Comparison of prevalence of avian pathogenic *Escherichia coli* in South African and US poultry

by

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MMedVet (Altil)

In the Department of Production Animal Studies,

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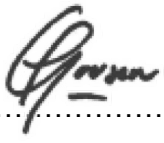
DECLARATION

I, Jaco Louis Goosen, hereby declare that this mini dissertation, which I hereby submit for the MMedVet (Altil) degree to the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, is my own work and that it has not been previously presented by me for degree purposes at any other tertiary institution.



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Dr. J.L. Goosen

DEDICATION

To my wife, Lorette, and our two boys, Jacques and Stephan, for all their support, encouragement and love during my study years. I could not have done it without you.

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SUMMARY

by

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Avian pathogenic *Escherichia coli* (APEC) as the etiological agent for Colibacillosis has extensively been described, with numerous research papers dissecting and elaborating on the prevalence and population dynamics of APEC throughout the world. The South African landscape however has not been elucidated to the same extent.

In this study, 3025 South African *E. coli* samples were analysed for the period 2017 – 2022. Data were also analysed to compare the prevalence of APEC in the United States of America (USA) versus South Africa (SA). The USA isolates were all from broiler operations, with SA isolates from different operations.

The study investigated the prevalence of APEC in SA, whether population differences occur for specific virulence - associated genes (VAGs) between operations, as well as the possible differences of virulence - associated gene (VAG) prevalence over time (2017 – 2022) within SA. The data available were also analysed to establish the potential difference of VAG prevalence between SA and USA. The extracted DNA was screened by a multiplex PCR for five APEC VAGs (*cva*C, *iss, iuc*C, *tsh* and *irp*2). The pathogenicity of each isolate was determined by comparing the number of genes detected in each isolate to a positive control. Isolates with two or more virulence genes were considered APEC positive.

This research provides supporting evidence for the theory that geographical and environmental factors influence the genetic diversity and subsequent virulence of APEC. It would therefore suggest that prophylactic measures would need to be tailored to regional needs as required by each operation for a specific period in time.

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ABBREVIATIONS

^{0}C……..Degrees Celsius

ANOVA…….Analysis of variance

APEC…….. Avian pathogenic *Escherichia coli*

BP……..Base pair

Buf……Buffer

CARY……Concentrated Area of Relocated Yankees

COVID 19……Corona virus disease of 2019

DOC……Day-old chick

DNA…… Deoxyribonucleic acid

DALRRD…….Department of Agriculture, Land Reform and Rural Development

E. coli…… *Escherichia coli*

ERIC……..Enterobacterial repetitive intergenic consensus

ExPEC……..…Extraintestinal pathogenic *E. Coli*

FTA……..Flinders Technology Associates

GIT…… Gastrointestinal tract

HSD……..… Honestly significant difference

HPAI……..Highly pathogenic avian influenza

Inf……..……Infinite hold

kV……..……Kilovolt

LSM…… Living standards measure

M1……..……..Marker

Min……Minutes

MLST……Multi-Locus sequence typing

MLVA……Multiple-locus variable number of tandem repeat analysis

NAE……..….Non antibiotics ever

NC…….. North Carolina

NICD……...National Institute for Communicable Diseases

PCR……. Polymerase chain reaction

PFGE……..Pulsed - field gel electrophoresis

RAPD…….. Randomly amplified polymorphic DNA

SA……. South Africa

SAPA…….South African Poultry Association

SAS……. Statistical analysis software

USA…… United States of America

UN……. United Nations

VAG…….....Virulence - associated gene

VAGs……...Virulence - associated genes

VG……Virulence genes

WI……Wash buffer

WP……..……Wash buffer

WGS……Whole genome sequencing

CHAPTER 1: BACKGROUND

1.1 General Introduction

According to the United Nations' department of economic and social affairs the world population was projected to peak at 8 billion people in November of 2022. Population growth is set to continue, all be it at a reduced pace and is estimated to reach 10.4 billion in 2100. A large proportion of this population increase will be seen in Africa as life expectancy increases and fertility rates remain high (UN, 2022).

Poultry meat consumption worldwide has seen a double-digit growth increase and is expected to form 47% of total protein consumed globally in 2031 (OECD - FAO, 2022). The South African poultry industry, according to an industry profile drafted by the South African Poultry Association (SAPA) in 2020, contributed 18% to the South African total gross product, with 110 000 people either directly or indirectly employed by the industry. Seventy four percent of all birds were used for meat production and 26% for egg production. The per capita consumption of poultry meat and eggs for South Africa in 2020 was 38.93kg and 9.29kg respectively, which included backyard poultry consumption (SAPA, 2020).

Consumer demand is currently driven by lower living standards measure (LSM) groups in search of more affordable poultry meat. The more health conscious, higher LSM markets in contrast are pushing for higher quality and improved welfare standards placing pressure on both sides of the bell curve (OECD - FAO, 2022). The Covid19 pandemic and the recent war in Ukraine have had a severe impact on the supply and logistic sectors, and have once again emphasised the importance of self-sufficiency (UF, 2023). Africa and closer to home, Southern Africa, will be hard pressed to ensure sustainable food supply for its ever-growing population. Production efficiency, flock health and welfare will need to be at an optimal level to meet the growing demand and remain commercially viable.

As production systems aim to become more cost competitive, early detection of pathogens, finding the root cause and the ability to treat specific flocks with success will increasingly become more relevant. Future success of poultry production will rely heavily on consumer confidence and will inevitably become progressively more focused on healthy nutritious animal protein. The future increased demand in poultry meat and eggs will undoubtably add pressure on quality systems, requiring decisive actions to ensure the highest quality products that are free from foodborne pathogens.

1.2 Literature review

1.2.1 Introduction to *Escherichia coli*

Escherichia coli (E.coli) as part of the *Enterobacteriaceae* family, is a non-spore forming, gram negative, rod - shaped, mostly flagellated, facultative anaerobe. The bacteria was first discovered by Theodor Escherich in 1885 and today, it is one of the best studied organisms and utilised as a model bacterium (Lim et al., 2010). *E.coli* can be a commensal or a pathogen in nature and forms part of the normal gut microbiota. *E.coli* can act as an opportunistic pathogen in a compromised host, crossing the epithelial barrier and inevitably cause infection. Pathogenic *E.coli* can generally be considered to be either diarrhoeal or extraintestinal pathogenic *E.coli* (ExPEC) (Leimbach et al., 2013).

1.2.2 Avian pathogenic *Escherichia coli* defined

The focus of this dissertation is on the ExPEC pathovar, avian pathogenic *Escherichia coli* (APEC) as the primary causative agent of the poultry disease, colibacillosis (Dho-Moulin and Fairbrother, 1999). This syndromic disease is endemic across the world, impacting all poultry production systems (Apostolakos et al., 2021; Shah et al., 2021). The South African poultry industry is not unique in this sense as they too experience high losses due to colibacillosis. APEC negatively affects production parameters by causing increased mortality and feed conversion, decreased growth rates, suboptimal

hatchability and condemnations at plant level (Dho-Moulin and Fairbrother, 1999; Krishnegowda et al., 2022).

The most important routes of APEC infection are thought to be the respiratory as well as the intestinal systems. Compromised epithelial tissue allows pathogens free entry into the bloodstream (Antão, 2008; Kemmett, 2013). Other potential routes of infection such as cutaneous abrasions could also allow APEC to enter and cause pathology (Kromann, 2022). Vertical introduction of APEC from parent to progeny will lead to infection of day old chicks, as confirmed by Giovanardi et al. in 2005. Breeder hens with salpingitis may lead to *in ovo* infection which will inevitably present as omphalitis, further complicated in many cases by septicaemia. Hatchery hygiene undoubtably also plays an extremely important role in the transmission of bacterial infections in day old chicks (Olsen et al., 2012).

APEC isolates generally belong to specific serogroups, namely O1, O2 and O78, as well as to a limited number of clones (Dho-Moulin and Fairbrother, 1999). A multitude of different virulent genes (VG) have been identified that could possibly contribute to the pathogenicity of APEC (Ewers et al., 2004; Johnson et al., 2008a; Ling et al., 2013; Mageiros et al., 2021; Mora et al., 2009; Rodriguez-Siek et al., 2005a; Skyberg J. A., 2003). The virulence - associated genes (VAGs) of chromosomal as well as plasmid origin assist and allow APEC colonisation and inevitably infection to occur in extraintestinal locations. The adhesion of the APEC pathogen is seen as the first step of its pathogenesis. Autotransporter proteins that ensure adhesion and invasion of APEC are encoded by VAGs such as the temperature sensitive haemagglutinin (*tsh*) gene of plasmid ColV (Dozois, 2000). Once it has crossed the epithelial barrier it becomes important for the APEC pathogen to evade the immune system. The increased serum survival gene (*iss*) of chromosomal or ColV plasmid origin is one of the genes identified that aids in the existence or survival of the pathogen (Horne et al., 2000; Awarded, 2017). Various cytotoxins are produced by APEC bacteria. The VAG *cva*C of ColV plasmid origin is one such gene that encodes for the cytotoxin microcin ColV, first discovered by Fantinatti *et al.* (1994) and by Parreira and Yano (1998). Iron acquisition is critical for most metabolic pathways, with VAGs *irp*2 of chromosomal

origin and *iuc*C of ColV plasmid origin, two of the genes responsible for the iron transport and aerobactin iron sequestering system, respectively (Kemmett, 2013; Janben et al., 2001). Several other VAGs exist and are continually being added, further enhancing our understanding of the pathogenicity of APEC. In this study APEC was defined using specific virulent genes (VG) previously described.

1.2.3 Classification of APEC

APEC population virulence in South Africa remains poorly defined despite numerous publications describing its prevalence across the world. The characterisation of APEC has further been complicated by the fact that no single molecular test is able to completely characterise APEC in its entirety. A number of different methods exist that can be utilised to classify *E. coli*. The following include but not limited to; phylogenetic typing, serological classification, enterobacterial repetitive intergenic consensus (ERIC), multi-locus sequence typing (MLST), randomly amplified polymorphic DNA (RAPD), whole genome sequence (WGS) and virulence genes (VG) (Awawdeh, 2017; Salehi et al., 2008). Each of these methods described, maintain their own particular strengths and weaknesses (Kemmett, 2013).

Serological and bacteriological methods are not sensitive enough to differentiate all bacterial isolates. Molecular differentiation of different *E. coli* strains may aid in distinguishing between the strains that are specifically pathogenic and can be used for epidemiological research (Salehi et al., 2008).

RAPD is a molecular technique that can be used as a DNA fingerprinting method to determine the diversity of APEC isolates. The advantages are that it is a simple, quick, easy and cost effective assay. Additionally, a low quantity of template DNA is needed and no sequence data for primer construction are required. One of the main disadvantages of RAPD is their low reproducibility. RAPD analysis require purified, high molecular weight DNA and precautions are needed to avoid contamination of DNA samples (Salehi et al., 2008).

It remains difficult to compare the various studies, as there is no specific definition of APEC based on any unique set of VAGs (Awawdeh, 2017). A study by Collingwood et al., 2014, further questions whether APEC isolated from infected chickens diagnosed with colibacillosis, harbours any VAGs. At present there is no literature that define APEC as harbouring 2 genes or more, however, collectively the literature states that APEC is a diverse group of ExPEC which can survive outside the intestinal tract. A study by (Skyberg J. A., 2003) however, concluded that the majority of faecal isolates from presumably healthy birds carried no more than one virulent gene. In this study two or more virulence - associated genes were used to describe APEC isolated from presumably healthy birds. Five specific VAGs (*cva*C, *iss, iuc*C, *tsh* and *irp*2) were used to characterise APEC prevalence in SA and USA.

1.2.4 APEC associated genes

A study by (Rodriguez-Siek et al., 2005a) highlighted the diversity and indicates the prevalence of 38 different VAGs obtained from chicken species in the USA. The APEC isolates showed a high prevalence of the following VAGs; *iuc*C (74.3%), *cva*C (63.4%), *iss* (84.9%), *tsh* (56.9%), and *irp*2 (58.8%). Other VAGs found in abundance were *omp* (70.6%), *feoB* (99.0%), *fimH* (98.3%), *traT* (73.6%), *sitA* (84.9%) to name a few. Another study performed in the USA conducted by (Johnson et al.(2008a) identified five VAGs (*iutA*, *iss*, *ompT*, *iroN* and *hlyF)* that were linked to APEC isolates. A study conducted by Van der Westhuizen and Bragg, in 2012 found a higher virulence gene prevalence in South African diseased chicken samples, compared to Zimbabwean chicken samples. Mbanga and Nyararai performed a study in 2015 on samples collected in Zimbabwe. It focused on the virulence profile of APEC isolated from confirmed cases with colibacillosis. The most prevalent genes in this case were found to be *iut*A (80%), *fim*H (33.3%) and *hly*F (24.4%). Very little research has since been conducted in this field in Southern Africa, let alone South Africa.

1.2.5 Factors affecting the prevalence of APEC

The diversity of the APEC pathotypes described permits one to question whether geographical differences are associated with APEC virulence prevalence (Lozica et al., 2021b; Xuhua et al., 2021). This then also forms the premise of the study as populations are exposed to different environmental systems, feed programs, selection programs, medication and vaccination strategies that could potentially alter the avian pathogenic virulence population (Grakh et al., 2022; Li et al., 2021; Lozica et al., 2021a).

In addition to the above mentioned, it is also suspected that APEC prevalence and specific virulent genes could be influenced by the differences between operations. Christensen et al., suggested in 2021 that a high degree of vertical transmission of virulence factors from parent to progeny farms could occur. Furthermore, horizontal gene transfer was also seen to contribute to the spread of pathogenicity (Ewers et al., 2004; Mageiros et al., 2021). It is known that medication and vaccination strategies differ between operations. Longer lived birds as an example are exposed to intensified immunisation programs during the rearing and laying period (Gottstein et al., 2019). Broiler vaccination programs in South Africa are usually focused on the most predominant respiratory and immunosuppressive viral infections such as Newcastle disease, Infectious bronchitis virus and Infectious bursal disease (MSD, 2023). In terms of medication, broiler populations are generally exposed to a range of different coccidiostats during the grow out period. Breeder and layer populations in contrast generally do not get exposed to coccidiostats. When we turn to antibiotic growth promoter usage, we also find differences between countries and operations. South Africa, unlike many other countries, are still allowed to include antibiotic growth promoters in poultry rations and it is thus found in most broiler poultry rations in South Africa. The inclusion of antibiotic growth promoters modulate and promote intestinal gut health, enhancing and improving performance parameters such as feed conversion, average daily gain and term mortality (Kleyn, 2014). The modulation and promotion of certain bacterial populations could potentially increase selection pressure on these populations to undergo change (Danzeisen et al., 2011).

Cleaning and disinfection mostly only occur at the end of the rearing or laying cycle and is in stark contrast to most broiler farms in South Africa which operate on a 42 to 45-day turnaround. Substandard biosecurity practices could potentially add to increasing microbial load, impacting on the APEC population (Awawdeh et al., 2022; Lutful Kabir, 2010). The fact remains, biosecurity practices differ between broiler, breeder and layer operations in South Africa. Variability in APEC population could potentially be influenced by the differences experienced between the various operations.

The zoonotic potential of APEC and the risk to human life cannot be underestimated. Several studies have indicated that human isolates express virulence factors similar to those identified in poultry strains, implying that specific isolates could be acquired from poultry (Ewers et al., 2005; Jeong et al., 2021; Liu et al., 2018; Mitchell et al., 2015; Rodriguez-Siek et al., 2005b; Subhashinie and Han, 2019; Zhuge et al., 2021). It therefore becomes even more important for extensive research to be conducted to better understand the prevalence and the potentially ever evolving genetic makeup of APEC in South Africa.

CHAPTER 2: HYPOTHESIS

2.1 Problem statement

The analysis aimed to add new insight to South Africa's APEC virulence prevalence, potential differences in APEC populations in broiler, breeder and layer operations and attempted to show a potential population shift over time. The study further compared APEC virulence - associated gene prevalence between South Africa and United States of America.

2.2 Hypothesis

2.2.1 There is a prevalence of APEC and its virulence genes in SA for the period 2017 – 2022.

2.2.2 There is a difference in APEC prevalence and its virulence factors between various poultry operations in SA for the period 2017 - 2022.

2.2.3 There is a population shift in APEC prevalence and its virulence factors in SA for the period 2017 – 2022.

2.2.4 There is a difference in APEC virulence gene prevalence in SA for the period 2018 – 2019 & 2022 compared to the USA in 2016.

2.3 Objectives

The objective is to characterise APEC in SA by analysing historical and current data generated from intestinal samples. The first objective is to determine the prevalence of APEC and its virulence factors ; *cva*C, *iss, iuc*C, *tsh* and *irp*2. The second objective is to determine whether there is a difference in APEC prevalence and its various virulence factors between poultry operations in SA. The third objective is to determine if a population shift has taken place over time. Lastly to determine the prevalence of APEC in SA in comparison to USA, specifically focusing on broiler data.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection

Samples were collected from presumably healthy birds by a Chemunique representative. The gastrointestinal tract (GIT) of each chicken from the gizzard through to the rectum was ligated and excised using sterile technique. These samples were immediately placed in a sterile Whirl-Pak® bag containing 10 ml of sterile saline and cooled on ice. Samples were then sent to Intertek testing laboratory (T0627) in South Africa for E.coli isolation and further processing.

3.2 Isolation of *E. coli*

The dataset ultimately comprised of isolates and flinders technology associates (FTA) card samples that were collected between 2016 and 2022. The South African samples were collected between 2017- 2022 and consisted of 3025 samples.

Table 13: Number of E.coli samples collected per operation in SA over time (2017 - 2022)

Number of *E.coli* samples collected per operation in SA over time (2017 - 2022)	
Operations	**Sample number**
DOC	386
Broiler	690
Breeder pullet	380
Breeder layer	1202
Layer pullet	314
Layer breeder	53

Table 14: Number of E.coli samples collected over time (2017 - 2022)

Number of *E.coli* samples collected over time (2017 - 2022)						
Year	2017	2018	2019	2020	2021	2022
Sample number	80	190	567	115	930	1143

Data analysed to compare the prevalence of APEC in the United States of America (USA) versus South Africa (SA) comprised of *E.coli* isolates, with 774 USA isolates collected in 2016 and 209 SA isolates collected between 2018 – 2019 and 2022. The USA isolates were all from broiler operations, with SA isolates from different operations. Each sample set yielded results of the five virulent genes used for APEC determination.

For each intestinal tract, the ascending portion of the duodenal loop, the ileum and 20 cm of the jejunum surrounding the Meckel's diverticulum were isolated and rinsed with sterile peptone solution to remove the contents. All sections were opened longitudinally to expose the mucosal surface and placed in a Whirl-Pak® bag. The weight of each GIT was recorded. Each GIT was homogenised using sterile peptone solution and subsequently serially diluted before being plated onto *E.coli* selective agar (CHROM agar, CHROMagar LTD, Paris, France). Plates were incubated under aerobic conditions at 37°C for 24 hours. After this time, up to 5 representative *E. coli* colonies were picked from each GIT sample for further analysis.

3.3 FTA card preparation

Each of the colonies isolated above were picked from agar and re-suspended in 25 µl of DNAzol® Reagent. The 25 µl of DNAzol® Reagent containing the bacterial suspension was then applied to the Indicating FTA® Classic Card (GE Healthcare Life Sciences Pittsburgh, USA). Samples were allowed to dry for at least one hour at room

temperature, after which the FTA cards were sent to DuPont Experimental station, Wilmington (permit number 136187, E353/010) in the United States.

3.4 DNA Recovery

Whatman Harris Micro Punch (GE Healthcare Life Sciences Pittsburgh, USA) was used to obtain a 2 mm punch from each FTA card isolate and placed in a 96 well block. The Micro-punch was sterilized with ethanol between punches. The Whatman FTA Elute procedure was followed, where DNA was eluted in Macharey-Nagel BE buffer 740306.100 to a final volume of 25 µl and stored at 4°C until further analysis.

3.5 Polymerase chain reaction (PCR) analysis

The conventional multiplex PCR used in this study, stems from work done by (Skyberg J. A., 2003). The irp2 gene was later added as a target for testing by Waukesha and confirmed by research performed by Ewers et al., (2005) and Janben et al.,(2001). Published multiplex PCR protocol was used to detect presence of putative virulence-linked gene loci colicin ColV operon (*cva*C), increased serum survival (*iss*), aerobactin iron sequestering system (*iuc*C), temperature sensitive haemagglutinin (*tsh*) and iron-repressible protein (*irp*2) in collected DNA samples and controls. The pathogenicity of each isolate was determined by comparing the number of genes detected in each isolate to a positive control. Isolates with two ≥ virulence genes were considered APEC positive. The PCR product was electrophoresed on Qiagen QIAxcel Advanced.

Table 15: APEC gel conditions used in virulent gene analysis

Method	Cartridge Type
DM150	DNA Fast Analysis

Method definition

Action	Voltage	Duration	Position
Purge	0.00kV	10 sec	WP
Inject	0.00kV	0 sec	WI
Inject	10.00kV	10 sec	M1
Inject	0.00kV	0 sec	WI
Sample inject	10.00kV	10 sec	None
Inject	0.00kV	0 sec	WI
Inject	0.00kV	0 sec	WI
Separate	10.00kV	150 sec	Buf
Purge	0.00kV	5 sec	WP

[1]

[1] kV – kilovolt, sec – seconds, WP & WI – Wash buffers, M1 – Marker, Buf – Run buffer

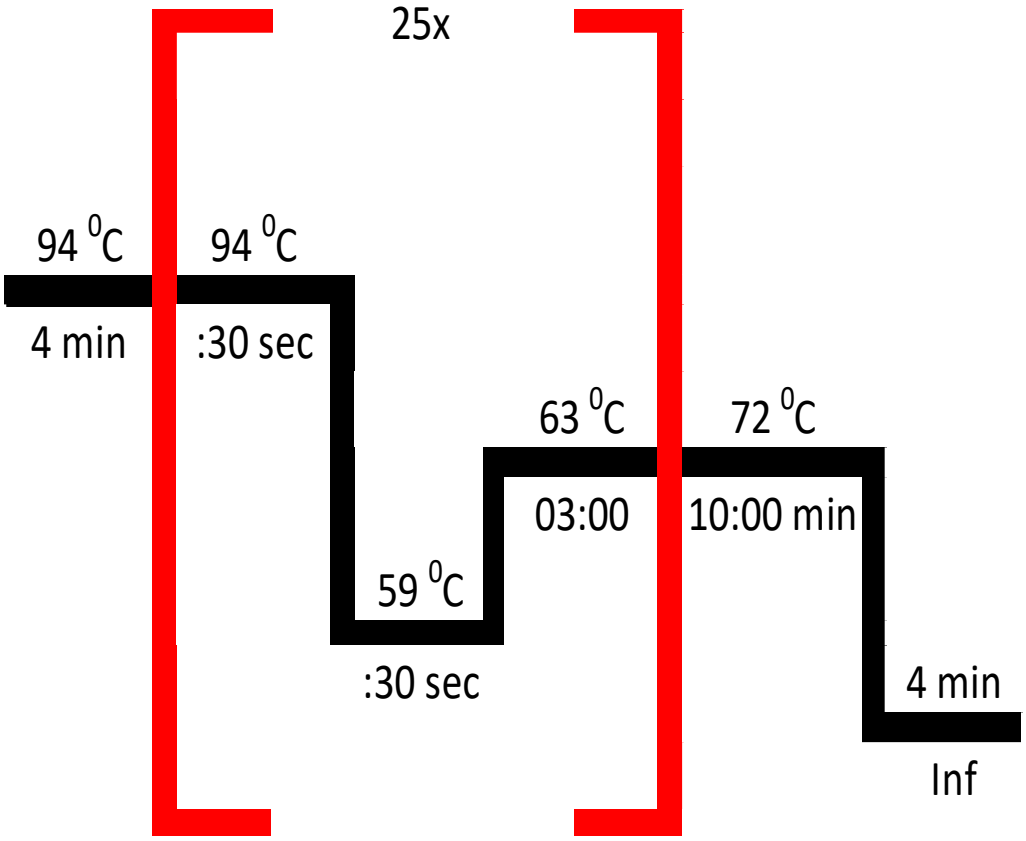

Figure 1: APEC cycler conditions.

[2]

[2] Temperature in centigrade – ^{0}C; Min – minutes ; Sec – Seconds; inf – Infinite hold; 25x – 25 cycles of the indicated temperatures.

Table 16: Primers used for detection and amplicons of virulence - associated genes and the generated amplicon sizes.

Gene	Protein	Primer	Amplicon (bp)
Iss	Increased serum survival	5'-GTGGCGAAAACTAGTAAAACAGC-3' 3'-CGCCTCGGGGTGGATAA-5'	760
iucC	Aerobactin iron sequestering system	5'-CGCCGTGGCTGGGGTAAG-3' 3'-CAGCCGGTTCACCAAGTATCACTG-5'	541
Tsh	Temperature sensitive haemagglutinin	5'-GGGAAATGACCTGAATGCTGG-3' 3'-CCGCTCATCAGTCAGTACCAC-5'	420
cvaC	Colicin ColV operon	5'-GGGCCTCCTACCCTTCACTCTTG-3' 3'-ACGCCCTGAAGCACCACCAGAA-5'	366
irp2	Iron transport	5'-AAGGATTCGCTGTTACCGGAC-3' 3'-TCGTCGGGCAGCGTTTCTTCT-5'	413

(Dozois et al., 1992; Ewers et al., 2005; Horne et al., 2000; Janben et al., 2001; Maurer et al., 1998; Rodriguez-Siek et al., 2005a; Skyberg J. A., 2003)

3.6 Diversity assessment

RAPD PCR was used as a DNA fingerprinting method to determine the diversity of APEC isolates. PCR amplifications were carried out in 25 µl volumes using Ready-to-Go RAPD Beads (Amersham Biosciences, Buckinghamshire, England). Primer 2 (5'd[GTTTCGCTCC]-3') was used for RAPD PCR analysis, and the PCR amplification program was conducted as per manufacturer's protocol. Amplifications products were resolved using QIAxcel Advanced capillary gel electrophoresis system using a DNA screening gel with the following electrophoresis conditions:

Table 17: RAPD gel conditions used for analysis

Method	Cartridge Type
*AM 320	DNA Screening

Method definition

Action	Voltage	Duration	Position
Purge	0.00kV	10 sec	WP
Inject	0.00kV	0 sec	WI
Inject	4.00kV	20 sec	M1
Inject	0.00kV	0 sec	WI
Sample inject	5.00kV	10 sec	None
Inject	0.00kV	0 sec	WI
Inject	0.00kV	0 sec	WI
Inject	0.00kV	0 sec	WI
Inject	0.00kV	0 sec	WI
Inject	0.00kV	0 sec	WI
Inject	0.00kV	0 sec	WI
Separate	6.00kV	320 sec	Buf
Purge	0.00kV	5 sec	WP

[3]

[3] Kv – kilovolt, sec – seconds, WP & WI – Wash buffers, M1 – Marker, Buf – Run buffer

Amplification products were determined using Qiaxcel Bio calculator V3.2 software and according to the manufacturer procedure using marker size 15bp-5000bp (Qiagen, USA). Data were exported to Bionumerics software (Applied Maths Inc., Austin, TX). RAPD profiles were analysed and compared using unweighted pair group method, arithmetic averages, and Dice similarity coefficient.

3.7 Statistical analysis

Data were analyzed with John's Macintosh project (JMP) 15.0 (Statistical analysis software (SAS) Institute, Concentrated Area of Relocated Yankees (CARY), North Carolina (NC)) using one-way analysis of variance (ANOVA) when data was continuous to determine effect of location and operation and year with block (sample type) included as a random effect. When the model was significant, means were separated using a protected Tukey Honestly Significant Difference (HSD) (Tukey, 1949) and significance was defined as $P \leq 0.05$. The categorical dataset was analyzed using a Rao-Scott chi-square (Lavassani et al., 2009). Means were separated using a Pearson pairs test ($P \leq 0.05$).

CHAPTER 4: RESULTS

4.1 Prevalence of APEC in South Africa for the period of 2017 - 2022

A total of 3025 isolates were analysed to establish the prevalence of APEC in South Africa. As indicated in Table 6, the majority of the isolates for the period 2017 to 2022 can be classified as non-pathogenic, with 58.8% (n=1780) regarded as non - APEC and 41.2% (n=1245) as APEC.

Table 18: APEC prevalence in South Africa for the period of 2017 – 2022. (APEC positive depicted as 1, APEC negative depicted as 0).

	Freq	APEC/NO		
	Share	0	1	Total Responses
Location	South Africa	1780	1245	3025
		58.8%	41.2%	

4.1.1 APEC gene percentage in South Africa

When analysing the percentage genes within the South African dataset one can clearly see that a high number of isolates, 40.3% (n=1219), tested negative for all five virulence genes and would therefore be regarded as non - APEC. Only 18.5% (n=561) of the isolates only had one virulence gene detected and, in this case, would form part of the non - APEC group. Isolates with two or more virulence - associated genes (VAGs) were regarded as APEC positive; 17.3% (n=522) had 2, 11.5% (n=348) had 3, 8.9% (n=270) had at least four and only 3.5% (n=105) isolates had five of the virulent associated genes that were targeted by the multiplex PCR.

Table 19: APEC gene percentage in South Africa for the period of 2017- 2022. (0 - 5 indicating number of VAGs).

	Freq	GENE %						
	Share	0	1	2	3	4	5	Total Responses
Location	South Africa	1219	561	522	348	270	105	3025
		40.3%	18.5%	17.3%	11.5%	8.9%	3.5%	

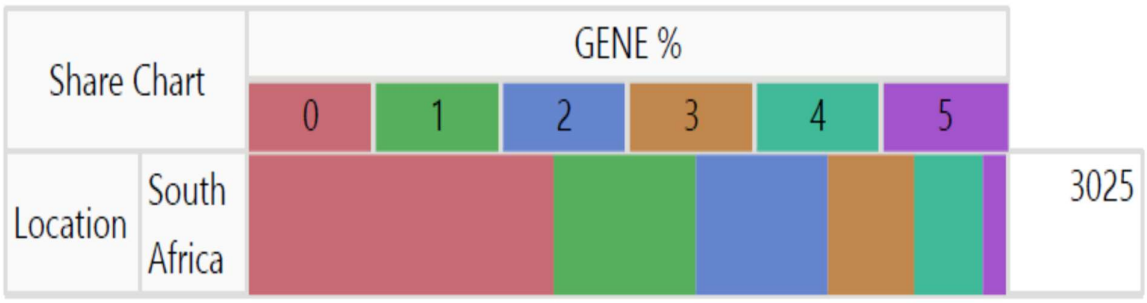

Figure 2: APEC gene percentage in South Africa for the period of 2017-2022. (0 – 5 indicating number of VAGs).

4.1.2 APEC gene expression in South Africa

The virulence genes *cva*C had the highest detection rate with 39.20% (n=1186) of the isolates testing positive for this specific gene. *Tsh* had the lowest detection frequency with only 22.8% (n=691) of the isolates testing positive. VAGs *iss*, *iuc*C and *irp*2 also had low positive detection rates at 25.80% (n=780), 29.50% (n=893) and 23.30% (n=707) respectively.

Table 20: APEC virulence gene (VAG) frequency identified in South Africa for the period of 2017 - 2022. (VAG positive depicted as 1, VAG negative depicted as 0).

Location : South Africa	APEC : Virulence associated genes (VAG)					
Positive/Negative	cvaC	iss	iucC	tsh	irp2	Total isolates
0	1839	2245	2132	2334	2323	3025
	60.80%	74.20%	70.50%	77.20%	76.70%	
1	1186	780	893	691	707	
	39.20%	25.80%	29.50%	22.80%	23.30%	

4.2 APEC in South Africa per operation for the period of 2017 - 2022

As can be seen in Table 9, 64.3% (n=773) group A / Breeder Layer isolates were found to be non - APEC and statistically different to groups C / Broiler, D / DOC, E / Layer Breeder and F / Layer Pullet. A further 35.7% (n=429) of the isolates were APEC positive and statistically higher compared to group B / Breeder Pullets. Group B / Breeder Pullets showed an even stronger shift towards non - APEC, with 80.0% (n=304) non - APEC, statistically significant difference to all other groups. A low number 20.0% (n=76) of APEC positives were noted in this group. The DOC category interestingly showed a completely different picture with 59.8% (n=231) classified as APEC positive, with statistically significant difference to groups A / Breeder Layer, B / Breeder Pullet, C / Broiler and F / Layer Pullet. Broiler isolates collected from birds close to slaughter age again come through strong with 45.7% (n=315) APEC positives, statistically different to groups A / Breeder Layer and B / Breeder Pullet and 54.3% (n=375) non - APEC isolates, statistically different to groups D / DOC and E / Layer Breeder.

On the table egg side of the industry, most of the Layer Breeders isolates were APEC positive with 69.8% (n=37) and statistically different to A / Breeder Layer, B / Breeder Pullet, C / Broiler and F / Layer Pullet. Layer Pullet isolates were equally distributed with 50.0% (n=157) APEC positive, statistically different to groups D / DOC and E / Layer Breeder and 50.0% (n=157) non - APEC, statistically different to groups A / Breeder Layer and B / Breeder Pullet.

Table 21: APEC vs non - APEC in South Africa by operation for the period of 2017 – 2022.

Frequency Share Comparisons			APEC / NO		
			0	1	Total Responses
Operations	Breeder Layer	A	773	429	1202
			64.30%	35.70%	
			C,D,E,F	B	
	Breeder Pullet	B	304	76	380
			80.00%	20%	
			A,C,D,E,F		
	Broiler	C	375	315	690
			54.30%	45.70%	
			D,E	A,B	
	DOC	D	155	231	386
			40.20%	59.80%	
				A,B,C,F	
	Layer Breeder	E	16	37	53
			30.20%	69.80%	
			*	A,B,C,F*	
	Layer Pullet	F	157	157	314
			50.00%	50.00%	
			D,E	A,B	

[4]

[4] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). APEC positive depicted as 1, APEC negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

4.2.1 APEC gene percentage in South Africa per operation

As indicated in Table 10, Group B / Breeder pullet had the highest frequency of non - APEC detections with 64.5% (n=245) and were statistically different to all other groups. Group A / Breeder layers also had a high percentage of non - APEC gene detections with 51.9% (n=624) and were statistically different to all groups except for group B / Breeder pullet. VAGs groups 2, 3 and 5 had statistically higher detection frequencies compared to group B / Breeder Pullet.

Group C / Broilers had the highest frequency (6.2% (n=86)) of isolates testing positive for all five APEC genes and were statistically different to groups A / Breeder Layer and B / Breeder Pullet. Group C / Broilers also then had the highest number of isolates (22.2% (n=307)) with four or more VAGs and were statistically different to all other groups. This group was similar to group E / Layer Breeder and had a higher percentage of isolates that tested positive for 3 and more VAGs and were significantly different to other groups. Group C / Broiler isolates (22.3% (n=309)) had two or more VAGs and were statistically different to groups A / Breeder Layer and B / Breeder Pullet.

Group D / DOC (11.9% (n=46)) like group F / Layer Pullet (12.4% (n=39)) had a high percentage of isolates testing positive for 4 or more of the VAGs and were statistically different to groups A / Breeder Layer, B / Breeder Pullet and E / Layer Breeder. Group D / DOC had 30.6% (n=118) isolates that tested positive for two or more of the VAGs and were significantly different to groups A / Breeder Layer, B / Breeder Pullet, C / Broiler and F / Layer Pullet. Group D / DOC VAG groups 3 and 5 were statistically different to group B / Breeder Pullet with 13% (n=50) and 4.4% (n=17) detections compared to 7.1% (n=27) and 1.8% (n=7) respectively. Group D / DOC (22.5% (n=87)) had 0 positive APEC genes and were statistically different to group C / Broiler and E / Layer Breeder. This group had 17.6% (n=68) isolates with at least one VAG and were statistically different to group A / Breeder Layer and C / Broiler.

Group E / Layer Breeder had the highest percentage of isolates that tested positive for gene percentage groups with 2 and 3 or more VAGs and were significantly different to groups A / Breeder Layer, B / Breeder Pullet, C / Broiler and F / Layer Pullet.

Group F / Layer Pullet had 30.9% (n=97) of the isolates that tested positive for one or more of the VAGs and was found to be statistically different to all groups except group E / Layer Breeder. This group had 20.7% (n=65) of isolates that were positive for two or more VAGs and were statistically different to groups A / Breeder Layer and B / Breeder Pullet. It was also found that 12.7% (n=40) of isolates had three or more VAGs and were statistically different to group B / Breeder Pullet. Group F / Layer Pullet had 12.4% (n=39) of the isolates that contained 4 or more VAGs and was statistically different to groups A / Breeder Layer, B / Breeder Pullet and E / Layer Breeder. This group then also had 19.1% (n=60) of the isolates with 0 VAG and was found to be statistically different to group C / Broiler.

Table 22: APEC gene percentage in South Africa per operation for the period of 2017 – 2022.

Frequency Share Comparisons			GENE %						
			0	1	2	3	4	5	Total Responses
Operations	Breeder Layer	A	624	149	179	131	64	55	1202
			51.90%	12.40%	14.90%	10.90%	5.30%	4.60%	
			C,D,E,F		B	B		B	
	Breeder Pullet	B	245	59	25	27	17	7	380
			64.50%	15.50%	6.60%	7.10%	4.50%	1.80%	
			A,C,D,E,F						
	Broiler	C	198	180	309	306	307	86	1386
			14.30%	13.00%	22.30%	22.10%	22.20%	6.20%	
					A,B	A,B,D,F	A,B,D,E,F	a,B	
	DOC	D	87	68	118	50	46	17	386
			22.50%	17.60%	30.60%	13.00%	11.90%	4.40%	
			C,e	A,C	A,B,C,F	B	A,B,E	b	
	Layer Breeder	E	6	10	21	15	0	1	53
			11.30%	18.90%	39.60%	28.30%	0.00%	1.90%	
			*	*	A,B,C,F*	A,B,D,F*	*	*	
	Layer Pullet	F	60	97	65	40	39	13	314
			19.10%	30.90%	20.70%	12.70%	12.40%	4.10%	
			C	A,B,C,D	A,B	B	A,B,E		

[5]

[5] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). Number of positive VAGs indicated as 0 – 5. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

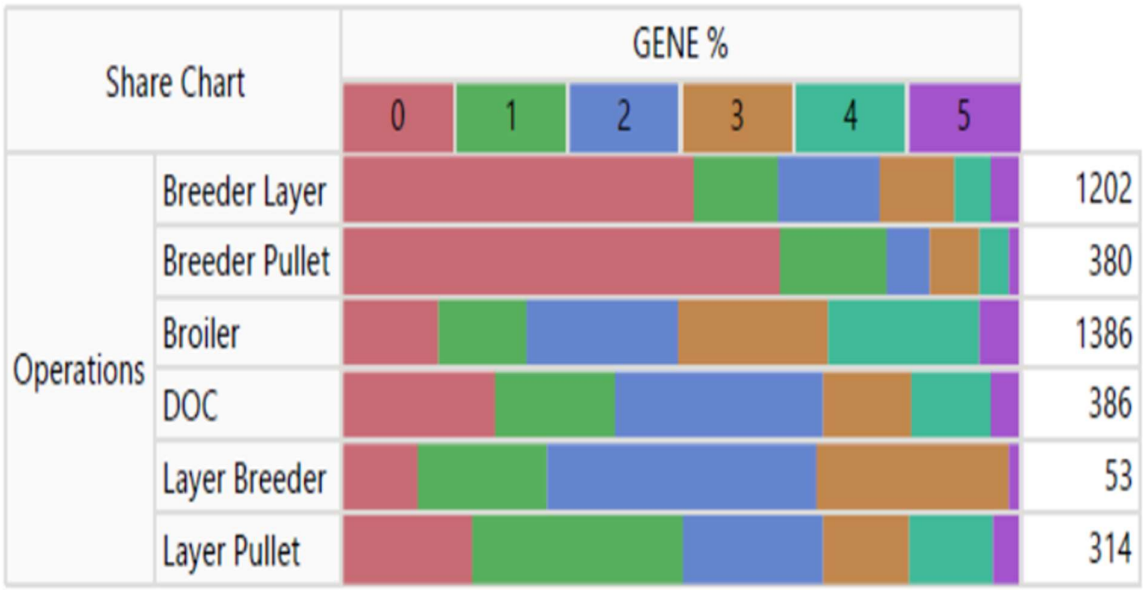

Figure 3: APEC gene percentage in South Africa per operation for the period of 2017 - 2022. Number of positive VAGs indicated as 0 – 5.

As per Table 11 the percentage positive APEC genes for the Breeder Pullet group was significantly different to all other groups. The Breeder layer group was seen to be significantly different to all groups except for the Broiler group. No significant difference was detected between DOC and Layer Breeder groups as well as between Layer Breeder and Layer Pullet groups.

Table 23: APEC gene percentage in South Africa per operation for the period of 2017 – 2022. (Operations not connected by the same letter are significantly different)

Operation	Letter indicating significant difference	Least Sq Mean
Breeder layer	C, D	6.0039203
Breeder Pullet	E	5.4333789
Broiler	D	5.209809
DOC	A	4.6275407
Layer Breeder	A, B	4.3325827
Layer Pullet	B	3.5958231

4.2.2 APEC gene expression per operation in South Africa

As per Table 12(a), groups E / Layer Breeder (47.2% (n=25)) and F / Layer Pullet (47.1% (n=148)) had statistically higher *tsh* VAG positive detections compared to other groups. Groups A / Breeder Layer and B / Breeder Pullet in contrast had significantly less positive *tsh* detections with 84.9% (n=1021) and 86.8% (n=330) testing negative. These two groups were statistically different in terms of negative detection rates

compared to the rest of the groups. Group C / Broiler also had a majority testing negative (70% (n=483)) for the *tsh* VAG, statistically different to groups E / Layer Breeder and F / Layer Pullet. This group had a 30% positive testing frequency, statistically different to groups A / Breeder Layer, B /Breeder Pullet and D / DOC. Group D / DOC saw an increase in negative detections at 79.3% (n=300), statistically different to groups C / Broiler, E / Layer Breeder and F / Layer Pullet. This group had 20.7% (n=80) positive detections of the *tsh* gene and were statistically different to groups A / Breeder Layer & B / Breeder Pullet.

Group E / Layer Breeder, statistically, had the highest detection rate of VAG *cva*C according to table 12(b). This group had 81.1% (n=43) positives, significantly higher than all other groups. Group D / DOC had the second highest detection of the *cva*C gene with 60.4% (n=233), statistically different to groups A / Breeder Layer, B / Breeder Pullet, C / Broiler and F /Layer Pullet. The negative detections (39.6% (n=153)) were statistically higher than group E / Layer Breeder. Group B / Breeder Pullet had the least amount of positive *cva*C gene detections. The number of negative detections in this group was statistically different to all other groups. Group A / Breeder Layers also had a significantly high number (64.2% (n=772) negative detections, statistically different to C / Broiler, D / DOC, E / Layer Breeder and F / Layer Pullet. Group A / Breeders Layers had a statistically higher number of positive detections (35.8% (n=430)) in comparison to Group B / Breeder Pullets with 15.3% (n=58). Group C / Broiler positives (58.7% (n=405)) were statistically higher than group A / Breeder Layers and B / Breeder Pullets. The negative detection rate (41.3% (n=205)) was statistically different to groups D / DOC and E / Layer Breeder.

Group E / Layer Breeder had the highest detection (41.5% (n=22)) of *Irp*2 VAG, statistically different to all other groups. Group D / DOC had the highest negative detection rate at 86.8% (n=335), statistically different from all other groups. Group C / Broiler had the second highest detection rate at 30.0% (n=207), statistically different to groups A / Breeder Layer, B / Breeder Pullet and D / DOC. The negatives in this group was statistically different from group E / Layer Breeders. Groups A / Breeder Layer and B / Breeder Pullet had high number of negatives, with 78.5% (n=943) and

78.9% (n=300) respectively and were significantly different to groups C / Broiler, E / Layer Breeder and F / Layer Pullet. The positive detections for group A / Breeder Layer (21.5% (n=259)) and Breeder Pullet (21.1% (n=80)) were statistically different to group D / DOC.

Group D / DOC had the highest positive detection rate for *iuc*C VAG at 49.7% (n=192), significantly different to all other groups. Group F / Layer Pullet also had a high detection rate with 41.7% (n=131) positives, significantly higher than groups A / Breeder Layer, B / Breeder Pullet, C / Broiler and E / Layer Breeder. Group B / Breeder Pullets had the highest frequency of negatives at 83.4% (n=317), significantly different to groups A / Breeder Layer, C / Broiler, D / DOC and F / Layer Pullet. Group A / Breeder Layers also had a high negative detection rate with 77.9% (n=936), significantly different to groups C / Broiler, D / DOC and F / Layer Pullet. This group had significantly higher detection of *iuc*C positives compared to group B / Breeder Pullet. Group C / Broilers also had a high detection rate with 33.6% (n=232), significantly different to groups A / Breeder Layer, B / Breeder Pullet and E / Layer Breeder. The negative detection for this group (66.4% (n=458)) were significantly different to groups D / DOC and F / Layer Pullet.

Group D / DOC had the highest detection of *iss* VAG at 43.3% (n=167) and were significantly different to all groups. Group C / Broilers had the second highest detection rate at 29.9% (n=206) and is statistically different from groups A / Breeder Layer, B / Breeder Pullet, E / Layer Breeder and F / Breeder Pullet. This group had 70.1% (n=484) negative detections, significantly higher than group D / DOC. Group E / Layer Breeder had the highest negative detection rate with 94.3% (n=50), statistically different to groups A / Breeder Layer, C / Broiler, D / DOC and F / Layer Pullet. Group B had the second highest negative detection for the *iss* gene, statistically different to groups A / Breeder Layer, C / Broiler, D / DOC and F / Layer Pullet. Group A / Breeder Layer had a slightly higher positive detection rate with 24.5% (n=295), statistically different to group B / Breeder Pullet and E / Layer Breeder. The negative detections (75.5% (n=907)) for this group were statistically different to group C / Broiler and D / DOC.

Table 24(a): APEC gene expression for virulence - associated gene *tsh* in South Africa per operation for the period of 2017 – 2022.

Frequency Share Comparisons			tsh		
			0	1	Total Responses
Operations	Breeder Layer	A	1021	181	1202
			84.90%	15.10%	
			C,D,E,F		
	Breeder Pullet	B	330	50	380
			86.80%	13.20%	
			C,D,E,F		
	Broiler	C	483	207	690
			70.00%	30.00%	
			E,F	A,B,D	
	DOC	D	306	80	386
			79.30%	20.70%	
			C,E,F	A,B	
	Layer Breeder	E	28	25	53
			52.80%	47.20%	
			*	A,B,C,D*	
	Layer Pullet	F	166	148	314
			52.90%	47.10%	
				A,B,C,D	

[6]

[6] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 12(b): APEC gene expression for virulence - associated gene *cva*C in South Africa per operation for the period of 2017 – 2022.

Frequency Share Comparisons			cvaC		
			0	1	Total Responses
Operations	Breeder Layer	A	772	430	1202
			64.20%	35.80%	
			C,D,E,F		
	Breeder Pullet	B	322	58	380
			84.70%	15.30%	
			A,C,D,E,F		
	Broiler	C	405	285	690
			58.70%	41.30%	
			D,E	A,B	
	DOC	D	153	233	386
			39.60%	60.40%	
			E,F	A,B,C,F	
	Layer Breeder	E	10	43	53
			18.90%	81.10%	
			*	A,B,C,D,F *	
	Layer Pullet	F	177	137	314
			56.40%	43.60%	
			D,E	A,B	

[7]

[7] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 12(c): APEC gene expression for virulence - associated gene *irp*2 in South Africa per operation for the period of 2017 – 2022.

Frequency Share Comparisons			irp2		
			0	1	Total Responses
Operations	Breeder Layer	A	943	259	1202
			78.50%	21.50%	
			C,E,F	D	
	Breeder Pullet	B	300	80	380
			78.90%	21.10%	
			C,E,f	D	
	Broiler	C	483	207	690
			70.00%	30.00%	
			e	A,B,D	
	DOC	D	335	51	386
			86.80%	13.20%	
			A,B,C,E,F		
	Layer Breeder	E	31	22	53
			58.50%	41.50%	
			*	A,B,C,D*	
	Layer Pullet	F	229	85	314
			72.90%	27.10%	
			E	A,b,D	

8

[8] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 12(d): APEC gene expression for virulence - associated gene *iuc*C in South Africa per operation for the period of 2017 – 2022.

Frequency Share Comparisons			iucC		
			0	1	Total Responses
Operations	Breeder Layer	A	936	226	1202
			77.90%	22.10%	
			C,D,F	B	
	Breeder Pullet	B	317	63	380
			83.40%	16.60%	
			A,C,D,F		
	Broiler	C	458	232	690
			66.40%	33.60%	
			D,F	A,B,E	
	DOC	D	194	192	386
			50.30%	49.70%	
				A,B,C,E,F	
	Layer Breeder	E	44	9	53
			83.00%	17.00%	
			C,D,F*	*	
	Layer Pullet	F	183	131	314
			58.30%	41.70%	
			D	A,B,C,E	

9

[9] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 12(e): APEC gene expression for virulence - associated gene *iss* in South Africa per operation for the period of 2017 – 2022.

Frequency Share Comparisons			iss		
			0	1	Total Responses
Operations	Breeder Layer	A	907	295	1202
			75.50%	24.50%	
			C,D,	B,E	
	Breeder Pullet	B	338	42	380
			88.90%	11.10%	
			A,C,D,F		
	Broiler	C	484	206	690
			70.10%	29.90%	
			D	A,B,E,F	
	DOC	D	219	167	386
			56.70%	43.30%	
				A,B,C,E,F	
	Layer Breeder	E	50	3	53
			94.30%	5.70%	
			A,C,D,F*	*	
	Layer Pullet	F	247	67	314
			78.70%	21.30%	
			C,D	B,E	

10

[10] *Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

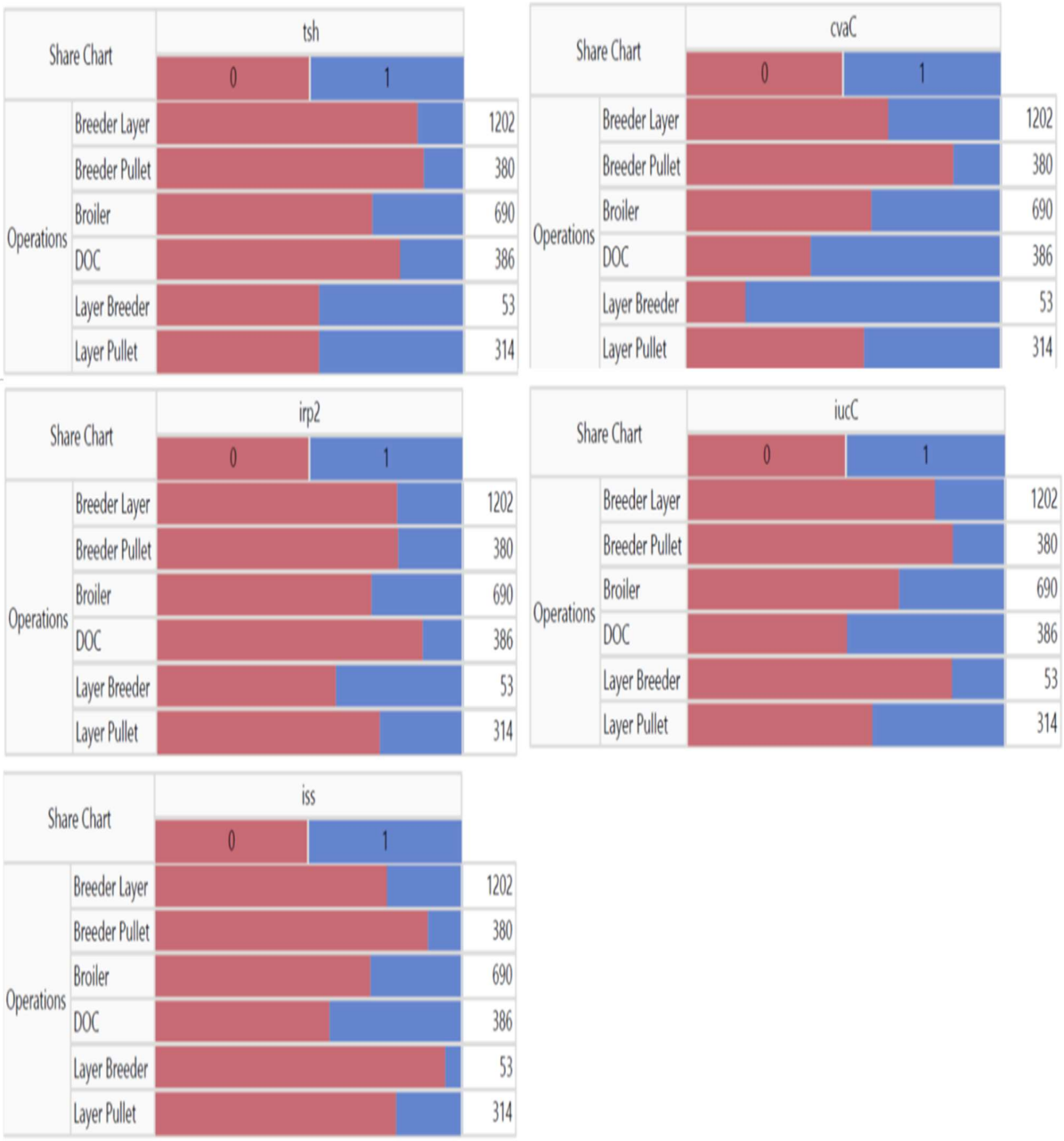

Figure 4: APEC gene expression for virulence - associated genes; tsh, cvaC, irp2, iucC and iss in South Africa per operation for the period of 2017 – 2022: Breeder Layer (Broiler breeder), Breeder Pullet (Broiler breeder rearing), Broiler, Broiler day old chick (DOC), Layer Breeder (Layer parent) and Layer Pullet (Layer rearing). VAG positive depicted as 1, VAG negative depicted as 0.

4.3 APEC prevalence in South Africa over time for the period of 2017 - 2022

As recorded in Table 13, a high percentage of APECs were isolated in 2017 with 70.0% (n=56) positives found. The high number of APEC positive in this year is statistically different to the number of APEC positive found in groups B – F, representing year 2018-2022. The years 2018 and 2019 saw a substantial reduction in percentage positive isolates with 41.1% (n=78) and 51.7% (n=298) respectively. APEC positive isolates in 2018 were found to be statistically higher in comparison to the year 2020, which recorded a mark decrease in APEC positive (22.6% (n=26)) in comparison to 2019. The percentage of non - APEC for the same year was seen to be statistically higher compared to groups A / 2017 and C / 2019. Group C / 2019 APEC positive isolates again were statistically higher compared to groups B, D, E and F. The subsequent years of 2020 - 2022 again showed a more favourable move towards a non - APEC majority. A strong shift back towards non-pathogenic was recorded in 2020 with 60.9% (n=70) of the isolates classified as non - APEC. This specific year had statistically less APEC positive isolates in comparison to all other groups (A, B, C, E & F). A slight shift back to the centre was recorded in 2021 with a variance of 16.1%. Group E / 2021 positive APEC percentage was statistically higher than group D / 2020. non - APEC isolations in 2021 were statistically higher than groups A / 2017 and C /2019. Group F / 2022 showed a similar picture with 37.8% pathogenic isolates. A statistically significant difference was detected compared to group D in terms of positive isolates and again significantly different for non - APEC isolates in comparison to groups A / 2017 and C / 2019.

Table 13: APEC prevalence in South Africa over time for the period of 2017 – 2022.

Frequency Share Comparisons			APEC / NO		
			1	FALSE	Total Responses
Year	2017	A	56	24	80
			70.00%	30.00%	
			B,C,D,E,F*	*	
	2018	B	78	112	190
			41.10%	58.90%	
			D	A,C	
	2019	C	293	274	567
			51.70%	48.30%	
			B,D,E,F	A	
	2020	D	26	89	115
			22.60%	77.40%	
				A,B,C,E,F	
	2021	E	360	570	930
			38.70%	61.30%	
			D	A,C	
	2022	F	432	711	1143
			37.80%	62.20%	
			D	A,C	

[11]

[11] *APEC positive depicted as 1, APEC negative depicted as False. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

4.3.1 APEC gene percentage in South Africa over time

Group A / 2017 as indicated in Table 14 had a statistically higher percentage APEC positives in comparison to the years 2018 - 2022. In comparison to groups C / 2019, D / 2020, E / 2021, F / 2022, group A / 2017 also had statistically more isolates (20.0%(n=16)), classified with three VAGs. Groups D / 2020, E/ 2021 & F / 2022 like group A /2017 also had statistically higher isolates classified with at least one VAG. Group B / 2018 and group D / 2020 had statistically higher percentage zero VAGs isolated in comparison to groups A / 2017, C / 2019, E / 2021 and F / 2022 with 54,2% (n=103) and 56.5% (n=65) isolates testing negative respectively. Group C / 2019 in comparison to groups A / 2017, B / 2018, D / 2020, E / 2021 and F / 2022, had statistically more isolates testing positive for all five VAGs with 9.7% (n=55) of the isolates testing positive. This group, as with group E / 2021 also had the highest percentage isolates classified with four VAGs at 11.1% (n=63) and 11.6% (n=108) respectfully. Group F / 2022 had the second highest percentage of isolates after group C / 2019 statistically higher than groups D / 2020 and E / 2021 with 3.2% (n=37) testing positive for all five VAGs. This group in comparison to groups B / 2018 and D /2020, and like group C / 2019 had statistically more isolates test positive for two of the VAGs.

Table 14: APEC percentage gene positive (# gene 1-5) in South Africa over time for the period of 2017 - 2022.

Frequency / Share / Comparisons			GENE %						
			0	1	2	3	4	5	Total Responses
Year	2017	A	6	18	36	16	3	1	80
			51.90%	12.40%	14.90%	10.90%	5.30%	4.60%	
			*	B,C*	B,C,D,E,F*	c,d,E,F*	*	*	
	2018	B	103	9	23	33	18	4	190
			54.20%	4.70%	12.10%	17.40%	9.50%	2.10%	
			A,C,E,F			d,E,F			
	2019	C	233	41	103	72	63	55	567
			41.10%	7.20%	18.20%	12.70%	11.10%	9.70%	
			A		b,D		A,D,F	A,B,D,E,F	
	2020	D	65	24	10	11	5	0	115
			56.50%	20.90%	8.70%	9.60%	4.30%	0.00%	
			A,C,E,F	B,C					
	2021	E	373	197	147	97	108	8	930
			40.10%	21.20%	15.80%	10.40%	11.60%	0.90%	
			A	B,C	d		A,D,F		
	2022	F	439	272	203	119	73	37	1143
			38.40%	23.80%	17.80%	10.40%	6.40%	3.20%	
			A	B,C	b,D			D,E	

12

[12] *Number of positive VAGs indicated as 0 – 5. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

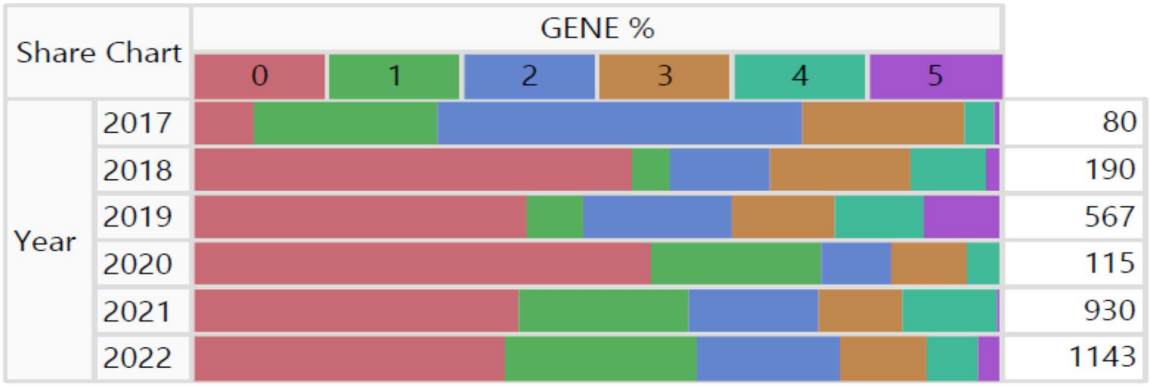

Figure 5: APEC gene percentage in South Africa over time for the period of 2017 - 2022. Number of positive VAGs indicated as 0 – 5.

4.3.2 APEC gene expression in South Africa over time

Gene expression for *tsh* VAG as per Table 15(a), were statistically lower in group A / 2017 in comparison to all other groups analysed, with 98% (n=79) negative detections for the specific gene. Groups B / 2018, C / 2019, D / 2020, E / 2021 and F / 2022 had statistically higher *tsh* VAG positive detections in comparison to group A / 2017. Groups B / 2018, C / 2019, D / 2020 on its part had statistically lower *tsh* positive detections in comparison to groups E / 2021 and F / 2022. Groups E / 2021 and F / 2022 had statistically higher *tsh* positives recorded at 25.2% (n=234) and 26.2% (n=299) in comparison to the rest of the groups.

Table 15(b) indicates that 52.5% (n=42) *cva*C VAG were detected in group A / 2017 and is seen to be significantly different to group B / 2018, D / 2020 and F / 2022. Groups C / 2019 and E / 2021 also saw high detection rates at 44.1% (n=250) and 44.5% (n=414) respectively and were significantly higher than groups D / 2020 and F / 2022. Groups B / 2018, D / 2020 and F / 2022 had significantly less *cva*C VAGs detected in comparison to group A / 2017. Group B / 2018 had 38.4% (n=73) *cva*C positive reactions and was significantly higher than group F / 2022. Group F / 2022 had 67.7% (n=774) of the isolates that were negative for *cva*C and was seen to be

significantly lower in comparison to all other groups except group D / 2020, which on its own was also significantly lower than groups A / 2017, C / 2019 and E / 2021.

A high frequency of i*rp*2 positive VAG (48.5% (n=39)) was detected in group A / 2017 with statistically significant difference to all other groups. Group F / 2022 had the second highest *irp*2 positive isolate frequency, statistically higher than groups B / 2018, C / 2019, D / 2020, E / 2021 followed by group C / 2019 which in turn was statistically higher than B / 2018, D / 2020 and E / 2021. Groups B / 2018 and E / 2021 was significantly higher than group D / 2020. Group D recorded the lowest frequency with 96.5% (n=111) isolates negative for *irp*2 VAG which is statistically lower than all other groups.

VAG *iuc*C detection were statistically lower in group D / 2020 in comparison to all other groups, with 93.9% (n=108) of the isolates testing negative. Group F / 2022 had the second lowest positive *iuc*C detections, statistically lower than groups A / 2017, C / 2019 and E / 2021. Groups B / 2018 and E / 2021 were statistically lower than groups A / 2017 and C / 2019. Groups A / 2017 and Group D / 2020 again had a statistically higher frequency that was detected compared to other groups, with 42.5% (n=34) and 43.2% (n=245) isolates testing positive for the VAG *iuc*C. Groups B / 2018 and F / 2022 had a statistically higher *iuc*C detection compared to group D / 2020. Group E / 2021, as with groups B / 2018 and F / 2022 had a statistically higher detection compared to group D / 2020. Group E / 2021 also had a statistically higher positive frequency of iucC VAG compared to group F / 2022.

VAG *iss* detection frequency for group A / 2017 was the highest at 48% (n=39) and group B / 2018 at 37.4% (n=71) positive were statistically higher than groups D / 2020, E / 2021 and F / 2022. Group C / 2019 with 44.8% (n=254) positive detections were statistically different to groups B / 2018, D / 2020, E / 2021 and F / 2022. Group F / 2022 had the least number of positive detections with 14.9% (n=170). This group had 85.1% (n=973) negative detections which is regarded as statistically different to all other groups evaluated. Group D and E had very low levels of *iss* positives, however still statistically higher than group F / 2022. These two groups with 74.8% (n=86) and

76.7% (n=713) negative detections respectively were statistically different to groups A / 2017, B / 2018 and C / 2019. Group B / 2018 also had a statistically lower detection of *iss* compared to group C / 2019.

Table 15(a): APEC gene expression for *tsh* in South Africa over time for the period of 2017 - 2022.

Frequency Share Comparisons			tsh		
			0	1	Total Responses
Year	2017	A	79	1	80
			98.80%	1.30%	
			B,C,D,E,F*	*	
	2018	B	161	29	190
			84.70%	15.30%	
			E,F	A	
	2019	C	458	109	567
			80.80%	19.20%	
			E,F	A	
	2020	D	96	19	115
			83.50%	16.50%	
			E,F	A	
	2021	E	696	234	930
			74.80%	25.20%	
				A,B,C,D	
	2022	F	844	299	1143
			73.80%	26.20%	
				A,B,C,D	

13

[13] VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference ($P < 0.05$) shown as uppercase alpha level, statistical difference ($P < 0.1$) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.

Table 15(b): APEC gene expression for *cva*C in South Africa over time for the period of 2017 - 2022.

Frequency Share Comparisons			cvaC		
			0	1	Total Responses
Year	2017	A	38	42	80
			47.50%	52.50%	
			*	B,D,F*	
	2018	B	117	73	190
			61.60%	38.40%	
			A	f	
	2019	C	317	250	567
			55.90%	44.10%	
				D,F	
	2020	D	77	38	115
			67.00%	33.00%	
			A,C,E		
	2021	E	516	414	930
			55.50%	44.50%	
				D,F	
	2022	F	774	369	1143
			67.70%	32.30%	
			A,b,C,E		

14

[14] *VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 15(c): APEC gene expression for *irp*2 in South Africa over time for the period of 2017 - 2022.

Frequency Share Comparisons			irp2		
			0	1	Total Responses
Year	2017	A	41	39	80
			51.30%	48.80%	
			*	B,C,D,E,F*	
	2018	B	170	20	190
			89.50%	10.50%	
			A,C,F	D	
	2019	C	435	132	567
			76.70%	23.30%	
			A,F	B,D,E	
	2020	D	111	4	115
			96.50%	3.50%	
			A,B,C,E,F		
	2021	E	807	123	930
			86.80%	13.20%	
			A,C,F	D	
	2022	F	757	386	1143
			66.20%	33.80%	
			A	B,C,D,E	

15

[15] *VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 15(d): APEC gene expression for *iuc*C in South Africa over time for the period of 2017 - 2022.

Frequency Share Comparisons			iucC		
			0	1	Total Responses
Year	2017	A	46	34	80
			84.90%	15.10%	
			*	B,D,E,F*	
	2018	B	137	53	190
			72.10%	27.90%	
			A,C	D	
	2019	C	322	245	567
			56.80%	43.20%	
				B,D,E,F	
	2020	D	108	7	115
			93.90%	6.10%	
			A,B,C,E,F		
	2021	E	664	266	930
			71.40%	28.60%	
			A,C	D,F	
	2022	F	855	288	1143
			74.80%	25.20%	
			A,C,e	D	

16

[16] *VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 15(e): APEC gene expression for *iss* in South Africa over time for the period of 2017 - 2022.

Frequency Share Comparisons			iss		
			0	1	Total Responses
Year	2017	A	41	39	80
			51.30%	48.80%	
			*	D,E,F*	
	2018	B	119	71	190
			62.60%	37.40%	
			c	D,E,F	
	2019	C	313	254	567
			55.20%	44.80%	
				b,D,E,F	
	2020	D	86	29	115
			74.80%	25.20%	
			A,B,C	F	
	2021	E	713	217	930
			76.70%	23.30%	
			A,B,C	F	
	2022	F	973	170	1143
			85.10%	47.10%	
			A,B,C,D,E		

17

[17] *VAG positive depicted as 1, VAG negative depicted as 0. Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

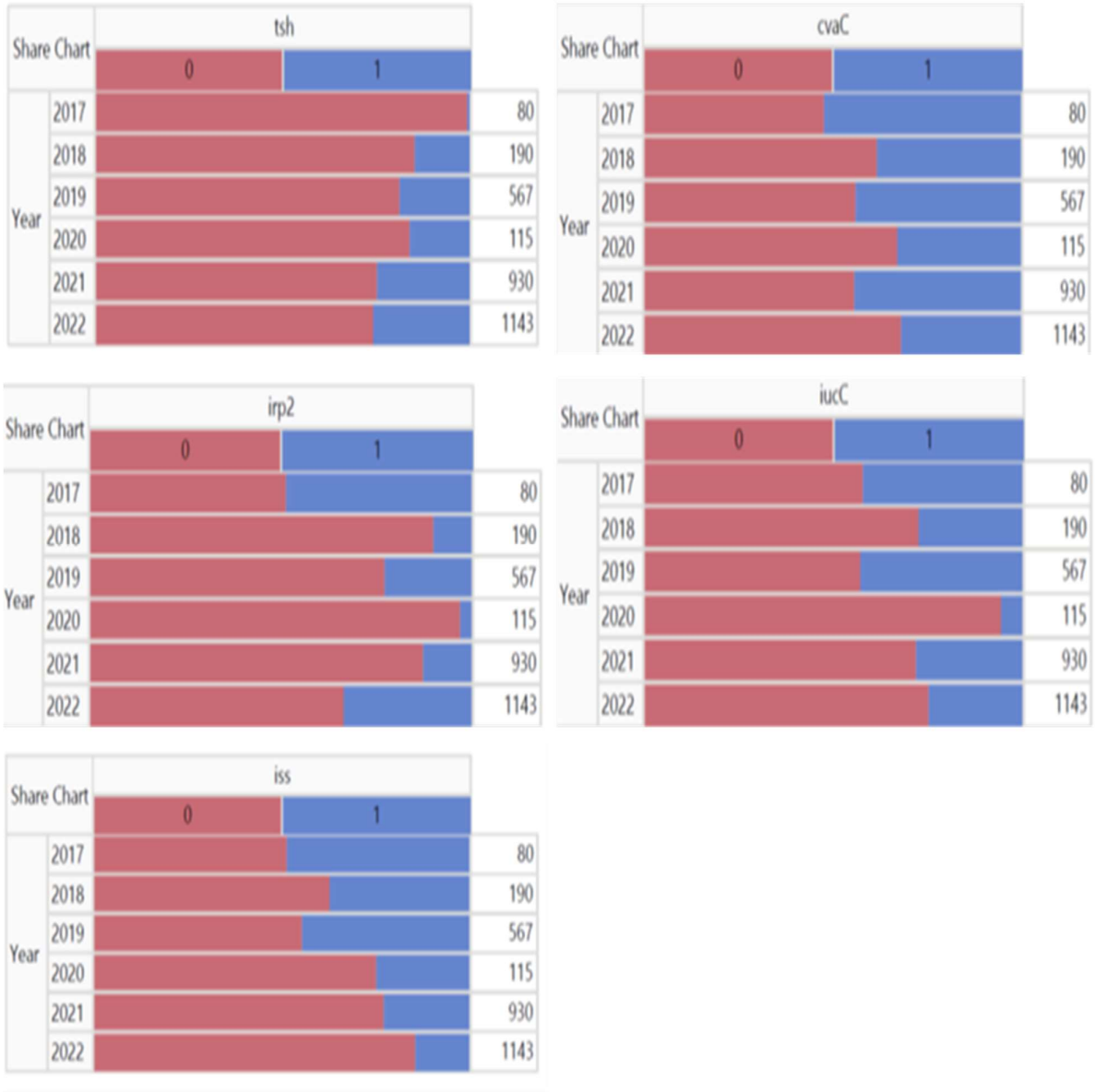

Figure 6: APEC gene expression for tsh, cvaC, irp2, iucC and iss in South Africa over time for the period of 2017-2022. VAG positive depicted as 1, VAG negative depicted as 0.

4.4 APEC prevalence in South Africa (2018 – 2019, 2022) versus United States of America (2016)

4.4.1 APEC gene percentage SA versus USA

A total 983 *E coli* isolates were analysed to determine the difference of APEC gene percentage in SA and USA. Two hundred and nine isolates from SA were compared to 774 isolates from USA.

As shown in Table 16, most South African APEC isolates (57 / 27.3%) had four virulence associate genes (VAGs). Most of the USA isolates had three or more VAGs with a frequency of 32.4% (n=251). Groups with zero and one VAGs were statistically different between SA and the USA. SA had 7.2% (n=15) of the isolates where none of the five VAGs were detected, in comparison to the USA with 0.1% (n=1) of isolates. SA had 9.6% (n=20) isolates that tested positive for at least one of the VAGs in comparison to 2 / 0.3% of the USA isolates. VAG groups two and three were also statistically different between SA and USA. SA had 22.0% (n=46) isolates positive for two or more VAGs in comparison to 28.7% (n=222) for the USA. SA had 24.9% (n=52) isolates in comparison to 32.4% (n=251) USA isolates that had three or more VAGs.

Table 16: APEC gene percentage - SA (2018 -2019, 2022) compared to the USA (2016).

Frequency / Share / Comparisons		GENE %						
		0	1	2	3	4	5	Total Responses
Location	SA	15	20	46	52	57	19	209
		7.20%	9.60%	22.00%	24.90%	27.30%	9.10%	
		B	B					
	USA	1	2	222	251	217	81	774
		0.10%	0.30%	28.70%	32.40%	28.00%	10.50%	
				a	A			

18

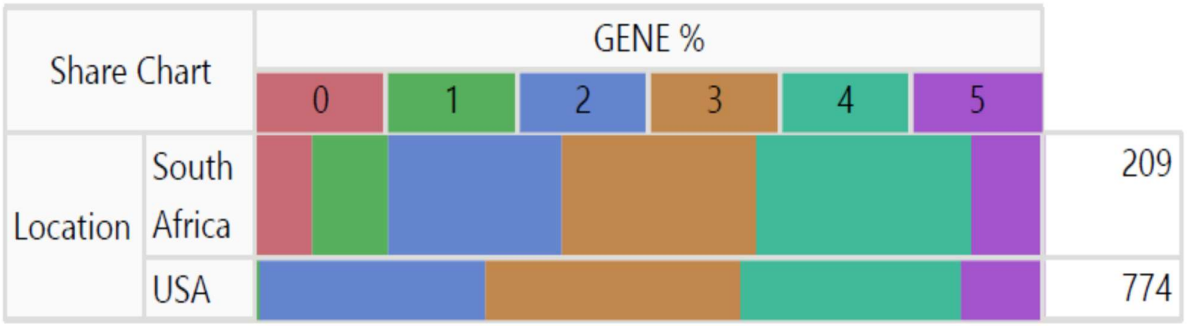

Figure 7: APEC gene percentage South Africa (2018 – 2019, 2022) vs USA (2016). Number of positive VAGs indicated as 0 – 5.

[18] Number of positive VAGs indicated as 0 – 5. Letter depicted in a category indicates significant difference from that group. Statistical difference ($P < 0.05$) shown as uppercase alpha level, statistical difference ($P < 0.1$) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.

4.4.2 APEC gene expression South Africa vs United States of America

The highest frequency as per Table 17(a) of *tsh* VAG were detected in the group B / USA with 74.2% (n=574) of the isolates testing positive. The two groups were found to be statistically different, with 59.8% (n=125) of group A / South Africa testing negative for *tsh.* *The* USA had 81.4% (n=630) of the isolates that tested positive for the VAG *cva*C in comparison to the 76.1% (n=159) South African isolates. Both positive and negative groups approached statistical significance when compared. No significant difference was noted between SA and USA isolates comparing *irp*2 gene positivity. Equal distribution between positive and negative detection was noted. No statistical difference between SA and USA isolates were noted for the VAG *iuc*C. The USA however had the highest number of positive detections with 63.3% (n=490). Statistical difference was noted between groups for *iss* VAG, with South African isolates having the highest positive rate at 56.9% (n=119).

Table 17(a): APEC gene expression for VAG *tsh* – South Africa (2018 – 2019, 2022) versus USA (2016). VAG positive depicted as 1, VAG negative depicted as 0.

Frequency Share Comparisons			tsh		
			0	1	Total Responses
Location	SA	A	125	84	209
			59.80%	40.20%	
			B		
	USA	B	200	574	774
			25.80%	74.20%	
				A	

19

[19] *Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 17 (b): APEC gene expression for VAG *cva*C – South Africa (2018 – 2019, 2022) versus USA (2016). VAG positive depicted as 1, VAG negative depicted as 0.

Frequency Share Comparisons			cvaC		
			0	1	Total Responses
Location	SA	A	50	159	209
			23.90%	76.10%	
			b		
	USA	B	144	630	774
			18.60%	81.40%	
				a	

20

[20] *Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 17(c): APEC gene expression for VAG *irp*2 – South Africa (2018 – 2019, 2022) versus USA (2016). VAG positive depicted as 1, VAG negative depicted as 0.

Frequency Share Comparisons			irp2		
			0	1	Total Responses
Location	SA	A	104	105	209
			49.80%	50.20%	
	USA	B	361	413	774
			46.60%	53.40%	

[21]

[21] *Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 17(d): APEC gene expression for VAG *iuc*C – South Africa (2018 – 2019, 2022) versus USA (2016). VAG positive depicted as 1, VAG negative depicted as 0.

Frequency Share Comparisons			iucC		
			0	1	Total Responses
Location	SA	A	85	124	209
			40.70%	59.30%	
	USA	B	284	490	774
			36.70%	63.30%	

22

[22] *Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Table 17(e): APEC gene expression for VAG *iss* – South Africa (2018 – 2019, 2022) versus USA (2016). VAG positive depicted as 1, VAG negative depicted as 0.

Frequency Share Comparisons			iss		
			0	1	Total Responses
Location	SA	A	90	119	209
			43.10%	56.90%	
	USA	B	409	365	774
			52.80%	47.20%	

23

[23] *Letter depicted in a category indicates significant difference from that group. Statistical difference (P < 0.05) shown as uppercase alpha level, statistical difference (P < 0.1) shown as lower case alpha level. Base count warning (100) indicated as *, Base count minimum (30) indicated as **.*

Figure 8: APEC gene expression for tsh, cvaC, irp2, iucC and iss – South Africa (2018 – 2019, 2022) vs USA (2016). VAG positive depicted as 1, VAG negative depicted as 0.

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 Discussion

This study, in accordance with previous research, has shown that intestinal *Escherichia coli* populations act as a reservoir for APEC. This could potentially serve as a threat to the health and wellbeing of commercial poultry including broiler, breeder and layer operations. (Ewers et al., 2009; Kemmett et al., 2014; Olsen et al., 2012).

The focus of this study was to characterise APEC prevalence in South Africa, determine APEC prevalence and potential virulent gene differences between the various poultry operations. The study also determined APEC prevalence and virulent gene changes over time. Available data was then also used to compare APEC VAG prevalence in SA to that of the USA.

Isolates were regarded as APEC positive if two or more VAGs were detected. Data analysed for SA indicated that 41.2% of the isolates were APEC positive and the majority (58.8%) of *Escherichia coli* isolates obtained from the GIT of healthy broilers, breeders and layers were indeed commensal and non-pathogenic in nature. Seventeen point three percent of the total APEC positive isolates had two or more, 11.15% had 3 or more, 8.9% had at least 4 genes and only 3.5% of the isolates had all 5 of the VAGs.

The prevalence of APEC in other African countries was somewhat lower compared to what was found in this study. Mtonga et al., 2021 for example reported 18.6% pathogenic *E. coli* isolated from chicken farms in Zambia. *E. coli* was classified using a phenotypical method and would therefore lead to a different outcome. The current study focused on virulent genes previously identified that aid in the pathogenicity of *E. coli*. The different detection methodologies again highlight the complexity of defining APEC.

The most prevalent VAG identified among South African *E. coli* isolates were *cva*C with a detection rate of 39.2%. This is in contrast with a number of studies where the VAG *iss* was found to be the most prominent virulence gene with detection frequencies of 84% for broilers in Pakistan, 82.7% in various poultry in Germany, 100% in broilers from India and 97.87% in chickens and ducks from China. All samples were from diseased or potentially infected birds, unlike for the samples in this particular study, that were from apparently healthy poultry (Azam et al., 2019; Ewers et al., 2004; Narasinakuppe Krishnegowda et al., 2022; Xuhua et al., 2021). A study performed by Paixão et al., (2016) on samples collected from dead broiler breeders and apparently healthy broilers from Portugal, found that both commensal and pathogenic *E. coli* contained VAGs to some degree or another. They concluded that *iss (*serum survival gene) and the iron uptake related genes such as *irp*2 were the most prominent for the 11 VAGs that was tested by using multiplex PCR.

In South Africa significant operational differences in terms of APEC positivity was detected, with statistically higher positive isolations recorded for broiler day old chicks (DOC). One reason for this could be the lack of heterophil function in day old chicks as described by (Wells et al., 1998). The Layer breeder group also had a statistically higher APEC positivity. Breeder pullets in contrast had the lowest number of APEC positive detections with 80% of the isolates found to be negative. The Breeder layer group also had a low negative detection rate of 64.3%, regarded to be significantly different to all other groups except for Breeder pullets. The marked difference in APEC prevalence between the operations can potentially be explained by host – microbial interactions as suggested by (Casadevall and Pirofski, 2001). These interactions can be influenced by various factors such as immune status of the birds, differences in production systems or possible feed variations (Awawdeh, 2017).

Significant differences were noted for the various VAGs between the different operations. Layer breeders as an example had a statistically different detection rate to all other groups with VAG *cva*C detection at 81.1%. Positive detection levels for VAG *irp*2 at 41.5% was also statistically different to all other operations. VAG *iuc*C and *iss* positive detection in the broiler DOC population of 49.7% and 43.3%

respectively was also seen to be significantly different to all other operations. Similar detection levels within the different operations for some of the VAGs were also noted.

APEC prevalence in 2017 was found to be significantly higher in comparison to subsequent years, with a marked decrease in prevalence noted in the years following up to 2022. The percentage positive APEC overtime fluctuated but showed an overall decrease from a high of 70% in 2017 to 37.8% positivity in 2022. The year 2020 in particular had an extremely low positive detection rate, with only 22.6% of the samples testing positive. Reasons for the decrease in APEC prevalence over time could possibly be attributed to improvements in husbandry and biosecurity practices. The first outbreak of highly pathogenic avian influenza (HPAI) in South African commercial poultry was recorded in 2017, followed by a second outbreak in 2021. This may have led to an industry wide revision of biosecurity protocols and subsequent tightening on-farm disease mitigation protocols (DALRRD, 2023). The COVID 19 pandemic reached its peak case incidence in mid-2020 in South Africa (NICD, 2021). This could possibly have contributed to an increased awareness of potential pathogens leading to the improvement of staff and poultry house hygiene and in-turn in a reduction of APEC prevalence in 2020. As humans could potentially act as vectors for APEC and spread it between flocks, as noted by (Johnson et al., 2008a), one could argue that the apparent improvements in personal hygiene could potentially also have contributed to the decrease in APEC prevalence recorded in 2020.

A statistically significant high number (45%) with 2 or more of VAGs were noted in 2017. A shift towards the right was recorded in 2019 with the highest frequency (9.7%) of isolates positive for all five VAGs. Statistically significant movement or variation over time was noticed for the specific VAGs. As an example, *tsh* detection rate moved from a low of 1.3% positive in 2017 to a high of 26.2% in 2022. VAG *iss* in contrast decreased from 48.8% positive in 2017 to a low detection of 14.9% in 2022. This is in agreement with (Johnson et al., 2008b) which concluded that progressive movement for certain virulence factors over time could be possible, as variation was observed for VAG *iss* between different isolates.

Notable differences were detected when comparing South African APEC data to that of the USA, providing supporting evidence for the perceived differences in prevalence. The USA population had a statistically higher percentage of isolates with two and three VAGs testing positive compared to SA. The USA also had a higher percentage detection for isolates with four and five VAGs. With a closer look at the specific VAGs, statistical differences for *tsh*, *cva*C and *iss* were established. Positivity rate for VAG *tsh* was significantly different between the two countries, with 74.2% detected in the USA population compared to 40.2% in SA. VAG *cva*C also came up strong with 81.4% detected for APEC isolates in the USA compared to 76.1% for SA. The South African population had a statistically significant higher positive detection rate for VAG *Iss* at 56.9% compared to the USA with 47.2%.

One explanation for the vast differences detected, could be the difference in time, seeing that all the USA samples were collected in 2016 compared to South African samples collected in 2018 - 2019 and 2022. There was also operational differences in the data analysed between the two countries. The USA data comprised of only broiler isolates whereas the SA data were made up of various different operations. A study by Fancher et al. in 2021 suggested that change of season and age of the bird could potentially affect virulence factors. They showed that the prevalence of all five of the VAGs tested for in spring (80.6%) was high compared to that in the summer period at 13.0%. VAG prevalence fluctuation specifically for *omp*T, *hly*F, and *iut*A were recorded at different ages. We then also know that environmental and management practices, such as stocking density and air quality in the chicken house could potentially play a role in the increase of virulence factors. Infections due to APEC could therefore also be secondary in nature (Kers et al., 2018; Fancher et al., 2021; Kathayat et al., 2021). The limited or complete removal of antibiotics (NAE) in USA as shown in a recent study has also led to an increase in APEC (Fancher et al., 2021).

Rapid diagnostics and appropriate control measures are increasingly becoming more important. This, in an effort to ensure the best possible meat hygiene is achieved to meet the ever increasing rise in consumer demand. Routine antibiotic use for therapeutic and or prophylactic measures have been implemented to control infectious

diseases in poultry production environment. The search however, for alternative solutions is gaining momentum, in light of the potential zoonotic threat and the ever increasing evidence supporting the high frequency of antibiotic resistant bacteria in poultry (Van den Bogaard et al., 2001). Some of the alternative measures include but are not limited to; vaccines, bacteriophages, endolysins, plant extracts, probiotics, specific algae, essential oils, bacteriocins, APEC virulence and growth inhibitors and antibody therapy (Van der Westhuizen, 2017; Kathayat et al., 2021). The success of these novel solutions will largely depend on the method of action as well as the economical feasibility of these actives. One such product is the modified live vaccine Poulvac® *E. coli,* with proven commercial value in broilers, breeders and layers. The gene *aroA* was deleted from an APEC strain, impairing the *curli fimbriae* production rendering it avirulent, while retaining the ability to elicit a sufficient immune response (Zoetis, 2023).

Characterisation and further elucidating of APEC prevalence in South Africa, as well as between operations within South Africa will assist in advanced diagnostics and further lead to improved measures to assist in prophylactic measures to control disease outbreaks specific to avian pathogenic *Escherichia coli* (Jeong et al., 2012; Van der Westhuizen, 2017; Wilczyński et al., 2022).

This study provides more insight into the epidemiology and population dynamics of APEC in SA as well as USA, but it raises a number of questions that could be addressed by further research. The link between the different operations; rearing, laying, hatchery and broiler farms would need to be investigated. This specific study was limited by the number of VAGs tested. Future research would benefit by increasing the number of VAGs, reinforcing the characterisation of APEC.

5.2 Conclusion

It is clear, as highlighted in this investigation, that the avian gastrointestinal tract serves as a reservoir for APEC colonisation in South African poultry. It can thus be confirmed that APEC and its virulence genes are prevalent in South Africa. Vast differences in APEC prevalence as well as its virulence factors were detected between various poultry operations in SA for the period analysed. A noticeable change in population dynamics were observed over time and we can thus conclude that a population shift over time (2017 – 2022) did occur. The data analysed for SA and USA also suggests that virulence gene prevalence differ between two countries.

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ADDENDUM A

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

4 November 2022

CONDITIONALLY APPROVAL

Ethics Reference No	**REC054-22**
Protocol Title	**Avian pathogenic Escherichia coli in South African poultry**
Principal Investigator	**Dr JL Goosen**
Supervisors	**Dr DBR Wandrag**

Dear Dr JL Goosen,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC054-22) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

Conditionally approved.

NOTE: Only historical/retrospective data to be used in the study. For any further sampling and/or experimental work, AEC approval will have to be obtained.

We wish you the best with your research.

Yours sincerely

100 YEARS OF VETERINARY EDUCATION

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YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

ADDENDUM B

CERTIFICATE OF ACCREDITATION

In terms of section 22(2) (b) of the Accreditation for Conformity Assessment, Calibration and Good Laboratory Practice Act, 2006 (Act 19 of 2006), read with sections 23(1), (2) and (3) of the said Act, I hereby certify that:-

INTERTEK TESTING LABORATORY (PTY) LTD

Co. Reg. No.: 1971/002229/07

Facility Accreditation Number: **T0627**

is a South African National Accreditation System accredited facility
provided that all conditions and requirements are complied with

This certificate is valid as per the scope as stated in the accompanying schedule of accreditation, Annexure "A", bearing the above accreditation number for

CHEMICAL AND MICROBIOLOGICAL ANALYSIS

The facility is accredited in accordance with the recognised International Standard

ISO/IEC 17025:2017

The accreditation demonstrates technical competency for a defined scope and the operation of a quality management system

While this certificate remains valid, the Accredited Facility named above is authorised to use the relevant accreditation symbol to issue facility reports and/or certificates

_____

Mr T Baleni
Acting Chief Executive Officer

Effective Date: 26 February 2020
Certificate Expires: 14 December 2024

ADDENDUM C

USDA

United States Department of Agriculture

Marketing and Regulatory Programs

Animal and Plant Health Inspection Service

4700 River Road
Unit 40
Riverdale, MD 20737

Compliance Inspection Checklist
Permit number 136187

Name of individual present during compliance check Michael Perry

Title of individual present during compliance check Laboratory Supervisor

How many shipments have been received this year? 1

Are conditions/restrictions on permit being met? Yes

Is the permittee in compliance? Yes or No (circle response)

If no, explain deficiencies or discrepancies _____

ANDREW JONES Digitally signed by ANDREW JONES Date: 2019.11.13 14:15:00 -05'00'

Signature of inspector

11/13/19
Date of inspection

Andrew Jones
Printed name of inspector

Veterinary Medical Officer
Title of Inspector