 3 days of life. Some piglets show hemorrhagic diarrhoea with staining of the hind quarters while some may be found dead. They might appear severely lethargic and may be crushed by

the sow. Discoloured reddish brown diarrhoeic faeces with very foul smelling and often contain shreds of necrotic intestinal mucosal lining.

2.2.3 Diagnosis

Isolation of *C. perfringens* type C is highly suggestive of clostridial infection but it is not enough to confirm a diagnosis as they may be found in the intestine of some healthy individuals (Uzal *et al*., 2023) unless the clinical findings and lesions are characteristic (Henry Too, 2019). Rapid post mortem changes and clostridia multiplication will occur right after death, hence live piglets should be submitted for laboratory diagnosis (Henry Too, 2019).

2.2.4 Treatment and control

Once clinical signs are shown in piglets, it is mostly acute and irreversible , high mortality can be observed or become stunted in growth even if it survives, hence treatment is often ineffective. However, parenteral or oral antibiotics can be given to piglets within 2 hours of birth as preventive measures. Besides, vaccination of pregnant sow with 2 doses of *C. perfringens* type C bacterins at 3 and 6 weeks of gestation respectively can pass the immunity to piglets at birth via colostrum. Vaccinated sows are recommended to receive another booster of vaccine at 3 weeks before each farrowing (Burrough, 2022).

2.3 Coronaviral gastroenteritis

Viral gastroenteritis is due to transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus (PEDV) and porcine deltacoronavirus (PDCoV). Cross-protection is absent although all three viruses belong to the same family. They are highly contagious intestinal infections that affect all ages of pigs (Burrough, 2022) and clinically associated with occasional vomiting, profuse diarrhoea, and dehydration in piglets less than 2 weeks of age. Since they are almost clinically indistinguishable, they will be discussed together.

2.3.1 Pathogenesis

Coronaviruses such as TGEV, PEDV and PDCoV infect and result in severe, segmental villous atrophy by affecting villus enterocytes of the jejunum and ileum. Then, malabsorption, osmotic diarrhoea, dehydration followed by metabolic acidosis will occur in a short time. Infected pigs excreted faeces that shed massive amounts of virus and transmitted rapidly by aerosol or contact exposure. These viruses survive at lower temperatures hence severe epidemics are more common during winter.

2.3.2 Clinical signs

According to Burrough (2022), the main clinical sign of coronaviral gastroenteritis in all age groups of pigs is watery diarrhoea. Mortality can exceed 60% in naive populations, where pre-weaned piglets infected during the first week of life often die within 3 or 4 days of infection. However, in growing and finishing pigs, clinical

signs are typically limited to diarrhoea, which may be profuse, that resolves in a few days with occasional vomiting. Morbidity is high compared to mortality which can be negligible in older pigs. In sows and gilts with no preexisting immunity, moderate severity may be observed and may exhibit varying combinations of anorexia, vomiting, diarrhoea, depression, and agalactia. The gross lesions including typical signs such as thin-walled, small-intestinal segments filled with watery contents and suckling piglets may have undigested milk in the colon. Severe segmental villus blunting and fusion, with near complete loss of villi in severe disease can be observed through microscope. Neutrophilic infiltration of the lamina propria may be observed in areas where enterocytes are sloughed (Burrough, 2022).

2.3.3 Diagnosis

For rapid detection of coronaviruses, PCR assays can be applied to faeces and population-level samples, such as oral fluids to detect PEDV, TGEV, and PDCoV. High quantities of virus in faeces are shed by affected pigs, and viral nucleic acid is often detectable by PCR assay even after clinical signs have resolved. To detect viruses using fixed tissue specimens, immunohistochemical tests and in situ hybridization can be carried out. (Burrough, 2022).

2.3.4 Treatment and control

According to Burrough (2022), there is no specific treatment available for porcine coronaviral gastroenteritis. Some supportive care measures can be taken during

an outbreak such as increasing the temperature of farrowing crates to minimise loss of body heat, and providing electrolyte solutions and free access to water to combat dehydration. Vaccination of gestating sows to boost lactogenic immunity which can pass immunity to piglets may be sufficient to protect neonates, however vaccines do not induce complete immunity in pig herds without a recent history of coronaviral disease because there is no cross-protection among different enteric coronaviruses. According to Jordan & Derbyshire (1995), the replication of transmissible gastroenteritis virus (TGEV), may be stopped by treatment with interferon (IFN) -alpha. Thus, it was discovered that pig enterocytes and swine testis (ST) cells both produced less TGEV when treated with natural porcine IFN-alpha.

2.4 Enteric salmonellosis

Enteric salmonellosis causes small and large intestines to inflame and necrosis, resulting in diarrhoea. Some serotypes of *Salmonella* can cause generalised sepsis too and pigs of all ages are susceptible especially in weaned and growing-finishing pigs (Burrough, 2022). According to De Lucia & Ostanello (2020), *Salmonella* infections are a concern in pig production due to two major reasons which are the clinical disease in pigs and the potential of contaminating pork products that may affect human health. In Europe, pork was the second most frequent food source of Salmonellosis outbreak in humans (2015-2019) after egg, with 7% and 33% respectively (Pinedo *et al.*, 2022).

2.4.1 Pathogenesis

According to Burrough (2022), *Salmonella enterica* serotype *Choleraesuis* is usually affecting pigs by producing necrotizing enterocolitis, and septicemic disease characterised by hepatitis and pneumonia.

2.4.2 Clinical signs

In pigs with enteric salmonellosis, reduced feed intake and diarrhoea with liquid yellow faeces that may contain necrotic debris followed by fever can be observed. The clinical signs usually last for three to seven days and it can reoccur (Burrough, 2022).

2.4.3 Diagnosis

According to Burrough (2022), faecal culture using selective media can isolate *Salmonella* spp. effectively in terms of time and cost. After that, serotyping is carried out to confirm the serotype involved in the culture. Nowadays, PCR assays with high serotype specificity are more available to save the time for final etiological diagnosis. Besides culture, histologic examination of affected tissue such as intestines and liver have high diagnostic value that can differentiate enteric salmonellosis from proliferative enteropathy and swine dysentery.

2.4.4 Treatment and control

Antimicrobial treatment can be done to acutely ill pigs and medication of the affected group via water or feed based on results of minimum inhibitory concentration testing to identify which antibiotic is the most suitable for the diseased pigs to avoid any antimicrobial resistance. Other than that, thorough cleaning and disinfection of contaminated facilities and elimination of the source of the organism such as subclinically infected animals that shed the organism intermittently to lower the chance of repeated occurance in farm. (Jaime & Higes, 2018).

2.5 Farm husbandry factors

Several risk factors are associated with gastrointestinal infections in pig production. The main risk factors can be grouped into four categories such as farm hygiene, feeding practices, herd management and health management. Under farm hygiene, biosecurity measures including cleaning and disinfection of inanimate objects such as boots, vehicle dips and manure management are the concerns. For feeding practices, the types of feeding and the timing introducing creep feed to weaning piglets are taken into account. Furthermore, herd management such as herd size, housing system including quarantine, all in/all out management are needed to be given attention to. Other than that, health management should not be forgotten. Farmers should always beware of the herd health and vaccination status (De Lucia & Ostanello, 2020).

CHAPTER 3: METHODOLOGY

3.1 Sample selection

Convenient sampling was carried out at a total of fifteen farms in peninsular Malaysia, which are ten farms of Perak, four farms of Selangor and one farm of Johor.

3.2 Sample collection

Only litters of pre-weaned piglets with diarrhoea were selected to take rectal samples following animal ethics guidelines (UPM/IACUC/AUP-U024/2023). From each farm, a minimum of 2 litters and maximum of 6 litters were chosen. Then, three piglets from each litter were randomly picked, sampled and pooled into one sample. These three samples from each farrowing pen (litter) were pooled into one sample. Total rectal samples collected from 183 pre-weaned piglets and pooled them into 61 samples (n=61). After collection the faecal samples were sealed in a sample bag with minimum air and kept in an ice box with ice packs and transferred into the refrigerator under 4°C for bacteria isolation and oocysts count procedure, and paired samples were kept into the freezer under -80°C for viral RNA detection. Samples were analysed within seven days.

3.3 Basic farm background parameter

Farm background parameter such as sow parity (1-10), litter size (8-15), farrowing unit type (open, close, mix), farrowing crate emptying time (3 days, 5 days, 7 days, as needed) and pre-weaned piglet mortality (<10%, <20%, >20%) are took from the farms.

3.4 Detection of common pathogens

Rectal samples that were collected previously were subjected to 3 different methods to detect different common pathogens. Firstly, bacteria isolation and identification was done to detect *Salmonella* spp. and *Clostridium perfringens*. Secondly, Modified McMaster technique was used to carry out oocysts count of *Cystoisospora suis*. Thirdly, viral detection (Nested Polymerase Chain Reaction) was done to detect *Coronaviridae* family.

3.4.1 Bacteria isolation and identification

3.4.1.1 *Salmonella* spp. isolation

The faecal sample of 1 ml was taken and placed in a container with 9 ml of autoclaved buffered peptone water to encourage growth. This mixture is then incubated at 37°C for 18 to 24 hours. Afterward, 1.0 ml of the sample solution is transferred into 9.0 ml of Rappaport-Vassiliadis (RV) enrichment broth for further incubation at 42°C for 24 hours. For selective isolation of certain bacteria, 100 µL of RVS solution is dispensed onto Xylose Lysine Deoxycholat (XLD) agar using a micropipette, specifically for *Salmonella* and *Shigella* species. The samples are applied to agar with a sterile swab. Then, it is incubated at 37°C for 18 to 24 hours to promote bacterial growth. Pink with black centred colonies with Hydrogen sulphide positive is produced by *Salmonella* spp.

3.4.1.2 *Clostridium perfringens* isolation

Direct selective culturing of samples was done on Tryptose Sulfite Cycloserine (TSC) agar. The samples are applied to TSC agar with a sterile swab. Then, it is incubated at 37°C for 18 to 24 hours, anaerobically. Characteristic black colonies are produced by *C. perfringens*.

3.4.2 Modified McMaster technique

Modified McMaster Technique was used to determine the number of Coccidia eggs per gram (EPG) of faeces. The faecal material was loosened, and the middle part of the faces were taken into the beaker. Four grams of faecal samples were weighed using an electronic scale in a 100ml beaker and added with sodium chloride (NaCI) supersaturated solution with specific gravity of 1.2 until it reached 60ml mark. The solution was mixed thoroughly and filtered using tea sieve into another 100ml beaker and the residues discarded. The filtrates were stirred and pipetted into the counting chamber of McMaster slides. The slides were allowed to stand for 10 seconds. Under light microscope, the X100 objective lens was used to visualise and count the oocyst in each chamber. EPG was calculated using the formula below:

$$\frac{Number\ of\ oocysts\ counted}{Weight\ of\ faeces\ (g)} \times \frac{Volume\ of\ NaCl\ used\ (60\ ml)}{2(0.15\ ml\ McMaster\ chamber)} = Oocysts\ per\ gram\ of\ faeces$$

3.4.3 Nested RT-PCR

The primers for screening polymerase chain reaction (PCR) assay of *Coronaviridae* family showing in Table 3.1 were selected based on study Watanabe *et al.* (2010). The assays target RNA-Dependent RNA Polymerase (RdRp), the region slightly more upstream from polymerase. This assay has been modified from the original publication to increase the sensitivity to detect more variant coronaviruses. The target amplicon to produce in Round 1 and Round 2 is 440 bp and 434 bp respectively. A second, heminested step has also been added to increase sensitivity. Before RNA extraction from a total of 61 rectal samples was done using innuPREP Virus DNA/RNA Kit according to manufacturer's protocol.

Table 3.1: Primer set for *Coronaviridae* gene

Reference	Kit	Round	Primer	Sequence (5' to 3')
Watanabe *et al.* (2010)	MyTaq™ One-Step RT-PCR kit (Bioline, UK)	1	Forward	GGTTGGGAYTAYCCHAARTGTGA
			Reverse	CCATCATCASWYRAATCATCATA
	MyTaq™ Red Mix Kit (Bioline, UK)	2	Forward	GAYTAYCCHAARTGTGAYAGAGC
			Reverse	CCATCATCASWYRAATCATCATA

Nested RT-PCR was done including Round 1 and Round 2. In round 1, each PCR reaction was carried out in PCR tubes by adding 17.5 µL MyTaq™ One Step RT-PCR

MasterMix, 1 µL of forward primer, 1 µL of reverse primer and 5 µL of template. 0.5 µL of RNase-free water was added to make the final volume in each tube 25 µL.

In round 2, each PCR reaction was carried out in PCR tubes by adding 12.5 µL MyTaq™ Red Mix, 1 µL of forward primer, 1 µL of reverse primer and 5 µL of template. 5.5 µL of RNase-free water was added to make the final volume in each tube 25 µL. The PCR amplification was performed using a thermal cycler (T100™ Thermal Cycler, Bio-rad, USA). The final protocol used to screen all samples was described in Table 3.2 for Round 1 and Table 3.3 for Round 2.

Table 3.2: Round 1 cycling of Nested RT- PCR for *Coronaviridae*

Step	Temperature (°C)	Duration	Cycle(s)
Reverse Transcription	**45**	**20 min**	**1**
Initial activation	**95**	**1 min**	**1**
Denaturation	**95**	**10 sec**	**39**
Annealing	**49**	**10 sec**	**39**
Extension	**72**	**30 sec**	**39**
Final extension	**72**	**5 min**	**1**

Table 3.3: Round 2 cycling of Nested RT- PCR for *Coronaviridae*

Step	Temperature (°C)	Duration	Cycle(s)
Initial activation	**95**	**1 min**	**1**
Denaturation	**95**	**15 sec**	**34**
Annealing	**50**	**15 sec**	**34**
Extension	**72**	**10 sec**	**34**
Final extension	**72**	**5 min**	**1**

Commercial agarose powder (Vivantis, USA #PC0701 – 500g) was used to make 2% (w/v) agarose gel for electrophoresis by mixing 2g of agarose powder into 100 ml of Tris-acetate-EDTA (TAE) buffer and boiled in a microwave (NN-SM332M, Panasonic, Malaysia) until it fully homogenized. The gel was prestained with 5 µL of RedSafe™ Nucleic Acid Staining Solution (iNtRON, South Korea). The agarose gel was poured into a gel mold with comb. and waited for 20 to 30 minutes until it fully solidified. Then, the solidified gel was removed from the tank gently and placed into the electrophoresis tank (Bio-Rad, USA). TAE buffer was added into the tank until the gel is submerged. 5 µL of GelPilot 100 bp Plus Ladder (Qiagen®, Germany) was pipetted into first well of the gel; 6 µL of PCR product was pipetted into following wells of the gel. Electrophoresis was proceeded by using the MS Power Supply MP200V (Major Science, USA) with 80 volts for 45 minutes or until the DNA ladder reached the bottom

end of the gel. Finally, the gels were visualised under UV transilluminator (GeneGenius Gel Imaging System, SynGene, UK) and the DNA bands were captured using GeneSnap Software (Syngene, United Kingdom).

3.5 Statistical Analysis

Data collected were analysed using IBM SPSS Statistic 23. To analyse the correlation between detection of common pathogens and the farm husbandry factors, Spearman's correlation is used. As the confidence interval of this study was 95%, the statistical results were only significant when $p<0.05$.

CHAPTER 4: RESULTS

4.1 Gel viewing

For Round 1, the designed forward and reverse primers successfully amplified a PCR product of 440 bp. The forward and reverse primers successfully amplified a 434 bp PCR product. The results for Round 2 were obtained by PCR amplification at 50°C with no non-specific PCR bands or primer dimers. This assay's gel electrophoresis of the non-template control and negative control did not produce any PCR bands, verifying that no contamination during preparation procedure or PCR amplification.

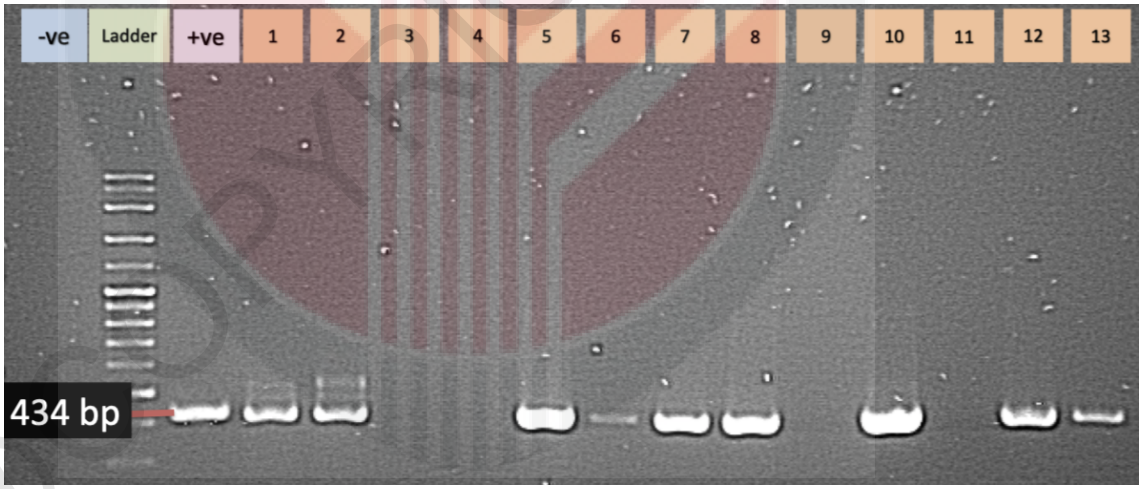

Figure 4.1: Detection of *Coronaviridae* using Nested RT-PCR (Ladder) 100bp marker; (+ve) positive control; (-ve) negative control; (1-2, 5-8, 10, 12, 13) positive samples; (3-4, 9, 11) negative sample; *Coronaviridae* at 434bp.

4.2 Descriptive analysis

In our study, 56 out of 61 (91.8%) litters were positive to at least one of the examined enteric pathogens (*C. perfringens*, *Salmonella* spp., *Coronaviridae* and *C. suis*). 11 out of 61 (18%) litters were positive for only one of these pathogens, 45 out of 61 pathogens were positive for more than one pathogen and finally, only 5 out of 61 (8.2%) were negative for all the examined pathogens.

The percentage of total positive sample for each pathogen are 97.96% of *C. perfringens*; 77.05% of *Coronaviridae*; 14.75% of *Cystoisospora suis*; 3.28% of *Salmonella* spp. as shown in Figure 4.2.

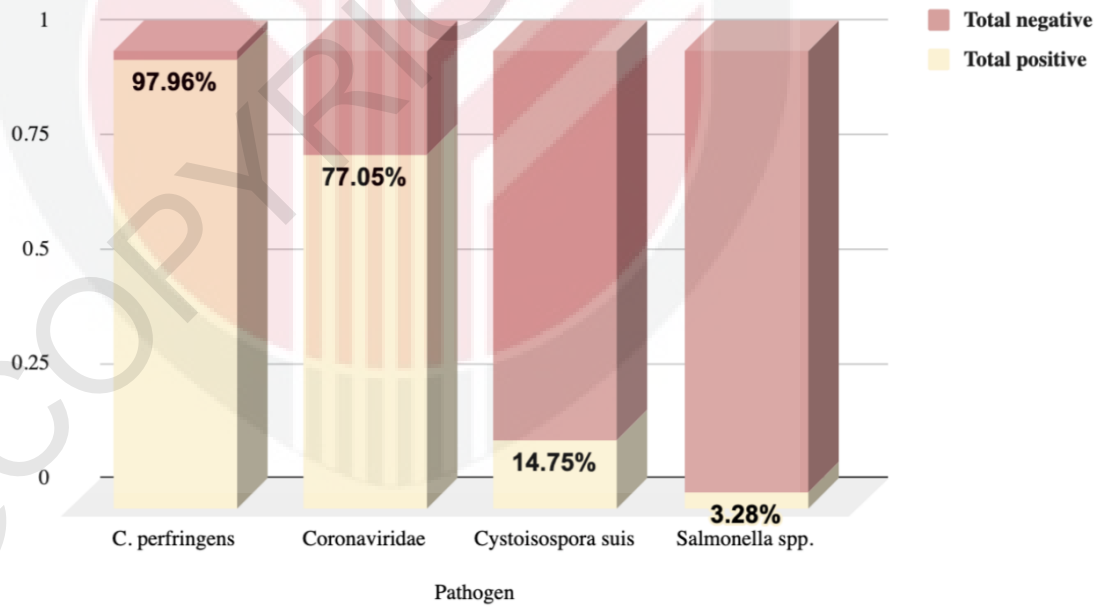

Figure 4.2: Total percentage of positive samples for each pathogen

The other finding (Figure 4.3) shows most of the farms are having co-infection with 2-3 pathogens. There is only 1 farm with only 1 pathogen (*Coronaviridae*) detected, 7 farms with 2 pathogens detected, 6 farms with 3 pathogens detected and 1 farm with 4 pathogens detected.

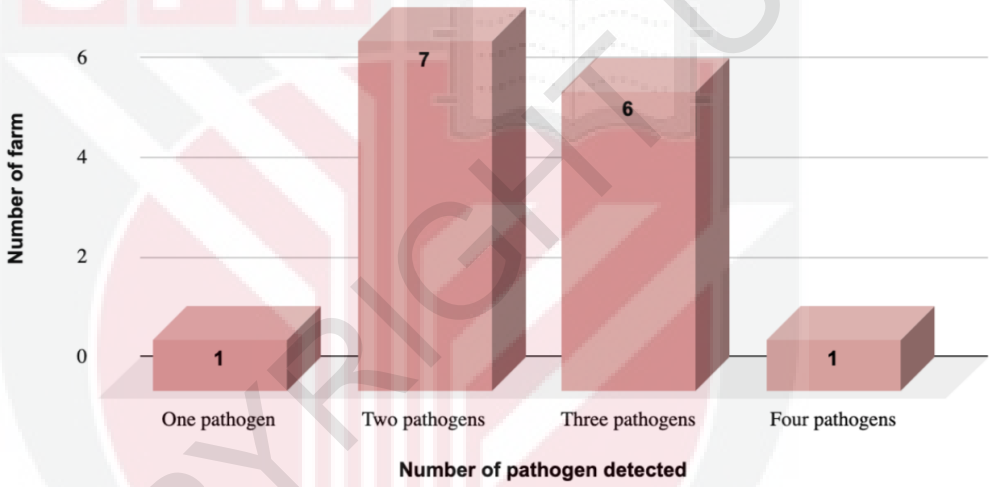

Figure 4.3: Total number of farms with number of pathogens detected

Besides, one of the findings (Figure 4.4) is that of the rectal samples that were only taken from piglets with pre-weaned diarrhoea, 49.2% are from the first and second parity of sow.

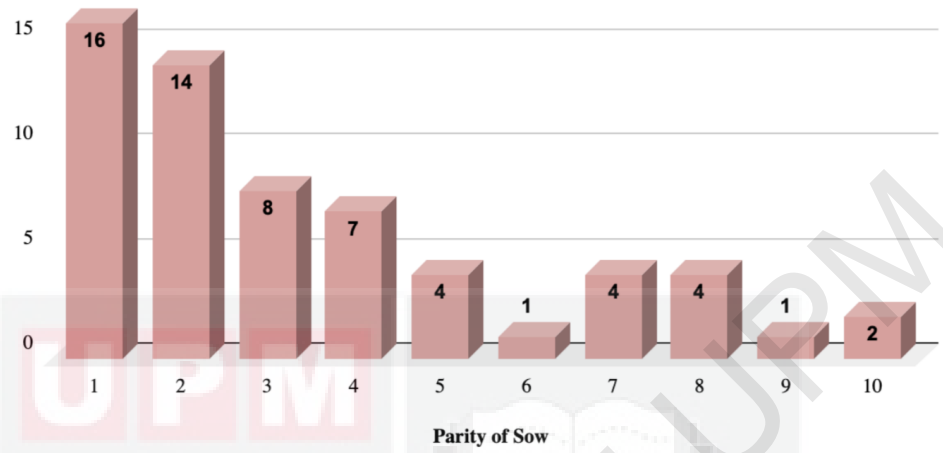

Figure 4.4: Number of rectal samples from different parity of sow

Subsequently, Figure 4.7 shows that the only two rectal samples with *Salmonella* spp. detected are both from the first parity of young sow, which further explain the low quality milk produced by young sows.

By looking at Figure 4.5, 63.4% of rectal samples from diarrhoiec piglets having litter size higher than 11 due to competition of sow milk among piglets.

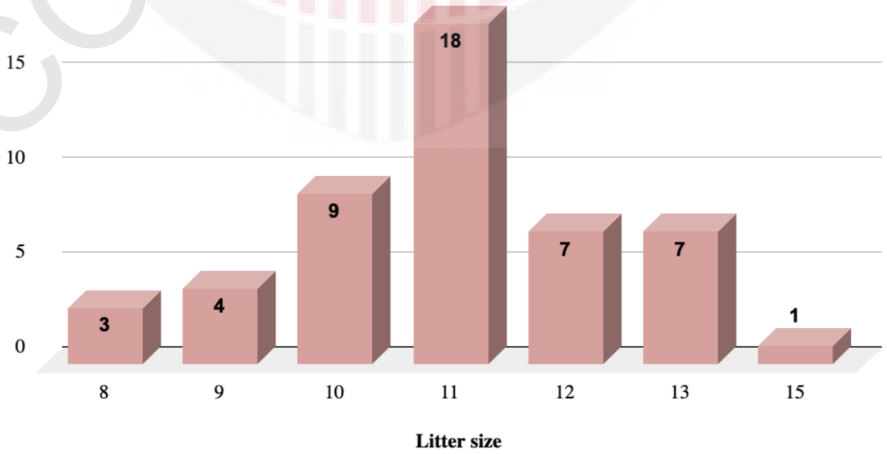

Figure 4.5: Number of rectal samples from different litter size

In terms of farrowing crate emptying time, samples with emptying time of 3 to 5 days have significantly lower percentage of co-infections compared to samples with shorter or longer emptying time (Figure 4.6, $p<0.05$).

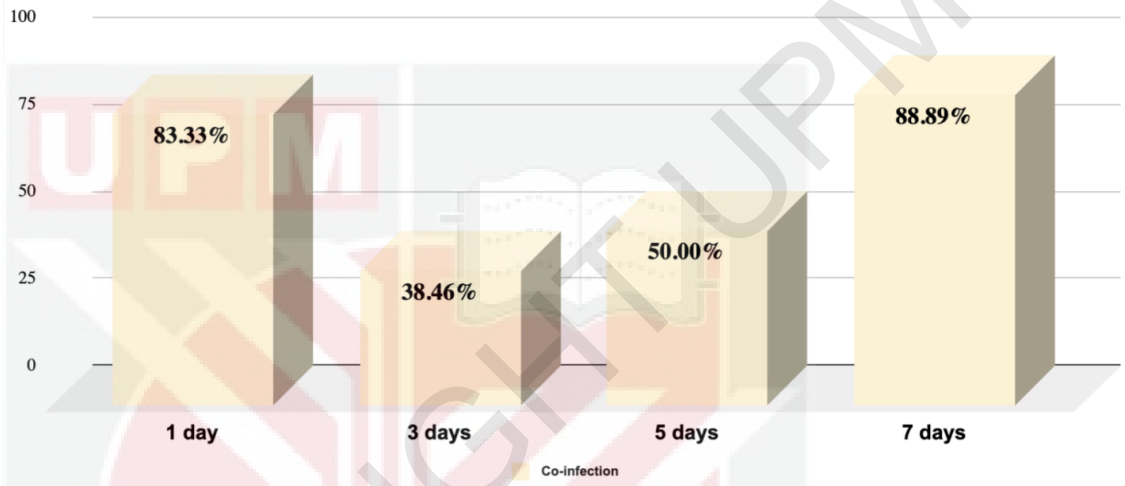

Figure 4.6: Percentage of co-infection with different farrowing crate emptying time

4.3 Spearmans' correlation test

To be more specific, the pathogens causing co-infection are mostly *Coronaviridae*, followed by *C. perfringens* as shown in Figure 4.7. There is a positive weak correlation ($r=0.586$, $p<0.05$) between detection of *C. perfringens* and *Coronaviridae* indicates when the detection of *C. perfringens* increases, the detection of *Coronaviridae* will be increased too.

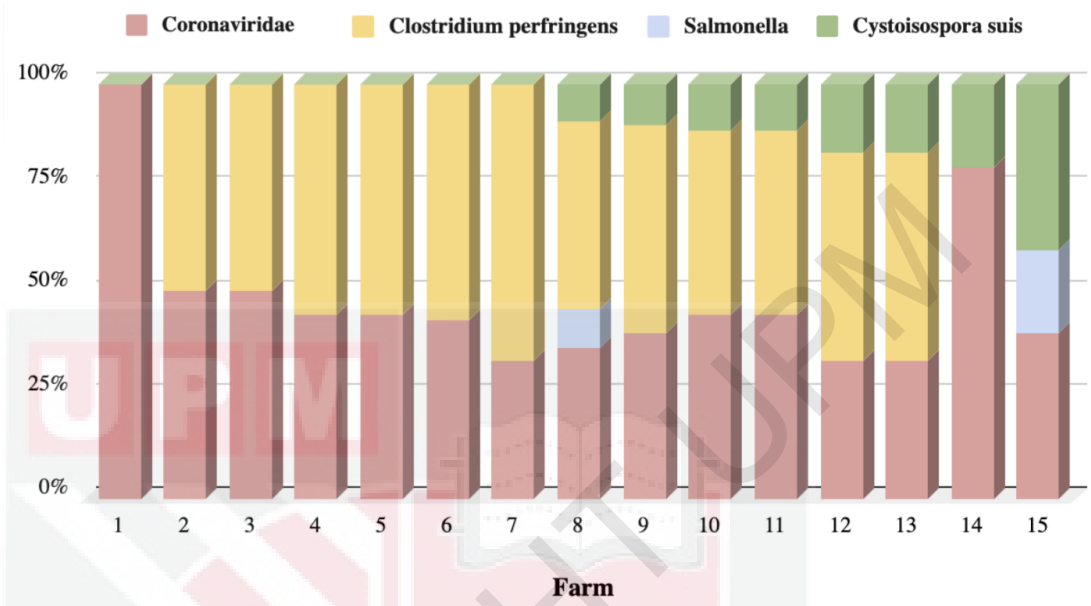

Figure 4.7: Types and percentage of pathogens detected in each farm

CHAPTER 5: DISCUSSION

In our study, the two most detected pathogens among all the rectal samples we took from diarrhoea pre-weaned piglet litters are *C. perfringens* (97.96%) followed by *Coronaviridae* (77.05%). This might be due to *C. perfringens* can be found ubiquitously in the pig intestinal tract (Mesonero-Escuredo *et al.*, 2018) and serotyping was not done in our study for this bacteria. High detection of *Coronaviridae* might be due to the properties of the virus which is high morbidity. These 2 pathogens co-infected 12 out of 15 (80%) farms in our study. This can be proved by a study by Mesonero-Escuredo *et al*. (2018) in Spain, 43.1% of pre-weaned piglet diarrhoea samples taken were having co-infection either bacterial, viral or protozoal with *C. perfringens* involved in all cases. Besides, according to Kylla *et al*. (2019), pre-weaned piglet diarrhoea, is often due to a complex of factors with mixture of infectious agents and other factors such as husbandry factors, environmental temperature and humidity, passive immunity transferred by colostrum and milk which play an important role in resisting infection in piglets, all contributing to its manifestation. This can explain our findings where 49.2% of rectal samples taken from diarrhoeic piglets are from the first or second parity of sow and the only 2 out of 61 samples detected with Salmonella spp. through bacterial culture, are from the first litter by young sows, which produced low quality of milk. Besides, in our study, husbandry factors such as farrowing crate emptying time of 3-5 days effectively lower the entry and transmission of enteric pathogens within litter by allowing sufficient downtime and disinfection process. Indeed, husbandry factors are important for farmers to control pre-weaned piglet diarrhoea occurrence effectively.

CHAPTER 6: CONCLUSION

In conclusion, four common pathogens examined (*Coronaviridae*, *C. perfringens*, *Salmonella* spp. and *C. suis*) are all detected in rectal samples we took from pre-weaned piglets with diarrhoea. The 2 most commonly detected pathogens are *Coronaviridae* and *C. perfringens* which potentially cause viral infection and bacterial infection in piglets respectively. Furthermore, these pathogens often co-exist in pre-weaned piglet diarrhoea which causes significant economic losses to farmers. Besides the pathogens themselves causing the losses, farm husbandry factors such as farrowing crate emptying time also contribute to pre-weaned piglet diarrhoea. Our study provides new insight into a better understanding of causes of pre-weaned piglet diarrhoea and encourages farmers to practise better farm management to prevent huge losses due to pre-weaned piglet diarrhoea.

It can recommend that severity of diarrhoea (Score F1-F4) should be included in the next research focus in order to precisely correlate the pathogenicity of each pathogen and severity of pre-weaned piglet diarrhoea. Besides, an economic impact assessment can be carried out to identify how significant the pre-weaned piglet diarrhoea is leading to economic loss of the farm. Other than that, additional pathogen sequencing should be done to specify the type and species of pathogen causing pre-weaned piglet diarrhoea. This can help veterinarians to gain more comprehensive insights and strategies to manage and prevent diarrhoeal diseases in pre-weaned piglets, thereby reducing economic losses.

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