EVALUATION AND APPLICATION OF MOLECULAR AND IMMUNOLOGICAL SEROTYPING TECHNIQUES FOR SELECTED AVIAN AND HUMAN *E. coli* STRAINS

BY

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Declaration of own work

I, Segomoco Lesego Ignatious Sebate, identity number (student number), hereby declare that this research project submitted to the Central University of Technology, Free State, for the degree Master of Health Sciences in Environmental Health, is my own independent work. This work complies with the Code of Academic Integrity as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State, and has not been submitted before to any institution by myself or any other person for the attainment of a qualification.

……..

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I certify that the above statement is correct.

……..

Dr Olga de Smidt (supervisor)

This achievement is dedicated to my beloved

late ***Grandparents:***

(Jessie Puleng Sebate (Nkoko Jesi) and Masopa

Mattheus Sebate (Ragolo Ntebe)), my late

Paretnts:

(Masetoki Lydia Sebate (Mmisa) and

Moriritlhane Johnny Seobi (NgiNgi)) and late

bestfriend:

Dr Lehlohonolo Mathengtheng.

May your souls rest in eternal peace!!!

Responding to life:

I had to make peace with was, surrender to what is AND have faith in what will be!!!

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ABSTRACT

A link exists between human and animal diseases caused by *E. coli*. Avian pathogenic *E. coli* (APEC) causes detrimental economic losses in the poultry industry due to infections in poultry such as colibacillosis and cellulitis. In 2003, poultry producers were made aware that some avian diseases could be transmitted to humans, particularly via the faecal-oral route. For the identification of these *E. coli* strains, surface antigens are used. Based on surface antigens namely somatic (O), capsular (K) and flagella (H) antigens, different *E. coli* strains can be serotyped. The most common APEC strains are O1, O2 and O78, but serotypes O8, O15, O18, O36, O88, O109, O115 and O116 have also been reported as *E. coli* isolates associated with cellulitis and colibacillosis in poultry. *E. coli* strains O1:K1:H7, O18:K1:H7 and O15:K52:H1 have been linked to diseases in both mammals and birds. In South Africa, only enumeration is performed routinely to monitor the presence of *E. coli* in poultry processing plants, thus no serotyping occurs in this setting. For this reason, it is important to know which serotypes are prevalent, especially in poultry abattoirs, to prevent the possibility of such pathogens infecting humans via poultry. It was therefore the aim of this study to develop a method that can identify these pathogens at serotype level from environmental samples. To identify the three laboratory strains, both genotypic (molecular) and phenotypic (immunological) characteristics were considered. When using multiplex-PCR as a molecular serotyping technique, targeted genes (*wzx-1*, *neuC*, *fliC*, *wzx-2* and *fumC*) were detected on the test strains. With the optimization of multiplex-PCR, it was possible to apply this technique to field isolates as well as to the environmental sample. One-step digestion of multiplex PCR allowed the differentiation of the O1:K1:H7, O18:K1:H7 and O15:K52:H1 APEC *E. coli* test strains. The method proved to be fairly simple and cost effective, yet such a method is currently not

available in the poultry sector. Conversely, an array of immunological techniques was able to detect only flagellin antigens on both the O1:K1:H7 and O18:K1:H7 strains using plate agglutination on the test strains. Additionally, K1 bacteriophage was able to detect K1 antigens again on both O1:K1:H7 and O18:K1:H7 using zone of inhibition, pour plate and broth clearing assays. However, counter current immunoelectrophoresis results were inconclusive due to negative results on the targeted antigen (i.e., K52 antigen).

LIST OF TABLES

		Page
Table 2.1:	Previous and current classifications of poultry abattoirs in South Africa	**19**
Table 2.2:	Selected MSA used for species detection	**29**
Table 3.1:	Oligonucleotide primer sequences for the reference strains from previously published works referred to in this study	**61**

LIST OF FIGURES

		Page
Figure 2.1:	Six categories of *E. coli* that can cause gastroenteritis in humans.	**19**
Figure 3.1:	The preparation of inoculum from preserved cultures (in 40% glycerol at -20°C) and incubated for 5 hours (at 37°C) with shaking by first preparing pre-inoculum incubated (at 37°C) with shaking for 16 hours.	**61**
Figure 3.2:	The three reference strains O1:K1:H7 (A), O15:K52:H1 (B) and O18:K1:H7 (C) cultivated on Violet Red Bile with MUG agar showing blue fluorescence light when observed under UV light.	**69**
Figure 3.3:	The three bacterial strains O1:K1:H7, O15:K52:H1 and O18:K1:H7 genomic products separated in a 0.8% agarose gel (85 V for 30 minutes) showing sufficient genomic DNA (gDNA) without evidence of degradation.	**70**
Figure 3.4:	Amplification of five genes: *fumC* (~140 bp), *fliC* (~550 bp), *neuC* (~676 bp), *wzx-1* (~1100 bp), and *wzx-2* (~551 bp) from reference strains in lanes A (O1:K1:H7), B (O15:K52:H1) and C (O18:K1:H7) for specificity with 16S rRNA gene product (~1300 bp) included in all reactions as internal control.	**71**
Figure 3.5:	All five primer pairs targeting *fumC* (~140 bp), *fliC* (~550 bp), *neuC* (~676 bp), *wzx-1* (~1100 bp), and *wzx-2* (~551 bp) genes were used for amplification of targeted gene products in O1:K1:H7 (lane 1), O15:K52:H1 (lane 2), and O18:K1:H7 (lane 3) and all the strains mixed in lane 4.	**72**

Figure 3.6: Amplification of all five primer pairs targeting *fumC* (~140 bp), *fliC* (~550 bp), *neuC* (~676 bp), *wzx-1* (~1100 bp), *wzx-2* (~551 bp) genes 16S rRNA targeting primer pair product (~1300 bp) in all three strains O1:K1:H7, O15:K52:H1 and O18:K1:H7, with increased concentrations of template (lane 1), dNTPs (lane 2), Taq polymerase (lane 3), primer pairs (lane 4), and $MgSO_4$ (lane 5). In lane 6, all individually increased reagents were mixed with all the strains. Lanes M and NTC represent the GeneRuler™ 1 kb DNA ladder (ThermoFisher Scientific) and the non-template control respectively. **73**

Figure 3.7: Multiplex PCR using gDNA (A) and whole cell (B) as a template of all the primer pairs against three reference strains individually in lanes 1, 2 and 3 for O1:K1:H7, O15:K52:H1 and O18:K1:H7 respectively, and against all the strains in lanes 4. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (ThermoFisher Scientific) and the non-template control respectively. **74**

Figure 3.8: In silico screening (using online NebCutter V2.0 software) of restriction enzyme digestions in *wzx-2*, *fliC*, 16S rRNA, *neuC*, *fumC* and *wzx-1* sequenced genes showing the restriction enzyme sites of *Bst*API digestion on *wzx-1* and *fliC* sequenced genes (in black boxes), and *Rsa*I and *Mnl*I digestion on *wzx-2* and *fliC* sequenced genes (in black boxes). **75**

Figure 3.9: Digestion of *wzx-2* and *fliC* by eight restriction enzymes with only two restriction enzymes (*Mnl*I and *Rsa*I) digesting *wzx-2* and only three digestions (*BstA*PI, *Mnl*I and *Rsa*I) observed in the digestion of *fliC*. Lanes M and NTC represent the GeneRuler™ 1 kb DNA ladder (ThermoFisher Scientific) and the non-template control respectively. **76**

Figure 3.10: Low melting temperature agarose gel (2% w/v) of optimized mPCR product with whole cells (O1:K1:H7, O15:K52:H1 and O18:K1:H7) as template of all the primer pairs listed in Table 3.1 (lane 1). One-step digestion of *fliC* fragment by *BstA*PI restriction enzyme in lane 2 (Mizani *et al*., 2017). Two fragments of ~400 bp and ~150 bp were observed and an additional two fragments of ~900 bp and ~200 bp were also observed from the digestion of the *wzx-1* mPCR product. Lane M represents the GeneRuler™ 1 kb DNA ladder (ThermoFisher Scientific). **77**

Figure 3.11: One-step digestion of seven selected field isolates (lanes 1, 3, 5, 7, 9, 11 and 13) with *BstA*PI restriction enzyme which yielded product observed in adjacent lanes (2, 4, 6, 8, 10, 12 and 14) (Mizani *et al*., 2017). Colonies in lanes 1, 9 and 11 yielded two fragments of ~400 bp and ~150 bp and another fragment of ~550 bp that remained intact after digestion for colony in lane 11 observed in lane 12. Lane M represents the GeneRuler™ 1 kb DNA ladder (ThermoFisher Scientific) and the non-template control. **79**

Figure 3.12: Repeat of one-step digestion (lane 2) of optimised mPCR (lane 1) (Figure 3.8) used as positive controls for one-step digestion of two environmental samples in lanes 3 and 4. Lanes M and NTC represent the GeneRuler™ 1 kb DNA ladder (ThermoFisher Scientific) and the non-template control respectively. **80**

Figure 4.1: The preparation of inoculum (A) from cultures preserved in 40% glycerol at -20°C and incubated for 5 hours (at 37°C) with shaking by first preparing pre-inoculum incubated (at 37°C) with shaking for growth monitoring over a period of 11 hours (B). **90**

Figure 4.2: Preparation of O-antigen suspension (O-AS) by steaming in autoclave and water bath. H-antigen suspension (H-AS) from the enumeration culture was adjusted with the inoculum up to OD of 0.1 at 600_{nm}. After incubation, O-AS bottles were allowed to cool (at room temperature for autoclaved O-AS and at 4°C for water bathed O-AS) and gently mixed and left for 1 hour before use. The contents of the H-AS bottle was mixed with an equal amount of 1% FS (1:1 v/v). **92**

Figure 4.3: Representative microtiter plate setup showing O1 and O18ab antisera testing. All 96 wells were filled with 50 µl of PBS. Diluted O1 antiserum (1:10 v/v) was added to column 1 rows A-D and O18ab antisera were added to column 1 rows E-H. After mixing, the contents of column 1 were serially diluted across the plate to column 12. The reference strains (50 µl) were added as follows: O1:K1:H7 in rows B and F (red), O15:K52:H1 in rows C and G (yellow), and O18:K1:H7 in rows D and H (blue). Rows A and E (green) served as controls and contained 50 µl of PBS only. **94**

Figure 4.4: An inoculum (1ml) prepared as in Figure 3.1 was mixed with LB broth (ten test tubes containing 9 ml) to 1:10 v/v concentration. K1 bacteriophage (100 µl) was pipetted into the first test tube and serially diluted across all ten test tubes. The contents in the test tubes in the red block (for Broth Clearing Assay, BCA) were transferred (100 µl) to respective test tubes containing 9.9 ml of molten agar (stored at 45°C). For BCA, the test tubes were incubated at 37°C overnight. The contents in the molten agar test tubes were poured onto respective thin layered LB agar plates. After solidifying, the plates were incubated at 37°C overnight to observe the plaque formation. **96**

Figure 4.5: Representative counter current immunoelectrophoresis setup showing three different antigens (Ag), which were three reference strains O1:K1:H7, O15:K52:H1 and O18:K1:H7 on the cathode terminal represented by O1, O15 and O18 respectively and tested against K52 antibody (Ab) on the anode terminal with O15 included as positive control strain. **97**

Figure 4.6: Growth curves of *E. coli* strains cultivated in LB broth at 37°C. The growth was monitored over 10 hours by measuring OD at 600_{nm}. Average values of three independent replicates were plotted. The error bars represent standard deviations. **99**

Figure 4.7: Representative microtitre plate results of serially diluted H7 monospecific H rabbit antiserum against all reference strains with lane A as the negative control. **100**

Figure 4.8: Zone of inhibition caused by K1 bacteriophage from the vertical phage stripe with O1, O15 and O18 representing the O1K:1:H7, O15:K52:H1 and O18:K1:H7 strains respectively. The red lines represent the area of growth inhibition by the phage. **101**

Figure 4.9: LB broth with *E. coli* reference strains O1:K1:H7 (A) and O1:K1:H7 (B) showing the clearing activity of the broth (MIC) by K1 bacteriophage. With *E. coli* reference strain O15:K52:H1 (C), all the test tubes were turbid showing the inability of K1 bacteriophage to clear the broth. **102**

Figure 4.10: LB agar plate overlaid with *E. coli* reference strain O15:K52:H1 showing an overgrowth of the strain on plate A (the arrow point at the edge of the cell growth) and plate B showing plate covered with plaque caused by the phage for both the O1:K1:H7 and O18:K1:H7 reference strains. **103**

TABLE OF CONTENTS

DECLARATION ..... **i**

DEDICATIONS ..... **ii**

ACKNOWLEDGEMENTS ..... **iii**

ABSTRACT ..... **iv**

LIST OF TABLES ..... **vii**

LIST OF FIGURES ..... **viii**

CHAPTER 1

1.1 Introduction **2**

1.2 Aim and Objectives **4**

1.3 Thesis Layout **5**

Chapter 1: **Introduction** 5

Chapter 2: **Literature Review** 5

Chapter 3: **Application of a multiplex PCR/Restriction enzyme digestion (mPCR/RED) approach for identifying *E. coli* serotype strains O1:K1:H7, O15:K52:H1 and O18:K1:H7** 6

Chapter 4: **Specificity and sensitivity of immunological serotyping assays on reference strains** 6

Chapter 5: **Concluding Remarks** 7

1.4 References **8**

CHAPTER 2

2.1 Introduction 11

2.2 The South African poultry industry (SAPI) 12

2.2.1 High-throughput abattoirs (HTPA) 13

2.2.2 Regulations and monitoring of SAPI 15

2.3 Poultry Pathogens 15

2.4 Avian Pathogenic *E. coli* (APEC) 20

2.5 Assays and Techniques Used for the Identification of *E. coli* 23

***2.5.1.1 Polymerase chain reaction (PCR)* 26**

2.5.2 Immunological identification 29

2.6 The strains of interest 31

2.7 Advantages of mPCR 31

2.8 References 33

CHAPTER 3

3.1 Introduction 57

3.2 Materials and Methods 59

3.2.1 Cultivation of *E. coli* strains 59

***3.2.1.1 Reference strains* 59**

***3.2.1.2 Field isolates* 59**

3.2.1.3 Environmental sample **60**

3.2.2 Procedure used to prepare inoculums 60

3.2.3 Bacterial cultivation on agar plates 61

3.2.4 Genomic material (gDNA) isolation from LB agar plate 61

3.2.5 Pre-optimized multiplex PCR conditions and primer specificity 62

3.2.6 Optimization of multiplex-PCR on reference strains' gene products 65

3.2.7 Purification of DNA fragments for sequencing 65

3.2.8 Sequencing and analysis 66

3.2.9 Selection of restriction enzymes and their application on the optimized mPCR product 67

3.2.10 Application of the optimized multiplex-PCR on the gene products of the reference strains 67

3.2.11 One-step digestion of optimized mPCR with BstAPI restriction enzyme 68

3.3 Results and discussion 68

3.3.1 Bacterial isolation 68

3.3.2 gDNA yields 69

3.3.3 Primer specificity analysis 70

3.3.4 Pre-optimized multiplex-PCR on the gene products of reference strains ... 71

3.3.5 Multiplex-PCR optimization 73

3.3.7 One-step digestion of optimized mPCR product to differential *fliC* and *wzx-2* 76

3.3.9 Application of mPCR/RED protocol on field isolates and environmental samples 77

3.4 Conclusions 81

3.5 References 82

CHAPTER 4

4.1 Introduction 88

4.2 Materials and Methods 90

4.2.1 Growth monitoring of reference strains 90

4.2.2 Microtiter plate agglutination 91

***4.2.2.1 Preparation of O- and H-antigen suspensions* 91**

***4.2.2.2 Plate agglutination* 92**

4.2.3 Plaque assay 94

***4.2.3.1 Cross-brush assay* 95**

***4.2.3.2 Broth clearing plate and pour assay* 95**

4.2.4 Counter current immunoelectrophoresis 97

4.3 Results and discussion 117

4.3.1 Sampling of reference strains using growth curve as reference for plate agglutination assay 98

4.3.2 Microtitre-plate agglutination 99

4.3.3 Plaque assay 100

4.3.4 Counter current immunoelectrophoresis 103

4.4 Conclusion 104

4.5 References 105

CHAPTER 5

5.1 Concluding remarks 110

CHAPTER 1
Introduction

1.1 Introduction

South Africa's poultry meat production exceeded the one million ton mark for the first time in 1998 and seemed to keep growing (Germishuis, 2000). In 2017, 47 025 employees were directly employed by the industry with 57% working in broiler processing (poultry abattoirs); 13% working in broiler distribution; and 30% working in broiler breeding, hatchery and rearing (SAPA, 2017). According to the Department of Agriculture, data for 2017 revealed that the total poultry meat (all saleable) production amounted to 1.658 million tons. Broiler production contributed approximately 92% to the total poultry meat production with the balance of 8% made up by cull layer hens and cull broiler breeders (SAPA, 2017).

The South African Meat Safety Act No. 40 of 2000 (South Africa, 2000) specifies that it is mandatory that high-throughput poultry abattoir environments be monitored for the presence of specific microorganisms. These organisms include bacteria such as *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens,* intestinal *Enterococci* and *Listeria*. *E. coli* can be found both inside (enteric *E. coli*) and outside (extraintestinal *E. coli*) the digestive tract (Bien *et al*., 2012). Extraintestinal *E. coli* (ExPEC) can further be subdivided into uropathogenic *E. coli* (UPEC), septicemic *E. coli* (SPEC), neonatal meningitis *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC). UPEC and NMEC can cause diseases such as urinary tract infections (UTI/pyelonephritis and neonatal meningitis respectively) when *E. coli* contaminated chicken is consumed (Bien *et al*., 2012).

APEC is an important extraintestinal pathogenic bacterial species as it contains harmful serotypes. APEC is known to cause infectious diseases in poultry such as colibacillosis and cellulitis (Lutful Kabir, 2010). The most common APEC strains are O1, O2 and O78; however, serotypes O8, O15, O18, O36, O88, O109, O115 and

O116 have also been reported to be associated with colibacillosis and cellulitis in poultry (La Ragoine *et al.,* 2000; Stordeur *et al.,* 2004). The occurrence of these infectious diseases has caused extensive losses in the poultry industry in South Africa (Germishuis, 2000). There is also evidence that humans who come into direct contact with poultry are at risk of contracting *E. coli* diseases from poultry (Ojeniyi, 1989). In 2015, Zweifel and co-workers reported that total viable counts of *E. coli* from plucked carcasses of three poultry abattoirs ranged from 2.9 to 3.3 log CFU/g in Zurich. It is therefore important to implement targeted and sustainable measures at selected stages of the poultry slaughter process to detect the presence of such microorganisms in poultry abattoirs. Presently, if *E. coli* strains are detected in South African abattoirs, they are not subjected to serotyping but are only enumerated; so the prevalence of strains of APEC in South African abattoirs is currently unknown. For many bacterial species there exists serotypes that are infectious or that produce harmful toxins. However, these harmful serotypes cannot be identified using the required legislated monitoring procedures that are applied in South Africa.

1.2 Aim and Objectives

To identify *E. coli*, multiple assays such as nucleic acid based methods (NABM, or molecular techniques) and immunological serotyping can be used. However, some methods are non-specific (microtiter plate agglutination and countercurrent immunoelectrophoresis) as well as expensive and laborious; for example, it is necessary to locate and purchase the antibodies and create a suitable environment to perform the assay, yet only limited positive results may be obtained.

The aim of this study was therefore to assess the usefulness of multiplex-PCR or immunology as techniques for serotyping selected laboratory and environmental APEC *E. coli* strains. To achieve this aim, it was important to find suitable primers, optimize multiplex, find suitable restriction enzymes, source suitable antibodies for immunology, and optimize immunology protocols and application using environmental isolates. A peripheral aim was to test the best performing assays and techniques on laboratory strains and to apply them on field and environmental samples collected from the effluent of a poultry abattoir. To achieve these aims, the following objectives were devised:

- To evaluate the specificity and sensitivity of multiplex PCR on the O1:K1:H7, O15:K52:H1 and O18:K1:H7 strains followed by optimization;
- To apply (or optimize) the multiplex PCR/Restriction enzyme digestion (mPCR/RED) approach for identifying *E. coli* serotypes O1:K1:H7, O15:K52:H1 and O18:K1:H7 on field and environmental samples collected at a high-throughput poultry abattoir; and

- To evaluate the specificity and sensitivity of microtitre-plate agglutination, plaque assay, and counter current immunoelectrophoresis.

1.3 Thesis Layout

Chapter 1: Introduction

In Chapter 1 the research project is introduced, the problem is specified and the aims and objectives are presented.

Chapter 2: Literature Review

Chapter 2 entails a review of related literature. Topics that are relevant to this research project are covered subsequent to the introduction such as the background of the South African poultry industry, the nature of high-throughput abattoirs, regulations and monitoring protocols for the South African poultry industry, challenges in the poultry industry, avian pathogenic *E. coli*, the identification of *E. coli* in poultry, and assays and techniques used for the identification of *E. coli* (e.g., immunological identification using agglutination and molecular identification using a polymerase chain reaction).

Chapter 3: Application of a multiplex PCR/Restriction enzyme digestion (mPCR/RED) approach for identifying *E. coli* serotype strains O1:K1:H7, O15:K52:H1 and O18:K1:H7

After a brief introduction to Chapter 3, the materials and methods that were utilised in this study are discussed. The topics that follow include the validation of the presence of extracted DNA products, primer specificity with unoptimized PCR conditions, unoptimized multiplex-PCR on reference strain gene products, optimization of multiplex-PCR on reference strain gene products, the application of the optimized multiplex-PCR on reference strain gene products, verification of *BstAPI* as the enzyme of choice from in silico screening, one-step digestion of optimized mPCR with *BstAPI* restriction enzyme, one-step digestion application on field isolates with *BstAPI* restriction enzyme, and the application of one-step digestion of optimized mPCR of environmental samples with the *BstAPI* restriction enzyme. A brief conclusion brings this chapter to a close.

Chapter 4: Specificity and sensitivity of immunological serotyping assays on reference strains

The introduction of this chapter is followed by a more extensive discussion of the materials and methods. Further topics include the results of bacterial isolation, bacterial preservation, growth studies, bacterial enumeration, microtitre-plate agglutination, plaque assay, and counter current immunoelectrophoresis.

Chapter 5: Concluding Remarks

Chapter 5 presents an overall discussion that covers the findings of Chapter 3 and Chapter 4. Recommendations are offered as future applications of the findings are suggested.

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CHAPTER 2

Literature Review

2.1 Introduction

A substantial portion of the human diet in many countries is made up of poultry meat. Globally, people consumed approximately 12 kg of poultry per person between 2000 and 2009 (DAFF, 2014; SAPA, 2016). At the time, the world average annual consumption (WAAC) rate of poultry exceeded that of approximately 9 kg per person of beef and was steadily approaching the WAAC value for pork of approximately 15 kg per person (Avarez-Fernandez *et al.*, 2013; SAPA, 2016). In South Africa, the relatively low price of poultry meat makes it a more attractive protein for consumers than beef. Poultry meat consumption in South Africa has increased from approximately 23 kg per person in 2004 to approximately 35 kg per person in 2013 (DAFF, 2014; Esterhuizen, 2015; SAPA, 2016). With this noteworthy increase in poultry consumption, the threat of poultry for human disease transmission is also high. For example, as far back as 1989, Ojeniyi documented the direct transmission of *E. coli* from poultry to humans. More recently, Jacob *et al*. (2003) found that colibacillosis infections caused by *E. coli* in poultry were transferred to humans via the faecal-oral route. Moreover, these researchers found that the infection was often food or water borne.

2.2 The South African Poultry Industry (SAPI)

The South African poultry industry comprises two sectors, namely the broiler and the egg layer sectors (SAPA, 2016). The broiler sector of the industry makes up 80% of the total industry, with the egg laying component comprising the remaining 20% (DAFF, 2014). The broiler sector of the industry is mostly centred around privately owned abattoirs. These abattoirs are spread throughout the country with most of them located in the provinces of Gauteng and KwaZulu-Natal (DAFF, 2014). These abattoirs slaughter more than 750 million chickens each year (DAFF, 2014).

South African legislation, more specifically the Meat Safety Act No. 40 of 2000 (South Africa, 2000), requires that all poultry abattoirs are registered (DARD, 2009). Registered poultry abattoirs are classified according to how many chickens are slaughtered each day. Originally, poultry abattoirs were graded from A to E (Table 2.1). However, currently registered poultry abattoirs are rated and classified as high-throughput abattoirs (HTPA), low-throughput abattoirs, and rural abattoirs. There are 322 registered poultry abattoirs in South Africa with 176 high-throughput abattoirs, 67 low-throughput abattoirs and 79 rural abattoirs (DARD, 2009). About 90% of these poultry abattoirs are privately owned, while about 5% are owned by the government (DARD, 2009). The other 5% are operated as community projects (Molapo, 2009).

Table 2.1: Previous and current classification of poultry abattoirs in South Africa

PREVIOUS CLASSIFICATION	CURRENT CLASSIFICATION	
Grading	**Classification**	**Maximum slaughter chicken per day**
A and B	High-throughput abattoirs	> 2000
C and D	Low-throughput abattoirs	< 2000
E	Rural abattoirs	< 50

Source: Adapted from Molapo, 2009

In South Africa, seven major producers are responsible for the majority of broiler production. These producers include Astral Foods, Countrybird, Daybreak, Fourie's Poultry Farms, Rainbow Limited, Rocklands, and Tydstroom (DAFF, 2014). Astral Foods and Rainbow Limited are the most productive and are responsible for the production of 46% of the broilers in South Africa (DAFF, 2014). These two companies produce more than 220 million of approximately 750 million broilers per annum (DAFF, 2014).

2.2.1 High-throughput abattoirs (HTPA)

In HTPA, more than 2000 chickens are slaughtered each day. In the slaughtering process, vast volumes of water are used to wash the chicken carcasses. This process culminates in large volumes of water being drained from abattoirs; in fact, the quantity of water that drains away from South African HTPA exceeds 10 billion litres annually. The volume of wastewater produced by abattoirs amounts to approximately 29 million litres daily, while approximately 14 litres of water is used per chicken daily (DAFF, 2014).

The processing of live chickens in an HTPA is a complex process which comprises two stages that are referred to as primary and secondary processing. Emphasis has been placed on the role that poultry abattoir processing plays in the development of ill health amongst workers exposed to various pathogens. This occurs during both primary (receiving, shackling, stunning, bleeding, scalding, de-feathering, evisceration, recovery) and secondary (portioning, brining, filleting, chilling, freezing, packaging and dispatching) processing operations (Harmse *et al*., 2017).

The feathers, feet, skin and gastrointestinal tracts of chickens harbour a large number of micro-organisms that enter a poultry abattoir (Pan & Yu, 2014), and abattoir workers may be exposed to these micro-organisms. Many of these micro-organisms are pathogenic and are referred to as animal-borne pathogens (ABP) (Meat Inspection Manual, 2007). According to the Advisory Committee on Dangerous Pathogens in the United Kingdom, most abattoir workers are unnecessarily exposed to chicken parts and residues which might contain ABP (ACDP, 2012).

2.2.2 Regulations and monitoring of SAPI

Open chicken carcasses introduce a high number of microorganisms that may by pathogenic to the environment, and workers are constantly exposed to this hazard. Rigorous monitoring is therefore required. South African poultry producers are regulated by a body called the South African Poultry Association (SAPA). SAPA is responsible for a code of practice that requires collection, compilation and distribution statistics; the promotion of positive aspects of the broiler industry's image; and adherence to food compliance and safety regulations. It also critically monitors imports; institutes and maintains protective import tariffs; and supports industry training (DAFF, 2014). This body is also responsible for monitoring chickens that are reared for human consumption (broilers) and ensuring that they are reared in healthy environments (SAPA, 2012).

However, current monitoring procedures involve only the enumeration of listed microorganisms that might be present in the meat in an abattoir (South Africa, 2000). For example, a survey revealed that, in five regions in South Africa, the enumeration of microorganisms ranged from 2.2×10^2 to 3.2×10^6 CFU on a plate count agar (PCA) plate (Mabote *et al.*, 2011). Moreover, the enumeration of microorganisms is performed only up to species level (South Africa, 2000).

2.3 Poultry Pathogens

In 2007, an abattoir hygiene manual was provided by the Veterinary Public Health Unit of the South African National Department of Agriculture. The tests that were conducted used predominantly meat carcasses. These tests were mainly conducted

to detect bacteria that are most frequently associated with food-borne diseases such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Clostridium*, *Salmonella* and *E. coli* (specifically O157:H7) (Meat Inspection Manual, 2007). The *E. coli* O157:H7 strain can cause serious food poisoning in humans and is occasionally responsible for product recalls (Yun Lee *et. al.*, 2009). However, O157:H7 is not the only strain associated with disease.

E. coli forms part of the complex community of microorganisms in poultry. It begins to colonize the gastrointestinal tract (GIT) within a few hours after hatching, reaching up to 10^6 colony forming units per gram ($CFU.g^{-1}$) of intestinal contents during the first few days of life (Dho-Moulin & Fairbrother, 1999). The complex community of microorganisms residing in or passing through the GIT is referred to as intestinal microbiota and these microbiota play an important role in metabolic, nutritional, physiological and immunological processes in the human body. For instance, they initiate important metabolic activities by extracting energy from otherwise indigestible dietary polysaccharides such as resistant starch and dietary fibres. These metabolic activities also lead to the production of important nutrients such as short-chain fatty acids, vitamins (vitamin K, vitamin B12 and folic acid) and amino acids, which humans are unable to produce themselves (Hamer *et al*. 2008). In addition, the intestinal microbiota participate in the defence against pathogens by mechanisms such as colonization resistance and the production of antimicrobial compounds.

Furthermore, intestinal microbiota are involved in the development, maturation and maintenance of the gastrointestinal functions (sensory and motoric), the intestinal barrier, and the mucosal immune system. These are just a few examples of the

functional contributions of intestinal microbiota to human health, which is a subject that has been regularly reviewed (Barbara *et al*. 2005; Cerf–Bensussan & Gaboriau–Routhiau, 2010; Sekirov *et al*., 2010; Zoetendal *et al*., 2008). In recent years, a sharp increase has been seen in the number of publications addressing intestinal microbiota, and these articles have provided various lines of evidence supporting a close link between microbiota and human health.

Infections associated with *E. coli* include intra-abdominal infections, acute bacterial meningitis, urinary tract infection, pyelonephritis, neonatal meningitis, gastroenteritis, and septicemia (Bauchart *et al*., 2010; Dai *et al*., 2010; Bien *et al*., 2012). The pathogenic *E. coli* strains are broadly classified as either enteric/diarrheagenic *E. coli* or extraintestinal *E. coli* (ExPEC). Strains of enteric *E. coli* can be grouped into six categories: enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and diffuse adherent *E. coli* (DAEC). The pathogenesis of these categories are presented in the summary below as well as in Figure 2.1.

1) The pathogenesis of ETEC diarrhoea involves two steps: intestinal colonization, followed by the elaboration of diarrheagenic enterotoxins (Baron, 1996).

2) EPEC serogroups bind intimately to the epithelial surface of the intestine, usually the colon, via the adhesive bundle-forming pili (BFP). The lesion caused by EPEC consists mainly of the destruction of microvilli (Baron, 1996).

3) Haemorrhagic colitis is caused by EHEC strains (most notably O157:H7) that colonise the gut by intimately adhering to the epithelial cells causing the condensation of actin and the effacement of the microvilli. They also produce

relatively large quantities of the bacteriophage-mediated Shiga-like toxin. Many strains of O157:H7 also produce a second cytotoxin (Shiga-like toxin 2, or Vero toxin 2) which is similar in effect but antigenically different (Baron, 1996; Belanger *et al*., 2011).

4) EIEC pathogenesis starts with the penetration of the bacteria into the epithelial cell which causes the lysis of the endocytic vacuole. The bacteria proliferate and move intracellularly and eventually spread laterally to the adjacent cells (Sansonetti, 1992; Goldberg & Sansonetti, 1993).

5) EAEC enhances the secretion of mucus which traps the bacteria to form a biofilm mucus layer and releases the cytotoxin (Tzipori *et al*., 1992).

6) DAEC strains induce finger-like projections that extend from the surface of infected cells. The projections wrap around the bacteria but do not effect complete internalization (Cookson & Nataro, 1996; Yamamoto *et al*., 1994).

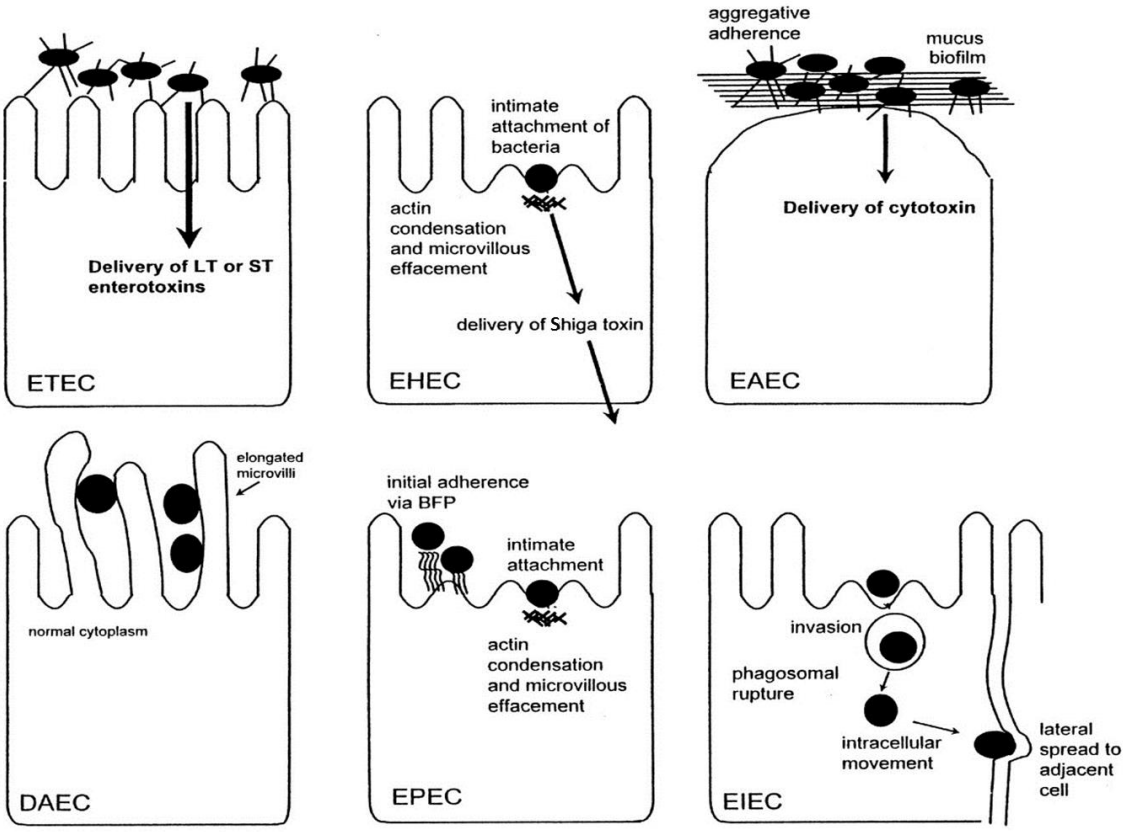

Figure 2.1: Six categories of *E. coli* that can cause gastroenteritis in humans. It should be noted that these descriptions are largely the result of in vitro studies and may not completely reflect the phenomena occurring in infected humans (Nataro & Kaper, 1998; Smith & Fratamico, 2005).

These pathogenic *E. coli* are serotyped on the basis of their O (somatic), H (flagella), and K (capsular) surface antigen profiles. Each of the six categories listed above has a different pathogenesis and comprises a different set of O:H serotypes (Nataro & Kaper, 1998). There are also *E. coli* strains that cause disease outside the GIT; these are known as extraintestinal pathogenic *E. coli* (ExPEC) and they include human uropathogenic *E. coli* (UPEC) (which is one of the most virile causes of bacterial infectious diseases in industrialized countries), avian pathogenic *E. coli* (APEC), and *E. coli* causing neonatal meningitis (NMEC) and septicemia (SPEC) (Andersen *et al*., 2012; Johnson & Stell, 2000; Russo & Johnson, 2003; Soto *et al*., 2008; Germon *et al*., 2005). ExPEC infections are among the most significant

infectious diseases in production birds and result in severe losses due to mortality, production losses, and condemnations (Ewers *et al*., 2009). Concurrently, infection with APEC generally begins as a localized infection of the air sacs commonly referred to as airsacculitis or 'the air sac disease' which, in turn, may spread to other internal organs resulting in systemic infection. This initial infection generally occurs in 4–9-week-old broiler chickens and in laying hens at the peak of egg production which occurs at week 30 (Antao *et al*., 2008). ExPEC, APEC and UPEC also share virulence-associated traits and have overlapping O serogroups and phylogenetic types (Rodriguez-Siek *et al.,* 2005; LeStrange *et al*., 2017). Nevertheless, serotyping is still a common method for estimating the pathogenic potential of APEC strains as it has been accepted for a long time that some serotypes, including serotypes O1, O2, O8, O18, and O78, are detected more frequently than other serotypes (Ewers *et al*., 2009).

2.4 Avian Pathogenic *E. coli* (APEC)

Human hosts are the point of origin for all sequenced pathogenic *E. coli* (Johnson *et al*., 2007; Johnson *et al*., 2012). This partiality has left a gap in our knowledge, as various *E. coli* strains cause significant and widespread diseases in animals, including in those raised for human consumption (Barnes *et al.*, 2003). Consequently, while the genomic analysis of *E. coli* strains from animals can be justified solely on the basis of *E. coli's* detrimental impact on animal agriculture, a broader rationalization would also include the potential link between animal-source *E. coli* and human disease. Links between human and animal disease caused by *E.*

coli have been well established by various studies (Johnson *et al*., 2007, Belanger *et al*., 2011; Bergeron *et al*., 2012; Harmse *et al*., 2017).

In contrast, APEC strains are commonly of the O1, O2 and O78 serotypes, but serotypes O8, O15, O18, O36, O88, O109, O115 and O116 have also been reported for *E. coli* isolates associated with cellulitis and colibacillosis in poultry (Blanco *et al*., 1998; La Ragoine *et al.,* 2000; Stordeur *et al.,* 2004). APEC is the most common bacterial pathogen that affects chickens (Barnes *et al*., 2008; Ghunaim *et al*., 2014). A considerable diversity of serogroups has been detected among poultry clinical isolates and only a small percentage of these isolates belongs to serotypes O1, O2 or O78. Therefore, no single *E. coli* serotype used as a bacterin can provide full protection against all the serotypes that cause avian *E. coli* infections. Although it is generally agreed that the immediate source of UPEC causing human UTIs is an individual's own colonic flora (Johnson & Stell, 2000; Johnson *et al.*, 2007; Chassin *et al*., 2011; Bien *et al.*, 2012), it is not completely understood how these virulent strains come to inhabit the colon. One hypothesis is that retail poultry harbouring avian pathogenic *E. coli* (APEC) represents a food-borne source of *E. coli* strains that are capable of causing human UTIs; or they could serve as a genetic pool for ExPEC strains (Ron, 2006; Ewers *et al*., 2009; Vincent *et al.,* 2010; Belanger *et al*., 2011; Manges & Johnson, 2012). Additionally, there is a well-documented history of transfer of *E. coli* strains and their plasmids from poultry to humans (Ojeniyi, 1989; Van Den Bogaard *et al.,* 2001), and one report has demonstrated that APEC plasmids can contribute to the urovirulence (cause of disease in the urinary system) of *E. coli* for mammalian hosts (Skyberg *et al.,* 2006). Virulence factors of *E. coli* include the ability to resist phagocytosis, utilization of highly efficient iron acquisition

systems, resistance to killing by serum, the production of colicins, and adherence to respiratory epithelium (Dziva & Stevens, 2008; Tufani *et al.,* 2011; Ahmed, 2013).

In poultry, *E. coli* causes colibacillosis (a collective term for several diseases such as pericarditis, perihepatitis, peritonitis, airsacculitis, septicemia and other mainly extraintestinal diseases) which is a widespread disease and is responsible for economic losses in the poultry industry globally. Infected chickens could be the source of animal disease from avian origin, because ExPECs cause extraintestinal disease, share virulence-associated traits, and have overlapping O serogroups and phylogenetic types (Barnes *et. al.,* 2003; Rodriguez-Siek *et. al.,* 2005; Ron, 2006; Fasaei *et al*., 2009; Lutful Kabir, 2010; Ghunaim *et al*., 2014; Jajarmi *et al.,* 2017). These economic losses are caused by increased mortality, increased carcass condemnation rates at the time of processing, decreased growth rate, and decreased feed conversion efficiency of affected birds. In 2000, APEC was responsible for 45.2% of condemned poultry carcasses in Brazil, which is the world's largest exporter of chicken meat (Fallavena *et al*., 2000). In addition to its negative economic impact, APEC is also considered a major source for spreading antimicrobial resistance to other bacteria (mainly through their plasmids) and the exchange of other genetic material (Gyles, 2008). This is prevalent in Europe, the USA and Australia, where up to 92% of avian *E. coli* isolates were found to be resistant to three or more antimicrobial drugs despite strict measures on antibiotic use in the poultry industry (Gyles, 2008).

The most prevalent bacteria in the intestinal tract in warm-blooded animals and humans is *E. coli*. They constitute approximately 10^6-10^9 CFU.g^{-1} of stool and can easily contaminate food products during animal evisceration after slaughter through contact with tainted water or during food handling (Alvarez-Fernandez *et al.,* 2013). *E. coli* is also considered as a major zoonotic agent, as it can be involved in intestinal and extra-intestinal infectious diseases (Lei *et al*., 2010).

2.5 Assays and Techniques Used for the Identification of *E. coli*

Serotyping is based on the fact that strains of the same species can differ in the antigenic determinants expressed on the cell surface. Surface structures such as lipopolysaccharides, membrane proteins, capsular polysaccharides, flagella and fimbriae display antigenic variations. Strains differentiated by antigenic differences are said to be serotypes. Serotyping for several Gram-negative and Gram-positive bacteria is performed using a number of serologic assays such as bacterial agglutination, latex agglutination, co-agglutination, and fluorescent and enzyme-labelling assays.

Of the 181 numbered *E. coli* O antigens, only a small subset is associated with disease in humans (Nataro & Kaper, 1998). According to the modified Kauffman scheme, *E. coli* is serotyped (serotype is distinct variations within a species) on the basis of their O (somatic), H (flagella), and K (capsular) surface antigen profiles (Lior, 1996). As many as 170 different O antigens, each defining a serogroup (group of serotypes/serovars with common antigens), are recognized currently. The presence of K-antigens was originally determined by means of bacterial agglutination tests. It was argued that an *E. coli* strain that was inagglutinable by O antiserum but became agglutinable when the culture was heated could be considered to have a K-antigen.

However, the discovery that several different molecular structures, including fimbriae, conferred the K phenotype led experts to suggest restructuring the K-antigen designation to include only acidic polysaccharides (Lior, 1996). Proteinaceous fimbrial antigens have therefore been removed from the K series and have been given F designations (Orskov *et al.,* 1982).

A specific combination of O and H antigens defines the serotype of an isolate. *E. coli* of specific serogroups can be associated reproducibly with certain clinical syndromes, but it is not in general the serologic antigens themselves that bestow virulence. Rather, the serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Whittam *et al.,* 1993). Molecular (PCR) and immunological (ELISA) methods have been described for the identification and serotyping of *E. coli* (McCarthy, 2003; Shaw & Bosley, 2005; Warburton, 2005; Kim *et al.,* 2006).

In 2017, Harmse and co-workers documented that there was a presence of hazardous biological agents in poultry abattoirs such as bacteria (Harmse *et al.,* 2017). Furthermore, all the production areas showed high levels of microbial agents above the permissible human infectious dose. The receiving, shackling and killing areas had the highest counts for the following species: *Pseudomonas*, *Listeria*, *Salmonella* and *Bacillus* (Harmse *et al*., 2017). Coliforms, including *E. coli,* were also found to occur throughout several production areas (Bohaychuk *et al*., 2009; Craven *et al*., 2000; Lindbald *et al*., 2006; Lutgring *et al*., 1997; Nonnenmann *et al*., 2010). However, the listed organisms had been identified by these studies only to species level, and it was therefore deemed vital to evaluate the methods used to serotype

strains of *E. coli* in poultry abattoirs, especially in wastewater that can be a reservoir of such pathogens.

2.5.1 Molecular identification

Molecular serotyping assays (MSAs) are based on the characteristics provided by the nucleic acid sequences of the microbial genome (Sanderson & Nichols, 2003), whereas all other methodologies rely on the expression of the phenotypic characteristics of the bacteria (Fung, 2002). Molecular identification uses MSAs for the identification of the organisms and they have also been applied for the verification of *E. coli* species. Although there is no 'gold standard' for the standardization of MSAs, some of these methodologies have been applied as standard methods, for example the Warnex™ semi-quantitative real-time PCR system and PCR (Shaw & Bosley, 2005). MSAs are highly specific and their specificity is determined by the selection of the primer pairs and the probes (Scheu *et al.*, 1998). Primers have been developed to indicate the presence of bacteria (McCabe *et al.*, 1999), but the level of specificity can be chosen for genus level, species level and serotype level. These methods can be applied to determine bacterial serotypes in addition to the immunological serotyping methods of microorganisms. Due to their specificity, some MSAs do not require the isolation and purification of the target microorganism prior to testing (Bettleheim & Beutin, 2003; Bell *et al.*, 2005). However, it should be noted that high concentrations of other microbiota can have an influence on the sensitivity of the PCR (Ramesh *et al.*, 2002). MSAs play an important role in the discrimination of microbial strains, even at levels lower than species level, during food-borne disease outbreak investigations (Barrett *et al*., 1994). These methods have been applied in the diagnosis of patients

suffering from food-borne illnesses during outbreaks, and they have also been used to test food and environmental samples to trace the source that caused the disease (Swaminathan *et al.*, 2001).

2.5.1.1 Polymerase chain reaction (PCR)

One of the most popular MSAs applied in the food industry is the polymerase chain reaction (PCR). This method forms the basis for most of the nucleic acid-based techniques and is also applied for rapid screening of microorganisms in samples from various origins (Merk *et al.*, 2001). Amplified fragments can also be verified by various methods, such as the ELISA combined with PCR (PCR-ELISA) southern blotting, and DNA-hybridisation and sequencing can also be applied to confirm the amplified DNA product (Fratamico *et al.*, 1995; Mo & Wang, 1997; Lehmacher *et al.*, 1998; Scheu *et al.*, 1998; Meyer, 1999; Hébert *et al.*, 2000; Gilligan *et al.*, 2000; Fach *et al.*, 2003; Mairena *et al.*, 2004). Another method, which is a modification of PCR applied for the verification of bacteria, is real-time PCR. With this method, the amplification of DNA is monitored in real time by the detection of probes that bind to the amplified DNA and no further detection or visualization of the amplified DNA fragments is required (Jothikumar *et al.*, 2003; Palomares *et al.*, 2003). This method can further be applied for the quantification of the amplified DNA (Roussel *et al.*, 2005).

Various successful applications of MSAs to verify the presence of food-borne organisms at species level, including *E. coli,* have been repeated in the past years (Agersborg *et al.*, 1997; Gouws *et al.*, 1998; Denis *et al.*, 2001; Hudson *et al.*, 2001; Agarwal *et al.*, 2002; Ramesh *et al.*, 2002; Bhaduri, 2003; Fratamico, 2003; Martín *et al.*, 2003; Jofré *et al.*, 2005; Isonhood *et al.*, 2006; Myint *et al.*, 2006). For example, different genes have been used to identify species in sexual organisms using population genetic theory and DNA sequences (Birky, 2013). In addition, Ramesh *et al.* (2002) applied multiplex PCR for the verification of *Staphylococcus aureus* and *Yersinia enterocolitica* from milk samples by the simultaneous amplification of the *nuc* (nuclease) and *ail* (virulence-associated attachment invasion locus) genes. *Yersinia enterocolitica* was also verified by Bhaduri (2003) by amplifying the *virF* (from the virulence plasmid) and *ail* genes. The amplification of various genes has also been targeted for *E. coli* serotype verification because testing for an organism up to species level has been found to be inadequate. It is therefore necessary to verify up to serotype level because there are different/multiple *E. coli* serotypes that are pathogenic.

The amplification of *stx* (Shiga toxins) and *eae* (intimin) genes, however, only verifies the presence of STEC and the intimin gene respectively and not the specific serotype (Guan & Levin, 2002). For this purpose, genes from the O-antigen (as they are the somatic antigens) gene cluster, such as the *wzy* (O-antigen polymerase) gene, contain sequences that are specific to the serotypes (Feng *et al.*, 2005). The O-antigen, which is part of the lipopolysaccharide (LPS), is a repeat unit polysaccharide that is an integral component of the Gram-negative bacterial outer membrane (Samuel & Reeves, 2003). The genes encoding the O-antigens are

usually located in a cluster containing three groups of genes that are involved in the nucleotide sugar biosynthesis, sugar transfer (glycosyl transferases) and O-antigen processing.

The advantages of PCR include high sensitivity *in situ* detection of target templates, it is not laborious, and it is highly specific. However, substances in faeces have been shown to interfere with the PCR, thus decreasing its sensitivity and making detection without prior isolation difficult (Panutdaporn *et al.,* 2004). Although several methods can be used successfully to remove such inhibitors (e.g., phenolic compounds, glycogen, fats, cellulose, constituents of bacterial cells, non-target nucleic acids and heavy metals) (Barnard *et al*., 2011), the additional processing will escalate expenditure for detection.

Table 2.2: Selected MSAs used for species detection

Method	As described by
PCR	Lorenz, 2012
Real Time PCR (RT-PCR)	Fraga *et al.,* 2008
Warnex™ semi-quantitative RT-PCR system	Shaw & Bosley, 2005
Multiplex PCR	Zheng *et al*., 2014
Reverse transcriptase PCR	Bürgmann *et al.,* 2003
Colony Hybridization	Hill *et al.,* 2001
DNA Hybridization − Hydrophobic-Grid Membrane Filter (HGMF)	Kaboré *et al*., 2009
PCR − ELISA for STEC detection	Ge *et al.,* 2002
Protein Profile	Docter *et al*., 2014
Plasmid Profile	Khadgi *et al*., 2013
Pulse Field Gel Electrophoresis (PFGE)	Herschleb *et al*., 2007
Ribotyping	Martinson *et al*., 2015
PCR − Restriction Fragment Length Polymorphism	Ota *et al*., 2007
Amplified Fragment Length Polymorphism	Fry *et al*., 2009

2.5.2 Immunological identification

Immunological serotyping assays (ISAs) depend on the interaction between antibodies and antigens from the intended organism (Baylis, 2003). One of the best known and most generally applied ISAs is the enzyme-linked immunosorbent assay (ELISA). This method has become available as a kit and some kits have also been accepted as standardized methods. The Meridian Premier EHEC kit (Meridian

Diagnostics) and the Assurance EHEC immunoassay (BioContol Systems Inc.) are examples of such kits (Warburton, 2005 & 2006). Although these methods are rapid to perform, multiple washing stages make them labour-intensive.

Lateral flow devices (LFDs) are also ISAs that are applied for the identification of *E. coli.* These LFDs are membrane-based tests that rely on immunochromatography, which is a combination of chromatography and antigen-antibody reaction. These test devices have been applied for food testing, and commercial kits are available for specific strains such as *E. coli* O157:H7, while the DuopathVerotoxins (Merck) are available for the detection of Shiga toxins (Stx1 [VT1] and Stx2 [VT2]) (Baylis, 2003).

Another ISA includes the agglutination test, where the interaction between the antigen and the subsequent antibody produces macroscopic clumping (Baylis, 2003). Kits are commercially available for the detection of specific pathogens such as *E. coli* O157:H7, and also for the detection of toxins produced by microorganisms such as the Verocytotoxigenic *E. coli*-Reverse Passive Latex Agglutination (VTEC-RPLA) kit available from Oxoid for the detection of *E. coli* toxins (Chart *et al.*, 2001; Oxoid, 2006).

Agglutination was first observed by Gruber and Durham when the serum antibody was found to react with the bacterial cells. The agglutination test was originally developed for the detection of antibody to *Brucella* (Wright & Smith, 1897). To date, a great deal of work has been done to improve diagnostics. Primarily, agglutination assay is the clumping of bacteria, erythrocytes, or cells due to the introduction of an antibody. This assay can be used either qualitatively or quantitatively. In the

qualitative method, the antibody is mixed with the particulate antigen and a positive test is indicated by the clumping of the particulate antigen. Agglutination can also be tested indirectly in a quantitative method where instruments such as a spectrophotometer and nephelometer are used to measure absorbed or scattered light from a very sensitive microsphere due to lattice/turbidity that is formed by antibody and antigen.

2.6 The strains of interest

The strains that were both pathogenic to poultry and humans were chosen based on information in the literature. The O1:K1:H7 strain (Mora *et al*., 2009), the O15:K52:H1 strain (Johnson *et al*., 2002), and the O18:K1:H7 strain (Johnson & Stell, 2000; Moulin-Schouleur *et al*., 2006) were selected. For molecular serotyping, the targeted genes were the flippase gene (*wzx*), the fumerase gene (*fumC*), the flagellin gene (*fliC*), and neuC.

2.7 Advantages of mPCR

Multiplex PCR has been firmly established as a general technique of choice because it has been shown that it can amplify multiple loci in the human dystrophin gene (Edwards & Gibbs, 1994). Internal controls and template quality and quantity are some of the advantages when using mPCR, and it is also efficient because the cost of reagents and preparation time are lesser in multiplex PCR than in systems where several tubes are used (Edwards & Gibbs, 1994). The most recent application of this technique was when it was used for the simultaneous detection of six sexually transmitted diseases (Vica *et al*., 2016). The inclusion of restriction enzymes makes

it even more advantageous in the mPCR because it can cleave DNA at specific sequences (https://www.addgene.org/protocols/restriction-digest/).

This study aimed to include the following as reference strains: O1:K1:H5, O15:K52:H1 and O18:K1:H7. Selective enrichment was also used for the reduction of competing organisms and this had an influence on the duration of the identification process. This led to the evaluation of the specificity and sensitivity of PCR on reference strains and finally the application of a multiplex PCR/restriction enzyme digestion (mPCR/RED) approach for identifying the *E. coli* strains from both on-field isolates and environmental samples (wild-type). The molecular assay was followed by the evaluation of specificity and sensitivity of immunological assays on reference strains.

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CHAPTER 3

Application of a multiplex PCR/Restriction enzyme digestion (mPCR/RED) approach for identifying *E. coli* serotypes O1:K1:H7, O15:K52:H1 and O18:K1:H7

3.1 Introduction

Microbial isolate identification is an important tool to use for the characterization of an organism using phenotypic and genotypic characteristics (Tang *et al*., 1998). Phenotypic characteristics mostly identify microorganisms up to species level. Understanding these characteristics allows the research world access to the body of knowledge that exists about a particular species (Mora *et al*., 2011). For example, knowledge is gained about economically important pathogens for humans or animals or about those that cause spoilage in food products or are the sources of a contaminant. This field of study is always growing because the ability to investigate the genetic relationship between isolates has been sustained in many communications (Whatmore *et al*., 2000; Jarraud *et al*., 2002).

Because phenotypic characteristics are the products of gene expression, it is possible to identify microorganisms using the information from microbial genomes using molecular assays to identify the genotypic characteristics (Emerson *et al*., 2008). Sequencing specific regions (genes) of the ribosomal RNA such as the sequenced genes by PCR can be compared to information that is presented in the database (Emerson *et al*., 2008). Using 16S rRNA as an example, many different species share the same 16S sequence. Therefore, the accuracy of PCR is highly dependent on the quality of the database against which the sequence is compared to (Wallon *et al*., 2010).

Many techniques have been used previously for species typing. The Kauffmann-White scheme for typing *Salmonella* species (about 2500 serovars) is a well-known technique in this regard (Wattiau *et al*., 2011). Others include phage typing, biotyping, bacteriocin typing, protein typing, and genotyping (Sridhar Rao, 2006).

With genotyping, amplification of genes by PCR has led to an explosion of published molecular assays over the past 25 years (Sobel *et al*., 2008). This technique has been widely recognised, not only in clinical microbiology, but in the pharmaceutical sector as well. A variety of techniques can be used for analysing extracted DNA and many techniques have been developed for typing. Some of the widely used methods include Pulsed-field gel electrophoresis (PFGE), Multilocus sequence typing (MLST), Multilocus variable number of tandem repeats analysis (MLVA), Ribotyping, DNA Microarrays, and multiplex PCR (mPCR) (Borucki *et al*., 2004). Multiplex PCR is an advantageous method due to its proficiency to provide more information with less sample. Moreover, it has high throughput, is cost effective and time saving, has increased accuracy of data analysis, and also performs well in a closed setting to decrease the possibility of contamination (Edwards & Gibbs, 1994). Numerous primer sets are described in the literature that are able to selectively amplify specific antigens or even strains of selected APEC *E. coli*. However, these primer sets have not been combined in multiplex PCR reactions to detect multiple strains simultaneously, which will allow molecular serotyping to be less tedious and time-consuming. Apart from optimization, PCR product length similarities in a multiplex approach can also be a hurdle.

Therefore, the aim of this chapter is to describe the use of a multiplex PCR approach followed by restriction enzyme digestions to differentiate among three laboratory APEC strains (O1:K1:H7, O15:K52:H1 and O18:K1:H7) that can be transferred from chickens to humans.

3.2 Materials and Methods

3.2.1 Cultivation of *E. coli* strains

3.2.1.1 Reference strains

The *E. coli* (reference strains) used in this study (O1:K1:H7, O15:K52:H1 and O18:K1:H7) were smooth strains supplied by Professor Johnson from the Veterans Administration Medical Center (Minnepolis, USA) (Shi *et al*., 1996; Cagnacci *et al*., 2008). The strains were transported as stab cultures in nutrient agar on dry ice. Cells were harvested from the stab cultures using an inoculation loop and transferred to sterile Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride) and incubated at 37ºC with shaking (180 rpm) for 12 hours. Revived strains were transferred to LB agar plates and incubated at 37ºC for 12 hours. Each strain was also grown on Violet Red Bile Agar with 4-Methylumbelliferyl ß-D-glucuronide (MUG) (Biolab Diagnostics) and viewed under UV light to confirm its identity as *E. coli*. Revived strains were also aliquoted in 2 ml Eppendorf tubes and centrifuged for 5 min at 5000 rpm. The supernatant was discarded and the cells were resuspended in 40% final glycerol concentration solutions and stored at -20ºC.

3.2.1.2 Field isolates

Wastewater/effluent was collected from a poultry abattoir, diluted, pipetted onto Chromocult® Coliform Agar (peptone 3 $g.l^{-1}$, sodium chloride 5 $g.l^{-1}$, sodium dihydrogen phosphate 2.2 $g.l^{-1}$, disodium hydrogen phosphate 2.7 $g.l^{-1}$, sodium pyruvate 1 $g.l^{-1}$, tryptophan 1 $g.l^{-1}$, sorbitol 1 $g.l^{-1}$, chromogenic mixture 0.4 $g.l^{-1}$, tergitol®7 0.15 $g.l^{-1}$ and agar 10 $g.l^{-1}$) (Merckmillipore) and spread on the agar plate using a sterilized hockey stick. The plates were incubated overnight at 37ºC. Seven

individual purple colonies on the Chromocult® Coliform Agar were randomly picked from different plates with the inoculation loop and resuspended in 1 ml of ultrapure water. The cell suspensions were later used as template in the optimized multiplex PCR protocol.

3.2.1.3 Environmental sample

Duplicate plates (3.2.1.2) were covered with 1 ml ultrapure water which was spread over the plate with a sterilized hockey stick to collect the entire colony growth on the surface of the agar plate. The cell suspensions were aspirated and 1 µl was used as whole cell template in optimized multiplex PCR assay.

3.2.2 Procedure used to prepare inoculums

Reference strains preserved in 40% glycerol at -20^{o}C were transferred to sterile LB broth (100 ml broth in a 500 ml Schott bottle) using a sterile inoculated loop. The bottle was incubated at 37ºC for 16 hours with shaking (180 rpm). This culture served as pre-inoculum of which 100 µl was transferred to a new bottle containing 100 ml sterile LB media. The freshly inoculated culture was incubated at 37ºC for 5 hours with shaking (180 rpm). This culture served as the inoculum (Figure 3.1).

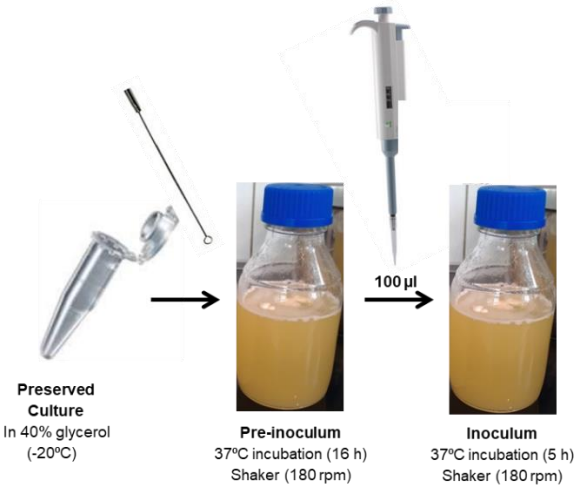

Figure 3.1: The preparation of inoculum from preserved cultures (in 40% glycerol at -20°C) and incubated for 5 hours (37°C) with shaking by firstly preparing pre-inoculum incubated (37°C) with shaking for 16 hours.

3.2.3 Bacterial cultivation on agar plates

The inoculum of the reference strains (O1:K1:H7, O15:K52:H1 and O18:K1:H7) was prepared as described in Figure 3.1. From the inoculum, a sterile inoculation loop was used to streak for single colonies isolation on the LB agar plates and incubated overnight at 37°C.

3.2.4 Genomic material (gDNA) isolation from LB agar plate

To extract genomic DNA, the RTP® Bacteria DNA Mini Kit (© QIAGEN 2013) was used according to the protocol for the organisms (O1:K1:H7, O15:K52:H1 and O18:K1:H7) as described in the manufacturer's instruction manual. Isolated single colonies (cells) after overnight incubation (at 37ºC) on LB agar plate were harvested with a sterilized inoculation loop (⅓ full) and suspended in sterile 1 500 µl distilled water and mixed thoroughly by vortexing. This was followed by centrifugation at

10 000 rpm for 3 minutes. Supernatant was aspirated without disturbing the pellet, and 400 µl of resuspension buffer (resuspension Buffer R) was added to the pellet and resuspended by an up and down pipetting motion. The resuspended sample was transferred to the extraction tube (extraction tube L) and vortexed briefly. The sample was placed in a 65ºC water bath for 10 minutes with periodic vortexing. The extraction tube was then transferred to 95ºC hotplate and incubated for further 10 min with periodic vortexing. Binding Buffer B6 (400 µl) was added to the sample followed by brief vortexing. The sample was loaded onto a RTA Spin Filter Set and incubated at 25°C for 1 min, then centrifuged at 12 000 rpm for 1 min. The filtrate was discarded. Bound DNA was washed twice with wash buffers I (400 µl) and II (600 µl) respectively. After washing the bound DNA, 3 min centrifugation at maximum speed was included as a final step for ethanol removal. The RTA Spin Filter was then placed into a new 1.5 ml Eppendorf tube and eluded the bound DNA with 100 µl of elution buffer (Elution Buffer D). The samples were stored at 4ºC until used. To confirm that stored gDNA was intact and that sufficient gDNA had been extracted, 0.8% (w/v) agarose gel electrophoresis with ethidium bromide was used for analysis. Electrophoresis was performed at 85 V for 30 minutes and visualized under UV light.

3.2.5 Pre-optimized multiplex PCR conditions and primer specificity

The primer pairs listed in Table 3.1 targeting the *wzx-1*, *neuC*, *fliC*, *wzx-2* and *fumC* genes (Integrated DNA Technologies, Inc. [IDT]) were amplified individually (single PCR reaction) as selective markers for the O1, K1, H7, O18 antigens and the O15:K52:H1 strain. The 16S rRNA gene was included as an internal reaction control

gene for the *E. coli* ThermoPol® buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.52 µM primers, 0.2 mM dNTP mixture, 1U of *Taq* DNA polymerase (New England BioLabs Inc.) and with 2% (v/v) of bovine serum albumin (BSA) used as a reagent to increase product yield in the reaction. The reaction conditions consisted of an initial denaturation cycle of 94°C for 10 min, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 68°C for 180 sec, followed by final extension of 68°C for 10 min. The products were analyzed by electrophoresis on 1% (w/v) agarose with ethidium bromide at 90 V for 45 min and visualized under UV light.

Table 3.1: Oligonucleotide primer sequences for the reference strains used in this study

Primer Name	Target Gene	Tm	Amplicon size (bp)	Sequence (5'-3')	Target Antigen/Strain	Reference
wl-14632F wl-14633R	*wzx-1*	54,9°C 54,8°C	1098	GTGAGCAAAAGTGAAATAAGGAACG CGCTGATACGAATACCATCCTAC	O1	Momtaz *et. al.,*2013
wl-14656 wl-14657	*wzx-2*	55,6°C 56,2°C	551	GTTCGGTGGTTGGATTACAGTTAG CTACTATCATCCTCACTGACCACG	O18	Momtaz *et al.,*2013
fumCF fumCR	*fumC*	68,4°C 47,4°C	138	GCTGCTGGCGCTGCGCAAGCAA CCGGAAATCTCCTGT	O15:K52:H1	Johnson *et al.,* 2004
H7f H7r	*fliC*	58,4°C 59,1°C	550	ACGATGCAGGCAACTTGACG GGGTTGGTCGTTGCAGAACC	H7	Zhao *et al.,*2009
neu1 neu2	*neuC*	57,2°C 59,6°C	676	AGGTGAAAAGCCTGGTAGTGTG GGTGGTACATCCCGGGATGTC	K1	Moulin-Schouleur *et al.,* 2006
27F 1392R	16S rRNA	55,2°C 57,4°C	1365	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	*E. coli*	Srinivasan *et al*., 2015

3.2.6 Optimization of multiplex-PCR on reference strains' gene products

Dimethyl sulfoxide (DMSO) replaced the BSA in the multiplex-PCR (mPCR) reactions and was used as a reagent for increasing product yield (Hardjasa *et al*., 2010). A gradient PCR (52°C-58°C) was performed on the C1000™ Thermal Cycler (BioRad, Singapore) to find the most suitable annealing temperature for each primer set. Touchdown mPCR (TdPCR) where reactions with two separate cycles were used was also adopted for the optimization. Working concentrations for reaction constituents were adjusted as follows: dNTPs from 0.2 mM to 0.3 mM, genomic DNA (template) from 1:50 dilution to 2:50 dilution, primers from 0.5 µM to 1 µM, *Taq* DNA polymerase from 1 U to 10 U, and $MgSO_4$ from 1.5 mM to 2 mM in individual reaction mixtures of 50 µl volumes. The thermal cycling program consisted of initial denaturation for 3 min at 94°C followed by TdPCR. The first cycle was 30 s denaturation (at 94°C), 45 s annealing (63°C), and 210 s extension (at 68°C) for 10 cycles. Before final extension, another 20 cycles of 30 s denaturation (at 94°C), 45 s annealing (at 58°C), and 210 s extension (at 68°C) followed with a final extension step at 68°C for 10 min. Amplicons were analyzed by electrophoresis on 1% (w/v) agarose with ethidium bromide at 90 V for 45 min and visualized under UV light.

3.2.7 Purification of DNA fragments for sequencing

The GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience, USA) protocol was followed for the purification of DNA from the gel. Bands were excised with the X-Tracta™ Agarose Gel Extraction Tool (LabGadget, Illinois) on a Dark Reader® (Clare Chemical Research), transferred to a pre-weighed 2 ml Eppendorf tube, and weighed. The weighed gel slices (79 mg for *fumC*, 90 mg *fliC*, 88 mg for

neuC, 92 mg for *wzx-2* and 83 mg for *wzx-2*) were transferred to individual 1.5 ml Eppendorf tubes. For each 10 mg of gel slice, 10 µl of capture buffer was added to the gel slices in the Eppendorf tubes and vigorously mixed by vortexing. The mixture was incubated in a water bath at 60°C until the gel agarose had completely dissolved (5-15 minutes). After the gel slice had completely dissolved, the liquid was centrifuged through a GFX column. The filtrate was discarded and the column washed with wash buffer (500 µl). The DNA was eluded by applying 50 µl of elution buffer. Extracted DNA was stored at -20°C until used.

3.2.8 Sequencing and analysis

PCR products purified from the gel were used as templates for sequencing. Sequencing was performed on both the sense and antisense strands on the ABI Prism 3130 XL genetic analyser using the Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). PCR was performed on the C1000™ thermal cycler (BioRad) with the same primers that had been used for generating the amplicons (25 cycles: 30 s at 94°C, 30 s at 63°C, 180 s at 68°C). DNA was precipitated with 25 mM EDTA and 70% ethanol. The sequences obtained were compared to the NCBI GenBank Database (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm for identification.

3.2.9 Selection of restriction enzymes and their application on the optimized mPCR product

Clustal Omega, EMBL-EBI (http://www.ebi.ac.uk) was used to perform multiple sequence alignments of sequenced DNA and database sequences of interest. In-silico restriction mapping was performed on sequenced products using NEBcutter V2.0 software (New England BioLabs) to allow and provide a predicted digestion pattern for all commercially available restriction enzymes. Restriction map analysis was carried out and the predicted restriction profiles were determined for each of the sequenced genes with eight enzymes: *Bfa*I, *Bsr*DI, *BstA*PI, *Bst*BI, *Mnl*I, *NmeA*III, *Rsa*I and *Sna*BI (New England Biolabs, USA).

3.2.10 Application of the optimized multiplex-PCR on the gene products of the reference strains

The optimization mPCR described in section 3.2.6 was followed for both genomic DNA material and whole cell as templates. The mPCR reaction was as follows: 16S rRNA gene (included as an internal reaction control gene for the *E. coli*), ThermoPol® buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.52 µM primers, 0.2 mM dNTP mixture, 10U of *Taq* DNA polymerase (New England BioLabs Inc.) and with 2% (v/v) DMSO. Both genomic DNA and whole cell products were analyzed by electrophoresis on 1% (w/v) agarose with ethidium bromide at 90 V for 45 min and visualized under UV light.

3.2.11 One-step digestion of optimized mPCR with BstAPI restriction enzyme

Optimized mPCR products were digested using one-step digestion (addition of restriction enzyme at only one stage) with restriction enzyme following the New England BioLabs protocol (Mizani *et al*., 2017). Optimized mPCR product (10 µl), 5 µl of 1X reaction (CutSmart®) buffer (50 mM Potassium Acetate, 20 mM Tris-Acetate, 10 mM Magnesium Acetate, 100 µg.ml^{-1} BSA, pH 7.9 at 25°C), 0.5 µl of selected restriction enzyme (*Bst*API) were mixed together, and distilled water was added to adjust to the volume to a final volume of 50 µl in a PCR reaction tube. The tube was incubated for 1 hour at 60°C. One-step digestion was analyzed by electrophoresis on 2% (w/v) agarose with ethidium bromide at 85 V for 40 min and observed under UV light.

3.3 Results and discussion

3.3.1 Bacterial isolation

The bacterial growth of the three reference strains appeared as smooth, shiny, creamy white, circular colonies with smooth edges (filiform), convex elevation and a repelling odour when cultivated on an LB agar plate (Madigan *et al.,* 2010). When cultivated on Violet Bile Red with MUG, all the reference strain colonies appeared red and emitted blue fluorescence when observed under UV light (Leclercq *et al*., 2002). In contrast, non-*E. coli* colonies produced red non-fluorescing colonies under UV light (Villari *et al*., 1997; Dogan *et al*., 2002).

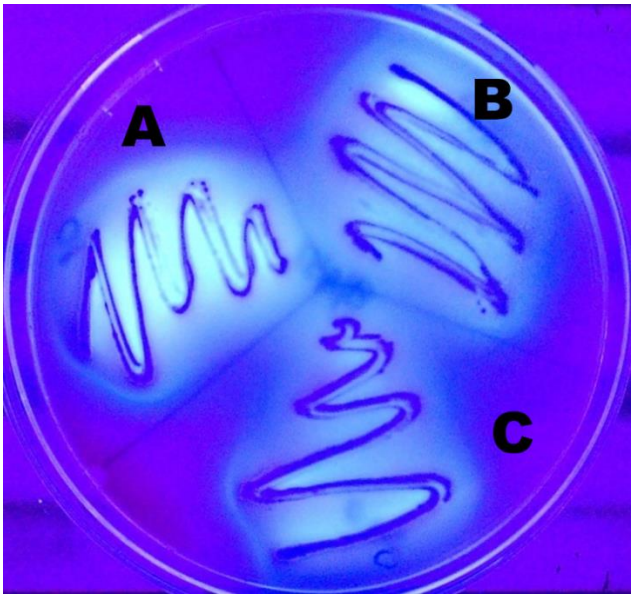

Figure 3.2: Reference stains O1:K1:H7 (A), O15:K52:H1 (B) O18:K1:H7 (C) cultivated on Violet Red Bile with MUG agar showing blue fluorescence light when observed under UV light.

The modified Solty's preservation method with 40% glycerol was used to preserve the cultures and all could be revived without incident.

3.3.2 gDNA yields

The gDNA yield of all three reference strains (O1:K1:H7; O15:K52:H1 and O18:K1:H7) was high and intact as was be observed on the 0.8% (w/v) agarose gel (Figure 3.3).

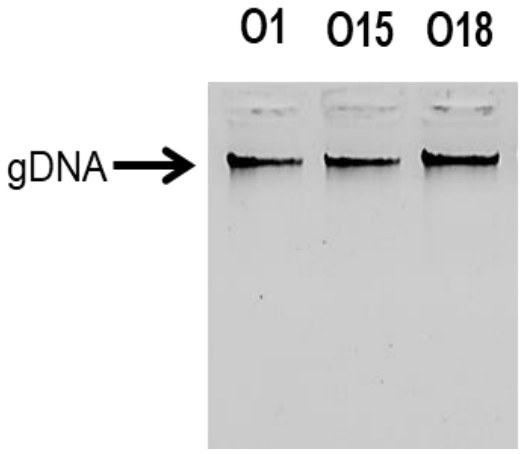

Figure 3.3: gDNA yield for O1 (O1:K1:H7), O15 (O15:K52:H1) and O18 (O18:K1:H7) separated in a 0.8% agarose gel (85 V for 30 minutes) showing sufficient gDNA without any degradation.

3.3.3 Primer specificity analysis

Successful amplification was confirmed for each reaction by the presence of a ≈1 300 bp fragment that represented the 16S rRNA internal control. Figure 3.4 depicts the PCR products produced by each primer pair listed in Table 3.1 used in single reactions (one primer pair and DNA from one reference strain). The expected 138 bp band for *fumC* was only amplified when O15:K52:H1 was included as DNA template (Figure 3.4, *fumC* lane B). No amplification was detected for O1:K1:H7 or O18:K1:H7. The *fliC* gene (550 bp) representative of H7 was amplified from O1:K1:H7 and O18:K1:H7 (Figure 3.4, *fliC* lanes A & C) and no product was obtained from O15:K52:H1. The *neuC* gene (676 bp) represented K1 and yielded results similar to *fliC*. The *wzx-1* and *wzx*-2 genes served as specific targets for O1 and O18 respectively. Primer pair specificity was confirmed by the presence of a 1098 bp only from O1:K1:H7 DNA (Figure 3.4, *wzx-1* lane A) and similarly a 551 bp fragment was observed only when O18:K1:H7 DNA was included as template

(Figure 3.4, *wzx-2* lane C). The *fumC* primer pair showed non-specific binding where ≈1200 bp and ≈700 bp fragments were also detected. This was not entirely unexpected as the fumC forward primer melting temperature of 68.4^{o}C was much higher compared to that of the other primers. To overcome this problem, TdPCR was adopted by increasing the initial annealing temperature for 10 cycles, followed by 20 cycles at a lower annealing temperature 58^{o}C (Hecker & Roux, 1996).

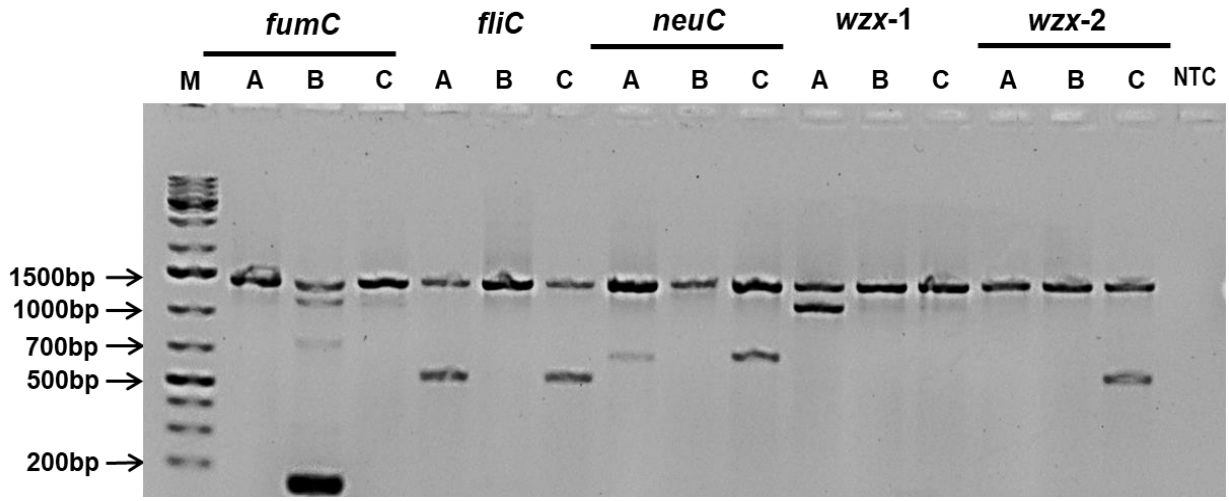

Figure 3.4: Single primer pair amplification of five genes; *fumC* (138 bp), *fliC* (550 bp), *neuC* (676 bp), *wzx-1* (1098 bp), and *wzx-2* (551 bp) in from *E. coli* reference strains − Lanes A (O1:K1:H7), B (O15:K52:H1) and C (O18:K1:H7). The 16S rRNA internal reaction control amplicon is represented by the ≈1300 bp present. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific) and the non-template control respectively.

3.3.4 Pre-optimized multiplex-PCR on the gene products of reference strains

Before modifying the PCR conditions, the primer pairs were tested using a multiplex approach. All five primer pairs for the targeted genes as well as the 16S rRNA internal control primer pair were pooled and used in single reactions. DNA from each

reference strain and all three combined strains were used as templates in separate reactions (Figure 3.5). Amplification profiles for O1:K1:H7, O15:K52:H1, O18:K1:H7 were as expected (Figure 3.5 lanes 1, 2 & 3), with the same non-specific binding still visible for *fumC* in O15:K52:H1. Not all the targeted genes were amplified when reference strain DNA was pooled and included as a template (Fig 3.5, lane 4). The internal control (≈1 300 bp) and *fumC* (138 bp) failed to amplify, presumably due to reaction competition (Hamajima *et al*., 2002). Systematic optimization was therefore required to achieve optimum amplification of all targeted genes in complex reaction conditions. Furthermore, the intense band present at ≈550 bp in lane 4 demonstrated that the amplification products of *fliC* (550 bp) and *wzx-2* (551 bp) could obviously not be resolved by agarose electrophoresis and thus required secondary processing for conclusive confirmation.

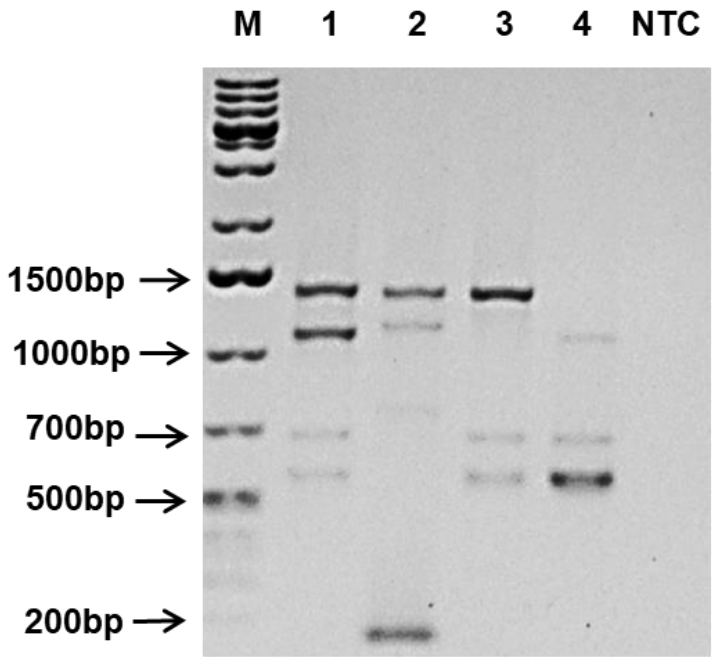

Figure 3.5: Multiplex PCR targeting all genes: *fumC* (138 bp), *fliC* (550 bp), *neuC* (676 bp), *wzx-1* (1098 bp), and *wzx-2* (551 bp). Template DNA from O1:K1:H7 (lane 1), O15:K52:H1 (lane 2), O18:K1:H7 (lane 3) as well as all three pooled strains (lane 4) were used. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific) and the non-template control respectively.

3.3.5 Multiplex-PCR optimization

PCR conditions were adapted to the TdPCR described in section 3.2.6. Figure 3.6 illustrates the resulting multiplex PCR amplification obtained with the different concentrations of template (gDNA), dNTPs, Taq polymerase, primers and $MgSO_4$ and showed that increasing the concentration of Taq was the only parameter that overcame competition and improved the sensitivity of detecting the five targeted genes in a multiplex approach (Figure 3.6, lane 3) (Roux, 2009; Apte & Daniel, 2009).

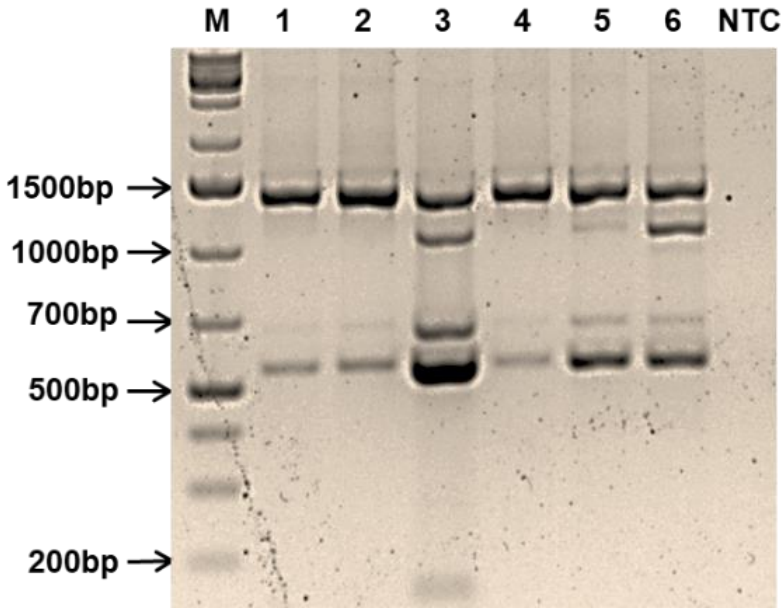

Figure 3.6: Representative gel of multiplex PCR demonstrating optimization attempts. Amplification of target genes *fumC* (138 bp), *fliC* (550 bp), *neuC* (676 bp), *wzx-1* (1098 bp) and *wzx-2* (551 bp) and internal control 16S rRNA gene (≈1300 bp) is shown. DNA from reference strains O1:K1:H7, O15:K52:H1 and O18:K1:H7 were pooled and used as template. Lane 1: increased concentrations of DNA template (4% v/v); lane 2: dNTPs (0.3 mM); lane 3: *Taq* polymerase (10 U); lane 4: primer pairs (1 µM); lane 5: $MgSO_4$ (2 mM); and in lane 6: all changes were incorporated in a single reaction. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific) and the non-template control respectively.

Optimized mPCR protocol was applied using gDNA and whole cells as template. Being able to use a whole cell means that a direct PCR approach for the detection of specific *E. coli* serotypes will be more economical and practical if the protocol is to be applied on field isolates. All target genes were detected using either DNA sources as template (Figure 3.6). Clearly, the extracted gDNA resulted in better resolution, but when using the whole cell, the direct PCR approach was able to adequately detect all the targeted genes (Figure 3.6 B) (Ruiz-Villalba *et al*., 2017).

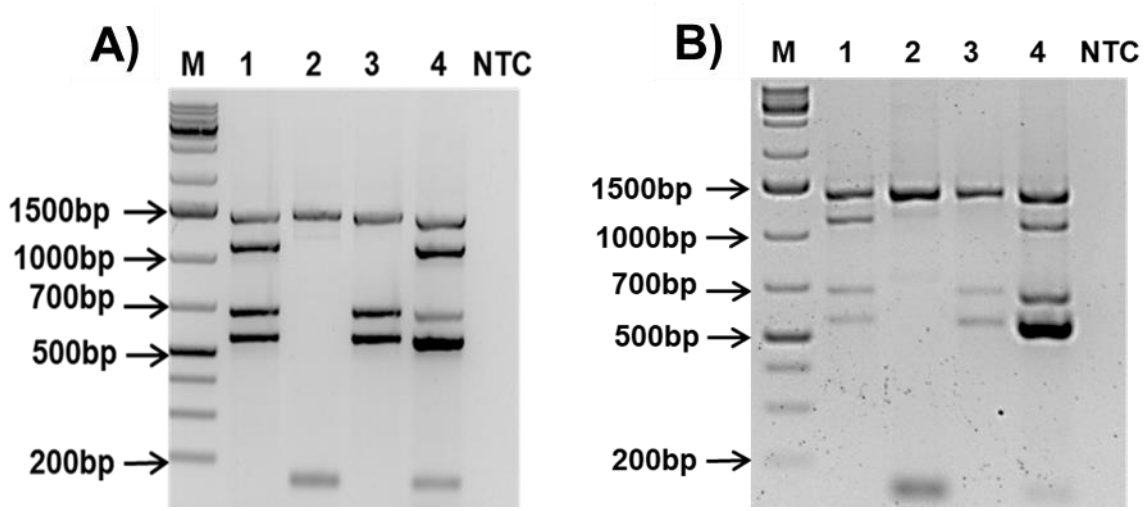

Figure 3.7: Multiplex PCR using gDNA (A) and whole cell (B) as a template to detect *fumC* (138 bp), fliC (550 bp), *neuC* (676 bp), *wzx-1* (1098 bp) and *wzx-2* (551 bp) genes in *E. coli* reference strains. Lanes 1, 2 and 3 contained DNA from O1:K1:H7, O15:K52:H1 or O18:K1:H7 respectively. Lane 4 contained pooled DNA from all three strains. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific) and the non-template control respectively.

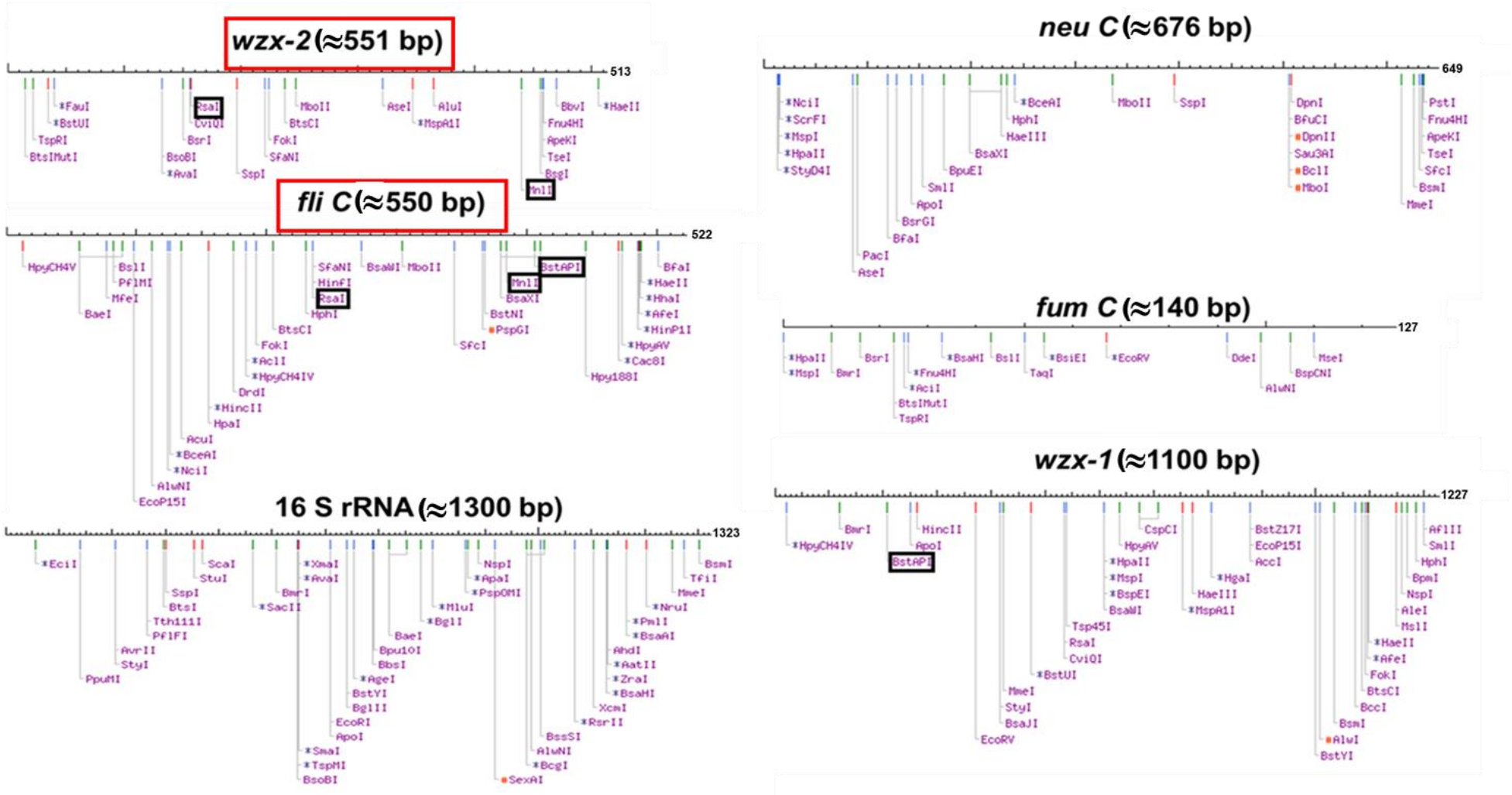

Figure 3.8: In-silico screening (using online NebCutter V2.0 software) of enzyme restriction sites in *wzx-2*, *fliC*, 16S rRNA, *neuC*, *fumC* and *wzx-1*. *Bst*API restriction sites on *wzx-1* and *fliC* and *Rsa*I and *Mnl*I sites on *wzx-2* and *fliC* are indicated by black boxes. Maps are not drawn to scale.

3.3.7 One-step digestion of optimized mPCR product to differential *fliC* and *wzx-2*

Online NebCutter V2.0 software was used with sequenced gene product data to search for restriction enzymes that were able to differentiate between the ≈550 bp (*fliC*) and ≈551 bp (*wzx-2*) (Figure 3.8). Eight restriction enzymes (*Bfa*I, *Bsr*DI, *Bst*API, *Bst*BI, *Mnl*I, *Nme*AIII, *Rsa*I and *SnaB*I) were identified for laboratory analysis.

The *wzx*-2 gene product was only digested by *RsaI* and *MnlI* to yield the expected ≈400 bp products (Figure 3.9). None of the other enzymes were able to digest this gene product. *Rsa*I, *Mnl*I and *Bst*API were able to digest *fliC,* yielding ≈270 & ≈280 bp and ≈400 & ≈150 bp (Figure 3.9). Only *Bst*API was able to digest the *fliC* gene but not the *wzx-2* gene. However, *wzx-1* also contained a *Bst*API restriction site (Figure 3.8) and the multiplex PCR product mix digested the 1098 bp *wzx-1* gene product into two fragments of ≈900 and ≈200 bp.

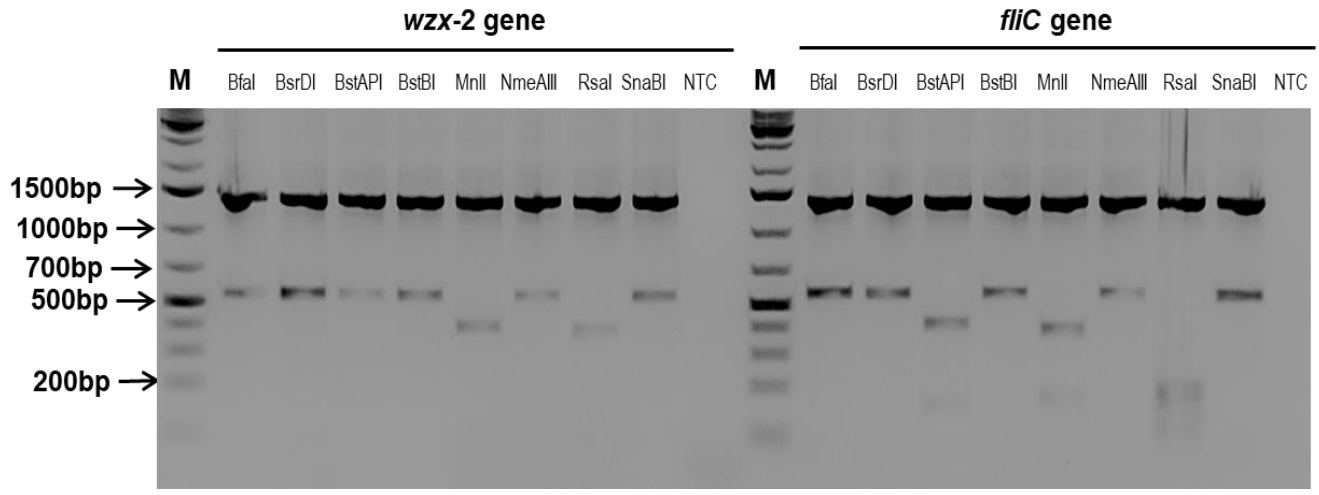

Figure 3.9: Restriction enzyme digestion profiles of the *wzx-2* and *fliC* genes. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific) and the non-template control respectively.

Digestion of mPCR products with *Bst*API resulted in the ≈1098 bp *wzx-1* gene product that was cut into two fragments of ≈850 and ≈250 bp (the latter is visible as a faint band in Figure 3.10, lane 2). Furthermore, the intense 550 bp product representing both *fliC* and *wzx-1* gene products was digested into two fragments of ≈400 bp and ≈150 bp (the latter overlapping with the *fliC* band in Figure 3.10, lane 2) leaving the ≈551 bp *wzx-1* fragment still intact. Other bands of ≈1300 bp (16S rRNA), ≈676 bp (*neuC*) and ≈138 bp (*fumC*) remained unaffected.

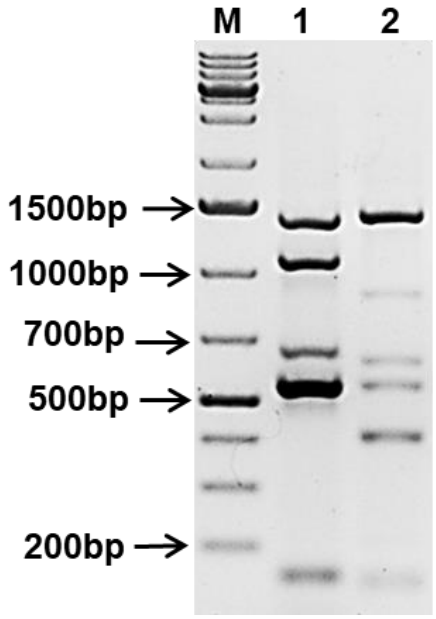

Figure 3.10: Restriction enzyme digestion profiles of multiplex PCR products amplified from whole cell, direct PCR. Lane 1: multiplex PCR products – undigested. Lane 2: one-step digestion with *BstAPI* restriction enzyme. Lane M: GeneRuler™ 1 kb plus DNA ladder (Thermo Scientific).

3.3.9 Application of mPCR/RED protocol on field isolates and environmental samples

Proof of concept for the mPCR/RED protocol for *E. coli* serotyping to detect serotypes O1:K1:H7, O15:K52:H1 and O18:K1:H7 was attempted on *E. coli* colonies

isolated from poultry abattoir effluent. Seven colonies were randomly picked from Chromocult® Coliform agar plates and subjected to whole cell, direct multiplex PCR. The resulting PCR products were digested with *Bst*API. Figure 3.11 shows the mPCR results and digestion profiles for each isolate. Internal control 16S rRNA bands were amplified in all seven isolates. None of the isolates harboured O1 antigen or could be classified as O15:K52:H1 serotype confirmed by the absence of ≈1098 bp (*wzx-1* gene) or ≈138 bp (*fumC* gene) PCR amplicons. Similarly, the K1 antigen was not detected because no amplification of the *neuC* gene (≈676 bp) was observed. All the isolates contained either O18 antigen or H7 antigen or both, judged by the presence of a ≈550 bp amplicon (Figure 3.11, lanes 1, 3, 5, 7, 9, 11 and 13). Digestion of the mPCR products showed that two isolates could be serotyped as K1:H7 (lanes 12 and 14) retaining a ≈550 bp fragment after digestion accompanied by two fragments ≈400 and ≈150 bp in length. Three isolates contained only the H7 antigen (lanes 2, 4 and 10) and two contained the O18 antigen (lanes 6 and 8).

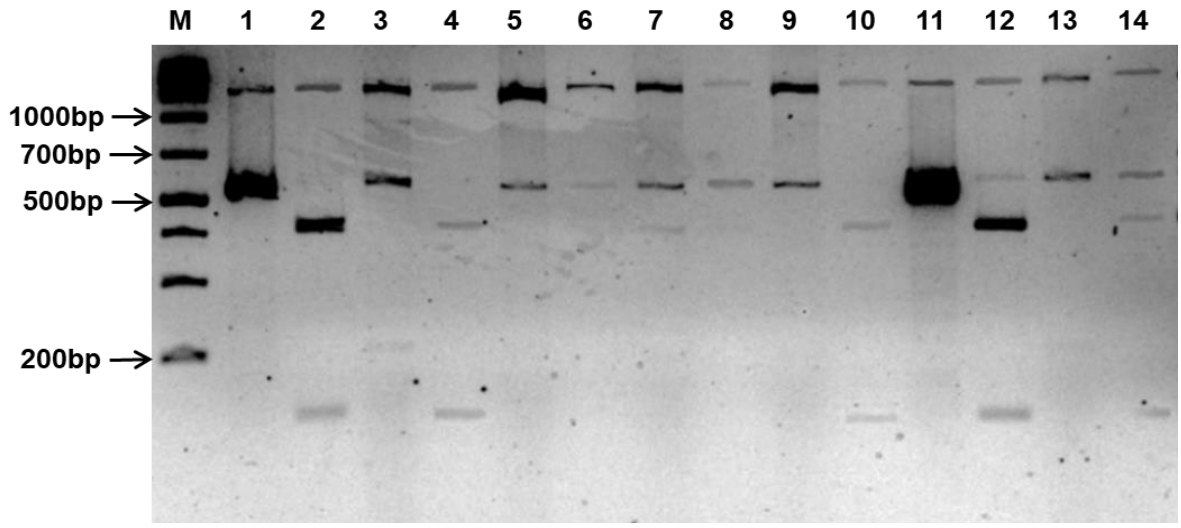

Figure 3.11: Application of mPCR/RED protocol on seven randomly selected *E. coli* isolates from poultry abattoir effluent grown on Chromocult® Coliform agar. Even numbered lanes represent undigested PCR products and uneven numbers represent their one-step *Bst*API digestion profiles. Lane M represents the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific).

A further attempt was made to apply the mPCR/RED protocol to environmental samples, as it was envisaged that serotyping would be possible from a mixture of colonies on an agar plate. A mixture of colonies from randomly selected plates was harvested in 1 ml ultrapure water and 1 µl of the cell suspension was used as template for mPCR. Figure 3.12 shows the absence of any profiles which was possibly due to the random selection of the plates. The other possible reasons could be the overload of DNA, as in this event underrepresented isolates will not be amplified because only 1 µl was used for PCR; so the distribution of serotypes could not play a role. Therefore more optimization could be required because it was no longer one colony.

One-step digestion of the optimized mPCR product of the reference strains (lane 1) is observed in lane 2, showing that the *wzx-1* fragment of ≈1100 bp (lane 1)

presented two fragments of ≈900 bp and ≈200 bp after digestion with *BstA*PI in lane 2 (Figure 3.12) (Mizani *et al*., 2017). Also, the *fliC* fragment (≈550 bp in lane 1) was digested into two fragments of ≈400 bp and ≈150 bp as observed in lane 2 (Figure 3.12). With optimized mPCR products of the two environmental samples (lanes 3 and 4), the internal control gene fragment (≈1300 bp) and a fragment of ≈550 bp (*fliC*) were observed in both lanes (Figure 3.12), which confirmed the presence of O18 antigen in the environmental samples.

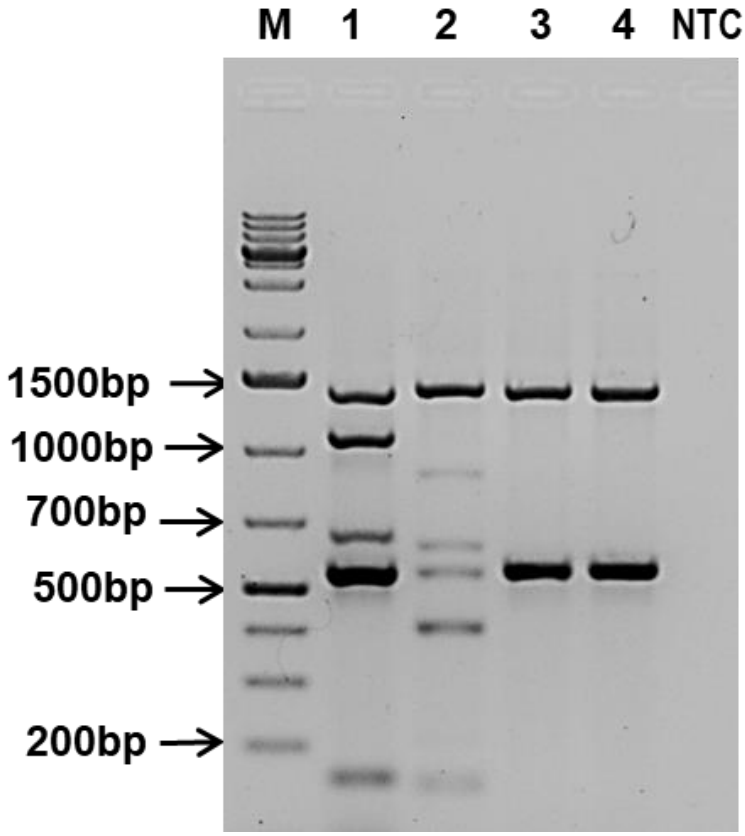

Figure 3.12: mPCR/RED protocol applied to reference strains and two environmental samples. Lanes 1 and 2 were already depicted in Figure 3.10 as mPCR products from a mixture of reference strains and one-step digestion with *BstA*PI respectively. Repeat of one-step digestion (lane 2) of optimized mPCR (lane 1) as depicted in Figure 3.8 was used as a positive control for one-step digestion of two environmental samples in lanes 3 and 4. Lanes M and NTC represent the GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific) and the non-template control respectively.

3.4 Conclusions

Primers as suggested by the literature for the selective detection of *E. coli* serotype O15:K52:H1 (targeting the *fumC* gene) and genes for the specific detection of antigens O1 (*wzx-1*), O18 (*wzx-2*), K1 (*neuC*) and H7 (*fliC*) were successfully used in a multiplex PCR application. The initial mPCR showed non-specific binding for *fumC* amplification. To produce the expected results, the mPCR was optimized by performing a gradient PCR to assist with the development of a touchdown PCR protocol to eliminate non-specific binding. Also, BSA was replaced by DMSO to increase PCR product yield. Furthermore, an increase in working concentration of *Taq* polymerase from 1 U to 10 U proved to be a necessary adjustment for mPCR improvement. After successful optimization of mPCR (for both gDNA and whole cell), the two fragments could not be distinguished from one another due to similar PCR product sizes: a somatic antigen gene product (O18) of 551 bp (*wzx-2* gene product) and a flagellin antigen gene product (H7) of 550 bp (*fliC* gene product). To distinguish between the two fragments, the *Bst*API restriction enzyme was selected *in silico* and used to digest the *fliC* gene product into ~400 bp and ~150 bp fragments while the *wzx-2* product was left undigested. Furthermore, the mPCR/RED protocol was tested on field isolates and it was demonstrated that O18:H7, O18 and H7 *E. coli* serotypes could be detected in poultry abattoir effluent.

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CHAPTER 4

Specificity and sensitivity of immunological serotyping assays for *E. coli* strains O1:K1:H7, O15:K52:H1 and O18:K1:H7

4.1 Introduction

Microorganisms possess unique growth stages that are important in serotyping (Maier, 2008). Growth stages are divided into the lag phase, exponential phase, stationary phase and death phase (Maier, 2008). In order for the growth phases to occur, the organism requires certain basic growth parameters for energy generation and cellular biosynthesis. Both physical (pH, temperature, osmotic pressure, hydrostatic pressure and moisture content) and chemical factors (quantity of carbon, nitrogen, sulphur, phosphorus and other elements provided by the medium) can affect the growth of an organism (Feher *et al*., 2012). For most microorganisms (bacteria), nutrient media contain most of the chemical factors required for bacterial growth. For *E.coli*, the Luria-Bertani (LB) medium is suitable for growth stages of this bacterium.

E. coli strains have many different serotypes based on long-standing observation of different antigenic determinants (O, K and H) expressed on the cell surfaces (Brenner *et al*., 2005; Ucieklak *et al*., 2017). Early in the 1940s, Kauffmann was able to subdivide *E. coli* into a number of O groups (Kauffmann, 1943). Using a boiled culture for O-antiserum production and as an antigen in agglutination tests, he was able to establish well-defined *E. coli* O groups (Kauffmann, 1944). Later, *E. coli* causing pyelonephritis O-antigens were serotyped using ELISA (Brauner *et al*., 1989). A problem of cross-reaction between O-antigen antisera and several O-antigens and partial cross-reaction with K-antigens (not only from *E. coli* but from other Enterobacteriaceae species) was encountered (Orskov *et al*., 1977; Brenner *et al*., 2005). K-antigens were earlier serotyped by passive hemagglutination, double diffusion in gel, and immunoelectrophoresis (Orskov *et al*., 1977). In contrast,

H-antigens were serotyped by the classical method of agglutination of flagellated whole bacterial cells with highly specific absorbed monovalent H-antigen antisera (Ratiner *et al*., 2003).

Some serotypes of *E. coli* cause various diseases both in animals and humans (Marshall & Levy, 2011). Strains such as O157:H7, O104:H4 and O104:H21 have caused serious disease outbreaks in different parts of the world (Piérard *et al*., 2012). Earlier, the O157:H7 strain was serotyped using monoclonal antibodies. Specificity and sensitivity of the antibodies proved to be important parameters to observe when differentiating between different antigenic determinants of the strain. Jin and co-workers (2012) used indirect-ELISA where the plates were coated with the capture antibody followed by the addition of the sample and a secondary antibody conjugated to an enzyme. This assay presented the specificity of the antibodies and reached 3×10^4 CFU.ml^{-1} sensitivity. The specificity of serotyping was also previously applied in the immune complexes for serotyping HIV based strains on the use of epitope-mimicking peptides (Shchelkanov *et al*., 2001). Currently, commercially available kits target only serotypes that are considered economically important, such as O157:H7.

The objective of this chapter is to present an evaluation of the specificity and sensitivity of immunological serotyping assays using plate agglutination assays, plaque assays, and counter current immunoelectrophoresis on three laboratory APEC strains (O1:K1:H7, O15:K52:H1 and O18:K1:H7) that can be transferred from chickens to humans.

4.2 Materials and Methods

4.2.1 Growth monitoring of reference strains

The inoculum was prepared as described in Figure 3.1. After a 5-hour incubation period, 100 ul was transferred to a fresh set of bottles containing 100 ml LB medium in which the growth was monitored over time. Optical density (600_{nm}) of the culture was measured every half hour for the first 5 hours and thereafter hourly for a further 6 hours. Growth monitoring was performed as three technical repeats.

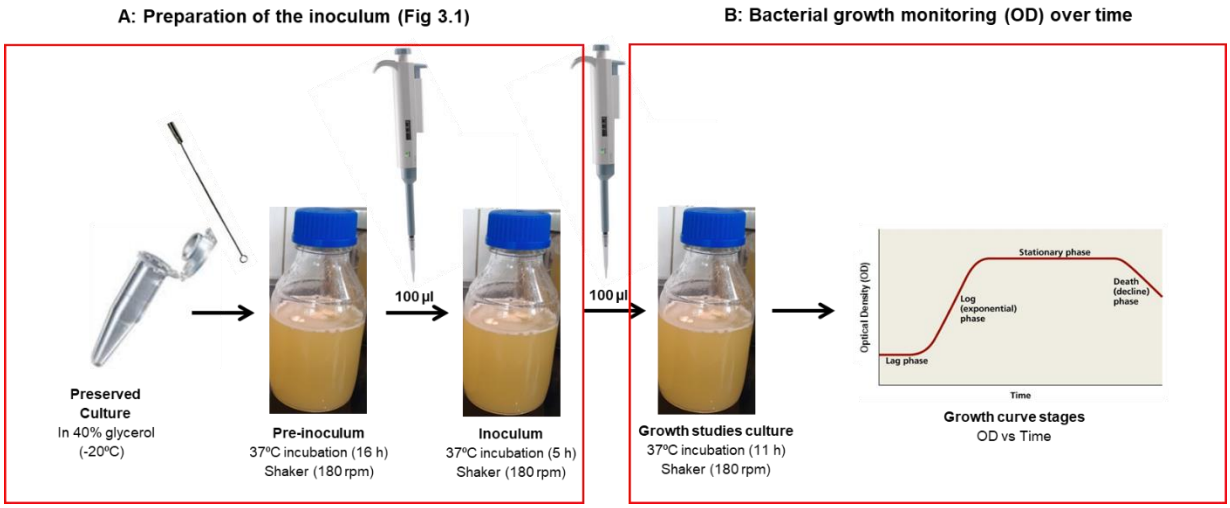

Figure 4.1: Preparation of inoculum (A) from cultures preserved in 40% glycerol at -20°C and incubated for 5 hours (37°C) with shaking (180rpm) by firstly preparing pre-inoculum incubated at 37°C with shaking (180rpm) for growth monitoring over a period of 11 hours (B).

Numbers of colonies forming units per millilitre ($CFU.ml^{-1}$) were determined by initially preparing the inoculum as illustrated in Figure 3.1. After 5 hours of incubation, fresh media bottles were inoculated with 100 µl of the inoculum until the optical density (600_{nm}) reading of 0.1 was reached. Inoculated bottles (enumeration culture) were incubated for 5 hours in a shaker incubator at 180 rpm at 37ºC and were serially diluted up to 10^{-6} (Madigan *et al*., 2010).

4.2.2 Microtiter plate agglutination

4.2.2.1 Preparation of O- and H-antigen suspensions

Antigen suspensions (ASs) for use in antisera testing were prepared as described in Figure 3.1 and Figure 4.1. The AS was diluted to a final concentration of 10^9 CFU.ml^{-1} by adding formaldehyde in saline (1% formol saline) according to McFarland standard (www.evaluations-standards.org.uk). Three bottles, each containing 100 ml of 10^9 CFU.ml^{-1} in 1% formol saline (1% FS), were prepared. To expose the somatic (O) antigens by removing the capsule on the reference strains, two methods were adopted for further preparation of the O-antigen suspension (O-AS). The first bottle of O-AS was steamed for 1 hour in a 95ºC closed water bath (to facilitate steaming) followed by cooling at 4ºC for 20 minutes. The second bottle of O-AS was autoclaved and left to cool overnight. Both bottles of O-AS were mixed gently by swirling and left for 1 hour to allow sedimentation. The contents of the O-AS bottles were diluted with an equal volume of sterile saline and used in plate agglutination (www.evaluations-standards.org.uk). For further preparation of H-antigen suspension (H-AS), an equal volume of H-AS was mixed with 1% FS (1:1 v/v). The suspension was left to stand overnight at 25°C until use (Figure 4.2).

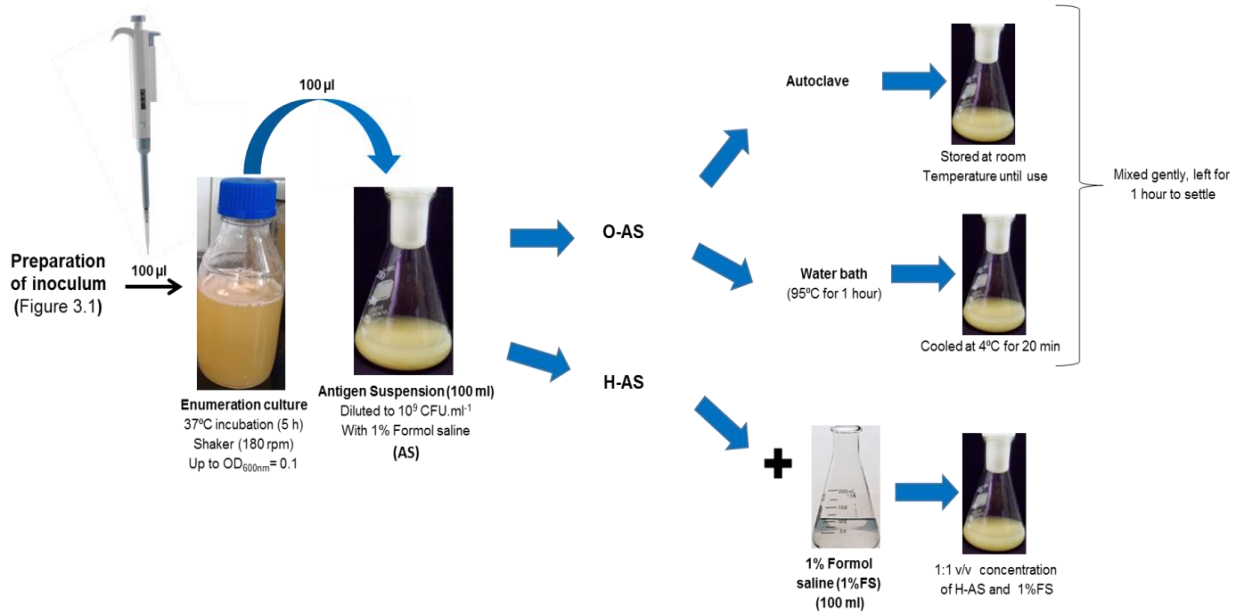

Figure 4.2: Preparation of O-antigen suspension (O-AS) by steaming in an autoclave and immersion in a water bath. H-antigen suspension (H-AS) from the enumeration culture was adjusted with the inoculum up to OD of 0.1 at 600_{nm}. After incubation, O-AS bottles were allowed to cool (at room temperature for autoclaved O-AS and at 4°C for water bathed O-AS) and gently mixed and left for 1 hour before use. The contents of H-AS bottle was mixed with an equal quantity of 1% FS (1:1 v/v) (www.evaluations-standards.org.uk).

4.2.2.2 Plate agglutination

Six undiluted antisera solutions were purchased from Statens Serum Institut, Denmark, namely: O1 monospecific for O1 antigen; O18ab unabsorbed for O18; O18ac monospecific for O18; O15 monospecific for O15 antigen; H1 monospecific H1; and H7 monospecific for H7 (SSI Diagnostica, 2008 & 2012a). All the reference strains were pre-screened for plate agglutination by placing a droplet of O-AS on a glass slide followed by the addition of an undiluted droplet of O antisera (O1 monospecific, O15 monospecific, O18ab unabsorbed, and O18ac monospecific). Stock of diluted 1:10 (v/v) antisera was prepared according to the Rockland-Inc.com protocol and tested independently against all three reference strains (O1:K1:H7;

O15:K52:H1 and O18:K1:H7). Two antisera were tested per 96 well plate. The plate setup was as follows: Rows A and E were used as controls and contained a phosphate buffered solution with a pH of 7.4 (PBS) only. ASs (Figure 4.2) of the different strains were added to rows B and F (O1:K1:H7), rows C and G (O15:K52:H1), and rows D and H (O18:K1:H7) (Figure 4.3). PBS was added to all 96 wells of a round welled microtiter plate. An equal volume of each antisera was added to column 1 A – D and column 1 E – F respectively (Figure 4.3). After carefully mixing the contents of column 1, eleven times successive serial dilution of antisera across the plate (columns) were performed and 50 µl from the last well was discarded. ASs (O-AS or H-AS) (50 µl) prepared as described in Figure 4.2 were added to the respective rows (Figure 4.3), except for rows A and E, where the volume was replaced by an equal volume of PBS and used as controls. O-AS plates were incubated at 50ºC overnight in a moist chamber and H-AS plates were incubated at 50ºC for 2 hours (SSI Diagnostica, 2008 & 2012a; www.evaluations-standards.org.uk).

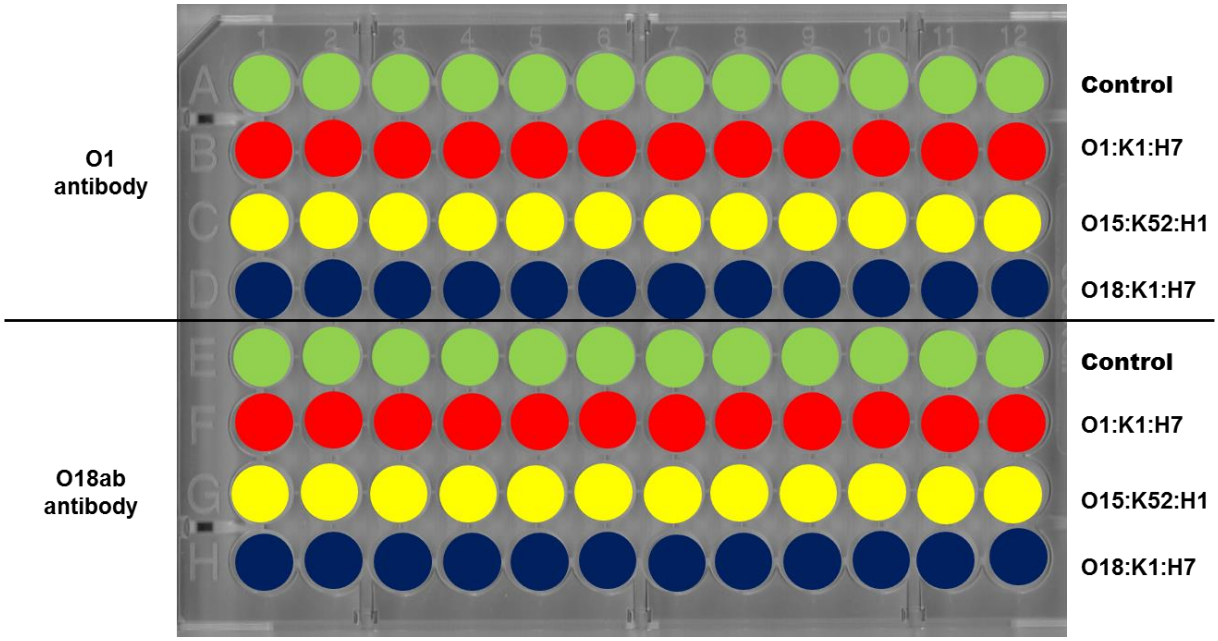

Figure 4.3: Representative microtiter plate setup showing O1 and O18ab antisera testing. All 96 wells were filled with 50 µl of PBS. Diluted O1 antiserum (1:10 v/v) was added to column 1 rows A-D and O18ab antisera was added to column 1 rows E-H. After mixing, the contents of column 1 were serially diluted across the plate to column 12. The test strains (50 µl) were added as follows: O1:K1:H7 to rows B and F (red), O15:K52:H1 to rows C and G (yellow), and O18:K1:H7 to rows D and H (blue). Rows A and E (green) served as controls and contained 50 µl of PBS only (SSI Diagnostica, 2008 & 2012 (a) (www.evaluations-standards.org.uk).

4.2.3 Plaque assay

Undiluted bacteriophage (K1) suspension was purchased from Statens Serum Institut, Denmark. Sensitivity and specificity were tested for this suspension using both the cross-brush and broth clearing assays against revived reference strains as described previously in Figure 4.1.

4.2.3.1 Cross-brush assay

A sterile yellow micropipette tip was used to aseptically transfer (10 µl) and streak one line of K1 bacteriophage suspension vertically across an LB agar plate. The plate was left for 10 min, or until the applied phage suspension had dried. Thereafter, all three reference strains were streaked horizontally across the line of bacteriophage and the agar plate was incubated overnight at 37ºC (SSI Diagnostica, 2012b).

4.2.3.2 Broth clearing plate and pour assay

Sterile sets of test tubes were prepared containing 9 ml of LB broth and 9.9 ml of molten LB agar (with molten LB agar stored at 45ºC in a water bath until use). An inoculum (1 ml) prepared as previously mentioned in Figure 3.1 was transferred to 10 test tubes containing 9 ml of LB broth resulting in a 1:10 (v/v) inoculum. Nine times successive serial dilution of 100 µl of K1 bacteriophage (phage) were performed from the first test tube across all ten test tubes containing 1:10 (v/v) concentration of inoculum in LB broth. From all ten test tubes 100 µl was transferred to respective ten molten LB agar test tubes (9.9 ml) and mixed thoroughly by pipetting. Before solidifying, the contents of the test tubes were poured evenly onto already prepared LB agar plates and allowed to solidify. The plates were incubated overnight at 37ºC. Each test tube containing 1:10 (v/v) inoculum concentration and successive serially diluted bacteriophage was also incubated overnight at 37ºC for the broth clearing assay (Figure 4.4).

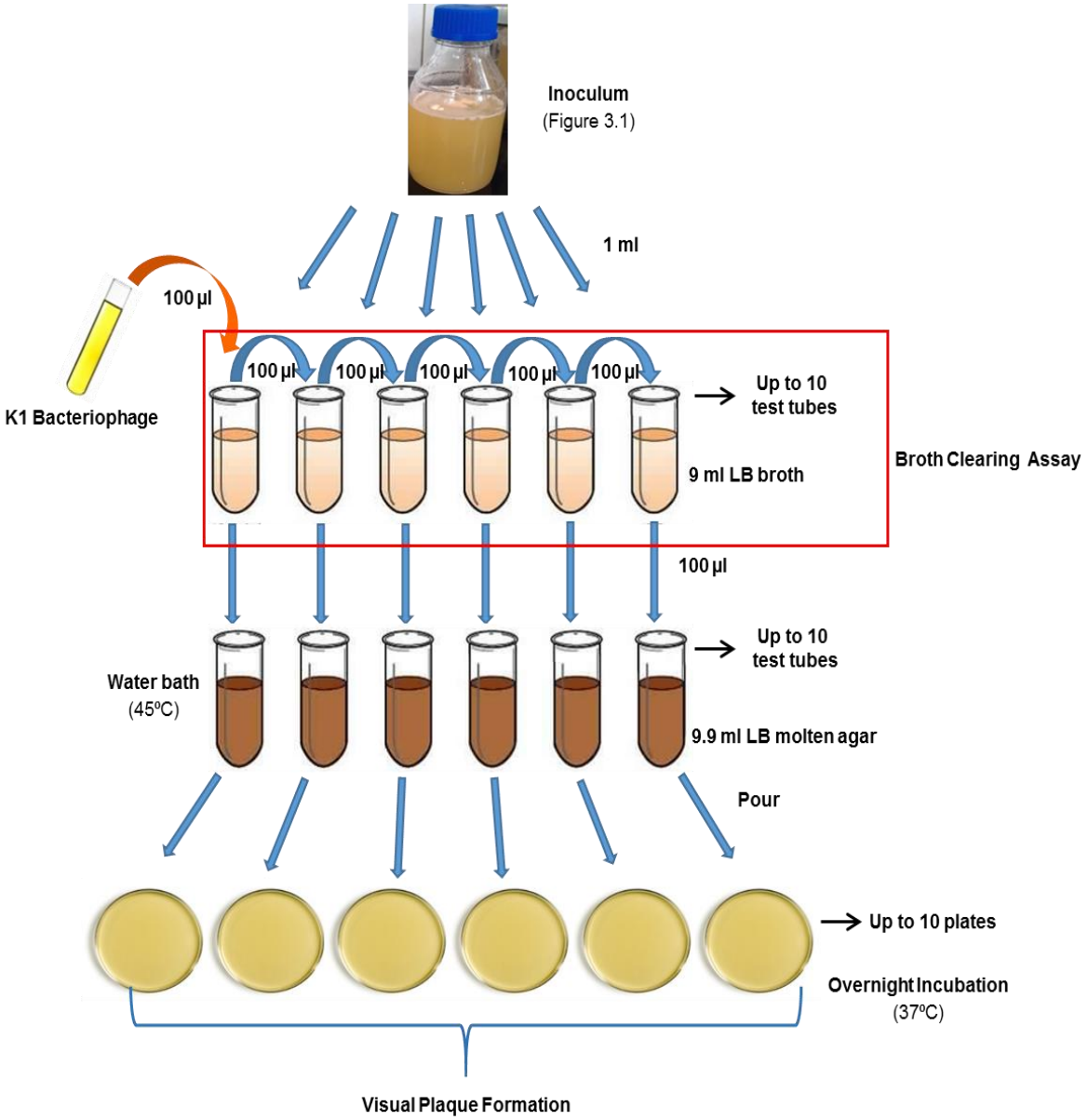

Figure 4.4: An inoculum (1ml) prepared as described in Figure 3.1 was mixed with LB broth (ten test tubes containing 9 ml) to 1:10 (v/v) concentration. K1 bacteriophage (100 µl) was pipetted into the first test tube and serially diluted across all ten test tubes. The contents in the test tubes in the red block (for broth clearing assay [BCA]) were transferred (100 µl) to respective test tubes containing 9.9 ml of molten agar (stored at 45°C). For BCA, the test tubes were incubated at 37°C overnight. The contents of the molten agar test tubes were poured onto LB agar plates and thinly layered. After solidifying, the plates were incubated at 37°C overnight to observe the plaque formation.

4.2.4 Counter current immunoelectrophoresis

A glass surface area (70 × 100 mm) was marked positive and negative at the bottom on the opposite ends and placed on an electrophoresis apparatus (submarine) with the positive end facing towards anode and the negative facing cathode. Molten agarose (2%) was evenly spread over a glass on a flat surface. Agarose was left for 15 minutes to solidify without disturbance. Wells that were not more than 50 mm apart were made using a yellow micropipette tip (two parallel columns of three wells). Welled agarose was submerged to just below the surface in single strength of Tris-acetate Ethylene-diamine-tetra-acetic acid (EDTA) buffer (1× TAE) (40 mM Tris, 20 mM acetic acid and 1 mM EDTA). A bacterial layer (colony) from LB agar was suspended in 1 ml of PBS (pH 7.4) for antigen preparation. An antigen (30 µl) was pipetted into the cathode well and 30 µl of antiserum (1:10 v/v) was added into the second well (anode). Counter current immunoelectrophoresis was conducted at room temperature for 20 min using a constant current of 40 V. The glass agarose was transferred to a moist chamber and incubated for 6 hours.

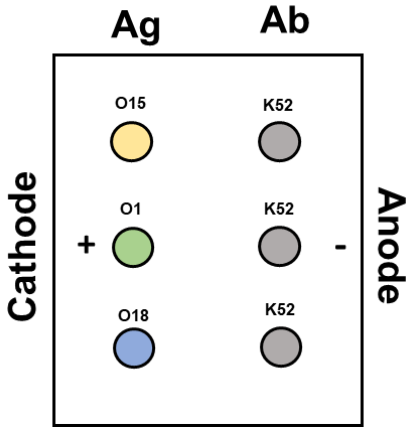

Figure 4.5: Representative **c**ounter current immunoelectrophoresis setup showing three different antigens (Ag) (three reference strains O1:K1:H7, O15:K52:H1 and O18:K1:H7) on the cathode terminal represented by O1, O15 and O18 respectively. These were tested against K52 antibody (Ab) on the anode terminal with O15 included as a positive control strain (Ørskov & Ørskov, 1984).

4.3 Results and discussion

4.3.1 Sampling of reference strains using growth curve as reference for plate agglutination assay

Cells in the exponential phase are in their healthiest state and this is of utmost importance because all the antigens will be well presented (Madigan *et al*., 2010). It was therefore important to understand the growth patterns of the O1:K1:H7, O15:K52:H1 and O18:K1:H7 strains. Optical density was measured as a guide to determine the appropriate time interval for harvesting cells while in the exponential phase and at a suitable concentration of 10^9 CFU.ml^{-1} for immunology testing. None of the three strains showed any difficulty adjusting to the new media as the growth curves did not show any lag phase (Figure 4.6). Concentrations of $\approx 10^9$ CFU.ml^{-1} were reached after 5 hours of growth; i.e., $8.23 \pm 5.55 \times 10^9$ CFU.ml^{-1}, $1.12 \pm 0.05 \times 10^{10}$ CFU.ml^{-1}, and $1.05 \pm 0.04 \times 10^{10}$ CFU.ml^{-1} were determined for O1:K1:H7, O15:K52:H1 and O18:K1:H7 respectively (Madigan *et al*., 2010). For microtitre-plate agglutination, 1×10^9 CFU.ml^{-1} was required to perform the assay according to the McFarland Standard (www.evaluations-standards.org.uk).

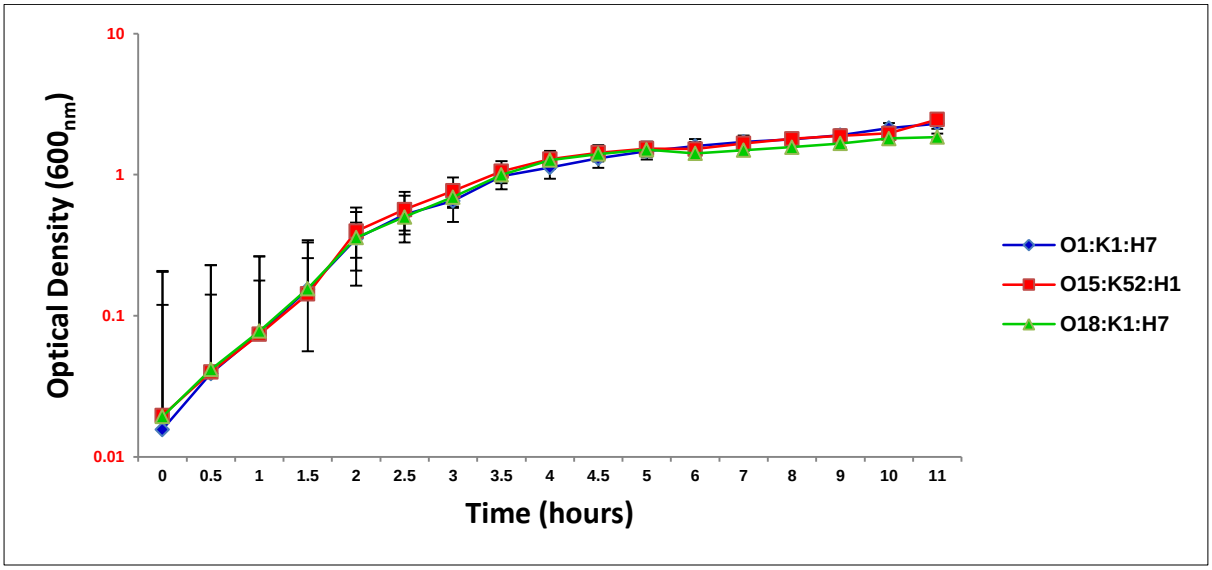

Figure 4.6: Growth curves of *E. coli* strains cultivated in LB broth at 37°C. The growth was monitored over 10 hours by measuring OD at 600_{nm}. Average values of three independent replicates were plotted and error bars represent standard deviations.

4.3.2 Microtitre-plate agglutination

Positive microtiter-plate agglutination results were observed as cells forming a lattice network with the antibodies formed a turbid blanket at the base of the well. Conversely, negative results were observed as cells sinking to the bottom of the well formed a spot (dot) at the bottom. No turbidity was observed in the wells where O1, O18ab, O18ac, O15 and H1 antisera were tested against the respective antigens and only dots that had formed at the bottom of the wells were observed. In contrast, H7 antiserum was specific and formed a lattice network with the O1:K1:H7 and O18:K1:H7 strains (Figure 4.7) with the maximum sensitivity represented by the last turbid well. Maximum sensitivity of H7 monospecific H rabbit antiserum was at 1/8 (Figure 4.7, lane B at row 3) for O1:K1:H7 and 1/16 for O18:K1:H7 (Figure 4.7, lane D at row 4).

These results could be explained by the false negative results concept which was reported by Kricka in 1999. Kricka stated that the cause of these observations was that the detection antibody was preventing reaction with the analyte. Also, it is possible that inhibitors were present in the antigen preparation media (Kricka, 1999; Kragstrup *et al*., 2013). It was therefore suggested that efforts be directed at improving existing methods for identifying and eliminating this type of analytical interference and such inhibitors.

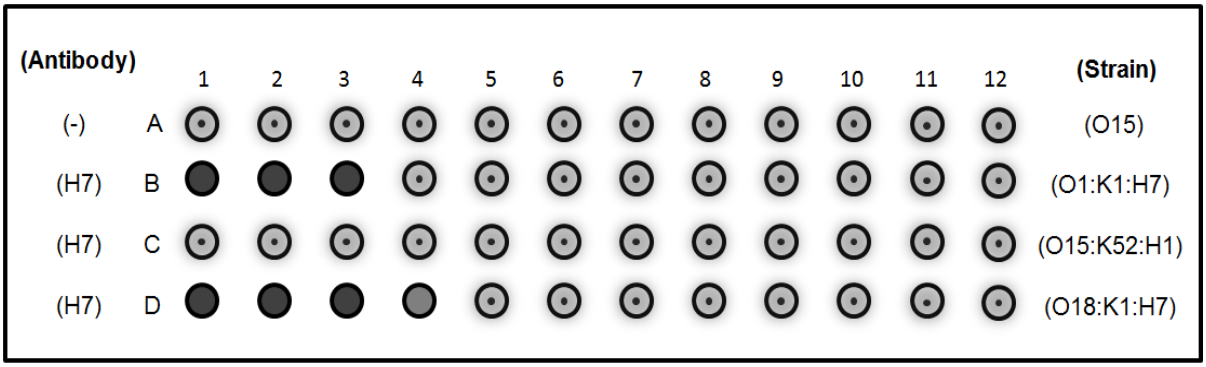

Figure 4.7: Representative microtitre plate results of serially diluted H7 monospecific H rabbit antiserum against all reference strains with lane A as the negative control.

4.3.3 Plaque assay

Cross-brush assay succeeded in showing specificity through growth inhibition of the O1:K1:H7 and O18:K1:H7 reference strains that were inhibited by K1 bacteriophage (phage) infection. The sensitivity of the strains is also clearly observable by the zone of inhibition which is represented by the size of the red line (the longer the red line the more sensitive the strain) in Figure 4.8. This was due to the cell lysis caused by the phage. Conversely, reference strain O15:K52:H1 managed to grow over the phage cross-brush which demonstrated the specificity of the phage because the targeted capsular antigen (K1) was not present on the strain (Ørskov & Ørskov,

1984). Specificity of the phage and sensitivity of the reference strains (O1:K1:H7 and O18:K1:H7) are further illustrated by the broth clearing assay in Figure 4.9. There was no broth clearing effect representing specificity because all the test tubes were turbid for the O15:K52:H1 reference strain (Figure 4.9C). However, Figure 4.9A and 4.9B show sensitivity as minimum inhibition concentrations (MICs) of 10^{-5} (0.1 ml.10^{4} ml^{-1}) for the O1:K1:H7 reference strain and 10^{-6} (0.1 ml.10^{5} ml^{-1}) for the O18:K1:H7 reference strain can be observed respectively.

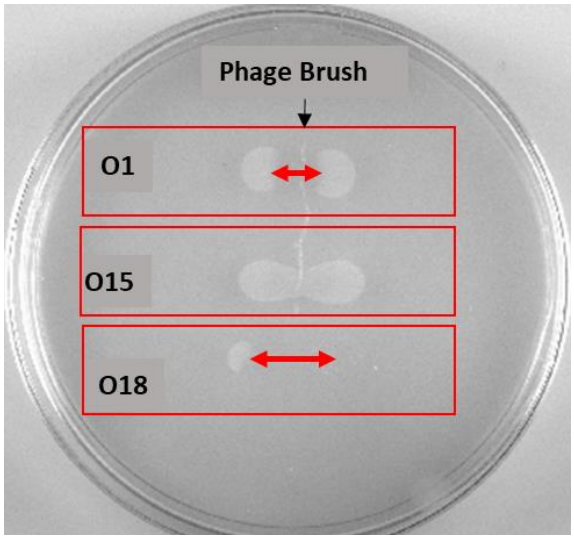

Figure 4.8: Zone of inhibition caused by K1 bacteriophage from the vertical phage stripe. Here, O1, O15 and O18 represent reference strains O1K:1:H7, O15:K52:H1 and O18:K1:H7 respectively. The red lines represent the area of growth inhibition by the phage (Ørskov & Ørskov, 1984).

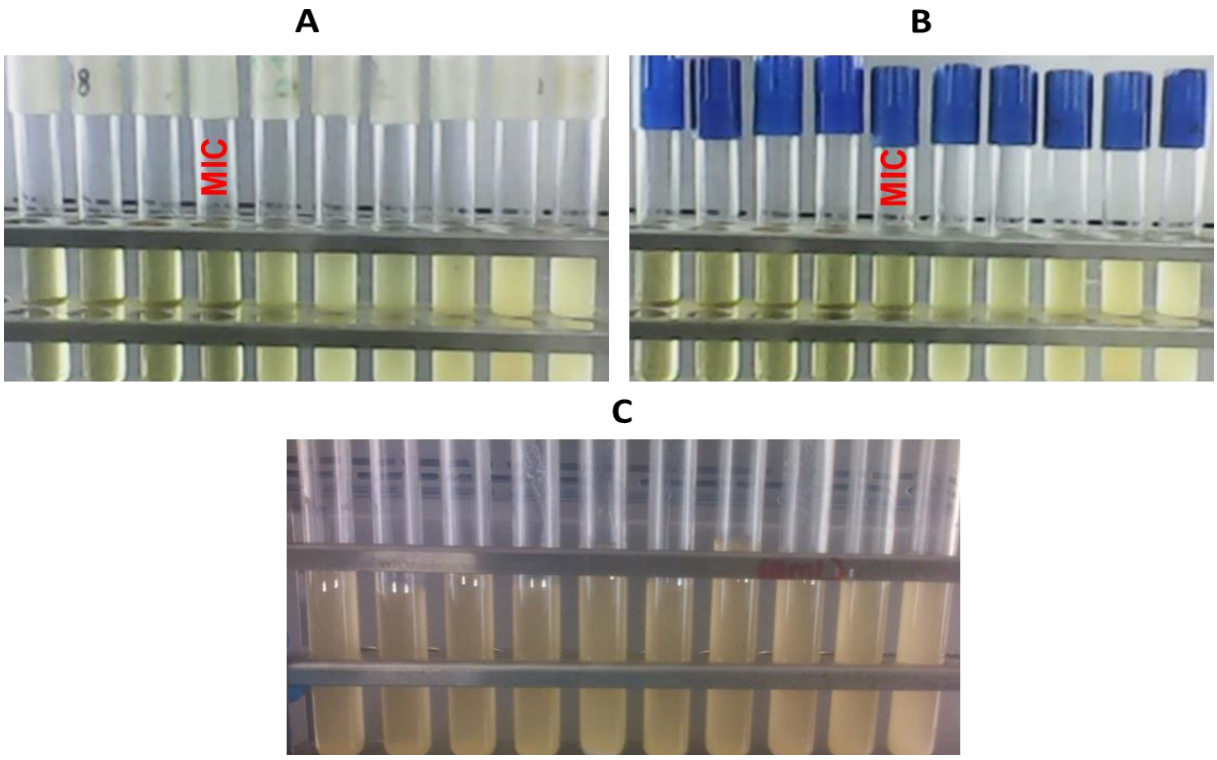

Figure 4.9: LB broth with *E. coli* reference strains O1:K1:H7 (A) and O1:K1:H7 (B) showing the clearing activity of the broth (MIC) by K1 bacteriophage. With *E. coli* reference strain O15:K52:H1 (C), all the test tubes were turbid showing the inability of K1 bacteriophage to clear the broth (Madigan *et al*., 2010). Specificity of the phage was further demonstrated by the pour plate assay.

Where clear spots or plaques (Figure 4.10B) were formed, they represented the growth inhibition of reference strains O1:K1:H7 and O18:K1:H7 due to ruptured cells caused by the phage. However, the plate of reference strain O15:K52:H1 was not inhibited (Figure 4.10A).

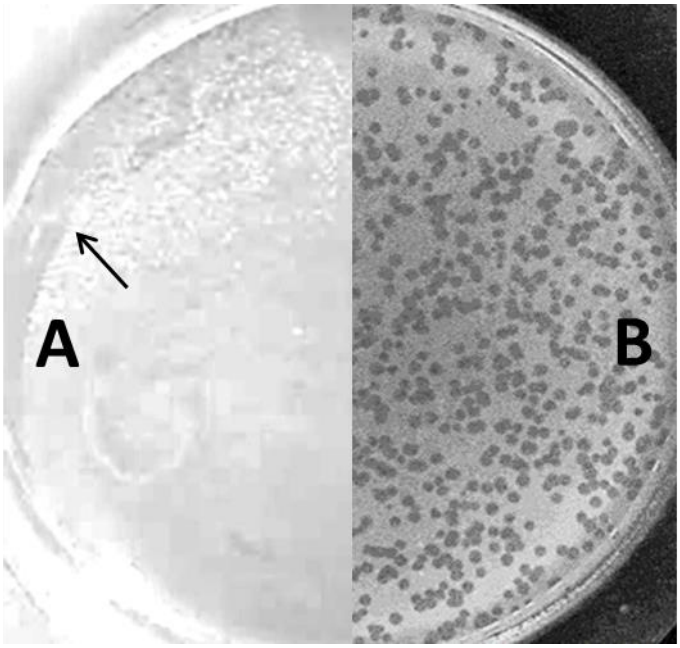

Figure 4.10: LB agar plate overlaid with *E. coli* reference strain O15:K52:H1 showing an overgrowth of the strain on plate A (the arrow points at the edge of the cell growth) and plate B, showing plate B covered with plaque caused by the phage for both reference strains O1:K1:H7 and O18:K1:H7.

4.3.4 Counter current immunoelectrophoresis

The presence of an immunoprecipitin line between the antigen and antisera indicates the presence of antibody for the antigen in the test sera. The presence of more than one immunoprecipitin indicates the heterogenicity of the antibody for the antigen in the test sera. In contrast, the absence of the immunoprecipitin line indicates the absence of any antibody for the antigen in the test sera. In all three the tested *E. coli* reference strains (O1:K1:H7, O15:K52:H1 and O18:K1:H7), no immunoprecipitin lines were observed between the antigen (reference strain) and the antibody (K52 monovalent K rabbit antiserum), including the O15:K52:H1 reference strain that possessed the K52 capsular antigen.

A possible explanation for these results is that the tested strain could have possessed positively charged capsular antigens (El-Refaie & Dulake, 1975). Sharma *et al*. (1997) also reported that counter current immunoelectrophoresis had low sensitivity for serodiagnosis of typhoid fever.

4.4 Conclusion

Growth curve data of the reference strains contributed significantly towards determining the cell concentration (1×10^9 CFU.ml^{-1}) that was used for immunological assays. H7 monospecific H rabbit antiserum effectively agglutinated with the target flagella antigens presented by two reference strains (O1:K1:H7 and O18:K1:H7), thereby showing its specificity with different levels of sensitivity (1/8 dilution for O1:K1:H7 and 1/16 dilution for O18:K1:H7). The other antisera (O1, O18ab, O18ac, O15 and H1) presented negative results. The specificity and different sensitivity levels (10^{-4} dilution and 10^{-5} dilution for O1:K1:H7 and O18:K1:H7 respectively) became evident with K1 bacteriophage on the O1:K1:H7 and O18:K1:H7 reference strains. Broth clearing proved to be the best method for determining the sensitivity of K1 bacteriophage against the two reference strains, whereas the zone of inhibition and plaque forming assays were the decisive methods for ascertaining the specificity of K1 bacteriophage on the targeted capsular antigens (K1). Counter current immunoelectrophoresis proved to be a tedious assay as it presented negative results even for the targeted capsular antigen (K52) present on the positive control (reference strain O15:K52:H1). Due to the predominantly negative results encountered with the immunological assays on the reference strains, no attempt was made to use these techniques on field isolates or environmental samples.

4.5 References

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CHAPTER 5

Concluding remarks

5.1 Concluding remarks

The poultry industry in South Africa is the largest individual agricultural sector in the country and contributes 15% to the GDP. An average high-throughput poultry abattoir employs about 29 workers. As with any food sector that works with raw animal products, microbial contamination, especially through faecal contamination, is prevalent. Because abattoir workers are working in close contact with open carcasses, the possibility of the transfer of hazardous biological agents (HBAs) from carcasses to humans is very high. The presence of these HBAs in abattoirs is presently enumerated and identified only at species level. This may be deemed a high risk oversight and thus the current study utilised molecular and immunological techniques to identify one such microorganism (*E. coli*) at serotype level.

By using molecular techniques, both the genomic material and whole cell were used as the template for the mPCR. For specificity, the *wzx-1*, *neuC*, *fliC*, *wzx-2* and *fumC* genes were amplified individually as selective markers for the O1, K1, H7, O18 antigens and the O15:K52:H1 strain. After optimization of mPCR, the mPCR with increased *Taq* DNA polymerase was able to detect different *E. coli* strains with different hosts' pathogenicities (i.e., different O, K and H antigens). Information about which primers to use in the reactions was found in the literature, but they had not previously been used in a mPCR.

It was found that the assay did not produce unique bands for all targeted genes, thus *BstAPI* was used to ascertain the presence of the *wzx* O18 product in the mPCR. In silico screening, online NebCutter V2.0 software was used to choose the correct restriction enzyme. For sensitivity, the application of the optimized mPCR on both field isolates and the environmental sample was adequately sensitive to identify the

targeted antigens. The findings suggest that the use of whole cell as a template will make application to field isolates much more efficient.

Using immunological techniques, only the flagellin antigen (H7) was able to show specificity by the formation of lattice with H7 monospecific H rabbit antiserum targeted on the O1:K1:H7 and O18:K1:H7 strains. Sensitivity of varying degrees (8-fold dilution factor for O1:K1:H7 and 16-fold dilution factor for O18:K1:H7) with the microtitre-plate agglutination (Figure 3.7) process was also achieved. Plate agglutination assay targeting other antigens (O1, O18, O15 and H1) was inconclusive. Furthermore, the specificity and sensitivity of K1 bacteriophage was observed by the zone of inhibition and pour plate and broth clearing assays. In counter current immunoelectrophoresis assay (CCIE), no precipitin lines were observed on both the targeted (O15:K52:H1) and the non-targeted (O1:K1:H7 and O18:K1:H7) strains when separated on agarose gel electrophoresis against K52 antiserum. These results indicated that the CCIE did not yield the expected results. The reasons for the inconclusive results from plate agglutination and CCIE assays may have been due to factors acting on the equilibrium constant such as temperature, pH, ionic strength, duration of incubation, and concentrations of antigen and antibody. For this reason, not even one serotype could be identified and it was thus not possible to apply the assay to field isolate identification.

For future research projects, it is recommended that an inter-laboratory validation of the optimization protocol to test more field isolates might be a suitable entity to focus on. It is also important to develop less costly methods such as second and third generation ELISAs which may be more suitable for routine testing.