Breed Susceptibility to Enterotoxigenic and Enteroaggragative *Escherichia coli* Strains in South African pigs

by

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Declaration

I, **Nyaradzo Stella Chaora,** vow that this dissertation has not been submitted to any other University other than the University of KwaZulu-Natal and that it is my original work conducted under the supervision of Prof. M. Chimonyo and Mr E.F Dzomba. All assistance towards the production of this work and all the references contained herein have been duly accredited.

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Abstract

Escherichia coli diarrhoea is the most important source of mortality in piglets. The most frequently isolated strain in enterotoxigenic *E. coli* diarrhoea is F4*ab/ac*. Recent studies in South Africa reported non-fimbrial strains such as PAA and EAST-1 to be prevalent. The objective of the study was to determine whether there are breed differences among pigs with respect to *E. coli* adhesion phenotypes and correlate them to polymorphisms at selected candidate genes in the South African population.

A total of 225 pigs aged 3-12 weeks of the imported (Large White, Landrace and Duroc), local and crossbreds, were sampled from the Eastern Cape and Limpopo provinces of South Africa and genotyped for PCR-RFLP polymorphisms at four candidate genes associated with *E. coli* F4*ab/ac* resistance/susceptibility. These genes were Mucin 4 (*MUC4*), Mucin 13, (*MUC13*), Mucin 20 (*MUC20*) and Transferrin Receptor (*TFRC*). The *TFRC* and *MUC13* genes were less polymorphic, the *C* allele was close to fixation and the homozygous *CC* genotype was the most frequent in all three pig populations. There was a significant difference ($P < 0.05$) in allelic and genotypic distribution amongst breeds for the *TFRC* locus. The g.8227G>C polymorphism in *MUC4* segregated in all three breeds and the marker was moderately polymorphic. There was a significant difference ($P < 0.05$) in genotypic distribution amongst breeds for *MUC4*.The g.191C>T polymorphism in *MUC20* segregated in the local and crossbred pigs and was close to fixation in the imported pigs. There was a significant difference ($P < 0.05$) in allelic and genotypic distribution amongst breeds for *MUC20*, which was moderately polymorphic. There was a reduction in heterozygosity in both the *TFRC* and *MUC13* loci, although *MUC4* and *MUC20* genes had higher heterozygosity levels. The *MUC4* gene had a negative F_{IS} value, indicating outbreeding at this locus. The *MUC20*, *MUC13* and *TFRC* genes had a positive F_{IS} value, indicating inbreeding at these loci. Overall, the studied population was outbred. Imported pigs in *TFRC* and *MUC20* deviated from Hardy-Weinberg equilibrium (HWE). All breeds were in HWE at the *MUC4* and *MUC13* genes. There was no linkage disequilibrium observed amongst the analysed loci.

A total of 109 piglets of three breeds (Large White, indigenous and crossbred) aged 3-5 weeks, were investigated for the susceptibility to *E. coli* F4, PAA strains and EAST-1 toxin. Adhesion tests were conducted on pig intestinal cells, which were viewed under a phase contrast microscope. Three phenotypes were identified as, adhesive, weakly adhesive and non-adhesive. There was a significant association ($P < 0.05$) between breed and level of adherence of the F4 and PAA strains. Highest frequencies of adhesion phenotypes were observed in the indigenous pigs for both F4 and PAA *E. coli* strains. Large White pigs had the lowest frequency of non-adhesion in F4 and PAA *E. coli* strains. The F4 strain had a higher ($P < 0.05$) level of adherence compared to PAA and EAST-1 in Large White pigs. Age of pigs had a significant effect on the level of *E.* coli adherence in indigenous and crossbred pigs ($P < 0.05$). Adhesion of F4 and EAST-1 was higher in weaned indigenous and crossbred pigs, respectively, than in suckling piglets. There was no significant difference between F4 adhesion and the genotypes at all four candidate genes genotypes.

The study showed that both imported and local pig populations carry receptors and are susceptible to F4, PAA and EAST-1 *E. coli* infections. Indigenous pigs were less susceptible than Large White to *E. coli* infection. Although polymorphic and segregating in the populations, the *MUC4* g.8227G>C and *MUC20* g.191C>T mutations were not associated with the adhesion phenotypes and cannot be used in the selection of susceptible animals.

Keywords: Pigs, candidate genes, susceptibility, enterotoxigenic *E. coli*

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Dedication

To: God who has brought me this far, my family and friends who have been great sources of motivation and inspiration, my mentors for developing me to my fullest potential and all those who believe in the richness of learning.

Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

Pork is the third most produced red meat in South Africa and is estimated to have contributed 16.3 % of the gross value of agricultural production in the country during the 2010/11 financial year (South African Pork Producers Organization, 2011). The current estimation on pork consumption has increased to 232 970 tonnes per year with an annual increment of 6.3 %. The pig industry in South Africa is comprised of a commercial intensive sector and the communal production system. Commercial production is estimated to have approximately 400 commercial farms (Department of Agriculture, Forestry and Fisheries, 2010). In South Africa most indigenous breeds are raised by smallholder farmers located in the marginal areas and are used to alleviate food insecurity challenges.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhoea in piglets. It is responsible for economic losses in the pig industry through mortality, morbidity, decreased growth rates and costs incurred through treatment. Diarrhoea has the highest morbidity and is responsible for over 10.8 % of pre-weaning mortality (Ngeleka *et al.,* 2003). Infection can occur anytime, but the highest incidence of disease occurs during the first few weeks of life. The most common clinical signs in colibacillosis are diarrhoea, progressive dehydration, roughening of coat hair, increased body temperature and shivering. In addition to these clinical signs, infection by *E. coli* can also reduce intake, bodyweight, feed conversion efficiency and increase leucocytes and erythrocytes counts.

Dietary management of pigs is considered as a potential control method, for example, feeding high fiber and low protein diets, supplementing the feed with spray-dried plasma, tryptophan or probiotics (Bertschinger and Fairbrother, 1999). Antibiotics such as tetracyclines, sulphur drugs, furazoledone drugs and penicillin have also been used to treat *E. coli* infection. Use of antibiotics can cause the bacteria to become resistant. Another disadvantage of using antibiotics is that farmers have to give a withdrawal

period if the antibiotics are administered before slaughter, causing a delay in the age at slaughter. The delay results in losses from being unable to provide a product to the market when it is required. Supplementation of a pig's diet and use of antibiotics are also not economically feasible because they are expensive. Parental vaccination with purified F4 fimbriae may prevent ETEC infection in suckling piglets, through the immunoglobulin A (IgA) antibodies that can be transmitted via colostrum and milk to suckling piglets. Vaccination is inefficient in preventing post weaning diarrhoea (PWD) that is no longer protected by passive lactogenic immunity, because it stimulates a systemic rather than F4 specific immune response (Duan *et al.,* 2011). Oral immunization of $F4R^+$ piglets with purified F4 fimbriae induces an F4 specific intestinal immune response that protects them against a subsequent ETEC challenge (Verdonck *et al.,* 2008). In contrast, oral immunization with F4 fimbriae purified from $F4^+$ ETEC mutants, in which the specific polymeric stability of the fimbriae is disrupted, reduces mucosal immune responses (Vendonck *et al.,* 2007), due to the polymeric stability of F4 fimbriae being important for its biologic activity (Devriendt *et al.,* 2010). A more sustainable approach to control *E. coli* infections is breeding for disease resistance.

The pathogenesis of enterotoxigenic *E. coli*-induced diarrhoea involves production of fimbriae that adhere to specific receptors present on the intestinal epithelium and aid the organism to resist flushing from the small intestine. This is the initial but imperative step in ETEC-induced diarrhoea. Two toxin types are subsequently released, namely, the heat stable (ST) and the heat labile (LT) toxins. The B subunits of LT bind to the epithelial cells and an A subunit which is translocated into the epithelial cell, activates adenylate cyclase. This is followed by a subsequent increase in cyclic AMP levels, which inhibits sodium, chlorine and water absorption by the villus cells and stimulates their loss from intestinal crypt cells (Van den Broeck *et al.,* 2000). Consequently, profuse watery diarrhoea occurs.

The most common fimbriae are F4, F5, F6, F18 and F41 (Duan *et al.,* 2011). The F4 strain is the most prevalent and occurs as three antigenic variants: F4*ab*, F4*ac* and F4*ad* (Baker *et al.,* 1997; Li *et al.,* 2007).

In addition to fimbrial adhesins, there are a number of potential non-fimbiral adhesins that cause ETEC infections. These include, adhesion involved in diffuse adherence (AIDA-1) (Benz and Schmidt, 1989), porcine attaching and effacing-associated factor (PAA) (Batisson *et al.,* 2003) and *E. coli* attaching and effacing factor (EAE) (Ngeleka *et al.,* 2003). Enterotoxins, namely, heat-stable (ST) and heat-labile (LT) disturb the intestinal fluid and cause diarrhoea in pigs (Vu Khac *et al.,* 2006). The heat stable enterotoxins are classified into STa and STb types (Duan *et al.,* 2011). The STb type is mostly found in porcine ETEC. The gene for STb (*est B*) is found in ETEC diarrhoea from post weaning pigs. Enteroaggragative heat-stable factor (EAST-1) (Savarino *et al.,* 1993) is an ST that is highly common in ETEC strains. To investigate the presence of receptors for specific *E. coli* strains on a pig's intestinal cell, an *in vitro* adhesion test can be carried out to identify the adhesion phenotypes of a particular strain. Mohlatlole *et al.* (2013), for example, reported the absence of F4*ab/ac* and F18 fimbrial adhesins in a South African population. Non-fimbrial adhesins such as, AIDA-1, PAA and EAST-1 were detected in 14.5, 17.9 and 20.2 % of the piglets respectively. Such findings necessitate further investigations on the presence of receptors for adhesion of these non-fimbrial strains. There is also a need to understand the susceptibility of the South African population to fimbrial strains commonly associated with the diarrhoeal infection in piglets. Information on the adhesion phenotypes of the South African pig population is a prerequisite for breeding for ETEC resistance.

There are three receptors specific for ETEC infection. These are high molecular-weight intestinal mucin-type sialoglycoproteins (IMTGPs) (Billey *et al.,* 1997), an enterocyte membrane-associated transferring (Grange and Mouricat, 1996) and an intestinal neutral glycosphingolipid (IGLad) receptor (Grange *et al.,* 1999). The receptor(s) for *F4ab/ac* are located on pig chromosome 13 (SSC13) (Edfors-Lilja *et al.,* 1995). To date, the causative mutations for these receptors remain unknown. After fine mapping the loci for F4*ab/ac* receptor(s), the region of interest is between markers *SW207* and *S0075* (Jorgensen *et al.,* 2010). Zhang *et al,* (2008) through transcriptome analysis of porcine Expressed Sequence Tags were able to identify several receptors within this region, such as Mucin-type (*MUC*) 4, *MUC13, MUC20* and

Transferrin receptor (*TRFC*), Activates CDC42 kinase 1 (*ACT1*), *KIAA0226,* Solute carrier family 12 (*SLC12A8*), Karyopherin alpha 1 (*KPNAI*) and Myosin light chain kinanse (*MYLK*). These are positional candidate genes because of their apical location in the small intestine. Susceptibility is determined by a single locus with a dominant susceptibility allele and a recessive resistant allele. A simple polymerase chain reaction (PCR) test that identifies the presence or absence of the specific mutation can, therefore, be used to select resistant pigs. Susceptibility to infection by pathogenic *E. coli* depends on the bacterial adherence or lack of the ability to adhere to brush border vesicles prepared from porcine intestinal epithelium (Rutter *et al.,* 1975; Gibbons *et al.,* 1977). To date, there are no studies that have been carried out to identify the genotypes of the candidate genes segregating in pigs in South Africa.

1.1 Justification

Increase in *E. coli* strains' resistance to various antibiotics and the increase in incidence of post-weaning diarrhoea have necessitated the exploration of alternative disease control methods. To date, there are no long-term strategies for protecting pigs against ETEC in South Africa. There is need to conduct *in vitro* adhesion tests that identify adhesion phenotypes and receptors in the intestinal tissues of the pigs to which the fimbrial and non-fimbrial adhesins attach to during infection. Analysis of receptors helps identify genes conferring resistance to ETEC infections in South African pigs. The presence of resistant pigs to these strains could help in developing resistant populations through marker-assisted selection methods. A reduction in diarrhoea caused by ETEC infection can be acquired through selection of ETEC resistant pigs.

1.2 Objectives

The main objective of the study was to determine breed susceptibility of South African pig populations to ETEC infections. The study also investigated the feasibility of using candidate genes to select pigs susceptible or resistant to F4 ETEC infections.

The specific objectives of the study were to:

a) Identify polymorphisms in *MUC4, MUC13, MUC20* and *TFRC* ETEC candidate genes in imported, local and crossbred pigs of the South African population;

b) Compare the adhesion of F4, PAA and EAST-1 ETEC strains to intestinal cells of Large White, indigenous and crossbred pigs of South Africa; and

c) Investigate associations between F4 adhesion phenotypes and genotypes of known candidate genes for ETEC resistance in the South African pigs.

1.3 Hypotheses

a) *MUC4, MUC13, MUC20* and *TFRC* candidate genes are polymorphic and segregate in the South African pig populations;

b) The South African pig population is more resistant to F4 ETEC strain in comparison to PAA and EAST-1 strains and indigenous breeds in South Africa are more resistant to the adhesion of ETEC strains compared to imported and crossbred pigs; and

c) Adhesion phenotypes do not correlate with susceptible genotypes of candidate genes.

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Chapter 2: Literature Review

2.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) - induced diarrhoea is a widespread form of colibacillosis in pigs which causes death and losses to the pig industry. This disease is evident through watery diarrhoea in the first few days after birth or after weaning in pigs. The current chapter discusses an overview of pig production, highlights the major diseases found post-weaning and the prevalence and pathogenic effects of ETEC. It also reviews positional candidate genes involved in ETEC infection and preventative strategies for the control of ETEC.

2.2 Overview of pig production in South Africa

Livestock production is increasing rapidly than any other agricultural sector worldwide (Faustein *et al.,* 2003). The total consumption of pork in the developing world has increased by 70 % between 1971 and 1995, while the consumption has increased by 26 % in the developed world (Delgado *et al.,* 1999). Over the past decade pork production in South Africa has increased extensively.

2.2.1 Pig production statistics

The South African pork industry provides 2.2 % of revenue to the agricultural sector in South Africa. Over the past ten years, the gross value of pork production has amounted to R 1.753 million and South Africa accounts for 0.2 % of the world pork production (Oyewumi and Jooste, 2006). The gross value of pork production depends on the quality produced and the price received by farmers. Pork is produced throughout the nine provinces in South Africa. SAPPO (1999) indicated that 80 % of the total pig numbers in South Africa are designated to commercial areas and 20 % to developing areas. The distribution of total pig numbers in the commercial areas on a per province basis is North West - 12 %, Western Cape – 18 %, Northern Cape – 2 %, Free State – 12 %, Eastern Cape – 4 %, KwaZulu Natal – 15

%, Mpumalanga – 15 %, Limpopo – 8 % and Gauteng – 14 %. The total number of pigs in commercial areas is 1 240 487 and these are usually Large White, South African Landrace and Duroc breeds (SAPPO, 1999). The distribution of total pig numbers in the developing areas on a per province basis is Eastern Cape – 69.1 %. Gauteng, Northern Cape and Western Cape - <1.0 %, North West- 3 %, Free State – 4 %, KwaZulu Natal – 10 %, Mpumalanga – 3 % and Limpopo – 10 %. The total numbers of pigs in communal production systems are 315 513 and these are usually indigenous breeds (SAPPO, 1999).

Pig production is commonly practised in areas close to the maize production areas (Visser, 2004). Areas distant to the maize production areas are more pressurised in terms of economic efficiency and sustainability. Approximately 400 commercial producers and 19 stud breeders are found in South Africa. The pork industry is estimated to employ 10 000 workers consisting of approximately 4 000 farm workers and 6 000 in the processing and abattoir sectors (DAFF, 2010).

The South African local market is divided to almost 1:1 between the fresh market and the processing meat market. The prices in the red meat industry are determined by demand and supply prices. Most of the pork produced in South Africa is exported to SADC countries, such as Mozambique, Mauritius, Angola, and Zambia. Pork production is profitable because it has a faster turnaround period than other red meats such as beef. The production of pork has increased over the past years, due to an increase in market prices and unavailability of red meat substitutes (DAFF, 2010). Pigs are also multipurpose and their products range from meat, cooking fats and bristles (Lekule and Kyvsgaard, 2003).

Despite these advantages, the pork industry has some weaknesses. The industry is susceptible to diseases such as diarrhoea, African swine fever and respiratory infections. Outbreaks such as African swine fever can drastically reduce sales due to the contagious nature of the disease. In areas where there is a shortage of water, there may be disturbances in cleaning pens, which will hinder producers to meet safety requirements. South Africa has never been a pork exporter of any substantial magnitude. South African Meat Industry Company (2000) indicated that 10 427 tonnes of pork were imported during the year 2000.

China dominated the world pork production, accounting for approximately 50 % of world production, followed by the European Union (18 %) and USA (10 %) (Visser, 2004). This increases competition in the South African pig industry, given its small structure. One of the biggest threats to the South African pig industry is the increased influx of poultry meat mainly from the USA (Mathis, 1999). Almost 50 % of all imported meat is poultry.

The pork industry has several opportunities. Pork is a vital source of protein for human health. The industry is also active in addressing consumer requirements and running promotions (DAFF, 2010). In order to have sustainable and profitable pig farming, there is need for monitoring and application of health measures, biosecurity programmes, transparent import and export protocols and most importantly sound technology development and research strategy.

2.2.2 Pig production systems

Pigs are produced under different production systems ranging from simple backyard systems to large scale systems with advanced biosecurity measures (Lekule and Kyvsgaard, 2003), depending on whether pigs are being kept for sale or for household consumption. The South African pig industry consists of different production systems, namely intensive, large scale outdoor and free-range production systems. In South Africa there are two common production systems which are expressed according to the scale of production, namely, small scale and large scale production systems.

Small scale systems comprise of scavenging and semi intensive systems. Small scale systems are usually practised by subsistence farmers of South Africa who produce pigs for their own consumption. The scavenging system is cheap and requires little capital investment and management. It is characterised by increased mortality, low litter sizes and low weights at weaning and slaughter, lack of or minimal biosecurity, limited health care, supplementary feeding and inadequate housing. A household keeps 1-3

sows which are allowed to scavenge for food. Pigs kept under the semi-intensive system are limited to a certain area, and it is the farmer's responsibility to provide feed for the pigs. Normally during the day the pigs are allowed to go in a fenced larger area where they can wallow and graze. Since this system is more managed than the scavenging system, litter numbers are improved. Indigenous breeds are common under this system, but you may also find crosses of indigenous and exotic breeds under this system.

Large scale production systems are characterised by improved breeds, feed concentrates and also better performance. Pigs kept under this system are usually kept in outside pens or indoors (Honeyman, 2005). The housing is generally made with concrete floors and asbestos roofing. Adequate feed, water, pen space and shade is given to pigs so as to meet their requirements. This system requires skilled management and veterinary protection against diseases and parasites. Mostly commercial breeds such as Large White, Landrace and Duroc are used under this system due to their high lean growth potential and reduced backfat thickness (Webb *et al.*, 2006). Some commercial farms in South Africa also practice multi-site rearing, where breeding sows, weaners and growers are kept on sites that are distant from each other, to prevent aerosol infection and spread of diseases and pests by birds (Kyriazakis and Whittemore, 2006). Some areas in South Africa have started practising commercial outdoor production (Honeyman, 2005), where sows are kept in paddocks and are provided with individual pens for shelter.

2.2.3 Exotic breeds

The origin of pigs in Southern Africa is unclear. There is one archaeological record of a pig introduced into the Eastern parts of South Africa between the 3rd and 7th centuries (Plug and Badenhorst, 2001). In recent centuries, pigs were bartered from Chinese trading ships which were passing through Southern Africa shores (Ramsay et al., 1994). A more recent phase of pig introduction into South Africa began in the 16th to 17th century when European breeds types like Large White, Duroc and Landrace were

introduced (Bester and Kusel, 1998). These breeds are normally termed imported pigs because they are not originally from South Africa.

There are three pure breeds farmed in South African registered with the Pig Breeder's Society (PBS) namely, South African Landrace, Large White and Duroc. The PBS keeps registration and performance records of the pedigrees of purebred sows and boars registered by the PBS (SAPPO, 1999). The Landrace breed was originally developed in Denmark and exported to different countries from 1949. This breed has become the second best commercial breed in South Africa and has been in South Africa since 1952(Visser *et al.,* 1993; Swart *et al.,* 2010). The Large White breed was first established in 1884 in England as a distinct breed (Briggs, 1983) and is one of the two major pig populations in the world (Ruvinsky and Rothschild, 1998). The Large white breed is the prominent commercial breed in South Africa and has been farmed in South Africa since 1903 (Swart *et al.,* 2010). The Duroc breed originated in the USA and is a vital terminal sire in most countries. Duroc pigs were imported to South Africa from Canada in 1980 mainly for use in crossbreeding programmes (Visser *et al.,* 1993). Approximately 75 % of all registered pigs in South Africa are involved in the National Pig Performance and Progeny Testing Scheme (NPPPTS) of the Agricultural research Council's Animal Improvement Institute (ARC) (Visser, 2004).

2.2.4 Indigenous breeds

Various indigenous breeds have been identified in South Africa but information on their origin is limited. Kolbroek pigs are an indigenous phenotype of South Africa of unknown origin (Swart *et al.,* 2004). Kolbroek pigs are very short with pricked ears and a squashed face. The breed is dark coloured, either black or brown and piglets are often striped at birth. These pigs are hardy, scavenge outside homesteads, have high disease tolerance, perform well on high fibre diets and are docile (Visser, 2004). This makes the breed ideal in rural areas where intensive farming is not possible.

The Windsnyer breed is similar to the ancient Egyptian breed which is small and has bristles that form a distinct mane. Like most of the indigenous animals these pigs have a large colour variation of black, reddish-brown, brown, black and white or spotted. Some of the young pigs have longitudinal stripes which are typical to the young bushpig. The Windsnyer pig is very hardy and scavenges for its food. It can convert food with a low nutrient content very efficiently, enabling it to survive on food such as the cereal by-products of brewing. Sows of this breed are good mothers, which results in few piglet deaths. The Windsnyer breed was also reported to be found in Mozambique and Northern Zimbabwe (Halimani *et al.*, 2010). It is closely related to an indigenous breed in Zimbabwe called the Mukota breed (Holness, 1991). Few studies have been done to characterise, the production environment and the livelihood strategies of those who depend on these pigs for food and income (Chimonyo and Dzama, 2007). Although the Mukota and Windsnyer breeds are phenotypically similar, the genetic link is not known (Halimani *et al.*, 2010). The above mentioned pigs are usually termed local pigs because they are presumed to have originated from Southern Africa.

At weaning young pigs are introduced for the first time to artificial food, a new environment which may result in alimentary and respiratory diseases. Approximately 70% of the pork industry losses, be it mortality, morbidity, culling, veterinary costs or abortion as a result of enteric and respiratory diseases (Varley and Wiseman, 2001).

2.3. Common post-weaning diseases in pigs

Post weaning pig diseases cause direct losses through morbidity and mortality. The most common diseases which occur post weaning in pigs are post weaning multi-systemic wasting syndrome, post weaning respiratory disease complex, swine dysentery and colibacillosis.

2.3.1. Post weaning multi-systemic wasting syndrome

Post weaning multi-systemic syndrome spreads at an alarming rate and leads to mortalities of 6 – 10 % (Segales and Domingo, 2002). It mostly affects the kidneys, lungs, liver and lymph nodes of pigs between 5 to 10 kg bodyweight. The main symptoms are diarrhoea, loss of weight, appetite, breathing difficulties, jaundice and blotched skin (Kyriazakis and Whittemore, 2006). Affected pigs have characteristic lesions on multiple tissues (multi-systemic), particularly in lymphoid organs (Harding and Clark, 1997). The disease is associated with a porcine circovirus (PVC) because its DNA is in the form of a ring (Clark, 1997). There are two serotypes, type 1 which does not cause disease and type 2 which causes disease which can be found in lesions and can be isolated in pure cultures. Post weaning multi-systemic syndrome tends to be a slow and progressive disease. Symptoms of the disease are sudden death, enlarged peripheral lymph nodes, diarrhoea, respiratory distress caused by interstitial pneumonia and incoordination. From an age of 6 - 8 weeks, weaned pigs lose weight and gradually become emaciated (Harding and Clark, 1997). Contributing factors are infected faeces, mechanical means via clothing, equipment, trucks, birds and rodents and high stocking densities (Segales and Domingo, 2002). Since the disease is caused by a virus it is difficult to treat. Prevention methods are improving hygiene and reducing stocking densities.

2.3.2 Post weaning respiratory disease complex

Post weaning disease complex occurs in weaners and common symptoms include increased mortality, coughing, reduced growth rates and difficulties in breathing. The disease results from a number of different respiratory diseases. Individual diseases involved include porcine reproductive respiratory syndrome (PRRS), porcine respiratory coronavirus (PRVS) and swine influenza (SI).

Porcine reproductive respiratory syndrome causes milk pneumonia. The disease is transferred horizontally through carriers and vertically to the foetus during gestation. Clinical signs are not shown upon infection,

and the disease is carried in syringe needles used for injections during the weaning period (Kyriazakis and Whittemore, 2006). The PRRS is followed by PRVS infection which attacks surviving macrophages, and reduces their ability to protect against bacteria, thereby causing mild pneumonia. Finally, at 3-4 months of age, influenza virus attacks the lining of the bronchi and trachea and reduces the ability of the respiratory tract to clear infection and produces mild pneumonia (Van Reeth, 1997).

Respiratory diseases may be treated through administering antibiotics through injections, in water medication and in feed medication. Treatment may also be through improvement of ventilation, reducing stocking density and reducing stress. Prevention may be through administering vaccines and antibiotics.

2.3.3 Swine dysentery

Swine dysentery is an acute mucohaemorrhagic disease in pigs (Jacobsen *et al.*, 2004) that is caused by stress. The symptoms of swine dysentery are diarrhoea, diarrhoea with blood, reduced growth rates and death. Treatment is through administration of antibiotics through injection, in water medication and in feed medication. Control of the disease may be through improving hygiene, reducing stocking density, controlling rodents and avoid the purchasing of infected pigs.

2.3.4 Enteric colibacillosis

Escherichia coli post weaning diarrhoea is also known as post weaning enteric colibacillosis. It is an organism with many serotypes which causes, septicaemia in neonatal pigs, diarrhoea in suckling piglets and in newly weaned piglets and oedema in growing pigs (Kyriazakis and Whittemore, 2006). Enteric colibacillosis result in decreased bodyweights (Nagy and Fekete, 1999; Fairbrother *et al.*, 2005; Lee *et al.*, 2008). The enteric strain of colibacillosis attacks the brush borders and reduces absorption, thereby causing diarrhoea and toxicity. Apart from diarrhoea other symptoms of the infection are blood and/or

black tarry faeces, dirty wet pens, rapid weight loss or occasional vomiting. At weaning the loss of a sow's milk and antibodies (secretory IgA) allows the *E. coli* to attach to the villi of the small intestines. The toxins produced cause acute diarrhoea, usually within five days of weaning (Kyriazakis and Whittemore, 2006). Animals suffering from colibacillosis experience loss of appetite, loss of weight and may die from dehydration. Colibacillosis can be treated through antibiotics by injection, oral administration or in water medication. Vaccinations can be administered against various serotypes. Gilts can be vaccinated twice before first parturition and pass one the built immunity to the litter (Kyriazakis and Whittemore, 2006). Control may be through improving hygiene, provide warmth to weaners and reduce stress at weaning.

2.4 Prevalence and pathogenesis of *E. coli* in weaner pigs

Pathogenic *Escherichia coli* strains are major causes of different intestinal illnesses. The *E. coli* species is a Gram negative bacterium which belongs to the *Enterobacteriaceae* family (Cassels and Wolf, 1995, Bardiau *et al.,* 2010). Post weaning diarrhoea is one form of an *E. coli* infection which causes deaths in weaned pigs. Consequently colibacillosis *causes* significant losses to the pig industry (Vu-Khac *et al.,* 2004; Zhang *et al.,* 2007). The most prevalent factors linked to ETEC in cases which result in diarrhoea are fimbiral adhesins, non-fimbrial adhesins and enterotoxins. The ETEC bacteria have fimbriae or pili which facilitate in attachment to specific receptors on the small intestines (Fairbrother *et al.,* 2005). These bacteria reproduce quickly to reach numbers as high as 10^9 colony forming units (CFU) per gram of tissue (Nagy & Fekete, 2005).

Apart from ETEC there are other pathogenic gastrointestinal strains of *E. coli* which are categorised according to their virulence properties: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive E. coli (EIEC), enteroaggregative *E. coli* (EAEC), "diffusely adherent" *E. coli* (DAEC) and necrotoxinogenic *E. coli* (NTEC) (Nataro and Kaper, 1998). This categorisation is

established on virulence factors of the bacteria like elaboration of toxins and colonising factors and/or specific interactions with intestinal epithelial cells (Cassels and Wolf, 1995). ETEC is the most studied of the categories of *E. coli* that cause diarrhoea.

Two processes should occur for pathogenesis of *E. coli* to take place: the pathogenic bacteria must attach to the cell surface and the attachment involves an interaction between an adhesion molecule and receptor molecule (Finlay and Falkow, 1989). The adhesion molecule is found on the microbe and the receptor molecule is found on the host's surface (Cassel and Wolf, 1995). The bacterium enters the host via oral route and colonise the distal part of the intestinal mucosa through fimbriae and other adhesins attaching to receptors on the small intestinal epithelium or in the mucus coating the epithelium (Fairbrother *et al.,* 2005). The bacterium release enterotoxins which have adenylate cyclase enzyme and cyclic AMP, which inhibits sodium, chlorine and water absorption by the villus cells, resulting in eatery diarrhoea (Fairbrother *et al.,* 2005). The extent of bacterial colonization of the small intestine influences whether or not disease results from infection.

Diarrhoea is responsible for 12-30 % of piglet deaths in China. *Escherichia coli* infection has resulted in 56 % cases of diarrhoea and 25 % mortality due to *E. coli* infections in China (Li *et al.,* 2007). Currently in SA there is limited information on *E. coli* diarrhoea and oedema in pigs. A total of 674 cases of *E. coli* infection linked with colibacillosis were reported from Onderstepoort Veterinary Institute from 1974-1991. From these cases that were reported 46 % displayed symptoms of inflammation of the small intestine, 50 % showed septicaemia and 12 pigs showed signs of oedema (Henton and Engelbrecht, 1997). In some areas in Central Vietnam, namely Huon Toan, Huon Van and Thuy Duong, incidences of diarrhoea were found to be higher in the rainy season (38, 65 and 32 % respectively) than in the dry season (30, 21 and 24 % respectively) (Hong *et al.,* 2006). In Southern Germany faecal samples from pigs infected with diarrhoea were collected from 24 farms. Out of the farms visited, 14 farms were recorded

positive for ETEC. A total of 278 piglets were examined from these 14 farms and 18 % were positive for ETEC (Weiler *et al.*, 2000).

2.5 Virulence factors and prevalence of ETEC in pigs

Adhesion is when different particles or surfaces stick to each other. Adhesins express different characteristics, but most frequently manifest as morphological structures called fimbriae (pili) and fibrillae. Fimbriae are non-flagellar filamentous appendages composed of repeating protein subunits (Cassels and Wolf, 1995). This appendage ranges between 4-10 nm in diameter and are found in most Gram-negative and some Gram-positive bacteria. Fimbriae are used by bacteria to adhere to animal host cells.

The common fimbrial adhesins found in ETEC from weaned pigs are F4 formerly known as K88 and F18 (Nagy and Fekete, 1999; Debroy and Maddox, 2001). The F18-induced diarrhoea is found in isolates from just weaned piglets, whereas F4 is found in both neonatal and just weaned piglets. The F-antigens (fimbrial) were previously described as K-antigens (kapsular), hence why F4 was formerly known as K88 in older literature. The F5 (K99), F6 (987P) and F41 ETEC strains are more common in ETEC causing neonatal diarrhoea (Wilson and Francis, 1986; Nagy and Fekete, 1999; Debroy and Maddox, 2001). ETEC colonising factors produce enterotoxins, heat stable (Sta or Stb) and/ or heat labile (LT), after attaching to the intestines (Nagy and Fekete, 1999; Vu Khac *et al.*, 2004). These toxins secrete water and electrolytes and/ or decrease fluid absorption in the intestinal lumen of the host, which then leads to diarrhoea. The ability of adhesion of ETEC to intestinal epithelial cells is primarily through the production of thin (3-7 nm) proteinaceous surface appendages (fimbriae or pili), which can differs morphologically, biologically and antigenically on several strains. Some of them are morphologically similar to the common fimbriae (’Type 1’ fimbriae or pili) of *E. coli.* (Duguid *et al.*,1955).

Potential virulence factors associated with ETEC infection have been identified. These are porcine attaching and effacing- associated factor (PAA) (Batisson *et al.,* 2003), new pili factor (type IV) (Pitchel *et al.,* 2002), enteroaggregative heat-stable factor (EAST-1) (Savarino *et al.,* 1993) and adhesin, involved in diffuse adherence (AIDA-1) (Benz and Schmidt, 1989). *Escherichia coli* which results in post weaning diarrhoea and oedema disease is restricted to O sero-groups, namely, O8, O45, O138, O139, O141, O147, O149, and O157 (Nagy and Fekete, 1999; Frydendahl *et al.,* 2002). The O antigen which has repetitions of an oligosaccharide unit (O unit) is part of the lipopolysaccharide in the outer membrane of Gram-negative bacteria and provides major antigenic properties to the cell surface (Han *et al.,* 2007). A total of 186 O-antigens forms are known for *E. coli* (Feng *et al.,* 2004).

2.5.1. Fimbrial adhesins

The common fimbrial adhesins are the F4, F18, F5, F6 and F41 strains.

2.5.1.1 F4

F4 fimbriae is encoded by single copies of the minor subunit *FaeC* at the tip of the fimbrium and around 100 copies of the major subunit *FaeG* forming the fimbrium (Joller *et al.,* 2009). Outbreaks of ETEC infection are usually associated with F4 fimbriae Zhang *et al.* (2007) reported that approximately 65 % of fimbriated *E. coli* isolates carry the F4 fimbria gene in USA. F4 fimbria is responsible for diarrhoea in nursing and weaned pigs (Fairbrother *et al.,* 2005; Duan *et al.,* 2011) and occurs as variants F4*ab*, F4*ac* and F4*ad* with, F4*ac* been the most common nowadays. The "a" stands for a common epitope and the "b", "c" and "d" represent specific epitopes. Previously F4*ab* was reported in many incidences and F4*ad* was reported in China (Guinee and Jansen, 1979) but F4*ac* is now dominant worldwide. Choi and Chae (1999) studied 44 F4-positive *E. coli* isolates from pigs with diarrhoea for presence of F4*ab*, F4*ac* and F4*ad*. From isolates examined, 96 % had F4*ac* fimbiral genes and 4 % had the F4*ab* gene. Likewise

Alexa *et al.* (2001) examined 237 F4-positive *E. coli* isolates from PWD and found F4*ac* present in 98 % of the isolates, F4*ab* present in 0.8 % and F4*ad* present in 1 %. Lee *et al*. (2008) examined 188 *E. coli* isolates, and of those studied, 7 % carried the F4 fimbiral gene. In Slovak Republic *E. coli* isolates were taken from 92 piglets that died from diarrhoea, 24 % carried a gene for F4 (Vhu-Khac *et al.,* 2004). Zhang *et al.* (2007) reported that out of 304 *E. coli* isolates submitted, 67 % were positive for F4. The three types of F4 variants not only represent antigenic variants, but also show differences in binding properties. Some pigs are susceptible to all types, some are susceptible to two types (*ad* and *ac* or *ad* and *ab*), while others are susceptible to one type (*ac* or *ab*) and some are resistant to all three types (Francis *et al.,* 1998). The genetic determinant for the biosynthesis of F4 is located on large and unconjugative plasmids (Van Den Broeck *et al.,* 2000). The F4 fimbriae comprises of numerous copies of the *faeG* major subunit, and one copy of the *faeC* minor subunit (Verdonck *et al.*, 2004).

The interaction of the F4 fimbriae with F4-specific receptors, allows for $F4^+$ ETEC to attach to the epithelial cells and colonise the small intestine of a pig (Van den Broeck et al., 2000). There are three receptors specific for $F4^+$ ETEC, the high molecular-weight intestinal mucin-type sialoglycoproteins (IMTGPs) Billey *et al.,* 1998), an enterocyte membrane-associated transferring (Grange & Mouricat, 1996) and an intestinal neutral glycosphingolipid (IGLad) receptor (Grange *et al.,* 1999). The host receptors have different structures and the F4 recognise these structures differently. This may account for the binding differences amongst the F4 variants (Duan *et al.*, 2011). The gene encoding for F4 receptor has not yet been identified. Moreover, since there are different F4 variants (F4*ab*, F4*ac* and F4*ad*), it is possible that there are different receptors responsible for F4 fimbriae. Identification of the mutations would assist in selecting against ETEC-F4 susceptible animals, and thereby reducing the incidences of diarrhoea outbreaks.

2.5.1.2 F18

F18 fimbria is usually responsible for PWD or oedema disease (ED) (Fairbrother *et al.,* 2005; Duan *et al.,* 2011). These fimbriae are long flexible appendages which show a distinct zigzag pattern (Nagy *et al.,* 1997). F18 has two types, namely, F18*ab* and F18*ac*. Most of the strains expressing F18*ac* are ETEC and belong to serogroups O141 and O157 which result in diarrhoea by producing enterotoxins, whereas, the F18*ab* variant is usually expressed in strains producing Shiga toxins and cause ED (Nagy *et al.,* 1997). F18 fimbriae are encoded by the *fed* gene and are made up of five genes. The *fedA* gene consists of the major subunit and the *fedE* and *fedF* encode the minor subunits (Duan *et al.,* 2011). F18-positive ETEC mostly produces heat-stable enterotoxins, *STa* and *STb,* and less frequently produces heat-labile enterotoxin (LT) (Francis, 2002). Frydendahl (2002) reported that 27 % of 173 samples of ETEC from PWD in Demark were positive for PCR for the *fedA* gene, while 89 % of the isolates from ED were F18 positive. In North Carolina, F18-positive for ETEC comprised of 53 % of the 175 *E. coli* samples isolated in PWD (Post *et al.,* 2000). Osek *et al.* (1999) reported on 62 % of strains from diarrhoea, been positive for F18 and 84 % of strains from ED been positive for F18 in Poland. In Canada, Amezcua *et al.* (2002) reported that only 7 % of 28 farms investigated were positive for F18 ETEC from PWD. Lee *et al*. (2008) examined 188 *E. coli* isolates, and of those studied, 15 % carried the F18 fimbiral gene. In Slovak Republic *E. coli* isolates were taken from 92 piglets that died from diarrhoea, 11 % carried a gene for F18 (Vhu-Khac *et al.,* 2004). Zhang *et al.* (2007) reported that out of 304 *E. coli* isolates submitted, 34 % were positive for F18.

Alfa (1, 2) fucosyltransferase 1 (*FUT1*) gene located on SSC6q11 has been identified as the receptor for the F18 (Schroyen *et al.,* 2012). The principle mutation for the discrepancy in susceptibility has been recognised as *FUT1* c.308A>G. The *FUT1* gene is responsible for synthesis of glycotransferase which is the enzyme that facilitates synthesis of carbohydrate structure which mediate the adhesion and the colonisation of bacterial adhesion. The polymorphism in the *FUT1* gene occurring due to a mis-sense mutation occurring on nucleotide 307 (M307) results in the substitution of adenine (A) for guanine (G) resulting in G/A causing the pig to be susceptible (Meijerink *et al.,* 1997). The G allele is dominant over

the A therefore making the *FUT1*$^{GG/AG}$ individuals susceptible and the *FUT1*^{AA} resistant (Frydendahl *et al.*, 2003).

2.5.1.3 F5 and F41

The K99 fimbriae aid in the attachment of ETEC to mucosal cells of calves, lambs and piglets. It is made up of eight genes, *fanA – fanH* with the major subunit been *fanC* (Duan *et al.,* 2011). The K99 fimbriae expression is affected by different environmental factors like the degree of aeration provided and the growth rate of cells (Duan *et al.,* 2011). Lee *et al*. (2008) examined 188 *E. coli* isolates, and of those studied, 30 % carried the K99 fimbiral gene. In Slovak Republic *E. coli* isolates were taken from 92 piglets that died from diarrhoea, 1 % carried a gene for K99 + F41 (Vhu-Khac *et al.,* 2004). Zhang *et al.* (2007) reported that out of 304 *E. coli* isolates submitted, 0.6 % were positive for K99. The receptor essential for K99 to bind to the host enterocytes is glycoprotein receptor N-glycolylneuraminic acid-GM3 (NeuGc-GM3). This ganglioside receptor which is located on the surface of bovine enterocytes is also found on equine red blood cells (Teneberg *et al.,* 1990).

F41 and K99 fimbriae are normally found on the same strains with serogroups O8 or O9 (Duan *et al.,* 2011). Similar to K99, F41 fimbriae expression is also influenced by environmental factors. Lee *et al*. (2008) examined 188 *E. coli* isolates, and of those studied, 4 % carried the F41 fimbiral gene. Zhang *et al.* (2007) reported that out of 304 *E. coli* isolates submitted, 0.57% were positive for F41. Glycoproteins from human erythrocytes and glycophorin have been found to act as an erythrocyte receptor for F41 fimbriae (Lindahl and Wadstrom 1986).

2.5.1.4 F6

987P is usually associated with *E. coli* causing neonatal diarrhoea (Wilson and Francis, 1986). Issacson and Richter (1981) described the 987P fimbriae as a long straight structure surrounding the bacterial cell. The 987P gene cluster comprises of eight genes (*fasA – fasH*), composed of one major subunit *fasA* and two minor subunits, *fasF* and *fasG* (Khan and Schifferli, 1994). These genes are adjacent to a *Tn1681*-like transpoon containing genes encoding the heat-stable enterotoxin STIa (Johnson and Nolan, 2009). The *fasG* minor subunit is responsible for the attachment of the bacteria to the porcine intestines. The *fasB* and *fasD* subunits are involved in exporting and assembling the structural components of the fimbriae (Duan *et al.,* 2011). 987P was found to be present in 10 % of weaned pigs in Korea (Fairbrother *et al*., 2005). Lee *et al*. (2008) examined 188 *E. coli* isolates, and of those studied, 6 % carried the 987P fimbiral gene. In Slovak Republic *E. coli* isolates were taken from 92 piglets that died from diarrhoea, 3 % carried a gene for 987P (Vhu-Khac *et al.,* 2004).

Zhu *et al.* (2005) identified histone H1 proteins as receptors for 987P. These receptors bind to the membrane and to 987P, thereby stabilising sulfatide-fimbriae interaction (Duan *et al.,* 2011). The 987P receptor expression increases with age which leads to the shedding of free receptors into the intestinal lumen to cover the fimbriae, thereby preventing adhesion (Duan *et al.,* 2011).

2.5.2 Non-fimbrial adhesins

The common non-fimbrial adhesins are AIDA-1 and PAA

2.5.2.1 Adhesin, involved in diffuse adherence (AIDA-1)

The AIDA-1 gene has the capacity to autoaggregate and form biofilms apart from its adhesive properties. Therefore it is classified under the Self-Associating Auto Transporter family (Duan *et al.,* 2011). AIDA-1 is encoded by *AidA (orfA)* and *aah (orfb)* genes. The precursor of AIDA-1 is coded by *AidA* and requires

the adjacent (autotransporter adhesion heptosyl-transferase) genes whose product add heptoses to the AIDA protein (Fairbrother *et al.*, 2005; Duan *et al.*, 2011). AIDA-1 has been associated with other virulence factors such as F18. The gene which encodes AIDA-1 is repeatedly found on the same plasmid as the *fed* gene cluster for F18 (Mainil *et al.*, 2002), indicating that AIDA-1 and F18 are somehow related. Niewerth *et al.* (2001) reported an association of AIDA-1 and F18 in *E. coli* isolated from pigs with ED and PWD. Zhang *et al.* (2007) examined 304 *E. coli* isolates and found 27 % positive for AIDA-1 gene. Lee *et al.* (2008) also examined 188 *E. coli* isolates and out of those 3 % were positive for AIDA-1.

2.5.2.2 Porcine attaching and effacing- associated factor (PAA)

The PAA gene has been reported that it is vital for the development of attaching and effacing (AE) lesion by human EPEC strains (Batisson *et al.*, 2003). The significance of PAA in ETEC associated diarrhoea is limited. Zhang *et al.* (2007) examined 304 *E. coli* isolated and found 60 % positive for PAA gene. Lee *et al.* (2008) also examined 188 *E. coli* isolates and out of those 7 % were positive for PAA.

2.5.3 Enterotoxins

Enterotoxins are extracellular proteins or peptides (exotoxins) which are able to avert their actions on the intestinal epithelium (Nagy and Fekete, 1999). ETEC is characterised by production of one or two of either large molecular weight (88 kDa) heat labile enterotoxin (LT) or small molecular weight (11-18 amino acid containing) heat stable peptide toxins (ST) (Nagy and Fekete, 1999). Heat labile enterotoxins are mainly produced by human and porcine ETEC, whilst ST enterotoxins are produced by bovine, porcine and human ETEC. The LT and ST enterotoxins do not produce pathological lesions or morphological alterations on the mucosa, but instead synthesise functional changes e.g., an increase in the secretion of water, sodium, chlorine and a reduction in liquid absorption (Nagy and Fekete, 1999). Consequently this leads to dehydration and acidosis.

2.5.3.1 Heat-labile enterotoxin (LT)

The heat-labile enterotoxin comprises of a catalytic A subunit (LTA) and a pentamer of receptor-binding B subunits (LTB) (Duan *et al.,* 2011). There are two types of LT enterotoxins, namely, LT-I and LT-II. LT-I is responsible for diarrhoea in both humans (LTh-I) and porcine (LTp-I) and LT-II (LTIIa and LTIIb) is associated with diarrhoea in animals only. Synthesis of the A and B subunits of LT takes place in the cytoplasm, and then they are transported through the inner membrane and finally assembled into holotoxin in the periplasm (Fairbrother *et al.,* 2005). The LT toxin is highly related to the cholera toxin and both of these toxins bind GMI ganglioside, however, LT enterotoxin can also bind other receptors. The LT receptor binds to receptors on the surface of intestinal epithelial cells. Apart from enterotoxicity LT has other functions. For instance, Horstman *et al.* (2004) suggested that LT enterotoxin can act as an adhesin that binds bacteria to GMI ganglioside on the epithelial cell surface. Berberov *et al.* (2004) reported that the eradication of the LT enterotoxin not only reduces incidences of diarrhoea, but also reduces chances of colonisation of the enterotoxin to the epithelial cells of gnotobiotic pigs. Heat-labile enterotoxins have better antigenicity when compared to ST enterotoxins (Nagy and Fekete, 1999).

2.5.3.2 Heat-stable enterotoxin (ST)

The ST enterotoxin is small and monomeric (Duan *et al.,* 2011). It is classified into two types, namely STa and STb. There are two STa variants: STaH which is found in human ETEC and STaP found in both humans and porcine. The STa is a low molecular weight peptide which comprises of 18 or 19 amino acids and 3 disulphide bonds (Fairbrother *et al.,* 2005). The STa gene for ETEC is *estA* (Duan *et al.,* 2011). It is water and methanol soluble, resists boiling for 15mins, its digestion is by proteolytic enzymes and it is inactivated by agents that destroy disulphide bonds (Fairbrother *et al.,* 2005). Enteroaggregative heat-stable factor (EAST-1) is an example of an enterotoxin which belongs to the STa enterotoxin family and is associated with ETEC diarrheal infection in humans and animals (Choi *et al.,* 2001). The EAST-1 gene

shares approximately 50 % protein identity with the STa toxin, but the effect of EAST-1 on induction of electrolyte loss from the intestine is limited (Duan *et al.,* 2011). The STa enterotoxin is responsible for diarrhoea in neonatal animals but is rarely found as a sole enterotoxin. In South Korea, 188 *E. coli* isolates from pigs with diarrhoea were examined and 10 % contained STa enterotoxin (Lee *et al.,* 2008). Frydendahl (2002) reported that out of 219 isolates, 27 % were positive for STa toxin gene. Zhang *et al.* (2007) also reported that out of 304 *E. coli* isolates, 27 % were positive for Sta. The main receptor for Sta is guanylate cyclase C (GC-C) which includes the atrial natriuretic peptide receptors GC-A and GC-B (Vaandrager, 2002). The GC-C receptor is found in the apical membrane of the intestinal epithelial cells and binding of ligands to the extracellular domain stimulates the intracellular enzymatic activity (Natara and Kaper, 1998). The GC-C receptor is used by Sta to cause diarrhoea.

The STb variant has not only been found in porcine *E. coli* isolates but in humans and other animals as well. The Stb gene (*estB*) (Duan *et al.,* 2011), is usually observed in isolates from PWD and is associated with AIDA-1 (Ngeleka *et al.,* 2003). The Stb enterotoxin is heat stable but susceptible to degradation by proteolytic enzymes (Fairbrother *et al.,* 2005). Although Stb is linked to pig diarrhoea, it has not been easy to experimentally reproduce a diarrheal disease which carries the Stb toxin alone (Duan *et al.,* 2011). The mechanism by which STb causes fluid build-up in the intestine is not known. Dubreuil (1997) reported that an increase in the level of prostaglandin E2 in the intestines results in production of fluid. The STb enterotoxin is commonly found amongst some virulent factors that cause ETEC like F4 and F18 (Francis, 2002). In earlier studies (Moon *et al.,* 1999; Post *et al.,* 2000), the gene for Stb (*estB*) was found to be the only enterotoxin which leads to ETEC to be isolated from PWD. More recently, Francis (2002) reported that no isolates from ETEC PWD comprised of STb alone, but that there were other genes present. This shows that there have been changes over time. Frydendahl (2002) examined of 219 isolates, 78 % were positive for the STb toxin gene. Zhang *et al.* (2007) reported that out of 304 *E. coli* isolates, 76 % were positive for Stb. In South Korea, 188 *E. coli* isolates from pigs with diarrhoea were examined and 21 % contained Stb enterotoxin (Lee *et al.,* 2008). The receptor for Stb is not known, it has been

presumed that STb may bind non-specifically to the plasma membrane prior to endocytosis (Chao *et al.*, 1997).

2.5.3.3 Enteroaggregative heat-stable factor (EAST-1)

Enteroaggregative heat-stable factor (EAST-1) is a low molecular weight enterotoxin, belonging to the STa family and has four cysteine residues (Fairbrother *et al.*, 2005). Initially, EAST-1 was found in human *E. coli*. The gene that encodes EAST1 (*estA*) is now also found in porcine ETEC (Frydendahl, 2002). The EAST1 enterotoxin is found in F4-positive ETEC and also F18-positive ETEC. Frydendahl (2002) reported that out of 219 isolates, 65.6 % were positive for the EAST-1 toxin gene. In the United States, Zhang *et al.* (2007) examined 304 *E. coli* isolates and 35 % contained EAST-1. In South Korea, 188 *E. coli* isolates from pigs with diarrhoea were examined and 42 % contained EAST-1 enterotoxin (Lee *et al.,* 2008). The significance of EAST-1 in the pathogenesis of ETEC is limited. The receptor gene for EAST-1 is the same as for the Sta enterotoxin, which is guanylate cyclase C (GC-C) (Vaandrager, 2002).

2.6 Positional candidate genes for *E. coli* susceptibility and resistance

The selection for lack of receptor molecules or for genetic variants of receptors molecules where a pathogen is unable to bind represents an attractive route to genetic resistance in animal science (Rampoldi *et al.,* 2011). The adhesion of ETEC fimbriae results in extremely high interaction between the fimbriae produced by bacteria and the receptors that are found on the brush borders of enterocytes (Bertshcinger and Fairbrother, 1999). Out of the five specific types of porcine fimbriae, F4 and F18 are most prevalent. The two variants for F18 (F18ab and F18ac) have the same receptors (Rippinger *et al.*, 1995) and those for F4 (F4ab, F4ac and F4ad) have different receptors (Bijlsma *et al.,* 1982). The mutation for F18 is known (Meijerink, 1997) which has given rise to studies on the elimination of *E. coli* F18 susceptibility

allele from the pig population in Switzerland and other countries, so as to increase the resistance of piglets in commercial populations (Luther *et al.*, 2009).

The ETEC F4 resistance and susceptibility is inherited by a monogenetic trait and that the susceptible allele is dominant over the resistant allele (Gibbons *et al.*, 1977). To date attempts that have been made to identify breeding stock that inherit resistance, have not been entirely positive. The ETEC F4*ab*/F4*ac* receptor gene (*F4bcR*) is mapped to chromosome 13 (SSC13) (Edfors-Lilja *et al.*, 1995; Python *et al.*, 2002). This region contains several positional candidate genes, such as Mucin-type (*MUC*) 4, *MUC13*, *MUC20* and Transferrin receptor (*TRFC*). These are positional candidates because of their apical location in the small intestine (Zhang et al., 2008). Later, Joller *et al.* (2009) mapped the ETEC F4ad/F4ac susceptibility locus to the interval *SW207-S0075* refined in a 5.7cM region around the *F4bcR* locus. Mucins are glycoproteins that cover the apical surfaces of epithelial cells in gastrointestinal and respiratory tracts, forming the first line of host defence against enteric pathogens (Zhang *et al.*, 2008). These proteins intercede with the interactions between epithelial cells and their milieu by changing cell adhesion, lubricating and protecting of mucosa, renewing and differentiation of epithelia and cell signal transduction (Ringel and Lohr, 2003). These functions position mucins in disease procedures like infectious and inflammatory diseases and cancer.

2.6.1 Mucin-type 4 (MUC4)

Mucin 4 (*MUC*4) is a large membrane-bound O-glycoprotein that is found on the surface of gastrointestinal epithelial cells. It plays a vital role in the lubrication and protection of mucosa (Moniaux *et al.*, 1999). It is restricted on human chromosome 3p29, which is orthologous to the region containing SW207 and SW083 including the F4ab/ac receptor locus (Jorgensen *et al.*, 2010). Jorgensen *et al.* (2010) reported a relationship between resistance and a mutation in intron 7 of the mucin 4 (*MUC4* g.8227 G> C) candidate gene. Nonetheless, information presented by Rasschaert *et al.* (2007) and Joller (2009) raised

reservations as to whether this mutation is not in complete linkage disequilibrium with the F4*ac* receptor locus. These doubts were answered by Rampoldi *et al*. (2011) who reported that six SNPs *ALGA0072075, ALGA0106330, MUC13-226, MUC13-813, DIA0000584* and *MARC0006918* were in complete linkage disequilibrium. Based on these findings Rampoldi *et al.* (2011) suggested that the locus for F4*acR* is located between *LMLN* locus and microsatellite S0283. Jorgensen *et al.* (2010) identified an *Xbal* polymorphism in intron 7 of porcine *MUC*4 in two unrelated populations. This polymorphism is in complete linkage disequilibrium with susceptibility to ETEC F4ab/ac and is used in in Danish pig breeding selection programmes (Jacobsen *et al.*, 2009).

2.6.2 Mucin-type 13 (MUC13)

Mucin 13 (*MUC*13) is a trans-memebrane mucin that is highly expressed in the gastrointestinal tract of humans (Williams *et al.*, 2001). The expression of *MUC*13 has been seen in gastric cancer (Shimamura *et al.*, 2005) and inflammatory bowel disease (Moehle *et al.*, 2006). Mucin 13 has been mapped to SSC13q41 where the receptor of ETEC Fab/ac is found. Therefore *MUC*13 is a positional candidate gene for ETEC F4ab/ac. Zhang *et al.* (2008) established the complete 2679-bp cDNA of pig *MUC*13, and found that it is highly expressed in the jejunum and moderately expressed in the trachea, stomach and liver. Their results showed that MUC13 is in strong linkage disequilibrium with the receptor for ETEC F4ab/ac, which therefore provides potential markers for selection of ETEC F4ab/ac resistant animals in pig breeding schemes.

2.6.3 Mucin-type 20 (MUC20)

Mucin 20 is a membrane-bound mucin localised on the plasma membrane and its mRNA expression is up-regulated in injured kidneys (Higuchi *et al.*, 2004). Mucin 20 expresses a shorter isoform which is localised in the plasma membrane. The longer isoform may be secreted. RT-PCR analysis performed by

Ji *et al.* (2011) showed that *MUC*20 mRNA was expressed most highly in the kidney, prostate, epididymis and bladder. Mucin 20 has been previously assigned to SSC13q41 where the ETEC F4ab/ac receptor is located, and is therefore considered a candidate gene for ETEC F4ab/ac receptor (Ren *et al.,* 2009). Nonetheless, *MUC*20 genomic structure and association with susceptibility to ETEC F4ab/ac is limited in pigs.

2.6.4 Transferrin Receptor (TFRC)

The locus controlling ETEC F4ab/ac susceptibility (SSC13q41) harbours positional candidate genes coding for some transferrins or mucin-like sialoglycoproteins (Grange *et al.,* 1999). Transferrin receptor (TFRC) was assigned to SSC13q41 by radiation hybrid mapping (Van Poucke *et al.,* 2001); hence it was considered as a positional candidate gene for ETEC f4ab/ac receptor (Python *et al.,* 2004). TFRC is responsible for transporting iron from the transferrin protein into the cell. The bacterium which carries *E. coli* relies on iron to survive and reproduce (Jacobsen *et al.,* 2011).

2.7 Control of post weaning diarrhoea caused by *Escherichia coli*

Diarrhoea caused by ETEC can be treated by oral administration, fluids and electrolytes and by parental antibiotics. In cases where the outbreak is severe, antibiotics can be administered soon after weaning. The treatment is usually given for 3-5 days and can be administered twice a day. Oral electrolyte solutions which have potassium, dextrose and sodium chloride are effective in treatment of diarrhoea in neonatal and weaned pigs (Nagy and Fekete, 1999). However, if feed is a channel of drug administration in weaned pigs, it is important to consider that weaned pigs have a reduced feed intake, and instead an individual administration is more appropriate. Resistance to antibiotic treatment of ETCE isolates which cause PWD is common, which is why it is crucial to modify preventive and treatment methods. The *E. coli* isolates show resistance to different antimicrobials including apramycin, trimethoprim-sulfonamide,

spectinomycin and neomycin (Fairbrother *et al.,* 2000), mainly due to the prophylactic use of antibiotics. It is interesting to note that in Scandinavia and Europe, the withdrawal of prophylactic antibiotics lead to an increase in diarrhoea, weight loss and mortality due to *E. coli* in post-weaning pigs (Casewell *et al.,* 2003). Changes in feeding regimes like the increase in protein levels at the beginning can lead to increased outbreaks of colibacillosis 3 weeks after weaning due to shock from the new feed (Fairbrother *et al.,* 2005). This goes to show that diet is an important factor that can either influence or prevents colibacillosis.

2.7.1 Prevention Strategies

The most important features to note in PWD *E. coli* prevention is correct management techniques like 'all in all out', clean and dry stys, removal of diarrheal faeces, reducing mastitis, metritis and agalactia syndrome (MMA in sows).

Supplementation of a diet that is rich in energy and milk products has been shown to reduce mortality and increase feed intake and therefore reduce the onset of clinical signs of ETEC (Tzipori *et al.,* 1980). Other products such as dried plasma added to feeds, aid in reducing diarrhoea (Van Beers-Schreurs *et al.,* 1992). This is in contrast to Dreau *et al.* (1994) who reported that the presence of soya beans in the feed increased PWD, possibly due to the presence of trypsin inhibitors or antigens that induce a localised immune response. Li et al. (1991) supported this and reported that soya beans could result to decreased villus height, deepening of crypts, and an increase in anti-soya immunoglobulin in the serum leading to production of *E. coli*. The presence of organic acidifiers in the feed can promote a higher mean daily weight gain, feed conversion, and decreased incidence of PWD (Giesting and Easter, 1985). Holm and Poulsen (1996) reported that the addition of zinc oxide at levels above 2400 ppm in the feed decreases the severity of PWD although zinc sulphate and organic zinc are potentially toxic.

Parental vaccination with purified F4 fimbriae may prevent ETEC infections in suckling piglets, due to the protective immunoglobulin A (IgA) antibodies which can be transmitted via colostrum and milk to suckling piglets. This method is not efficient in preventing post weaning diarrhoea that is not protected by passive lactogenic immunity, because it stimulates a systemic rather than F4-specific immune response (Duan *et al.,* 2011). Oral immunization of $F4R^+$ piglets with purified F4 fimbriae induces a F4 specific intestinal immune response that protects them against a subsequent ETEC challenge (Verdonck *et al.,* 2008). In contrast oral immunization with F4 fimbriae purified from $F4^+$ ETEC mutants, in which the specific polymeric stability of the fimbriae is disrupted, results in reduced mucosal immune responses (Vendonck *et al.*, 2007), due to the polymeric stability of F4 fimbriae being very important for its biologic activity (Devriendt *et al.,* 2010).

Other vaccination strategies are the oral immunisation of pigs with live wild-type virulent *E. coli* strain carrying the fimbrial adhesions (Fairbrother *et al.,* 2005). These can be administered in drinking water to weaned pigs and orally to pre-weaned pigs a week before diarrhoea is expected to occur. This method encourages intestinal colonization by bacteria which induces production of intestinal antibodies which will block bacterial adhesion and therefore prevent diarrhoea (Fairbrother *et al.,* 2005). This system confirms to be successful in control of F4 and F18 *E. coli* associated PWD.

2.8 Breeding of resistant pigs

The existence of *E. coli* strain resistant loci in pigs through breeding is a potential way of preventing PWD. The practicality of breeding for disease resistance can be illustrated by the *E. coli* F18-associated PWD. Frydendahl *et al.* (2003) showed that a PCR-RFLP test that detected FUT1 M307 polymorphism which is linked to the gene controlling expression of the *E. coli* F18 receptor, could be a useful tool for selection of resistant animals in large scale. The use of a PCR-RFLP test in Switzerland increased the proportion of resistant Large White pigs and reduced the percentage of susceptible pigs from 44-18%

(Vogeli *et al.,* 2002). Some genotyping methods for the detection of pigs resistant to F4-positive ETEC have been implemented based on genetic polymorphisms in the porcine gene for *MUC*4 in the region of chromosome 13, which carries receptors for F4*ab/ac* (Jorgensen *et al.,* 2004). Selection for F4 resistance has challenges since resistant sows (F4 receptor negative sows) do not transfer antibodies in their colostrum to piglets (Fairbrother *et al.,* 2005). Therefore heterozygous piglets do not acquire passive immunity to neonatal diarrhoea caused by ETEC strains.

Other methods for preventing *E. coli* infection which have been implemented, such as supplementation of diets with different nutrient levels, use of antibiotics, use of probiotics, require large capital injections which may not be affordable to most. Therefore, breeding of resistant pigs is not only a more affordable preventative method, but it is also a more effective way to control and prevent *E. coli* infection in pigs.

2.9 Summary

Escherichia coli infection in pig production is threatening the pig industry. Information regarding *E. coli* strains, susceptibility amongst breeds, distribution and prevalence of the infection in pigs found in South Africa is limited. There is a need, therefore to determine the different *E. coli* strains found in local and imported breeds and quantify the susceptibility of such strains in these breeds. The broad objective of this study was to determine whether there are breed differences in adhesion phenotypes and correlate them to polymorphisms at selected candidate genes and finally associate the identified phenotypes with the candidate gene genotypes.

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Chapter 3: Investigation of polymorphisms at the *MUC4, MUC13, MUC20* and *TFRC* candidate genes for F4*ab/ac* resistance in South African pig populations

Abstract

Selection for *E. coli* F4*ab/ac* resistance has become common due to the increasing resistance of the bacteria to antibiotics. Four candidate genes were studied in three South African breeds, imported (Large White, Landrace and Duroc), crossbred and local, in order to identify polymorphisms conferring resistance to *E. coli* F4*ab/ac*. A total of 225 pigs aged 3-12 weeks were genotyped to target restriction sites in *MUC4, MUC13, MUC20* and *TFRC* candidate genes. Four polymorphisms of c.8227G>C for *MUC4,* c.576C>T for *MUC13,* g.191C>T for *MUC20* and c.291C>T for *TFRC* were detected. The susceptible allele *C* was close to fixation at over 90 % in all three breeds for the *TFRC* and *MUC13* loci and there was a genic and genotypic significant difference ($P < 0.05$) amongst breeds for the *TFRC* loci. The resistant *TT* genotype was found in less than 2 % of the entire population for the *TFRC* locus and was not found in any pigs for the *MUC13* locus. Both *TFRC* and *MUC13* were not polymorphic in the studied population. The *MUC4* and *MUC20* genes were polymorphic in the population. The resistant alleles *G* for *MUC4* and *C* for *MUC20* were present in the population with the highest frequency observed in the imported pigs. There was a significant difference in genotypic distribution amongst breeds at the *MUC20* and *MUC4* loci ($P < 0.05$). An excess of homozygotes in *TFRC* and *MUC20* was observed, leading to a deviation from HWE in the imported pigs at these loci. All three breeds were in HWE at the *MUC4* loci although an excess in heterozygotes was observed. The subpopulations at the *TFRC, MUC13* and *MUC20* loci were inbred and those at the *MUC4* locus were outbred. There was no significant linkage disequilibrium observed amongst the loci analysed. The results showed that *MUC4* and *MUC20* were informative and the presence of the resistant alleles makes it possible to use them as markers for selection against susceptibility to F4 *E. coli*.

Keywords: Pigs, polymorphisms, susceptibility, *E. coli* F4

3.1 Introduction

Diarrhoea remains an important cause of morbidity and mortality in livestock and is one of the most common diseases in suckling and post weaning piglets. Enterotoxigenic *Escherichia coli* (ETEC) also known as enteric colibacillosis expressed by the F4 fimbriae are a major cause of diarrhoea and death in neonatal and weaner pigs (Nagy and Fekete, 1999; Fairbrother *et al.,* 2005). In South Africa the prevalence of *E. coli* is found to be high in pigs (44-50 %) than in cattle (5-20 %) and humans (8 %) and the prevalence is higher in commercial (51 %) than in the communal (44 %) pigs (Ateba *et al.,* 2008). There is an increased incidence of ETEC especially in weaned pigs due to stress of weaning, lack of antibodies from the sow's milk and dietary changes (Henton and Englebrecht, 1997).

Various control strategies for ETEC include use of antibiotics and vaccines, supplementation of pig's diets with egg yolk antibodies and use of zinc and/or spray-dried plasma, bacteriophages and probiotics (Fairbrother *et al.,* 2005). These strategies short term remedies and uneconomical. In South Africa the most common strategies used against *E. coli* are the use of vaccinations and antibiotics (Henton and Englebrecht, 1997). Continual use of antibiotics leads to the development of resistance in animals. Previous studies (Maynard *et al.,* 2003; Yang *et al.,* 2009) have reported on resistance of *E. coli* isolates to Oxtetracycline, Spectinomycin and Trimethoprim-sulfonaide drugs due to continual use over time. Furthermore, farmers need to give a withdrawal period before slaughter after administration of some drugs, which hinders the farmers from meeting the target market at the required time. Hence, it is important for the development of long term control strategies like genetic control.

Genetic improvement of *E. coli* resistance has become increasingly popular in the pig industry. *Escherichia coli* carries many fimbrial adhesins and, F4*ab/ac* is frequently isolated from neonatal and weaned pigs showing signs of diarrhoea (Francis, 2002; Fairbrother *et al.,* 2005; Zhang *et al.,* 2008). The F4 fimbria facilitates the bacterial attachment to F4 variant specific receptors on the brush border of the

intestines, thereby leading to the colonization of the pig's small intestine (Baker *et al.*, 1997; Zhang *et al.*, 2008). These receptors are not present on every pig, and their absence in some pigs lead to resistance to F4 ETEC induced diarrhoea (Sellwood *et al.*, 1975). Expression of these receptors is genetically determined and inherited as an autosomal dominant trait (Gibbons *et al.*, 1977; Van den Broeck *et al.*, 2000).

The gene underlying resistance to F4ab/ac ETEC has been assigned by linkage analysis to pig chromosome SSC13q41 (Edfors-Lilja *et al.*, 1995; Python *et al.*, 2002; Jorgensen *et al.*, 2004). Positional candidate genes have been identified that are most likely important in coding for some transferrins or mucin-like sialoglycoproteins (Python *et al.*, 2002; Jorgensen *et al.*, 2004). Mucins are large glycoproteins that cover the apical surfaces of epithelial cells in the gastrointestinal and respiratory tract, forming the first line of host defense against enteric pathogens. The mucin 4 (*MUC4*) gene in intron 7 found on pig chromosome 13 was identified, with the *C* allele associated with susceptibility, dominating the resistant *G* allele (Jorgensen *et al.*, 2004). The polymorphism was in complete linkage disequilibrium with the phenotype of susceptibility for ETEC F4*ab/ac* and is used in genetic tests in the Danish pig breeding industry (Schroyen *et al.*, 2012). Apart from *MUC4*, *MUC13* and *MUC20* are other mucin genes on chromosome SSC13q41 that have been proposed as candidate genes for the ETEC F4*ab/ac* receptor (Zhang *et al.*, 2008; Ji *et al.*, 2011). The *C* allele for *MUC13* was associated with susceptibility and the *T* allele with resistance in a White Duroc x Erhualian population (Zhang *et al.*, 2008). The *C* allele for *MUC20* was associated with resistance and the *T* with susceptibility in a White Duroc x Erhualian population (Ji *et al.*, 2011). Transferrins are responsible for transporting iron from the transferrins into the cell, therefore, the gene encoding for the receptor is an interesting candidate gene for susceptibility of ETEC F4*ab/ac*. Transferrin receptor (*TFRC*) is found on chromosome SSC13q41 and the *C* allele was found to be associated with F4*ab/ac* susceptibility in a White Duroc x Erhualian population as well (Wang *et al.*, 2007). This association was more distinct for the F4*ac* than with the F4*ab* receptors.

The increase in antimicrobial resistance of *E. coli* isolates gives rise for a need to promote genetic improvement in South African pigs. Hence, why there is need to identify candidate genes that code for transferrins or mucin-like sialoglycoproteins in the South African population. To date, none of these candidate genes have been researched in the South African pigs. There is no information available on how polymorphic these genes are or how they segregate in the different pig breeds used in the country. This information could be used when breeding for resistance to *E. coli* infection. Sustainability of genetic control strategies is beneficial to the South African pig population, particularly to smallholder farmers who cannot afford antibiotics and depend on livestock for their livelihood.

The objective of the study was therefore to assess polymorphisms and the level of genetic variation of *MUC4*, *MUC13*, *MUC20*, and *TFRC* genes in the South African pig population. The study aimed to determine the potential of these genes that have been reported as important in conferring resistance/susceptibility to ETEC *F4ab/ac* in other populations, and use them as candidate genes in selection and breeding for *E. coli* resistance in the South African pig industry. Since these genes were polymorphic in other populations, they may also be polymorphic and segregating in the South African pigs. The presence of resistant alleles for the investigated genes could be of great importance in designing selection strategies against colibacillosis in pigs and was therefore investigated.

3.2 Materials and Methods

3.2.2 Pigs

A total of 225 neonatal to weaning phase pigs from Eastern Cape (n = 170) and Limpopo (n =56) provinces were used. The pigs were sampled from imported (Large White, Landrace and Duroc), local (indigenous) and crossbred (cross between imported and local pigs) pigs with sample sizes of 82, 112 and 32 for each population, respectively. The imported pigs had access to the sow's milk before weaning and

were fed on a commercial diet after weaning. The local and crossbred pigs also suckled before weaning and scavenged and fed on household kitchen leftovers after weaning. Imported pigs were housed in a commercial setup 24 hours a day with controlled management procedures. The local and crossbred pigs were kept in a free range setup during the day and enclosed at night.

3.2.3 Blood collection

Blood was collected from the jugular vein using a needle and syringe set (Promex syringe, 0.8 × 16mm BP Microlana needles). About of 2 ml of venous blood was injected directly into EDTA-vacutainer tubes labeled with the pig's identification number and breed. Upon collection, the blood was stored in ice-filled cooler boxes and then transported to the laboratory where it was stored at -20º C until DNA extraction.

3.2.4 DNA extraction and quantification

The blood collected from the pigs was used to extract DNA using a DNeasy Blood and Tissue kit (Qiagen Gmbh, D-40724 Hilden). The DNA concentration was measured using a Qubit 2.0 fluorometer (Molecular probes- invitrogen).

3.2.5 Polymerase chain reaction

The primer sequences, associated polymorphism and the expected product sizes of the genes under investigation are given in Table 3.1.

Table 3.1: Primer sequences and the expected PCR products of the four ETEC F4*ab/ac* candidate genes

Gene	Mutation	Primer	Sequence	Product size	Restriction sites	Digestion Enzyme	Reference
MUC4	g.8227 G>C	F	GTG CCT TGG GTG AGA GGT TA	367 bp	367, 216, 151	*Xbal*	Jensen *et al.*, 2006
		R	CAC TCT GCC GTT CTC TTT CC				
MUC13	c.576 C>T	F	ATG TGG AAG AAC AGA ACT TGA TTG AG	536 bp	317, 186, 33	*Hhal*	Zhang *et al.*, 2008
		R	ATA GTC AGG GCG GGG TAT ACT ACC				
MUC20	g.191 C>T	F	CGT GAT AAT CCA AGA GGC AAG TG	175 bp	175, 127, 48	*Alul*	Ji *et al.*, 2011
		R	CAA CAA GAA CTG AGA CCA GCA CC				
TFRC	c.291 C>T	F	TGT CTG CTA TGG GAT TAT TGC	667 bp	352, 231, 169, 146	*Alul*	Wang *et al.*, 2007
		R	TCT GCT TCG AAA GTT TCT GTC				

3.2.5.1 TFRC gene

The polymorphisms of *TFRC* gene were determined using methods described by Wang *et al.* (2007). The thermal cycles were done for 3 min at 94 ºC, followed by 35 cycles of 10s at 94 ºC, 30 s at 53 ºC, 1 min 30s at 72 ºC and a final extension of 10 min at 72 ºC. A total of 167 pigs were used in this analysis, 54 imported, 84 local and 29 crossbred pigs. An *AluI* PCR- RFLP method was used to genotype the 291 C>T polymorphism. The susceptible *C* allele is characterized by fragments of 121, 146, 169 and 231 bp and the *T* allele by 169, 146 and 352 bp.

3.2.5.2 MUC13 gene

The polymorphism of *MUC13* was determined as described by Zhang *et al.* (2008). The thermal cycles were for 15 min at 95 ºC, 35 cycles of 15 s at 95 ºC, 52 ºC for 30 s 72 °C for 1 min and a final extension of 72 °C for 10 min. A total of 160 pigs were used in this analysis, 60 imported, 70 local and 30 crossbred pigs. A *Hhal* PCR-RFLP assay was used to genotype a c.576C>T polymorphism. The susceptible *C* allele is detected by 317, 186 and 33 bp fragments and the resistant *T* allele by 317 and 219 bp fragments.

3.2.5.3 MUC4 gene

The polymorphism for *MUC4* was determined using the method described by Jensen *et al.* (2006). The thermal cycling were done for 5 min at 95°C, followed by 35 cycles of 15s at 95°C, 30s at 60°C, 1 min at 72°C and a final extension of 10 min at 72°C., 63 imported, 105 local and 29 crossbred pigs. The PCR product was digested at 37°C overnight with the *XbaI* restriction enzyme to genotype the g.8227 G>C polymorphism and then checked with 2.5 % agarose gel. The resistant allele *G* is indigestible by Xbal. The susceptible allele *C* is digested into 151 and 216 fragments.

3.2.5.4 MUC20 gene

The *MUC20* polymorphism was determined using the methods described by Ji *et al.* (2011). The thermal cycle was done for 15 min at 95 ºC, followed by 35 cycles of 95 ºC for 15s, 54 ºC for 30s, 72 ºC for 1 min with a final extension at 72 ºC for 10 min. A total of 149 pigs were used in this analysis, 51 imported, 77 local and 21 crossbred pigs. An *AluI* PCR-RFLP was used to genotype a g.191C>T polymorphism. The resistant *C* allele is characterized by two fragments 48 and 127 bp and the *T* allele is the uncut fragment 175 bp.

Digestion for all four genes (*MUC4, 13, 20* and *TFRC*) was done overnight at 37 ºC. The digested products were stained with ethedium bromide, separated using 2.5 % agarose gel electrophoresis and visualized using a Bio Rad UV transilluminator.

3.2.6 Statistical analyses

The alleles and allele frequencies were calculated using the GENEPOP Software (v4.201) (Rousset, 2008). Polymorphism information content (PIC) of each locus was calculated using a microsatellite tool-kit according to the formula:

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} P_i^2 - \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Where:

P_i = The frequency of allele i

J = The allele n having codominance with allele i

N = The number of individuals of one population

Observed heterozygosity (Ho) and expected heterozygosity (He) for each locus per population were also calculated with GENEPOP. The He was calculated according to Nei (1978):

$$He = 1 - \sum_{i=1}^{k} pi^2$$

Where p_i is the frequency of the i^{th} of k alleles

The Hardy-Weinberg Equilibrium (HWE) were also analysed using GENEPOP. The accurate values of statistical significance were estimated by the Markov Chains method by running 10 000 dememorization, 150 batches and 5 000 iterations/batch (Raymond and Rousset, 1995). The P-values were calculated by complete enumeration and the global test across loci was constructed using Fisher's method. The F-statistics were computed following Wright (1943). These indices were represented by F_{IS} (inbreeding coefficient of an individual per subpopulation), F_{ST} (average inbreeding of the subpopulation), F_{IT} (inbreeding coefficient in the total population). The linkage disequilibrium (LD) test was also computed for pairs of loci for the four candidate genes. The test assumed that genotypes at one locus were independent from genotypes at the other locus. The test computes the association between diploid genotypes at two loci following methods described by Weir (1996).

3.3 Results

3.3.1 Restriction sites in the RFLP fragments

Digestion of the *TFRC* c.291 C>T polymorphism using *AluI* produced three genotypes, *CC* – 231, 169 and 146 bp, *CT* – 352, 231, 169 and 146 bp and *TT* – 352, 169 and 146 bp fragments (Figure 3.1). The digestion of *MUC13* c.576C>T polymorphism with *HhaI* produced two genotypes, *CC* - 317, 186 (3.2a) and 33bp and C*T* - 317, 219 and 186 bp fragments (3.2b). Digesting the *MUC4* c.8227G>C gene PCR product with *XbaI* restriction enzyme produced three genotypes, *GG* – 367 bp, *GC* - 367, 216 and 151 bp

and *CC* – 216 and 151 bp fragments (Figure 3.3). Digestion of the *MUC20* g.191 C>T polymorphism using the *AluI* restriction enzyme produced three genotypes, *CC* - 127 and 48 bp, *CT* – 175, 127 and 48 bp and *TT* - 175 bp fragments (Figure 3.4).

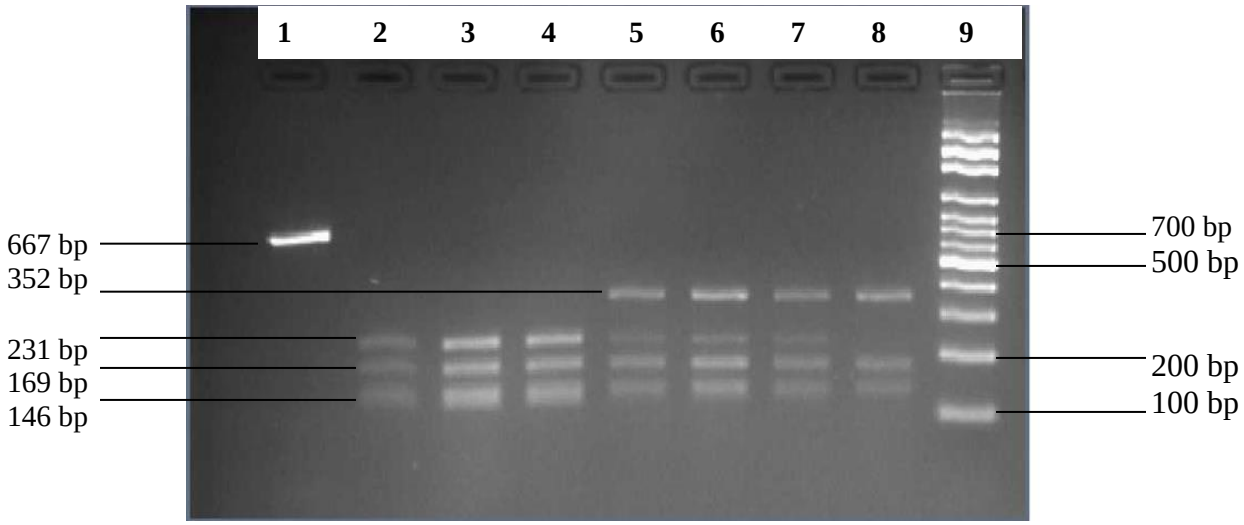

Figure 3.1: Restriction fragment length polymorphism genotypes showing *TFRC* c.291C>T polymorphism. Lane 1: PCR Product; Lanes 2- 4: *CC*; Lanes 5 - 7: *CT*; Lane 8: *TT*; Lane 9: 100bp ladder

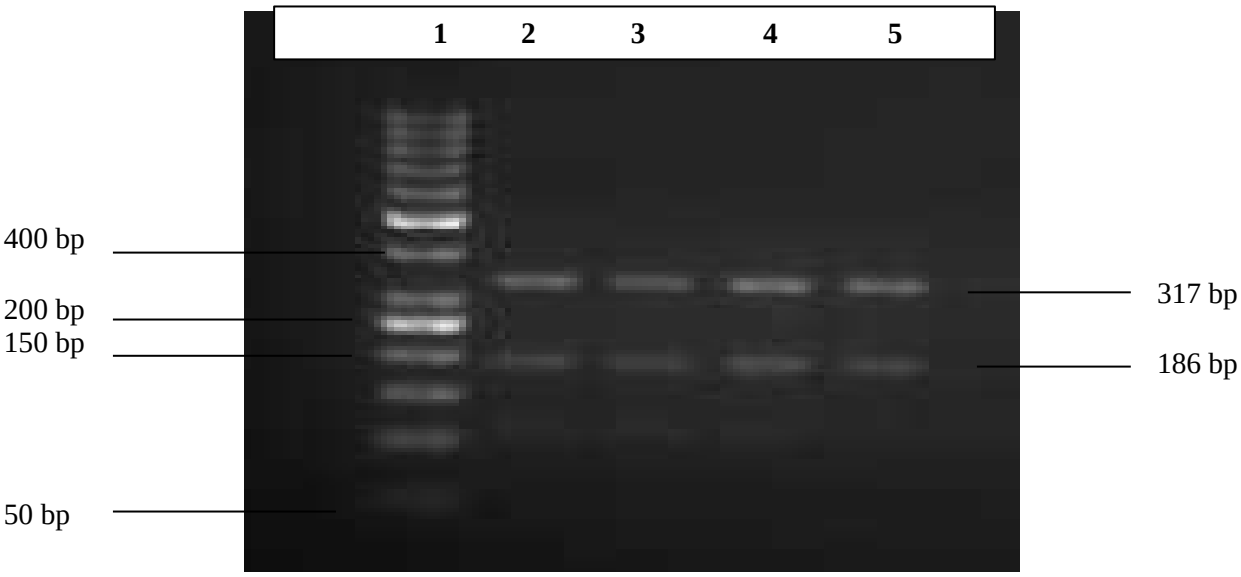

Figure 3.2a: Restriction fragment length polymorphism genotypes showing *MUC13* c.576C>T polymorphism. Lane 1: 50 bp ladder; Lanes 2-5: *CC* genotype.

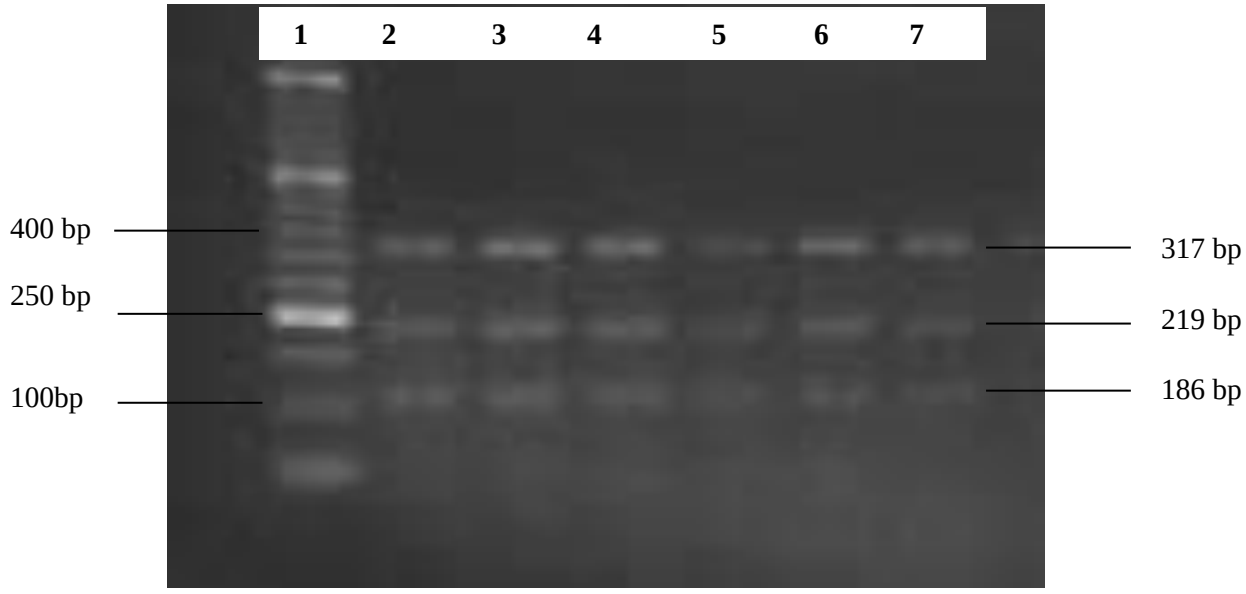

Figure 3.2b: Restriction fragment length polymorphism genotypes showing *MUC13* c.576C>T polymorphism. Lane 1: 50 bp ladder; Lanes 2-7: *CT* genotype.

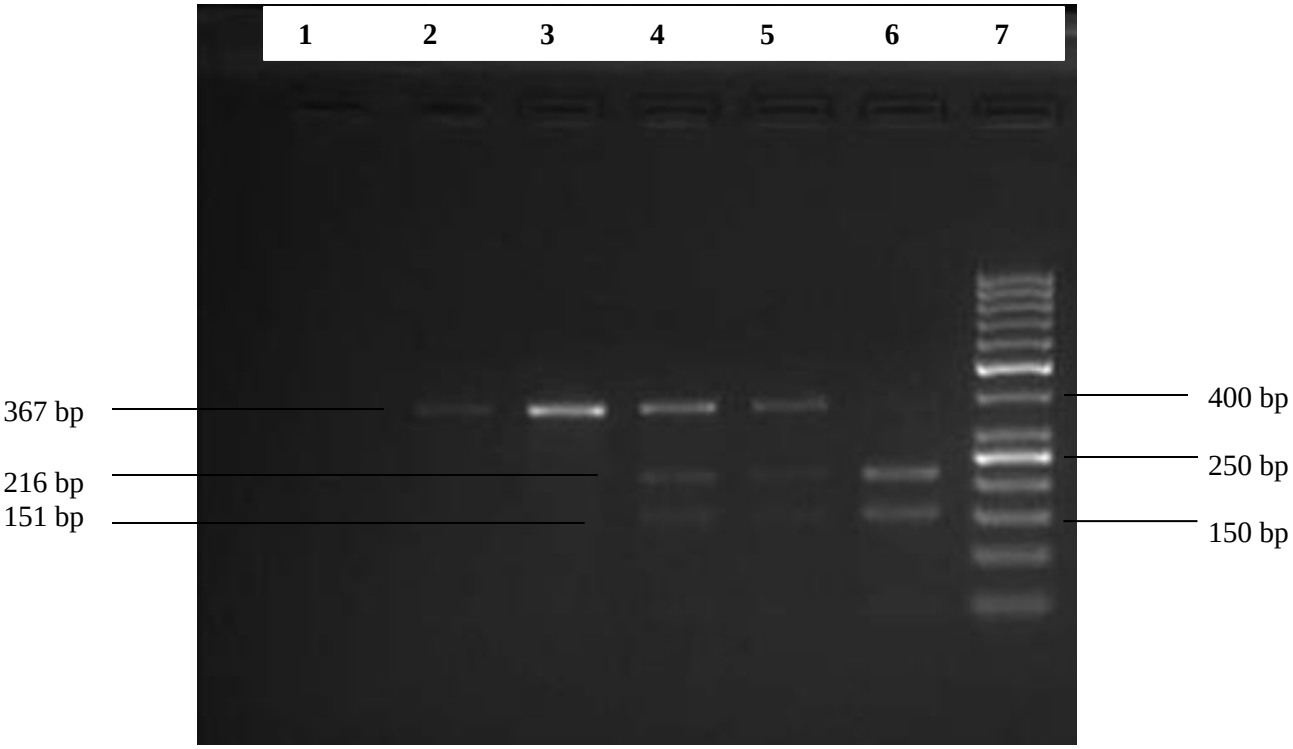

Figure 3.3: Restriction fragment length polymorphism genotypes showing *MUC4* c.8227G>C polymorphism. Lane 1: Negative control; Lane 2: PCR Product; Lane 3: *GG*; Lane 4 and 5: *GC*; Lane 6: *CC*; Lane 7: 50bp ladder.

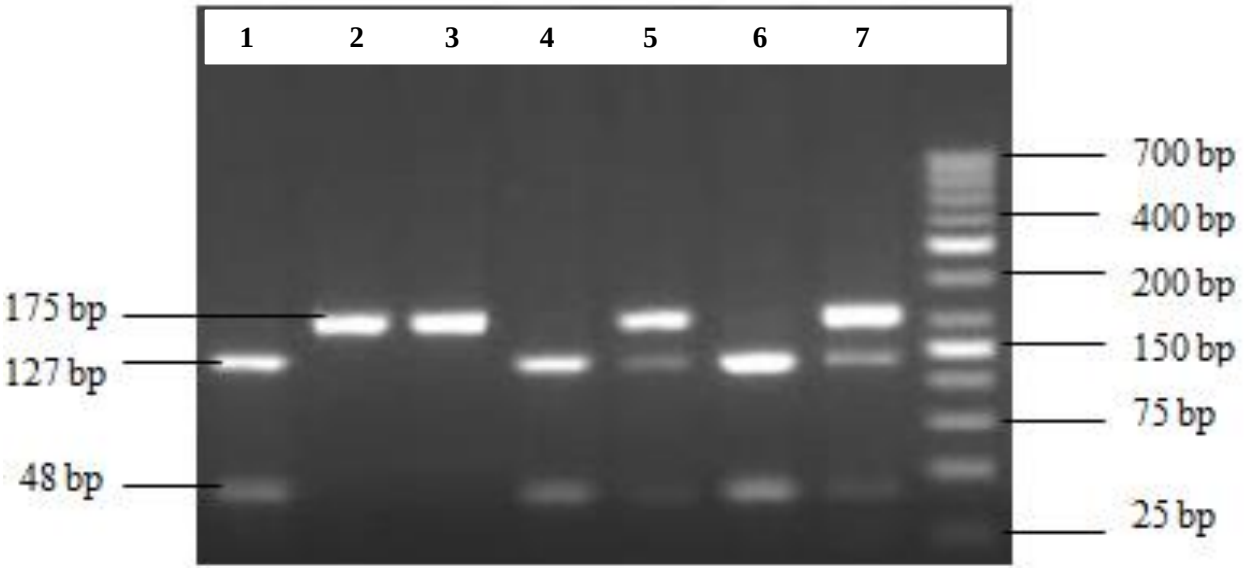

Figure 3.4: Restriction fragment length polymorphism genotypes showing *MUC20* g.191 C/T polymorphisms. Lanes 1, 4 and 6: *CC* genotype; Lane 2: PCR product; Lane 3: *TT* genotype; Lanes 5 and 7: *CT*; Lane 8: 25 bp ladder.

3.3.2 Allelic and genotypic distribution

The results show that the allelic frequencies in *TFRC* for the susceptible allele *C* were significantly higher (P < *0.05*) than those of the resistant allele *T* in all three breeds (Table 3.2). Allele *C* was close to fixation and had a frequency of over 90 % in all three breeds. The homozygote *CC* genotype was at a frequency of over 90% in the imported and crossbred pigs. In the local breed, the susceptible homozygous *CC* genotype was at a frequency of 84.3 %. There was a significant difference (P *<0.05*) in allelic and genotypic distribution amongst the breeds with the unfavorable genotype being most frequent in all breeds. In the analysed pig population the susceptible allele *C* in *MUC13* was fixed in imported and crossbred pigs. Only a small proportion of the local pigs carried the resistant allele *T.* All of the imported and crossbred pigs carried the susceptible *CC* genotype. Over 90 % of the local pigs carried the susceptible *CC* genotype and 2 % carried the heterozygous *CT* genotype (Table 3.2). There was no significant difference in both the allelic and genotypic distribution.

Allele frequencies for *MUC4* are shown in Table 3.2. The resistant *G* allele was at a higher frequency than the susceptible *C* allele. There was, however, no allele fixation in all three breeds. There was no significant difference in the allelic distribution. The *MUC4* genotypes segregated in all three breeds. There was increased variation in *MUC4* and this was shown by the heterozygous *GC* genotype having the highest frequency in all three breeds, followed by the homozygous resistant *GG* genotype then the susceptible *CC* genotype. There was a significant difference (P *<0.05*) in the genotypic distribution, with the heterozygous genotype being most frequent in all breeds. The resistant *C* allele in *MUC20* was close to fixation at over 90 % in the imported pigs, whilst in the crossbred and local pigs it was at a frequency of 71.4 % and 75.3 %, respectively. The resistant *CC* genotype was also predominant in the imported pigs, followed by the *CT* and *TT* genotype. The resistant *CC* genotype was found at a frequency of 57.1 % and 58.7 % in the crossbred and local pigs, respectively (Table 3.2). There was a significant statistical difference (P *<0.05*) in the allelic and genotypic distribution amongst the three breeds.

Table 3.2: Genic and genotypic frequencies of the four loci among imported, local and crossbred pig populations

Locus	Breed	N	Allele frequency		P-value	Genotype frequency			[a]P-value
			C	*T*		*CC*	*CT*	*CC*	
	Imported	78	0.972	0.028		0.962	0.018	0.018	
TFRC	Cross	32	1	0		1	0	0	
	Local	115	0.915	0.084	*	0.843	0.144	0.012	*
			C	*T*		*CC*	*CT*	*TT*	
	Imported	78	1	0		1	0		
MUC13	Cross	32	1	0		1	0		
	Local	115	0.985	0.015	NS	0.970	0.029		NS
			C	*G*		*CC*	*CG*	*GG*	
	Imported	78	0.746	0.253		0.493	0.507		
MUC4	Cross	32	0.586	0.413		0.207	0.758	0.344	
	Local	115	0.689	0.31	NS	0.3786	0.6213		*
			C	*T*		*CC*	*CT*	*TT*	
	Imported	78	0.931	0.069		0.902	0.059	0.039	
MUC20	Cross	32	0.714	0.322		0.571	0.286	0.143	
	Local	115	0.753	0.247	*	0.587	0.333	0.080	*

[a]P-value - <*0.05* significant

3.3.3 Observed and expected heterozygosity, Inbreeding coefficient (F_{IS}) Polymorphic information content (PIC) and Hardy-Weinberg equilibrium

The average observed heterozygosity was lower than the expected heterozygosity in the imported and local pigs for the *TFRC* locus (Table 3.3). The observed and expected heterozygosity values of the crossbred pigs were all equal to zero; furthermore, all breeds had a positive F_{IS} value indicating inbreeding. PIC values in all breeds were low at this locus. According to the X^2 goodness-of-fit test, the crossbred and local pigs were in Hardy-Weinberg equilibrium. Imported pigs deviated from HWE (P *<0.05*). The local breed had a higher observed heterozygosity than expected, and all other breeds had values of zero for observed and expected heterozygosity. The average inbreeding coefficient for all three breeds was negative, although it was not significantly lower than zero for the *MUC13* locus. All three breeds were in HWE for *MUC13* and the PIC value for all breeds was low. All the breeds were in HWE for *MUC4* gene. The observed heterozygosity was higher than the expected heterozygosity in all three breeds and the all breeds were moderately polymorphic. All breeds at this locus indicated a considerable level of outbreeding due to negative F_{IS} values. The *MUC20* locus showed a higher expected heterozygosity than the observed heterozygosity in the imported and crossbred pigs (Table 3.3). The observed heterozygosity was higher than the expected heterozygosity in the local pigs. All three breeds were inbred at this locus. The imported pigs were lowly polymorphic, whilst the crossbred and local pigs were slightly polymorphic. The crossbred and local breeds were in HWE, while the imported pigs deviated from HWE (P *<0.05*).

3.3.4 Wright's fixation indices (F_{IS}, F_{ST} and F_{IT})

The mean estimates of F-statistics for the *TFRC* loci were 0.2055 for F_{IT} (inbreeding coefficient of an individual relative to the total population), due to 0.1827 for F_{IS} (within population inbreeding coefficient inter-individual) and 0.0279 for F_{ST} (between population breeding coefficient in a subpopulation) (Table 3.3).

Table 3.3: Expected Heterozygosity (He), Observed Heterozygosity (Ho), Inbreeding coefficient (F_{IS}) Polymorphic information content (PIC), Hardy-Weinberg equilibrium (HWE) and F-statistics (F_{IS}, F_{ST} and F_{IT}) values between individuals and among populations

Locus	Breed	Ho	He	F_{IS}	PIC	Breed [a]P-value	HWE (P)	F_{IS}	F_{ST}	F_{IT}
							*	0.1827	0.0279	0.2055
	Imported	0.019	0.054	0.6624	0.05	0.028				
TFRC	Cross	0	0	0	0	NS				
	Local	0.142	0.153	0.0699	0.14	NS				
							NS	-0.0053	0.0033	0.0019
	Imported	0	0	0	0	NS				
MUC13	Cross	0	0	0	0	NS				
	Local	0.029	0.028	-0.0075	0.03	NS				
							NS	-0.4290	0.0159	-0.4064
	Imported	0.507	0.381	-0.3333	0.31	NS				
MUC4	Cross	0.759	0.493	-0.5516	0.37	NS				
	Local	0.619	0.429	-0.4468	0.34	NS				
							*	0.2184	0.0716	0.2744
	Imported	0.059	0.129	0.5468	0.12	0.0103				
MUC20	Cross	0.286	0.418	0.3220	0.33	NS				
	Local	0.325	0.380	0.1097	0.31	NS				
All loci		0.230	0.210	-0.0800			NS	-0.1627	0.0342	-0.1229

[a]P-value- *<0.05* is significant

The fixation indices at the *MUC13* loci were all low. The inbreeding coefficient of the total population was 0.0019 for *MUC13* and this was due to a low within population inbreeding coefficient (-0.0053). The F_{IT} value amongst all population for the *MUC4* was negative and this was influenced by a low value in F_{IS}. The *MUC20* locus had the highest total inbreeding coefficient (0.2744) in comparison to the other three loci, which was due to a high within population coefficient value (0.2184). Overall, loci population variation was -0.1229 and this was accounted for mostly by within breed variation (-0.1627).

3.3.5 Linkage disequilibrium and association between loci

There was no significant linkage disequilibrium among any loci. Therefore, there was no evidence of violation of independent assortment of loci analysed in this study (Table 3.4). The standard error showed lower deviation from the probability test values.

3.4 Discussion

To identify potential markers that can be used against ETEC F4*ab/ac* in the South African pigs, four candidate genes, *TFRC*, *MUC13*, *MUC20* and *MUC4* were used to genotype individual pigs from three different breeds, namely, imported, crossbred and local. The objective was to identify polymorphisms segregating in a South African pig population, so as to ascertain whether these genes are potential markers that could be used in breeding programs. These genes were used because they were found to be polymorphic in other populations. The above genes have been mapped to chromosome 13q41 and have been used as candidate genes for ETEC F4*ab/ac* in White Duroc x Erhualian intercross pigs.

Table 3.4: Association between different loci in South African imported, local and crossbred pig breeds

Locus 1	Locus 2	P-value	Standard Error
TFRC	*MU13*	1.0000	0.0000
TFRC	*MUC4*	0.6681	0.0006
MUC13	*MUC4*	0.4936	0.0004
TFRC	*MUC20*	0.7454	0.0015
MUC13	*MUC20*	1.0000	0.0000
MUC4	*MUC20*	0.0161	0.0017

Wang *et al.* (2007) reported that in *TFRC* the *C* allele was most frequent in pigs susceptible to ETEC F4 and the *T* allele in resistant pigs to ETEC infections. For *MUC13*, Zhang *et al.* (2008) showed that the *C* allele was predominant in sows susceptible to ETEC F4*ab/ac* and *T* in resistant pigs. Previously, Ji *et al.* (2011) reported that the *C* allele in *MUC20* was most frequent in resistant pigs and the *T* allele in susceptible pigs to ETEC F4*ab/ac*. For *MUC4* the *G* allele was associated with resistance to ETEC F4*ab/ac* in Large White pigs and the *C* allele with susceptibility according to previous studies (Jorgensen *et al.*, 2003).

Mucins are interesting candidate genes. They are large glycoproteins expressed as glycocalyx on the intestinal enterocytes or expressed to form the mucosal layer on the epithelial cells, which forms the barrier between those epithelial cells and their environment (Dekker *et al.*, 2002). The most widespread studied polymorphism in relation to ETEC F4ab/ac susceptibility is the SNP at position 8227 in intron 7 of mucin 4 (*MUC4*). Transferrin receptor (*TFRC*) is a potential candidate gene because of its relation to ETEC F4 susceptibility (Grange and Mouricout, 1996) and also its location on SSC13q41. The transferrin receptor is needed for the uptake of transferrin, therefore the gene encoding for the receptor is an interesting candidate gene for ETEC F4*ab/ac* (Schroyen *et al.*, 2012).

The susceptible allele in *TFRC* gene was found at a high prevalence in all three breeds in the current population. This trend was similar in the genotypic frequencies, where the homozygous *CC* genotype was most frequent in all breeds except for the local breed where the genotypes slightly segregated. The resistant allele and genotype were at a low frequency in all three breeds, which makes it difficult to use *TFRC* as a potential marker for selection against susceptibility to ETEC F4*ab/ac* in the current pigs, because overall the homozygous susceptible genotype was dominant in the studied population. Breed variation could also influence allele combinations, which may be the reason why the crossbred pigs did not have individual pigs which carried the resistant genotype at this locus. There was a significant difference found in the allele and genotypic frequencies for all breeds.

The c.291C>T polymorphism was not found to be a causative mutation in previous reports (Wang *et al.*, 2007). It has significant linkage disequilibrium with ETEC F4*ab/ac*, especially F4ac receptor.

The unfavorable *C* allele in *MUC13* was also highly prevalent in all three breeds. No mutation was found in the *MUC13* c.576C>T gene for both the imported and crossbred pigs, which suggests that these pigs were monomorphic at that region, leading to allele fixation in these two breeds. A similar trend was seen in the genotypic frequencies. Moreover, all breeds were not significant at the allelic and genotypic distribution. The *MUC13* gene was reported to be is in strong linkage disequilibrium with ETEC F4*ab/ac* receptor (Zhang *et al.*, 2008), but it is not a potential marker for selection of ETEC F4*ab/ac* resistant animals in the current population. Joller *et al.* (2009) performed an *in vitro* adhesion test and phenotyped five piglets as resistant and one susceptible to ETEC F4*bc*. The genotype for marker *MUC13* was homozygous *CC* for the five resistant pigs and heterozygous *CT* for the one susceptible piglet to ETEC F4*bc* and that for marker *MUC4* the genotypes were *GC* and *CC*, respectively. They concluded that the locus for ETEC F4*bc*R was located distal to *MUC4* and that the causative mutation for ETEC F4*ab/ac* susceptibility was probably located around the region containing the *MUC13* gene. The high frequency of the *C* allele known to confer susceptibility to ETEC F4 in the current study, suggests that *TFRC* and *MUC13* cannot not be used as a potential candidate gene against susceptibility to ETEC F4 in the current population.

A large proportion of the current population carried the resistant *C* allele for the *MUC20* g.191C>T polymorphism, increasing the chances of selecting resistant individual pigs that can be used in breeding programs. The imported pigs had the highest frequency in comparison to the crossbred and local pigs. Furthermore, the imported pigs had high genotypic frequencies amongst the three breeds. There was a significant difference amongst breeds in allelic and genotypic distribution for the *MUC20* locus. The *MUC4* g.8227 polymorphism which was proposed as a candidate gene for F4*ab/ac* adhesion phenotype (Joller *et al.*, 2009) was investigated in three breeds of South Africa. The imported pigs showed the

highest level of the resistant allele *G*, with a frequency of 74.6 %. The *MUC4* allele segregated in the crossbred and local pigs, although there was no significant difference in allelic distribution. The *MUC4* gene segregated in all three breeds and the heterozygous genotype had the highest frequency in all three breeds resulting in an increased variation for the locus. There was also a significant difference in the genotypic distribution. In addition, the imported and local pigs did not carry the susceptible *GG* genotype. A decrease in homozygosity at this locus leaves room for selection against ETEC infection for the *MUC4* locus. High frequencies of the resistant alleles in *MUC4* and *MUC20*, enables these two genes to be used as markers in the studied population.

Reasons for the imported pigs having the highest frequency of the resistant *G* allele for *MUC4* and the resistant *C* allele for *MUC20* in the current population, could be attributed to the fact that these pigs were kept in a commercial setup were pigs are selected in favor of production traits like birth weight, average gain weight and carcass weight which could have influenced the resistant alleles. The F4*bc*R has been associated with production traits such as birth weight, growth rate and carcass weight in Duroc x Erhualian population (Yan *et al.,* 2009). Pigs carrying the F4*ab*R and F4*ac*R have a greater birth weight, average daily gain and carcass weight. Consequently, these selection traits could have resulted in the imported pigs having a high frequency of the resistant allele.

The *MUC4* candidate gene was also studied in previous studies. Filistowicz and Jasek (2006) detected the resistant allele of *MUC4* at frequencies of 0.162, 0.875 and 0.857 in Polish Landrace, Belgian Landrace and Duroc, respectively. The susceptible *CC* allele was at a frequency of 0.220, 0 and 0, respectively. Cirera *et al.* (2004) identified the *GG* resistant genotype in Yorkshire, Landrace, Duroc and Hampshire at a frequency of 0.002, 0.200, 0.883 and 0.979, respectively. Previously, this polymorphism has been found to be in complete linkage disequilibrium with the phenotype for susceptibility to ETEC F4*ab/ac* and is currently been used as a genetic test in the Danish pig breeding industry (Schroyen *et al.*, 2012).

The discriminating power of markers was calculated using the Polymorphism information content (PIC). The PIC shows how polymorphic a marker in a given population is. It is often used to measure the informativeness of a genetic marker for linkage studies. The index is useful for establishing through linkage analysis whether the marker is near a gene or another marker of interest. Therefore, a marker is highly informative for linkage studies if any individual chosen at random is likely to be heterozygous for that marker. The PIC values range between 0 and 1 and a population is said to be highly polymorphic if PIC greater than 0.5 and lowly polymorphic if PIC is below 0.25 (Bostein *et al.,* 1980). The *TFRC* locus was found at a low of 5 %, which suggests that overall this gene did not segregate in the current population. Similar trends were detected for the *MUC13* locus, which was lowly polymorphic in all three breeds. The *TFRC* and *MUC13* genes were either close to fixation of fixed in all three breeds, which in turn resulted in these two genes not been polymorphic. The *MUC4* and *MUC20* loci were moderately polymorphic in the studied population with an average PIC of 0.3 for both loci, which makes these two loci more informative to use as candidate genes against ETEC infection in the studied population than *TFRC* and *MUC13*. In addition pigs at the *MUC4* and *MUC20* loci carried high frequencies of the resistant alleles.

Heterozygosity is a measure used to assess genetic diversity. Takezaki and Nei (1996) determined that for a marker to be useful for measuring genetic variation; it should have an average heterozygosity of between 0.3 and 0.8. The fixation indices showed a low level of genetic diversity among individuals of the same breeds and low genetic similarities amongst the three breeds for the *TFRC* locus. This was indicated by a deficit in heterozygosity in all three breeds. The average heterozygosity for all three breeds was at a low of 0.05 at this locus. The results also showed that the crossbred pigs had a lower diversity as compared to the local and imported pigs, and this was shown by values of zero for observed and expected heterozygosity in the crossbred pigs. Similar trends of low genetic diversity were found for the *MUC13* gene, especially in the imported and crossbred pigs, which had heterzygosity values of zero. The *MUC20* gene showed low diversity in the imported and crossbred pigs. The local pigs had a higher diversity and

this was shown by a heterozygosity value of 0.32, compared to the imported and crossbred pigs which had values of 0.05 and 0.2.

The *MUC4* gene had an excess of heterozygotes which was shown by higher observed heterozygosity values in all three breeds. The average heterozygosity for all three breeds was 0.6 at the *MUC4* locus. The highest genetic diversity was detected in crossbred pigs, followed by the local and imported pigs. There was increased genotypic variation, which was shown by an excess in heterozygotes. Historical information on origins, breeding and selection strategies applied to the indigenous pig populations of southern Africa is very limited. The favorable values in the local and crossbred populations could reflect low selection pressures, natural patterns of mating, and the presence of genetic substructure due to the many small communal farms sampled or a diverse founding history.

Overall the South African population studied showed low genetic diversity because it had an average heterozygosity of 0.23 for all three breeds at all loci. Swart *et al.* (2010) reported on high heterozygosity values of 0.522, 0.584, 0.504 and 0.537 for South African landrace, Large White, Duroc and indigenous pigs, respectively. The Duroc was introduced in South Africa only thirty years ago and is still kept only by a limited number of farmers. The South African Landrace has been in the country since 1952 and is the second largest commercial breed. The most prominent commercial breed is the large white which has been farmed since 1903 (Swart *et al.*, 2010).

F_{IS} is another tool used to measure genetic diversity and measures heterozygosity deficiencies (e.g., F_{IS} = (He- Ho)/He) in a population. The *TFRC*, *MUC13* and *MUC20* loci had a positive F_{IS} value in all three breeds, indicating a considerable level of inbreeding for these two loci. The local and crossbred pigs were sampled from communal areas, were farmers have small population sizes that are close to each other. This results in inbreeding due to farmers exchanging breeding animals with their neighbors. For *MUC4* candidate gene, all three breeds had a negative F_{IS} value for all breeds. Overall the current population was

outbred, which was shown by a negative F_{IS} value for all loci. The *MUC4* gene contributed to the low F_{IS} value of the entire population due to the low F_{IS} values in subpopulations at this locus.

The Hardy-Weinberg equilibrium (HWE) states that allele and genotype frequencies at a polymorphic locus will not change from generation to generation in the absence of other evolutionary influences (Winter, 2005). The influences comprise of random mating, mutations, selection, genetic drift, gene flow and small population sizes. The crossbred and locals pigs for *TFRC* and *MUC20* genes were in HWE whilst the imported pigs deviated. The local and crossbred pigs sampled were from communal areas where farmers keep small population sizes. Furthermore, due to small population sizes mating is random in the populations resulting in inbreeding. There is also no selection pressures for productive traits like growth rate in the local and crossbred pigs kept in communal areas. All these factors could have influenced the local and crossbred pigs being in HWE. The imported pigs deviated from HWE at the *TFRC* and *MUC20* loci. Imported pigs are kept in a commercial setup with controlled management and breeding programs. Imported pigs are selected for production traits like growth rate, lean meat, weaning weight and backfat thickness. This increases the chances of non-random mating because individuals that are less productive are culled from the population. There is a high level of gene flow in imported pigs from importation of breeds with favorable production characteristics from other countries and the practice of Artificial Insemination. In addition, pigs kept in a commercial setup are characterized by a large population size. These factors could have influenced the deviation of the imported pigs from HWE. All pigs for the *MUC13* and *MUC4* loci were in HWE.

Wright's fixation indices are useful tools for studying the genetic differentiation of populations. Three fixation indices were developed to evaluate population subdivision: F_{IS} (individuals within subpopulations) + F_{ST} (subpopulations) = F_{IT} (total population variation) (Nei and Chesser, 1983). The F stands for fixation index, fixation being increased homozygosity resulting from inbreeding. The subdivision in populations leads to the loss of genetic variation within subpopulations and this is

measured by heterozygosity. Genetic variation within subpopulations is as a result of small population sizes and genetic variation acting within each of the populations. In the studied population the total variation was highest in the *MUC20* locus, followed by *TFRC* and *MUC13*. The lowest was in *MUC4*. In all loci total variation was influenced by F_{IS}, high values in the *MUC20*, *TFRC* and *MUC13* genes and a negative F_{IS} value in the *MUC4* gene. The *C* allele in *TFRC*, *MUC13* and *MUC20* (imported breeds) genes was close to fixation resulting in increased homozygosity at these loci and therefore, inbreeding. A large proportion of the sampled local and crossbred pigs were from communal areas characterized by small populations which encourage incidences of inbreeding. Furthermore, breeds kept in communal areas are selected to adapt to a diet low in protein content and local ecologies. The selection pressure on growth rate in communal pigs is weak and their growth rates are generally low, resulting in allele sharing in these pigs because they share the same selection pressures. All breeds in the *MUC4* locus had increased genetic variation which was shown by high observed and expected heterozygosity values at this locus.

Linkage disequilibrium (LD) is the non-random association of alleles at two or more loci, which descend from single ancestral chromosomes (Reich, 2001). Linkage disequilibrium is also the occurrence of some combinations of alleles or genetic markers in a population more often than would be expected from a random formation of haplotypes from alleles based on their frequencies. Factors that can affect LD include selection, gene mutation and factors that affect HWE like migration and random drift (Daly *et al.* 2001). The results in the current study did not show LD amongst the four loci that were experimented. These results are in contrast with other findings, where a strong LD was found between loci c.191C>T of *MUC20* and loci c.575C>T of *MUC13* in a White Duroc and Erhualian intercross (Ji *et al.,* 2011). The reason for no LD in the current study could be due to distance between loci, and/or the factors that affect HWE such as migration, selection, random mating and mutations.

Generally high prevalence of the susceptible alleles for F4 ETEC were observed in all three breeds at the *TFRC* and *MUC13* loci analysed. This implies that the population is highly likely to be affected by ETEC

if there is an outbreak. The *MUC4* and *MUC20* genes had high frequencies in the resistant alleles, meaning the population can be selected against ETEC susceptibility. The resistance in the population, however, needs to be confirmed by an *in vitro* adhesion experiment. The resistance genotypes detected in the imported breeds for *MUC4* and *MUC20* loci could be because they are commercial breeds, which undergoes selection to improve their reproduction and production traits, thereby affecting their immunity traits as well (Lemus-Flores *et al.*, 2001).

3.5 Conclusions

The South African pig population investigated showed a high frequency of resistant *MUC4* and *MUC20* alleles and genotypes to ETEC F4. The *TFRC* and *MUC13* resistant genotypes were at a very low frequency in all three breeds. The presence of the *G/C* and *C/T* mutation in the *MUC4* and *MUC20*, respectively, in the studied population facilitates the selection against ETEC F4. There is need to conduct *in vitro* adhesion tests in the current population, so as to ascertain adhesion phenotypes of the current population.

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Chapter 4: Adhesion of *Escherichia coli* in piglets and association of phenotypes to known candidate genes in South African breeds

Abstract

Enterotoxigenic *Escherichia coli* is a major pathogenic bacterium that causes diarrhoea in pre-weaned and post-weaned piglets. The adhesion of *E. coli* to the brush borders of the epithelial cells of piglets is a prerequisite for effective colonisation leading to diarrhoea. Successful adhesion occurs in the presence of the *E. coli* receptors that are found on the brush borders of the epithelial cells. The objective of the study was to compare the susceptibility of South African breeds to enterotoxigenic *E. coli* strains. An *in vitro* adhesion experiment was carried out for F4, PAA and EAST-1 *E. coli* strains using intestinal brush borders from 109 pigs of three South African pig breeds. Large White, indigenous and crossbred pigs that were 3-12 weeks old were used. The results showed significant differences ($P < 0.05$) in adhesion frequencies of receptors among the three breeds. Adhesion phenotypes, adhesive, weakly adhesive and non-adhesive were found in all breeds. The F4 and PAA strains adhered in all three breeds. The Indigenous pigs had the highest frequency of non-adhesive intestines and over 70 % of the Large White pigs were adhesive to all strains. In indigenous and crossbred pigs, adhesion was higher in suckling piglets than in weaners. The *TFRC, MUC13, MUC4* and *MUC20* genotypes were not associated with adhesion phenotypes. The South African population studied carried receptors for all strains measured. If there is an outbreak of *E. coli* carrying the above strains, the South African population is most likely to be affected. The indigenous pigs of the South African population studied were more resistant to F4, PAA and EAST-1 *E. coli* strains compared to Large White and crossbred pigs.

Keywords: *E. coli* strains, piglets, adhesion, susceptibility

4.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major pathogenic cause of diarrhoea in neonatal and weaned piglets and causes significant losses to pig industries worldwide. There has been an increase of

ETEC, particularly in South Africa resulting in an increase of colibacillosis (Henton, 2010). The bacterium has two main virulence factors, fimbrial and non-fimbrial adhesins and enterotoxins. The fimbriae which have finger-like projections facilitate the colonization of the piglet's small intestine through the attachment of ETEC strains to specific receptors present on the microvilli of epithelial cells (Li *et al.,* 2007). Colonising bacteria synthesise enterotoxins which stimulate the small intestine to secrete massive fluids and electrolytes into the gut lumen, thereby causing diarrhoea (Van den Broeck *et al.,* 2000). Adhesion of *E. coli* to the epithelial cells of the small intestine is an essential precondition for the bacteria to effectively colonise and cause diarrhoea.

Adhesion of *E. coli* is measured by an *in-vitro* test, is used to quantify the number of bacterial cells which will have adhered to pig intestinal cells. Most ETEC strains from piglets with diarrhoea possess the fimbrial adhesins for F4, F5, F6, F18 or F41, among which F4 is the most prevalent one that causes piglet diarrhoea during the pre-weaning and post-weaning period (Moon *et al.,* 1977; Nagy Fekete, 1997; Li *et al.,* 2007). The F4 strain has three variants which are F4*ab*, F4*ac* and F4*ad* and F4*ab/ac* is most commonly isolated from piglets with diarrhoea (Sellwood *et al.,* 1975; Choi and Chae, 1999). Lately non-fimbrial virulence factors of ETEC have been isolated from pigs, including adhesion involved in diffuse adherence (AIDA-1) (Benz and Schimdt, 1989), enteroaggregative heat-stable enterotoxin 1 (EAST-1) (Savarino *et al.,* 1993), new pili factor (type IV) (Pitchel *et al.,* 2002) and porcine attaching and effacing-associated factor (PAA) (Batisson *et al.,* 2003).

Susceptibility and resistance to infection have been correlated with bacterial adherence or lack thereof, to specific bacterial adhesion receptors on the small intestines epithelial cells of the host animal. Pigs that are resistant to infection with ETEC positive strains do not express intestinal receptors for the particular strain (Rutter *et al.,* 1975; Sellwood *et al.,* 1979). The adhesive phenotype behaves in a simple dominance Mendelian fashion (Gibbons *et al.,* 1977). The locus encoding *E. coli* receptors for F4*ab/ac* receptors was initially mapped to pig chromosome SSC13q41 (Edfors-Lilja *et al.,* 1995). The *MUC4*,

MUC13, *MUC20* and *TFRC* candidate genes were assigned to SSC13q41 and considered to be candidate genes for ETEC F4*ab/ac* receptor. In Chapter three we analysed the prevalence of resistant and susceptible alleles of these four candidate genes in a South African population. These candidate genes are assumed to be very close to the receptors of *E. coli* (Schroyen *et al.,* 2012), which is why we indirectly analysed the diversity of *E. coli* receptors through the above candidate genes. The *MUC4* and *MUC20* candidate genes segregated and were polymorphic in the population, however, the *MUC13* and *TFRC* candidate genes were close to fixation and were not polymorphic.

There is limited information available on the prevalence and effects of ETEC diarrhoea in pigs in South Africa. From 1971 to 1991, 674 cases of *E. coli* colibacillosis were reported at the Onderstepoort Veterinary Institute. Of these, 46 % had enteritis or inflammation of the small intestine and 47 % showed septicemia of blood poisoning (Henton and Engelbrecht, 1997). These infections were high in weaners than in suckling pigs. A recent study (Mohlatlole *et al.,* 2013) investigating the prevalence of colibacillosis in South African pigs observed the absence of F4 and F18 fimbrial adhesins. Non-fimbrial adhesins such as, AIDA-1, PAA and enterotoxin EAST-1 were detected in 15, 18 and 20 % of the piglets respectively. Such findings necessitate further investigations on the adherence of these fimbrial and non-fimbrial strains in the South African pig populations. The knowledge of ETEC strain adhesion phenotypes, will give us information on the presence of receptors in a South African pig population. There is need to test if the candidate genes we studied in Chapter three are associated with the ETEC receptors carried by pigs from the different breeds of South Africa.

The objectives of the study were to:

a) Compare the susceptibility of South African Large White, indigenous and crossbred pigs to F4, PAA and EAST-1 ETEC strains; and

b) Determine the association between adhesion phenotypes to genotypes of known ETEC F4 candidate genes.

The results will help build an understanding in the susceptibility of the South African pig population and possible genetic control strategies for breeding for resistance to diarrhoea.

4.2 Materials and methods

2.1 Pigs and sampling of intestines

A total of 109, 3-12 week old pigs randomly selected from small holder and commercial farms in the Eastern Cape Province were used in the study. The suckling piglets were aged 3-5 weeks and the weaned piglets were 6- 12 weeks old. Pigs of each breed were selected from Alice, Port St Johns and Mthatha based on availability of indigenous, cross and Large White breeds in those areas. A total of 20, 66 and 23 pigs of Large White, indigenous and crossbred pigs were sampled respectively. The Large White pigs were reared in a large-scale production system, where they were enclosed 24 hours a day. The indigenous and crossbred pigs were kept in a small scale system, where they were allowed to scavenge during the day and enclosed at night.

4.2.2 Preparation of epithelial cells

Approximately 10-20 cm of jejunal specimens were cut from sampled pig intestines within 30 minutes post euthanisation. The specimens were taken between 3.5 and 7.5 distal the *Arteria mesentrica cranialis*. The segments were rinsed of all content in EDTA solution before being placed in bottles containing 80 ml of cold EDTA solution and stored at 4 ºC till further processing. The epithelial cells were prepared according to methods by Joller *et al.* (2009). A 4 cm piece of chilled small intestine was scrapped to obtain a superficial layer of the small intestine and collected in 50 ml centrifuge tubes which contained 30

ml PBS-formaldehyde. The suspension was stirred vigorously with forceps for 1 min and stored at 4 °C for 15 min to sediment large sell fragments. The supernatant was decanted and stored at 4 °C for 20 min again for sedimentation of the cells. Thereafter the supernatant was centrifuged at 1 200 rpm for 10 min. The pellet was carefully suspended in 10 ml PBS and centrifuged again.

4.2.3 Bacterial strains

The fimbriated bacterial strain used in the adherence assay was an F4-positive strain. Non-fimbriated isolates used in the adherence test were PAA strain and EAST-1 toxin, which were found prevalent in a South African population (Mohlatlole *et al.,* 2013). All three strains were obtained from the Bacteriology Laboratory at the Ondesterpoort Veterinary Institute of Agricultural Research Council. The bacterial strains were prepared using methods described by Joller *et al.* (2009). Confluent growth was picked from blood agar plates and grown for 24 h at 37 ºC in Tryptocase Soy Broth (TBS) in test tubes a day before use. Before the strains were used, 1 ml of the bacterial culture was diluted in pre-warmed TBS at a proportion of 1: 10 and incubated at 37 ºC for 90 min to achieve maximum growth rate of the bacteria.

4.2.4 Microscopic adhesion test

The adherence assay test of ETEC expressing F4 and PAA strains and EAST-1 toxin to brush borders prepared from the intestines of pigs was done as described by Joller *et al*. (2009). Briefly, 1 ml of re-suspended enterocytes was incubated in 6-well macroplates at 37 °C for 30 min with 1 ml freshly grown culture from each of the ETEC strains. Subsequently, 20 well-separated and intact enterocytes was scored for each sample under a phase contrast microscope with a 100 x magnification. According to criteria proposed by Baker *et al.* (1997), specimens were classed as adhesive (susceptible) to ETEC if at least 10 % of the brush borders bound more than two bacteria. Specimens with fewer than 10 % of the brush borders binding more than two bacteria, yet more than 10 % binding one or two bacteria were considered

weakly positive. Specimens were all brush borders bound no bacteria were judged non-adhesive (resistant). The genotypes of the pigs at *MUC4, MUC13, MUC20* and *TFRC* candidate genes were adopted from Chapter three to associate adhesion phenotypes and genotypes from the above candidate genes.

4.2.5 Statistical analyses

The data on adhesion: adhesive, weakly adhesive and non-adhesive were analysed using the SAS version 9.2 statistical package. For each strain the association between breed, age of animal and adherence was analysed using the chi-square test for association of SAS version 9.2. An association between adhesion phenotypes and genotypes of the four candidate gene was also performed using a chi-square test in SAS.

4.3 Results

4.3.1 Association of breed and adherence by strain

For each strain, we observed three adhesion phenotypes, adhesive (Figure 4.1a), weakly adhesive (Figure 4.1b) and non-adhesive (Figure 4.1c) were observed. Most of the pigs with an adhesive phenotype had all their brush borders binding more than two bacteria.

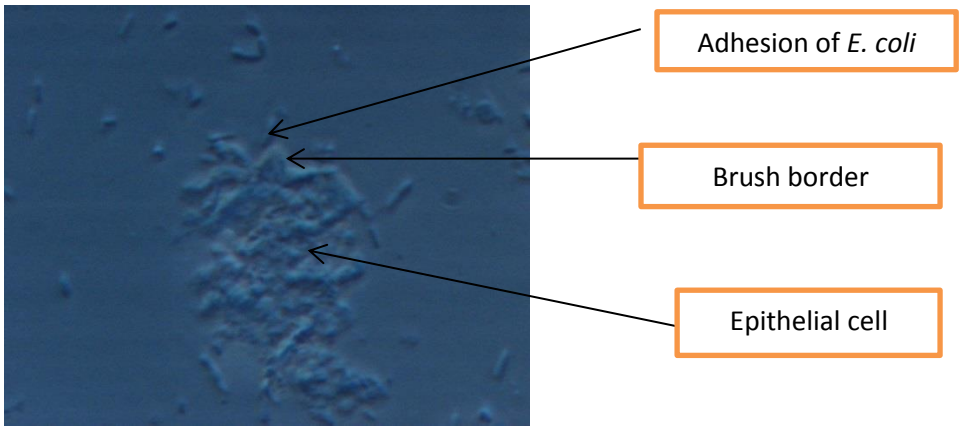

Figure 4.1a: Microscopic adhesion patterns in adhesive pigs

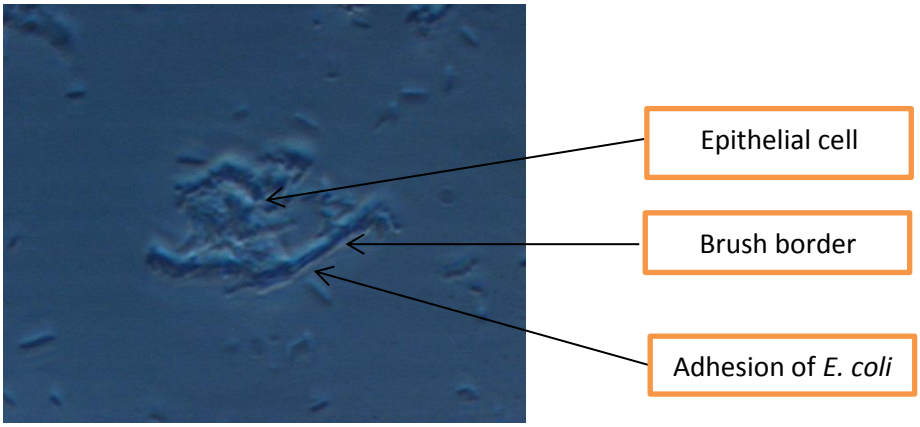

Figure 4.1b: Microscopic adhesion patterns in weakly adhesive pigs

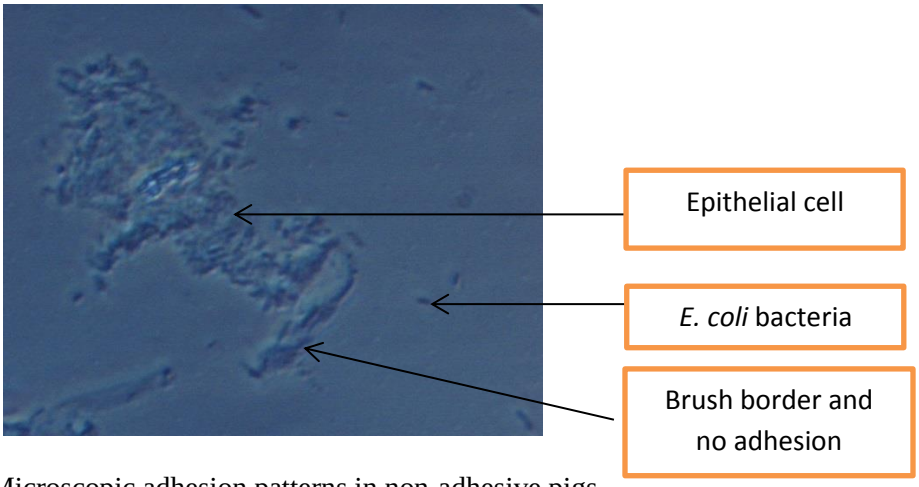

Figure 4.1c: Microscopic adhesion patterns in non-adhesive pigs

There was no association between breed and adherence observed for the EAST-1 ETEC toxin. Significant association of breeds were observed for F4 and PAA strains ($P < 0.05$) as shown in Table 4.1. Majority of the pigs' intestines were adhesive to F4 ETEC across all breeds, with relatively fewer non-adhesive and weakly adhesive phenotypes observed. Adhesion of PAA to pig intestines followed a similar trend across all breeds. In the F4 ETEC strain, indigenous pigs had the highest frequency of intestines which were adhesive followed by crossbred then Large White pigs. Similarly, indigenous pigs also had the highest frequency of intestines which were weakly and non-adhesive, followed by Large White then crossbred pigs. Large white pigs had no individual pigs which were non-adhesive. Therefore, all Large White pigs adhered to F4 regardless of level of adherence. For the PAA ETEC strain, indigenous pigs had the highest frequency of intestines which were adhesive, followed by crossbred then Large White pigs. Indigenous pigs also had the highest frequency of intestines which were weakly adhesive to PAA strain, followed by Large White then crossbred pigs. Similarly, crossbred pigs also had the lowest frequency of intestinal cells which were non-adhesive and indigenous pigs had the highest non-adhesive frequency to PAA. Therefore, a large proportion of the crossbred pigs were adhesive to PAA.

4.3.2 Association of strain and adherence by breed

There was a significant ($P < 0.05$) association between Large White pigs and ETEC strain adherence (Table 4.2). The F4 ETEC strain had the highest frequency of adhesive Large White intestinal cells, followed by EAST-1 toxin then PAA strain. This trend was similar in the weakly adhesive phenotype, F4 strain had the highest frequency, followed by PAA strain then EAST-1 toxin. The highest frequency of non-adhesive Large White pig intestinal cells was in PAA strain followed by EAST-1 toxin. The F4 ETEC strain had no Large White individual pigs which had cells which were non-adhesive. Large White pigs were either adhesive or weakly adhesive to F4.

Table 4.1: Frequencies (%) and numbers (in parentheses) of the three adhesive phenotypes

Adhesive phenotype	F4				EAST-1				PAA			
	Total	**IND[d]**	**LW[e]**	**CR[f]**	**Total**	**IND**	**LW**	**CR**	**Total**	**IND**	**LW**	**CR**
AD[a]												
	62.39	32.11	14.68	15.60	68.81	42.20	11.93	14.68	66.06	36.70	10.09	19.27
	(68)	(35)	(16)	(17)	(75)	(46)	(13)	(16)	(72)	(40)	(11)	(21)
WAD[b]												
	18.35	11.93	3.67	2.75	8.26	4.59	0.92	2.75	12.84	10.09	1.83	0.92
	(20)	(13)	(4)	(3)	(9)	(5)	(1)	(3)	(14)	(11)	(2)	(1)
NAD[c]												
	19.27	16.51	0.00	2.75	22.94	13.76	5.50	3.67	21.10	13.76	6.42	0.92
	(21)	(18)	(0)	(3)	(25)	(15)	(6)	(4)	(23)	(15)	(7)	(1)
Total												
	109	66	20	23	109	66	20	23	109	66	20	23
[g]P- value		*				NS				*		

[a] AD: Adherence, [b] WAD: Weak adherence, [c] NAD: Non-adherence, [d] IND: Indigenous pigs, [e] LW: Large White pigs, [f] CR: Crossbred pigs, [g] P-value *<0.05*

Table 4.2: Frequencies (%) and numbers (in parentheses) of the three adhesive phenotypes in each breed for three ETEC strains

	Indigenous			Large White			Cross		
Strain	**AD[a]**	**WAD[b]**	**NAD[c]**	**AD**	**WAD**	**NAD**	**AD**	**WAD**	**NAD**
F4									
	17.68	6.57	9.09	26.67	6.67	0.00	24.64	4.35	4.35
	(35)	(13)	(18)	(16)	(4)	(0)	(17)	(3)	(3)
EAST-1									
	23.23	2.53	7.58	21.67	1.67	10.00	23.19	4.35	5.80
	(46)	(5)	(15)	(13)	(1)	(6)	(16)	(3)	(4)
PAA									
	20.20	14.65	24.24	18.33	3.33	11.67	30.43	1.45	1.45
	(40)	(29)	(48)	(11)	(2)	(7)	(21)	(1)	(1)
Total									
	61.11	14.65	24.24	66.67	11.67	21.67	78.26	10.14	11.59
	(121)	(29)	(48)	(40)	(7)	(13)	(54)	(7)	(8)
P-value[d]		NS			*			NS	

[a] AD: Adherence, [b] WAD: Weak adherence, [c] NAD: Non-adherence, [d] P-value *<0.05*

4.3.3 Association of age and adherence by strain

There was a significant (P *<0.05*) association between age and level of adherence in indigenous pigs for the F4 ETEC strain. There was no association in the EAST-1 toxin and PAA strain (Table 4.3). In the F4 ETEC strain, indigenous weaned pigs had a higher frequency of adhesive cells than suckling piglets. This trend was similar for the weakly adhesive phenotype. Suckling indigenous pigs had a higher frequency of non-adhesive intestines to F4 strain than weaned pigs. There was a significant (P *<0.05*) association between age and adherence in crossbred pigs for the EAST-1 toxin, but there was no association in the F4 and PAA strains. Weaned crossbred pigs had a higher frequency of adhesive cells to EAST-1 toxin, in comparison with suckling crossbred pigs. Weaned crossbred pigs also had a higher percentage of intestinal cells that had a weak adhesive phenotype to EAST-1 toxin than suckling pigs. Suckling crossbred piglets had the highest frequency of intestinal cells that were non-adhesive to EAST-1 toxin. Weaned pigs had no individual intestinal cells that were non-adhesive to EAST-1 strain. There was no association between age and adherence in Large White pigs for all strains.

4.3.4 Association of candidate gene polymorphisms with susceptibility to ETEC F4

The c.291C>T (*TFRC*), c.576C>T (*MUC13*), c.8277G>C (*MUC4*) and g.191C>T (*MUC20*) polymorphisms which confer resistance to *E. coli* F4 did not show significant (P *>0.05*) association with adhesion to *E. coli* F4 (Table 4.4).

Although all results were non-significant, the c.291C>T polymorphism for *TFRC* showed that the *CC* homozygous genotype was predominantly observed in susceptible (adhesive) pigs, followed by weakly adhesive then non-adhesive.

Table 4.3: Frequencies (%) and numbers (per strain) of the three adhesive phenotypes before weaning (BW) and after weaning (AW)

	F4				EAST-1				PAA			
	AD[a]	WAD[b]	NAD[c]	Total	AD	WAD	NAD	Total	AD	WAD	NAD	Total
Age	**INDIGENOUS**											
BW												
	15.15 (10)	3.03 (2)	16.67 (11)	34.85 (23)	19.70 (13)	1.52 (1)	13.64 (9)	34.85 (23)	19.70 (13)	4.55 (3)	10.61 (7)	34.85 (23)
AW												
	37.88 (25)	16.67 (11)	10.61 (7)	65.15 (43)	50.00 (33)	6.06 (4)	9.09 (6)	65.15 (43)	40.91 (27)	12.12 (8)	12.12 (8)	65.15 (43)
P-value[d]		*				NS				NS		
	LARGE WHITE											
BW												
	50.00 (10)	10.00 (2)	0.00 (0)	60.00 (12)	35.00 (7)	5.00 (1)	20.00 (4)	60.00 (12)	35.00 (7)	10.00 (2)	15.00 (3)	60.00 (12)
AW												
	30.00 (6)	10.00 (2)	0.00 (0)	40.00 (12)	30.00 (6)	0.00 (0)	10.00 (2)	40.00 (8)	20.00 (4)	0.00 (0)	20.00 (4)	40.00 (8)
P-value		NS				NS				NS		
	CROSS											
BW												
	21.74 (5)	8.70 (2)	8.70 (2)	39.13 (9)	17.39 (4)	4.35 (1)	17.39 (4)	39.13 (9)	39.13 (9)	0.00 (0)	0.00 (0)	39.13 (9)
AW												
	52.17 (12)	4.35 (1)	4.35 (1)	60.87 (14)	52.17 (12)	8.70 (2)	0.00 (0)	60.87 (14)	52.17 (12)	4.35 (1)	4.35 (1)	60.87 (14)
P-value		NS				*				NS		

[a] AD: Adherence, [b] WAD: Weak adherence, [c] NAD: Non-adherence, [d] P-value *<0.05*

Table 4.4: Association of genotypes at polymorphic sites with ETECF4 adhesion phenotypes

		ETEC F4			
Locus	**Genotype**	**Adhesion**	**Weak adhesion**	**Non-adhesion**	**[a]P-value**
Sample size			109		
c.291C>T	*CC*	50	10	8	NS
	CT	3	4	1	
	TT	1	0	0	
c.576C>T	*CC*	50	10	10	NS
	CT	1	1	0	
	TT	0	0	0	
c.8227G>C	*GG*	6	1	3	NS
	GC	53	19	15	
	CC	1	0	0	
c.191C>T	*CC*	21	9	6	NS
	CT	15	5	4	
	TT	4	0	2	

[a]P- value <*0.05*

The heterozygous *CT* genotype was observed in eight pigs, majority of which were of a weakly adhesive phenotype, followed by adhesive then non-adhesive phenotypes. Only one pig had the homozygous *TT* genotype and this pig was adhesive to ETEC F4. The *CC* genotype for *MUC13* was predominantly observed in adhesive pigs and this genotype had the same number of animals which were weakly adhesive and non-adhesive. The *CT* genotype was observed in two pigs, one which had an adhesive phenotype and another with a weakly adhesive phenotype. The *TT* genotype was not observed in any pigs.

The c.8277G>C polymorphism for *MUC4* showed that the heterozygous *GC* genotype was predominantly observed in susceptible pigs, followed by weakly adhesive then non-adhesive. The *CC* genotype was only observed in one pig which was adhesive to ETEC F4. The *GG* genotype was observed in a few animals, with the highest number of pigs having an adhesive phenotype, followed by non-adhesive then weakly adhesive phenotype. The c.191C>T polymorphism for *MUC20* showed that the *CC* genotype was predominantly observed in adhesive pigs, followed by weakly adhesive then non-adhesive pigs. The *CT* genotype was observed in 24 pigs and the highest number had an adhesive phenotype, followed by weak adhesion then non-adhesive. The *TT* genotype was observed in 10 animals, eight having an adhesive phenotype and two having a non-adhesive phenotype.

4.4 Discussion

The susceptibility of South African pigs to *E. coli* infection was investigated in this study through an *in vitro* adhesion test of the prevalent ETEC pathotypes of the pig's intestines. The prevalence of ETEC pathotypes in a South African population was previously tested (Mohlatlole *et al.,* 2013). The F4 fimbrial strain was not observed, however, non-fimbrial PAA strain and EAST-1 toxin were observed. This is why we used F4, PAA and EAST-1 for adhesion in the current study instead of other colonising factors. The results from the current population showed that pigs in the South African population contain receptors for both non-fimbriated PAA strain and EAST-1 toxin and the fimbriated F4 ETEC strain.

There was a significant difference in the level of adherence of F4 ETEC strain. In indigenous pigs the adhesive phenotype had highest frequency followed by the non-adhesive then weakly adhesive phenotype. This trend was similar in crossbred pigs. In Large White pigs, the non-adhesive phenotype had the lowest frequency. In fact no Large White individual pig intestinal cells carried the phenotype. Overall, indigenous pigs had the highest frequency of adhesive, weakly adhesive and non-adhesive intestinal cells followed by crossbred then Large White pigs. Considering that the Large White pigs sampled were reared in a commercial setup with strict management procedures, it is also possible that the parents of the Large White pigs in the current study were vaccinated with a purified F4 vaccine to prevent ETEC infections in suckling piglets, because the immunoglobulin A (IgA) can be transmitted via colostrum and milk in suckling piglets (Duan *et al.,* 2011). This method is not effective in weaned pigs, which may have resulted in some of the Large White pigs adhering to F4 but, at a lower frequency than indigenous and crossbred pigs. Furthermore, these Large White pigs may have been treated for diarrhoea after birth, which could have resulted in a lower frequency of adhesive intestinal cells.

The indigenous and crossbred pigs were kept in a small scale system where the pigs are allowed to scavenge in an open environment, and where there are no biosecurity measures taken. Therefore, there are no vaccinations or any precautions taken against diseases which could have increased the frequency of adherence by the F4 strain in both breeds. The F4 strain adhered to the brush borders of all pig breeds. This shows that the population of pigs contains intestinal receptors for F4 ETEC strain, which facilitate the attachment of ETEC to the small intestine of a pig.

The level of adherence of the PAA strain was also significantly associated with the breed. All breeds had intestines which adhered to the PAA strain. Similar to the F4 strain, in indigenous pigs, the highest frequency was in the adhesive, followed by non-adhesive then weakly adhesive phenotypes. The Large white and crossbred pigs followed a similar trend. Overall, the indigenous pigs had the highest frequency of adhesive, weakly adhesive and non-adhesive pig intestinal cells amongst the three breeds. Therefore,

the pigs in this population had receptors for PAA ETEC strain. As observed the highest frequency of PAA adhesion was in the indigenous breed. These results agree with Romer *et al.* (2012). About 3 PAA ETEC strain adhered to one cell from a wild boar pig and 0.49 PAA strains adhered to one cell from domestic pigs reared in a commercial system. The PAA strain is a new and potential virulent non-fimbrial ETEC strain (Batisson *et al.,* 2003) and its level of adherence has never been tested in the South African pig population.

The level of adherence of EAST-1 toxin was not associated with the breed. Enteroaggregative heat-stable enterotoxin 1 (EAST-1) is an ETEC toxin and is only released after an ETEC strain attaches to the receptors on a pig's intestinal cell, which may explain the lack of association between EAST-1 toxin adherence and breed. These enterotoxins release water and electrolytes which result in diarrhoea. Osek (2003) have reported that EAST-1 toxin is found in isolates that also contain F4 and F18 ETEC strains. Ngeleka *et al*. (2003) also reported that EAST-1 toxin contributes to pathogenicity only in combination with other virulence genes. Therefore, for EAST-1 toxin to be a significant threat, it has to be in the presence of an ETEC strain. There are other cases were EAST-1 toxin has been isolated on its own (Choi *et al.,* 2001).

Indigenous pigs had the highest frequency of intestinal cells that were non-adhesive to F4 (17 %) and PAA (14 %), therefore, they were more resistant when compared to crossbred and Large White pigs. According to the results, Large White pigs were more susceptible to F4 amongst the three breeds because there were no individual pig intestinal cells that were non-adhesive. Indigenous pigs are known to be hardy, disease resistant and can adapt well to environmental conditions. If the Indigenous pigs had picked up ETEC F4 in the environment they were reared in, they may have developed resistance. The Large White pigs were either adhesive or weakly adhesive, which makes it difficult to select any of the Large White pigs to breed against ETEC F4. The crossbred pigs were more susceptible to PAA when compared to Large White pigs, because of the low frequency (0.9 %) of non-adhesive crossbred cells.

The indigenous and crossbred pigs, had intestines with a non-adhesive phenotype for both F4 and PAA strains, suggesting that a proportion of the indigenous and crossbred pigs did not contain the receptors for F4 and PAA and therefore could be selected to be used in F4 and PAA ETEC breeding programs. Our results agree with Li *et al*. (2007), who reported that Large White pigs were more susceptible to F4 than Chinese Songliao Black indigenous pigs. In another study Yan *et al.* (2009), reported that indigenous Chinese pigs were highly resistant to F4 as compared to the Duroc, Large White and Landrace.

In the Large White breed which was the most susceptible amongst all breeds, the F4 strain had the highest frequency of adhesive pig intestinal cells in comparison to EAST-1 toxin and PAA strain. These results were to be expected because the F4 strain is the most common *E. coli* strain isolated from pigs affected by ETEC diarrhoea (Moon *et al.*, 1977; Nagy & Fekete, 1997; Li *et al.*, 2007). Our findings are in contrast to a study carried out in a South African population on the prevalence of colibacillosis. The F4 strain was not prevalent, but PAA and enterotoxin EAST-1 were detected in 18 and 20 % of the piglets, respectively (Mohlatlole *et al.,* 2013). The EAST-1 toxin adhered at a higher frequency than PAA strain in Large White pig intestinal cells, which agrees with Mohlatlole *et al* (2013).

There was a significant association between age and adhesion. These results agree with Willemsen and Graaf (1992) who reported that the presence of receptors on brush border cells is dependent on age. In indigenous pigs, there was an association of F4 ETEC strain and age and in crossbred pigs the association was in the EAST-1 toxin. In both indigenous and crossbred pigs, the frequency of adhesion was higher in post-weaned than suckling pig intestinal cells in F4 and EAST-1, respectively. Henton and Engelbrecht (1997) also found higher cases of *E. coli* diarrhoea in weaned South African pigs than suckling ones. Diarrhoea caused by *E. coli* infection is prevalent in both suckling and weaned pigs (Debroy and Maddox, 2001; Li *et al.*, 2007; Andrea *et al.*, 2011), but it is most commonly found in weaned pigs (Fairbrother *et al.*, 2005; Duan *et al.*, 2011) leading to post-weaning diarrhoea. *Escherichia coli* infection is also increased in weaned pigs due to stress from separation from the sow, introduction to a new environment

and the introduction to a new diet (Henton and Engelbrecht, 1997). In Large White pigs, adhesion of F4, PAA and EAST-1 toxin was independent of age.

The results showed that the South African population studied contained receptors for F4 and PAA ETEC strains and EAST-1 toxin in all three breeds and if there is an outbreak of *E. coli* containing these strains, then the population is most likely to be affected. The population showed that PAA had the highest adhesive frequency than F4 in all three breeds. This agrees with Mohlatlole *et al.* (2013), were the F4 strain was less prevalent than PAA strain and EAST- toxin. The adhesion results also show that the current population was not significantly associated to EAST-1 toxin adherence, which disagrees with Mohlatlole's *et al.* (2013) prevalence study, were EAST-1 toxin was more prevalent than F4 and PAA strains.

Considering that *TFRC*, *MUC4*, *MUC13* and *MUC20* are positional candidate genes for ETEC F4 receptors on SSC13q41, we aimed to evaluate the associations between adhesion phenotypes and genotypes of these candidate genes in a South African population. There was no significant association between F4 adhesion phenotypes and candidate gene genotypes in the current population. The candidate genes were previously tested in a South African population in Chapter three and alleles in *TFRC* and *MUC13* candidate genes were close to fixation or fixed. Furthermore, the genes were lowly polymorphic in *TFRC* and *MUC13* and moderately polymorphic in *MUC4* and *MUC20* genes. These factors could have influenced a non-significant association, because not all candidate genes segregated in the population and the genes did not have enough discriminatory power resulting from some of the genes being fixed or close to fixation.

For the TFRC c.291C>T polymorphism, the *CC* genotype was predominant in pigs susceptible to F4, which is in agreement with Wang *et al.* (2007), who reported that the *CC* genotype was observed in pigs susceptible to F4. For the *MUC13* c.576C>T polymorphism, two genotypes *CC* and *CT* were found and

where in both resistant and susceptible pigs. The *C* allele was predominant in susceptible pigs; however we did not identify the causal mutations that absolutely distinguished susceptible pigs form resistant pigs. This is in agreement with Zhang *et al.* (2008) who failed to identify the causal mutation for ETEC F4. They identified a strong association of *MUC13* haplotypes with susceptibility/resistance to ETEC F4. For the *MUC20* g.191C>T polymorphism, allele *C* was predominant in animals that were susceptible to ETEC F4 and there was no significant association between adhesion phenotypes and *MUC20* genotypes. This is in contrast with Ji *et al.* (2011), where allele *C* was predominant in resistant pigs and also there was a strong association between the *MUC20* polymorphism and ETEC F4 adhesion phenotypes. Rampoldi *et al.* (2011) observed a Large White boar with a recombination between the ETEC F4 receptor and the polymorphism in *MUC4* and suggested the causative mutation to be more downstream of the chromosome 13 and possibly located around the region of *MUC13* and *MUC20*. Zhang *et al.* (2007) and Ji *et al.* (2011) both came to the same conclusion that the polymorphisms in *MUC13* and *MUC20* are not causative mutations, but are good markers for ETEC F4. For the *MUC4* g.8227 G>C polymorphism, the heterozygous *GC* genotype was predominant in susceptible pigs. These results agree with Jorgensen *et al.* (2004), were the *GC* genotype was heterozygous susceptible in a genotyping test.

4.5 Conclusions

Pigs in all breeds contained receptors for F4 and PAA strains and EAST-1 toxin which lead to the significant adherence of these isolates. Indigenous pigs were more resistant to F4 and PAA ETEC strains in comparison to Large White and crossbred pigs. All Large White pigs were susceptible to F4 ETEC infection. The level of adherence in Large White pigs was dependent on the strain, and F4 *E. coli* had the highest level of adherence in Large White pigs. The EAST-1 toxin is more likely to cause diarrhoea in pigs if it is associated together with an ETEC strain. In Large White pigs, adhesion occurred at a high level, regardless of age of pigs. In indigenous and crossbred pigs, adhesion was higher in weaned pigs

than in suckling pigs. The detected *MUC4*, *MUC13*, *MUC20* and *TFRC* polymorphisms were not significantly associated to ETEC F4 adhesion phenotypes.

4.6 References

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Chapter 5: General Discussion, Conclusions and Future Research

5.1 General discussions

Colibacillosis is one of the most important pre-weaning diseases in pig production in South Africa (Henton, 2010). Enterotoxigenic *Escherichia coli* is the major cause of colibacillosis affecting both neonatal and weaning piglets (Li *et al.,* 2007). Colibacillosis leads to significant losses to the pig industry due to mortality, increased use of antibiotics, excessive weight loss and frequent vaccinations. Breeding for disease resistance is becoming increasingly important and could reduce losses due to *E. coli* infections in the pig industry.

The onset of diarrhoea is usually caused by the interaction of the host and pathogens. The interaction involves three steps, which are, adhesion of fimbriae to specific receptors on the small intestine, colonization of the small intestines and production of toxins. The most frequently isolated *E. coli* strain from cases of diarrhoea is F4 (Chen *et al.,* 2004) and *in-vitro* adhesion tests have associated F4*ab/ac* with receptor ECF4*bc*R (Baker *et al.,* 1997). The receptors for F4 are closely related to sialoglycoproteins and mucin type glycoproteins. Molecular studies have positioned the locus for this receptor on pig chromosome (SSC) 13q41. The actual causative mutation remains unknown (Ren *et al.,* 2009). Mucin genes, *MUC4, MUC13, MU20* and Transferrin receptor (*TFRC*) have been identified as candidate genes for resistance to *E. coli* F4 since they have been mapped in the same region as the ECF4*bc*R (Ren *et al.,* 2009). The polymorphisms of these genes showed a strong association with F4*ab/ac E. coli* adhesion phenotypes in White Duroc x Erhualian populations (Jorgensen *et al.,* 2004; Wang *et al.,* 2007; Zhang *et al.,* 2008; Ji *et al.,* 2011).

South Africa has a diversity of pig breeds and their genetic status to ETEC F4 disease resistance is unknown. Huge sums of money are spent on antibiotics for colibacillosis control. The genetic variation in the South African pig population for the *MUC4*, *MUC13*, *MUC20*, and *TFRC* genes which have been

reported as important in conferring resistance/susceptibility to *E. coli F4ab/ac* was reported in Chapter 3. Three breeds were studied, imported (Large White, Landrace and Duroc), local (indigenous) and crossbred pigs. The *C* allele was close to fixation at over 90 % in *TFRC* and *MUC13* genes, showing allele fixation at these two loci. Furthermore, these two genes did not segregate in the studied population and were not polymorphic. The *C* allele and *CC* genotype which were shown to confer susceptibility in previous studies (Wang *et al.*, 2007; Zhang *et al.*, 2008), are the most frequent in the South African population. The lower levels of heterozygosity at these two genes and the dominant prevalence of the *C* allele leaves no room for selection using the *TFRC* and *MUC13* candidate genes in these populations.

The *MUC4* gene was moderately polymorphic in the South African pigs. The *G* allele which confers resistance to ETEC F4 (Jorgensen *et al.*, 2004) had the highest frequency. The imported pigs had the highest frequency of the favorable *G* allele in all three breeds. Imported pigs from the current population were reared under a commercial setup, which is influenced by intense selection for a high lean growth rates. These effects of selection could have influenced a high frequency of the resistant *G* allele because unhealthy animals that do not perform well may have been culled from the population. The heterozygous *GC* genotype had the highest frequency in all three breeds showing increased variation for the *MUC4* marker. The higher PIC and heterozygosity values for *MUC4* would make *MUC4* a potential marker for the selection against susceptibility to F4 ETEC infections in this population. For the *MUC20* locus, the favorable *C* allele which confers resistance to ETEC F4 (Ji *et al.*, 2011) had the highest frequency and was segregating in the crossbred and local pigs. The *CC* genotype also had the highest frequency in all breeds and this gene was moderately polymorphic. There is therefore, also room for selection for the *MUC20* gene in the studied pig population.

The *TFRC* and *MUC13* candidate genes both had low observed and expected heterozygosity values resulting in a deficit in heterozygotes, signifying low levels of diversity in all breeds at these loci. The imported breeds at the *MUC20* locus had a higher expected than observed heterozygosity, resulting in low

diversity in the imported pigs. However, the crossbred and imported pigs had higher observed heterozygosity values resulting in higher diversity. Therefore, *MUC20* can be an informative marker for crossbred and local pigs in the studied population. The *MUC4* locus could also be a useful marker in the South African population because it had high average heterozygosity values in all three breeds. The *TFRC, MUC13* and *MUC20* genes had positive F_{IS} values, suggesting inbreeding at these loci. The *MUC4* gene had negative F_{IS} values indicating that the subpopulations were outbred at these loci. Overall the population had a negative F_{IS} value, and the gene that contributed to outbreeding in the overall population was *MUC4*.

All breeds were in HWE for the *MUC13* and *MUC4* genes. For *TFRC* and *MUC20* genes, the imported breeds deviated from HWE. Factors that may result in deviation from HWE are migration, presence of mutations, selection, non- random mating, gene flow and also large population sizes. The imported breeds are reared in a commercial production system where there is selection for productive traits like growth rate, which eliminates poor performing individual pigs. Selection of productive traits influences gene flow from Artificial Insemination and importation of breeds with favorable traits from other countries. The presence of selection also eliminates non-random mating. Furthermore, pigs in a commercial production system are kept in large numbers.

In the current South African population there was no significant association amongst the four loci. Populations were combinations of alleles or genotypes are not in expected proportions are not found to be in linkage disequilibrium. The level of linkage disequilibrium is influenced by selection, rate of recombination, rate of mutation, genetic drift, non-random mating and population structure.

In Chapter 3, South African pigs were genotyped in order to analyse the prevalence of resistant and susceptible alleles in four candidate genes and ascertain whether these genes can be used in the population against ETEC infection. In Chapter 4, the susceptibility of South African pigs to ETEC strains was tested

in order to determine if the population carried receptors that could facilitate the adhesion of ETEC. There was a significant difference in the level of adherence of the F4 and PAA strains. The level of adherence for F4 was highest in the indigenous pig intestinal cells and lowest in Large White. Furthermore, Large White pigs all adhered to F4 and indigenous pigs had the highest frequency of non-adhesive intestinal cells. For the PAA strain, indigenous pigs had the highest frequency of intestinal cells which adhered to the strain and Large White pigs had the lowest. Indigenous pigs also had the highest frequency of intestines which did not adhere to PAA and Large White had the lowest frequency. Indigenous pigs are known to be hardy and disease resistant, which could have resulted in a high frequency of pig intestinal cells which were non-adhesive. Large white pigs face selection pressures and are selected for productive traits like growth rate and lean meat, which could have influenced a low adhesive frequency than in indigenous pigs, because poorly performing individuals in commercial breeds are usually culled from the population. The selection pressure on growth rate in indigenous pigs in South Africa is generally low. This may be the reason why indigenous pigs had the highest frequency of F4 and PAA adhesion in comparison to Large White pigs.

The association between breed and strain of ETEC was determined. There was a significant difference in the Large White pigs and the highest frequency of adherence was found in the F4 strain. The F4 strain also had the lowest frequency of Large White pig intestinal cells which were non-adhesive. This confirms previous reports that F4 is the most frequently isolated strain of ETEC diarrhoea and that Large White, Landrace and Duroc pigs are more susceptible to *E. coli* infection than indigenous pigs (Li *et al.,* 2007). There was a significant difference in the level of adherence and age in the indigenous and crossbred pigs. For the F4 strain there was a significant difference in the age at which adherence occurred in indigenous pigs. The level of adherence was higher in weaned than suckling pig intestinal cells. In addition, the highest frequency of non-adhesive indigenous pig cells was found in suckling than weaned pigs. Similarly, in the crossbred pigs there was a significant difference between age and the level of adhesion of the EAST-1 toxin. The frequency of adherence was higher in weaned pig cells with the highest frequency

of non-adhesive pigs being recorded in suckling crossbred intestinal cells. Suckling pigs receive immunity through antibodies from the sow's milk, which in turn protects them from infection. After weaning this immunity is lost because weaners no longer receive suckling milk. This could have resulted in a higher frequency of non-adhesion in suckling pig cells and a high frequency of adhesion in weaned pig cells.

We also associated the candidate genes in Chapter 3 and the F4 adhesion phenotypes in Chapter 4, in order to determine whether the genotypes in Chapter 3 are associated with the ETEC receptors in South African pigs. There was no significant difference between the candidate gene genotypes and F4 adhesion phenotypes. These results may have been attributed to the low polymorphic values, allele fixation and genotype dominance in *TFRC* and *MUC13* genes.

5.2 Conclusions

The *TFRC* and *MUC13* candidate genes were fixed or close to fixation in all breeds and were also lowly polymorphic. Therefore, they are not good markers to be used in the South African population. The *MUC4* and *MUC20* candidate genes are potential genes which can be used against *E. coli* infection in the South African population, since they were moderately polymorphic and also segregated. However, there was no significant difference between F4 adhesion phenotypes and the genotypes from these two genes, which also makes them unsuitable for use in selection programs. The South African pig population carries receptors which facilitate the adhesion of F4, PAA strains and EAST-1 enterotoxin. These receptors however, are not linked to the candidate genes investigated in this study. The indigenous pigs were more resistant to adhesion and the Large White pigs were more susceptible to adhesion of all the three *E. coli* strains. Adhesion in Large White pigs occurred regardless of age, but adhesion in indigenous and crossbred pigs was higher in post-weaned pigs.

5.3 Future research

Future research in the South African pig population should focus on other candidate genes that are polymorphic and which carry genotypes that are associated to F4 ETEC adhesion phenotypes. A genome wide association study can provide higher marker density, where many single nucleotide polymorphisms (SNPs) can be tested simultaneously. Studies on the susceptibility of pigs from other regions using *in vitro* adhesion tests should also be carried out to have a better understanding of the status of the pigs in the region.

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