# BREED EFFECTS ON THE VIRULENCE GENE PROFILES AND GENETIC DIVERSITY AT *FUT1*, *MUC4*, *MUC13* AND *MUC20* CANDIDATE GENES FOR CONTROLLING DIARRHOEA-CAUSING *ESCHERICHIA COLI*

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Submitted in fulfillment of the requirements for the degree of Master of Science (Genetics) in the School of Life Sciences, University of KwaZulu-Natal

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# ABSTRACT

*Escherichia (E) coli* infections result in diarrhoea and oedema in growing pigs. Enterotoxigenic (ETEC), shigatoxin producing (STEC) and enteroaggregative (EAEC) *E. coli* have been identified as the principal causes of colibacillosis in most pig production systems. These *E. coli* use fimbrial and non-fimbrial adhesins to adhere to the intestines and cause infection. Absence or presence of the receptors on the intestinal walls determines the resistance or susceptibility of the host to the *E. coli.* In other populations, candidate genes linked to the receptors have been found to be associated with resistance/susceptibility to infection and are used in marker-assisted selection programs. This study investigated the presence and prevalence of ETEC, STEC and EAEC and the associated virulence genes in 263 *E. coli* isolates sampled from Landrace, Large White, Duroc and Indigenous piglets from the Animal Production Institute of the Agricultural Research Council (ARC) in Irene and Middledrift farm in Eastern Cape Province. The study also investigated polymorphisms at six candidate genes associated with two *E. coli* receptors in the same pig populations. Over 39 % of the isolates tested positive for the *E. coli* virulent genes investigated. None of the samples had fimbrial adhesins. The mode of attachment of the investigated *E. coli* was through non-fimbrial adhesins which were found in 49.06% of the isolates. The 106 *E. coli* isolates were categorized into 25 pathotypes carrying definable and unique combinations of *E. coli* virulence factors.

The resistant allele for *Alfa (1) fucosyltransferase 1* (*FUT1*) *M307*, a candidate gene for *FI8R*, was present in less than 1 % of the population. Various mutations of mucin genes *MUC4 g.8227*, *MUC20 c1600* and *g.191* were found in the population. Their respective alleles for controlling *F4ab/ac E. coli* adhesion in pigs were predominant in both breeds. Three loci (*FUT1*, *MUC20 g.191* and *MUC20 c.1600*) deviated from Hardy Weinberg equilibrium (HWE) in the Indigenous and the Large White breeds. Heterozygotes deficiency and high levels of within breed diversity was observed in these two breeds at the mentioned loci. Overall, the study observed a wide range of toxin and colonisation factors (CFs) giving rise to diverse pathotypes in South African pigs. The absence of fimbrial adhesins suggests a different colibacillosis control program from that previously used. The presence of the resistant alleles in most of the loci investigated was low, however their presence suggest it is possible to use them to generate a resistant population using marker assisted selection. This study serves as a foundation for future pig colibacillosis control and immunity studies in the South African pig herds.

# DECLARATION – PLAGIARISM

I, **Ramadimetja Prescilla Mohlatlole**, declare that:

1. The research reported in this thesis is my original work, and where the work of others has been used the source is acknowledged accordingly.
2. This dissertation has not been submitted to any other University other than the University of Kwazulu-Natal
3. The experimental work described in the dissertation was conducted by me under the supervision of Mr E.F. Dzomba and Prof. M. Chimonyo.
4. All assistance towards the production of this work and all the references contained herein have been duly accredited.

**Ramadimetja Prescilla Mohlatlole: _____ Date: _____**

Approved as to style and content by:

**Mr E.F. Dzomba: _____ Date: _____**

**Prof M. Chimonyo: _____ Date: _____**

# PUBLICATIONS

Details of contribution to publications that include research presented in this thesis:

1. Ramadimetja Prescilla Mohlatlole, Evelyn Madoroba, Farai Catherine Muchadeyi, Michael Chimonyo, Arnold Tapera Kanengoni and Edgar Farai Dzomba, 2013. Virulence profiles of enterotoxigenic, shigatoxin and enteroaggregative *Escherichia coli* in South African pigs. Tropical Animal Health Production, 45, 1399-1405.
2. R. P. Mohlatlole, E. Madoroba, F. C. Muchadeyi, A. T. Kanengoni, M. Chimonyo, and E. F. Dzomba, 2012. Diversity of *Escherichia coli* virulence genes for colibacillosis in piglets. In Proceedings of the 33rd conference of the 'International Society for Animal Genetics'. Cairns, Australia. (Abstract)

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Adenine |
| AA | Amino acid |
| ADG | Average daily gain |
| A/E | Attaching and effacing |
| AEEC | Attaching and effacing *Escherichia coli* |
| *AIDA-1* / *aidaA* | Adhesin involved in diffuse adherence factor or gene |
| *AluI* | Restriction enzyme of the *Arthrobacter luteus* origin |
| ARC | Agricultural Research Council |
| *astA* | Enteroaggregative heat-stable enterotoxins type 1 encoding gene |
| bp | Base pair |
| C | Cytosine |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanidine monophosphate |
| CFU | Colony forming units |
| CF (s) | Colonisation factor/ s |
| cM | Centi Morgan |
| CP | Crude protein |
| D | Day |
| DAFF | Department of Agriculture, Fisheries and Forestry |
| DE | Digestible Energy |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleoside triphosphates |
| *EAE/ eae* | Attaching and effacing factor or gene |
| EAEC | Enteroaggregative *Escherichia coli* |

| | |
|---|---|
| *EAST-1* | Enteroaggregative heat-stable toxin |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetra acetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| *elt/ eltAB* | Heat-labile toxin gene |
| *estI/A* | Heat-stable toxin variant 1 encoding gene |
| *estII/ B* | Heat-stable toxin variant 2 encoding gene |
| *et al* | (*et alii*) and others |
| EtBr | Ethidium bromide |
| ETEC | Enterotoxigenic *Escherichia coli* |
| *FbaI* | restriction enzyme sourced from *Flavobacterium balustinum* |
| *F* | Fimbrial antigen |
| F | Forward |
| $F_{is}$ | Inbreeding coefficient |
| $F_{it}$ | Average inbreeding coefficient of the individuals relative to the population |
| $F_{st}$ | Average inbreeding of the subpopulation relative to the whole population |
| *FaeG* | *F4* fimbriae gene |
| *FanA* | *F5* fimbriae encoding gene |
| *FasA* | *F6* fimbriae encoding gene |
| *fedA* | Fimbriae *FI8*encoding gene |
| *fim41a* | Fimbriae *F41* encoding gene |
| *FUT-1* | Alfa (1) fucosyltransferase |
| *FUT-2* | Alfa (2) fucosyltransferase |
| *FI8R* | *E. coli FI8* surface receptor |
| *F4bcR* | *E. coli F4ab/ac* surface receptor |

| | |
|---|---|
| G | Guanine |
| g | Gram |
| Gb4 | Globotetraosylceramide |
| Gp119 | A 119 kDa surface glycoprotein (*AIDA-1* surface receptor) |
| h | Hour |
| H | Flagella components antigen |
| $H_e$ | Expected heterozygosity |
| $H_o$ | Observed heterozygosity |
| *Hin6I* | restriction enzyme sourced from *Haemophilus influenza* |
| *HlyA* | haemolysin gene |
| HWE | Hardy-Weinberg equilibrium |
| IFA | Immunoflourescent assay |
| IMViC | Indole, Methyl red, Voges-Proskauer, Citrate |
| K | Capsular or surface component antigens |
| KDa | Kilo Daltons |
| *LMLN* | Leishmandysin-like gene |
| LEE | Locus of enterocyte effacement |
| *LT* | Heat-labile enterotoxin |
| mg | Milligram |
| MgCl2 | Magnesium Chloride |
| Min | Minute |
| MJ | Mega Joule |
| ml | Millilitre |
| mM | Millimole |
| *MUC* | Mucin genes |

| | |
|---|---|
| n/ N | Sample size |
| ND | Neonatal diarrhoea |
| *O* | Somatic cell wall component antigens |
| $^{o}$C | Degrees Celsius |
| *paa/PAA* | Porcine attaching and effacing–associated factor encoding factor or gene |
| PCR | Polymerase Chain Reaction |
| PWD | Post weaning diarrhoea |
| R | Reverse |
| RFLP | Restriction fragment length polymorphisms |
| *RYR-1* | Ryanodine receptor type 1 |
| SA | South Africa |
| SAS | Statistical Analysis System |
| SAT | Serum agglutination test |
| SNP (s) | Single nucleotide polymorphism/s |
| *ST* | Heat-stable enterotoxins |
| *STa/ I* | Heat-stable enterotoxin variant 1 |
| *STb/ II* | Heat-stable enterotoxin variant 2 |
| STEC | Shigatoxin producing *Escherichia coli* |
| *Stx* | Shigatoxin |
| *Stx2e* | Oedema associated shigatoxin |
| *stx1/ I* | Shiga toxin variant 1 encoding gene |
| *stx2/ II* | Shiga toxin variant 2 encoding gene |
| *stx2e* | Gene encoding shigatoxin associated with porcine oedema |
| T | Thymine |
| TAE | Tris-acetate |

| | |
|---|---|
| *Taq* | *Thermus aquaticus* |
| *Tir* | Traslocated intimin receptor |
| *TRFC* | Transferrin receptor |
| Tris-HCl | Trishydroxymethyl aminomethane-Hydrochloric acid |
| U | Uracil |
| USA | United State of America |
| UV | Ultraviolet |
| V | Voltage |
| VF (s) | Virulence factor /s |
| $X^2$ | Chi-square test |
| *XbaI* | Restriction enzyme sourced from *Xanthomonas badrii* |
| ZnO | Zinc oxide |
| - | Negative |
| + | Positive |
| Mg | Microgram |
| µl | Microlitre |
| µM | Micromole |
| % | Percentage |
| 1 U | Unit |

# CHAPTER 1

## General Introduction

### 1.1 Introduction

Pork is the third most produced meat in South Africa, coming second after beef and mutton. According to a report released by department of agriculture, forestry and fisheries (DAFF), South Africa produces approximately 1,599 million pigs annually, the majority of which come from commercial farms. Large-scale pig farmers mainly keep exotic breeds such as Duroc, Large White and composite breeds among which the South African Landrace is the most common. An annual pork consumption of 232,970 tonnes per year was recorded in the 2011/2012 production year, classifying pork as one of the important sources of animal protein for the local population (DAFF, 2012).

Apart from large commercial farms, small-scale pig farming plays a significant role in the South African economy (DAFF, 2012). Smallholder farmers keep indigenous pigs which they use as both an alternative investment and source of income especially in rural areas where poverty levels are high (Ironkwe and Amefule, 2008). Kolbroek and Windsnyer are popular breeds kept by smallholder farmers in the Eastern Cape and Limpopo provinces of South Africa (Halimani et al., 2010). The Mukota breed which is native to the Mukota area of north-eastern Zimbabwe is another breed reared in the small-scale farming sectors (Halimani et al., 2010).

Colibacillosis is a general term used to refer to *E. coli* infection symptoms in animals. The symptoms manifests as severe watery diarrhoea, oedema and neurological disorders in young pigs (Bosworth and Vögeli, 2003). Failure to control the disease results in poor weight gains and death which consequently

represents a loss to pork producers and the pig industry (Francis, 1999; Kemm, 1993). Three *E. coli* groups known as ETEC, STEC and EAEC have been commonly associated with colibacillosis in pigs (Chen et al., 2004; Choi et al., 2001). Current information on the prevalence of *E. coli* diarrhoea and oedema in pigs in South Africa is limited. The last documented cases of colibacillosis were from 1971 to 1991 which were reported by the Onderstepoort Veterinary Institute. Of the colibacillosis cases reported, 45.5 % of the piglets had inflamed small intestines, 46.9 % showed blood poisoning and only 1.8 % showed oedema signs (Henton and Engelbrecht, 1997). It was also reported that the *E. coli* infections were observed more frequently in weaners than in suckling piglets.

Antibiotics are the common control strategies used to treat bacterial infections (Bosworth and Vögeli, 2003). However, over the years, *E. coli* isolated from piglets affected by colibacillosis has shown resistance to the commonly used antibiotics (Hammerum and Heuer, 2009), thus increasing the probability of *E. coli* infections. Some *E. coli* are zoonotic, therefore, transmission from pigs to humans through contaminated meat and other products is possible. Meat contamination by *E. coli* occurs during slaughter and it has been reported that colonies of *E. coli* from farms and other animal sources have been found in retail meat (Hammerum and Heuer, 2009). *Escherichia coli* infections occur even when other conventional control strategies such vaccinations are used and strict biosecurity is implemented (Bilkei and Biro, 2000; Pittman, 2010).

Selection for disease resistance is one alternative strategy that could be used to control diseases such as *E. coli*. Animals do not show the same response when exposed to infections (Bilkei and Biro, 2000). Several factors can contribute to the development of disease symptoms and these include age, stress levels, animal biological status, immunological status as well as the genetic profiles of the animal (Rothschild et al., 2007; Pittman, 2010 ). Since most of the DNA sequence variations in animals are heritable, selective breeding has been used as an important strategy to increase disease resistance (Bertschinger et al., 2004; Rothschild et al., 2007). Advances in molecular biology have provided the research tools necessary to detect small changes in the base sequences of the DNA between animals. These sequence variations in

animals have been studied in association with responses to infectious diseases and markers have been developed for predicting the occurrence of diseases (Stone and Cundiff, 1985).

Resistance to *E. coli F4* and *FI8* infections in pigs is controlled by the presence or absence of receptors in the host intestinal tissues. Receptors for *F4* and *FI8 E. coli* have not yet been characterized but linkage studies have identified candidate genes which are associated with the presence or absence of these receptors (Sellwood, 1983; Meijerink et al., 1997; Jensen et al., 2006). The candidate gene alpha-1fucosyltransferase (*FUT1*) controls the resistance to *FI8* fimbriated *E. coli* that causes oedema and diarrhoea in Swiss Landrace and Large White pigs (Meijerink et al., 1997). Several Mucin genes (*MUC4, MUC13* and *MUC20*) have been found to reduce susceptibility to *F4 E. coli* in Danish crossbreed and White Duroc × Erhualian intercrosses (Jensen et al., 2006; Zhang et al., 2008; Ji et al., 2011)

## 1.2 Justification

In any industry, production is more profitable when an efficient production system is applied and the costs of production are lower than the marginal returns. In meat production, medication and disease control programmes contribute significantly to the share of the inputs used in production and, therefore, affect profitability (Otte and Chilonda, 2000). Since disease outbreaks are spontaneous and unpredictable, the actual figure of disease control costs as a proportion of the total inputs used in pig production cannot be estimated. This is also likely to increase if the potential threats of diseases are not taken in to account at early stages of production.

Finding a sustainable and cost-effective method to reduce the effect of colibacillosis infections in piglets is one of the major challenges facing the pig industry worldwide (Bosworth and Vögeli, 2003). Current control strategies are based on antimicrobials and vaccines, which incorporate fimbrial antigens, have proven to be ineffective. Antimicrobials lose effectiveness over time when bacteria develop resistance and vaccines do not induce full immunity (Wong et al., 1995; Bilkei and Biro, 2000). Over the years,

molecular studies have led to the discovery of genetic resistance of pigs against *E. coli* infections (Sellwood, 1983; Meijerink et al., 1997). Polymorphisms in *FUT1* gene have been associated with *FI8R* genotypes and have been widely used in selection for *FI8 E. coli* resistance in pigs. Mucin genes (*MUC4, MUC13* and *MUC20*) have also been proposed for the identification of the presence or absence of the *F4bcR* and have shown significant association with *F4 E. coli* adhesion and non-adhesion phenotypes.

Although *E. coli* is one of the most important diseases in pig production in South Africa (Henton, 2010), pork producers have been relying mainly on conventional control measures. No studies investigating infectious *E. coli* disease susceptibility or resistance have been carried out in the South African pig population. Selection of the breeding stock against *F4* and *FI8 E. coli* infections using the proposed genes could reduce costs associated with antibiotic and vaccine purchases. The variability in the time taken by pigs to reach slaughter age will be reduced. Loss due to piglet mortality will also decrease.

There is a need for effective genetic control programmes to determine the characteristics of the pathogen and its distribution, the aetiology and pathogenecity of the affected population. An understanding of the genetics of the host population is also imperative as it gives information on the immunity status of the pigs, as well as an indication of the candidate genes that could be successfully used in genetic control or treatment programs.

## 1.3 Objectives

The objectives of the study were to determine the prevalence of pathogenic *E. coli* and their associated fimbriae and non-fimbrial adhesins in Large White, Duroc, Landrace and indigenous pig populations of South Africa. Polymorphisms in candidate genes used for selection against the *F4ab/ac* and *FI8 E. coli* were also investigated in the same pig populations.

The specific objectives were to:

1. determine the prevalence of Enterotoxigenic, Shiga toxin and Enteroaggregative *E. coli* in South African pigs;
2. investigate the mode of colonisation of the prevalent *E. coli* in the South African pig population;
3. determine the existing *E. coli* pathotypes and their distribution in the South African pigs; and
4. investigate polymorphisms at *E. coli F4* candidate genes *MUC4*, *MUC13* and *MUC20* and *E. coli FI8* candidate gene *FUT1* in the exotic and indigenous South African pig breeds.

## 1.4 References


Bertschinger, H. U., Hofer, A., Stranzinger, G. and Vögeli, P. 2004. Breeding pigs resistant to *Escherichia coli FI8* in the field- A progress report from Switzerland. International Society for Animal Hygiène, 11-13 October 2004, Saint-Malo, France.

Bilkei, G. and Biro, O. 2000. Postpartum booster vaccination of sows against *E. coli* infections. *Berliner und Münchener Tierärztliche Wochenschrift*, 113, 67-70.

Bosworth, B. T. and Vögeli, P. 2003. Methods and compositions to identify swine genetically resistant to *FI8 E. coli* associated diseases. EP Patent 0,985,052.

Chen, X., Gao, S., Jiao, X. and Liu, X. F. 2004. Prevalence of serogroups and virulence factors of *Escherichia coli*strains isolated from pigs with postweaning diarrhoea in eastern China. *Veterinary Microbiology*, 103, 13-20.

Choi, C., Kwon, D. and Chae, C. 2001. Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *E. coli* isolated from diarrheic piglets. *Journal of Veterinary Diagnostic Investigation*, 13, 26-29.

DAFF. 2012. *Trends in the Agricultural sector 2011* [Online]. South Africa, Pretoria: Department of Agriculture, Forestry and Fisheries. Available: Available: http://www.daff.gov.za/docs/statsinfo/Trends_2011.pdf [Accessed 19 November at 11:23 am 2012].

Francis, D. H. 1999. Colibacillosis in pigs and its diagnosis. *Swine Health Production*, 7, 214-244.

Haesebrouck, F., Pasmans, F., Chiers, K., Maes, D., Ducatelle, R. and Decostere, A. 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? . *Veterinary Microbiology*, 100, 255-268.

Halimani, T., Muchadeyi, F., Chimonyo, M. and Dzama, K. 2010. Pig genetic resource conservation: The Southern African perspective. *Ecological Economics*, 69, 944-951.

Hammerum, A. and Heuer, O. 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, 48, 916-921.

Henton, M. 2010. *Zoonotic diseases of pigs* [Online]. Available: http://www.sapork.biz/zoonotic-diseases-of-pigs-2/ [Accessed 29 July 2012 at 11:59 am].

Henton, M. and Engelbrecht, M. 1997. *Escherichia coli* serotypes in pigs in South Africa. *The Onderstepoort Journal of Veterinary Research*, 64, 175-187.

Ironkwe, M. O. and Amefule, K. U. 2008. Appraisal of indigenous pig production and management practices in River State, Nigeria. *Journal Of Agriculture and Social Research*, 8, 1-7.

Jensen, G. M., Frydendahl, K., Svendsen, O., Jorgensen, C. B., Cirera, S., Fredholm, M., Nielsen, J. P. and Moller, K. 2006. Experimental infection with *Escherichia coli O*149: *F4ac* in weaned piglets. *Veterinary Microbiology*, 115, 243-249.

Ji, H., Ren, J., Yan, X., Xiang Huang, X., Zhang, B., Zhang, Z. and Huang, L. 2011. The porcine *MUC20* gene: molecular characterization and its association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Molecular Biology Reports*, 38, 1593-1601.

Kemm, E. 1993. *Pig production in South Africa*, Agricultural Research Council (ARC) Bulletin, 427. V and R Printers, South Africa, Pretoria.

Meijerink, E., Fries, R., Vögeli, P., Masabanda, J., Wigger, G., Stricker, C., Neuenschwander, S., Bertschinger, H. U. and Stranzinger, G. 1997. Two α (1,2) fucosyltransferase genes on porcine

Chromosome 6q11 are closely linked to the blood group inhibitor and *Escherichia coli FI8* receptor loci. *Mammalian Genome*, 8, 736-741.

Muggli-Cockett, N. E., Cundiff, L. V. and Gregory, K. E. 1992. Genetic analysis of bovine respiratory disease in beef calves during the first year of life. *Journal of Animal Science*, 70, 2013-2019.

Otte, M. J. and Chilonda, P. 2000. Animal Health Economics: An Introduction. Livestock Information. Sector Analysis and Policy Branch, Animal Production and Health Division, FAO, Rome, Italy. ftp://ftp.fao.org/docrep/fao/010/ag275e/ag275e.pdf.

Pittman, J. S. 2010. Enteritis in grower-finisher pigs caused by *FI8*-positive *Escherichia coli*. *Journal of Swine Health and Production*, 18, 81-86.

Rothschild, M. F., Hu, Z. and Jiang, Z. 2007. Advances in QTL mapping in pigs. *International Jounal of Biological Science*, 3, 192-197.

Sellwood, R. 1983. Genetic and immune factors in the susceptibility of piglets to *Escherichia coli* diarrhoea. *Annales De Recherches Veterinaires*, 14, 512-518.

Simianer, H., Solbu, H. and Schaeffer, L. R. 1991. Estimated genetic correlations between disease and yield traits in dairy cattle. *Journal of Dairy Science*, 74, 4358-4365.

Wong, I., Moreno, M., Valderrama, S., Joglar, M., Horrach, M., Bover, E., Borroto, A., Basulto, B., Calzada, L., Hernandez, R., Herrera, L. and De La Fuente, J. 1995. Immunity and protection elicited by a recombinant vaccine against enterotoxigenic *Escherichia coli*. *Biotecnologia Aplicada*, 12, 9-15.

Zhang, B., Ren, J., Yan, X., Huang, X., Ji, H., Peng, Q., Zhang, Z. and Huang, L. 2008. Investigation of the porcine *MUC13* gene: isolation, expression, polymorphisms and strong association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Animal Genetics*, 39, 258–266.

# CHAPTER 2

## Literature Review

### 2.1 Introduction

Diseases are major determinants of profitability in animal production. *Escherichia coli* infections in pigs have been ranked among the most important diseases in the pig industry. Piglets affected by *E. coli* show severe watery diarrhoea, oedema, dehydration, depression, anorexia and neurological symptoms such as staggering (Kemm, 1993; Francis, 1999;Wang et al., 2010). *Escherichia coli* is a gram negative, and rod shaped group of bacteria forming part of the microflora found in the gastro intestinal tract of animals and humans. There exist pathogenic strains that could cause diarrhoea and death in neonatal and weaned pigs. Although a number of pathogenic *E. coli* exist, three main classes are frequently associated with colibacillosis in pigs. Enterotoxigenic *E. coli* is the leading cause of diarrhoea and oedema in growing pigs. Recently, EAEC has been frequently isolated from pigs affected by diarrhoea. Shigatoxin producing *E. coli* plays a minor role in diarrhoea infections, but have been isolated more frequently in oedema cases.

Colonization factors are a primary requirement for a successful colonization of the *E. coli* in the pig's gastrointestinal tract. Fimbriae are one type of CF expressed by most ETEC, STEC and EAEC. In pigs, *E. coli* expressing fimbriae types *F4* (*K88*), *F5* (*K99*), *F6*, *FI8* and *F41* are the most prevalent (Nagy et al., 1999; Souza Da Silva et al., 2001; Fekete et al., 2002; Nagy and Fekete, 2005). The second type of colonization factor is known as afimbrial adhesins, and these are shared among *E. coli* that affect most animals including pigs and humans. Three of the non-fimbriae adhesins include adhesin involved in diffuse adherence (*AIDA-1*), the porcine attaching and effacing-associated factor (*PAA*) and attaching and effacing factor (*EAE*) and have been frequently isolated from *E. coli* from pre-weaned and weaned pigs affected by diarrhoea.

Successful initiation and progression of colibacillosis is a complex process which requires fimbriae, designated receptors on the lining of the small intestines of the host, the optimum bacterial load and finally the production of toxins. As such, designing a successful prevention strategy or control method against an *E. coli* infection in pigs will require sound knowledge of the pathogen as well as its interaction with the host. Vaccines, antibiotics as well as breeding for genetic resistance are some of the methods used to prevent colibacillosis outbreaks in pigs (Sellwood, 1983; Wong et al., 1995).

This review is aimed at characterising pathogenic *E. coli* involved in swine colibacillosis, investigating the pathogenesis of *E. coli* in the development of the disease, the effect of the disease on the pig, pork producers and the pig industry. The prevalence and the status of the existing pathogenic *E. coli* causing diarrhoea in pig herds will also be highlighted. The methods used to control *E. coli*, their efficacy and the potential future control strategies will also be discussed.

## 2.2 Virulence factors and classification of *E. coli*

Pathogenic *Escherichia coli* could be classified using several characteristics among which serotype, colonisation factors and toxin genes are the common ones. Types or classes of *E. coli* may result in different clinical manifestation because of the types of the VFs they possess. For example, specific VFs are involved in the generation of diarrhoea and oedema-like symptoms. While colonisation factors aid in the first steps of pathogenesis, the toxins play an important role in the generation of the symptoms. The role and importance of various virulence factors is explained below.

### *2.2.1 Serotype*

*Escherichia coli* can be classified by blood group antigens or by serotype. There are three major antigens which include the somatic cell wall phospholipid-polysaccharide complexes (*O*), the surface or capsular

antigens that are acidic (*K*) and components of the flagella (*H*). The *O*, *K* and *H* antigens are further divided into subtypes which are numerically designated and are differentiated by specific antibodies (Whitfield et al., 1988; Osek et al., 1999). The *O* and the *H* are mostly associated with diarrhoea while the *K* antigen is associated with complex intestinal infections in mammalian diseases. About 53 *H* antigens of *E. coli* are recognised (Whitfield et al., 1988) and 179 *O* serotypes exist (Mattsson and Melin, 2004).

### *2.2.2 Colonisation factors*

In addition to the serogroups, CFs have been used to distinguish *E. coli* strains. Two types of CFs consisting of fimbrial and non-fimbrial adhesin factors have been documented. Fimbrial antigens are CFs that aid bacteria to adhere to the intestinal wall of piglets, before they proliferate and produce toxins intestine (Nagy et al., 1999). *Escherichia coli* bacteria without fimbriae would not be able to adhere to the specific receptors found in the intestinal lining of the host. This adhesion is a key to a successful infection and results from a highly specific interaction between the fimbriae produced by the bacteria and the receptor present on the lining of the small intestine (Nagy et al., 1999). The EAEC, STEC and ETEC, however, do not always possess the common CFs. Other modes of attachment of *E. coli* other than fimbriae include: *AIDA-1*, *PAA* and *EAE* (Yamamoto and Nakazawa, 1997; Vu-Khac et al., 2006; Lee et al., 2009).

#### *2.2.2.1 Fimbrial adhesins*

Fimbrial adhesins are long thin filaments expressed on the surface of the bacteria and they are allow the secure attachment of bacteria to the host intestinal receptors (Nagy et al., 1999). Each fimbriae has a specific receptor to which it binds to. Non-expression of the receptor by the host results in failure of the *E. coli* to securely attach to the intestinal walls. They are expressed by both the pathogenic and non-pathogenic *E. coli*. The same *E. coli* isolate can express more than one type of fimbrial adhesin (Hu et al.,

1994). Fimbriae have been intensively studied in pigs and the most common fimbrial adhesins found in *E. coli* isolated from the swine colibacillosis studies include *F4*, *F18*, *F41*, *F5* and *F6*.

*2.2.2.1.1 F4*

*F4* was previously known as *K88* and exists in three antigenic variants *ab*, *ac* and *ad*. The *F4ac* is the most common in worldwide but *F4ad* is most prevalent in China (Wang et al., 2006). Out of the 36 *E. coli* isolates from Chinese piglets suffering from *F4* diarrhoea used in the study, 88.9 % was found to contain *F4ad* fimbrial adhesin. Choi and Chae (1999) could not detect *F4ad* fimbriae in *E. coli* isolates from diarrheic piglets in Korea collected between 1995 and 1998 but found *F4ab* and *F4ab* in 5.4 % of the isolates. The receptors for *F4ab/ac* fimbriae are closely related to sialoglycoproteins and mucin glycoproteins (Francis et al., 1998). Li et al. (2007) identified 8 phenotypes of *F4* variants combinations *in vitro* using jejunum samples from piglets. *Escherichia coli* expressing *F4* fimbriae are predominant in neonatal piglet infections (Hu et al., 1993; Do et al., 2006b).

Moon et al. (1999) found more *E. coli F4* from faecal and tissue isolates of diarrheic piglets less than three weeks (84 %) than in older piglets (64 %).

*2.2.2.1.2 FI8*

In addition to *F4*, *FI8* (*F107*, *2134*) is also prominent fimbriae in colibacillosis. The *FI8* is available as antigenic variants *ab* and *ac*. The *FI8* is mostly associated with post weaning infections (Souza Da Silva et al., 2001; Fekete et al., 2002; Bao et al., 2008). *Escherichia coli* isolates from faecal and tissue of infected piglets showed that *FI8* was most common in older piglets (>3 weeks) (29 %) than those three weeks and younger (11 %) (Moon et al., 1999). Chen et al. (2004) also found that, in China, the *FI8 E. coli* (24.2 %) was more prevalent in weaned piglets than *F4 E. coli* (9.8 %). Souza da Silva et al. (2001) found that 76 % of the *E. coli* isolates from 34 pigs in Brazil were of the *FI8ab* fimbriae type.

#### *2.2.2.1.3 F41*

*F41* harbouring strains are mannose-resistant haemaglutinin and electron microscopy analysis of the strains shows a structural presentation of thin and flexible pili. *F41* plays an important role in colonisation in the lower section of the small intestines (To, 1984). To (1984) found that newly born piglets and cattle infected with *F41* expressing *E. coli* showed clinical symptoms of colibacillosis. The post-mortem analysis of the infected animals showed that the ileum was heavily colonized with *E. coli F41*. The role of *F41* in pig infections is limited compared to *F4* and *FI8* expressing strains. *F41* was found in 22 % of the isolates from diarrheic Landrace and Large White piglets in Zimbabwe (Madoroba et al., 2009). Zhang et al. (2007) detected *F41* in only 0.57 % of the isolates taken from diarrheic piglets in the United States of America.

#### *2.2.2.1.4 F5* and *F6*

The other *E. coli* strains including *F5* and *F6* are not the main cause of mortality, and their prevalence in pigs is marginal (Yang et al., 2009). Colonisation factors *F5* and *F6* were previously designated to fimbriae antigens *K99* and *987P*, respectively and are associated with colibacillosis in pigs (Do et al., 2005). Madoroba et al. (2009) identified *F5* and *F6* virulence genes in 22.5 and 1.5 % of the piglets showing signs of scours, respectively. Zhang et al. (2007) found that only 0.57 % of the isolates from diarrheic piglets in the Unites States had adhesin *F5* while none carried the *F6* adhesin.

### *2.2.2.2 Non-fimbrial adhesins*

Non-fimbrial adhesins are outer membrane proteins that *E. coli* uses to secure an attachment to the host's intestinal surfaces. They have a capsule-like appearance (Goldhar, 1996). Non-fimbrial adhesins are becoming increasingly important in *E. coli* infections. They serve as alternative attachment method in the absence of fimbriae but could also occur concurrently with fimbriae. It has been observed quiet often that

*E. coli* isolated from enteric colibacillosis in pigs lack expression of common attachment factors, but express non-fimbrial adhesins *AIDA-1*, *EAE* and *PAA* (Ngeleka et al., 2003).

*2.2.2.2.1 AIDA-1*

Laarmann and Schmidt (2003) described the *AIDA-1* as an adhesin belonging to a family of autotransporter proteins. The *AIDA*-associated heptosyltransferase gene together with the *AIDA-1* gene which encodes a pre-pro-protein of 132 kDa which later matures into the adhesin *AIDA-1* is necessary for full adherence activity. The study investigated the binding of the *AIDA-1* to several mammalian cells and identified a 119 kDa surface glycoprotein (gp119) as an *AIDA-1* receptor. Niewerth et al. (2001) observed an increased frequency of *AIDA-1* in *E. coli* isolates taken from oedema affected piglets. The occurrence of *AIDA-1* among colibacillosis infections in piglets was 2.7 % in South Korea and 26.9 % in the USA (Zhang et al., 2007; Lee et al., 2009).

*2.2.2.2.2 EAE*

Some *E. coli* uses the attaching and effacing (A/E) mechanism to colonise their host and the genes involved in this type of colonisation are clustered in a chromosomal pathogenecity island called the locus of enterocyte effacement (LEE). This category of *E. coli* is termed attaching and effacing *E. coli* (AEEC). The *EAE* and *PAA* form part of the LEE genes involved in the A/E mechanism (Batisson et al., 2003). The intimin encoding gene *eae* is a 94-kDa outer membrane protein which plays a role in the ability of *E. coli* to attach to epithelial cells, efface the microvillus surface, and then disrupts the underlying cytoskeleton (Nataro and Kaper, 1998).

The role of the *EAE* adhesin was confirmed by Donnenberg and Kaper (1991) using *eae* gene deletion and re-introduction infection model. The A/E ability was restored upon the re-introduction of the *eae* gene back in the plasmid. Later studies (Donnenberg et al., 1993; Zhu et al., 1994) also confirmed previous reports on the role of *EAE* adhesin as the production of characteristic intimate adherence to the intestinal

wall with effacement of microvilli. Necroscopic analysis of newly born piglets infected with *EAE* positive strains showed intimate attachment of bacteria to enterocytes and microvillus effacement but no diarrhoea 48 h post infection. The adherence of the bacteria was observed mostly in the lower section of the gut (Donnenberg et al., 1993). Zhu et al. (1994) found that *E. coli O*45 strain positive for *EAE* induces A/E lesions in gnotobiotic piglets. The receptor for the *EAE E. coli* is known as the translocated intimin receptor (*Tir*) and like *eae* and *paa* is also found in the LEE (Nataro and Kaper, 1998; Marchès et al., 2000).

*2.2.2.2.3 PAA*

Battison et al. (2003) characterised the *paa* gene as a 27.6 kDa protein that is involved in the development of A/E lesions during the colonisation of AEEC. An *in vivo* study in new born piglets showed that *PAA* and *EAE* positive *E. coli* induced diarrhoea within 24 h post inoculation (p.i) and a microscopic assessment of intestines 120 h following infection revealed a tight attachment of the bacteria to epithelial cell surfaces and effacement of microvilli (Batisson et al., 2003). The severity of the A/E lesions is associated with the onset and the severity of diarrhoea. Porcine attaching and effacing-associated factor targets primarily the ileum and it is alleged that it uses heparan sulphate receptors to attach to the host cell surface (Batisson et al., 2003).

An association exists between the *eae* and *paa* genes. The *PAA* protein is involved in the early stages of bacterial colonization since *PAA* negative strains showed less adherence and infected piglets had no diarrhoea, or delayed commencement of diarrhoea (Nataro and Kaper, 1998). Battison and co-workers (2003) found that *EAE+/PAA-* isolates failed to induce diarrhoea and lesions. In some piglets, mild diarrhoea and non-severe or no A/E lesions developed 83 h p.i.

### *2.2.3 Toxin genes*

Apart from the serogroups and CFs, the three pathogenic *E. coli* ETEC, EAEC and STEC are grouped according to the toxins they produce. Enterotoxigenic *E. coli* is characterised by the release of toxins known as heat-stable enterotoxin (*ST*) and heat-labile toxins (*LT*). The *ST* exists in two variants *STa* and *STb*. Shigatoxin *E. coli* produces shiga toxins (*Stx*) or verotoxins (Wang et al., 2010). The enteroaggregative *E. coli* strains are characterised by the release of the enteroaggregative heat stable toxin (*EAST-1*) which is encoded for by the *astA* gene (Zhu et al., 1994).

#### *2.2.3.1 LT*

Although the *STa* toxin is common among *E. coli* causing colibacillosis strains, *LT* toxin is the most important factor causing a significant outcome of the disease. Chen et al. (2004) found that 60.5 % of the isolated from diarrheic piglets in China contained only the *LT* toxin. Heat-labile toxin acts on the adenylatecyclase, which is found on the intestinal epithelial cells, by increasing the production of intracellular cyclic adenosine monophosphate (cAMP). Elevated levels of cAMP in the epithelial cell result in reduced absorption of electrolytes and water from the lumen that eventually results in osmotic diarrhoea (Nataro and Kaper, 1998).

Erume et al. (2008) used gnotobiotic piglets to determine the role of *STb*, *LT* and *STa* on the progression of *E. coli* diarrhoea in pigs. The results showed that the deletion of the *eltAB* gene which encodes the *LT* toxin from the plasmid resulted in the reduction in weight loss, dehydration and metabolic acidosis. The introduction of the gene resulted in severe diarrhoea, dehydration and weight loss. The deletion of the *estA* and *estB* which are genes encoding *STa* and *STb* toxins, respectively, showed no significant effect on the colibacillosis and the introduction of the Δ*estB* had no effects on weight loss.

*2.2.3.2 STa*

The pathogenic mechanism of the *STa* is similar to that of *LT* except that it acts on the guanylatecyclase C enzyme (Nataro and Kaper, 1998). Parma et al. (2000) found that the *STa* gene was more prevalent in the ETEC isolates from diarrheic piglets. Chen et al. (2004) found that the *STa*, *STb* and *LT* enterotoxins are common in most *E. coli* isolated from post-weaning diarrhoea. The prevalence of individual toxins varies with geographical region and time frame.

*2.2.3.3 STb*

Heat stable toxin variant b is also one of the most prevalent *ST* toxins (Chen et al., 2004). The mechanisms of pathogenesis and the intestinal receptors of the *STb* toxin are not fully understood. The *STb*, however, causes partial degeneration of villi and has also been associated with increased secretion of bicarbonate and calcium from intestinal cells (Nataro and Kaper, 1998; Fairbrother et al., 2005). Parma et al. (2000) found *STb* toxins in ETEC isolates of pigs that had diarrhoea, delayed growth and oedema.

*2.2.3.4 Stx*

Shigatoxins, *Stx1* and *Stx2* are the most prominent in the STEC isolated from piglets showing clinical symptoms of colibacillosis. Genes, *stxI* and *stxII* encode for *Stx1* and *Stx2* toxins, respectively. The antigenic forms of shiga toxins *Stx1* and *Stx2* are classified according to their amino acid content. The shiga toxin 2 also occurs as variant (*Stx2e*) which is encoded for by *stx2e* gene and associated with *FI8* fimbrial adhesin which prevails in isolates from pigs with oedema (Osek et al., 1999; Chen et al., 2004). Shiga toxin variant 2e is found in only a small portion of *E. coli* causing post weaning diarrhoea (PWD) but considered an important factor in oedema producing strains (Parma et al., 2000).

Shigatoxins recognises the cell surface receptor globotriaosylceramide with the exception if *Stx2e* of which's surface receptor is globotetraosylceramide (Gb4) in most mammals (Paton and Paton, 1998). The *Stx2e* toxin binds to a Gb4 receptor and destruct the protein synthesis, resulting in death of endothelial

cells, intestinal epithelial cells and any cells having a Gb4 receptor (Nataro and Kaper, 1998). This consequently results in oedema clinical symptoms.

#### *2.2.3.5 EAST-1*

Enteroaggregative heat-stable toxin is becoming an important virulent factor in pig *E. coli* infections. Choi et al. (2001) found that 22.7 % of the *E. coli* isolates collected from 14 d diarrheic piglets carried the *astA* gene. Enteroaggregative heat stable toxin is a heat stable enteroxin and shares the same host receptor (cGMP) as the *STa*, suggesting that their pathogenic mechanism could be similar. Although the exact mechanism of *EAST-1* in *E. coli* infections is not fully understood, affected animals elicit characteristic histopathological lesions (Veilleux and Dubreuil, 2006). In addition, a common association of *EAST-1* with the *STa* and *F4* genes has been revealed (Choi et al., 2001).

### *2.2.4 Association between toxins and colonisation factors*

Zhang et al. (2007) found that *F4* and *FI8* positive isolates contributed 64.6 and 34.3 %, respectively. Most of the fimbriae *F4* and *FI8* positive isolates contained more than one toxin, with 42.5 % of the *F4* isolates contained *LT* and *STb* toxins while 23.9 % contained *LT, STb* and *EAST-1*. A combination of *STa, STb* and *EAST-1* toxins was found in 31.7 % of the *FI8* positive isolates. Out of 720 *E. coli* strains isolated from diarrheic piglets in China, Choi et al. (2001), found that *EAST-1* was found in 22.7 % of the isolates. In addition, these *EAST-1* positive isolates also carried genes associated with fimbriae *F4* (16 %) and toxin gene *STa* (46 %). The study further identified six major pathotypes *EAST-1*, *EAST-1/STa*, *EAST-1/STa/STb*, *EAST-1/STa/F5*, *EAST-1/STa/F4*, and *EAST-1/STb/F4*. The number of pathotypes found in isolates from colibacillosis infected pigs varies from place to place and depends on the number of virulence genes involved in the infection induction.

## 2.3 Pathogenesis and prevalence of *E. coli* infections in pigs

Neonatal diarrhoea may be visible from day 0-10 of life, while the post-weaning diarrhoea occurs 4-10 d after weaning (weaning at 21-28 d). Two processes are important for *E. coli* bacteria to successfully cause oedema or diarrhoea in the host; adhesion and production of toxins (Frydendahl et al., 2003). Upon entering the host via the oral route, *E. coli* adheres to the brush borders of the small intestines and colonise the host gastrointestinal tract (Imberechts et al., 1992; Francis, 2002). This is subsequently followed by production of enterotoxins that act on the ion transport system within the lumen and this result in water and electrolytes excretion. The excessive loss of water results in diarrhoea and may lead to metabolic acidosis, dehydration and, if severe, may result in death. In the case of oedema, the toxins are absorbed through the lumen into the blood stream and cause damage to the vascular cells. Severe damage of these cells may cause oedema of the caecal and colonic submucosa, lesions in the brain which result in central nervous system damage followed by imbalance, paralysis of hind legs and death (Clugston et al., 1974; Francis et al., 1989; Imberechts et al., 1992).

Ironkwe and Amefule (2008) indicated that *E. coli* colibacillosis is a significant cause of loss in the Nigerian small scale pig production. In China, 56.2 % of the diarrhoea and 24 % deaths in piglets were a result of *E. coli* infections (Li et al., 2007). Wong et al. (1995) found that *E. coli* infections accounted for 21 % of the deaths in non-vaccinated piglets. Bilkei and Biro (2000), however, published a 7.5 % and 10.7 % pre-weaning mortality in piglets from two groups of vaccinated sows were the second group was given a booster vaccine after farrowing.

There is limited information on the prevalence of *E. coli* diarrhoea and oedema in pigs in South Africa. From 1971 to 1991, 674 cases associated with colibacillosis due to *E. coli* were reported in pigs at the Onderstepoort Veterinary Institute. Of these, 45.5 % of the piglets had inflamed small intestines and 46.9 % showed blood poisoning and only 12 pigs showed oedema signs (Henton and Engelbrecht, 1997). The *E. coli* infections were observed more frequently in weaners than in suckling piglets.

## 2.4 Diagnostic tools for *E. coli* infections

Methods for detection of the serotype and virulence factors can be antigen or DNA based. A range of tests can then be performed to determine the fimbriae type of the *E. coli* of interest and these include serum agglutination test (SAT), enzyme linked immunosorbent assay (ELISA), indirect immunoflourescent assay (IFA) (Choi and Chae, 1999). A smear or a frozen section of the infected intestine sample is prepared from cultures of the bacteria and viewed under a fluorescent microscope for the IFA method. The SAT method identifies the fimbrial antigen by the use of the monoclonal antibodies. The monocloclonal antibodies A-3, EK409, EPN3, L10-5 and 19B4 are specific to *F4*, *F5*, *F6*, *F4I* and *FI8* respectively (Chen et al., 2004). There is, however, downfalls associated with ELISA and the IFA methods, and these are that the cultured organisms may not always express fimbriae. Also encapsulated organism may yield false positives. The IFA method is more sensitive and specific than the SAT and ELISA methods as they need more antigen production to obtain efficient results and they are not always reliable.

The other methods involve taking faecal isolates of infected pigs and using deoxyribonucleic acid (DNA) probes and polymerase chain reactions (PCR) to evaluate the presence of DNA sequence consistent with those that encodes for virulence determinants of the fimbriae of interest (Mullaney et al., 1991; Francis, 1999). The PCR test is reliable, sensitive and specific, thus allowing identification of variants of a given fimbriae (Wang et al., 2006). The conventional PCR method, however, has limitations such as the inability to amplify different parts of the genome simultaneously, thereby making the process lengthy. The other limitation is that the gel electrophoresis used for post-PCR detection is difficult to handle because of its shape and size and is fairly insensitive and difficult to automate. The insensitivity is due to the fact that DNA material in individual wells can overlap to neighbouring wells, thus requiring extra caution during well fill (Call et al., 2001).

Mullaney et al. (1991) used DNA based method as a reference to compare results for detection of *K88, K99* and *987P* fimbriae antigens obtained using SAT, ELISA and IFA. Any positive obtained the three methods but negative when using the DNA probes was considered a false positive. The SAT produced the highest number of false positives with 40 % average for the *K99, K88* and *987P* probes. On average, the ELISA yielded 28.3 %, and the IFA method 13.3 % false positives of the three fimbrial antigens. Over the years, although the conventional PCR is still used, the PCR procedure has been modified, resulting in the development of multiplex PCR. Multiplex PCR is the most preferred and has been used in various studies for diagnosis of ETEC infections associated with diarrhoea and oedema (Madoroba et al., 2009). The multiplex PCR was developed to reduce the amount of time spent on analysis by the conventional method. This method has the ability to target genes located on different parts of the genome, resulting in simultaneous detection of the presence of distinct PCR products based on the inter-product DNA sequence differences (Call et al., 2001).

## 2.5 Control strategies against *E. coli* bacterial infections in pigs

Several control strategies have been developed over the years to try to combat and reduce *E. coli* infections. Conventional methods such as antibiotics and vaccines are generally used to control colibacillosis in pigs. With advances in research new control methods involving diet additives such as bacteriophages and zinc oxide have been proposed and tried out. Another method, which could be feasible in the long run, is the use of the diagnostic tool for genetic resistance to *E. coli* when selecting a pig herd. The potential benefits and the disadvantages of the current and the new methods are discussed below.

### *2.5.1 Antibiotics*

Antibiotics have always been used as therapeutics for *E. coli* infections in pigs. Currently, most of the antibiotics used to treat *E. coli* infections in South Africa contain ampicillin, tetracycline, streptomycin,

neomycin, sulfamethaxazole and lycomycin. Due to concern of human safety the antibiotic use in animals used for human consumption is limited and only a given range of registered antibiotics can be used in livestock (Hammerum and Heuer, 2009). This has created a huge challenge as a recent report shows that *E. coli* has since developed resistance against some of the antimicrobial reagents used in pig production (Moneoang and Bezuidenhout, 2009), thus increasing the probability of *E. coli* contaminated meat.

Moneoang and Bezuidenhout (2009) reported that between 88.5 and 100 % of the *E. coli* isolated in both commercial and communal pigs of the Mafikeng region in the North West province of South Africa were resistant to erythromycin, oxytetracycline and sulphamethoxazole. Most of the farmers used tetracycline, which is the broad spectrum antibiotic as a therapeutic agent against the *E. coli* infections thus the outcome of the *E. coli* being resistant to such a wide range of antibiotics was expected. The situation could become worse in South Africa because pork consumption doubled from the year 2000 to 2008. Hence the pressure is placed on the pig industry to increase production. South Africa already consume (208 million kg) more pork than it produces (189 million kg), indicating that it relies on imports for the extra pork needed (DAFF, 2010).

The misuse of antibiotics as antimicrobrial growth promoters in livestock farming in South Africa has already been reported (Maritz, 2006). The pressure placed on the pig industry may result in a tremendous increase in the use of antibiotics to enhance growth in pigs. Unlike the European countries, South Africa has no strict regulation against the use of antimicrobials as growth promoters. As such, there are greater risks of antimicrobial resistance by *E. coli* (Stein, 2002). In South Africa, more emphasis was placed on exported meat as it has to meet the international standards and less effort was done on locally consumed meat products. The antimicrobial residual levels in exported meat is controlled under Foodstuff, Cosmetic and Disinfectants Act which is the legal framework used by the DAFF. The food safety and quality assurance directorate controlled by the Meat Safety Act of 2000, is currently implemented to control antibiotic residual levels in meat consumed locally (Maritz, 2006). This is, however, only useful for animals slaughtered in abattoirs. Small scale sectors slaughters are not covered under the Act.

Antimicrobial resistant *E. coli* isolated from infected pigs have been reported in other countries. Multiple antimicrobial resistance (≥ 5 antimicrobials) of *E. coli* isolates evaluated from 2002 to 2008 in China was found to be 89 %. The frequencies of resistance to sulfamethaxazole, tetracycline, and streptomycin were 95, 94 and 84 %, respectively (Wang et al., 2010). Boerlin et al. (2005) also found *E. coli* isolates from diarrheal piglets were resistant to several antibiotics including sulfonamides, apramycin, tetracycline and gentamicin. About 79.4 % of ETEC isolates from diarrheal pigs in Vietnam showed resistance to 3 or more of the following microbial agents, tetracycline, streptomycin, amoxicillin and sulfamethoxazole (Do et al., 2006). There is, therefore, an urgent need for a more effective remedy or control method.

### *2.5.2 Immunisations or vaccines*

In South Africa, the vaccines incorporating fimbrial antigens *F4ac*, *F4ab*, *F5* and *F6* are used in pregnant gilts and sows to control colibacillosis caused by *E. coli* expressing these fimbriae in neonatal piglets (Intervet, 2010; Onderstepoort, undated; Pharmavet, undated). Some important immunoglobulins cannot pass through the placenta to the developing piglets during pregnancy. As such, vaccinations of pregnant sows during late pregnancy have been done to induce *E. coli* tolerance in piglets. The vaccine results in the production of serum antibodies which are concentrated in the colostrum and induce protection at neonatal stage. However, the vaccine efficiency depends on the amount of colostrum consumed and the degree of the pathogen invasion. It, therefore, does not provide any protection against the PWD (Haesebrouck et al., 2004).

Wong et al. (1995) achieved 92 % protection against *E. coli* infections in neonatal piglets by using VACOLI$^{TM}$ recombinant vaccine that contained *E. coli K88ab* and *K99* antigens. At this phase, although colibacillosis was observed in some piglets, no mortalities occurred, whereas during post weaning 0.37 % of the vaccinated piglets died. Bilkei and Biro (2000) used Porcovac Plus in two groups of sows. The first group was given a single dose of vaccine in late pregnancy, whereas the second group had two doses of

vaccine at the same time and a booster vaccine at 2 to 7 d postpartum. Piglets from both sow groups still experienced pre-weaning diarrhoea of 16.1 % (group 1) and 23.3 % (group 2).

### *2.5.3 Diet incorporated control methods*

Ever since the identification of *E. coli* colibacillosis as a major threat to economic losses in pig farming, inclusion of zinc oxide, bacteriophages and egg yolk antibodies have been incorporated in the pig rations. Dietary inclusion of pharmacological doses of zinc oxide doses post-weaning reduced the incidence of diarrhoea (Sargeant et al., 2010). Zinc oxide influences the expression of genes associated with inflammation. Trevisi et al. (2010) found that supplementation of infected piglets with L-tryptophan maintains feed intake and weight gain. The pigs increased intake to compensate for the first limiting amino acid. Supplementation with L-tryptophan may affect lean to fat ratio and is, therefore, not economically feasible.

Other methods include using egg-yolk antibody product in the piglets feed. Chickens produce high immunoglobulins thus when vaccinated with a specific *E. coli* fimbrial antigen, they produce a high amount of immunoglobulins in their yolks. The egg-yolk antibody inclusion should be more than 3.2 % of the feed, although Chernysheva et al. (2004) found this level did not induce immunity in all the piglets. This shows that the egg-yolk antibody is not a feasible method in protecting piglets against ETEC infections.

Bacteriophages destroy target bacteria by feeding on it as such could be used as therapeutic agents against *E. coli* infections in pigs. After identifying the target bacteria in an *in vitro* adhesion, tests should be performed to determine phages that are efficient against those bacteria. Jamalludeen et al. (2009) successfully prevented colibacillosis in piglets by oral administration of bacteriophages shortly after the challenge with *E. coli O*149:*H*10:*F4*. The bacteriophage is, however, susceptible to low stomach pH. As

such, sodium carbonate must be given before administration. Administration of phages after the onset of diarrhoea is not encouraged since the excessive water excretion may result in phages being washed out.

### *2.5.4 Disease resistance and genetic approach*

There is a huge concern of zoonotic transmission of pathogenic *E. coli* from animals to humans. Meat contamination of ETEC occur during slaughter and colonies of *E. coli* from animal origin have been found in retail meat (Hammerum and Heuer, 2009). So far, it is not safe to treat the bacteria using the conventional method. More effective and feasible methods are needed. Selection for absence of receptor molecule or for genetic variants of receptor molecules to which the pathogen cannot bind would is an attractive route for genetic resistance studies. Breeding for resistance of pigs against *E. coli* infections remains the best possible solution to reduce loss of production due to colibacillosis, high cost of treatment and to ensure animal welfare and consumer safety.

A number of studies have been performed on the porcine genome in anticipation to find the genetic control of the adhesin of *E. coli*. There are two main receptor genes which control the expression *F4* and *FI8* in pigs which are the *F4bcR* and *FI8R,* respectively. The *in vitro* adhesion method that involves the brush border suspension plus the *E. coli* bacteria of known fimbriae was used to detect the phenotype of the pigs. However, it was found that the method is not sound for selection of breeding animals; as such the selection is done using candidate genes from closely linked locus.

#### *2.5.4.1 FI8 E. coli receptor*

The expression of ETEC *FI8ab* and *FI8ac* receptors in pigs is controlled by the *FI8bcR* gene. This gene has been mapped on SSC6 and is closely linked to blood group inhibitor locus *S* on the same gene (Meijerink et al., 1997; Vögeli et al., 1997; Meijerink et al., 2000). The resistance to ETEC *FI8ab/ac*

adhesin is inherited in an autosomal recessive Mendelian pattern. The dominant allele *B* controls the susceptibility and resistance is controlled alternatively by the recessive allele *A* (Alexa et al., 2002; Yan et al., 2009). Alpha fucosyltransferase 1 and 2 (*FUT1, FUT 2*) and ryanodine receptor 1 (*RYR1*) have been studied to determine the candidate genes for selection against the *FI8bc* susceptibility. Several studies on the Swiss Landrace and Large White pigs showed that there is mutation at base pair 307 (*M307*) of the *FUT1* gene that was linked to the *E. coli FI8* susceptibility and resistant locus (Vögeli et al., 1997; Meijerink et al., 2000).

*2.5.4.1.1 FUT1 gene*

Meijerink et al. (1997) idetified *FUT1* and *FUT2* genes located on SSC6q11 as the possible candidates for the *FI8R* detection. These two genes form part of the blood group antigens and are found on surface of the cell membrane. *FUT2* gene controls the amount of mRNA in the mucosa of the small intestine. The *FUT1* gene is responsible for synthesis of glycotransferase which is the enzyme that facilitates synthesis of carbohydrate structure which mediate the adhesin and the colonisation of bacterial adhesin. Linkage analysis studies revealed that *FUT1* gene polymorphism is >1 cM from S and the *FI8R* loci. It is, thus, a good candidate gene for controlling the expression of *FI8R*. Meijerink et al. (2000) revealed that the polymorphism in the *FUT1* gene was solely responsible for the *FI8* adhesin. A polymorphism in the *FUT1* gene occurring due to a mis-sense mutation in nucleotide 307, result in the substitution of adenine (*A*) by guanine (*G*). The resulting *G/A* genotype causes 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 (Bosworth and Vögeli, 2003; Frydendahl et al., 2003; Bao et al., 2008).

The *FUT1* polymorphism at *M307 G/A* provides a non-invasive method for detecting resistance and susceptibility against the *FI8* ETEC bacteria in pigs (Bosworth and Vögeli, 2003; Frydendahl et al., 2003). Frydendahl et al. (2003) confirmed that *FUT1* gene polymorphism is important for *FI8ab/ac* adhesin in piglets. A total of 27 pigs were given ETEC *FI8* inoculum orally and only 1 pig out of 17

*FUT1AA* genotype developed diarrhoea, while 71.4 % of the ten susceptible pigs developed diarrhoea. There were no other microbes in the resistant pigs that developed diarrhoea and it was concluded that there might have been another mode of attachment other than *FI8* since the *ST* and *LT* can only be produced after adhesion. Results from Swiss blood testing lab where 400 to 800 pigs were examined per year showed that the *A* allele in Large White increased from 0.31 (1996) to 0.54 (2004) and from 0.07 to 0.24 in Landrace pig population (Bertschinger et al., 2004). Bao et al. (2008) also confirmed the *FUT1* as the candidate gene for selection against the *FI8* adhesins

Lemus-Flores et al. (2009) investigated the frequency of the *FUT1* gene polymorphisms in two Mexican pig breed populations of Yorkshire and Creole pigs. The Creole pig, which is indigenous to Mexico, showed a high frequency of the resistant genotype *AA* (0.39) compared to the Yorkshire (0.1). A Romanian study on *FUT1* gene showed resistant allele frequency of 0.63 and 0.30 in Mangalitsa and Bazna, respectively (Ciobanu et al., 2001).The Bazna pig breed is crossbreed produced from crossing the Mangalitsa and Berkshire, which further confirm that native or indigenous breeds could be more adapted or tolerant to diseases than newly developed breeds used for commercial purposes. The resistance allele for the *FUT1 M307* polymorphism was found to be more prevalent in Zlotnicka spotted (0.63) and the F1 Zlotnicka spotted × Polish Landrace (0.58) than in the Polish Landrace (0.22) and Zlotnicka White (0.21) (Klukowska et al., 1999).

Filistowicz and Jasek (2006) found resistant genotype for *FUT1* was 2.9, 12.5 and 7.2 % for the Polish Landrace, Belgian Landrace and Duroc, respectively. Large White boars were more resistant than the Landrace in the Czech Republic, with *GG* genotype of 0.625 and 0.789 respectively (Vrtková et al., 2007). Styanova (2009) reported a prevalence of the resistance allele of 0.147 in Danube breed of the Czech Republic. The frequencies of *FUT1* resistant genotype were 0.05 and 0.07 in German Landrace boars and their progeny respectively, 0.26 and 0.22 in Large White and 0.33 and 0.25 in Pietrain breeds of the German Republic (Binder et al., 2005).

*2.5.4.2 F4ab/ac Receptor*

Several attempts have been made to locate the specific gene responsible for the resistance against the ETEC *F4* (*F4bcR*). Python et al. (2005) indicated that the *F4ab* and *F4ac* receptor was controlled by the *F4bcR* gene which was mapped on the SCC13q41 (Ren et al., 2009). Python et al.(2002) used serum transferrin gene and 10 microsatellites linked to *F4abR* gene and located it between *S0068* and *Sw1030*. Python et al. (2005) subsequently study refined the locus to region between *Sw207* and *S0283*. Joller et al. (2009) used the microsatellite markers *HSA125gt* and *MUC4gt* and found the *F4bcR* locus was between *SW207-S0075* interval. In a recent study, the locus for the *F4bcR* was refined to region between leishmanolysin-like gene *LMLN g.15920* and *S0283* (Rampoldi et al., 2011). Another study showed that *F4ab* and *F4bc* receptors are controlled by two different but closely linked loci. It was further revealed that *F4abR* gene is located between *S0283 Sw1833*, a 4.8 cM region and locus for *F4bcR* is 1.6 cM region between *S0283* and *Sw1876* (Niu et al., 2011).

The resistance for *F4ab/ac E. coli* adhesin is an autosomal recessive trait (Sellwood, 1983) and susceptible pigs genotype is given by allele combination *SS* and *SR* and the resistant genotype is given by a *RR* (Fairbrother et al., 2005; Rasschaert et al., 2007). The genes associated with this locus are those encoding for some transferrins or mucin-like siaglycoproteins. A number of studies have been done on these groups of genes in anticipation to find a causative mutation for *F4ab/ac* adhesin. Some of the genes identified are transferrin receptor (Van Poucke et al., 2009), *MUC4* (Jorgensen et al., 2003; Jensen et al., 2006; Peng et al., 2007; Rasschaert et al., 2007), *MUC13* (Zhang et al., 2008), *MUC20* (Ren et al., 2009; Ji et al., 2011) and Solute carrier organic anion transporter family member 2A1 (Van Poucke et al., 2009). For the purpose of this study, only the association of the Mucin genes with the *F4bcR* would be discussed.

#### *2.5.4.2.1 Mucin genes*

Mucins are characterised by large glycoproteins that are bound to the membrane and found on the surface of the epithelial cells of the gastrointestinal tract. Furthermore, mucins play an important role in controlling cell adhesin, the lubrication and protection of the mucosa (Fairbrother et al., 2005; Jensen et al., 2006). Three genes belonging to this family of glycoproteins have been identified as candidate genes for predicting the presence or absence of *F4bcR* in pigs. These genes are expressed in abundance in the intestinal tissues (Jacobsen et al., 2011). Ren et al. (2009) used the comparative radiation hybrid map and found three candidate genes for *F4acR* from the human genome which are *MUC4, MUC13* and *MUC20*. The three genes encodes the membrane bound O-glycoproteins, there was a strong association between the *F4acR* locus and the *MUC4* gene. Jacobsen and co-workers (2009) further confirmed the association of *MUC4, MUC13* and *MUC20* in the susceptibility to ETEC *F4ab/ac* using inter-crosses between two Wild Boars and eight Swedish Large White sows. The polymorphisms in the three genes, however, show an incomplete association with ETEC *F4ab/ac* genotype. None of these polymorphisms were found in the regulatory regions and did not result in any substitutions of crucial amino acids, or disrupts splice sites, and thus none are strong causal candidates.

The three genes coding for these mucin proteins are located on the porcine chromosome 13 and highly associated with resistance against *F4ab/ac* ETEC adhesin. Despite the association of the three mucin genes with the *F4bcR*, the causative gene and mutation remains unknown (Zhang et al., 2008; Ji et al., 2011). In addition, the characterisation of the genomic structures of the three mucin genes is still underway (Ji et al., 2011). The association of *MUC4* with ETEC *F4ab/ac* susceptibility/resistance has been intensively studied (Jorgensen et al., 2003; Jensen et al., 2006; Rasschaert et al., 2007; Joller et al., 2009; Rampoldi et al., 2011), but there exist few studies on association of ETEC *F4ab/ac* with *MUC13* and *MUC20* (Zhang et al., 2008; Ji et al., 2011).

Zhang et al. (2008) found three polymorphisms in *MUC13* which revealed a significant association with ETEC *F4ab/ac* susceptibility in White Duroc × Erhualian. *In vitro* ETEC *F4ab/ac* adhesin phenotypes showed a strong association with *c.576 C/T, c.908 A/G* and *c.935 A/C* polymorphisms genotypes. Ji et al. (2011) investigated seven single nucleotide polymorphisms (SNPs) in *MUC20* in White Duroc × Erhualian cross and revealed a strong association of ETEC *F4ab/ac* adhesin phenotypes with two polymorphisms. These polymorphisms are the *FbaI g.191 C/T* in intron 5 and the *Hin6I c.1600 C/T* in exon 6 of *MUC20* gene.

Jorgensen et al. (2003) reported an *XbaI* polymorphism of *MUC4* gene that could be useful for selection against susceptibility to *F4ab/ac* adhesin. Rasschaert et al. (2007) confirmed the *XbaI* polymorphism in intron 7 *MUC4, g.8227 C/G* as a possible causative mutation for the *F4ab/ac* adhesin. The resistant allele *A* was not digested by *XbaI*, and the susceptible allele *G* was digested into 151bp and 216bp fragments. The gene was, however, absent in 30.2 % of the pigs which were positive for *in vitro* villous adhesin test. It was concluded that there is another locus responsible for the *F4ab/ac E. coli* adhesin. Joller (2009) also confirmed *g.8227 C/G* polymorphism in the *MUC4* gene was strongly associated with the *F4ab/ac* adhesins.

*MUC4* genotypes are only partially associated with the expression of the *F4bcR*. Jensen et al. (2006) found that about 20 % of the *E. coli F4* resistant genotype piglets had diarrhoea. In a recent study using 45 offspring of a Large White boar with a *F4bcR* and *MUC4* recombination from Switzerland, the *MUC4* gene was found to have no linkage with the *F4bcR* locus (Rampoldi et al., 2011). The frequency of resistant genotype for *MUC4* in Belgian Landrace, Duroc and Polish Landrace were found to be 87.5, 85.7 and 16.2 %, respectively (Filistowicz and Jasek, 2006). Vrtkva et al. (2007) found that resistance allele is higher in native breed Prestice black-pied (0.8) than in Large White (0.52) and Landrace (0.26) of the Czech Republic. Li et al. (2008) reported a that the frequency of the resistance genotype of the *MUC4* gene was 20.2, 52.3 and 100 % in Chinese Landrace, Large White and Siaglo Black breeds, respectively.

*2.5.4.3 Methods for detection of receptors and their candidate gene*

The diagnosis of the *F4* and *FI8* receptor genotype has been performed using DNA based markers while the phenotype detection is based on the *in vitro* adhesin testing. The human genome has been extensively studied and the knowledge of the genetic control of *E. coli* diarrhoea known to occur in both humans and pigs allowed the study of the genetic control of this disease in pigs. Performing linkage analysis using the logarithm of odds ratio between the *F4* and *FI8* receptor genes and their respective markers allowed the identification of these receptors loci on the pig genome (Ren et al., 2009). The use of microsatellites and markers to locate the *F4bcR* and *FI8R* genes is very complicated and time consuming thus would not be feasible for selection of livestock for breeding. As such the polymorphism in the *FUT1* and Mucin genes (*MUC4*, *MUC13* and *MUC20*) that has been identified in several studies is now widely used to diagnose the *FI8R* and *F4bcR* genotypes.

The *in vitro* villous adhesion test has been performed to determine specific receptors in pigs. The phenotypes obtained are then compared to the alternative genotypes to verify the reliability of the candidate gene used in the selection process (Jensen et al., 2006). The *in vitro* villous adhesion test requires brush border cell of the pig under study as well as the sample of the reference *E. coli* bacteria. These samples are suspended in mannose for 30 min at room temperature. A drop of suspension is then viewed under the electron or a phase contrast microscopy to quantify the bacteria attached to the brush border. If five bacterial adhere to the brush border, then the epithelial cell is considered adhesive (Li et al., 2007; Rasschaert et al., 2007; Yan et al., 2009). The quality of an adhesion can be classified into 3 types known as adhesive where most bacteria adhere to most epithelial cells, non-adhesive and weak adhesin (where few bacteria adhere to most epithelial cells).

Hu et al. (1993) successively used the *in vitro* villous adhesion method and found that it was possible for one pig to express no or more than one fimbrial receptor at once. A total number of four adhesin phenotypes were identified. Yan et al. (2009) found eight phenotypes of the *F4* fimbrial adhesin

combinations occurred in pig breeds in China. The method is difficult to incorporate in breeding programmes as it requires a major surgery or the slaughter of the experimental animals (Francis, 1999; Rasschaert et al., 2007). Another problem associated with the *in vitro* villous adhesion method is that that age of the animal affects the *F4ab/ac* adhesion because the villous length decreases with age, therefore, resulting in older pigs being less susceptible.

DNA-based methods are sensitive and they allow precise genotyping of living animals. The allele specific (AL) and the restricted fragment length polymorphism (RFLP) PCR are among other DNA-based methods used to determine the susceptibility and resistance to *F4* and *FI8 E. coli* adhesin in pigs. Although most studies use RFLP-PCR, Lee et al. (2002) used both AL-PCR and RFLP-PCR and was able to find a 765 bp product of same mutation of *FUT1* gene at *M307* for both methods. The standard PCR method has been used over the years and allows amplification of one target gene at a time. The procedure, although sensitive and successfully used to identify the Mucin genes and *FUT1* gene polymorphisms, still have limitations. For example, it takes longer to amplify target genes due to the inability to amplify a number of target genes simultaneously. The limitations lead to the development of the multiplex PCR (Call et al., 2001). The other limitation involves the gel electrophoresis which is used for post-PCR detection.

*2.5.4.4 Application of candidate genes in pig breeding*

Knowledge of the genotype of these genes could mark the end of low profitability due to *E. coli* infections by selecting against the susceptible pigs and breeding of the resistant pigs in herds. This strategy was introduced in Switzerland in 1996 and after five years, the resistance allele of *FUT1* gene in Large White breed was increased from 0.31 to 0.54 and while the A allele in the Swiss Landrace tripled (0.07 to 0.24) (Bertschinger et al., 2004). A follow up study indicated that the resistant genotype of *FUT1* (*AA*) in Swiss Large White increased from 11 to 33 % and Duroc susceptibility was decreased from 86 to 51 %. The Landrace susceptibility was reduced by 12 % in a period of five years (Stranzinger, 2004 ).

Jensen et al. (2006) investigated the effect of the susceptibility and resistant genotype of the *XbaI* polymorphism in *MUC4* gene and found that when the piglets are challenged with *O*149:*F4ac* ETEC strains, 65 % of the *GG/AG* piglets developed diarrhoea and none of the *AA* piglets had diarrhoea on the first day of the challenge.

The *FUT1* gene is located in the region around the *RYR1* gene which is associated with the Malignant hyperthermia syndrome (Meijerink et al., 1997; Meijerink et al., 2000; Binder et al., 2002). Coddens et al. (2008) found no association between *FUT1* and the *RYR1* genes. Binder et al. (2002; 2005) investigated the effect of *FUT1* gene on production traits such as average daily gain (ADG), side fat thickness, carcass length, meat colour, pH of musculus longssimus dorsi and found no significant effects. Haung et al. (2008) also investigated *FUT1* and *RYR1* genes in Landrace and Duroc pig breeds in Taiwan and found no association between the two genes. The *FUT1* gene also showed no significant effect on the ADG, backfat thickness and feed conversion ratio but the *RYR1* gene had an effect on the ADG. Styanova (2009) also found that *FUT1* and *RYR* gene have no significant effects on backfact thickness and body weight gain. Buske et al. (2006) showed that *FUT1* is also located in a region associated with litter size and it was suggested that it may have pleiotropic effect on litter size and could be useful in selection for this reproductive trait. The results of the study were not significant and could not exclude that *FUT1* gene has pleiotropic effect on litter size.

### 2.6 *E. coli* colibacillosis in South African pigs

There is inadequate information published on the prevalence of *E. coli* in South Africa. No cases of colibacillosis outbreaks in South African pigs have been documented since 1997. Henton and Engelbrecht (1997) reported a 45.5 % prevalence for enteritis, where the colonization factor *F4* was associated with 46.9 % of the isolates from 674 cases reported from 1971 to 1991. The study also revealed that *E. coli* makes a contribution of 16.2 % of all diseases affecting pigs in South Africa and 70 % of the cases were from the province previously known as Transvaal. The reasons for this could be because the former

Transvaal area which comprises Limpopo, Gauteng, the eastern part of North West and Mpumalanga provinces is the main swine production area (DAFF, 2012). This part of the country is also well known for its warm temperatures, which may be favourable for multiplication of *E. coli*. The pathogenic factors important in colibacillosis were however not investigated.

Other related studies include a characterisation of antibiotic susceptibility of the *E. coli*, in which the *E. coli* isolates obtained from pigs were found to have multi-antibiotic resistance property (Ateba and Bezuidenhout, 2008; Monoeang et al., 2009). Another related study targeted a specific strain of *E. coli* (*O*157) type of *STEC* investigating *stx1*, *stx2* and *eaeA* genes but did not include ETEC and *EAST-1* toxin producing strains (Ateba et al. 2008; Ateba and Mbewe, 2011). The studies revealed that there was a level of pork contamination at sales points and that pigs are a higher source of the pathogen compared to cattle. The results suggest that since a number of South Africans rely on pork as a source of meat, they may acquire infections as a result of undercooked meat. Other than these studies, available information is web based and only includes a brief importance of *E. coli* infections in pig diseases and mortality and how to deal with such issues (Henton, 2010).

The other disadvantage facing the South African pig industry is the lack of advanced genetic improvement. Current genetic programs are mainly focused the improvement of the pork production and reproductive traits (Haesebrouck et al., 2004), with less emphasis on disease control. As such, no studies have been conducted on the genetic disease susceptibility status of colibacillosis in the South African pig population.

## 2.7 Summary

*Escherichia coli* infections remain the major cause of loss in pig production. The direct and indirect costs incurred by these infections are as a result of low growth rates, increased use of therapeutic agents, mortality and the increased proportions of condemned meat. Although different measures have been tried

as therapeutics against *E. coli*, antibiotics and vaccines are mostly used worldwide. However for as long as antibiotics are used, humans' safety will always be compromised as *E. coli* has developed resistance to most well-known antimicrobials. Vaccines may be a potential short term solution to *E. coli* infections. However they have been found to not induce full immunity and may last only for a given time frame thus requires repeated doses which may not be financially feasible.

Colibacillosis has been reported to break through even in situations where farm biosecurity is very strict. Therefore knowledge on the pathogenesis of *E. coli* is the most important in determining an effective control strategy. DNA based methods remain the best diagnostic procedure for *E. coli* suspicious infections as they are able to detect the causal genotype and therefore make the therapeutic measure easier to design. The selection of genetically resistant line of pigs, which is done by removal of susceptible individuals from the herd, remains the best possible solution. This method is mostly possible if one locus is involved in the control of the disease. The broad objective of the study is, therefore, to determine the prevalence of pathogenic *E. coli* and existence of polymorphisms in four candidate genes used for selection against the *F4ab/ac* and *FI8 E. coli* pig populations of South Africa.

## 2.8 References


Alexa, P., Stouracová, K., Hamrík, J. and Salajka, E. 2002. Gene typing of the colonisation factors *FI8* of *Escherichia coli* isolated from piglets suffering from post-weaning oedema disease. *Veterinarni Medicina-Praha*, 47, 132-136.

Bao, W., Wu, S., Musa, H., Zhu, G. and Chen, G. 2008. Genetic variation at the alpha 1 fucosyltransferase (*FUT1*) gene in Asian wild boar and Chinese and Western commercial pig breeds. *Journal of Animal Breeding and Genetics*, 125, 427-430.

Batisson, I., Guimond, M.P., Girard, F., An, H., Zhu, C., Oswald, E., Fairbrother, J. M., Jacques, M. and Harel, J. 2003. Characterization of the Novel Factor *Paa* Involved in the Early Steps of the

Adhesin Mechanism of Attaching and Effacing *Escherichia coli*. *Infection and Immunity*, 71, 4516-4525.

Bertschinger, H. U., Hofer, A., Stranzinger, G. and Vögeli, P. 2004. Breeding pigs resistant to *Escherichia coli FI8* in the field-A progress report from Switzerland. International Society for Animal Hygiène, 11-13 October 2004, Saint-Malo, France.

Bilkei, G. and Biro, O. 2000. Postpartum booster vaccination of sows against *E. coli* infections. *Berliner und Münchener Tierärztliche Wochenschrift*, 113, 67-70.

Binder, S., Götz, K., Thaller, G. and Fries, R. 2002. Effects of variation in the *FUT1* gene on various traits in swine.*Technische Universität München* 0, E006.

Binder, S., Götz, K., Thaller, G. and Fries, R. 2005. Investigations on the impact of genetic resistance to oedema disease on performance traits and its relation to stress susceptibility in pigs of different breeds. 56th Annual meeting of the European Association for Animal Production, 5-8 June 2005, Uppsala, Sweden.

Boerlin, P., Travis, R., Gyles, C. L., Reid-Smith, R., Heather Lim, N. J., Nicholson, V., Mcewen, S. A., Friendship, R. and Archambault, M. 2005. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Applied and Environmental Microbiology*, 71, 6753-6761.

Bosworth, B. T. and Vögeli, P. 2003. Methods and compositions to identify swine genetically resistant to *FI8 E. coli* associated diseases. EP Patent 0,985,052.

Buske, B., Sternstein, I., Reissmann, M., Reinecke, P. and Brockmann, G. 2006. Analysis of association of *GPX5*, *FUT1* and *ESR2* genotypes with litter size in a commercial pig cross population. *Archiv Tierzucht/Archives Animal Breeding*, 49, 259-268.

Call, D. R., Brockman, F. J. and Chandler, D. P. 2001. Detecting and genotyping *Escherichia coli O*157:*H*7 using multiplexed PCR and nucleic acid microarrays. *International Journal of Food Microbiology*, 67, 71-80.

Chen, X., Gao, S., Jiao, X. and Liu, X. F. 2004. Prevalence of serogroups and virulence factors of *Escherichia coli*strains isolated from pigs with postweaning diarrhoea in eastern China. *Veterinary Microbiology*, 103, 13-20.

Chernysheva, L. V., Friendship, R. M., Dewey, C. E. and Gyles, C. L. 2004. The effect of dietary chicken egg-yolk antibodies on the clinical response in weaned pigs challenged with a *K88+ Escherichia coli* isolate. *Journal of Swine Health and Production*, 12, 119-122.

Choi, C. and Chae, C. 1999. Genotypic prevalence of *F4* variants (*ab*, *ac*, and *ad*) in *Escherichia coli* isolated from diarrheic piglets in Korea. *Veterinary Microbiology*, 67, 307-310.

Choi, C., Kwon, D. and Chae, C. 2001. Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *E. coli*isolated from diarrheic piglets. *Journal of Veterinary Diagnostic Investigation*, 13, 26-29.

Ciobanu, D. C., Day, A. E., Nagy, A., Wales, R., Rothschild, M. F. and Plastow, G. S. 2001. Genetic variation in two conserved local Romanian pig breeds using type 1 DNA markers. *Genetics Selection Evolution* , 33, 417-432.

Clugston, R., Nielsen, N. and Smith, D. 1974. Experimental edema disease of swine (*E. coli* enterotoxemia) III. Pathology and pathogenesis. *Canadian Journal of Comparative Medicine*, 38, 34-43.

Coddens, A., Verdonck, F., Mulinge, M., Goyvaerts, E., Miry, C., Goddeeris, B., Duchateau, L. and Cox, E. 2008. The possibility of positive selection for both *FI8+ Escherichia coli* and stress resistant pigs opens new perspectives for pig breeding. *Veterinary Microbiology*, 126, 210-215.

DAFF. 2010. *PORK MARKET VALUE CHAIN PROFILE* [Online]. South Africa, Pretoria: Department of Agriculture, Forestry and Fisheries. [Accessed 14 Novemember at 15:03 2012].

Do, T., Stephens, C., Townsend, K., Wu, X., Chapman, T., Chin, J., Mccormick, B., Bara, M. and Trott, D. 2005. Rapid identification of virulence genes in enterotoxigenic *Escherichia coli* isolates associated with diarrhoea in Queensland piggeries. *Australian Veterinary Journal*, 83, 293-299.

Do, N., Cu, H., Nguyen, N., Nguyen, X., Au, X., Van, T., Vu, N. and Trott, D. 2006a. Antimicrobial resistance phenotypes of ETEC isolates from piglets with diarrhea in North Vietnam. *Annals of the New York Academy of Sciences*, 1081, 543-545.

Do, T. N., Cu, P. H., Nguyen, H. X., Au, T. X., Vu, Q. N., Driesen, S. J., Townsend, K. M., Chin, J. J. C. and Trott, D. J. 2006b. Pathotypes and serogroups of enterotoxigenic *Escherichia coli* isolated from pre-weaning pigs in north Vietnam. *Journal of Medical Microbiology*, 55, 93-99.

Donnenberg, M. and Kaper, J. B. 1991. Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infection and Immunity*, 59, 4310-4317.

Donnenberg, M., Tzipori, S., Mckee, M. L., O'brien, A. D., Alroy, J. and Kaper, J. B. 1993. The Role of the eae Gene of Enterohemorrhagic *Escherichia coli* in Intimate Attachment *In Vitro* and in a Porcine Model. *The Journal of Clinical Investigation*, 92, 1418-1424.

Erume, J., Berberov, E. M., Kachman, S. D., Scott, M. A., Zhou, Y., Francis, D. H. and Moxley, R. A. 2008. Comparison of the contributions of heat-labile enterotoxin and heat-stable enterotoxin b to the virulence of enterotoxigenic *Escherichia coli* in *F4ac* receptor-positive young pigs. *Infection and Immunity*, 76, 3141-3149.

Fairbrother, J. M., Nadeau, É. and Gyles, C. L. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, 6, 17-40.

Fekete, P. Z., Gerardin, J., Jacquemin, E., Mainil, J. and Nagy, B. 2002. Replicon typing of *FI8* fimbriae encoding plasmids of enterotoxigenic and verotoxigenic *Escherichia coli* strains from porcine postweaning diarrhoea and oedema disease. *Veterinary Microbiology*, 85, 275-284.

Filistowicz, M. and Jasek, S. 2006. Preliminary study on the effect of *FUT1* and *MUC4* loci on the fertility of sows and on bredding success in piglets. *Acta fytotechnica et zootechnica (online), Mimoriadne-Special*, 9, 23-26.

Francis, D., Moxley, R. and Andraos, C. 1989. Edema disease-like brain lesions in gnotobiotic piglets infected with *Escherichia coli* serotype *O*157: *H*7. *Infection and Immunity*, 57, 1339-1342.

Francis, D. H., Grange, P. A., Zeman, D. H., Baker, D. R., Sun, R. and Erickson, A. K. 1998. Expression of Mucin-Type Glycoprotein *K88* Receptors Strongly Correlates with Piglet Susceptibility to *K88* Enterotoxigenic *Escherichia coli*, but Adhesin of This Bacterium to Brush Borders Does Not. *Infection and Immunity*, 66, 4050-4055.

Francis, D. H. 1999. Colibacillosis in pigs and its diagnosis. *Swine Health Prodution*, 7, 214-244.

Francis, D. 2002. Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis. *Journal of Swine Health Production*, 10, 171-175.

Frydendahl, K., Kare Jensen, T., Strodl Andersen, J., Fredholm, M. and Evans, G. 2003. Association between the porcine *Escherichia coli FI8* receptor genotype and phenotype and susceptibility to colonisation and postweaning diarrhoea caused by *E. coli O*138: *FI8*. *Veterinary Microbiology*, 93, 39-51.

Goldhar, J. 1996. Nonfimbrial Adhesins of *Escherichia coli*. *Advances in Experimental Medicine and Biology*, 408, 63-72.

Haesebrouck, F., Pasmans, F., Chiers, K., Maes, D., Ducatelle, R. and Decostere, A. 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? . *Veterinary Microbiology*, 100, 255-268.

Hammerum, A. and Heuer, O. 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, 48, 916-920.

Henton, M. and Engelbrecht, M. 1997. *Escherichia coli* serotypes in pigs in South Africa. *The Onderstepoort Journal of Veterinary Research*, 64, 175-187.

Hu, Z., Hasler-Rapacz, J., Huang, S. and Rapacz, J. 1993. Studies in swine on inheritance and variation in expression of small intestinal receptors mediating adhesin of the *K88* enteropathogenic *Escherichia coli* variants. *Journal of Heredity*, 84, 157-165.

Huang, S. Y., Chung, M. T., Tsou, H. L. and Li, H. L. 2008. Association of polymorphism in the alpha (1, 2) fucosyltransferase gene with growth performance in two Western pig breeds in Taiwan. *Livestock Science*, 114, 336-340.

Imberechts, H., De Greve, H. and Lintermans, P. 1992. The pathogenesis of edema disease in pigs. A review. *Veterinary Microbiology*, 31, 221-233.

Intervet, S.-P. 2010. *Vaccines* [Online]. Available: http://www.intervet.com/species/pigs/vaccines.aspx [Accessed 29 November at 1: 23 pm 2012].

Ironkwe, M. O. and Amefule, K. U. 2008. Appraisal of indigenous pig production and management practices in River State, Nigeria. *Journal Of Agriculture and Social Research*, 8, 1-7.

Jacobsen, M., Cirera, S., Joller, D., Esteso, S., Kracht, S. S., Edfors, I., Bendixen, C., Archibald, A. L., Voegeli, P., Neuenschwander, S., Bertschinger, H. U., Rampoldi, A., Andersson, L., Fredholm, M. and Jorgensen, C. B. 2011. Characterisation of five candidate genes within the ETEC *F4ab/ac* candidate region in pigs. *BMC Research Notes*, 4, 225-232.

Jamalludeen, N., Johnson, R. P., Shewen, P. E. and Gyles, C. L. 2009. Evaluation of bacteriophages for prevention and treatment of diarrhea due to experimental enterotoxigenic *Escherichia coli O*149 infection of pigs. *Veterinary Microbiology*, 136, 135-141.

Jensen, G. M., Frydendahl, K., Svendsen, O., Jorgensen, C. B., Cirera, S., Fredholm, M., Nielsen, J. P. and Moller, K. 2006. Experimental infection with *Escherichia coli O*149: *F4ac* in weaned piglets. *Veterinary Microbiology*, 115, 243-249.

Ji, H., Ren, J., Yan, X., Huang, X., Zhang, B., Zhang, Z. and Huang, L. 2011. The porcine *MUC20* gene: molecular characterization and its association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Molecular Biology Reports*, 38, 1593-1601.

Joller, D., Jørgensen, C. B., Bertschinger, H. U., Python, P., Edfors, I., Cirera, S., Archibald, A. L., Bürgi, E., Karlskov-Mortensen, P., Andersson, L., Fredholm, M. and Vögeli, P. 2009. Refined localization of the *Escherichia coli F4ab/F4ac* receptor locus on pig chromosome 13. *Animal Genetics*, 40, 749-752.

Jorgensen, C. B., Cirera, S., Archibald, A., Andersson, L., Fredholm, M. and Edfors-Lilia, I. 2003. Porcine polymorphisms and methods for detecting them. Patent US7785778.

Kemm, E. 1993. *Pig production in South Africa*, Agricultural Research Council (ARC) Bulletin, 427. V and R Printers, Pretoria, South Africa.

Klukowska, B. J., Urbaniak, B. and Wito Ski, M. 1999. High frequency of *M307A/G* mutation at *FUT1* locus, causing resistance to oedema disease, in an autochtonous Polish pig breed, the Zlotnicka Spotted. *Journal of Animal Breeding and Genetics*, 116, 519-524.

Laarmann, S. and Schmidt, M. A. 2003. The *Escherichia coli AIDA* autotransporter adhesin recognizes an integral membrane glycoprotein as receptor. *Microbiology*, 149, 1871-1882.

Lee, S., Cho, K., Kang, S., Kim, C., Park, H., Choy, Y. and Choi, Y. 2002. Detection of pigs resistant to post weaning diarrhoea, oedema disease and porcine stress syndrome by allele specific polymerase chain reaction. *Animal Genetics*, 33, 237-239.

Lee, S. I., Rayamahji, N., Lee, W. J., Cha, S. B., Shin, M. K., Roh, Y. M. and Sang, H. Y. 2009. Genotypes, antibiogram, and pulsed-field gel electrophoresis profiles of *Escherichia coli* strains from piglets in Korea. *Journal of Veterinary Diagnostic Investigation*, 21, 510-516.

Lemus-Flores, C., Mejía-Martínez, K., Rodríguez-Carpena, J., Barreras-Serrano, A., Herrera-Haro, J. and Alonso-Morales, R. 2009. Genetic diversity and variation of *ESR*, *RBP4* and *FUT1* genes in Mexican creole and Yorkshire pig populations. *Journal of Biological Sciences*, 9, 878-883.

Li, H., Li, Y., Qiu, X., Niu, X., Lui, Y. and Zhang, Q. 2008. Identification and screening of gene (s) related to susceptibility to Enterotoxigenic *Escherichia coli F4ab/ac* in piglets. *Asian-Australasian Journal of Animal Sciences*, 21, 489-493.

Li, Y., Qiu, X., Li, H. and Zhang, Q. 2007. Adhesive patterns of *Escherichia coli F4* in piglets of three breeds. *Journal of Genetics and Genomics*, 34, 591-599.

Madoroba, E., Van Driessche, E., De Greve, H., Mast, J., Ncube, I., Read, J. and Beeckmans, S. 2009. Prevalence of enterotoxigenic *Escherichia coli* virulence genes from scouring piglets in Zimbabwe. *Tropical Animal Health Production*, 41, 1539–1547.

Marchès, O., Nougayrède, J.-P., Boullier, S., Mainil, J., Charlier, G., Raymond, I., Pohl, P., Boury, M., De Rycke, J., Milon, A. and Oswald, E. 2000. Role of *Tir* and *Intimin* in the Virulence of Rabbit Enteropathogenic *Escherichia coli* Serotype *O*103: *H*2. *Infection and Immunity*, 68, 2171-2182.

Maritz, J. 2006. Antimicrobial growth promoters in animal production- The situation locally and internationally. *Animal Feed Manufacturer Association Matrix*, 15, 3-7.

Mattsson, S. and Melin, L. 2004. An enhanced technique to serotype *Escherichia coli* and the prevalence of serotypes among Swedish pigs. Proceedings of the 18th International Pig Veterinary Society Congress, 27 June-1 June 2004, Hamburg, Germany.

Meijerink, E., Fries, R., Vögeli, P., Masabanda, J., Wigger, G., Stricker, C., Neuenschwander, S., Bertschinger, H. U. and Stranzinger, G. 1997. Two α(1,2) fucosyltransferase genes on porcine Chromosome 6q11 are closely linked to the blood group inhibitor and *Escherichia coli FI8* receptor loci. *Mammalian Genome*, 8, 736-741.

Meijerink, E., Neuenschwander, S., Fries, R., Dinter, A., Bertschinger, H., Stranzinger, G. and Vögeli, P. 2000. A DNA polymorphism influencing a (1, 2) fucosyltransferase activity of the pig *FUT1* enzyme determines susceptibility of small intestinal epithelium to *Escherichia coli FI8* adhesin. *Immunogenetics*, 52, 129-136.

Moneoang, M. S. and Bezuidenhout, C. C. 2009. Characterisation of enterococci and *Escherichia coli* isolated from commercial and communal pigs from Mafikeng in the North-West Province, South Africa. *African Journal of Microbiology Research*, 3, 088-096.

Moon, H. W., Hoffman, L. J., Cornick, N. A., Booher, S. L. and Bosworth, B. T. 1999. Prevalences of some virulence genes among *Escherichia coli* isolates from swine presented to a diagnostic laboratory in Iowa. *Journal Veterinary Diagnostic Investigation*,11, 557–560.

Mullaney, C. D., Francis, D. H. and Willgohs, J. 1991. Comparison of seroagglutination, ELISA, and indirect fluorescent antibody staining for the detection of *K99*, *K88*, and *987P* pilus antigens of *Escherichia coli*. *Journal of Veterinary Diagnostic Investigation*, 3, 115-118.

Nagy, B., Wilson, R. A. and Whittam, T. S. 1999. Genetic diversity among *Escherichia coli* isolates carrying *FI8* genes from pigs with porcine postweaning diarrhea and edema disease. *Journal of Clinical Microbiology*, 37, 1642-1645.

Nagy, B. and Fekete, P. Z. 2005. Enterotoxigenic *Escherichia coli* in veterinary medicine. *International Journal of Medical Microbiology*, 295, 443-454.

Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, 11, 142-201.

Niewerth, U., Frey, A., Voss, T., Le Bouguenec, C., Baljer, G., Franke, S. and Schmidt, M. A. 2001. The *AIDA* Autotransporter System Is Associated with *FI8* and *Stx2e* in *Escherichia coli* Isolates from Pigs Diagnosed with Edema Disease and Postweaning Diarrhea. *Clinical and Diagnostic Laboratory Immunology*, 8, 143–149.

Niu, X., Li, Y., Ding, X. and Zhang, Q. 2011. Refined mapping of the *Escherichia coli F4ab/F4ac* receptor gene (s) on pig chromosome 13. *Animal Genetics*, 42, 552-555.

Onderstepoort, B. P. undated. *Oil-Emulsion Vaccine Esherichia coli vaccine for cattle and sheep* [Online]. Available: http://www.obpvaccines.co.za/disease_goatsinfo.htm [Accessed 04 April at 4:15 pm 2011].

Osek, J., Gallien, P., Truszczy Ski, M. and Protz, D. 1999. The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland. *Comparative Immunology, Microbiology and Infectious Diseases*, 22, 163-174.

Parma, A., Sanz, M., Viñas, M., Cicuta, M., Blanco, J., Boehringer, S., Vena, M., Roibon, W., Benitez, M. and Blanco, J. 2000. Toxigenic *Escherichia coli* isolated from pigs in Argentina. *Veterinary Microbiology*, 72, 269-276.

Paton, J. C. and Paton, A. W. 1998. Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections. *Clinical Microbiology Reviews*, 11, 450-479.

Peng, Q. L., Ren, J., Yan, X. M., Huang, X., Tang, H., Wang, Y. Z., Zhang, B. and Huang, L. S. 2007. The *g. 243A> G* mutation in intron 17 of *MUC4* is significantly associated with susceptibility/resistance to ETEC *F4ab/ac* infection in pigs. *Animal Genetics*, 38, 397-400.

Pharmavet, S. A. undated. *Pharmavet South Africa Information Brochure* [Online]. Available: www.pharmavet-sa.com/Pharmavet%20brochure.pdf [Accessed 24 March at 12:25 pm 2011].

Python, P., Jörg, H., Neuenschwander, S., Asai Coakwell, M., Hagger, C., Bürgi, E., Bertschinger, H., Stranzinger, G. and Vögeli, P. 2005. Inheritance of the *F4ab*, *F4ac* and *F4ad E. coli* receptors in swine and examination of four candidate genes for *F4acR*. *Journal of Animal Breeding and Genetics*, 122, 5-14.

Python, P., Jörg, H., Neuenschwander, S., Hagger, C., Stricker, C., Bürgi, E., Bertschinger, H. U., Stranzinger, G. and Vögeli, P. 2002. Fine-mapping of the intestinal receptor locus for enterotoxigenic *Escherichia coli F4ac* on porcine chromosome 13. *Animal Genetics*, 33, 441-447.

Rampoldi, A., Jacobsen, M. J., Bertschinger, H. U., Joller, D., Burgi, E., Vogeli, P., Andersson, L., Archibald, A. L., Fredholm, M., Jorgensen, C. B. and Neuenschwander, S. 2011. The receptor locus for *Escherichia coli F4ab/F4ac* in the pig maps distal to the *MUC4-LMLN* region. *Mammalian Genome*, 22, 122-129.

Rasschaert, K., Verdonck, F., Goddeeris, B. M., Duchateau, L. and Cox, E. 2007. Screening of pigs resistant to *F4* enterotoxigenic *Escherichia coli* (ETEC) infection. *Veterinary Microbiology*, 123, 249-253.

Ren, J., Tang, H., Yan, X., Huang, X., Zhang, B., Ji, H., Yang, B., Milan, D. and Huang, L. 2009. A pig–human comparative RH map comprising 20 genes on pig chromosome 13q41 that harbours the ETEC *F4ac* receptor locus. *Journal of Animal Breeding and Genetics*, 126, 30-36.

Sargeant, H. R., Mcdowall, K. J., Miller, H. M. and Shaw, M. A. 2010. Dietary zinc oxide affects the expression of genes associated with inflammation: Transcriptome analysis in piglets challenged with ETEC *K88*. *Veterinary Immunology and Immunopathology*, 137, 120-129.

Sellwood, R. 1983. Genetic and immune factors in the susceptibility of piglets to *Escherichia coli* diarrhoea. *Annales De Recherches Veterinaires*, 14, 512-518.

Souza Da Silva, A., Valadares, G. F., Penatti, M. P. A., Brito, B. G. and Da Silva Leite, D. 2001. *Escherichia coli* strains from edema disease: *O* serogroups, and genes for Shiga toxin, enterotoxins, and *FI8* fimbriae. *V eterinary Microbiology*, 80, 227-233.

Stein, H. H. 2002. Experience of feeding pigs without antibiotics: a European perspective. *Animal Biotechnology*, 13, 85-95.

Stranzinger, G. F. 2004 Gene mutations conditioning some diseases in pigs and their application in breeding programmes in Switzerland. *Animal Science Papers and Reports*, 22, 127-129.

Styanova, S. 2009. Performance test traits in Danube White pigs with different *RYR*, *ESR* and *FUT1* genotypes. *Agricultural Science and Technology*, 1, 113-116.

To, S. C. M. 1984. *F41* Antigen Among Porcine Enterotoxigenic *Escherichia coli* Strains Lacking *K88*, *K99*, and *987P* Pili. *Infection and Immunity*, 43, 549-554.

Trevisi, P., Corrent, E., Messori, S., Casini, L. and Bosi, P. 2010. Healthy newly weaned pigs require more tryptophan to maximize feed intake if they are susceptible to *Escherichia coli K88*. *Livestock Science*, 134, 236-238.

Van Poucke, M., Melkebeek, V., Erkens, T., Van Zeveren, A., Cox, E. and Peelman, L. J. 2009. Molecular cloning and characterization of the porcine prostaglandin transporter (*SLCO2A1*): evaluation of its role in *F4* mediated neonatal diarrhoea. *BMC Genetics*, 10, 1-9.

Veilleux, S. and Dubreuil, D. J. 2006. Presence of *Escherichia coli* carrying the *EAST1* toxin gene in farm animals. *Veterinary Research*, 37, 3-13.

Vögeli, P., Meijerink, E., Fries, R., Neuenschwander, S., Vorländer, N., Stranzinger, G. and Bertschinger, H. 1997. A molecular test for the detection of *E. coli FI8* receptors: a breakthrough in the struggle against edema disease and post-weaning diarrhea in swine. *Schweizer Archiv für Tierheilkunde*, 139, 479-484.

Vrtková, I., Matoušek, V., Stehlík, L., Šrubařová, P., Offenbartel, F. and Kernelová, N. 2007. Genomic markers important for health and reproductive traits in pigs. *Research in Pig Breeding*, 1, 4-6.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J., Mora, A., Dahbi, G., López, C. and González, E. 2006. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia. *BMC Veterinary Research*, 2, 1-8.

Wang, J., Jiang, S. W., Chen, X. H., Liu, Z. L. and Peng, J. 2006. Prevalence of fimbrial antigen (*K88* variants, *K99* and *987P*) of enterotoxigenic *Escherichia coli* from neonatal and post-weaning piglets with diarrhea in central China. *Asian-Australasian Journal of Animal Sciences*, 19, 1011-2367.

Wang, X. M., Jiang, H. X., Liao, X. P., Liu, J. H., Zhang, W. J., Zhang, H., Jiang, Z. G., Lu, D. H., Xiang, R. and Liu, Y. H. 2010. Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiology Letters*, 306, 15-21.

Whitfield, C., Walker, S. G., Atkinson, C. F., Lam, J. S., Macdonald, L. A. and Beveridge, T. J. 1988. Serotype-specific monoclonal antibodies against the *H*12 flagellar antigen of *Escherichia coli*. *Microbiology*, 134, 1747-1753.

Wong, I., Moreno, M., Valderrama, S., Joglar, M., Horrach, M., Bover, E., Borroto, A., Basulto, B., Calzada, L., Hernandez, R., Herrera, L. and De La Fuente, J. 1995. Immunity and protection elicited by a recombinant vaccine against enterotoxigenic *Escherichia coli*. *Biotecnologia Aplicada*, 12, 9-15.

Yamamoto, T. and Nakazawa, M. 1997. Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. *Journal of Clinical Microbiology*, 35, 223-227.

Yan, X., Huang, X., Ren, J., Zou, Z., Yang, S., Ouyang, J., Zeng, W., Yang, B., Xiao, S. and Huang, L. 2009. Distribution of *Escherichia coli F4* adhesin phenotypes in pigs of 15 Chinese and Western breeds and a White DurocxErhualian intercross. *Journal of Medical Microbiology*, 58, 1112-1117.

Yang, B., Huang, X., Yan, X., Ren, J., Yang, S., Zou, Z., Zeng, W., Ou, Y., Huang, W. and Huang, L. 2009. Detection of quantitative trait loci for porcine susceptibility to enterotoxigenic *Escherichia coli F41* in a White Duroc × Chinese Erhualian resource population. *Animal*, 3, 946-950.

Zhang, W., Zhao, M., Ruesch, L., Omot, A. and Francis, D. 2007. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Veterinary Microbiology*, 123, 145-152.

Zhang, B., Ren, J., Yan, X., Huang, X., Ji, H., Peng, Q., Zhang, Z. and Huang, L. 2008. Investigation of the porcine *MUC13* gene: isolation, expression, polymorphisms and strong association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Animal Genetics*, 39, 258–266.

Zhu, C., Harel, J., Jacques, M., Desautels, C., Donnenberg, M., Beaudry, M. and Fairbrother, J. 1994. Virulence properties and attaching-effacing activity of *Escherichia coli O*45 from swine postweaning diarrhea. *Infection and Immunity*, 62, 4153-4159.

# CHAPTER 3

## Prevalence of enterotoxigenic, shiga toxin and enteroaggregative *Escherichia coli* in South African pigs

*Published in Tropical Animal Health and production (see Appendix 1)*


**Abstract**

*Escherichia coli*, particularly the enterotoxigenic (ETEC) and shiga toxin (STEC) producing strains, are important causes of colibacillosis in neonatal and early post-weaning piglets. Recently, there has been reported increase in the occurrence of enteroaggregative *E. coli* (EAEC) heat-stable enterotoxin 1 (*EAST-1*) strains in other pig populations across the world. The objective of the current study was to determine the presence and prevalence of enterotoxins (*LT*, *STa* and *STb*), shiga-toxins (*Stx*1, *Stx*2, *Stx2e*) and enteroaggregative heat stable *E. coli* (*EAST-1*) in South African pig populations. *Escherichia coli* strains were isolated from South African Landrace (n = 24), Large White (n = 126), Duroc (n = 28) and South African indigenous (n = 85) piglets aged between 9 and 136 days. Multiplex PCR (mPCR) and simplex PCR were used to detect the presence of the ETEC, STEC and *EAST-1* virulence genes in the 263 isolates. Of these isolates, 39.6 % were positive for *E. coli* virulent genes. Most of the positive isolates belonged to the ETEC (55.1 %) and EAEC (49.1 %) and STEC was the least recovered (2.8 %). The individual genes were found in the following proportions: *STb* (49.1 %), *LT* (0.9 %), *STa* (8.5 %), *Stx2e* (2.8 %) and *EAST-1* (50.0 %) toxins. The presence of the *EAST-1* in the current isolates suggests that there is wide variety of virulent gene combinations associated with colibacillosis in South African pig populations. The study provides a reference to estimate current and/or compare future observations on the occurrence of ETEC, EAEC and STEC in South African pig farms



**Keywords:** ETEC, STEC, EAEC, pigs, diarrhoea, toxins, oedema

## 3.1 Introduction

Diarrhoea has the highest morbidity estimated to be over 10 % among pre-weaning diseases in pigs in a number of regions (Ngeleka et al., 2003). *Escherichia coli*, particularly the enterotoxigenic and shiga toxin producing strains, are important causes of colibacillosis in South Africa (Kemm, 2002; Henton, 2010) and in many countries worldwide, affecting piglets at both neonatal and weaning phases (Nagy et al., 1999; Li et al., 2007; Rampoldi et al., 2011). Colibacillosis in piglets result in remarkable losses to the pig industry due to excessive weight loss, increased use of antibiotics, frequent vaccinations and mortality (Bosworth and Vögeli, 2003).

Enterotoxigenic *E. coli* is mainly associated with diarrhoea, while Shiga toxin producing *E. coli* causes oedema in infected piglets (Osek et al., 1999; Chen et al., 2004; Wang et al., 2010b). The enterotoxigenic strains are characterised by their release of heat-labile toxins (*LT*) and heat-stable toxins (*ST*) (Nataro and Kaper, 1998; Parma et al., 2000; Fairbrother et al., 2005;). Heat-stable toxins consist of variants *STa*/*STI* and *STb*/*STII* which have been isolated from diarrheic piglets (Parma et al., 2000). Shiga toxin-producing strains are recognised by their release of Shiga toxins, *Stx*1and *Stx*2 which also exist in variant *Stx2e* which is considered an important toxin for oedema in pigs (Chen et al., 2004). In addition to the enterotoxins and shiga toxins, EAEC has been implicated in porcine diarrhoea (Choi and Chae, 1999; Veilleux and Dubreuil, 2006). Enteroaggregative heat-stable enterotoxin 1 was found to be widely prevalent in Korean piglets and is considered to be an important virulence factor in colibacillosis of pre-weaned pigs, together with other ETEC and STEC virulence factors (Choi et al., 2001).

Colibacillosis due to *Escherichia coli* has been ranked among the most important diseases in pig production by the South Africa Pork Producers Organisation (Henton, 2010). Currently, one of the major challenges facing the pig industry is to find a sustainable and cost effective method to eradicate or reduce the effect of colibacillosis infections in piglets. Colibacillosis control strategies include antibiotics, vaccines incorporating known fimbriae antigens, zinc oxide (ZnO) and probiotics (Wong et al., 1995;

Owusu-Asiedu et al., 2003; Chernysheva et al., 2004; Jamalludeen et al., 2009; Sargeant et al., 2010). The success of any control or treatment programme for colibacillosis would depend on good knowledge of the aetiology of the disease, characteristics of the pathogens, pathogenecity as well as their distribution. There is paucity of information on the prevalent colibacillosis causing *E. coli* in South Africa.

Henton and Englebrecht (1997) observed 45.5 % incidence of occurrence for enteritis and colonization factor *F4* (46.9 %) from 674 cases reported over a period of 20 years from 1971 to 1991. This was one first study for South African ETEC after which there was no follow up studies. The prevalence of particular virulence gene combinations or pathotypes are bound to change with time (Vu-Khac et al., 2006) due to new mutations arising and others being selected out of the population by use of vaccines and antibiotics (Doughari et al., 2009). Moneoang and Bezuidenhout, (2009) described the antibiotic susceptibility of the South African *E. coli* without giving an indication of its prevalence and distribution in the country. Other studies by Ateba and co-workers (2008; 2011) targeted specific strains of *E. coli* 0157 and did not include ETEC and *EAST-1* toxin producing strains.

The objective of the study was to determine the prevalence of ETEC and STEC and EAEC in Duroc, Landrace and Indigenous pig breeds of South Africa. Genes that encode *LT*, *STa*, *STb*, *Stx1*, *Stx2*, *Stx2e* and *EAST-1* virulence toxins were investigated.

## 3.2 Materials and methods

### *3.2.1 Pigs*

Two hundred and sixty three neonatal (1-28 d) and post-weaned (29-136 d), healthy pigs of the indigenous (n = 85), Large White (n = 126), Duroc (n = 28) and Landrace breeds (n = 24) were sampled from Agricultural Research Council (ARC) Livestock unit, Irene and Middledrift Farm in Eastern Cape

Province. The exotic breeds were maintained on pelleted creep feed (19 % crude protein (CP)) *ad libitum* from age of two to five weeks. From five weeks, these weaners were fed on a mash diet (18 % CP, 14 MJ Digestible Energy (DE)/kg) and from six to eight weeks the diet composition was reduced to 16 % CP and 13.2 MJ DE/kg. The indigenous sows were maintained on a mash diet (13 % CP, 13 MJ DE/kg) and the piglets had access to the sows' feed. After weaning, the indigenous piglets were maintained on the same diet as the sows. Boars and pregnant sows were maintained on the same diet and they received 2kg feed supplement daily.

### *3.2.2 Faecal swab collection and microbiological analysis*

Rectal swabs were collected from the piglets using COPAN$^{R}$ faecal swabs and were immediately stored in a transport medium. The samples were labelled with the pig identification number, breed and sex, placed in ice filled cooler bags and transported to the laboratory immediately. A total of 213 faecal swabs were collected from piglets at ARC, Livestock Production Unit, located at Irene. Fifty additional samples were collected from the Middledrift Pig Farm in the Eastern Cape Province.

The samples were transported to the Feed and Food Analysis Laboratory at the ARC - Onderstepoort Veterinary Institute, (Onderstepoort, Pretoria) for further processing. The rectal swabs were streaked onto MacConkey and Blood Tryptose agar plates (Selecta-media-BTA Agar) followed by an overnight aerobic incubation at 37 $^{\circ}$C. Presumptive *E. coli* produced red or rose pink colonies on the MacConkey agar and these were subjected to biochemical tests. These isolates were tested using the indole, methyl red, Voges Proskauer, and Simmons citrate (IMVic) tests for confirmation of *E. coli*.

Eleven reference samples known to contain the enterotoxins and the fimbrial antigens were obtained from ARC-Onderstepoort veterinary institution, Bacteriology culture collection. The strains included B41 (*F5/F41/STa*), 1883-1 (*FI8*/*STa*), 1883-2 (*F41/ F5/ STa* ), 1883-3 (*F41/F5/ F6/ STa*), 1883-4 (*F4/F5/LT/STb*), *E. coli* ATCC 25922 (Negative control), *E. coli* 2020 (negative control), 1474 *K12*-

*K88ac* Geel (*F4/ F5/LT/STb*), 1474 *K12-K99* Pienk (*F5/STa*), 1474 *K12-K99* Geel (*F5/STa*) and *K99* (*F5/STa*).

### *3.2.3 DNA extraction*

Loopfuls of pure colonies from nutrient agar were dissolved in 1ml distilled water and boiled at 99 $^{0}$C on an Accublock$^{TM}$ digital dry bath (Labnet international Inc.) for 20 min to induce bacterial cell lysis for the release of DNA. Using an 18 G × 1½″ syringe needle (Terumo, R.B. Non-pyrogenic, Terumo Corporation), the tube lids were pricked to prevent the lids from popping due to pressure build up during heating. Samples were left to cool at room temperature and then centrifuged at 13 rpm (revolutions per min) for 5 min using an Eppendorf Mini Spin (Eppendorf, Germany). Supernatants that contained crude DNA were used for all the PCR reactions.

### *3.2.4 DNA amplification and gene identification*

Crude DNA extracts were subjected to multiplex PCR containing various combinations of primer sets. Oligo primers for enterotoxins (*LT*, *STa* and *STb*), shiga toxins (*Stx*1, *Stx*2 and *Stx2e*), were used for the polymerase chain reactions (PCR). The primer sequences and expected amplicon sizes are shown in Table 3.1. The 25 µl PCR reactions for each set of virulence genes consisted of 10µM of each primer, 5µl of DNA, 12.5 µl of 2X PCR Master mix (DreamTaq$^{TM}$ Green PCR Master Mix, Fermentas) and PCR water (Fermentas). Eppendorf AG 22331 Hamburg or Eppendorf AG 22331 Vapo. Protect thermocylcers (Eppendorf, Germany) were used for PCR and the cycling conditions were as follows: 10 min initial denaturation at 94 $^{\circ}$C, followed by 30 cycles of denaturation for 30 s at 94 $^{\circ}$C, annealing phase of 30 s at 56$^{\circ}$Cand 1 min extension at 72 $^{\circ}$C. The 30 cycles were followed by 7 min of extension at 72 $^{\circ}$C. For amplification of *EAST-1* toxin, a single PCR was conducted using the similar conditions as the above mentioned multiplex PCR with the volume of water adjusted accordingly.

### *3.2.5 Electrophoresis*

Seven (7) µl of PCR amplicons were electrophoresed through a 2 % agarose gel (Seakem$^{R}$ LE Agarose, Lonza, Rockland ME, USA) stained with 4µl per 100 ml of Ethidium bromide (EtBr) in 1X TAE Electrophoresis Buffer (40 mM Tris, 20 mM Acetic acid, 1mM EDTA) (Fermentas, Life Science, V.Graiiuno 8, LT-02241 Vilnius, Lithuania). The samples were electrophoresed for 1.5 to 2h, followed by visualization of the gels using a gel documentation system (Bio-Rad Trans-UV, Japan). The amplicon sizes were estimated using a 100 bp plus DNA ladder (Fermentas, Life sciences).

### *3.2.6 Statistical analysis*

The prevalence of the different virulence genes were calculated using SAS (2012). Differences in the prevalence of the individual virulence genes, gene combination among the breeds and between sites of collection were assessed using Chi-square ($\chi^2$) tests (SAS, 2012). A *P*-value of less than 0.05 was considered significant.

**Table 3. 1:** The primer sequences and amplicon sizes of the virulence genes/ factors of ETEC, STEC and *EAST-1*

| mPCR primer set | VFs | Primers sequences (5'-3') (2,3) | Target gene | Size (bp) | References |
|---|---|---|---|---|---|
| Enterotoxins | *STa*-F | GGG TTG GCA ATT TTT ATT TCT GTA | | | (Cai et al, 2003; Ngeleka et |
| | *STa*-R | ATT ACA ACA AAG TTC ACA AGC AGT A | *estI* | 183 | al., 2003; Cheng et al., 2010 ) |
| | *STb*-F | ATG TAA ATA CCT ACA ACG GGT GAT | | | (Cai et al, 2003; Ngeleka et |
| | *STb*-R | TAT TTG GGC GCC AAA GCA TGC TCC | *estII* | 360 | al., 2003; Cheng et al., 2010 ) |
| | *LT*-F | TAG AGA CCG GTA TTA CAG AAA TCT GA | | | (Cai et al, 2003; Ngeleka et |
| | *LT*-R | TCA TCC CGA ATT CTG TTA TAT ATG TC | *Elt* | 282 | al., 2003; Cheng et al., 2010 ) |
| Enteroaggregative | *EAST-1*-F | TCG GAT GCC ATC AAC ACA GT | | | |
| | *EAST-1*-R | GTC GCG AGT GAC GGC TTT GTA G | *astA* | 125 | (Cheng et al., 2006) |
| Shigatoxins | *Stx1*-F | ATT CGC TGA ATG TCATTC GCT | | | (Cai et al., 2003; Cheng et al., |
| | *Stx1*-R | ACG CTT CCC AGA ATT GCA TTA | *StxI* | 664 | 2010) |
| | *Stx2*-F | GAA TGA AGA AGA TGT TTA TAG CGG | | | (Cai et al., 2003; Cheng et al., |
| | *Stx2*-R | GGT TAT GCC TCA GTC ATT ATT AA | *StxII* | 281 | 2010) |
| | *Stx2e*-F | GAA TGA AGA AGA TGT TTA TAG CGG | | | (Cai et al., 2003; Cheng et al., |
| | *Stx2e*-R | TTT TAT GGA ACG TAG GTA TTA CC | *Stx2e* | 454 | 2010) |

## 3.3 Results

### *3.3.1 Microbiological results*

All the 263 faecal swab samples showed pure cultures of lactose-fermenting colonies on MacConkey agar, which is presumptive for *E. coli*. All 263 presumptive *E. coli* were gram negative short rods. The isolates were indole and methyl red positive, motile, and negative for Vogues Proskauer and citrate tests. Taken together, the results indicated that *E. coli* was isolated from all the 263 faecal swabs. All the 263 samples were identified as *E. coli* and these were genotyped.

### *3.3.2 Genotyping results*

All the primers for enterotoxigenic (Figure 3.1), shigatoxin and *EAST-1* (Figure 3.2) yielded PCR product of the expected size from all positive controls. Of the 263 *E. coli* isolates examined 40.3 % (106) were identified as purely enterotoxigenic (18.6 %) or shigatoxin (0.4 %) and enteroaggregative (17.5 %). About 30.4 % of the isolates were classified as both ETEC and EAEC, while two isolates carried genes associated with both STEC and ETEC. The virulence genes associated with the three *E. coli* types did not occur concurrently in any of the positive isolates (Table 3.2). The ETEC and EAEC were predominant in the tested isolates. No statistical difference was detected for the distribution of the three *E. coli* categories among the pig breeds.

Among the enterotoxins that were detected, the most frequently encountered toxin gene was *STb* (19.8 %), while the frequencies for the *STa* and *LT* genes were 3.4 % and 0.4 % respectively. Enteroaggregative heat stable toxin gene was identified in 20.5 % while *Stx2e* was carried by 1.1 % of the 263 isolates carrying virulence genes. Shiga toxin 2e was the only gene of the three shigatoxin genes evaluated that was detected and the *EAST-1* was the only enteroaggregative gene that was investigated (Table 3.2). A fraction of 4.9 % of isolates carried two of the toxic genes simultaneously. However none of the isolates

were positive for three or more of the toxic genes at the same time. Heat stable variant b toxin gene was recovered in the only *LT* positive isolates and in two of the *Stx2e*, 0.8 % *STa* and 1.9 % of the *EAST-1* positive isolates. In two isolates the *STa* gene was detected concurrently with the *EAST-1* gene.

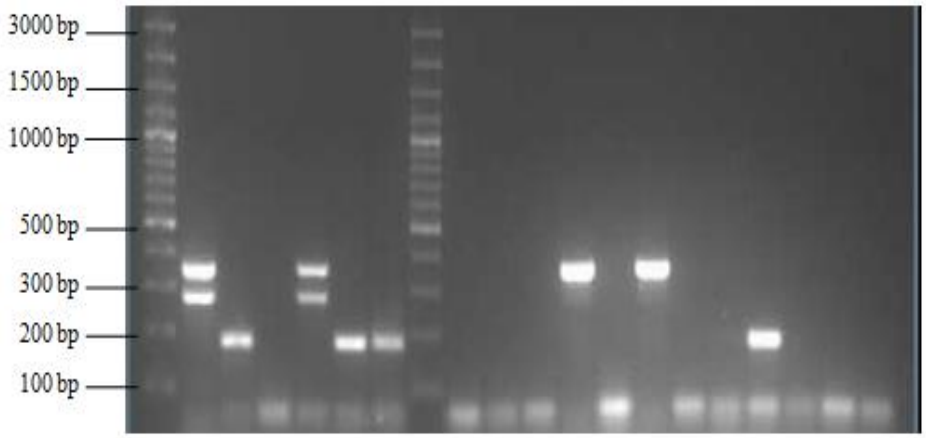


**Figure 3.1:** Typical gel picture showing ETEC virulence genes *STa*, *STb* and *LT*. Lanes 1 and 8:100 bp plus DNA ladder; Lane 2: *K88*/*STb*/*LT* positive reference strain; Lane 3: *K99* Pienk, *STa* positive reference strain; Lane 4: *E. coli* ATCC 25922, negative control strain; Lane 5: field *E. coli* isolate positive for *STb* and *LT* genes; Lanes 6, 7, 17: field *E. coli* isolates positive for *STa*; Lanes 12 and 14: field *E. coli* isolates positive for *STb*; Lanes 9, 10, 11, 13, 15, 16, 18, 19: field *E. coli* isolates negative for tested VFs; and Lane 20: no-DNA template control.

Table 3.3 shows that a large number of isolates obtained from Large White possessed most of the tested toxins. However, no statistical difference was obtained and this implies that breed did not affect the distribution of the individual toxins among the four pig breeds used in the study ($P > 0.05$) (Table 3.4).

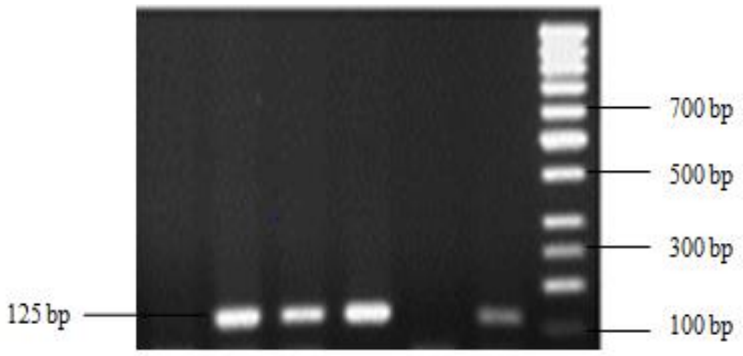


**Figure 3.2:** Gel picture of EAEC isolates showing virulence gene *EAST-1*. Lanes 1: negative isolate, Lanes 2, 3, 4 and 6: *EAST-1* positive isolate, Lane 5: no template control, Lane 7: 100 Plus DNA ladder.

**Table 3.2:** Proportion of the individual virulence genes in the enterotoxigenic, shigatoxin and enteroaggregative *E. coli*

| **Virulence genes** | **n (/263)** | **Prevalence (%)** |
|---|---|---|
| *LT* | 1 | 0.4 |
| *Sta* | 9 | 3.5 |
| *STb* | 50 | 19.0 |
| *Stx2e* | 3 | 1.1 |
| *EAST-1* | 53 | 20.2 |

**Table 3.3:** Effect of breed on the distribution of toxin genes among the tested pig population

| Virulence genes | Duroc | Indigenous | Landrace | Large White | P value |
|---|---|---|---|---|---|
| LT | 0 | 0 | 0 | 1 (0.9 %) | 0.8501 |
| STa | 2 (1.9 %) | 0 | 0 | 7 (6.6 %) | 0.0868 |
| STb | 7 (6.6 %) | 15 (14.2 %) | 1 (0.9 %) | 29 (27.4%) | 0.1892 |
| Stx2e | 0 | 1 (0.9 %) | 0 | 2 (1.9 %) | 0.9011 |
| EAST | 5 (4.7 %) | 20 (18.9 %) | 5 (4.7 %) | 24 (22.6 %) | 0.1845 |

## 3.4 Discussion

*Escherichia coli* bacteria are a members of *Enterobacteriaceae* which is a family of bacteria characterised as gram-negative, rod shaped, oxidase-negative, catalase-positive and ferments lactose (Adams and Moss, 2000). Other biochemical characteristics used to differentiate *E. coli* from other *Enterobacteriaceae* include determining the ability of the bacteria to produce indole from tryptophan (I), sufficient acid to reduce the medium pH below 4.4, the break point of the indicator methyl red (M), acetoin (V) and the ability to utilize citrate (C). *E. coli* is therefore characterised by IMViC type ++-- (Adams and Moss, 2000). All the lactose fermenting cultures subjected to the IMViC biochemical tests showed characteristics of *E. coli* cultures.

Only 40.3 % of the tested samples were positively identified as the EAEC, STEC and ETEC strains from which ETEC and EAEC were predominant groups. The results agree with findings by Do et al. (2005) where 89.7 % of the isolates collected from slaughtered piglets in Australia were enterotoxigenic. Similarly Zajacova et al. (2011) identified 7.8 % and 36.5 % of *E. coli* isolates from diarrheic piglets in Czech Republic collected between 2005-2009 as STEC and ETEC respectively. Lee et al. (2009) found that 90.9 % of the *E. coli* isolated from Korean pigs were enterotoxigenic. These results suggest that ETEC strains are the main pathogens responsible for *E. coli* induced colibacillosis in South African

piglets as well as in most countries worldwide. Clinical signs of ETEC infected pigs are characterized by severe, watery and in some cases uncontrolled and projectile diarrhoea (Francis, 2002; Pittman, 2010). The presence of the ETEC in the host does not necessarily mean a successful infection will take place. However factors such as stressors that may cause an upset in the gut microflora and elevated temperatures and diet together with poor immunity can result in the induction of colibacillosis by ETEC infection (Pittman, 2010). The notion that South African piglets have high prevalence of ETEC, therefore, subjects the industry to a potential excessive economic loss in case one of the stressors breaks through.

The economic loss will be as a result of the fact that ETEC is highly transmittable by direct contact especially in commercial sectors where pigs are housed in large groups. Pittman (2010) observed that the diarrheic faeces could project as far as one meter and can spread over the floor area, be smeared along walls and in the aisles separating the pens housing pigs diagnosed with ETEC pathotype *FI8/LT/STb/EAST-1/Stx2e*. Furthermore, Pittman (2010) observed the presence of partially digested feed particles in the stools which imply there is insufficient nutrient uptake by the host which could result in low weight gain or increased weight loss. Also the excessive loss of water through the diarrhoea would result in dehydration which may eventually lead to death.

The enteroaggregative *E. coli* has been identified among the major groups of *E. coli* implicated in swine diarrhoea (Choi et al., 2001). The results of the current study show that EAEC was the second most prevalent *E. coli* after ETEC. The results are in accordance with several studies on swine colibacillosis suggesting that EAEC can be regarded as a potential threat to the pig industry. Choi et al. (2001) identified 22 % of the *E. coli* strains isolated from diarrheic pre-weaned piglets in Korea from 1995 to 1998 as EAEC. Out of the 800 faecal swab isolates collected from piglets in Czech Republic 36.5 % were identified as EAEC (Zajacova et al., 2011). In addition to the 17.5 % pure EAEC strains isolated in the current study, 10 of the isolates were identified as both EAEC and ETEC, while two carried both ETEC and STEC associated genes. Choi et al. (2001) showed that *EAST-1* is not restricted to EAEC but can also be found in high prevalence in ETEC and enterohaemorrhagic *E. coli* strains isolated from diarrheic

piglets. Zajacova et al. (2011) also found that 74 % of the strains carrying the *EAST-1* belonged to the ETEC group while 12.9 % were also positive STEC.

Enteroaggregative *E. coli* is a group of bacteria characterised by aggregative adherence to intestinal mucosa of their host as their initial step of pathogenesis and have been found frequently in diarrhoea patients. The *EAST-1* toxin is believed to play a role in the pathogenesis of this group of *E. coli*, however not all the EAEC carry *EAST-1* gene (Nataro and Kaper, 1998; Veilleux and Dubreuil, 2006). Tzipori et al. (1985) investigated the effect of EAEC on gnotobiotic piglets and found that not all the piglets were able to develop diarrhoea. Histological studies on these gnotobiotic piglets revealed an unusual adherence of mucus enclosing densely packed aggregative bacteria on the epithelium of the small intestines (Tzipori et al., 1985). The high prevalence of EAEC in the current study suggests that it is an important potential pathogen that may induce colibacillosis or contribute to the increase in the virulence level of an already progressing colibacillosis in young pre-weaned and weaned piglets. As such there is a necessity to study the role of this group of *E. coli* in swine colibacillosis. The *EAST-1* toxin has been frequently isolated from diarrheal cases in humans (Veilleux and Dubreuil, 2006), suggesting that accidental contamination of meat by the pathogen during slaughter may also pose a potential transmission threat to humans.

The current results of low STEC prevalence agree with reports by other authors such as Moon et al. (1999), Vu-Khac et al. (2004; 2007) and Vidotto et al. (2009) who studied pig colibacillosis in United States, Slovakia and Brazil respectively. Vu-Khac et al. (2004; 2007) detected STEC associated genes in only 1.1 % and 4 % of the *E. coli* strains isolated from diarrheic piglets. In addition, Moon et al. (1999) and Vidotto et al. (2009) classified 4 % and 3 % of the *E. coli* isolates from diseased piglets as STEC respectively. Several factors have been proposed to contribute to the low prevalence of the STEC strains in the current study, one of which could be the method of sample collection, duration of collection and the period of collection.

The STEC isolated from the current pig population differs from that associated with the *E. coli* strains isolated from the human diseases. The *Stx1* and *Stx2* genes that are normally associated with zoonotic STEC could not be detected in the isolates. The results show that the experimental pig population is not a reservoir for STEC pathogenic to humans.

From the enterotoxin genes included in the study, the *STb* gene was the most prevalent followed by the *STa* and the *LT* genes had low prevalence. Results from similar studies show that *STb* and *STa* toxins are the most prevalent in ETEC isolated from diarrhoeic swine (Blanco et al., 1997; Osek et al., 1999). Blanco et al. (1997) found that 78.4 % of the isolates collected from diarrheic and healthy piglets in Spain contained *STb* gene and 62.16 % were positive for *STa* and 25.7 % had the *LT* gene. Osek et al. (1999) detected *STb* and *STa* in 77.5 % and 72.5 % of the *E. coli* isolates obtained from diseased pigs from farms in Poland. The results are however in contrast with other studies, suggesting that geographical region plays a role in recovery of virulent genes. Wang et al. (2011) obtained virulence gene frequencies of 67, 32 and 13 % for *Stx2e*, *STa* and *STb*, respectively from *E. coli* isolates collected from diseased swine herds in China between 2003 and 2008. Vidotto et al. (2009) found that out of the 300 *E. coli* isolates from diarrheic piglets in Brazil, *LT* (71 %) was the most prevalent compared to the *STa* (44 %), *STb* (47 %) and *Stx2e* (3 %). Celemin et al. (1995) used DNA probes to investigate prevalence of occurrence of ETEC in diseased pig isolates in Spain and found the following gene frequencies, *LTa* (31 %), *LTb* (71 %), *ST* (77 %) and *Stx* (47 %).

Heat labile, *STb* and *STa* toxins are used to identify the ETEC groups which are considered principal pathogenic *E. coli* isolated in swine colibacillosis studies. *LT*, *STa* and *STb* toxins cause in excessive water excretion and mal-absorption of electrolytes in the lumen (Nataro and Kaper, 1998) and result in dehydration and diarrhoea which is the most profound clinical symptom of colibacillosis.

Shigatoxin variant 2e is the only STEC toxin recovered in this study, signifying the importance of this toxin in South African porcine STEC infections. Fratamico et al. (2004) found that 70 % of the 689

isolates from faecal samples collected from pigs in United States contained both *Stx1* and *Stx2* genes, and 80 % of the *Stx2* positive isolates were positive for *Stx2e*. Blanco et al. (1997) also confirmed the importance of *Stx2e* in porcine STEC infections using oedema disease manifested piglets in Spain. Out of the 12 isolates obtained from piglets with oedema, 91.7 % were positive for *Stx2e* gene and one contained the *Stx1* gene (Blanco et al., 1997). Ateba and Bezuidenhout (2008) found that out of the 20 *E. coli* isolates from pigs in the North West Province of South Africa, none contained the *Stx1* and the *Stx2* genes. Most of the referred studies did not indicate the season or the time of collection and according to Wu et al. (2007) this could have affected the STEC shedding rate.

The *Stx2e* toxin cause oedema clinical and subclinical signs, such as massive destruction of the endothelial cells, haemorrhaging in the stomach wall, ileum, spiral colon, terminal colon, and caecum, oedema in eyes and vital organs including the brain which develop as a result of the absorption of the toxin through the intestinal walls into the blood stream (Francis, 2002; Matise et al., 2003; Oanh et al., 2012). Clinical symptoms of oedema can be observed within 4-9 days following oral inoculation of $10^{10}$ colony forming units (CFU) of *Stx2e* strain in 14-16 days old piglets (Matise et al., 2003). Oanh et al. (2012) recorded 100 % mortality of all six piglets born to non-immunised sows two days after an intravenous challenge with 50 ng *Stx2e* toxin. The piglets from which the *Stx2e* toxin genes were isolated in the present study did not show any signs of oedema. Matisse et al. (2003) suggested that one of the factors influencing the pathogenesis was the rate of the toxin transport into the blood stream. The rate of the toxin transport is influenced by the number of *Stx2e* toxin producing *E. coli* colonising the intestinal mucosa of the pig. A low bacterial population will yield low levels of *Stx2e* toxin which will result in vascular lesions in subclinical pigs even without detectable *Stx2e* in the blood. Since the clinical development of the oedema is depended on the dose of STEC present in the gut, there is a possibility of delayed development of oedema in the study population. The herds were not revisited following sample collection thus this has not been confirmed.

In this study 54 (22.5 %) of the *E. coli* positive isolates carried the *EAST-1* gene indicating the importance of this gene in swine colibacillosis. These results are comparable with studies by Vu-Khac et al. (2004), Lee et al. (2008) and Zajacova et al. (2011) who reported the prevalence of *EAST-1* gene in 53.3, 42.0 and 34.6 % of the isolates taken from diarrheic piglets in Korea and Czech Republic respectively. The role of the *EAST-1* toxin in swine diarrhoea is yet to be discovered (Francis, 2002; Ngeleka et al., 2003), the toxin is however believed to have a similar pathogenic mechanism as the *STa* toxin (Nataro and Kaper, 1998; Veilleux and Dubreuil, 2006; Yamamoto and Nakazawa, 1997). Frequent isolation of the *EAST-1* gene from *E. coli* isolated in swine diarrheal studies, including the current study suggest that this toxin may be a potential threat to the South African pig industry and there is a need for further investigation in the mechanism of its pathogenecity.

The results for the current study are not in agreement with the results by Henton and Englbrencht (1997), which reported the pathogenecity of *E. coli* in South African pigs. The study reported that the *ST* antigen was positive in 66.7 % of the isolates and *LT*, *EAST-1* and *Stx* virulence factors were not tested. The indigenous pigs have been previously shown to be more tolerant to diseases compared to the commercial pigs (Halimani et al., 2010). The incidence of the virulence genes are therefore expected to be less frequent in this group compared to the commercial breeds. Results from other studies also suggests breed difference in the resistance to *E. coli* with native breeds being more resistant in comparison to exotic breeds (Chappuis et al., 1984). The results of the current study are in contrast with previous studies and shows that there were no breed differences in the distribution of individual toxin genes as well as *E. coli* categories. Toxin distribution was not found to be affected by breed difference. This could be due to limited number of Duroc and Landrace populations to make a strong comparison among the breeds.

## 3.5 Conclusions

In conclusion, the study indicated the significance of ETEC and EAEC in *E. coli* infections in the South African pig industry. Virulence gene *STb* was more frequent than *STa* and *LT*. The *EAST-1* is also becoming increasingly an important factor in colibacillosis in young pigs. Shigatoxin *2e* gene was the only STEC variant isolated. The results suggest that there is a wide variety of virulence genes associated with diarrhoea in piglets. There was no breed differences in the *E. coli* isolated indicating that both the indigenous and exotic breeds are equally susceptible to colibacillosis.

## 3.6 References


Adams, M. R. and Moss, M. O. 2000. *Food Microbiology*, Royal Society of Chemistry, Cambridge.

Amezcua, R., Friendship, R. M., Dewey, C. E., Gyles, C. and Fairbrother, J. M. 2002. Presentation of postweaning *Escherichia coli* diarrhea in southern Ontario, prevalence of hemolytic *E. coli* serogroups involved, and their antimicrobial resistance patterns. *Canadian Journal of Veterinary Research*, 66, 73-78.

Ateba, C. N. and Bezuidenhout, C. C. 2008. Characterisation of *Escherichia coli O*157 strains from humans, cattle and pigs in the North-West Province, South Africa. *International Journal of Food Microbiology*, 128, 181-188.

Ateba, N. C. and Mbewe, M. 2011. Detection of *Escherichia coli O*157: *H*7 virulence genes in isolates from beef, pork, water, human and animal species in the North West province, South Africa: public health implications. *Research in Microbiology*, 162, 240-248.

Blanco, M., Blanco, J. E., Gonzalez, E. A., Mora, A., Jansen, W., Gomes, T., Zerbini, L. F., Yano, T., De Castro, A. and Blanco, J. 1997. Genes coding for enterotoxins and verotoxins in porcine

*Escherichia coli* strains belonging to different *O*: *K*: *H* serotypes: relationship with toxic phenotypes. *Journal of Clinical Microbiology*, 35, 2958-2963.

Bosworth, B. T. and Vögeli, P. 2003. Methods and compositions to identify swine genetically resistant to *FI8 E. coli* associated diseases. EP Patent 0,985,052.

Cai, H. Y., Archambault, M., Gyles, C. L. and Prescott, J. F. 2003. Molecular genetic methods in the veterinary clinical bacteriology laboratory: current usage and future applications. *Animal Health Research Reviews*, 4, 73-94.

Celemín, C., Rubio, P., Echeverria, P. and Suárez, S. 1995. Gene toxin patterns of *Escherichia coli* isolated from diseased and healthy piglets. *Veterinary Microbiology*, 45, 121-127.

Chappuis, J., Duval-Iflah, Y., Ollivier, L. and Legault, C. 1984. *Escherichia coli K88* adhesin: A comparison of Chinese and Large White piglets. *Genetic Selection Evolution*, 16, 385-390.

Chen, X., Gao, S., Jiao, X. and Liu, X. F. 2004. Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China. *Veterinary Microbiology*, 103, 13-20.

Cheng, D., Sun, H., Xu, J. and Gao, S. 2006. PCR detection of virulence factor genes in *Escherichia coli* isolates from weaned piglets with edema disease and/or diarrhea in China. *Veterinary Microbiology*, 115, 320-328.

Cheng, D., Yuan, Z. S., Lang, C. X., Pan, G. X., Wei, D. W. and Chang, S. H. 2010. Rapid diagnosis of ETEC and HPI-harboring *Escherichia coli* infection in newborn piglets with diarrhea. *African Journal of Microbiological Reseach*, 4, 575-580.

Chernysheva, L. V., Friendship, R. M., Dewey, C. E. and Gyles, C. L. 2004. The effect of dietary chicken egg-yolk antibodies on the clinical response in weaned pigs challenged with a *K88+ Escherichia coli* isolate. *Journal of Swine Health and Production*, 12, 279-284.

Choi, C. and Chae, C. 1999. Genotypic prevalence of *F4* variants (*ab*, *ac*, and *ad*) in *Escherichia coli* isolated from diarrheic piglets in Korea. *Veterinary Microbiology*, 67, 307-310.

Choi, C., Kwon, D. and Chae, C. 2001. Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *E. coli* isolated from diarrheic piglets. *Journal of Veterinary Diagnostic Investigation*, 13, 26-29.

Do, T., Stephens, C., Townsend, K., Wu, X., Chapman, T., Chin, J., Mccormick, B., Bara, M. and Trott, D. 2005. Rapid identification of virulence genes in enterotoxigenic *Escherichia coli*isolates associated with diarrhoea in Queensland piggeries. *Australian Veterinary Journal*, 83, 293-299.

Doughari, J. H., Ndakidemi, P. A., Human, I. S. and Bennade, S. 2009. Shiga toxins (Verocytotoxins). *African Journal of Microbiology Research*, 3, 681-693.

Fairbrother, J. M., Nadeau, É. and Gyles, C. L. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, 6, 17-40.

Francis, D. H. 2002. Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis. *Journal of Swine Health and Production*, 10, 171-175.

Fratamico, P. M., Bagi, L. K., Bush, E. J. and Solow, B. T. 2004. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* in swine feces recovered in the National Animal Health Monitoring System's Swine 2000 study. *Applied and Environmental Microbiology*, 70, 7173-7178.

Halimani, T., Muchadeyi, F., Chimonyo, M. and Dzama, K. 2010. Pig genetic resource conservation: The Southern African perspective. *Ecological Economics*, 69, 944-951.

Henton, M. 2010. *Zoonotic diseases of pigs* [Online]. Available: http://www.sapork.biz/zoonotic-diseases-of-pigs-2/ [Accessed 29 July 2011 at 11:59 am.

Henton, M. and Engelbrecht, M. 1997. *Escherichia coli* serotypes in pigs in South Africa. *The Onderstepoort Journal of Veterinary Research*, 64, 175-187.

Jamalludeen, N., Johnson, R. P., Shewen, P. E. and Gyles, C. L. 2009. Evaluation of bacteriophages for prevention and treatment of diarrhea due to experimental enterotoxigenic *Escherichia coli O*149 infection of pigs. *Veterinary Microbiology*, 136, 135-141.

Kemm, E. H. 2002. *Guide to small-scale pig farming* [Online]. South Africa, Pretoria. Available: http://www.cd3wd.com/cd3wd_40/lstock/001/SA_InfoPaks/docs/pigs1/index.htm.

Lee, S. I., Kang, S. G., Kang, M. L. and Yoo, H. S. 2008. Development of multiplex polymerase chain reaction assays for detecting enterotoxigenic *Escherichia coli* and their application to field isolates from piglets with diarrhea. *Journal of Veterinary Diagnostic Investigation*, 20, 492-496.

Lee, S. I., Rayamahji, N., Lee, W. J., Cha, S. B., Shin, M. K., Roh, Y. M. and Sang, H. Y. 2009. Genotypes, antibiogram, and pulsed-field gel electrophoresis profiles of *Escherichia coli* strains from piglets in Korea. *Journal of Veterinary Diagnostic Investigation*, 21, 510-516.

Li, Y., Qiu, X., Li, H. and Zhang, Q. 2007. Adhesive patterns of *Escherichia coli F4* in piglets of three breeds. *Journal of Genetics and Genomics*, 34, 591-599.

Matise, I., Cornick, N. A., Samuel, J. E. and Moon, H. W. 2003. Binding of Shiga toxin 2e to porcine erythrocytes *in vivo* and *in vitro*. *Infection and Immunity*, 71, 5194-5201.

Moon, H. W., Hoffman, L. J., Cornick, N. A., Booker, S. L. and Bosworth, B. T. 1999. Prevalences of some virulence genes among *Escherichia coli* isolates from swine presented to a diagnostic laboratory in Iowa. *Journal of Veterinary Diagnostic Investigation*, 11, 557-560.

Nagy, B., Wilson, R. A. and Whittam, T. S. 1999. Genetic diversity among *Escherichia coli* isolates carrying *FI8* genes from pigs with porcine postweaning diarrhea and edema disease. *Journal of Clinical Microbiology*, 37, 1642-1645.

Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, 11, 142-201.

Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D. M. and Fairbrother, J. M. 2003. Isolation and association of *Escherichia coli AIDA-I/STb*, rather than *EAST1* pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. *Journal of Veterinary Diagnostic Investigation*, 15, 242-253.

Oanh, T. K. N., Nguyen, V. K., De Greve, H. and Goddeeris, B. M. 2012. Protection of piglets against edema disease by maternal immunization with *Stx2e* toxoid. *Infection and Immunity*, 80, 469-473.

Osek, J., Gallien, P., Truszczy Ski, M. and Protz, D. 1999. The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland. *Comparative Immunology, Microbiology and Infectious Diseases*, 22, 163-174.

Owusu-Asiedu, A., Nyachoti, C. and Marquardt, R. 2003. Response of early-weaned pigs to an enterotoxigenic *Escherichia coli* (*K88*) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic. *Journal of Animal Science*, 81, 1790-1798.

Parma, A., Sanz, M., Blanco, J., Blanco, J., Viñas, M., Blanco, M., Padola, N. and Etcheverria, A. 2000. Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. *European Journal of Epidemiology*, 16, 757-762.

Pittman, J. S. 2010. Enteritis in grower-finisher pigs caused by *FI8*-positive *Escherichia coli*. *Journal of Swine Health and Production*, 18, 81-86.

Rampoldi, A., Jacobsen, M. J., Bertschinger, H. U., Joller, D., Bürgi, E., Vögeli, P., Andersson, L., Archibald, A. L., Fredholm, M. and Jørgensen, C. B. 2011. The receptor locus for *Escherichia coli F4ab/F4ac* in the pig maps distal to the *MUC4–LMLN* region. *Mammalian Genome*, 22, 122-129.

Sargeant, H. R., Mcdowall, K. J., Miller, H. M. and Shaw, M. A. 2010. Dietary zinc oxide affects the expression of genes associated with inflammation: Transcriptome analysis in piglets challenged with ETEC *K88*. *Veterinary Immunology and Immunopathology*, 137, 120-129.

Tzipori, S., Robins-Browne, R. M., Gonis, G., Hayes, J., Withers, M. and Mccartney, E. 1985. Enteropathogenic *Escherichia coli* enteritis: evaluation of the gnotobiotic piglet as a model of human infection. *Gut*, 26, 570-578.

Veilleux, S. and Dubreuil, D. J. 2006. Presence of *Escherichia coli* carrying the *EAST1* toxin gene in farm animals. *Veterinary Research*, 37, 3-13.

Vidotto, M. C., De Lima, N. C. S., Fritzen, J. T. T., De Freitas, J. C., Venancio, M. J. and Ono, M. A. 2009. Frequency of virulence genes in *Escherichia coli* strains isolated from piglets with diarrhea in the North Parana State, Brazil. *Brazilian Journal of Microbiology*, 40, 199-204.

Vu-Khac, H., Holoda, E. and Pilipčinec, E. 2004. Distribution of virulence genes in *Escherichia coli* strains isolated from diarrhoeic piglets in the Slovak Republic. *Journal of Veterinary Medicine, Series B*, 51, 343-347.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J., Mora, A., Dahbi, G., López, C. and González, E. 2006. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia. *BMC Veterinary Research*, 2, 1-8.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J. E., Dahbi, G., Mora, A., López, J., González, E. and Blanco, J. 2007. Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhea in Slovakia. *The Veterinary Journal*, 174, 176-187.

Wang, X. M., Jiang, H. X., Liao, X. P., Liu, J. H., Zhang, W. J., Zhang, H., Jiang, Z. G., Lü, D. H., Xiang, R. and Liu, Y. H. 2010. Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiology Letters*, 306, 15-21.

Wang, X. M., Liao, X. P., Liu, S. G., Zhang, W. J., Jiang, H. X., Zhang, M. J., Zhu, H. Q., Sun, Y., Sun, J., Li, A. X. and Lui, Y.-H. 2011. Serotypes, Virulence Genes, and Antimicrobial Susceptibility of *Escherichia coli* Isolates from Pigs. *Foodborne Pathogens and Disease*, 8, 687-692.

Welinder-Olsson, C. and Kaijser, B. 2005. Enterohemorrhagic *Escherichia coli* (EHEC). *Scandinavian Journal of Infectious Diseases*, 37, 405-416.

Wong, I., Moreno, M. and Del C, M. 1995. Immunity and protection elicited by a recombinant vaccine against enterotoxigenic *Escherichia coli*. *Biotecnologia Aplicada*, 12, 9-15.

Wu, X. Y., Chapman, T., Trott, D. J., Bettelheim, K., Do, T. N., Driesen, S., Walker, M. J. and Chin, J. 2007. Comparative analysis of virulence genes, genetic diversity, and phylogeny of commensal

and enterotoxigenic *Escherichia coli* isolates from weaned pigs. *Applied and Environmental Microbiology*, 73, 83-91.

Yamamoto, T. and Nakazawa, M. 1997. Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. *Journal of Clinical Microbiology*, 35, 223-227.

Zajacova, Z. S., Konstantinova, L. and Alexa, P. 2011. Detection of virulence factors of *Escherichia coli* focused on prevalence of *EAST1* toxin in stool of diarrheic and non-diarrheic piglets and presence of adhesin involving virulence factors in *astA* positive strains. *Veterinary Microbiology*, 154, 369-375.

# CHAPTER 4

## Prevalence of fimbrial adhesins (*F4, F5, F6, FI8* and *F41*) and non-fimbrial adhesins (*AIDA-1, EAE* and *PAA*) genes in South African pigs

*Published in Tropical Animal Health and production (see Appendix 1)*


### Abstract

*E. coli* is a major aetiological agent among different colibacillosis causing pathogens in the occurrence of digestive system diseases of newly born and weaned piglets. The objective of the current study was to determine the mode of attachment of the ETEC, EAEC and STEC pathogenic *E. coli* recovered in Chapter 3. A total of 106 isolates that were positive for ETEC, EAEC and STEC were tested for the presence of fimbrial genes *faeG, fanA, fim41a, fasA* and *fedA* and non-fimbrial adhesin genes *aidA, eae* and *paa*. None of the isolates tested positive for the fimbriae genes tested. Non-fimbrial adhesin factors were however, detected in 19.8 % of the 263 isolates which makes it 49.1 % of ETEC, STEC and *EAST1* positive strains. Of these non-fimbrial adhesins, *PAA* (17.9 %) was the most prevalent followed by *AIDA-1* and *EAE* genes that were detected in 14.5 % and 2.8 % of the isolates, respectively. The remaining 14.2 % carried more than one of the adhesins. There was no association between the incidences of occurrence of any particular adhesin genes with the four breeds of pigs. There is need for an intensive study on the role of *AIDA-1, EAE* and *PAA* in porcine diarrhoea. The absence of fimbrial genes suggests that a revision of the current colibacillosis control programme is necessary.



**Keywords:** colonisation factors, fimbriae, non-fimbrial adhesins, attachment

## 4.1 Introduction

Colonisation factors are involved in the first step of pathogenesis of ETEC, EAEC and STEC, which is securing attachment of the bacteria to its host intestinal epithelium (Nagy et al., 1999). Without the CFs, *E. coli* will be washed out with the gut contents due to continuous peristaltic movement of the gut. Failure of the initial step of pathogenesis means that subsequent processes involved such as multiplication of the organism to the required bacterial concentration of colony factor units and production of toxins will not occur. *E. coli* lacking CFs will therefore fail to induce an infection (Adlerberth et al., 2000).

Colonisation factors *F4*, *F5*, *F6*, *F41* and *FI8* are associated with colibacillosis in pigs (Do et al., 2005). These fimbrial antigens are CFs that aid *E. coli* to adhere to the intestinal wall of piglets, before it proliferate and produce toxins. The *F4* fimbrial antigen exists in three variants *ab*, *ac* and *ad*, while the *FI8* has two variants *ab* and *ac*. Although fimbrial adhesins are considered as important virulence factors in pathogenesis of *E. coli*, the non-fimbrial adhesins have also been frequently observed in *E. coli* infection in pigs. It was found that increasing numbers of cases of enteric colibacillosis in pigs lack expression of common attachment factors (*F4*, *F5*, *F6*, *FI8*, and *F41*), but express the non-fimbrial adhesins (Ngeleka et al., 2003). Adhesin involved in diffuse adherence, *PAA* and *EAE* are the most common adhesins found in pigs with neonatal and post weaning diarrhoea (Yamamoto and Nakazawa, 1997; Vu-Khac et al., 2006; Lee et al., 2009).

Knowledge of the CFs is important in efficient colibacillosis control programmes. Currently, antibiotics have been used in neonatal and post weaning diarrhea and oedema cases. With the increase antibiotic resistance shown by *E. coli* isolated from diseases and healthy pigs (Kwon et al., 1999; Moneoang and Bezuidenhout, 2009), vaccines have shown to be the most reliable method. Vaccines that target specific adhesins have been and are still used in colibacillosis control programs. In South Africa, vaccines incorporating fimbriae adhesins *F4ac*, *F4ab*, *F5* and *F6* are used in pregnant gilts and sows to control colibacillosis caused by *E. coli* expressing these fimbriae in neonatal piglets (Intervet, 2010). Use of

vaccines targeting specific CFs is, however, done without knowledge of the current prevailing adhesin factors. Prevalent colonization factors will change over time as a result of new mutations and old mutations disappearing because of selection pressures imposed by vaccines and antibiotics (Vu-Khac et al., 2006; Doughari et al., 2009).

The objective of the current study was, therefore, to determine the mode of colonization as well as the distribution of CFs in *E. coli* strains isolated from four South African pig breeds. Virulence factors investigated were fimbrial adhesins *F4, F5, F6, FI8ab, FI8ac* and *F41* and non-fimbrial adhesins *AIDA-1*, *PAA* and *EAE*. The study intended to determine CFs specific to STEC, EAEC and ETEC found in the South African pigs, and is a first step in providing an important database for the South African pork and veterinary drugs producers.

## 4.2 Materials and methods

### *4.2.1 DNA amplification and gene identification*

Only DNA of isolates that were positive for any of the *LT* and or *STa*, *STb*, *Stx2e* and *EAST-1* toxin genes (Chapter 3) were subjected to multiplex PCR for fimbriae set 1 (*F4*, *F5* and *F41*), fimbriae set 2 (*FI8+* and *F6*) and for adhesins (*AIDA-1*, *PAA* and *EAE*) using the same PCR and cycling conditions described previously in section 3.2.4 with the exception of the annealing temperatures. The annealing temperatures were 56 for both fimbriae set 1 and 2 and 58 for the non-fimbrial adhesin genes. The primer sequences for the fimbriae and adhesins are shown in Table 4.1. The electrophoresis of the PCR products followed the same procedure described in section 3.2.5.

**Table 4. 1** Primer sequences and PCR product sizes for individual colonisation factors

| mPCR primer set | VFs | Primers sequences (5'-3') (2,3) | Target gene | Size (bp) | Reference |
|---|---|---|---|---|---|
| Fimbriae 1 | *F4* (*K88*)-F | GAT GAA AAA GAC TCT GAT TGC A | | | (Cai et al, 2003; Cheng et al., |
| | *F4* (*K88*)-R | GAT TGC TAC GTT CAG CGG AGC G | *FaeG* | 841 | 2006; Cheng et al., 2010 ) |
| | *F5* (*K99*)-F | CTG AAA AAA ACA CTG CTA GCT ATT | | | (Cai et al, 2003; Cheng et al., |
| | *F5* (*K99*)-R | CAT ATA AGT GAC TAA GAA GGA TGC | *fanA* | 543 | 2006; Cheng et al., 2010 ) |
| | *F41*-F | GAT GAA AAA GAC TCT GAT TGC A | | | (Cai et al, 2003; Cheng et al., |
| | *F41*-R | TCT GAG GTC ATC CCA ATT GTG G | *fim41a* | 682 | 2006; Cheng et al., 2010 ) |
| Fimbriae 2 | *F6* (*987P*)-F | GTT ACT GCC AGT CTA TGC CAA GTG | | | (Cai et al, 2003; Cheng et al., |
| | *F6* (*987P*)-R | TCG GTG TAC CTG CTG AAC GAA TAG | *fasA* | 463 | 2006; Cheng et al., 2010 ) |
| | *FI8*-F1 (b) | ATG AAA AGA CTA GTG TTT ATT TCT T | | | |
| | *FI8*-F2 (c) | CGT GAA CGG TAA AAC ACA GGG | | 513 or | |
| | *FI8*-R | TTA CTT GTA AGT ACC GCG TAA GCC | *fedA* | 516 | (Cheng et al., 2010) |
| Adhesin factors | *AIDA-1*-F | ACA GTA TCA TAT GGA GCC A | | | |
| | *AIDA-1*-R | TGT GCG CCA GAA CTA TTA | *aidA* | 585 | (Ngeleka et al., 2003) |
| | *EAE*-F | CAT TAT GGA ACG GCA GAG GT | | | |
| | *EAE*-R | ATC TTC TGC GTA CTG CGT TCA | *eae* | 790 | (Ngeleka et al., 2003) |
| | *PAA*-F | ATG AGG AAC ATA ATG GCA GG | | | |
| | *PAA*-R | TCT GGT CAG GTC GTC AAT AC | *paa* | 360 | (Ngeleka et al., 2003) |

### *4.2.2 Statistical analysis*

The prevalence of fimbrial and non-fimbrial adhesins was determined using the procedure MEANS of SAS (2012) and the effects of breed on adhesin type was investigated using Chi-square ($\chi^2$) tests of the FREQ procedure in SAS (2012). A *P*-value of less than 0.05 was considered significant.

## 4.3 Results

### *4.3.1 DNA amplification and gene identification*

Of all the *E. coli* isolates that tested positive for *LT*, *STa*, *STb*, *EAST-1* and *Stx2e*, none of the isolates carried any of the tested fimbriae (*F4*, *F5*, *F6*, *FI8+* and *F41*). The PCR products that were obtained for the various reference strains used corresponded to the expected amplicon sizes. The PCR product for the *F4* gene produced a 854 bp band that is common to all the three variants. The *FI8* primers also produced an expected band sized ~513/516 which could represent one the variants *ab* or *ac*. The other positive controls (*F5* and *F41*) also showed the expected amplicon sizes (Figure 4.1). The non-fimbrial adhesin factors genes also produced the product sizes as specified in Table 4.1 and Figure 4.2. Non-fimbrial adhesins were detected as indicated by the presence of the *EAE*, *PAA* and *AIDA-1* genes of 790, 360 and 585 bp band sizes, respectively.

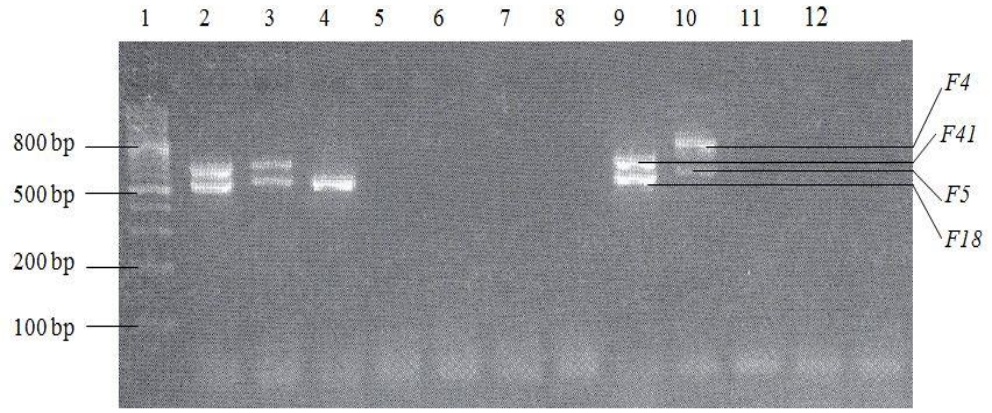


**Figure 4. 1**: Typical gel picture showing reference strains showing positive amplification of the fimbrial genes, *F4*, *F5*, *F18* and *F41*. Lane 1: 100 bp plus DNA ladder; Lanes 2 and 9: *FI8* and *F41* positive control. Lanes 3: *K99* Pienk *F41* and *F5* positive control; Lane 4: *FI8* positive control. Lane 8: No-template control; Lane 10: *K88ac* Pienk, *F4* and *F5* positive control; Lanes 5, 6, 7, 11, 12 and 13: Negative isolates.

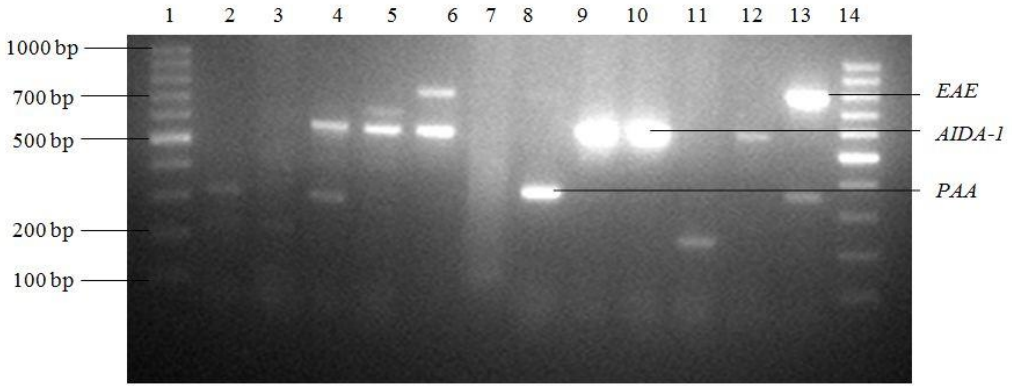


**Figure 4. 2:** Amplification of adhesin factors genes of ETEC, EAEC and STEC isolates. Lane 1 and 14: 100 bp plus DNA marker Lanes 2, 5, 6: Positive controls for *PAA*, *AIDA-1* and *EAE* respectively. Lane 3: A negative control. Lanes 4, 8, 13 *PAA* positive isolates. Lanes 4, 6, 9, 10, 12 *AIDA-1* positive isolates. Lanes 6 and 13: *EAE* positive. Lanes 11: An isolate negative for all adhesin factors.

### *4.3.2 Prevalence of colonisation factors*

The non-fimbrial adhesin factors were detected in 19.8 % of the 263 samples which accounts for only 49.1 % of the 106 isolates carrying toxin genes. Of the 19.8 % adhesin positive isolates, the *PAA* (7.2 %) and the *AIDA-1* (5.7 %) were the most common. The *EAE* gene was detected in only 1.14 % of the isolates. A smallest proportion of 5.7 % of the *E. coli* isolates carried more than one of the adhesin genes, of which 1.1 % was positive for both *EAE* and *AIDA-1*. The remaining 4.2 % of the isolates carried both *AIDA-1* and the *PAA*, and all three adhesin factors were detected in only 1 isolate (Table 4.2). The occurrence of mixtures of adhesin factors means that co-infections by several *E. coli* strains are possible.

**Table 4. 2:** Proportion of the positive for adhesin factors *EAE*, *AIDA-1* and *PAA*

| **Adhesin** | **Frequency (/106)** | **Percentage** | **P value** |
|---|---|---|---|
| *EAE* | 3 | 2.8 | <.0001 |
| *AIDA-1* | 15 | 14.5 | <.0001 |
| *PAA* | 19 | 18.0 | <.0001 |
| *EAE*/*AIDA-1* | 3 | 2.8 | <.0001 |
| *AIDA-1*/*PAA* | 11 | 10.4 | <.0001 |
| *EAE*/*AIDA-1*/*PAA* | 1 | 0.9 | <.0001 |

Breed had no significant effect (P > 0.05) on the distribution of adhesin genes *PAA*, *EAE* and *AIDA-1* (Table 4.3).

**Table 4.3:** The distribution of adhesin factors among the four breeds

| Adhesin gene | Duroc (n =28) | Indigenous (n =85) | Landrace (n =24) | Large White (n = 126) | P value |
|---|---|---|---|---|---|
| *EAE* | 0 | 3 | 0 | 4 | 0.6376 |
| *AIDA-1* | 4 | 11 | 1 | 14 | 0.7909 |
| *PAA* | 4 | 9 | 3 | 15 | 0.4155 |
| *EAE/AIDA-1* | 0 | 1 | 0 | 2 | 0.8539 |
| *AIDA-1/PAA* | 2 | 2 | 1 | 6 | 0.5703 |
| *EAE/AIDA-1/PAA* | 0 | 1 | 0 | 0 | 0.5879 |

## 4.4 Discussion

Colonisation factors are the principal step in *E. coli* infections as they aid bacteria to adhere to the intestinal wall of piglets, before they proliferate and produce toxins (Nagy et al., 1992). The fimbrial adhesins *F4*, *F5*, *F6* and *FI8* are the most common CFs associated with *E. coli* strains isolated from pigs with colibacillosis (Zhang et al., 2007; Madoroba et al., 2009). Non-fimbrial adhesins *AIDA-1*, *PAA* and *EAE* have also been implicated in *E. coli* infections in pigs (Ngeleka et al., 2003; Vu-Khac et al., 2006).

The use of PCR to evaluate the presence of fimbriae genes has been used in several studies (Wang et al., 2006; Madoroba et al., 2009). The PCR products for respective fimbrial and non-fimbrial adhesin genes obtained in the current study are consistent with those detected from studies also investigating the same fimbriae and afimbrial adhesins in pigs (Ngeleka et al., 2003; Cheng et al., 2010).

Fimbrial adhesins facilitate attachment of bacteria to the surface of host epithelium cells and are therefore considered the principal factor in the initiation of bacterial colonization (Francis, 2002; Zhang et al., 2007). The role of fimbriae in colibacillosis progression has been verified in several studies. *E. coli* bacteria carrying fimbrial adhesins recognise specific or non-specific mucous receptors on the epithelial

lining of the small intestines of their host to which they attach to following ingestion by the piglets (Gibbons et al., 1977; Jensen et al., 2006). The *F4* fimbrial adhesin has been associated with the development of more severe diarrhoea in suckling piglets, while the *FI8* adhesin has been constantly isolated from oedema and post weaning diarrheal cases (Moon et al., 1999; Chen et al., 2004). The other three fimbriae *F5, F6* and *F41* have been associated mostly with colibacillosis in neonatal pigs, although they occur less frequently (Ha et al., 2004). A study on 1044 replacement gilts in Cuba revealed a higher prevalence rate for *F4*+ (33 %) and *FI8*+ (32.4 %) *E. coli* (De La Fe Rodriguez et al., 2011).

Despite the fact that *F4* and *FI8* fimbriae have been found to be the most common occurring in ETEC and STEC implicated diarrhoea (Vidotto et al., 2009; De La Fe Rodriguez et al., 2011), results of the present study indicated otherwise as none of the ETEC, STEC and EAEC positive isolates had any of the investigated fimbrial adhesin genes. The findings are also in contrast with a previous South African study on swine colibacillosis that indicated a high prevalence of the *F4* antigens (Henton and Engelbrecht, 1997).

The absence of fimbrial genes in STEC and ETEC is however not a novel discovery. Several porcine diarrhoea and oedema studies have shown the absence of fimbrial antigens in toxic *E. coli.* For example Post et al. (2000) found that out of all the *E. coli* isolates positive for enterotoxigenic genes, only 94.9 % had one of the fimbrial genes in the United States. The other 5.1 % failed to show the fimbrial genes. Similarly Do et al. (2005) in Australia found 13 of the isolates that contained enterotoxins having none of the fimbrial genes while Vidotto et al. (2009) found fimbrial colonization factors in only 60 % of the toxin positive samples in Brazil. It has been suggested that the absence in ETEC in previous swine diarrhoea and oedema studies (Do et al., 2005; Vidotto et al., 2009) was probably due to the use of fimbrial vaccines (Nagy et al., 1992) and changes in genetic elements resulting in previously uncommon types emerging as new pig pathogenic *E. coli* (Do et al., 2005). Other fimbriae such as *F1*, *F165*, *F42* and *F17* that were not investigated in the current study could be involved.

The absence of the tested fimbriae in the *E. coli* isolates suggested that fimbriae are not the only adhesin factors important in the colonisation needed for a successful induction of diarrhoea in piglets. Therefore the presences of the aidA, eae and paa genes encoding non fimbrial colonisation factors AIDA-1, EAE and PAA respectively were tested in all the ETEC, STEC and EAEC positive isolates of this study population. *E. coli* harbouring these types of adhesin factors is known as the Attaching and effacing *E. coli* and is characterised by intimate attachment to epithelial cells and effacement of the microvillus surface in the small intestines of the host (Nataro and Kaper, 1998). It has been suggested that changes in the genetic elements of *E. coli* could result in previously uncommon types emerging as new pig pathogenic *E. coli* (Do et al., 2005)

The *AIDA-1* (14.2 %) and the *PAA* (17.9 %) were the most prevalent while the *EAE* was found in only 2.8 % of the positive isolates. Three combinations of these adhesin genes were also detected in 0.9 % of the isolates. Other studies have also shown that the *AIDA-1* and the *PAA* genes are more frequent than the *EAE* adhesin factor. In one study, *AIDA-1* and *PAA* were recovered in 26.9 % and 60 % of the isolates while *EAE* adhesin was found in only 1.1 % of the isolates from diarrhoeic piglets in the USA (Zhang et al. (2007). In Slovakia, the *EAE* gene was recovered at very low prevalence of 0.9 % in diarrhoeic pig population (Vu-Khac et al., 2006). In contrast, there were no *PAA* and *EAE* genes found in the toxin gene bearing *E. coli* isolated from slaughtered pigs in Switzerland (Zweifel et al. (2006), while in Canada, a prevalence of 8.8 % of the isolates collected from diarrhoeic and 7.5 % from non-diarrhoeic piglets were observed (Ngeleka et al., 2003).

The *EAE* and *AIDA-1* adhesin factors that were previously mostly associated with human *E. coli* infections are now frequently isolated from pig colibacillosis cases. Chapman et al. (2006) found that in addition to the common virulence genes found in ND and PWD *E. coli* isolates, the *AIDA-1* is also significant in distinguishing between the commensal and the pathogenic *E. coli*. Fang et al. (2008) performed an *in vitro* association of *AIDA-1* isolated from *E. coli* and found that it has a high binding affinity with two *AIDA* binding proteins (p65 and p120) from intestinal mucosa of pigs. Ngeleka et al.

(2003) also indicated the significance of *AIDA-1* adhesin in neonatal diarrhoea. Inoculation of colostrum deprived gnotobiotic piglets with 1 ml of $10^9$ CFU *AIDA-1/STb/EAST-1* and *AIDA-1/STb E. coli* strains resulted in diarrhoea in the piglets 20 h p.i and the post-mortem intestinal analysis showed bacterial colonies close adherence to the brush border of the epithelial cells along the ileum down to the colon. On the other hand the *EAST-1* strains alone failed to induce diarrhoea 72 h p.i, indicating the significance of the initial step of attachment by the *AIDA-1* in infection induction.

The *EAE* genes encodes for a 94 kDa protein intimin which is responsible for intimate attachment to membranes, and in pigs the attachment of *EAE* positive *E. coli* to the intestinal membrane resulted in effacement of microvilli and destruction of enterocytes and caused diarrhoea between 24 h and 35 h of inoculation in 78 % of infected pigs (Zhu et al., 1994). The host pathogen interaction of the colonisation of the *EAE* adhesin *E. coli* in pigs is yet to be verified but it is believed to follow similar mechanism to that of humans. In humans it is facilitated by the insertion of the translocated intimin receptor (*Tir*) on the epithelial layer of the small intestines (Law, 2000). The existing knowledge on the colonisation mechanism of *EAE* adhesin does not allow the selection of host resistant to the *EAE* carrying *E. coli*.

In addition to the *EAE* genes, the *PAA* encoding porcine protein has also been found to contribute to early stages of attaching and effacing lesions (Zweifel et al., 2006; Zajacova et al., 2011). The lesions found in pigs infected with these strains produce lesions similar to those produced by the enteropathogenic *E. coli* in humans (Vu-Khac et al., 2004). This shared property of porcine and human *EAE* and *PAA* producing strains suggest a potential threat of zoonotic transmission of this pathogen in humans from pigs and vice versa. The role of *PAA* adhesin in pigs has been demonstrated by the insertion of a transposon mutant (Tn*phoA*) in the *PAA* gene which was then analysed for adhesin on the ileal villi of piglets *in vitro*. The Tn*phoA* mutant showed reduction in the adherence to the intestinal epithelium compared to the wild-type *PAA E. coli* (Batisson et al., 2003). Ngeleka et al. (2003) found that *EAE* and *EAE/EAST-1/PAA E. coli* strains resulted in clinical signs of diarrhoea at 12 and 16 h p.i. respectively, in colostrum-deprived newly born piglets.

The *AIDA-1* (14.1 %) and the *PAA* (17.9 %) were the most prevalent in the 106 *E. coli* isolates investigated while the *EAE* was found in only 3.5 % of the positive isolates. Three combinations of these adhesin genes were also detected in 0.9 % of the isolates. The presence of different adhesin genes in one isolate is not a novel discovery. It has also been reported for fimbrial antigens (Jung et al., 2011). The concurrent occurrence of different adhesin factors result in the complexity and severity of colibacillosis caused by STEC, ETEC and EAEC infections in piglets (Taillon et al., 2008). Multiple adhesin isolates have been recovered from piglets showing diarrhoea in Canada and Slovakia (Ngeleka et al., 2003; Vu-Khac et al., 2004). Nataro and Kaper (1998) also indicated that the isolation of the *EAE* and *PAA* genes in the same isolates was common in colibacillosis studies.

Other studies have also shown that the *AIDA-1* and the *PAA* genes are found more frequently than the *EAE* adhesin factor. Zhang et al. (2007) recovered the *AIDA-1* and *PAA* in 26.9 and 60 % of the isolates while *EAE* adhesin was found in only 1.1 % of the isolates collected from January 2004 to August 2006 from diarrheic piglets in United States. Vu-Khac et al. (2006) also recovered the *EAE* gene at a very low prevalence of 0.9 % in diarrheic pig populations in Slovakia. Although the prevalence of genes encoding *PAA*, *EAE* and *AIDA-1* are in accordance with some of the studies, they are also contradicting with others. Zweifel et al. (2006) found no *PAA* and *EAE* genes in the toxin gene bearing *E. coli* isolated from slaughtered pigs in Switzerland. Ngeleka et al. (2003) found that *PAA* was present in 8.8 and 7.5 % of the isolates collected from diarrheic and non-diarrheic piglets respectively in Canada. Jung et al. (2011) found that the prevalence rate for the adhesin factor *PAA* was 30.3 % in Korean pigs and Wang et al. (2011) recovered the *EAE* gene in 52 % of the isolates collected from diseased pig herds in Guangdong Province, China between 2003 and 2008.

A comparison of the current study and other previous studies shows that there is no particular pattern of the fimbrial and non-fimbrial adhesins recovery. Since the studies were undertaken in different locations, it is sensible to conclude that geographical region may have an effect on the type and the distribution of

CFs found in *E. coli* involved in ND and PWD. This study identified that non-fimbriated adhesin factors *PAA* and *AIDA-1* were the most prevalent in the tested population of South African pigs.

Current vaccination programs against colibacillosis are based on the fimbrial adhesins. Vaccines incorporating fimbrial adhesins *F4ac, F4ab, F5* and *F6* are used in pregnant gilts and sows to control colibacillosis caused by *E. coli* expressing these fimbriae in neonatal piglets (Intervet, 2010; Onderstepoort, undated). This control method has been applied for years without the knowledge of the prevailing adhesin factors. In brief the results of the current experiment suggest a need for new vaccination programs to protect piglets from colibacillosis. The *PAA*, *AIDA-1* and the *EAE* antigens should be taken into consideration when formulating vaccines to protect neonatal and weaned piglets. In addition, our results imply that the use of vaccines in this sampled pig population may have placed a selection pressure against the fimbriated *E. coli*. At the moment the use of antibiotics remains the only feasible method to reduce the effect of infections that may arise due to the existing *E. coli* virulence genes profiles.

Swine diarrheal studies mostly focus on the detection and characterisation of pathogenic *E. coli* recovered from pig herds in general. Associations between several CF and the age as well as well as individual pig genetic makeup have been made. For example the *F4* has been associated frequently with sucking piglets while *FI8* has been isolated mostly from PWD cases (Moon et al., 1999; Coddens et al., 2007). There is also evidence that some pigs are susceptible to *E. coli* expressing *FI8* and *F4* while others show a degree of tolerance to these types. Piglets expressing *F4* and *FI8* receptors are susceptible to *E. coli* expressing these fimbriae (Gibbons et al., 1977; Bao et al., 2011). Even with the advancement in pig colibacillosis studies, there is relatively little research addressing the effect of breed on the recovery rate of individual CFs or fimbrial antigens. It would be interesting to know if there exists an association between the tested afimbrial genes with their shedding rate in different breeds. This association between the adhesin factors and the four breeds was investigated in the current study and the statistical evidence revealed no difference in the recovery rate of individual adhesin factors.

Also previous exposure of vaccines and other prevention strategies as well as treatment options for other bacterial diseases could have affected the recovery rate of the CFs in the breeds. Management of the indigenous and the exotic breeds' sows were different and this could have affected the piglets' immunity status at the neonatal phase. The iron dose given to the exotic breeds may place them at more risk of transmitting the infectious bacteria than the Indigenous breeds and this could be the reason why the Large White appears to be shedding a large number of the toxin carrying *E. coli*. Future studies reporting the relatedness between adhesin factors and specific pig breeds are therefore worth undertaking.

### 4.5 Conclusions

Potential pathogenic *E. coli* recovered in the current study did not carry genes for the investigated fimbriae. This shows that fimbriae are not a prominent CF in the current pig population. This pattern could be due to routine use of fimbrial antigen vaccines, resulting in common fimbriae being selected out of the population. The *E. coli* could also be using some type of fimbriae not included in the current study. The non-fimbrial adhesins, particularly *PAA* were the most prevalent CFs. Multiple CF combinations were also identified. The *AIDA-1*, *EAE* and *PAA* adhesins have also been implicated in human diarrheal studies, as such horizontal transmission between pigs and humans is possible. The results suggest that a revision of the current colibacillosis control program is necessary, and that construction of vaccines and antibiotics targeting non-fimbrial adhesins should be considered. The study determined, for the first time, the potential involvement of non-fimbrial adhesins in porcine diarrhoea in South Africa.

## 4.6 References


Adlerberth, I., Cerquetti, M., Poilane, I., Wold, A. and Collignon, A. 2000. Mechanisms of colonisation and colonisation resistance of the digestive tract part 1: bacteria/host interactions. *Microbial Ecology in Health and Disease*, 12, 223-239.

Bao, W., Ye, L., Zhu, J., Pan, Z., Zhu, G., Huang, X. and Wu, S. 2011. Polymorphism of *M307* of the *FUT1* Gene and Its Relationship with Some Immune Indexes in Sutai Pigs (Duroc × Meishan). *Biochemical Genetics*, 49, 665-673

Batisson, I., Guimond, M.-P., Girard, F., An, H., Zhu, C., Oswald, E., Fairbrother, J. M., Jacques, M. and Harel, J. 2003. Characterization of the Novel Factor *Paa* Involved in the Early Steps of the Adhesin Mechanism of Attaching and Effacing *Escherichia coli*. *Infection and Immunity*, 71, 4516-4525.

Cai, H. Y., Archambault, M., Gyles, C. L. and Prescott, J. F. 2003. Molecular genetic methods in the veterinary clinical bacteriology laboratory: current usage and future applications. *Animal Health Research Reviews*, 4, 73-94.

Chapman, T. A., Wu, X. Y., Barchia, I., Bettelheim, K. A., Driesen, S., Trott, D., Wilson, M. and Chin, J. J. C. 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Applied and Environmental Microbiology*, 72, 4782-4795.

Chen, X., Gao, S., Jiao, X. and Liu, X. F. 2004. Prevalence of serogroups and virulence factors of *Escherichia coli*strains isolated from pigs with postweaning diarrhoea in eastern China. *Veterinary Microbiology*, 103, 13-20.

Cheng, D., Sun, H., Xu, J. and Gao, S. 2006. PCR detection of virulence factor genes in *Escherichia coli* isolates from weaned piglets with edema disease and/or diarrhea in China. *Veterinary Microbiology*, 115, 320-328.

Cheng, D., Yuan, Z. S., Lang, C. X., Pan, G. X., Wei, D. W. and Chang, S. H. 2010. Rapid diagnosis of ETEC and HPI-harboring *Escherichia coli* infection in newborn piglets with diarrhea. *African Journal of Microbiological Research*, 4, 575-580.

Coddens, A., Verdonck, F., Tiels, P., Rasschaert, K., Goddeeris, B. and Cox, E. 2007. The age-dependent expression of the *FI8+ E. coli* receptor on porcine gut epithelial cells is positively correlated with the presence of histo-blood group antigens. *Veterinary Microbiology*, 122, 332-341.

De La Fe Rodriguez, P. Y., Coddens, A., Del Fava, E., Cortiñas Abrahantes, J., Shkedy, Z., Maroto Martin, L. O., Cruz Muñoz, E., Duchateau, L., Cox, E. and Goddeeris, B. M. 2011. High prevalence of *F4+* and *FI8+ Escherichia coli* in Cuban piggeries as determined by serological survey. *Tropical Animal Health and Production*, 43, 937-946.

Do, T., Stephens, C., Townsend, K., Wu, X., Chapman, T., Chin, J., Mccormick, B., Bara, M. and Trott, D. 2005. Rapid identification of virulence genes in enterotoxigenic *Escherichia coli* isolates associated with diarrhoea in Queensland piggeries. *Australian Veterinary Journal*, 83, 293-299.

Doughari, J. H., Ndakidemi, P. A., Human, I. S. and Bennade, S. 2009. Shiga toxins (Verocytotoxins). *African Journal of Microbiology Research*, 3, 681-693.

Fang, Y., Ngeleka, M., Middleton, D. and Simko, E. 2008. Isolation and identification of *AIDA-I* receptors in porcine intestinal mucus. *Veterinary Microbiology*, 126, 345-355.

Francis, D. H. 2002. Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis. *Journal of Swine Health and Production*, 10, 171-175.

Gibbons, R., Sellwood, R., Burrows, M. and Hunter, P. 1977. Inheritance of resistance to neonatal *E. coli* diarrhoea in the pig: examination of the genetic system. *Theoretical and Applied Genetics*, 51, 65-70.

Ha, S. K., Choi, C., Jung, K., Kim, J., Han, D. U., Ha, Y., Lee, S. D., Kim, S. H. and Chae, C. 2004. Genotypic prevalence of the adhesin involved in diffuse adherence in *Escherichia coli* isolates in pre-weaned pigs with diarrhoea in Korea. *Journal of Veterinary Medicine. B, Infectious diseases and public health*, 51, 166-168.

Henton, M. and Engelbrecht, M. 1997. *Escherichia coli* serotypes in pigs in South Africa. *The Onderstepoort Journal of Veterinary Research*, 64, 175-187.

Intervet, S.-P. 2010. *Vaccines* [Online]. Available: http://www.intervet.com/species/pigs/vaccines.aspx [Accessed 29 March at 1: 23 pm 2011].

Jensen, G. M., Frydendahl, K., Svendsen, O., Jorgensen, C. B., Cirera, S., Fredholm, M., Nielsen, J. P. and Moller, K. 2006. Experimental infection with *Escherichia coli O*149: *F4ac* in weaned piglets. *Veterinary Microbiology*, 115, 243-249.

Jung, B. Y., Byun, J.-W., Kim, H.-Y., Shin, D.-H., Kim, D. and Lee, O.-S. 2011. Serotypes and virulence genes of *Escherichia coli* isolated from diarrheic pigs in Korea. *Proceedings of the 5th Asian Pig Veterinary Society Congress*, 7-9 March 2011, Pattaya, Thailand.

Kim, J., Choi, C. and Chae, C. 2001. Prevalence of *eaeA*$^{+}$*Escherichia coli* isolated from pigs with diarrhea. *Journal of Veterinary Diagnostic Investigation*, 13, 355-356.

Kwon, D., Kim, O. and Chae, C. 1999. Prevalence of genotypes for fimbriae and enterotoxins and of *O* serogroups in *Escherichia coli* isolated from diarrheic piglets in Korea. *Journal of Veterinary Diagnostic Investigation*, 11, 146-151.

Law, D. 2000. A REVIEW: Virulence factors of *Escherichia coli O*157 and other Shiga toxin-producing *E. coli*. *Journal of Applied Microbiology*, 88, 729-745.

Lee, S. I., Rayamahji, N., Lee, W. J., Cha, S. B., Shin, M. K., Roh, Y. M. and Sang, H. Y. 2009. Genotypes, antibiogram, and pulsed-field gel electrophoresis profiles of *Escherichia coli* strains from piglets in Korea. *Journal of Veterinary Diagnostic Investigation*, 21, 510-516.

Madoroba, E., Van Driessche, E., De Greve, H., Mast, J., Ncube, I., Read, J. and Beeckmans, S. 2009. Prevalence of enterotoxigenic *Escherichia coli* virulence genes from scouring piglets in Zimbabwe. *Tropical Animal Health and Production*, 41, 1539-1547.

Moneoang, M. S. and Bezuidenhout, C. C. 2009. Characterisation of enterococci and *Escherichia coli* isolated from commercial and communal pigs from Mafikeng in the North-West Province, South Africa. *African Journal of Microbiology Research*, 3, 088-096.

Moon, H. W., Hoffman, L. J., Cornick, N. A., Booker, S. L. and Bosworth, B. T. 1999. Prevalences of some virulence genes among *Escherichia coli* isolates from swine presented to a diagnostic laboratory in Iowa. *Journal of Veterinary Diagnostic Investigation*, 11, 557-560.

Nagy, B., Arp, L., Moon, H. and Casey, T. 1992. Colonization of the small intestine of weaned pigs by enterotoxigenic *Escherichia coli* that lack known colonization factors. *Veterinary Pathology*, 29, 239-246.

Nagy, B., Wilson, R. A. and Whittam, T. S. 1999. Genetic diversity among *Escherichia coli*isolates carrying *FI8* genes from pigs with porcine postweaning diarrhea and edema disease. *Journal of Clinical Microbiology*, 37, 1642-1645.

Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, 11, 142-201.

Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D. M. and Fairbrother, J. M. 2003. Isolation and association of *Escherichia coli AIDA-I/STb*, rather than *EAST1* pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. *Journal of Veterinary Diagnostic Investigation*, 15, 242-253.

Onderstepoort, B. P. undated. *Oil-Emulsion Vaccine Esherichia coli vaccine for cattle and sheep* [Online]. Available: http://www.obpvaccines.co.za/disease_goatsinfo.htm [Accessed 04 April at 4:15 pm 2011].

Post, K. W., Bosworth, B. T. and Knoth, J. L. 2000. Frequency of virulence factors in *Escherichia coli* isolated from pigs with postweaning diarrhea and edema disease in North Carolina. *Swine Health and Production*, 8, 119-120.

SAS 2012. SAS User's Guide Version 9.2 ed., SAS Institute, Cary, NC, USA.

Taillon, C., Nadeau, É., Mourez, M. and Dubreuil, J. D. 2008. Heterogeneity of *Escherichia coliSTb* enterotoxin isolated from diseased pigs. *Journal of Medical Microbiology*, 57, 887-890.

Vidotto, M. C., De Lima, N. C. S., Fritzen, J. T. T., De Freitas, J. C., Venancio, M. J. and Ono, M. A. 2009. Frequency of virulence genes in *Escherichia coli* strains isolated from piglets with diarrhea in the North Parana State, Brazil. *Brazilian Journal of Microbiology*, 40, 199-204.

Vu-Khac, H., Holoda, E. and Pilipčinec, E. 2004. Distribution of virulence genes in *Escherichia coli* strains isolated from diarrhoeic piglets in the Slovak Republic. *Journal of Veterinary Medicine, Series B,* 51, 343-347.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J., Mora, A., Dahbi, G., López, C. and González, E. 2006. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia. *BMC Veterinary Research*, 2, 1-8.

Wang, J., Jiang, S. W., Chen, X. H., Liu, Z. L. and Peng, J. 2006. Prevalence of fimbrial antigen (*K88* variants, *K99* and *987P*) of enterotoxigenic *Escherichia coli* from neonatal and post-weaning piglets with diarrhea in central China. *Asian-Australasian Journal of Animal Sciences*, 19, 1011-2367.

Wang, X. M., Liao, X. P., Liu, S. G., Zhang, W. J., Jiang, H. X., Zhang, M. J., Zhu, H. Q., Sun, Y., Sun, J., Li, A. X. and Lui, Y.-H. 2011. Serotypes, Virulence Genes, and Antimicrobial Susceptibility of *Escherichia coli* Isolates from Pigs. *Foodborne Pathogens and Disease,* 8, 687-692.

Yamamoto, T. and Nakazawa, M. 1997. Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. *Journal of Clinical Microbiology*, 35, 223-227.

Zajacova, Z. S., Konstantinova, L. and Alexa, P. 2011. Detection of virulence factors of *Escherichia coli* focused on prevalence of *EAST1* toxin in stool of diarrheic and non-diarrheic piglets and presence of adhesin involving virulence factors in *astA* positive strains. *Veterinary Microbiology*, 154, 369-375.

Zhang, W., Zhao, M., Ruesch, L., Omot, A. and Francis, D. 2007. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Veterinary Microbiology*, 123, 145-152.

Zhu, C., Harel, J., Jacques, M., Desautels, C., Donnenberg, M., Beaudry, M. and Fairbrother, J. 1994. Virulence properties and attaching-effacing activity of *Escherichia coli O*45 from swine postweaning diarrhea. *Infection and Immunity*, 62, 4153.

Zweifel, C., Schumacher, S., Beutin, L., Blanco, J. and Stephan, R. 2006. Virulence profiles of Shiga toxin 2e-producing *Escherichia coli* isolated from healthy pig at slaughter. *Veterinary Microbiology*, 117, 328-332.

# CHAPTER 5

## Prevalence of 25 pathotypes identified in *E. coli* isolates taken from young Duroc, Large White, Landrace and indigenous pigs in South Africa

*Published in Tropical Animal Health and production (see Appendix 1)*


**Abstract**

A pathotype is defined by the presence or absence of one or more definable *E. coli* virulence factors and generated by the number and the nature of genes occurring concurrently in the same isolate. *E. coli* strains isolated from piglets and previously identified as ETEC, EAEC and STEC, were used in this experiment. Pathotypes were derived from the 106 isolates that were positive for toxin genes *STa*, *STb*, *LT*, *EAST-1* and *Stx2e* and non-fimbrial adhesins, *AIDA-1*, *EAE* and *PAA*. The isolates were grouped according to their respective pathotypes depending on the number and nature of virulent and adhesin genes they contained. In total, 25 pathotypes were recovered and the results show a significantly a higher prevalence of *EAST-1* (30.2 %), *STb* (13.2 %) and *STb/AIDA-1* (10.3 %). The pathotype *STb/EAST-1/EAE/AIDA-1/PAA* which was found in 0.9 % of the isolates contained the most number of virulent genes and might be the most pathogenic pathotype. The occurrence pattern of the current pathotypes is different from those obtained in other countries and, therefore, suggests that geographical region may influence pathotype distribution. The reason for the presence of the current pathotypes could be as a result of genetic change of *E. coli* and its adaptation in the host and environment overtime. These results suggest that a different colibacillosis control programme is required in colibacillosis treatment.



**Keywords:** pathotype, virulence genes association, virulence genes

## 5.1 Introduction

The potential effect of *E. coli* infections can be evaluated by examining the number of virulent genes occurring concurrently in an isolate. Different gene combinations or profiles exist in *E. coli* due to the presence or absence of one or more virulence factors and are known as pathotypes (Estrada-Garcia et al., 2008). According to Singleton and Sainsbury (1987), a pathotype is an infrasubspecific classification of a pathogen distinguished from other subspecies by its pathogenecity for a specific host. A pathotype, therefore, reflects the association of different virulence genes found in each individual isolate. This also represents the relationship between the virulence genes for toxins, fimbrial and non-fimbrial adhesins.

*Escherichia coli* pathotypes do not follow a uniform pattern of occurrence. The number of gene combination is not limited and can vary depending on the number of virulent genes isolated. Geographical regions variation of pathotypes has been portrayed in a number of studies. In addition, some pathotypes have been associated with the growth phase of the piglets in some regions (Jung et al., 2011; Toledo et al., 2011). Pathotypes harbouring *F18* and *F4* fimbrial genes have been found predominantly in weaned and neonatal piglets, respectively. Toledo et al. (2012) recently found 47 pathotypes in *E. coli* isolates from diarrheic pre-weaned piglets in Mexico. *F41/EAST-1* (4 %) pathotype was the most prevalent in this group. An additional 66 pathotypes were identified in isolates from weaned piglets with *F41/STa* (6 %) and *F41/EAST-1* (4 %) found most frequently. In contrast, Jung et al. (2011) found a total of 16 *E. coli* pathotypes in Korean pigs. *F4/LT/STb* was the most prevalent in neonatal diarrhoea and *F18/LT/STa/Stx2e* in post-weaning diarrhoea was the most predominant.

There are no reports on gene profiling of *E. coli* implicated in colibacillosis in South African pigs. Since gene profiles determine the pathogenecity of *E. coli* strains, a good outlook on the existing *E. coli* pathotypes would be a useful tool in improving pig colibacillosis control programs. The objective of the current study was, therefore, to determine the existing *E. coli* pathotypes and their distribution in the

South African pig population. Pathotypes were determined from the *E. coli* isolates that were previously characterised to carry virulence genes by multiplex and monoplex PCR in chapters 3 and 4.

## 5.2 Materials and methods

### *5.2.1 Determination of pathotypes*

Pathotypes were generated based on the number, type and the nature of the virulence genes identified within each isolate. The *E. coli* isolates having a similar virulence gene combination pattern were classified under the same pathotype. The 106 isolates positive for toxin genes *STa*, *STb*, *LT*, *EAST-1* and *Stx2e* and non-fimbrial adhesins, *AIDA-1*, *EAE* and *PAA* identified in chapter 3 and 4 were used for this experiment.

### *5.1.1 Statistical analysis*

Frequencies of resulting pathotypes and the $\chi^2$ test for association of toxin genes and adhesins were estimated using the procedure FREQ in SAS (2012). A 95 % confidence level was used in this analysis.

## 5.2 Results

A total of 25 pathotypes of different gene combination were identified from the 106 ETEC, EAEC and STEC strains. The major pathotypes classified by proportion of samples identified were *EAST-1* (30.2 %), *STb* (13.2 %) and *STb/AIDA-1* (10.38 %). A large proportion (57.8 %) of the positive isolates had a pathotype with at least two gene combinations. The remaining percentage showed the presence of a single virulent gene profiles, namely *EAST-1*, *STa* and *STb* (Table 5.1).

**Table 5.1:** Proportion of individual pathotypes (toxin and adhesin factor gene combination) identified from the 106 virulent gene positive *E. coli* strains

| Pathotype | n (/106) | Prevalence (%) |
|---|---|---|
| *EAST-1* | 32 | 30.19 |
| *EAST/EAE* | 2 | 1.89 |
| *STb/AIDA-1* | 11 | 10.38 |
| *STb/AIDA-1/PAA* | 5 | 4.72 |
| *STb/PAA* | 8 | 7.55 |
| *EAST-1/PAA* | 8 | 7.55 |
| *STb* | 14 | 13.21 |
| *STb/Stx2e/PAA* | 1 | 0.94 |
| *Stx2e/PAA* | 1 | 0.94 |
| *STb/EAE* | 2 | 1.89 |
| *STb/EAST-1/AIDA-1/PAA* | 1 | 0.94 |
| *STa/PAA* | 2 | 1.89 |
| *STa/STb* | 1 | 0.94 |
| *STa/EAST-1/AIDA-1/PAA* | 2 | 1.89 |
| *EAST-1/EAE/AIDA-1* | 1 | 0.38 |
| *Sta* | 3 | 2.83 |
| *STb/EAST-1/AIDA-1* | 1 | 0.94 |
| *STa/STb/AIDA-1/PAA* | 1 | 0.94 |
| *EAST-1/AIDA-1/PAA* | 1 | 0.94 |
| *STb/EAE/AIDA-1* | 2 | 1.89 |
| *STb/EAST-1* | 2 | 1.89 |
| *EAST-1/AIDA-1* | 2 | 1.89 |
| *LT/STb/AIDA-1/PAA* | 1 | 0.94 |
| *STb/Stx2e* | 1 | 0.94 |
| *STb/EAST/EAE/AIDA-1/PAA* | 1 | 0.94 |

*$P < 0.0001$ for the overall frequency of all pathotypes

The associations between individual toxin genes with each adhesin recovered were also investigated and the results are illustrated in table 5.2 below. Results revealed a strong association of 60.5 % between *STb* and *AIDA-1* while 44.4 % of the samples carrying *EAE* adhesin also carried the *EAST-1* toxin gene. *EAST-1* and *STb* were found in almost similar proportions in the isolates carrying the *PAA* adhesin gene ($P < 0.0001$).

**Table 5.2:** Association between individual toxin genes and adhesin factor genes in 19 pathotypes carrying both adhesin and toxin genes

| | **Adhesin genes** | | | |
|---|---|---|---|---|
| **Toxin genes** | *AIDA-1* | *PAA* | *EAE* | *P value* |
| *LT* | 2.6 | 2.5 | 0 | <.0001 |
| *STa* | 10.5 | 20.0 | 22.2 | <.0001 |
| *STb* | 60.5 | 37.5 | 33.3 | <.0001 |
| *Stx2e* | 0 | 5.0 | 0 | <.0001 |
| *EAST-1* | 26.3 | 35.0 | 44.4 | <.0001 |

The effect of breed on the pathotype recovery was also tested and the results revealed a significant effect in the distribution of only four pathotypes between the four breeds ($P < 0.05$) (Table 5.3).

**Table 5.3:** The distribution of the 25 pathotypes among the four pig breeds of South Africa

| Pathotype | Duroc (n = 10) | Indigenous (n = 34) | Landrace (n = 10) | Large White (n = 52) | P value |
|---|---|---|---|---|---|
| *EAST-1* | 2 (1.89 %) | 12 (11.32 %) | 2 (1.89 %) | 16 (15.09 %) | NS |
| *EAST-1/EAE* | 0 | 0 | 0 | 2 (1.89 %) | NS |
| *STb/AIDA-1* | 1 (0.94 %) | 5 (4.72 %) | 0 | 5 (4.72 %) | NS |
| *STb/AIDA-1/PAA* | 1 (0.94 %) | 1 (0.94 %) | 1 (0.94 %) | 2 (1.89 %) | NS |
| *STb/PAA* | 2 (1.89 %) | 2 (1.89 %) | 0 | 4 (3.77 %) | NS |
| *EAST-1/PAA* | 0 | 4 (3.77 %) | 2 (1.89 %) | 2 (1.89 %) | ** |
| *STb* | 0 | 4 (3.77 %) | 0 | 10 (9.43 %) | NS |
| *STb/Stx2e/PAA* | 0 | 0 | 1 (0.94 %) | 0 | NS |
| *Stx2e/PAA* | 0 | 1 (0.94 %) | 0 | 0 | NS |
| *STb/EAE* | 1 (0.94 %) | 0 | 0 | 1 (0.94 %) | NS |
| *STb/EAST-1/AIDA-1/PAA* | 0 | 1 (0.94 %) | 0 | 0 | NS |
| *STa/PAA* | 0 | 0 | 0 | 2 (1.89 %) | NS |
| *STa/STb* | 0 | 0 | 0 | 1 (0.94 %) | ** |
| *STa/EAST-1/AIDA-1/PAA* | 1 (0.94 %) | 0 | 0 | 1 (0.94 %) | ** |
| *EAST-1/EAE/AIDA-1* | 0 | 1 (0.94 %) | 0 | 0 | NS |
| *STa* | 0 | 0 | 3 (2.83 %) | 0 | NS |
| *STb/EAST-1/AIDA-1* | 1 (0.94 %) | 0 | 0 | 0 | ** |
| *STa/STb//AIDA-1/PAA* | 0 | 0 | 1 (0.94 %) | 0 | NS |
| *EAST/AIDA-1/PAA* | 0 | 0 | 0 | 1 (0.94 %) | NS |
| *STb/EAE/AIDA-1* | 0 | 0 | 0 | 2 (1.89 %) | NS |
| *STb/EAST-1* | 1 (0.94 %) | 0 | 0 | 1 (0.94 %) | NS |
| *EAST/AIDA-1* | 0 | 2 (1.89 %) | 0 | 0 | NS |
| *LT/STb/AIDA-1/PAA* | 0 | 0 | 0 | 1 (0.94 %) | NS |
| *STb/Stx2e* | 0 | 0 | 0 | 1 (0.94 %) | NS |
| *STb/EAST-1/EAE/AIDA-1/PAA* | 0 | 1 (0.94 %) | 0 | 0 | NS |

NS – Not significant *(P>0.05)*

** Significant *(P<0.05)*

## 5.3 Discussion

*E. coli* isolates from the piglets used in this study could be classified under 4 general groups known as ETEC, EAEC, STEC and AEEC. These groups are generated based on the specific virulence factors produced by a strain (Jadranka et al., 2010). An isolate can be classified under more than one group if it carries genes associated with the groups. ETEC is characterised by the production of heat-stable and heat-labile toxins, while STEC strains produce shigatoxins (Chen et al., 2004; Erume et al., 2008). AEEC are the strains carrying adhesin factors *AIDA-1*, *PAA* and *EAE* genes since they result in an attaching and effacing pattern during colonisation (Nataro and Kaper, 1998; Kim et al., 2001; Batisson et al., 2003). The EAEC group carries the *EAST-1* gene and are characterised by an enteroaggregative attachment (Ngeleka et al., 2003). In addition to group classification, *E. coli* can be classified using gene profiles of individual strains known as pathotypes.

In the current study, 25 pathotypes were observed of which different gene combinations with *EAST-1*, *STb* and *STb/AIDA-1*were the most prevalent. The effect of different *E. coli* pathotypes should be dependent on the nature of the virulence genes contained within the isolate. Ngeleka et al. (2003) has shown that pathotypes *AIDA-1/EAST-1/STb* and *AIDA-1/EAST-1* are more pathogenic than pathotype *EAST-1*, which failed to induce diarrhoea after 72 h following inoculation. A pattern of increased severity should also be expected as the number of the virulence genes in a given *E. coli* pathotype increases. The pathotype *EAST*-1 was found to be the most prevalent in this study. A possible explanation for *EAST-1* predominance could be selection pressure on the pathogenic *E. coli* strains. The frequent the use of vaccines and antibiotics could be reducing prevalence rates of other pathotypes making *EAST-1* the only pathotype that is more adapted to survival and proliferation under the existing host environment.

These findings are similar to those obtained in Korea (Choi et al., 2001) and Canada (Ngeleka et al., 2003) which showed that the *EAST-1* pathotype frequency in diseased pigs was 8.6 and 13.5 %, respectively. Piglets carrying *E. coli* of this pathotype showed signs of oedema and diarrhoea. This

suggests that even though its key function is yet to be discovered, *EAST-1* does have a role in colibacillosis infections. Yamamoto and Nakazawa (1997) also found that the *EAST-1* was found in most ETEC strains isolated from pigs from England, Ireland and Japan. In contrast, Wang et al. (2010) found *Stx2e/EAE* to be the most prevalent in China, while between *LT/STb/F4* was the most common pathotype in the US (Zhang et al., 2007). In addition to these studies, Do et al. (2010) investigated virulence factors in *E. coli* isolates from pigs with diarrhoea, sudden death or oedema disease symptoms from Vietnam and Québec province of Canada and discovered variation in the pathotypes predominant in the two countries. *STa/PAA/F5* and *LT/STb/EAST-1/PAA/F4* were most prevalent pathotypes generated from 20 virulence genes in Vietnam and Québec, respectively. Between 1995 and 1997, the most common pathotype isolated from diarrheic piglets was *STa/F6* (Kwon et al., 1999), while Jung et al. (2011) identified *F4/LT/ST* and *STb/EAST-1/PAA* to be highly prevalent between 2008 and 2009. The results show that there is emergence of new pathotypes and disappearance of old ones in different geographical regions. This could be triggered by some environmental factors at the specific time when the pathotype predominates.

Previous studies show that the number and types of *E. coli* pathotypes are dependent on the virulent genes present in particular geographical locations (Wang et al., 2010; 2011; Jung et al., 2011). Depending on the number and type of virulence genes investigated and those present, variable types and number of pathotypes can be generated. Zajacova et al. (2011) was able to detect 17 different pathotypes from 40 % of the 800 *E. coli* isolates taken from faeces of diarrheic piglets tested for 11 virulence genes in the Czech Republic between 2005 and 2009. Toledo et al. (2011) identified a total of 97 pathotypes in *E. coli* isolates where 13 virulence genes were investigated from diarrheic pre-weaned and weaned piglets in Mexico.

In contrast, Jung et al. (2011) found a total of 16 *E. coli* pathotypes using 12 different virulence genes in Korean pigs. In China, Wang et al. (2010) recovered 64 pathotypes using 10 virulence genes in 167 *E. coli* isolates taken from diseased and dead piglets from 52 herds between 2002 and 2008. In another

study, 10 *E. coli* pathotypes were obtained from 15 virulence genes tested in faecal swabs collected from 102 pigs with diarrhoea and oedema in China (Wang et al., 2011). By comparing the results of the current study with previous studies, it can be seen that the number of virulence genes, isolates as well as the geographical region greatly affects the number of pathotypes found in the different studies. No two countries from these studies have the same number of pathotypes. The two studies undertaken in China validate the important role played by the number of virulence genes, number of isolates tested, and period of collection as well as disease symptoms in the number of pathotypes recovered in swine colibacillosis cases.

In the current study, six pathotypes lacking potential CFs: *Sta* (1.14 %), *STb* (5.32 %), *EAST-1* (12.2 %), *STa/STb* (0.4 %), *STb/EAST-1* (0.8 %) and *STb/Stx2e* (0.4 %) were recovered. The absence of a CF in these pathotypes leads to less severity in infection or an absolute failure to induce an infection since the principal step of adhesion will not be possible. There is a possible action of these isolates being washed out of the gut during normal peristaltic movements of digesta in the host. Ngeleka et al. (2003) found that *E. coli* with the pathotypes *EAST-1* failed to induce diarrhoea in piglets even after 72 h of inoculation. The recovery of *E. coli* pathotypes lacking potential CFs is becoming more common in swine colibacillosis studies. Similarly, Vu-Khac et al. (2006) also found pathotype *EAST-1* (15.8 %) was more prevalent in *E. coli* isolates taken from piglets with post weaning diarrhoea in Slovakia, while pathotypes *STb/EAST-1* and *STa/STb* were each recovered in 1.0 % of the isolates only. In another, Slovakia study 1.6 % and 14.4 % of the *STb/EAST-1* and *EAST-1* pathotypes were recovered from diseased and healthy piglets aged 14 days old and younger (Vu-Khac et al., 2007).

In contrast to the current study Wang et al. (2010) recovered *STa* (7 %) was recovered more frequently than *STb* (6 %) pathotype, while *EAST-1* (39 %) and *STb/EAST-1* (6 %) and were found in 39 and 6 % of the diseased and dead piglets at neonatal phase in China between 2002 and 2008. In another study from the same country (China) *STa*, *STb*, *EAST-1* were recovered in the following proportions 7, 3 and 13 % from *E. coli* isolates collected from diseased piglets between 2003 and 2008 which is different from the

study conducted previously (Wang et al., 2011). Variability in pathotype prevalence in the same country has been documented and this is as a result of different times and durations of sample collection. Another reason resulting in these outcomes could be due to samples being collected from different districts of the country in question.

Pathotypes *STb/AIDA-1, EAST-1/EAE, STb/PAA, EAST-1/PAA, STb/EAE, STa/PAA, EAST-1/AIDA-1 and Stx2e/PAA, STb/Stx2e/PAA and STb/EAST-1/AIDA-1* carried at least one toxin gene and a colonisation factor gene. The presence of the colonisation factor gene implies a possibility of a successful infection induction when the favourable external factors such as elevated temperatures, transportation stress and diet change during weaning and lack of immunity exist (Pittman, 2010). In contrast to the current study, Ngeleka et al. (2003) found *EAST-1/PAA, EAST-1/EAE, STb/AIDA-1* and *STb/EAST-1/PAA* in 0.3, 3.3, 4.7 and 4.1 %, respectively in *E. coli* isolates collected from healthy and diarrheic piglets in Canada between 2000 and 2001. In the same study, it was also found that the occurrence of the toxin gene together with the adhesin genes is important for disease development. One day old piglets showed clinical signs of diarrhoea after 20 h of inoculation with *E. coli* carrying pathotypes *STb/EAST-1/AIDA-1* and *STb/AIDA-1* (Ngeleka et al., 2003).

In another study, *STb/AIDA-1* was important in the development of diarrhoea in new born colostrum-deprived piglets and in addition, *AIDA-1* lacking enterotoxin genes was able cause diarrhoea in pigs (Ravi et al., 2007). The results of the above study show that the presence of a CF in the pathotypes in the present study implies that they have the ability to cause a successful infection when stressful conditions prevail. The pathotypes carrying the *AIDA-1* gene in the current study have the potential to cause an infection regardless of the toxin gene present in the isolate. The piglets might have been asymptotic carriers and might have developed diarrhoea later. However, this could not be confirmed by post sample collection observation.

The development of colibacillosis in piglets is fairly simple when both the toxin and the adhesin factor are present in *E. coli*. Following ingestion, the adhesin factors are used to attach the bacteria to the epithelial cells of the small intestines for a successful colonisation. In the case of the AEEC, typical attaching and effacing lesions causes the reorganization of the enterocytes and the destruction of the brush border. The bacteria however remain in intimate contact with the cells (Nataro and Kaper, 1998). Once colonisation is established, an optimum concentration $10^9$ CFU per gram of tissue (Nagy & Fekete, 2005) of the available toxins has to be produced in order to initiate clinical symptoms. ETEC and EAEC results in poor absorption of water and electrolytes while STEC strains cause the destruction of protein synthesis, which eventually results in cell death and water retention of cells and organs in question (Nataro and Kaper, 1998). Typical clinical symptoms manifested by piglets affected by colibacillosis include watery and sometimes bloody diarrhoea, vomiting, dehydration, weight loss, oedema, incoordination, moribund and eventually death in cases left untreated (Parma et al., 2000; Fairbrother et al., 2005; Erume et al., 2008).

The concurrent presence of two or more adhesin factors in *E. coli* from swine colibacillosis has been reported (Ngeleka et al., 2003; Duan et al., 2011; Jung et al., 2011). Pathotypes *STb/AIDA-1/PAA*, *STb/EAST-1/AIDA-1/PAA*, *EAST-1/EAE/AIDA-1*, *STa/STb/AIDA-1/PAA*, *EAST-1/STb/AIDA-1/PAA*, *STb/EAE/AIDA-1*, *LT/STb/AIDA-1/PAA* and *STb/EAST-1/EAE/AIDA-1/PAA* from the current study carry at least one adhesin factor. These pathotypes have been recovered at minor prevalence rates in this as well as other studies. *STb/EAE/AIDA-1* and *EAST-1/EAE/AIDA-1* were found in 4.1 and 0.6 % of isolates recovered from piglets in Canada, respectively (Ngeleka et al., 2003). There is evidence that the presence of every extra virulence gene in a pathotype results in increased virulence of that particular strain.

Ngeleka et al. (2003) found that compared to *EAST-1/AIDA-1* and *EAST-1/STb/AIDA-1* pathotypes, *LT/STb/EAST-1/F4/PAA* was more virulent as it caused clinical signs of diarrhoea in a day old piglets within 4 h of inoculation, which only showed signs after 20 h. From the pathotypes recovered in this study, *STb/EAST-1/EAE/AIDA-1/PAA* pathotype will be expected to be the most pathogenic than the other

pathotypes. There are no reports on these 7 pathotypes in literature and thus the significance of these pathotypes to swine colibacillosis in South African piglets requires further investigation. The presence of multiple genes in a strain also suggests that the control measures against the strain should be more intense because the interaction between different virulence factors could result in an even more pathogenic strain.

Current results show a strong association between adhesin gene *AIDA-1* and *STb*. In addition 44 % of the isolates carrying the *EAE* adhesin factor also carried *EAST-1*. No significant difference was found between the presence of *EAST-1* and *STb* in the isolates carrying the adhesin *PAA*. The reason for this outcome is unknown and it could be due to the type and prevalence of individual virulence genes in different geographical regions. Ngeleka et al. (2003) also found that the *AIDA-1* gene is frequently isolated with *STb* in *E. coli* from Canadian piglets with colibacillosis. Other studies however found conflicting results to those obtained here. Taillon et al. (2008) reported a high recovery rate of the *EAST-1* gene together with the *AIDA-1* and *PAA* adhesin genes in isolates known to carry the *STb* gene. Ha et al. (2004) also reported a positive association of *EAST-1* and *AIDA-1* of 73.9 % in diarrheic piglets Korea. The degree of association suggests that *EAST-1/PAA*, *STb/PAA*, *STb/AIDA-1* and *EAST-1/EAE* may therefore be important colibacillosis markers in South African pigs.

Most of the 25 pathotypes found in the current study were distributed almost evenly among the four breeds investigated with the exception of *EAST-1/PAA*, *STa/PAA*, *STa/EAST-1/AIDA-1/PAA* and *STb/EAST-1/AIDA-1*. For most of the pathotypes all the breeds would be expected to show the same tolerance if any, to each type of the recovered pathogenic strains. As such the pigs should receive similar attention when it comes to treatment or control measures against colibacillosis. Pathotype *EAST-1/PAA* was recovered more frequently in the Indigenous breed while the other 3 pathotypes were found only in the Duroc breed. The reason for this distribution pattern is unknown but could be due to individual characteristics of the hosts. Small sample size of the Duroc breed could also have affected these results. There are insufficient studies involving pathogenetic analyses of *E. coli* pig samples and breeds as such there is lack of standard reference of natural populations (Girardini et al., 2012). Although breed has not

been intensively investigated, factors such as the age of the pigs have been associated with particular pathotypes in different countries. In Korea it was found that *F4/LT/STb* and *F18/LT/STa/Stx2e* predominated in neonatal and post-weaning diarrhoea, respectively (Jung et al., 2011). Toledo et al. (2012) identified *F41/EAST-1* (4 %) and *F41/STa* (6 %) as the most prevalent in *E. coli* isolates from diarrheic pre-weaned and weaned piglets in Mexico, respectively. The prevalence of pathotypes and their distribution among breeds in different countries is expected to vary as did the prevalence of pathotypes among different age groups.

Swine colibacillosis is in constant evolution, as such there is a possibility of years when few cases are reported as well as pandemic years. Countries have shown the emergence of new and disappearance of old pathotypes over years. Old studies showed that the presence of *F4* and *F18* positive pathotypes were more prevalent while recent studies show that pathotypes carrying the AE factor genes is becoming more common in recent studies. The AE strains have been reported to be similar to those found in *E. coli* isolates recovered from human diarrhoea. There is therefore a chance of direct transmission if contamination can occur during slaughter. Since *E. coli* infections are foodborne and have direct impact on consumer health (El-Rami et al., 2012), more emphasis should be placed on the safety of the pig herds particularly those used for meat production since the potential virulence strains in this study were recovered from healthy appearing pigs. With the current results it can be inferred that transfer of virulent strains from pork to humans is possible because the *E. coli* pathotypes discovered share virulence genes with strains known to previously affect humans.

Recent improvement in feeds and biosecurity measures by pig farmers can minimise neonatal diarrhoea and post-weaning colibacillosis on some farms but would not get rid of its occurrence on farms. *E. coli* infections are possible whenever favourable conditions prevail. The results of the current study provide baseline information for development of vaccines against the existing *E. coli* types to prevent high losses due to mortality, lowered performance, treatment costs and handling of sick older animals in case of colibacillosis outbreak. This protocol could be very useful for a short period of time since *E. coli*

pathotypes are going through constant evolution as such there is a need for frequent testing of pathogenic *E. coli* in pigs so as to keep up with the emerging pathotypes and develop new control measures.

## 5.4 Conclusions

The virulence gene profile of South African pigs is different from that reported in other countries and this suggests that geographical region plays a huge role on the pathogenecity of *E. coli* associated with colibacillosis. This means that pathogenic reports of one country cannot be used to design a control programme for another. The current findings could, therefore, be used as a baseline for diagnostic purposes and designing of vaccines in South Africa. In addition, the presence of different gene profiles in the present *E. coli* suggests that the pathogenecity of *E. coli* is different, ranging from lethal to less toxic.

## 5.5 References

Batisson, I., Guimond, M.-P., Girard, F., An, H., Zhu, C., Oswald, E., Fairbrother, J. M., Jacques, M. and Harel, J. 2003. Characterization of the Novel Factor *Paa* Involved in the Early Steps of the Adhesin Mechanism of Attaching and Effacing *Escherichia coli*. *Infection and Immunity*, 71, 4516-4525.

Chen, X., Gao, S., Jiao, X. and Liu, X. F. 2004. Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China. *Veterinary Microbiology*, 103, 13-20.

Choi, C., Kwon, D. and Chae, C. 2001. Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *E. coli* isolated from diarrheic piglets. *Journal of Veterinary Diagnostic Investigation*, 13, 26-29.

Do, T. N., Trott, D. J., Nadeau, É., Desautels, C. and Fairbrother, J. M. 2010. Comparison of the Pathotypes and Virotypes of Pathogenic *Escherichia coli* in Diseased Pigs in Vietnam and Quebec, Canada.Proceedings of the 21st International Pig Veterinary Society Congress, 18-21 July 2010, Vancouver, Canada.

Duan, Q., Yao, F. and Zhu, G. 2011. Major virulence factors of enterotoxigenic *Escherichia coli* in pigs. *Annals of Microbiology*, 1-8.

El-Rami, F. E., Rahal, E. A., Slelman, F. T. and Abdelnoor, A. M. 2012. Identification of Virulence Genes among Antibacterial-Resistant *Escherichia coli* Isolated from Poultry. *Advanced Studies in Biology*, 4, 385-396.

Erume, J., Berberov, E. M., Kachman, S. D., Scott, M. A., Zhou, Y., Francis, D. H. and Moxley, R. A. 2008. Comparison of the contributions of heat-labile enterotoxin and heat-stable enterotoxin b to the virulence of enterotoxigenic *Escherichia coli* in *F4ac* receptor-positive young pigs. *Infection and Immunity*, 76, 3141-3149.

Estrada-Garcia, T., Lopez-Saucedo, C., Thompson-Bonilla, R., Abonce, M., Lopez-Hernandez, D., Santos, J. I., Rosado, J. L., Dupont, H. L. and Long, K. 2008. Diarrheagenic *Escherichia coli* Pathotypes association with Infection and Diarrhea among Mexican Children: Atypical Enteropathogenic *E. coli* is Associated with Acute Diarrhea. *Journal of Clinical Microbiology*, 47, 93-98.

Fairbrother, J. M., Nadeau, É. and Gyles, C. L. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, 6, 17-40.

Girardini, L. K., Siqueira, F. M., Krewer, C. C., Krewer, C. C., Da Costa, M. M. and De Vargas, A. C. 2012. Phylogenetic and pathotype analysis of *Escherichia coli* swine isolates from Southern Brazil. *Pesquisa Veterinaria Brasileir*, 32, 374-378.

Ha, S. K., Choi, C., Jung, K., Kim, J., Han, D. U., Ha, Y., Lee, S. D., Kim, S. H. and Chae, C. 2004. Genotypic prevalence of the adhesin involved in diffuse adherence in *Escherichia coli* isolates in

pre-weaned pigs with diarrhoea in Korea. *Journal of Veterinary Medicine*. *B, Infectious diseases and public health*, 51, 166-168.

Jadranka, Ž., Jelena, A., Mišić, D., Dobrila, J. D., Milić, N., Ružica, A., Dragica, S. and Žutić, M. 2010. Isolation of ETEC strains from piglets with diarrhea in the neonatal period and their typization based on somatic and fimbrial antigens. *Acta Veterinaria*, 60, 497-506.

Jung, B. Y., Byun, J.-W., Kim, H.-Y., Shin, D.-H., Kim, D. and Lee, O.-S. 2011. Serotypes and virulence genes of *Escherichia coli* isolated from diarrheic pigs in Korea. Proceedings of the 5th Asian Pig Veterinary Society Congress, 7-9 March 2011, Pattaya, Thailand.

Kim, J., Choi, C. and Chae, C. 2001. Prevalence of *eaeA*$^{+}$ *Escherichia coli* isolated from pigs with diarrhea. *Journal of Veterinary Diagnostic Investigation*, 13, 355-356.

Kwon, D., Kim, O. and Chae, C. 1999. Prevalence of genotypes for fimbriae and enterotoxins and of *O* serogroups in *Escherichia coli* isolated from diarrheic piglets in Korea. *Journal of Veterinary Diagnostic Investigation*, 11, 146-151.

Nagy, B. and Fekete, P.Z., 2005. Enterotoxigenic *Escherichia coli* (ETEC) in veterinary medicine. *International Journal of Medical Microbiology*, 295: 443-454.

Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, 11, 142-201.

Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D. M. and Fairbrother, J. M. 2003. Isolation and association of *Escherichia coli AIDA-I/STb*, rather than *EAST1* pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. *Journal of Veterinary Diagnostic Investigation*, 15, 242-253.

Parma, A., Sanz, M., Blanco, J., Blanco, J., Viñas, M., Blanco, M., Padola, N. and Etcheverria, A. 2000. Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. *European Journal of Epidemiology*, 16, 757-762.

Pittman, J. S. 2010. Enteritis in grower-finisher pigs caused by *FI8*-positive *Escherichia coli*. *Journal of Swine Health and Production*, 18, 81-86.

Ravi, M. B., Ngeleka, M., Kim, S. H., Gyles, C., Berthiame, F., Mourez, M., Middleton, D. and Simko, E. 2007. Contribution of *AIDA-1* to the pathogenicity of porcine diarrheagenic *Escherichia coli* and to intestinal colonization through biofilm formation in pigs. *Veterinary Microbiology*, 120, 308-319.

Singleton, P. and Sainsbury, D. 1987. *Dictionary of Microbiology and Molecular Biology. 2nd Edition*, John Wiley & Sons, New York.

Taillon, C., Nadeau, É., Mourez, M. and Dubreuil, J. D. 2008. Heterogeneity of *Escherichia coli STb* enterotoxin isolated from diseased pigs. *Journal of Medical Microbiology*, 57, 887-890.

Toledo, A. A., Gómez, D., Cruz, C., Carreón, R., López, J., Giono, S. and Castro, A. M. 2012. Prevalence of virulence genes in *Escherichia coli* strains isolated from piglets in the suckling and weaning period in México. *Journal of Medical Microbiology*, 61, 148-156.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J. E., Mora, A., Dahbi, G., López, C., González, E. A. and Blanco, J. 2006. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia. *BMC Veterinary Research*, 2, 1-8.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J. E., Dahbi, G., Mora, A., López, J., González, E. and Blanco, J. 2007. Serotypes, virulence gens, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhea in Slovakia. *The Veterinary Journal*, 174, 176-187.

Wang, X. M., Jiang, H. X., Liao, X. P., Liu, J. H., Zhang, W. J., Zhang, H., Jiang, Z. G., Lü, D. H., Xiang, R. and Liu, Y. H. 2010. Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiology Letters*, 306, 15-21.

Wang, X. M., Liao, X. P., Liu, S. G., Zhang, W. J., Jiang, H. X., Zhang, M. J., Zhu, H. Q., Sun, Y., Sun, J., Li, A. X. and Lui, Y.-H. 2011. Serotypes, Virulence Genes, and Antimicrobial Susceptibility of *Escherichia coli* Isolates from Pigs. *Foodborne Pathogens and Disease*, 8, 687-692.

Yamamoto, T. and Nakazawa, M. 1997. Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. *Journal of Clinical Microbiology*, 35, 223-227.

Zajacova, Z. S., Konstantinova, L. and Alexa, P. 2011. Detection of virulence factors of *Escherichia coli* focused on prevalence of *EAST1* toxin in stool of diarrheic and non-diarrheic piglets and presence of adhesin involving virulence factors in *astA* positive strains. *Veterinary Microbiology*, 154, 369-375.

Zhang, W., Zhao, M., Ruesch, L., Omot, A. and Francis, D. 2007. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Veterinary Microbiology*, 123, 145-152.

# CHAPTER 6

## *MUC4, MUC13, MUC20* and *FUT1* gene polymorphisms and the prevalence of *E. coli* resistant candidate genotypes in South African pig populations


**Abstract**

Four candidate genes associated with susceptibility to adhesion of *E. coli* to the pigs' intestinal walls were investigated in South African exotic and indigenous pigs. The *FUT1 M307 A/G* polymorphism has been used as marker for *F18R* which facilitates the adhesion of *F18 E. coli* to the host intestines. The resistance genotype *AA* was not detected in the current pig population and the allele *A* was found in less than 1 % of the population with frequencies of 5.5 and 8.1 % Large White and indigenous pigs, respectively. There were no significant differences in gene *(P > 0.05)* in gene and genotype frequencies among populations. The mutations at *MUC4 g.8227 C/G* and *MUC20 c1600 C/T* and *g.191 C/T* were found in the population and alleles conferring *F4 E. coli* adhesins were predominant in both populations for all loci. There was heterozygotes deficiency and high levels of within breed diversity as a proportion of the total genetic variation ($F_{it}$). Both populations deviated from HWE with an excess of homozygotes observed at all three loci. No linkage disequilibrium was found between the analysed loci implying independent assortment of alleles during mating. The results suggest that using candidate genes for selection to resistance of *F4* and *FI8 E. coli* could be possible since the resistant alleles were present.



**Keywords:** candidate genes, polymorphism, *E. coli*, pigs

## 6.1 Introduction

Colibacillosis due to *E. coli* is one of the most important diseases occurring in piglets (Meijerink et al., 2000; Bosworth and Vögeli, 2003). *Escherichia coli*-associated diarrhoea causes major losses because of reduced weight gain and mortality. Increased use of antibiotics and vaccines to manage the diseases also increases cost of production and lowers profit margins. The progression of the *E. coli* infections into diarrhoea and oedema upon ingestion or transmission of the pathogen into the host is mainly facilitated by stress factors such as weaning and diet changes and external factors such as poor bio-security management (Amass et al., 2003; Pittman, 2010). Host-pathogen interactions also determine the successful colonisation of *E. coli* to cause infections.

In order to attain a successful colonisation, *E. coli* uses colonisation factors known as fimbriae and in some cases afimbrial adhesins to secure attachment to its host intestinal epithelium via the host intestinal receptor (Meijerink et al., 1997; Nagy et al., 1999). The host-pathogen interaction involves two primary steps namely (i) adhesion of fimbriae/ other adhesins to specific receptors on the gut walls and (ii) production of toxins (Billey et al., 1998). Receptors are the binding substrates found on the brush borders of the intestines (Van Den Broeck et al., 2000). Without relevant host receptors *E. coli* will fail to colonise and multiply to the required bacterial concentration of colony factor units to produce toxins and induce an infection. The continuous peristaltic movement of the gut contents will cause any unattached bacteria to be washed out before it establishes itself in the host (Adlerberth et al., 2000). The presence/absence of receptors has therefore been used to determine the resistance or susceptibility of pigs to different *E. coli* pathotypes (Bijlsma et al., 1982; Hu et al., 1993; Baker et al., 1997; Coddens et al., 2007).

The receptors for *F4ac* fimbrial antigen were characterised as having a molecular mass of 210 and 240 kDa and are closely related to sialoglycoproteins (Billey et al., 1998) and mucin type glycoproteins (Francis et al., 1998). Molecular studies positioned the locus for this receptor on pig chromosome (SSC)

13q41 although the causative mutation remains unknown (Ren et al., 2009; Rampoldi et al., 2011). Mucin genes *MUC4*, *MUC13* and *MUC20* have been mapped to the same region as the *F4bcR* (Ren et al., 2009) and the gene polymorphisms showed a strong degree of association with *F4 ab/ac* ETEC adhesion phenotypes (Jacobsen et al., 2009; Joller et al., 2009; Ren et al., 2009; Ji et al., 2011). These genes have therefore been suggested as candidate genes for the identification of animals susceptible/resistant to *F4 ab/ac* ETEC adhesion.

The *F18* receptor (*F18R*) has been identified on SSC6 and is closely linked to blood group inhibitor locus *S* on the same chromosome (Vögeli et al., 1997; Meijerink et al., 2000). The biochemical structure of *F18R* is yet to be characterised but a mutation at base pair 307 (*M307*) of the alpha 1 fucosyltransferase (*FUT1*) gene has been associated with *F18* adhesion phenotypes in Swiss Large White and Landrace populations (Meijerink et al., 2000). This gene has been used as a candidate gene for selection against the ETEC *F18+* susceptibility in Swiss and Chinese pig populations (Bosworth and Vögeli, 2003; Frydendahl et al., 2003; Bao et al., 2008). The dominant *B* allele controls susceptibility, whilst resistance was controlled by the recessive *A* allele (Alexa et al., 2002; Yan et al., 2009).

South Africa hosts a diversity of pig breeds of which their genetic status to ETEC *F4* and *F18* disease resistance is unknown. These breeds include Chester White, Duroc, Large Black, Large White, Hampshire, Hamline, Pietrain, Robuster, South African Landrace and Welsh. Kolbroek and Windsnyer are popular in the smallholder farming sector while in commercial pig production, Duroc, Landrace and Large White are the most common breeds (DAFF, 2006). *E. coli* infections have been ranked among the most important diseases in pig production by South Africa Pork Producers Organisation (Henton, 2010). In addition, it has also been previously documented that most of the oedema and diarrhoea cases reported by Onderstepoort Veterinary Institute between 1971 and 1991 were as a result of *E. coli* infections (Henton and Engelbrecht, 1997).

Current *E. coli* colibacillosis control measures in South Africa include antibiotics and vaccines incorporating fimbrial antigens *F4ac*, *F4ab*, *F5* and *F6* (Intervet, 2010). Since human safety is a concern, antibiotics use in animals used for human consumption should be limited (Hammerum and Heuer, 2009). A recent study showed that *E. coli* has developed resistance against some of the antimicrobial reagents used in pig production in South Africa (Moneoang and Bezuidenhout, 2009). Antibiotic resistance increases the chances of recovering *E. coli* in meat products from infected animals. In addition, antibiotics do not prevent the outbreak of the disease but work as therapeutic agents and are often administered after the onset of the symptoms. On the other hand, vaccines are administered to sows in gestation and, as such, their efficiency depends on the amount of colostrum consumed. The protection against colibacillosis decreases as the piglets grow (Haesebrouck et al., 2004). The loss of immunity at later phases of growth suggests additional costs to reverse the symptoms in case of the pig succumbing to the disease. Overall, considerable expenses are incurred on antibiotics and vaccines to control colibacillosis in the pig industry.

The introduction of genetic control strategies could be beneficial to the South African pig producers. Genetic improvement for disease resistance is increasingly gaining importance because the effect of *E. coli* infections is detrimental in pig herds and costs of reversing the symptoms affect the profits greatly (Buske et al., 2006). In Switzerland, the *FUT1 M307* polymorphism has been used successfully as a diagnostic test for the selection for resistance to oedema and post weaning diarrhoea caused by *E. coli F18* (Stranzinger, 2004 ). The effect of selection in the Switzerland resulted in an increase in the frequency of the resistance allele *A* by 22, 1 and 7 % in Large White, Landrace and Duroc breeds, respectively during the five years of selection between 1996 and 2001 (Bertschinger et al., 2004). This diagnostic procedure has been applied for over eight years and the pure *AA* herds generated through artificial insemination have not yet experienced problems relating to *FI8 E. coli* colibacillosis (Bertschinger et al., 2004; Stranzinger, 2004). Bao et al. (2011c) found that selection substantially

increased the favourable allele *A* of the *FUT1 M307* polymorphism from 0.046 in 2008 to 0.540 in 2011 in the Sutai pigs of China.

There is currently no documented study involving genetic resistance to *E. coli* infections in South Africa. As such, there is no information on the existing receptors and the role of the candidate genes in SA pig populations. The objective of the study was, therefore, to investigate polymorphisms of the *FUT1*, *MUC4*, *MUC13* and *MUC20* genes and determine the prevalence of alleles resistant to *F18+* and *F4 ab/ac* ETEC in the SA pig population. The level of polymorphism at these genes will influence their utility in selection programs while the presence of the resistance alleles implies resistance of the SA population to *F18+* and *F4ab/ac* ETEC.

## 6.2 Material and methods

### *6.2.1 Sample collection*

Blood samples were obtained from 153 neonatal, weaned and reproductive pigs from Large White (n = 91) and indigenous (n = 62) experimental pigs kept at the ARC Livestock unit in Irene in the Gauteng Province. The two breeds were reared in isolation from each another and selective breeding was practiced with no random mating. A 5 ml sample of blood was collected aseptically from the superficial veins around the chest area into EDTA Vacuette tube, which was stored at -20 ºC until further use.

### *6.2.2 DNA extraction*

Genomic DNA was extracted from the blood using an Whatman® FTA Elute card as described by Zhou et al. (2006). Approximately 100 µl of defrosted blood was pipetted onto an FTA card, labelled with the corresponding sample number and allowed to completely dry at room temperature. A 2 mm diameter disc of the dried blood was then punched into a 0.2 ml PCR tube. The tube was filled with 200 µl of 20 mM NaOH (pH 8.0) and this was incubated for 30 min while the contents were occasionally slightly shaken.

The solution was discarded and the disc was then rinsed with 200 µl of $TE^{-1}$ solution (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) for 5 min duration. The $TE^{-1}$ was also discarded leaving the disc in the tube. The disc contained the genomic DNA which was dried and stored at room temperature until further analysis.

### *6.2.3 Gene amplification and PCR-RFLP genotyping*

The *FUT1, MUC4, MUC13 and MUC20* primer sequences were obtained from previous studies as outlined in Table 6.1 and synthesized by Inqaba Biotech Company. The *FUT1M307* region was indicated by the presence of a 161 bp fragment PCR product. The mutation at this region was recognized by the *Hin6I* enzyme which digests the product into 117 and 44 bp when the susceptible allele *G* is present and an undigested 161bp fragment if it is the resistant allele *A* (Bao et al., 2008).

The PCR product for the *MUC4 g.8227 locus* was 367 bp. When digested with *XbaI,* it may yield an undigested resistant allele *G* (367 bp) and/or susceptible allele *C* digested to give 216 and 151 bp fragments (Jensen et al., 2006).The amplification of *MUC13* resulted in a 176 bp fragment. *BclI (FbaI)* and *MspI* were used for the genotyping of the *c.908* and *c.935* polymorphisms, respectively. The susceptible allele (*C*) for *MUC13 c.908* was given by 122 and 54 bp and the undigested fragment represented the resistant allele *A*. For the *MUC13 c.935* candidate gene, the susceptible allele was represented by an undigested 176 bp fragment and *MspI* restriction site for the resistant allele *C* resulting in 150 and 26 bp fragments (Zhang et al., 2008).

*MUC20* gene, *g.191* and *c.1600* yielded PCR products of 175 bp and 163 bp and these were digested with the *AluI* and *Hin6I* to detect their respective polymorphisms. The resistant allele *C* was characterized by two fragments 48 and 127 bp and *T* allele was the uncut fragment (175 bp) for the *g.191* polymorphism, while the *c.1600* polymorphism yielded resistant allele *C* (83 and 80 bp) and 163 bp amplicon for susceptibility allele *T* (Ji et al., 2011).

A 25 µl reaction mixture containing the DNA template on a 2mm FTA card disc, 0.5 µM of each primer, 1× Buffer (including 1.5 mm $MgCl_2$), 200 mM dNTPs and 0.25 U Taq DNA polymerase (Thermo-Scientific) was used for each PCR. Additional reagents and volume adjustments are outlined in Table 6.1. An Applied Biosystems GeneAmp® PCR system 2700 thermocycler was used and the cycling protocol was 5 min at 95 °C, 35 cycles of denaturing at 95 °C for 30 s, annealing temperatures ranging from 54-58 °C for 40 s, extending at 72 °C for 30 s, with a final extension at 72 °C for 7 min.

The PCR product was run on a 2 % EtBr stained agarose gel in TAE (40 mM Tris, 20 mM Acetic acid, 1mM EDTA) buffer for 1.5 h at 120 V and the gels were viewed under UV light using BioRAD transilluminator and the picture captured with imaging software. Restriction digestion was performed for each loci using enzymes specific for each mutation following the manufacturer's guidelines. Electrophoresis and the imaging of the gel followed the same procedure as above.

**Table 6. 1:** Primer sequences and the expected PCR products of the four ETEC *F4ab/ac* and *FI8* candidate genes

| Gene | Mutation | Primer | Sequence (5'-3') | Annealing T (°C) | Extra $MgCl_2$ (mM) | Reference |
|---|---|---|---|---|---|---|
| *FUT1* | *M307 A/G* | F | CCA ACG CCT CCG ATT CCT GT | 57 | 0.0 | Bao et al. 2008;2011 |
| | | R | GTG CAT GGC AGG CTG GAT GA | | | |
| *MUC 4* | *g.8227 C/G* | F | GTG CCT TGG GTG AGA GGT TA | 54 | 0.5 | Jensen et al. 2006 |
| | | R | CAC TCT GCC GTT CTC TTT CC | | | |
| *MUC13* | *c.908A/G* and *c.935A/C* | F | TGA GCA AGA TGA GTG CCC CAG T | 58 | 1.0 | Zhang et al. 2008 |
| | | R | TAG CCA GGC AGG CAC AAG CA | | | |
| | *c.1600 C/T* | F | AAG TGC CAC CTG CTC CTT TTG C | 57 | 0.5 | Ji et al. 2011 |
| | | R | GTT CAG GTC ACA GGC AGC GAT G | | | |
| *MUC20* | *g.191 C/T* | F | CGT GAT AAT CCA AGA GGC AAG TG | 56 | 0.0 | Ji et al. 2011 |
| | | R | CAA CAA GAA CTG AGA CCA GCA CC | | | |

### *6.2.4 Statistical analysis*

The GENEPOP Software (v4.201) (Rousset, 2008) was used to calculate allelic frequencies and population genetic parameters such as allelic diversity, expected heterozygosity ($H_e$), observed Heterozygosity ($H_o$) for each locus. The inbreeding coefficient ($F_{is}$), genetic differentiation ($F_{st}$) and the Hardy–Weinberg Equilibrium (HWE) were also analysed. Accurate values of statistical significance were estimated using the Markov Chains method by running 10000 dememorization, 150 batches and 5000 iterations/batch (Raymond and Rousset, 1995). The test for HWE assumed random union of gametes. Both the complete enumeration method, as described by Louis and Dempster (Louis and Dempster, 1987) and the exact *P-value* were calculated by complete enumeration and the global test across loci was constructed using Fisher's method.

The F-statistics parameters were computed following Weir and Cockerham (1984) method. $F_{it}$ represented the average inbreeding coefficient of the individuals relative to the whole population, $F_{st}$ was taken as the average inbreeding of the subpopulation relative to the whole population and $F_{is}$ as the inbreeding coefficient of the individual relative to its own subpopulation (Falconer and Mackay, 1996). The composite linkage disequilibrium was also computed for pairs of loci for the Mucin genes. The linkage disequilibrium (LD) test assumed that genotypes at one locus were independent from genotypes at the other locus. The test computed the association between diploid genotypes at two loci following methods described by Weir (1996).

## 6.3 Results

### *6.3.1 Genotyping*

The amplification of the six loci was successful in all the samples analysed. Two of the loci *MUC13 c.935* and *c.908* were found to be monomorphic while four other loci were successfully genotyped and showed an adequate level of polymorphism. The digested PCR product yielded the expected fragments sizes for

*FUT1 M307 (*Figure 6.1), *MUC4 g.8227*, *MUC20 c.1600* and *g.191* as well as *FUT1 M307* regions (Figures 6.2, 6.4 and 6.5, respectively). Only four of the six loci investigated showed polymorphic properties and were therefore included in the further analyses. The linkage disequilibrium tests and association analysis indicated an independent segregation of these loci.

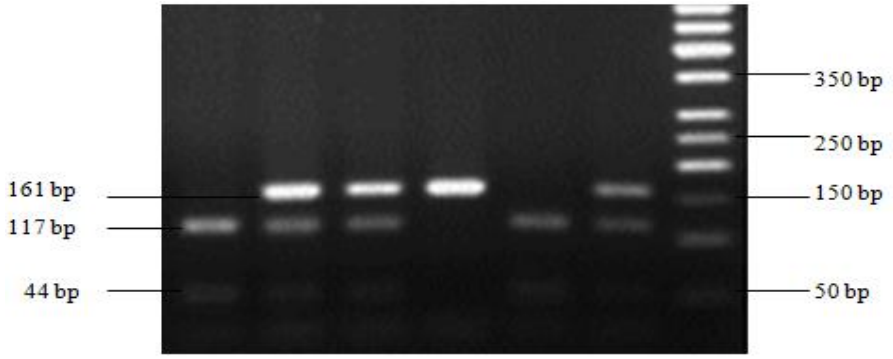


**Figure 6.1:** RFLP genotypes showing *FUT1 M307 A/G* polymorphisms. Lane 1 and 5: *GG*; Lane 2, 3 and 6: *AG*; Lane 4: undigested PCR product; Lane 7: 50 bp Ladder.

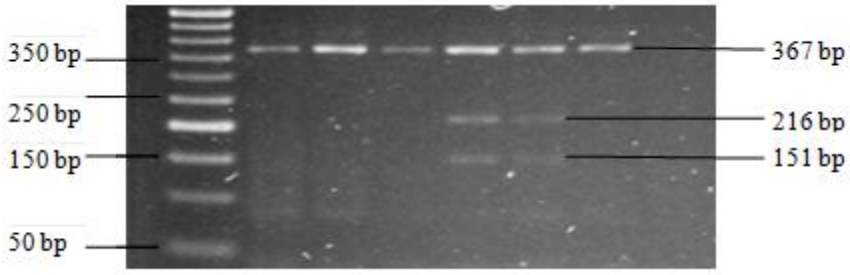


**Figure 6.2 :** Genotyping of *MUC4 g.8227 C/G* polymorphism. Lane 1: 50 bp Ladder; Lanes 2 -4 and 7: *GG*; Lanes 5 and 6: *CG*; Lane 8: no template control.

No polymorphisms for *MUC13 c.908 /c.935* loci were detected in the analysed breeds' samples. Only homozygous genotype *AA* was found in the sampled genotypes (Figure 6.3). These two loci were

dropped from subsequent analyses for this study. Analyses and conclusions are based on the four polymorphic loci that were genotyped successfully in all populations.

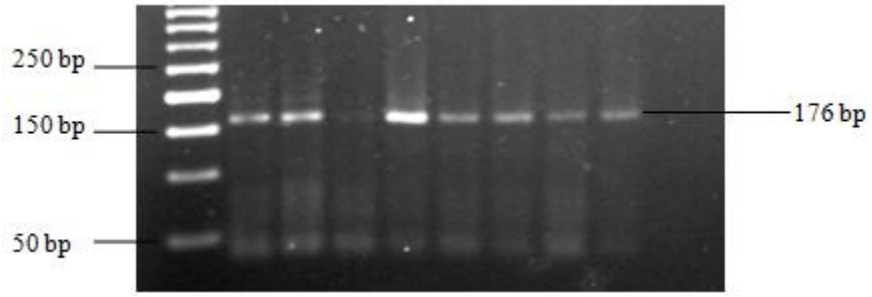


**Figure 6.3:** Genotyping results of *MUC13 c.908 A/G* and *c.935 A/C* polymorphisms. Lane 1: 50 bp Ladder; Lanes 2 -6: *c.908 AA*; Lanes 7-8: *AA*; Lanes 9: negative control.

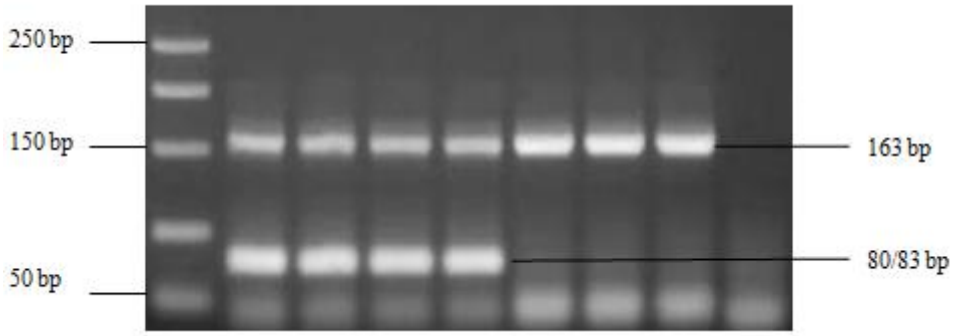


**Figure 6.4:** Genotyping RFLP genotypes for *MUC20 c.1600* gene. Lane 1: 50 bp Ladder; Lanes 2-5: *CT* genotypes; Lanes 6-7: genotypes *TT*; Lane 8: undigested PCR product; Lane 9: no template control.

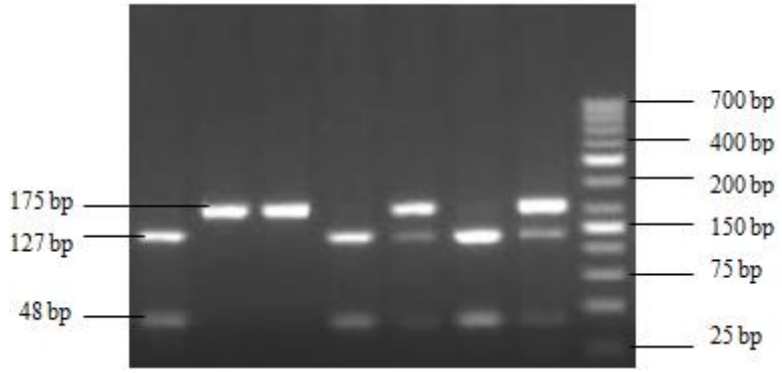


**Figure 6.5:** Genotyping of *MUC20 g.191 C/T* polymorphism. Lanes 1, 4 and 6: *CC* genotype; Lanes 2 and 3: *TT* genotype; Lanes 5 and 7: *CT*; Lane 8: 25-700 bp low range Ladder.

### *6.3.2 Genetic analysis*

#### *6.3.2.1 Allele and genotype frequencies*

Results showed that allele frequencies for the *FUT1 M307 A/G* resistant allele *A* were significantly lower than those of the susceptible allele ($P < 0.05$). *A* was found in only 8.1 and 5.5 % of the indigenous and Large White populations, respectively. Homozygote *AA* was not detected in these populations. The *AG* genotype was present in the populations with frequencies of 13 and 7 % in indigenous and Large White pigs, respectively (Table 6.2). The average frequencies of genotypes *AG* and *GG* in the two pig breeds were 13.5 and 86.5 %, respectively. No significant difference was found between the allele and genotypic frequencies among breeds ($P > 0.05$). Allele frequencies of the *MUC4 g.8227 C/G* SNP in two pig breeds are reported in Table 6.2. The resistance allele *C* was shown to be predominant in both breeds, with frequencies of 0.890 and 0.863 in the indigenous and Large White, respectively. In addition, the results show that less than 5 % of the populations carry the susceptibility genotype *GG*, which was found in 5.1 % of the indigenous and 0.33 % of the Large White pigs. The variation of the gene and genotype distribution among breeds was not statistically significant ($P > 0.05$). Only allele *A* and *AA* genotypes for the *MUC13 c.908 A/G and c.935 A/C* loci were detected in both breeds

In the analysed pig population the frequency of *MUC20 c.1600 C* allele was found to be the most with prevalent, with a frequency of 88.3 % in the indigenous and 99.5 % in Large White pigs. Only a small proportion of the population carried the susceptibility allele *T* and it was found in only 11.7 and 0.5 % of the indigenous and the Large White pigs, respectively. Approximately 90 % of the two breeds combined were found to carry the resistance genotype *CC*. The others genotypes *CT* and *TT* were found in 0.033 and 0.10 in the indigenous population while the Large White population lacked the *TT* genotype completely. There is a significant difference in the genotype and gene distribution with favourable genotypes found frequently in Large White than the indigenous pigs ($P < 0.05$). Alleles *C* and *T* for *MUC20 g.191 C/T* are found in 0.960 and 0.040 of the indigenous pigs respectively while in 87.9 % and

12.1 % of Large white carried the *T* and *C* alleles, respectively. The susceptibility genotype is found only in the Large White pigs with a frequency value of 0.033. The *CC* genotype is predominant in all the breeds and followed by the *CT* and then the *TT* genotype. The highest resistant genotype frequency occurred in the indigenous (91.9 %) and in only 79.1 % of Large White (Table 6.2). There was a significant statistical difference in the gene and genotype frequencies between the breeds ($P < 0.05$).

**Table 6.2:** Genic and genotypic frequencies of the four loci between the two pig populations

| **Locus** | **Breed** | **N** | **Allele frequency (%)** | | ***P value*** | **Genotype frequency (%)** | | | ***P value*** |
|---|---|---|---|---|---|---|---|---|---|
| | | | *A* | *G* | | *AA* | *AG* | *GG* | |
| *FUT1* | Indigenous | 62 | 8.1 | 91.9 | | - | 16.1 | 83.9 | |
| | Large White | 91 | 5.5 | 94.5 | NS | - | 11.0 | 89.0 | NS |
| | | | *C* | *G* | | *CC* | *CG* | *GG* | |
| *MUC4* | Indigenous | 62 | 89.0 | 11.0 | | 83.1 | 11.9 | 5.1 | |
| | Large White | 91 | 86.3 | 13.7 | NS | 75.8 | 20.9 | 3.3 | NS |
| | | | *C* | *T* | | *CC* | *CT* | *TT* | |
| *MUC20-C1600* | Indigenous | 62 | 88.3 | 11.7 | | 86.7 | 3.3 | 10.0 | |
| | Large White | 91 | 99.5 | 0.5 | ** | 98.9 | 1.1 | - | ** |
| | | | *C* | *T* | | *CC* | *CT* | *TT* | |
| *MUC20-G191* | Indigenous | 62 | 96.0 | 4.0 | | 91.9 | 8.1 | 0 | |
| | Large White | 91 | 87.9 | 12.1 | ** | 79.1 | 17.6 | 3.3 | ** |
| All loci | | | | | ** | | | | ** |

NS – Not significant ($P > 0.05$)

** – Significant ($P < 0.05$)

*6.3.2.2 Observed and expected heterozygosities, inbreeding coefficients and HWE*

Overall, $H_o$ and $H_e$ for all loci between breeds was 0.126 and 0.142, respectively. The results presented an overall heterozygote deficiency. $F_{is}$ was found to be 0.120 and no deviation from HWE was observed. The values of the observed heterozygosity for *FUT1 M307 locus* were 0.161 and 0.110 for indigenous pigs and Large White respectively and they were higher than the corresponding expected heterozygosity (Table 6.3). According to the $\chi^2$ goodness-of-fit test, both the Large White and the indigenous populations were in Hardy–Weinberg equilibrium.

The indigenous population deviated from HWE ($P < 0.05$) while the Large White population was in HWE on the *MUC4* locus. For both breeds the expected heterozygosities for the *MUC4* gene were higher than the $H_o$ with the values of 0.198 and 0.239 for indigenous and Large White pigs, respectively. The highest $F_{is}$ value for the *MUC4* gene was found in the indigenous population (0.402) and the Large White group showed a moderate inbreeding coefficient of 0.124.

The indigenous pigs had the highest value of $H_e$ (0.2093) for the *MUC20 c.1600* locus while the $H_o$ was 0.033. The $H_o$ of the Large White breed was similar to the expected heterozygosity value with a value of 0.011 (Table 6.3). The highest $F_{is}$ value was found in the indigenous pigs (0.841) than in the Large White, and this could be the contributing factor towards the deviation from HWE by the breed at this locus.
The observed and expected heterozygosities for *MUC20 g.191 C/T* were 0.081 and 0.078, respectively for the indigenous pigs. This locus showed higher expected heterozygosity (0.214) than the $H_o$ (0.176) in the Large White breeds (Table 6.3). The populations were in HWE at this locus. The average $F_{is}$ show a moderate level of inbreeding.

**Table 6. 3:** Genetic diversity at breed level and observed and expected heterozygosities

| Locus | Breed | $H_o$ | $H_e$ | $F_{is}$ | *P-value* |
|---|---|---|---|---|---|
| *FUT1* | Indigenous | 0.161 | 0.150 | -0.080 | NS |
| | Large White | 0.120 | 0.104 | -0.053 | NS |
| *MUC4* | Indigenous | 0.120 | 0.200 | 0.402 | ** |
| | Large White | 0.210 | 0.239 | 0.124 | NS |
| *MUC20-C1600* | Indigenous | 0.033 | 0.209 | 0.841 | ** |
| | Large White | 0.011 | 0.011 | 0 | NS |
| *MUC20-G191* | Indigenous | 0.081 | 0.078 | -0.034 | NS |
| | Large White | 0.176 | 0.214 | 0.179 | NS |
| All loci | | 0.126 | 0.142 | 0.110 | |

NS – Not significant ($P > 0.05$)

** – Significant ($P < 0.05$)

*6.3.2.3 F statistics*

Overall loci population variability was 0.2412 and this was accounted for mostly by within breed variation (0.2217) than between population variation (0.025). The mean estimates of F-statistics of the *FUT1* loci were -0.067 for $F_{it}$ which was due to -0.066 $F_{is}$ (Within population inbreeding estimate) and -0.001 for $F_{st}$ (estimate for population differentiation) (total inbreeding estimate). The average F-statistics values for the two breeds were negative although the number is not that significantly lower than zero ($P > 0.05$). The genetic differentiation between all populations for *MUC4 g.8227 C/G* was -0.005 and the average $F_{it}$ was 0.218. Much variation was from within population ($F_{is}$ = 0.223). The $F_{st}$ value between the two populations for *MUC20 c.1600 C/T* was 0.112 meaning that 11.2 % of the diversity is accounted for by genetic differentiation among populations. The total inbreeding estimate at the *c.1600* locus was 0.164. The $F_{st}$ for *MUC20 g.191 C/T* values indicated 2.51 % diversity among populations. The average level of $F_{it}$ was found to be 0.162.

**Table 6.4:** Genetic diversity between individuals, among populations and in the overall population

| Locus | HWE (*P*) | $F_{is}$ | $F_{st}$ | $F_{it}$ |
|---|---|---|---|---|
| *FUT1* | NS | -0.066 | -0.001 | -0.067 |
| *MUC4* | ** | 0.222 | -0.005 | 0.218 |
| *MUC20-C1600* | ** | 0.778 | 0.120 | 0.803 |
| *MUC20-G191* | NS | 0.137 | 0.033 | 0.164 |
| All loci | ** | 0.222 | 0.025 | 0.241 |

NS – Not significant ($P > 0.05$)

** – Significant ($P < 0.05$)

*6.2.3.4 Linkage disequilibrium and Association test between loci*

There was no evidence of linkage disequilibrium among any loci with no association between independent loci ($P > 0.05$) (Table 6.5).

**Table 6.5:** Association between different loci in South African Large White and Indigenous pig breeds

| Locus 1 | Locus 2 | P-value | S.E |
|---|---|---|---|
| *FUT1* | *MUC4* | 0.3473 | 0.0035 |
| *FUT1* | *MUC20-C1600* | 0.8778 | 0.0035 |
| *MUC4* | *MUC20-C1600* | 0.1704 | 0.0046 |
| *FUT1* | *MUC20-G191* | 0.2866 | 0.0036 |
| *MUC4* | *MUC20-G191* | 0.8683 | 0.0038 |
| *MUC20-C1600* | *MUC20-G191* | 0.9009 | 0.0030 |

## 6.3 Discussion

The diagnosis of the *F4* and *FI8* receptor phenotype detection is based on the candidate gene method. Development of DNA based methods has been a major breakthrough as they are sensitive and allow precise genotyping of living animals. *FUT1* has been mapped to SSC6 and has been used as a candidate gene for *F18R* since 1996 (Bertschinger et al., 2004). It has been suggested that the substitution of *G* by *A* at base pair 307 could alter the conformation and function of the protein, which leads to changes in the erythrocytes enzyme system and affects sensitivity to *F18+* ETEC (Vögeli et al., 1997). The resistant gene was found in low prevalence in the current pig population. No significant difference was found in the distribution of allele and genotypic frequencies in both breeds. The favourable allele *A* was found in less than 10 % of the pig population. There was an absence of the resistance genotype in both breeds. These results suggest that the South African pigs lack immunity to the *F18* ETEC and this is probably due to individual and breed variation. Resistance to disease could be affected indirectly by an association with other characteristics, such as growth rate or reproductive traits which have received major emphasis in genetic improvement programs in most domestic animals (Simianer et al., 1991).

The low frequencies of the *A* allele and *AA* genotype have been found in the Large White and Indigenous breeds. Wang et al. (2012) reported frequencies of the *AA* genotype and the resistance allele to be 0.075 and 0.287, respectively in the Large White breed in China. In addition, only the *GG* genotype was found in the Chinese indigenous breeds. In addition, only the *GG* genotype was found in the Chinese indigenous breeds. In Duroc and Landrace breeds of Taiwan, the frequency of the *AA* was 0.06 in both breeds and the *A* allele was found in 0.26 and 0.21 of the populations, respectively. Genotypic frequencies were significantly different and the Landrace was more susceptible than the Duroc breed (Huang et al., 2008).

The *MUC4 gene* has been reported in several studies as a candidate gene for *F4ab/ac* receptors. Both *F4ac* and *F4ab* receptors are controlled by the same locus (Python et al., 2005). Linkage analysis revealed *MUC4* gene is located on SSC13 (Rampoldi et al., 2011) and has the most significant linkage with

*F4acR* (Ren et al., 2009). In this study, the *MUC4 g.8227* polymorphisms which were proposed as the candidate locus for *F4ab/ac* adhesion phenotype (Joller et al., 2009) were investigated in the Large White and Indigenous breeds of South Africa. Both breeds showed significantly high levels of the resistant allele *C*, with frequencies of 0.890 and 0.863 in the Indigenous and Large White, respectively. In addition, the results show that less than 5 % of the population carry the susceptibility genotype *GG*, which found in 5.1 % of the Indigenous and 0.33 % of the Large White pigs.

The current results are in contrast with other studies. Filistowicz and Jasek (2006) identified the resistant genotype of the *MUC4 gene* in 16.2, 87.5 and 85.7 % in Polish Landrace, Belgian Landrace and Duroc respectively. The resistance allele was found in 0.47, 0.94 and 0.93 respectively. The overall resistant genotype and allele were found in 37.8 and 0.61 %, respectively. Only 51 % of the Swiss experimental population comprising of Large White, Landrace and crosses of the two breeds were found to carry the resistance genotype *CC*. The study confirmed the association of *MUC4 g.8227 as* a candidate gene for *F4bcR* but could not exclude *MUC4 as* a causal mutation. It was suggested that *F4ab/ac* adhesion/non-adhesion could be controlled by different loci which are closely related (Rampoldi et al., 2011).

The *MUC13* and *MUC20* genes have been analysed in a few studies reporting high association between the gene and the *F4bcR* (Ren et al., 2009; Ji et al., 2011). These genes form part of the mucin group genes that play a role in controlling cell adhesion, lubrication and protection of the mucosa (Fairbrother et al., 2005; Jensen et al., 2006). Ji et al. (2011) found that the *g.191 C/T* polymorphism in intron 5 and the *c.1600 C/T* polymorphism in exon 6 were significantly associated with the in vitro ETEC *F4ab/ac* adhesion phenotypes in the White Duroc × Erhualian population.

No polymorphisms were found in the *MUC13 c.908/c.935* gene for both the Indigenous and Large White breeds. The results suggested that these pigs were monomorphic at these regions. The resultant genotypes were all *AA* for the *c.908* and *c.935* loci. The *A* allele at *c.908* locus represents a resistance genotype while *A* at *c.935* corresponds to susceptibility to *F4 E. coli*. Since the genotypes were monomorphic, the

two loci were not included in further statistical analysis. The majority of the population in both pig breeds carried the resistance genotypes *c.1600 CC* and *g191*. The results show that there is a significant difference in the genotype and gene distribution with favourable genotypes found frequently in Large White than the Indigenous breed ($P < 0.05$).

The fixation indices showed a generally high level of genetic diversity among individuals of the same breed but high genetic similarities between the two breeds at the *M307* locus. This was sustained by the excess heterozygosity found in both populations. The mutation at this locus could have originated from the same ancestor. The populations were however in HWE which was brought about by the isolation of Large White from Indigenous breeds studied. This also demonstrates that selection at the *FUT1* locus could be natural and bidirectional favouring both the *A* and *G* alleles. Binder et al. (2005) reported that *AA* genotype frequencies were in HWE for the German Landrace, Large White and Pietrain breeds. The breeds had genotypic frequencies of 0.05, 0.22 and 0.3 for the AI Boars and their respective progeny showed the following frequencies 0.07, 0.22 and 0.25, respectively. However, when selective breeding is practiced, the genotypic frequencies are bound to change over time. Artificial selection for the favourable allele *A* of *FUT1* gene was practiced in the Swiss pig breeds for 8 years between 1996 and 2004. By 2004, the resistance allele *A* was increased from 0.31 to 0.51 in Large White and from 0.07 to 0.24 in Landrace pigs (Bertschinger et al., 2004). In China, 3 years of substantial selection resulted in a significant deviation of the *M307* genotypes in Sutai breed from HWE. Before selective breeding, there was no *AA* genotype but *AG* genotype was 0.092 with the *A* allele being 0.046. However, after selection the frequencies were 0.235 (*AA*) and 0.540 (*A*) (Bao et al., 2011b; 2011c).

The presence of the *A* allele assumed to confer resistance to *F18 E. coli* in the present study suggest that the implementation of selection against the *F18* ETEC adhesion genotypes using *FUT1* gene in the studied group could be rewarding in the long run. The *FUT1 M307* polymorphism is not only beneficial as a candidate gene for *F18 E. coli* adhesion/non-adhesion but has also been reported to be associated with production and reproductive traits in pigs. Bao et al. (2011b) found that average growth,

development and reproductive trait measures of *AA* genotype pigs were better than those of the *AG* or *GG* genotypes. Other studies have suggested that selection on the *FUT1* gene could affect the stress susceptibility of pigs because the *RYR1* gene is found on the same chromosome but no interactions between the 2 genes are reported elsewhere (Huang et al., 2008).

Results showed that the Indigenous population has deviated from HWE while the Large White population was in HWE. The deviation from the HWE in the Indigenous pigs could be as a result of inbreeding. The average $F_{is}$ value indicates that 22.17 % of the alleles are common within individuals of the same breed. The low gene and genotypic variation was shown by heterozygote deficiency. The heterozygote deficiencies could be explained by the inbreeding. The average $F_{is}$ value for the *MUC4* locus was 0.222 and the $F_{it}$ was 0.220. Apart from inbreeding favouring the presence of the favourable allele *C*, the high genotypic frequencies could also be explained by selection at another locus linked to the current locus. The *F4bcR* has been associated with production traits such as birth weight, growth rate and carcass weight in Duroc × Erhualian population (Yan et al., 2009). *F4abR* and *F4acR* animals have a greater birth weight, average daily gain, carcass weight and body length than animals lacking the receptors. The *F4bcR* have a beneficial influence on the production traits during the fattening period.

The populations show a deviation from HWE by the breed at this locus. This was caused by the high inbreeding levels shown by high fixation indices in both breeds and heterozygotes deficiency in the indigenous pigs (Laval et al., 2000). Positive $F_{st}$ values suggest existence of high levels of genetic differentiation between the two breeds and the *c.1600* and *g.191* mutation could have resulted from the same ancestor. Selection of breeding stock using phenotypic traits has been the main method for important production traits in pig herds worldwide (Rothschild, 2008). In some populations, deviations of genotypes and gene frequencies from the HWE should be expected for loci which are linked to the traits under selection (Goliášová and Wolf, 2004). Overall, high prevalence of the resistance alleles for *F4 E. coli* were observed in both breeds at the *MUC4* and *MUC20* loci analysed. This implies that the population might be resistant to *F4 ab/ac E. coli* but this need to be confirmed with adhesion experiments.

There is no active selection for colibacillosis resistance at ARC Livestock unit, as such selection could not have changed allele frequencies. The susceptibility of the Large White breeds in most of the loci analysed could also be due to the fact that they are a commercialised breed. Most commercial breeds undergo selection to improve their reproduction and production traits and these could affect their immunity traits (Lemus-Flores et al., 2001).

Only a small proportion of the total genetic variation ($F_{it}$) was due to differences among breeds value for the two populations at both loci. The $F_{st}$ values were 0.112 and 0.033 for *c.1600* and *g.191,* respectively. Positive $F_{st}$ values suggest that there exist high level of genetic differentiation between the two breeds and the *c.1600* and *g.191* mutation could have resulted from the same ancestor. Selection of breeding stock using phenotypic traits has by far has been the main method for important production traits in pig herds worldwide and South African (Rothschild, 2008). In some populations deviations of genotypes and gene frequencies from the HWE should be expected for loci which are linked to the traits under selection (Goliášová and Wolf, 2004). Overall high prevalence of the resistance alleles for *F4 E. coli* were observed in both breeds at the *MUC4* and *MUC20* loci analysed. This implies that the population might be resistant to *F4 ab/ac E. coli* but needs to be confirmed with adhesin experiments. There is no active selection for colibacillosis resistance at ARC Livestock unit; as such selection could have not brought about the shifting allele frequencies. The susceptibility of the Large White breeds in most of the loci analysed could also be due to the fact that they are a commercialised breed. Most commercial breeds undergo selection to improve their reproduction and production traits and these could affect their immunity traits (Lemus-Flores et al., 2001).

Linkage disequilibrium is defined as the non-random association between alleles at different loci and it is mainly affected by population history and breeding system (Slatkin, 2008). Allele associations were found to be mainly because of distal proximity but distant alleles could also be in LD. Linkage disequilibrium could also be affected by selection, gene mutation and other factors affecting the HWE (Daly et al., 2001) and allows for the selection of SNPs affecting important performance traits in a

rational way (Rothschild, 2008). The results of the current study did not show any LD and association between the four loci included in the analysis. Studies have shown a tight LD between the *MUC20 c.1600* and *g.191* loci as well as the *MUC13 c.935* genotypes (Ji et al., 2011). There was however no association found between the *MUC20 g.191 C/T* and *MUC13 c.908A/G* White Duroc × Erhualian pig population. Filistowicz and Jasek (2006) found that there was no significant interaction between *MUC4* and *FUT1* loci. The reasons for no LD in the current population could be due to distance between loci, and/or as a result of the same factors affecting the HWE, such as non-random mating and selection at other loci in close proximity to the loci investigated. The non-linkage in all the investigated genes suggest that using genotyping and marker assisted selection would be easier since there would be no interference between the loci.

## 6.4 Conclusions

The current study presents the first comprehensive genetic analysis of candidate genes for *F4* and *FI8 E. coli* in two pig populations of South African. The pig populations investigated showed a high level of resistance genotypes and alleles to the *F4 E. coli* especially for the *MUC4* and the *MUC20 g.191* loci. However, further studies needs to done to confirm the association of these genotypes with the adhesion/non-adhesion by *F4ab/ac E. coli.* The *E. coli FI8* resistance genotype was very low with the resistant allele found in less than 1 % of the population. The presence of the *A/G* mutation in the present pig population provides an opportunity for future herd improvement through the selection against the *ETEC FI8.*

## 6.5 References


Adlerberth, I., Cerquetti, M., Poilane, I., Wold, A. and Collignon, A. 2000. Mechanisms of colonisation and colonisation resistance of the digestive tract part 1: bacteria/host interactions. *Microbial Ecology in Health and Disease*, 12, 223-239.

Alexa, P., Stouracová, K., Hamrík, J. and Salajka, E. 2002. Gene typing of the colonisation factors *FI8* of *Escherichia coli* isolated from piglets suffering from post-weaning oedema disease. *Veterinarni Medicina-Praha*, 47, 132-136.

Amass, S. F., Halbur, P. G., Byrne, B. A., Schneider, J. L., Koons, C. W., Cornick, N. and Ragland, D. 2003. Mechanical transmission of enterotoxigenic *Escherichia coli* to weaned pigs by people, and biosecurity procedures that prevented such transmission. *Journal of Swine Health and Production*, 11, 61-67.

Baker, D. R., Billey, L. O. and Francis, D. H. 1997. Distribution of *K88 Escherichia coli*-adhesive and nonadhesive phenotypes among pigs of four breeds. *Veterinary Microbiology*, 54, 123-132.

Bao, W., Wu, S., Musa, H., Zhu, G. and Chen, G. 2008. Genetic variation at the alpha 1 fucosyltransferase (*FUT1*) gene in Asian wild boar and Chinese and Western commercial pig breeds. *Journal of Animal Breeding and Genetics*, 125, 427-430.

Bao, W., Ye, L., Pan, Z., Zhu, J., Du, Z., Zhu, G., Huang, X. and Wu, S. 2011a. Microarray analysis of differential gene expression in sensitive and resistant pig to *Escherichia coli FI8*. *Animal Genetics* (2011). doi:10.1111/j.1365-2052.2011.02287.x.

Bao, W., Ye, L., Pan, Z. Y., Zhu, J., Zhu, G. U. O. Q., Huang, X. and Wu, S. L. 2011b. Beneficial genotype of swine *FUT1* gene governing resistance to *E. coli FI8* is associated with important economic traits. *Journal of Genetics*, 90, 315-318.

Bao, W., Ye, L., Zhu, J., Pan, Z., Zhu, G., Huang, X. and Wu, S. 2011c. Polymorphism of *M307* of the *FUT1* Gene and Its Relationship with Some Immune Indexes in Sutai Pigs (Duroc × Meishan). *Biochemical Genetics*, 49, 665-673.

Bao, W., Ye, L., Zi, C., Su, X., Pan, Z., Zhu, J., Zhu, G., Huang, X. and Wu, S. 2012. Study on the age-dependent tissue expression of *FUT1* gene in porcine and its relationship to *E. coli FI8* receptor. *Genetics* (2012).doi:10.1016/j.gene.2012.01.035.

Bertschinger, H. U., Hofer, A., Stranzinger, G. and Vögeli, P. 2004. Breeding pigs resistant to *Escherichia coli* FI8 in the field-A progress report from Switzerland. International Society for Animal Hygiène, 11-13 October 2004, Saint-Malo, France.

Bijlsma, I. G. W., De Nijs, A., Van Der Meer, C. and Frik, J. F. 1982. Different Pig Phenotypes Affect Adherence of *Escherichia coli* to Jejunal Brush Borders by *K88ab*, *K88ac*, or *K88ad* Antigen. *Infection and Immunity*, 37, 891-894.

Billey, L. O., Erickson, A. K. and Francis, D. H. 1998. Multiple receptors on porcine intestinal epithelial cells for the three variants of *Escherichia coli K88* fimbrial adhesin. *Veterinary Microbiology*, 59, 203-212.

Binder, S., Götz, K., Thaller, G. and Fries, R. 2005. Investigations on the impact of genetic resistance to oedema disease on performance traits and its relation to stress susceptibility in pigs of different breeds. 56th Annual meeting of the European Association for Animal Production, 5-8 June 2005, Uppsala, Sweden.

Bosworth, B. T. and Vögeli, P. 2003. Methods and compositions to identify swine genetically resistant to *FI8 E. coli* associated diseases. EP Patent 0,985,052.

Buske, B., Sternstein, I., Reissmann, M., Reinecke, P. and Brockmann, G. 2006. Analysis of association of *GPX5*, *FUT1* and *ESR2* genotypes with litter size in a commercial pig cross population. *Archiv Tierzucht/Archives Animal Breeding*, 49, 259-268.

Coddens, A., Verdonck, F., Tiels, P., Rasschaert, K., Goddeeris, B. M. and Cox, E. 2007. The age-dependent expression of the *FI8+ E. coli* receptor on porcine gut epithelial cells is positively correlated with the presence of histo-blood group antigens. *Veterinary Microbiology*, 122, 332-341.

DAFF. 2006. *South African Country Report on Farm Animal Genetic Resources* [Online]. South Africa, Pretoria: Department of Agriculture, Forestry and Fisheries. Available: ftp://ftp.fao.org/docrep/fao/011/a1250f/.../CountryReports/SouthAfrica.pdf [Accessed 23 March at 12:13 pm 2011].

Daly, M. J., Rioux, J. D., Schaffner, S. F., Hudson, T. J. and Lander, E. S. 2001. High resolution haplotype structure in the human genome. *Nature Genetics*, 29, 229-233.

Fairbrother, J. M., Nadeau, É. and Gyles, C. L. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, 6, 17-40.

Falconer, D. S. and Mackay, T. F. C. 1996. *Introduction to Quantitative Genetics*. Longman Group Ltd, Harlow.

Filistowicz, M. and Jasek, S. 2006. Preliminary study on the effect of *FUT1* and *MUC4* loci on the fertility of sows and on breeding success of piglets. *Acta fytotechnica et zootechnica (online)*, *Mimoriadne Special*, 9, 23 -26.

Francis, D. H., Grange, P. A., Zeman, D. H., Baker, D. R., Sun, R. and Erickson, A. K. 1998. Expression of Mucin-Type Glycoprotein *K88* Receptors Strongly Correlates with Piglet Susceptibility to *K88* Enterotoxigenic *Escherichia coli*, but Adhesin of This Bacterium to Brush Borders Does Not. *Infection and Immunity*, 66, 4050-4055.

Francis, D. H. 1999. Colibacillosis in pigs and its diagnosis. *Swine Health Production*, 7, 214-244.

Frydendahl, K., Jensen, T. K., Andersen, J. S., Fredholm, M. and Evan, G. 2003. Association between the porcine *Escherichia coli FI8* receptor genotype and phenotype and susceptibility to colonisation and postweaning diarrhoea caused by *E. coli O138: FI8*. *Veterinary Microbiology*, 93, 39-51.

Goliášová, E. and Wolf, J. 2004. Impact of the *ESR* gene on litter size and production traits in Czech Large White pigs. *Animal Genetics*, 35, 293-297.

Gutiérrez, J. P., Marmí, J., Goyache, F. and Jordana, J. 2005. Pedigree information reveals moderate to high levels of inbreeding and a population genetic structure in the Catalonian donkey breed. *Journal of Animal Breeding and Genetics*, 122, 378–386.

Haesebrouck, F., Pasmans, F., Chiers, K., Maes, D., Ducatelle, R. and Decostere, A. 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? *Veterinary Microbiology*, 100, 255-268.

Hammerum, A. and Heuer, O. 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, 48, 916-921.

Henton, M. 2010. *Zoonotic diseases of pigs* [Online]. Available: http://www.sapork.biz/zoonotic-diseases-of-pigs-2/ [Accessed 29 July 2011 at 11:59 am.

Henton, M. M. and Engelbrecht, M. M. 1997. *Escherichia coli*serotypes in pigs in South Africa. *The Onderstepoort Journal of Veterinary Research*, 64, 175-187.

Hu, Z. L., Hasler-Rapacz, J., Huang, S. C. and Rapacz, J. 1993. Studies in Swine on Inheritance and Variation in Expression of Small Intestinal Receptors Mediating Adhesin of the *K88* Enteropathogenic *Escherichia coli* Variants. *Journal of Heredity*, 84, 157-165.

Huang, S. Y., Chung, M. T., Tsou, H. L. and Li, H. L. 2008. Association of polymorphism in the alpha (1, 2) fucosyltransferase gene with growth performance in two Western pig breeds in Taiwan. *Livestock Science*, 114, 336-340.

Intervet, S.-P. 2010. *Vaccines* [Online]. Available: http://www.intervet.com/species/pigs/vaccines.aspx [Accessed 29 March at 1: 23 pm 2011].

Jacobsen, M., Kracht, S. S., Esteso, G., Cirera, S., Edfors, I., Archibald, A. L., Bendixen, C., Andersson, L., Fredholm, M. and Jørgensen, C. B. 2009. Refined candidate region specified by haplotype sharing for *Escherichia coli F4ab/F4ac* susceptibility alleles in pigs. *Animal Genetics*, 41, 21-25.

Jensen, G. M., Frydendahl, K., Svendsen, O., Jorgensen, C. B., Cirera, S., Fredholm, M., Nielsen, J. P. and Moller, K. 2006. Experimental infection with *Escherichia coli O*149: *F4ac* in weaned piglets. *Veterinary Microbiology*, 115, 243-249.

Ji, H., Ren, J., Yan, X., Xiang Huang, X., Zhang, B., Zhang, Z. and Huang, L. 2011. The porcine *MUC20* gene: molecular characterization and its association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Molecular Biology Reports*, 38, 1593-1601.

Joller, D., Jørgensen, C. B., Bertschinger, H., Python, P., Edfors, I., Cirera, S., Archibald, A., Bürgi, E., Karlskov- Mortensen, P. and Andersson, L. 2009. Refined localization of the *Escherichia coli F4ab/F4ac* receptor locus on pig chromosome 13. *Animal Genetics*, 40, 749-752.

Laval, G., Iannuccellia, N., Legault, C., Milana, D., Groenen, M. a. M., Giuffrad, E., Anderssond, L., Nissene, P. H., Jârgensene, C. B., Beeckmann, P., Geldermann, H., Foulley, J., Chevaleta, C. and Ollivier, L. 2000. Genetic diversity of eleven European pig breeds. *Genetic Selection and Evolution*, 32, 187-203.

Lee, S., Cho, K., Kang, S., Kim, C., Park, H., Choy, Y. and Choi, Y. 2002. Detection of pigs resistant to post weaning diarrhoea, oedema disease and porcine stress syndrome by allele specific polymerase chain reaction. *Animal Genetics*, 33, 237-239.

Lemus-Flores, C., Ulloa-Arvizu, R., Ramos-Kuri, M., Estrada, F. J. and Alonso, R. A. 2001. Genetic analysis of Mexican hairless pig populations. *Journal of Animal Science*, 79, 3021-3026.

Li, Y., Qiu, X., Li, H. and Zhang, Q. 2007. Adhesive Patterns of *Escherichia coli F4* in Piglets of Three Breeds. *Journal of Genetics and Genomics*, 34, 591-599.

Louis, E. J. and Dempster, E. R. 1987. An exact test for Hardy-Weinberg and multiple alleles. *Biometrics*, 43, 805-811.

Meijerink, E., Fries, R., Vögeli, P., Masabanda, J., Wigger, G., Stricker, C., Neuenschwander, S., Bertschinger, H. U. and Stranzinger, G. 1997. Two α (1,2) fucosyltransferase genes on porcine Chromosome 6q11 are closely linked to the blood group inhibitor and *Escherichia coli FI8* receptor loci. *Mammalian Genome*, 8, 736-741.

Meijerink, E., Neuenschwander, S., Fries, R., Dinter, A., Bertschinger, H., Stranzinger, G. and Vögeli, P. 2000. A DNA polymorphism influencing α (1, 2) fucosyltransferase activity of the pig *FUT1* enzyme determines susceptibility of small intestinal epithelium to *Escherichia coli FI8* adhesin. *Immunogenetics*, 52, 129-136.

Moneoang, M. S. and Bezuidenhout, C. C. 2009. Characterisation of enterococci and *Escherichia coli* isolated from commercial and communal pigs from Mafikeng in the North-West Province, South Africa. *African Journal of Microbiology Research*, 3, 088-096

Nagy, B., Wilson, R. A. and Whittam, T. S. 1999. Genetic diversity among *Escherichia coli* isolates carrying *FI8* genes from pigs with porcine postweaning diarrhea and edema disease. *Journal of Clinical Microbiology*, 37, 1642-1645.

Pittman, J. S. 2010. Enteritis in grower-finisher pigs caused by *FI8*-positive *Escherichia coli*. *Journal of Swine Health and Production*, 18, 81-86.

Python, P., Jörg, H., Neuenschwander, S., Asai- Coakwell, M., Hagger, C., Bürgi, E., Bertschinger, H., Stranzinger, G. and Vögeli, P. 2005. Inheritance of the *F4ab*, *F4ac* and *F4adE. coli* receptors in swine and examination of four candidate genes for *F4acR*. *Journal of Animal Breeding and Genetics*, 122, 5-14.

Rampoldi, A., Jacobsen, M. J., Bertschinger, H. U., Joller, D., Bürgi, E., Vögeli, P., Andersson, L., Archibald, A. L., Fredholm, M. and Jørgensen, C. B. 2011. The receptor locus for *Escherichia coli F4ab*/*F4ac* in the pig maps distal to the *MUC4–LMLN* region. *Mammalian Genome*, 22, 122-129.

Rasschaert, K., Verdonck, F., Goddeeris, B. M., Duchateau, L. and Cox, E. 2007. Screening of pigs resistant to *F4* enterotoxigenic *Escherichia coli* (ETEC) infection. *Veterinary Microbiology*, 123, 249-253.

Raymond, M. and Rousset, F. 1995. An exact test for population differentiation. *Evolution*, 49, 1283-1286.

Ren, J., Tang, H., Yan, X., Huang, X., Zhang, B., Ji, H., Yang, B., Milan, D. and Huang, L. 2009. A pig–human comparative RH map comprising 20 genes on pig chromosome 13q41 that harbours the ETEC *F4ac* receptor locus. *Journal of Animal Breeding and Genetics*, 126, 30-36.

Rothschild, M. F., Hu, Z. and Jiang, Z. 2007. Advances in QTL mapping in pigs. *International Jounal of Biological Science*, 3, 192-197.

Rothschild, M. F. 2008. Swine Genetic Challenges of the Future: One man's thoughts. Proceedings of National Swine Improvement Federation Annual Conference and Symposium, 6-7 December 2008, St. Loius, Missouri.

Rousset, F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources*, 8, 103-106.

Sellwood, R. 1983. Genetic and immune factors in the susceptibility of piglets to *Escherichia coli*diarrhoea.*Annales De Recherches Veterinaires*, 14, 512-518.

Simianer, H., Solbu, H. and Schaeffer, L. R. 1991. Estimated genetic correlations between disease and yield traits in dairy cattle. *Journal of Dairy Science*, 74, 4358-4365.

Slatkin, M. 2008. Linkage disequilibrium - understanding the evolutionary past and mapping the medical futire. *Nature Reviews Genetics*, 9, 477-485.

Stranzinger, G. F. 2004. Gene mutations conditioning some diseases in pigs and their application in breeding programmes in Switzerland. *Animal Science Papers and Reports*, 22 127-129.

Toro, M. A., Rodrigáñez, J., Silió, M. and Rodríguez, C. 2000. Genealogical analysis of a close herd Black Hairless Iberian pigs. *Conservation Biology*, 14, 1843-1851.

Van Den Broeck, W., Coxa, E., Oudegab, B. and Goddeeris, B. M. 2000. The *F4* fimbrial antigen of *Escherichia coli* and its receptor. *Veterinary Microbiology*, 71, 223-244.

Vögeli, P., Meijerink, E., Fries, R., Neuenschwander, S., Vorländer, N., Stranzinger, G. and Bertschinger, H. 1997. A molecular test for the detection of *E. coli FI8* receptors: a breakthrough in the struggle against edema disease and post-weaning diarrhea in swine. *Schweizer Archiv für Tierheilkunde*, 139, 479-484.

Wang, S. J., Liu, W. J., Yang, L. G., Sargent, C. A., Liu, H. B., Wang, C., Liu, X. D., Zhao, S. H., Affara, N. A., Liang, A. X. and Zhang, S. J. 2012. Effects of *FUT1* gene mutation on resistance to infectious disease. *Molecular Biology Reports*, 39, 2805-2810.

Weir, B. S. 1996. *Genetic Data Analysis II*. *Methods for Discrete Population Genetic Data*. Sinauer Associates, Sunderland, Mass.

Weir, B. S. and Cockerham, C. C. 1984. Estimating F-statistics for the analysis of the population structure. *Evolution*, 38, 1358-1370.

Yan, X., Huang, X., Ren, J., Zou, Z., Yang, S., Ouyang, J., Zeng, W., Yang, B., Xiao, S. and Huang, L. 2009. Distribution of *Escherichia coli F4* adhesin phenotypes in pigs of 15 Chinese and Western breeds and a White Duroc x Erhualian intercross. *Journal of Medical Microbiology*, 58, 1112-1117.

Zhang, B., Ren, J., Yan, X., Huang, X., Ji, H., Peng, Q., Zhang, Z. and Huang, L. 2008. Investigation of the porcine *MUC13* gene: isolation, expression, polymorphisms and strong association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Animal Genetics*, 39, 258-266.

Zhou, H., Hickford, J. and Fang, Q. 2006. A two-step procedure for extracting genomic DNA from dried blood spots on filter paper for polymerase chain reaction amplification. *Analytical Bochemistry*, 354, 159-161.

# CHAPTER 7

## General discussion, Conclusions and Recommendations

### 7.1 General discussion

*Escherichia coli* infections remain the major cause of loss in pig production. The direct and indirect costs incurred by these infections are as a result of low growth rates, increased use of therapeutic agents, mortality and the increased proportions of condemned meat. Colibacillosis outbreaks have been observed even in cases where vaccines have been used (Wong et al., 1995). This dissertation characterises three types of pathogenic *E. coli* (ETEC, EAEC and STEC) isolated from South African pig breeds and their associated colonisation factors. This study determined the prevalence of multiple toxins and colonisation factors which form pathotypes in pig *E. coli* isolates. The study could be used as a reference for future studies and could also aid in the development of vaccines for colibacillosis prevention. In addition, the presence of the polymorphisms of four candidate genes known to confer *E. coli F4* and *FI8* resistance in the South African Large White and Indigenous breeds was also confirmed.

In Chapter 3, the different toxin genes and *E. coli* types that could result in colibacillosis in South African pigs were identified. STEC, EAEC and ETEC were found to have an important contribution in *E. coli* infections in South Africa. Of all the toxin genes investigated, the *EAST-1* was found to be the most predominant. It was expected that the ETEC would be found in higher frequencies and STEC in low frequencies in the South African pigs. The previous study on *E. coli* colibacillosis in pigs (Henton and Engelbrecht, 1997) showed a high prevalence of ETEC than the STEC. The high prevalence of the *EAST-1* gene was not anticipated even though the results are in accordance with several studies on swine colibacillosis. In South Africa, it has not been reported before, although it has been suggested, that the *EAST-1* was a potential threat to the pig industry. The level of its severity and contribution to the

progression of colibacillosis in pigs is yet to be determined. However, it needs to be considered when developing disease control programs for South African pigs because it is a potential threat.

In Chapter 4, colonisation factors were investigated and non- fimbrial adhesin were the possible factors used by the ETEC, EAEC and STEC to attach to the brush borders in intestines of the host. The absence of the investigated fimbriae in the current isolates was not expected. Because of possible evolution and of genetic material making up pathogenic *E. coli* and the continuous use of fimbriae incorporating vaccines in pigs it was acknowledged. The findings demonstrated the importance of *AIDA-1*, *PAA* and *EAE* as CFs and the importance of routine diagnosis for identification of virulence genes present in pig herds. The *AIDA-1*, *EAE* and *PAA* adhesins have also been implicated in human diarrheal studies, which imply that if pathogen shedding occurs, a horizontal transmission between pigs and humans might be possible. The results suggest that a revision of the current colibacillosis control program is necessary, and that manufacture of vaccines and antibiotics targeting non-fimbrial adhesins should be considered. The number and the nature of genes occurring concurrently in the same *E. coli* isolate were identified and used to generate 25 unique pathotypes. Of the 25 pathotypes recovered, *EAST-1* (30.2 %), *STb* (13.2 %) and *STb/AIDA-1* (10.3 %) were found in most of the isolates. To our knowledge, this study was the first to identify such a diversity of *E. coli* pathotypes in piglets in South Africa. Some of the pathotypes reported of in this study have not been reported elsewhere and this confirms that geographical region influences pathotypes distribution. The presence of the new pathotypes could be resulting from the genetic change of the *E. coli* and its adaptation in the host and environment overtime (Do et al., 2005).

The findings of the study were different from those obtained previously of *E. coli* infections in pigs where *F4* was found in almost half of the cases of diarrhoea and oedema-related symptoms in pigs (Henton and Engelbrecht, 1997). It has been shown that overtime *E. coli* may acquire new virulence genes associated with colibacillosis due to genetic mutation and other forces of evolution (Vu-Khac et al., 2006). This will consequently change their pathotypes and the proportion of different gene frequencies in *E. coli* (Nataro and Kaper, 1998; Wu et al., 2007; Wang et al., 2011). Wu et al. (2007) further indicated that overtime

existing pathogenic *E. coli* could respond to host selection pressure and evolve into new strains with increased virulence. In addition, Chen et al. (2004) found that use of vaccines results in selection pressure and subjects the associated *E. coli* pathotype to reduced prevalence. In contrast to vaccines, a recurring use of antibiotics results in pathogenic *E. coli* developing resistance against particular antibiotic(s) and emergence of more pathogenic strains (Amezcua et al., 2002; Wang et al., 2010). Subsequently, these pathogenic strains could have accounted for the difference of the current results with the previous ETEC and STEC studied in South African pigs. However, the question of origins of selection pressures that resulted in the absence of common fimbrial adhesins and the presence of the new pathotypes in South African *E. coli* could not be resolved by this current study.

Knowledge on the pathogenesis of *E. coli* is important in determining an effective control strategy. Host-pathogen interactions also determine the successful colonisation of *E. coli* to cause infections. The most important step to a successful *E. coli* infection is the adhesin of fimbriae to specific receptors (Billey et al., 1998) which are found in the intestinal epithelium of the host (Meijerink et al., 1997; Nagy et al., 1999). Receptors are the binding substrates found in the brush border of the intestines (Van Den Broeck et al., 2000). In Chapter 6, polymorphisms at four candidate genes (*FUT1*, *MUC4*, *MUC13* and *MUC20*) that are linked to *F4bcR* and *FI8R* receptors were investigated in the Large White and Indigenous breeds of South Africa. These candidate genes have been used previously and they have shown strong association with adhesin and non-adhesin of *E. coli* carrying the *FI8* and *F4* fimbriae. In Switzerland, the *FUT1* gene has been used since 1996 as a candidate gene for *FI8E. coli* receptor and the pig herds there have since shown a remarkable improvement (Bertschinger et al., 2004). The use of Mucin genes as candidate genes has been shown to reduce the attachment of *E. coliF4ab/ac* to piglets intestinal brush boarders in several studies (Jensen et al., 2006; Ji et al., 2011; Zhang et al., 2008).

The population was highly susceptible to *E. coli FI8*. However, the presence of the resistance allele *A* of the *FUT1 M307* locus, although proportionally small, provides an opportunity for selection towards the *FI8 E. coli* resistance. The *E. coli* isolated in the present study did not possess both the *F4* and *FI8*

fimbriae genes, suggesting that the genetic immunity to these *E. coli* types is not important for the pigs. However since *E. coli* evolves, the *F4* and *FI8* fimbrial adhesins may resurface in the future and then genetic immunity would be necessary. Therefore it makes sense to invest in selection for absence of these receptors. The absence of the *F4* fimbrial adhesin could also be as a result of the predominant absence of the *F4bc* receptors (shown by mucin resistant alleles) resulting in *E coli F4ab/ac* having no susceptible host and therefore being wiped out of populations. Using assisted selection the resistance to *FI8 E. coli* has been increased in the Swiss pig herd for over 8 years (Bertschinger et al., 2004; Stranzinger, 2004). This diagnostic procedure has been a successful tool in generating pig herds resistant to *FI8 E. coli* and could be beneficial for South African pig farmers in the long run if the association of the *FUT1* genotype with the *E. coli FI8* adhesin phenotypes coincides with those found in studies by Bao et al. (2008) and Meijerink et al. (2000). The presence of the *MUC4* and *MUC20* resistance genotypes was found to be very high in the present population. However, adhesin phenotypes still needs be done to investigate the level of association of the 3 polymorphic regions with the *F4bcR*. It has been reported by Jacobsen et al. (2009) that the receptor expression could be controlled by multiple loci.

The presence of the *AIDA-1*, *EAE* and *PAA* adhesins in the current population means that the pigs would be susceptible to ETEC, STEC and EAEC carrying these colonisation factors. Receptors for *AIDA-1* and *PAA* have been identified, meaning that a host has to lack the receptors in order to be resistant. There is a need to study these receptors to confirm possible genetic control mechanisms against *E. coli* carrying these adhesins.

### 7.2 Conclusions

Virulence genes in ETEC, STEC and EAEC isolated from piglets were found to play an important role in infections in the South African pig industry. The results suggest that there is a wide variety of virulence genes (*STb, STa, LT, EAST-1* and *Stx2e*) associated with diarrhoea in piglets. It was found that fimbriae are not a prominent colonisation factor for *E. coli* infection in the South African pigs examined. Non-

fimbrial adhesins, *AIDA-1*, *EAE* and *PAA* were found to predominate in the current population. *PAA* was particularly the most prevalent colonisation factor. Multiple colonisation factor combinations were also identified.

The current study presents the first comprehensive genetic analysis of candidate genes for *F4* and *FI8 E. coli* in two pig breeds. A high level of resistance genotypes and alleles to the *F4 E. coli*, especially the *MUC4* and the *MUC20 g.191* loci, was found in both the Large White and Indigenous pigs. The *E. coli FI8* resistance genotype was not found in the current population but the resistant allele *A* was found in small proportions in both breeds.

### 7.3 Recommendations and further research

The absence of fimbrial adhesins implies that the use of fimbriae incorporating vaccines would not be useful in this pig population. There is a need for new vaccination programs to protect piglets from colibacillosis and the *PAA*, *AIDA-1* and the *EAE* adhesins should be taken into consideration when formulating these vaccines.

The use of antibiotics remains the only feasible method to reduce the effect of infections that may arise due to the existing *E. coli* virulence gene profiles.

There is a need for pig herds to be screened routinely for CFs. This will keep farmers up to date with the CFs that may potentially cause colibacillosis and assist veterinarians with knowledge of the exact adhesin factors to immunise against. This would simplify control strategies against swine colibacillosis making them more effective and capable of targeting the exact causative pathogenic *E. coli* and reduce costs.

Further studies should focus on the following:

i. Further collection of samples from all parts of South Africa, to best estimate results that represent the entire population of the South African pigs

ii. Investigate the effects of age and season on the recovery rate of EAEC, ETEC and STEC variants in the South African pigs.

iii. Assess the role of the *EAST-1* toxin and *EAE*, *PAA* and *AIDA-1* adhesins in colibacillosis infections in South African pigs.

iv. Determine the role of different pathotypes especially those found in higher frequencies in the infection of pigs.

v. The association of the *FUT1* and Mucin gene polymorphisms with the adhesin phenotypes to determine the actual contribution of these genes to the immunity of the pigs to *F4 ab/ac* and *FI8+ E. coli*.

vi. Linkage disequilibrium studies between the Mucin genes and reproduction and productive traits to determine their association.

## 7.4 References

Amezcua, R., Friendship, R. M., Dewey, C. E., Gyles, C. and Fairbrother, J. M. 2002. Presentation of postweaning *Escherichia coli* diarrhea in southern Ontario, prevalence of hemolytic *E. coli* serogroups involved, and their antimicrobial resistance patterns. *Canadian Journal of Veterinary Research*, 66, 73-78.

Bao, W., Wu, S., Musa, H., Zhu, G. and Chen, G. 2008. Genetic variation at the alpha 1 fucosyltransferase (*FUT1*) gene in Asian wild boar and Chinese and Western commercial pig breeds. *Journal of Animal Breeding and Genetics*, 125, 427-430.

Bertschinger, H. U., Hofer, A., Stranzinger, G. and Vögeli, P. 2004. Breeding pigs resistant to *Escherichia coli FI8* in the field-A progress report from Switzerland. International Society for Animal Hygiène, 11-13 October, Saint-Malo, France.

Billey, L. O., Erickson, A. K. and Francis, D. H. 1998. Multiple receptors on porcine intestinal epithelial cells for the three variants of *Escherichia coli K88* fimbrial adhesin. *Veterinary Microbiology*, 59, 203-212.

Chen, X., Gao, S., Jiao, X. and Liu, X. F. 2004. Prevalence of serogroups and virulence factors of *Escherichia coli*strains isolated from pigs with postweaning diarrhoea in eastern China. *Veterinary Microbiology*, 103, 13-20.

Do, T., Stephens, C., Townsend, K., Wu, X., Chapman, T., Chin, J., Mccormick, B., Bara, M. and Trott, D. J. 2005. Rapid identification of virulence genes in enterotoxigenic *Escherichia coli* isolates associated with diarrhoea in Queensland piggeries. *Australian Veterinary Journal*, 83, 293-299.

Henton, M. M. and Engelbrecht, M. M. 1997. *Escherichia coli* serotypes in pigs in South Africa. *The Onderstepoort Journal of Veterinary Research*, 64, 175-187.

Jensen, G. M., Frydendahl, K., Svendsen, O., Jorgensen, C. B., Cirera, S., Fredholm, M., Nielsen, J. P. and Moller, K. 2006. Experimental infection with *Escherichia coli O*149: *F4ac* in weaned piglets. *Veterinary Microbiology*, 115, 243-249.

Ji, H., Ren, J., Yan, X., Xiang Huang, X., Zhang, B., Zhang, Z. and Huang, L. 2011. The porcine *MUC20* gene: molecular characterization and its association with susceptibility to enterotoxigenic *Escherichia coliF4ab/ac*. *Molecular Biology Reports*, 38, 1593-1601.

Meijerink, E., Fries, R., Vögeli, P., Masabanda, J., Wigger, G., Stricker, C., Neuenschwander, S., Bertschinger, H. U. and Stranzinger, G. 1997. Two α(1,2) fucosyltransferase genes on porcine Chromosome 6q11 are closely linked to the blood group inhibitor and *Escherichia coli FI8* receptor loci. *Mammalian Genome*, 8, 736-741.

Meijerink, E., Neuenschwander, S., Fries, R., Dinter, A., Bertschinger, H., Stranzinger, G. and Vögeli, P. 2000. A DNA polymorphism influencing α(1, 2) fucosyltransferase activity of the pig *FUT1* enzyme determines susceptibility of small intestinal epithelium to *Escherichia coli FI8* adhesin. *Immunogenetics*, 52, 129-136.

Nagy, B., Wilson, R. A. and Whittam, T. S. 1999. Genetic diversity among *Escherichia coli*isolates carrying *FI8* genes from pigs with porcine postweaning diarrhea and edema disease. *Journal of Clinical Microbiology*, 37, 1642-1645.

Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, 11, 142-201.

Stranzinger, G. F. 2004 Gene mutations conditioning some diseases in pigs and their application in breeding programmes in Switzerland. *Animal Science Papers and Reports*, 22 127-129.

Van Den Broeck, W., Coxa, E., Oudegab, B. and Goddeeris, B. M. 2000. The *F4* fimbrial antigen of *Escherichia coli* and its receptor. *Veterinary Microbiology*, 71, 223-244.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J., Mora, A., Dahbi, G., López, C. and González, E. 2006. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhoea in Slovakia. *BMC Veterinary Research*, 2, 1-8.

Wang, X. M., Jiang, H. X., Liao, X. P., Liu, J. H., Zhang, W. J., Zhang, H., Jiang, Z. G., Lü, D. H., Xiang, R. and Liu, Y. H. 2010. Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiology Letters*, 306, 15-21.

Wang, X. M., Liao, X. P., Liu, S. G., Zhang, W. J., Jiang, H. X., Zhang, M. J., Zhu, H. Q., Sun, Y., Sun, J., Li, A. X. and Lui, Y.-H. 2011. Serotypes, Virulence Genes, and Antimicrobial Susceptibility of *Escherichia coli* Isolates from Pigs. *Foodborne Pathogens and Disease*, 8, 687-692.

Wong, I., Moreno, M. and Del C, M. 1995. Immunity and protection elicited by a recombinant vaccine against enterotoxigenic *Escherichia coli*. *Biotecnologia Aplicada*, 12, 9-15.

Wu, X. Y., Chapman, T., Trott, D. J., Bettelheim, K., Do, T. N., Driesen, S., Walker, M. J. and Chin, J. 2007. Comparative analysis of virulence genes, genetic diversity, and phylogeny of commensal and enterotoxigenic *Escherichia coli* isolates from weaned pigs. *Applied and Environmental Microbiology*, 73, 83-91.

Zhang, B., Ren, J., Yan, X., Huang, X., Ji, H., Peng, Q., Zhang, Z. and Huang, L. 2008. Investigation of the porcine *MUC13* gene: isolation, expression, polymorphisms and strong association with susceptibility to enterotoxigenic *Escherichia coli F4ab/ac*. *Animal Genetics*, 39, 258-266.

# APPENDIX

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REGULAR ARTICLES

# Virulence profiles of enterotoxigenic, shiga toxin and enteroaggregative *Escherichia coli* in South African pigs

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**Abstract** Enterotoxigenic *Escherichia coli* (*ETEC*) and shiga toxin *E. coli* (*STEC*) are important causes of colibacillosis in piglets. Recently, enteroaggregative *E. coli* heat-stable enterotoxin 1 (*EAST-1*) has been implicated in pig diarrhoea. This study investigated the prevalence of enterotoxin [heat-labile toxins (*LT*), heat-stable toxin a (*STa*), heat-stable toxin b (*STb*)], shiga toxins (*Stx1*, *Stx2*, *Stx2e*), enteroaggregative heat-stable *E. coli* (*EAST-1*), associated fimbriae (*F4*, *F5*, *F6*, *F41*, *F18ab*, *F18ac*) and non-fimbrial adhesins [adhesin involved in diffuse adherence 1 (*AIDA-1*), attaching and effacing factor, porcine attaching- and effacing-associated factor] in South African pigs. A total of 263 *E. coli* strains were isolated from Landrace ($n$=24), Large White ($n$=126), Duroc ($n$=28) and indigenous ($n$=85) breeds of piglets aged between 9 and 136 days. PCR was used in the analysis. Virulent genes were detected in 40.3 % of the isolates, of which 18.6, 0.4 and 17.5 % were classified as *ETEC*, *STEC* and enteroaggregative *E. coli* (*EAEC*), respectively. Individual genes were found in the following proportions: *STb* (19.01 %), *LT* (0.4 %), *STa* (3.4 %), *St2xe* (1.1 %) and *EAST-1* (20.2 %) toxins. None of the tested fimbriae were detected in *ETEC* and *STEC* isolates. About one third of the *ETEC* and *STEC* isolates was tested negative for both fimbrial and non-fimbrial adhesins. Twenty-five pathotypes from *ETEC*-, *EAEC*- and *STEC*-positive strains were identified. Pathotypes *EAST-1* (30.2 %), *STb* (13.2 %) and *STb*/*AIDA-1* (10.4 %) were most prevalent. The study provided insight on possible causes of colibacillosis in South African pigs.

**Keywords** Diarrhoea · Oedema · *Escherichia coli* · Pigs

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**Abbreviations**

| | |
|---|---|
| AIDA-1 | Adhesin involved in diffuse adherence 1 |
| CF | Colonisation factors |
| EAE | Attaching and effacing factor |
| EAEC | Enteroaggregative *Escherichia coli* |
| EAST-1 | Enteroaggregative *E. coli* heat-stable enterotoxin |
| ETEC | Enterotoxigenic *E. coli* |
| LT | Heat-labile toxins |
| PAA | Porcine attaching- and effacing-associated factor |
| PCR | Polymerase chain reaction |
| STa | Heat-stable toxin a |
| STb | Heat-stable toxin b |