EVALUATION OF RAPID *SALMONELLA* IMMUNOASSAYS AND CHARACTERIZATION OF BACTERIAL ISOLATES THAT CAUSE FALSE-NEGATIVE AND FALSE-POSITIVE RESULTS IN THE TESTS

BY

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Master of Science
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DEDICATED TO MY BELOVED PARENTS, SISTER, FRIENDS

AND ALL MY TEACHERS

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LIST OF ABBREVIATIONS

API	American Proficiency Institute
ASPC	L'Agence de la santé publique du Canada
BGS	Brilliant Green Sulfa Agar
BLAST	Basic Local Alignment Search Tool
BPW	Buffered Peptone Water
BS	Bismuth Sulfite Agar
BSA	Brilliance *Salmonella* Agar
CDC	Center for Disease Control and Prevention
CDS	Coding Sequence
CFU	Colony Forming Unit
D.E.Te.CT	Diagnostic, Enrichment, Testing and Characterization
DEFT	Direct epifluorescent-filter technique
DNA	Deoxyribonucleic acid
ECPT	*E.coli* Phage Technology
EFSA	European Food Safety Authority
ELFA	Enzyme Linked Fluorescent Assay
ELISA	Enzyme-linked Immunosorbent Assays
FDA	Food and Drug Administration
GAP	Good Agricultural Practices
HE	Hektoen Enteric Agar
HRP	Horse Radish Peroxidase
IBIS	Institut de Biologie Intergrative et des Systems
LFA	Lateral Flow Assays
LIA	Lysine Iron Agar
LPS	Lipopolysaccharide
PCR	Polymerase Chain Reactions
PHAC	Public Health Agency of Canada
RAST	Rapid Annotation using Subsystem Technology
RBP	Receptor Binding Proteins
RFV	Relative Fluorescence Value
RNA	Ribonucleic acid
rRNA	ribosomal RNA

RT-PCR	Real-time PCR
RV	Rappaport-Vassiliadis
RVS	Rappaport-Vassiliadis Soya Peptone
SNP	Single Nucleotide Polymorphisms
SPR	Solid Phase Receptacle
SPT	*Salmonella* Phage *Technology*
STEC	Shiga Toxin producing *Escherichia coli*
TBG	Tetrathionate Brilliant Green
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron Agar
TT	Tetrathionate
VIDAS	Vitek Immuno Diagnostic Assay System
WGS	Whole Genome Sequencing
WHO	World Health Organisation
XLD	Xylose Lysine Desoxycholate
XLD	Xylose Lysine Desoxycholate Agar
XLT4	Xylose Lactose Tergitol 4

ABSTRACT

Salmonella spp. is a widely distributed Gram-negative foodborne pathogen that is a major cause of foodborne outbreaks in North America. The Public Health Agency of Canada (PHAC) estimates that approximately 88,000 Canadians are affected by foodborne *Salmonellosis* annually. In recent years, contaminated fresh produce has emerged as an important source of salmonellosis. Standardized culture methods for *Salmonella* spp. are considered as the "gold standard" in food diagnostics and are still in use today; however, they are laborious, time-consuming and must be confirmed by secondary biochemical tests. Immunoassays are the most commonly used rapid methods for the detection of *Salmonella* in food, and presumptive results are available within 8 to 24 hours. However, a common issue observed using immunoassays to test fresh produce for the presence of *Salmonella,* is a high percentage of false-positive test results due to the misidentification of closely related, non-*Salmonella* bacteria such as *Citrobacter* spp., *Hafnia* spp. and *Proteus* spp. In addition, there is also the chance of false-negative test results, due to high variation in surface antigens of *Salmonella enterica*. In this study, two commercially available immunoassays, the VIDAS UP *Salmonella* Phage Technology (SPT) Assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and an antibody-based lateral-flow test, the Reveal 2.0 *Salmonella* Assay (Neogen Corporation, Lansing, Michigan, United States) were evaluated for their accuracy in detecting *Salmonella*. VIDAS UP *Salmonella* (SPT) assay correctly identified 52/54 (96.3%) of the *Salmonella* isolates that were tested. The Reveal 2.0 *Salmonella* Assay identified 43/54 (79.63%) of the *Salmonella* isolates correctly. However, both assays failed to identify one isolate each of *Salmonella enterica* serovars Hull and Duesseldorf. Several VIDAS UP *Salmonella* (SPT) enrichment samples were obtained from a fresh produce grower. These enrichments had previously tested presumptive positive for *Salmonella,* however, confirmatory tests did not indicate the presence of *Salmonella*. The enrichments were

analyzed in order to obtain pure isolates of bacteria from the enrichment mixture that were responsible for the false positive test results. Three bacterial isolates that caused false-positive VIDAS UP *Salmonella* (SPT) reactions were isolated and subjected to whole genome sequencing and bioinformatic analysis. Blast analysis of the three false-positive isolates identified *Citrobacter amalonaticus* as the likeliest organism. The two isolates (one each) of *S*. Hull and *S*. Duesseldorf that produced false-negative results were analyzed in order to identify surface exposed components that are used as diagnostic targets in *Salmonella* immunoassays. Analysis of *S*. Duesseldorf showed that the flagella genes *fljB* and *fliC* differed significantly from other *Salmonella* isolates that tested positive, and the three *C.amalonaticus* isolates that caused false-positive test results. Analysis of the *S*. Hull genome identified a gene encoding a putative repression of phase I flagellin, which was located on a cryptic incomplete prophage. Both of these observations are likely responses for the lack of complete flagella on the surface of the *S*. Duesseldorf and *S*. Hull isolates, which may be responsible for the false-negative test results. The results of this work have identified the potential basis for false-positive and false-negative test results in rapid *Salmonella* immunoassays. The development of more selective immunoassays based on more specific monoclonal antibodies, identification of new antigens that are more specific to *Salmonella* and development of more selective enrichment media will lead to improved fresh produce testing and enhanced food safety in Canada.

RÉSUMÉ

Salmonella spp. est un agent pathogène Gram négatif largement distribué qui est une cause majeure d'éclosion d'origine alimentaire en Amérique du Nord. L'Agence de la santé publique du Canada (ASPC) estime qu'environ 88,000 Canadiens sont touchés par la salmonellose d'origine alimentaire chaque année. Au cours des dernières années, les fruits et légumes contaminés sont reconnus comme une source importante de salmonellose. Les méthodes de culture normalisées pour *Salmonella* spp. sont considérées comme « l'étalon d'or » à fin de diagnostic alimentaire et sont toujours utilisées aujourd'hui; cependant, ces méthodes sont laborieuses, longues et les résultats doivent être confirmés par des tests biochimiques secondaires. Les tests à base immunologique sont les méthodes les plus couramment utilisées pour la détection de *Salmonella* dans les aliments, et les résultats présomptifs sont disponibles à l'intérieur de 8 à 24 heures. Cependant, un problème communément observé en utilisant ces tests immunologiques pour tester les fruits et légumes pour la présence de *Salmonella* est un pourcentage élevé de résultats faussement positifs en raison de l'identification erronée de bactéries non-*Salmonella* étroitement apparentées, telles que *Citrobacter* spp., *Hafnia* spp. et *Proteus* spp. En outre, il existe également la possibilité de résultats faussement négatifs, en raison de la forte variation des antigènes de surface de *Salmonella enterica*. Lors de cette étude, deux tests immunologiques disponibles sur le marché, le test VIDAS *Salmonella* (SPT) utilisant la technologie de phage (BioMérieux, Montréal, Québec, Canada) et le test à immunochromatographie Reveal *Salmonella* 2.0 (Neogen Corporation, Lansing, Michigan, ÉU) ont été évalués pour leur précision dans la détection de *Salmonella*. Le test VIDAS (SPT) a correctement identifié 52/54 (96.3%) des isolats de *Salmonella* qui ont été testés. Le test Reveal 2.0 a identifié correctement 43/54 (79.63%) des isolats de *Salmonella*. Cependant, aucun des deux tests n'a permis d'identifier les isolats de *Salmonella enterica* serovars Hull ou Duesseldorf. Plusieurs échantillons

d'enrichissement VIDAS (SPT) ont été obtenus auprès d'un producteur de produit frais. Ces enrichissements avaient précédemment donné des résultats présumés positifs pour *Salmonella*, mais les tests de confirmation n'indiquaient pas la présence de *Salmonella*. Les trois isolats bactériens qui ont provoqué des résultats faussement positifs lors de la détection à partir du VIDAS (SPT) ont été isolées et soumises à un séquençage génomique complet et à une analyse bioinformatique. L'analyse par Blast des trois isolats faussement positifs a identifié *Citrobacter amalonaticus* comme l'organisme le plus probable. Deux isolats ayant donné des résultats faussement positifs (*S*. Hull et *S*. Duesseldorf) ont été analysés afin d'identifier les composants exposés à la surface qui sont utilisés comme cibles de diagnostic dans les tests immunologiques de *Salmonella*. L'analyse de *S*. Duesseldorf a montré que les gènes de la flagelles, *fljB* et *fliC*, diffèrent significativement des autres isolats de *Salmonella* qui ont testé positifs ainsi que les trois isolats de *Citrobacter amalonaticus* ayant provoqué des résultats faussement positifs. L'analyse du génome de *S*. Hull a identifié un gène présumé d'encoder un répresseur de la flagelline de phase I, qui est situé sur un prophage cryptique. Ces deux observations suggèrent possiblement que l'absence de flagelles entiers à la surface des isolats de *S*. Duesseldorf et *S*. Hull peut être responsable des résultats des tests faussement négatifs. Les résultats de ce travail ont identifié la base potentielle pour les résultats faussement positifs et négatifs de *Salmonella*. Le développement des tests immunologiques plus sélectif basé sur des anticorps monoclonaux plus spécifiques, l'identification de nouvelles cibles plus spécifiques pour *Salmonella* ainsi que le développement de milieu d'enrichissement sélective, conduira à une amélioration des tests sur les fruits et légumes et à une meilleure sécurité alimentaire au Canada.

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Thesis Format

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following

(a) A table of contents;

(b) An abstract in English and French;

(c) An introduction which clearly states the rational and objectives of the research;

(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);

(e) A final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers".

CONTRIBUTIONS OF AUTHORS

The following are the manuscripts prepared for publication:

1. **Raman, Reshmi**; Goodridge, D. Lawrence. 2017. "Rapid Diagnostics for Detection of *Salmonella* in Foods– A Review" **(Draft prepared)**
2. **Raman, Reshmi**; D'Souza, Yasmin; Tang Sik Fon, Marie; Goodridge, D. Lawrence, 2017. "Evaluation of the VIDAS UP *Salmonella* (SPT) Assay and Reveal 2.0 *Salmonella* Assay affinity-based methods for sensitivity and specificity in the detection of *Salmonella* spp." **(Draft prepared)**
3. **Raman, Reshmi**; Colavecchio, Anna; Barrere, Virginie; Levesque C. Roger; Goodridge, D. Lawrence, 2017. "Molecular Characterization of bacteria that cause false-positive and false-negative test results on the VIDAS UP *Salmonella* (SPT) affinity assay" **(Draft prepared)**

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The work reported here was done in the Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec, Canada. The entire research work was carried out in the Diagnostic, Enrichment, Testing and Characterization (D.E.Te.CT) laboratory, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec, Canada and the whole genome sequencing was carried out

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CHAPTER I

GENERAL INTRODUCTION

Outbreaks of foodborne disease continue to be a public health concern. They are caused by ingestion of food that is contaminated with chemicals, physical agents or microorganisms (Behravesh et al., 2012). Microbial foodborne illness affects approximately 1 in 6 persons annually in the United States and 1 in 8 people in Canada (Scallan et al., 2011; Thomas and Murray, 2014). Globally, the total number of foodborne illnesses is increasing, due to large multinational outbreaks caused by foodborne pathogens. Thus, emphasis on food safety is considered essential from farm to fork (Tauxe, 2006).

Foodborne pathogens commonly implicated in outbreaks include *Listeria monocytogenes,* Shiga toxin producing *Escherichia coli* (STEC), *Campylobacter* spp. and *Salmonella* spp. (Valderrama et al., 2016). *Salmonella* spp., a Gram-negative bacterium which belongs to the *Enterobacteriaceae* family, is considered as one of the leading foodborne pathogens in Canada and causes approximately 5% illnesses, 24% hospitalizations and 16% deaths, through consumption of *Salmonella* contaminated food in Canada (PHAC, 2016). Symptoms of salmonellosis include fever, diarrhea and abdominal cramps. Food vehicles implicated in outbreaks of salmonellosis include poultry, red meat, chocolate, flour, and increasingly fresh fruits and vegetables (Wu et al., 2017). This is because fruits and vegetables are grown in soil, where animals, non-potable water, soil alterations and harvest equipment act as sources of contamination for foodborne pathogens to spread (Jung et al., 2014). Additionally, there is no effective kill step to destroy pathogens once they have contaminated fresh produce. As a result, most foodborne illnesses now occur due to consumption of contaminated fresh produce and *Salmonella* accounts for approximately 50% of fresh produce related illnesses (Denis et al., 2016).

Advances in various microbiological and molecular techniques have played a significant role in the development of rapid tests for the food industry (Tauxe, 2006). Historically, conventional microbiological techniques such as standard culture methods were commonly used to isolate and enumerate foodborne pathogens in various foods. These methods are inexpensive and are considered as the gold standard to detect the foodborne pathogens; however, there are several limitations to cultural methods including the fact that it often takes 5-7 days to obtain a test result (Feng et al., 2007). Hence, rapid detection methods including, nucleic acid-based methods and immunoassays are routinely employed to speed up the detection process (Bell et al., 2016; Swaminathan and Feng, 1994). However, even though rapid methods have overcome the limitations observed with traditional culture methods, it is important to note that a positive result for a foodborne pathogen is always considered as a "presumptive" positive, until confirmed by the cultural methods (Feng, 1995). While most rapid methods can perform a sample analysis in 8-24 hours but the rapid methods are known to have issues with specificity. For example, a 14-year study showed that rapid tests used in the food industry to test for *Salmonella* had a false-positive rate of 3.9% and a false-negative rate of 4.9% (Cowan-Lincoln, 2013).

In North America, immunoassays are the most common type of rapid method used to test foods for the presence of foodborne pathogens (**Figure 1.1**) (Weschler, 2014). Immunoassays are based on the binding of an antibody to an antigen by means of an immunological reaction (Mandal et al., 2011). There are several types of immunoassays including, Enzyme-linked Immunosorbent Assays (ELISA), Lateral Flow Assays (LFA) and Latex Agglutination tests (Valderrama et al., 2016). In addition, variations of the typical immunoassay in which bacteriophage (phage) tail components are used in place of antibodies have also gained prominence (Odumeru and León-Velarde, 2012). One such assay, the BioMérieux VIDAS UP *Salmonella* (SPT) Assay, can deliver results within 24

hours and can be used to initially screen food (including fresh produce) for foodborne pathogens, including *Salmonella* spp. *E.coli* O157:H7 and *Listeria monocytogenes*. However, a common issue with rapid detection of foodborne pathogens from fresh produce samples is the interference from the sample matrix that could potentially produce false-positive or a false-negative test results (Goodridge et al., 2011). For example, the presence of other closely related bacteria, principally *Citrobacter* spp., *Proteus* spp., and *Hafnia* spp. can cause false-positive test results when immunoassays are used to detect *Salmonella* spp.. Therefore, there is a need to identify isolates within these genera that cause false-positive test results, as well as to characterize the reasons for the cross reactivity, as a first step in identifying solutions that can improve the specificity of immunoassays. In addition, there is the possibility of false-negative test results due to the inability of the immunoassays to detect all *Salmonella* isolates (Banada and Bhunia, 2008; Goodridge et al., 2011; Hahm and Bhunia, 2006; Hoorfar, 2011). Thus, in this study, an emphasis was placed on isolation and identification of *Salmonella* spp. and closely related non-*Salmonella* spp. that cause false-positive or false-negative test results when immunoassays (VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* Assay) are used to test fresh produce samples. Further, genomic and bioinformatic analysis of the whole genomes of bacteria that caused false-negative and false-positive test results were conducted to determine the reasons for these results.

1.1 General Hypothesis

False-positive and false-negative test results may be caused by the presence and absence of surface exposed proteins, which are targets for detection. The cross-reactivity in immunoassays might be due to the presence of similar cell surface exposed antigenic structures (flagella, outer membrane proteins, lipopolysaccharide components or fimbriae) of the *Salmonella* and non-*Salmonella* bacteria.

1.2 Objectives

1.2.1 Overall Objective

The overall objective is to analyse the reasons for false-positive and false-negative test results in rapid immunoassays and analysis of whole genome sequences of *Salmonella* and closely related bacteria, to identify characteristics of cell surface exposed proteins that are responsible for the test results.

1.2.2 Specific Objectives

1) To evaluate two commercially available affinity-based assays VIDAS UP *Salmonella* (SPT) Assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and Reveal 2.0 *Salmonella* Assay (Neogen Corporation, Lansing, Michigan, United States), for their ability to correctly identify *Salmonella enterica* isolates, and non-*Salmonella* bacteria belonging to closely associated genera of the *Enterobacteriaceae* including *Citrobacter*, *Proteus* and *Hafnia* spp.

2) To identify cell surface exposed proteins, belonging to closely associated genera of the *Enterobacteriaceace* including *Citrobacter*, *Proteus* and *Hafnia*, that share high levels of homology with *Salmonella enterica*, and to understand the reasons for false-positive and false-negative diagnostic results of VIDAS UP *Salmonella* (SPT) Assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and Reveal 2.0 *Salmonella* Assay (Neogen Corporation, Lansing, Michigan, United States) assays.

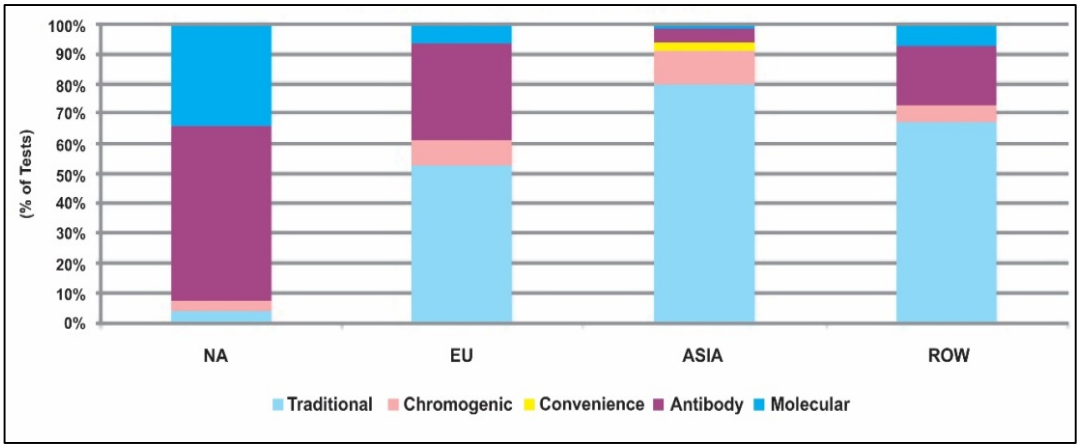

Figure 1.1. Comparison of food microbiology testing practices in North America (NA), Europe (EU), Asia and Rest of the World (ROW) (Adapted from, Weschler, 2014).

CHAPTER II

RAPID DIAGNOSTICS FOR DETECTION OF *SALMONELLA* IN FOODS – A REVIEW

2.1 Introduction

Foodborne illnesses continue to pose a threat to public health. *Salmonella* is one of the major foodborne pathogens causing 5% of illnesses in Canada (PHAC, 2016). The Public Health Agency of Canada (PHAC) reported nearly 2,500 *Salmonella* serovars, of which the top 5 accounts for 65% of the total *Salmonella* infections in Canada. The most common serovars that cause salmonellosis in Canada are listed in **Table 2.1** (PHAC, 2012).

Furthermore, in recent years, an increasing number of *Salmonella*-related outbreaks have been related to fresh produce consumption (Denis et al., 2016; Harris et al., 2003). A wide range of fresh produce items has made an impact on outbreaks and illnesses in North America. For example, fresh produce commodities such as leafy greens, lettuce and spinach were identified as potential sources of bacterial infections (FAO/WHO, 2008). Also, a recent U.S. Food and Drug Administration (FDA) survey identified *Salmonella* in 143 imported samples of strawberries. There is a high probability of finding foodborne pathogens from imported commodities of fresh produce as Canada imports approximately 88% of fruits and 41% of vegetables every year. According to 2011 import data, the main sources of produce imported into Canada were from the USA, which includes the products such as leafy greens, soft fruits, citrus fruits, grapes, cauliflower, broccoli, onions, beans and carrots; peppers, tomatoes, avocados, cucumbers and asparagus from Mexico; a range of fresh produce from Chile, Peru, Honduras, Guatemala, Costa Rica and China (Agriculture and Agri-Food Canada, 2014a; Agriculture and Agri-Food Canada, 2014b).

Apart from these incidences, a variety of produce including tomatoes, melons, spinach and sprouts have been associated with multiple *Salmonella* outbreaks (Hanning et al., 2009). Particularly, several outbreaks occurred between April and August 2008 in United States and Canada. The Center for Disease Control and Prevention (CDC) reported that *Salmonella enterica* serovar Saintpaul caused 1,442 cases and 2 deaths (Behravesh et al., 2011). In addition, multiple raw produce items including fresh jalapeno peppers, serrano peppers, and raw tomatoes were implicated in this outbreak. Additionally, multiple *Salmonella* outbreaks due to consumption of Roma tomatoes were reported in United States and Canada in the summer of 2004, resulting in 561 illnesses with a 30% hospitalization rate (Center for Disease Control and Prevention, 2005). As with other industrialized countries, Canada has seen an increased number of foodborne illness outbreaks linked to domestic fresh produce. Examples of more recent salmonellosis outbreaks that occurred in Canada include an outbreak linked to domestic green onions, which resulted in 20 cases of foodborne illness in 2010 and another caused by *S.* Newport, *S.* Hartford, *S.* Oranienburg*, and S.* Saintpaul in chia seeds and sprouted chia seed powder (Denis et al., 2016; Harvey et al., 2017).

2.1.1 Fresh produce outbreaks of *Salmonella*

During the last three decades, the number of fresh produce associated foodborne pathogen outbreaks have been increasing, as per CDC report. Approximately, 48% of these outbreaks were caused by *Salmonella* spp (Chen et al., 2016). Several major outbreaks occurred between 2004 and 2011, and were mainly associated with fresh produce items including cilantro, cucumbers, cantaloupes and peppers accounts a total of 629 outbreaks leading to almost 20,000 illnesses (Doyle and Buchanan, 2012). A variety of produce items including melons, sprouts, tomatoes, spinaches and peppers have also been implicated in multiple *Salmonella* outbreaks (Hanning et al., 2009). In March 2013, a

report from the U.S. CDC revealed that 46% of all foodborne illnesses that led to hospitalization or death occurred during 1998-2008 were attributed to fresh produce (Painter, 2013). *Salmonella* is found in the intestine of warm-blooded animals and contaminates produce when it is contacted in fecal matter (Franz et al., 2005; Ongeng et al., 2011). Contamination can occur and be amplified during pre-harvest, harvest, processing and distribution due to unclean equipment surfaces, poor hygiene of handlers and ineffective disinfection methods (Hanning et al., 2009; Harris et al., 2003). The development of Good Agricultural Practices (GAPs) can decrease the risk due to *Salmonella* contamination of fresh produce. Testing the product is an additional approach to determine the presence of *Salmonella.*

2.2 Methods to detect *Salmonella* species

Foodborne pathogens can be detected using either (i) Cultural/Conventional methods, or (ii) Rapid methods (Zadernowska and Chajęcka, 2012). In spite of the tedious process involved in cultural methods to identify foodborne pathogens, they are still considered as the "gold standard" due to their consistency in identifying the pathogens. Cultural methods employ an initial enrichment step in selective media to increase the numbers of the target bacteria (which are typically present in low numbers in foods), followed by plating on selective and differential media (Goodridge and Bisha, 2011; Silliker and Gabis, 1973). Enrichment techniques are also useful for recovery of injured bacterial cells present in stressed conditions in some foods. There are many cultural methods available for identification of *Salmonella* spp. These cultural methods take advantage of the unique aspects of *Salmonella* physiology or biochemistry compared to the other members within the *Enterobacteriaceae* family (Siegrist, 2009).

Due to the fact that they are less laborious and time consuming, rapid methods are used for routine microbiological analysis of foods. However, some inherent limitations continue to pose a challenge in the rapid detection of foodborne pathogens in different foods (Feng et al., 2007). One such challenge is the interference of background microflora in food samples, the presence of which may yield false-positive or false-negative test results because they contribute to the incorrect identification of target pathogens (Ge and Meng, 2009; Goodridge and Bisha, 2011). Additionally, the compounds present in food samples may interfere with the chemical or functional composition of the test reagents during pathogen detection procedures, which, in turn, give rise to false-negative or false-positive results. The two classes of rapid methods currently employed in the food industry are immunoassays and molecular methods.

2.2.1. Conventional Methods

Traditionally, culture-based methods were used exclusively for microbiological analysis. Culture-based methods increase the concentration of the target microorganisms in selective or differential culture media under controlled laboratory conditions (Doyle and Buchanan, 2012) in order to isolate the target bacterial cells from the contaminated food (Mandal et al., 2011). Identification of the bacterial species is carried out by observing the colony morphology, as well as through biochemical analysis (Merker, 1999; Nataro and Kaper, 1998).

The advent of rapid methods for routine testing in foods has largely confined the use of conventional methods to confirmatory testing. This conventional method of testing is used to confirm the test results from rapid tests that are considered to be presumptive. In Canada, the official cultural method for *Salmonella*, which is used to confirm presumptive positive test results from rapid methods, is contained within the Compendium

of Analytical Methods within the Bureau of Microbial Hazards, Food Directorate, of Health Canada. The procedure consists of six distinct stages including 1) non-selective enrichment (pre-enrichment) in nutrient broth of buffered peptone water, to favour the repair and growth of stressed or sub lethally-injured *salmonellae* arising from exposure to heat, freezing, desiccation, preservatives, high osmotic pressure or wide temperature fluctuations; 2) selective enrichment, in which replicate portions of each pre-enrichment culture are inoculated into two enrichment media (Tetrathionate Brilliant Green (TBG) broth and Rappaport-Vassiliadis Soya Peptone (RVS broth)) to enhance the proliferation of *salmonellae* through a selective inhibition of the growth of competing microflora; 3) in the selective plating step, enriched cultures are streaked onto two selective and differential agars (any two of these media, Bismuth Sulfite (BS) Agar, Brilliant Green Sulfa (BGS) Agar, and Brilliance *Salmonella* Agar (BSA)) for the isolation of *salmonellae*; 4) presumptive *Salmonella* isolates are purified on MacConkey agar plates to eliminate the possibility of viable but inhibited organisms from the selective agars contaminating the culture in further tests; 5) biochemical screening is then applied to identify suspected *Salmonella* colonies; and 6) serological identification using polyvalent and/or grouping somatic antisera are used to support the identification of isolates as *Salmonella*.

In the USA, the FDA Bacterial Analytical Manual (BAM) describes the procedures for isolation of *Salmonella* from foods (Andrews et al., 2007). For fresh produce, the sample is initially incubated in a non-selective broth including buffered peptone water for tomatoes and mangoes, universal pre-enrichment broth for cantaloupes, or lactose broth for leafy green vegetables and herbs (baby spinach, Romaine lettuce, cilantro, curly parsley, Italian parsley, cilantro, cabbage, and basil). For selective enrichment of *Salmonella*, aliquots of the initial non-selective enrichment are inoculated into Rappaport-Vassiliadis (RV) broth and Tetrathionate (TT) broth. Next, the selective enrichments are

plated on BS agar, Xylose Lysine Desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. Colonies with typical or atypical *Salmonella* morphology are picked from the agar plates. The colonies are used to incubate Triple sugar iron agar (TSI) and Lysine iron agar (LIA) slants. All cultures that give an alkaline but in LIA, regardless of TSI reaction, or cultures that give an acid but in LIA and an alkaline slant and acid but in TSI are considered to be potential *Salmonella* isolates. These isolates are submitted for biochemical and serological tests to confirm identification (Hammack et al., 2002; Pereira et al., 2006; Waltman, 2000).

2.2.2 Disadvantages of Cultural Media

The main limitations associated with cultural methods include, tediousness of the procedures, labour intensiveness, the time to test result (5-7 days) and the need for secondary biochemical confirmatory tests. An additional disadvantage is the fact that cultural methods are designed to only detect bacteria, since many agents of foodborne diseases, such as toxins and viruses (hepatitis A and norovirus) cannot be enriched (D'Souza et al., 2007; Jaykus and Escudero-Abarca, 2010) . The labour intensiveness and time to test result in particular, prelude the use of cultural methods for routine analysis of foods. Therefore, rapid methods, which are much less intensive, and deliver test results within 24 hours or less, have emerged as the assays commonly used to assess foods for the presence of foodborne pathogens.

2.2.3 Rapid Methods

Rapid and sensitive methods for detecting foodborne pathogens are essential to prevent foodborne infections. The quick detection of foodborne pathogens can be accomplished through various rapid methods including immunoassays and molecular methods. Immunoassays are mainly based on antibodies, affinity probe-biosensors and

cell-based assays. On the other hand, molecular methods or nucleic acid-based assays are based on Polymerase Chain Reactions (PCR) and DNA hybridization approaches.

2.2.3.1 Immunoassays

Immunoassays rely on the interaction between an antibody and an antigen in order to qualitatively identify the target foodborne pathogen. Qualitative (after enrichment or presence/absence test) identification of foodborne pathogens is less time consuming and laborious compared to conventional methods. Various types of immunoassays exist for rapid detection of foodborne pathogens (Crowther, 2008). The two main assays are the enzyme-linked immunosorbent assay (ELISA) and lateral flow assays (LFAs) (Jasson et al., 2011).

Several variations of the traditional antibody-antigen immunoassay have been developed. For example, antibodies have been replaced by bacteriophage tail components, which can be superior to antibodies in terms of specificity and binding kinetics. For example, in the case of *E. coli*, *beta*-galactosidase combines with the landscape of a phage (as probe) displaying an array of peptide binders on the surface, which in turn helps in identifying the species that is similar to antibody-antigen interaction (Emanuel et al., 2000; Petrenko and Vodyanoy, 2003). Similarly, a fluorescent-bacteriophage hybrid method with immunomagnetic separation was developed using modified direct epifluorescent-filter technique (DEFT) to estimate bacterial concentrations (Goodridge et al., 1999).

2.2.3.1.1 Enzyme linked immunosorbent assay (ELISA)

The enzyme linked immunosorbent assay (ELISA), known as a “sandwich” assay, is an immunological method widely used to detect microorganisms and toxins in food. The ELISA test is usually conducted in a 96-well microtiter plate, the wells of which are

coated with a specific antibody. When loaded into a microtiter plate well, the target pathogen binds to the specific antibody. A secondary antibody (with an enzyme attached) is added such that it binds to the target pathogen, thus forming a sandwich structure. After several washes to remove non-target bacteria, present due to nonspecific binding, an enzyme substrate is added, which generates a detectable signal (visible, fluorescence or luminescent) by reacting with the bound enzyme on the secondary antibody (Crowther, 2008). The ELISA technique has been widely used due to its simplicity and quickness for detecting *Salmonella* since the 1970s (Carlsson et al., 1975). However, common drawbacks associated with ELISA include its limited sensitivity and low specificity. Additionally, because the detection limit of the ELISA is between 10^4 and 10^5 CFU/ml, enrichment is always needed to improve its ability to detect foodborne pathogens, which are often present in foods in low numbers (Feng et al., 2007).

2.2.3.1.2 Lateral Flow Assays (Rapid Antibody-Based Assays)

The lateral flow assay (LFA) is a membrane-based method for the detection of foodborne pathogens. These assays are popularly known as "home pregnancy tests." Following bacterial enrichment, a drop of the enrichment culture is placed on a test device and the results are displayed within 5–30 mins. A typical lateral flow test strip consists of overlapping membranes that are mounted on a backing card. The sample (bacterial enrichment) is applied to one end of the strip at a sample portal, and is absorbed onto a sample pad, which is impregnated with buffer salts and surfactants that make the sample suitable for interaction with the detection system. The sample then migrates through the conjugate release pad, which contains antibodies that are specific to the target analyte and are conjugated to coloured or fluorescent particles. The sample, together with the conjugated antibody bound to the target analyte, migrates along the strip into the detection zone. This is a porous membrane with antibodies immobilized in lines, and the antibodies

react with the analyte bound to the conjugated antibody. Recognition of the sample analyte results in the formation of a test line, while a response on a second control line indicates the proper liquid flow through the strip. The read-out, represented by the lines appearing at the test line and the control line with different intensities, can be assessed by eyes or using a dedicated reader (Koczula and Gallotta, 2016).

The Reveal 2.0 *Salmonella* (Neogen) is an improved assay of the original Reveal *Salmonella* lateral flow immunodiagnostic test. This method may be used to detect *Salmonella enterica* serogroups (A–E) in food and environmental samples (Hoerner et al., 2011). The Reveal 2.0 *Salmonella* provides results within 24 hours, and has been modified in a couple of ways to improve the detection of certain *Salmonella* serovars. The modification includes the addition of a polyclonal antibody to the test. The lateral flow device architecture is incorporated to utilize a "naked strip" format without a plastic housing (Hoerner et al., 2011). In identifying foodborne pathogens like *Salmonella*, Reveal 2.0 *Salmonella* utilizes a proprietary medium called Revive. This media provides readily available nutrients to *Salmonella* and other components required for *Salmonella* to be recovered from stressed or injured conditions. The recovered *Salmonella* from Revive media is transferred to a brief enrichment Rappaport-Vassiliadis (RV) broth. This enrichment broth favors the growth of *Salmonella* which is detected by the Reveal 2.0 *Salmonella* device.

2.2.3.2 Bacteriophage Detection Methods

Bacteriophages (phages) are viruses that infect bacteria. By definition, they are obligate intracellular parasites. Phages recognize their hosts through bacteriophage receptor binding proteins (RBP), which bind to a specific receptor on the bacterial cell surface. These proteins are also called tail spikes, tail fibers or spike proteins. RBPs have

been recently exploited as an alternative to antibodies for the detection of foodborne pathogens. RBPs offer several advantages over antibodies including greater stability, ligand specificity, and affinity against carbohydrate epitopes, which are not typically recognized by most antibodies effectively (Miletic et al., 2016). The Vitek Immuno Diagnostic Assay System Phage Technology (VIDAS PT) is a commonly used assay in the food industry that employs RBP in combination with antibodies to effect detection of *Salmonella* spp. and other pathogens.

2.2.3.3 Vitek Immuno Diagnostic Assay System (VIDAS)

Introduced by BioMérieux in 2011, the VIDAS UP *Salmonella* Phage Technology (SPT) assay is an automated enzyme linked immunoassay that uses fluorescent technology (ELFA) for the detection of *Salmonella* in food. VIDAS is based on the application of RBP's, which are specific for *Salmonella* spp. This method is a two-step sandwich assay that uses a mixture of monoclonal antibodies and recombinant phage proteins. The principle used by the VIDAS assay is the Enzyme Linked Fluorescent Assay (ELFA) technology. Somatic and flagellar antigens are targeted, which allow for the detection of both motile and non-motile strains of *Salmonella* (Feng, 1992). A pipette tip-like unit (a Solid Phase Receptacle or SPR) serves as the solid phase during the process. The SPR is coated with polyclonal anti-*Salmonella* antibodies and reagents for the assays are sealed in reagent strips. Enrichment broth is placed into the reagent strip, after which the sample and reagents are cycled sequentially in and out of the SPR for a specific time until the fluorescence is detected by the instrument. Although studies that have evaluated the VIDAS assay, have demonstrated that it is highly sensitive, false-positive results may be observed in assays (Odumeru and León-Velarde, 2012; Zadernowska and Chajęcka, 2012).

2.2.3.4 Disadvantages of Rapid Antibody-Based Assays

Rapid immunoassays are sensitive, specific and easy to perform, thus making routine microbiological testing possible. Affinity-based assays such as immunoassays are capable of detecting toxins; however, these immunoassays are not as specific as nucleic acid-based assays due to the interference of background microflora (Goodridge and Bisha, 2011). The reactivity of these assays is influenced by the incubation conditions used and the components of the enrichment medium. Additionally, similarities within the cell surface exposed proteins that are targets of immunoassays may lead to false-positive test results due to the presence of closely related bacteria in the enrichment media. False-negative results may also occur when target antigens are not expressed (McQuiston et al., 2011). Understanding and characterizing the reasons for the cross reactivity are important first steps to identifying solutions that can improve the specificity of affinity-based assays (Zadernowska and Chajęcka, 2012).

2.2.4 Molecular Methods

Since the 1980s, advances in basic DNA research have aided in the development of various nucleic acid based techniques for foodborne pathogen detection, and contributed to the emergence of molecular-based pathogen detection methods (Bell et al., 2016; Ferrato et al., 2017; Gilbert and Dupont, 2010; Jay et al., 2005; Shariat and Dudley, 2013). Particularly, methods that involve nucleic acid amplification and hybridization are recognized to be rapid, sensitive, highly specific, automated and reproducible (Wang R.A. et al., 1997). These desirable features of molecular-based methods have resulted in their wide usage in microbiological analysis. Two common molecular approaches for detecting foodborne pathogens are the Polymerase Chain Reaction (PCR) and DNA hybridization (Wang.H, 2002).

2.2.4.1 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) for amplifying the target DNA was developed by Kary Mullis in the 1980s (Mullis, 1990). The PCR assay is a non-cultural technique that is based upon primer-meditated enzymatic amplification of specific segments of DNA for the detection of foodborne pathogens including *Salmonella* (Mckillip and Drake, 2004). The method employs a thermostable DNA polymerase to synthesize new strands of DNA complementary to a template strand that is derived from the sample to be tested. PCR assays exponentially amplify a single DNA fragment by over a million-fold within 2 to 3 hours. In conventional PCR, amplicons are amplified in a thermocycler and then detected following amplification by gel electrophoresis. Such end point detection systems are laborious and increase the time to detect, and are seldom used for rapid detection of foodborne pathogens. More recently, a variation of conventional PCR, termed real-time PCR (RT-PCR) has been developed, and this approach allows for visualization of the PCR amplicon as it is being produced in real time using fluorescent reporter molecules. This technique has revolutionized the microbiology related research in detecting and quantifying the microbial flora from the targeted samples (Filion, 2012). While, in theory, the PCR assay can amplify the DNA target from a single cell, the presence of PCR inhibitors in foods and environmental samples means that enrichment of the target bacteria prior to detection must still be employed.

The real-time PCR (RT-PCR) system detects accumulated PCR product by monitoring the increased fluorescence signal during amplification, through the use of a real-time thermocycler and fluorescence detector system. Several commercial RT-PCR systems are available for detection of foodborne pathogens, including the ABI Prism 7500 (Applied Biosystems, Warrington, UK), Probelia (Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France), BAX system (DuPont Qualicon, Wilmington, Delaware, USA),

TaqMan (PE-Applied Biosystems, Foster City, California ,USA), Gene-Trak (Neogen Corporation, Lansing, Michigan, USA), iQ-Check™ PCR (BioRad Laboratories, Hercules, California, USA), LightCycler (Roche Diagnostics, Manheim, Germany), and SmartCycler (Cepheid Inc., Sunnyvale, California, USA). In addition, the decreased time in detection afforded by RT-PCR due to real-time detection of amplicons, another advantage of RT-PCR over conventional PCR assays is the reduced chance for false-positive detection caused by DNA contamination from the environment. For example, several RT-PCR systems are automated, allowing for DNA extraction, and loading of the PCR tubes or detection plates to be facilitated by robotics. This has helped to overcome the problem of false-positive results posed by amplicon contamination (Maurer, 2011). The use of systems in which all PCR reagents come supplied pre-mixed also reduces the chances of contamination. For example, the BAX system was the first commercially available RT-PCR method to combine all PCR reagents (primers, thermostable DNA polymerase, and deoxyribonucleotides) into a single tablet, which decreases contamination that could occur when these reagents are added separately to the reaction tube. RT-PCR assays also include an internal standard, which is used to ensure that the assay has worked correctly, and did not fail due to the presence of PCR inhibitors.

While PCR and RT-PCR assays are generally regarded as more specific than immunoassays due to the ability to precisely identify specific DNA sequences within target cells, these assays still suffer from detection of false-positive targets when fresh produce is assessed for the presence of *Salmonella*. In one recent study, 49 target and 29 non-target strains were included to assess inclusivity, exclusivity and limit of detection using 7 commercial RT-PCR systems (Margot et al., 2013). All systems evaluated were able to correctly identify the 49 *Salmonella* strains. Nevertheless, false-positive results were obtained due to incorrect identification of *Citrobacter murliniae* strains as

Salmonella. Other groups have also reported false-positive *Salmonella* RT-PCR test results due to *Citrobacter* and *Proteus* spp. (Malorny et al., 2003; Moore and Feist, 2007).

2.2.4.2 DNA hybridization

A DNA probe hybridization assay uses a labelled DNA probe with a sequence complementary to the target sequence of a DNA or RNA molecule in the target organism (de Boer and Beumer, 1999; Fung, 2002; Mozola, 2006). A gene probe consists of either an entire gene or a fragment of the gene with a known function (Laird et al., 1991). In this assay, following an enrichment step, target cells are lysed and the resulting nucleic acids are purified prior to hybridization with a DNA probe specific for the target region of choice. Any unbound probe is removed by washing. The DNA-probe hybrid can then be detected using different detection techniques, such as enzymatic reactions, in a manner analogous to the ELISA method.

One advantage of DNA probe hybridization assays over other molecular methods such as RT-PCR is the fact that, depending on the format of the assay, large number of samples can be rapidly screened for the presence of foodborne pathogens such as *Salmonella*. DNA hybridization assays are not widely used in the food industry to detect *Salmonella* and other foodborne pathogens, but there are several commercial assays available. These include the Gene-Trak® *Salmonella* Assay (Neogen Corporation, Lansing, Michigan, United States) is the first introduced commercial assay, which is based on a hybridization format of two probes, a polyadenylic acid (poly dA) tail on the capture probe and a fluorescein labeled detector probe, specific for *Salmonella* ribosomal RNA (rRNA), and direct labeled enzyme-mediated colorimetric detection, as the probes are labeled with horse radish peroxidase. A higher throughput microwell format assay is also available under the name GeneQuence™ *Salmonella* Test System (Neogen Corporation,

Lansing, Michigan, United States). DNA hybridization assays are not as sensitive as RT-PCR assays and conventional culture methods. Since the assays require higher detection limits of enrichment culture for positive signal, they need a longer enrichment time to increase the concentration of *Salmonella* prior to detection. However, these methods may reduce issues with false-positive detection due to the lack of DNA amplification during detection (where any contaminating DNA may be amplified and contribute to a false-positive result) (Flowers et al., 1987; Zhang et al., 2011).

Table 2.1. The list of common *Salmonella* serovars causing salmonellosis in Canada, PHAC, 2012

***Salmonella* serovar**	**No. of Infections reported**	**Total (%)**
S.Enteriditis	2117	30
S.Heidelberg	1071	15
S.Typhi	814	12
*S*sp.|4, (5),12:i	281	4
S.Thompson	265	4
S.Infantis	184	3
S.Newport	153	2
*S*sp 4,5	108	2
S.Braenderup	98	1
Total	**5091**	**73**

Source: PHAC Report (2012)

CONNECTING TEXT

A comprehensive review of the scientific literature revealed the potential for rapid *Salmonella* immunoassays to cause false-positive test results due to the presence of closely related bacteria. The VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays are immunoassays commercially used for rapid testing of fresh produce samples for the potential presence of *Salmonella*. In the present study, the VIDAS UP *Salmonella* (SPT) assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and Reveal 2.0 *Salmonella* (Neogen Corporation, Lansing, Michigan, United States) immunoassay methods were evaluated for their reliability (sensitivity and specificity) in detecting *Salmonella*.

CHAPTER III

EVALUATION OF THE VIDAS UP *SALMONELLA* (SPT) ASSAY AND REVEAL 2.0 *SALMONELLA* AFFINITY-BASED METHODS FOR SENSITIVITY AND SPECIFICITY IN THE DETECTION OF *SALMONELLA* SPP.

3.1 ABSTRACT

Salmonella continues to be one of the leading foodborne pathogens. More illnesses are being associated with the consumption of *Salmonella* contaminated fresh produce than ever before. Recently, Centre for Disease Control and Prevention (CDC) estimated that 46 to 50% of the fresh produce is contaminated with *Salmonella* species. Sensitive and rapid detection methods are essential for testing the *Salmonella enterica* contaminated fresh produce. Numerous commercial rapid testing methods were developed for *Salmonella* species detection from contaminated food samples. However, precautions are to be taken while testing *Salmonella* contaminated fresh produce during antibody based immunoassays, as they have a high percentage of false-positive test results. The closely related, non-*Salmonella* bacteria such as *Citrobacter* spp., *Hafnia* spp. and *Proteus* spp. are the main cause of these false-positive results in immunoassays. In addition, false-negative results are also observed as certain cell surface exposed components are not expressed. The advanced immunoassay diagnostic methods are more sensitive and accurate methods for *Salmonella* identification. However, validation of such methods is essential to avoid *Salmonella* outbreaks. Therefore, the current study was conducted to evaluate VIDAS UP *Salmonella* (SPT) assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and Reveal 2.0 *Salmonella* (Neogen Corporation, Lansing, Michigan, United States) immunoassay methods, used for the detection of *Salmonella* in fresh produce samples. A total of 117 bacterial isolates (54 *Salmonella* and 63 non-*Salmonella*) were

evaluated in this study. From the 54 isolates of *Salmonella* tested using the rapid assays in the current study, the VIDAS UP *Salmonella* (SPT) assay correctly identified 52/54 (96.3%) of the *Salmonella* isolates and Reveal 2.0 *Salmonella* assay identified 43/54 (79.63%) of the *Salmonella* isolates. Of the 63 non-*Salmonella* isolates, the VIDAS UP *Salmonella* (SPT) assay incorrectly identified 3 isolates as *Salmonella*. None of the non-*Salmonella* isolates tested positive in the Reveal 2.0 *Salmonella* assay. *Salmonella enterica* Hull and *Salmonella enterica* Duesseldorf were not identified by both rapid assays. Though the rapid methods are able to correctly identify 80-96% of the *Salmonella* isolates, these results must be validated by comparing the genomic sequence differences between closely related *Salmonella* spp. to avoid presumptive results.

3.2 Introduction

One in ten people fall sick due to the consumption of contaminated food, which in turn leads to 420,000 deaths globally (WHO, 2015). In the United States alone, *Salmonella* is estimated to cause nearly one million illnesses, 190,000 hospitalizations and around 300 deaths every year (Center for Disease Control and Prevention, 2015). While in Europe, it is observed that 90,000 salmonellosis cases are reported yearly. European Food Safety Authority (EFSA) has estimated the overall economic burden of human salmonellosis to be as high as 3 billion EUR per year (EFSA, 2015). This holds good for Canada too, as salmonellosis outbreaks have been on an incline since 1998 till 2011 (PHAC, 2015). Developed and under-developed countries are facing major foodborne disease outbreaks due to *Salmonella* contaminated food (WHO, 2014). These data and implications indicate that the foodborne disease outbreaks are a major threat to global food safety and to human health. The most common serovars associated with human illness are *S. typhimurium* and *S. enteritidis* (Lee et al., 2015; Park et al., 2014). Although salmonellosis is considered to be a self-limiting, gastroenteritis and treatments are available to cure salmonellosis. Salmonellosis can be fatal to the immuno-suppressed, infants and elderly people depending on the serovars, strains and infectious dose of the pathogens (Alakomi and Saarela, 2009).

During the last decade, several large outbreaks of *Salmonella* in various fresh produce commodities have focused attention on methods to improve the safety of these raw, ready to eat products (Wu et al., 2017). One consequence of the increasing outbreaks due to contaminated fresh produce has been an increase in the amount of testing conducted in order to identify the presence of foodborne pathogens. However, the importance of selecting a properly validated method cannot be overstated, especially since there was little historical interest in testing fresh produce, and many rapid testing methods have not been

specifically validated for fresh produce applications (United Fresh Produce Association Food Safety & Technology Council, 2014).

Affinity based assays are the most used class of rapid testing methods in North America to test foods for foodborne bacterial pathogens (Weschler, 2014). Two of the most popular affinity based assays are the Enzyme Linked Immunosorbent Assay (ELISA) and the Lateral Flow Assay (LFA). The ELISA is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. In the context of *Salmonella* detection, a sample with an unknown amount of antigen is immobilized on a solid support (usually the walls and floor of wells of a microtiter plate). After the antigen is immobilized, a detection antibody linked to an enzyme such as Horse Radish Peroxidase (HRP) is added, forming a complex with the antigen. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate (ABTS or 3,3',5,5'-tetramethylbenzidine) to produce a visible signal (colorimetric or fluorescent product) due to the enzymatic cleavage of the substrate. Colorimetric equipment is used to measure the signal indicating colorimetric equipment indicating the presence of target antigen in the sample (Magliulo et al., 2007; Odumeru and León-Velarde, 2012).

There are many commercially available ELISA assays for use in testing foods for fresh produce and *Salmonella*. One of the more common assays is the Vitek Immuno Diagnostic Assay System (VIDAS) (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.), which is an automated qualitative enzyme-linked fluorescent immunoassay (ELFA) for the detection of *Salmonella* in food and food ingredients. The VIDAS instrument performs all of the assay steps automatically. In contrast to the manual manipulation required for microtiter plate based ELISA systems, a pipette tip-like disposable unit (a solid phase

receptacle or SPR) serves as the solid phase as well as a pipetter during the process. The SPR is coated with polyclonal anti-*Salmonella* antibodies and reagents for the assay are sealed in reagent strips. An aliquot of the enrichment broth is placed into the reagent strip and the sample and reagents are sequentially cycled in and out of the SPR for a specific length of time until the instrument detects fluorescence. The VIDAS assay was modified by replacement of the secondary, enzyme linked antibody with the tail fiber protein of a bacteriophage that is specific for the target pathogen. The tail fiber protein was produced as a recombinant peptide, labelled with a fluorescent dye and included into the VIDAS sandwich assay technology. These new VIDAS Phage Technology (PT) assays harness the specificity of bacteriophage receptor binding proteins to improve the specificity of existing ELISA based approaches (Odumeru and León-Velarde, 2012; Zadernowska and Chajęcka, 2012).

Lateral flow assays (LFAs) are another popular affinity assay format. LFAs typically have a sandwich type ELISA format with polyclonal antibodies as a capture antibody and a monoclonal antibody as the detection antibody. The antibodies are fixed on a hydrophobic polyvinylidine difluoride-based membrane. A drop of an enrichment sample is placed in a reaction window and travels by capillary action across the membrane to react with the antibodies and provide a colour change. Commercially available lateral flow immunoassays for the detection of *Salmonella* include: DuPont™ Lateral Flow System *Salmonella* (DuPont Qualicon, Wilmington, Delaware, USA), Singlepath *Salmonella* (Merck, Kenilworth, New Jersey, USA), and the Reveal® 2.0 *Salmonella* lateral flow (Neogen, Lansing, Michigan, United States) assay (Odumeru and León-Velarde, 2012). Continuous testing of various fresh produce samples highlighted the propensity for the VIDAS UP *Salmonella* Phage Technology (SPT) Assay and the Reveal 2.0 *Salmonella* lateral flow assay to produce presumptive positive test results, which could

not be confirmed as positive for *Salmonella*. The objective of this study was to evaluate both affinity based assays for their ability to detect pure isolates of *Salmonella* (specificity) and isolates of bacteria from closely related bacterial species (sensitivity).

3.3 Materials and Methods

3.3.1 Bacterial strain and culture conditions

A total of 54 strains of *Salmonella* **(Table 3.1)** and 63 strains of non-*Salmonella* bacteria **(Table 3.2)** were evaluated in both assays. The *Salmonella* isolates represented a total of common and rare (defined as isolates belonging to serovars outside of the top 100 serovars that cause human illness in Canada) serovars within *Salmonella enterica*. The non-*Salmonella* isolates belonged to (*Proteus* spp., *Hafnia* spp. and *Citrobacter* spp.) Metadata for all isolates can be found in the *Salmonella* Foodborne Syst-OMICS Database (SalFoS), which can be accessed at https://salfos.ibis.ulaval.ca/.

Frozen stock cultures were maintained in 40% glycerol and stored at -80°C until use. Prior to experiments, fresh bacterial cultures were prepared by inoculating the frozen stock cultures onto Xylose Lactose Tergitol 4 (XLT4) and Xylose Lysine Desoxycholate Agar (XLD) agar, followed by incubation overnight at 37°C. Isolated colonies were then inoculated in assay specific enrichment broth, as described below.

3.3.2 Isolation of bacteria causing false-positive results on the VIDAS UP *Salmonella* (SPT) and VIDAS UP *E. coli* (ECPT) platforms

Bacteria that were responsible for the false-positive test results were isolated from enrichment cultures of fresh produce samples from a grower that had previously tested as presumptive positive on either the VIDAS UP *Salmonella* (SPT) or (ECPT) platforms, but failed to be confirmed as *Salmonella*. In order to isolate the bacteria from the enrichment

that were responsible for the false positive test results, upon arrival at our laboratory, all enrichment cultures were immediately re-enriched, and tested on the VIDAS UP *Salmonella* (SPT) or (ECPT) platform to reproduce the false-positive test results. Any re-enriched cultures that produced a false-positive result on the VIDAS UP *Salmonella* (SPT) were serially diluted (10 fold), and each dilution was plated in duplicate on tryptic soy agar (TSA). Following overnight incubation at 37˚C, the TSA plates representing the highest dilution that allowed for isolated colonies were removed from the incubator. All colonies from one of the duplicate plates were removed, collectively enriched, and then tested on the VIDAS UP *Salmonella* (SPT). If the enrichment was positive, then all individual colonies from the other duplicate plate were tested individually on the VIDAS UP *Salmonella* (SPT). All colonies that resulted in positive test results were subjected to biochemical analysis, using the VITEK Compact 2 Instrument (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) in order to identify the bacteria species, but no definitive identifications were obtained. The isolated colonies were subjected to whole genome sequencing and bioinformatic analysis for identification purposes.

3.3.3 Evaluation of the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* Affinity Based Assays

Two affinity based assays (VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella*) were evaluated for their ability to detect *Salmonella* (specificity) and non-*Salmonella* (sensitivity) bacteria. All manufacturer's procedures were followed when conducting the assays.

3.3.3.1 Neogen Reveal 2.0 *Salmonella* procedure

To conduct the Reveal 2.0 *Salmonella* assay, a well isolated colony was incubated in 5ml of Revive broth (Neogen) (the broth had been pre-incubated at 42°C) for 4 hours at

37°C. After 4 hours, 5ml of Rappaport Vassiliadis (RV) broth (pre-incubated at 42°C) was added to the tubes to enhance the recovery of injured *Salmonella* cells. The samples were then incubated for 24 hours at 41.5°C. All rehydrated media was used within 6 hours of preparation and sterile water was to be warmed to 42°C when rehydrating Revive and 36°C when rehydrating 2*RV.

The enriched samples were removed after 24 hours of incubation, vortexed well, and 200 µl aliquot pipetted into a sample cup. One Reveal 2.0 *Salmonella* test dipstick was placed inside the cup containing the sample, with arrows facing downwards and incubated under ambient temperature for 15 minutes.

A positive result was indicated by two red lines, one each in both the control and test zones, while a negative result showed a line only in the control zone. The formation of any lines after 15 minutes was considered inaccurate and thus ignored. If no line appeared in the control zone, the test was considered as invalid.

3.3.3.2 VIDAS UP *Salmonella* (SPT) Assay

Pure isolates of *Salmonella* and non-*Salmonella* bacteria were enriched in buffered Peptone Water (BPW), that had been pre-warmed to 42 ± 1°C, and to which the *Salmonella* supplement had been added. These bacteria were from the culture collection of Dr. Goodridge, or had been isolated from enrichment cultures that had previously tested as false positive for the presence of *Salmonella*, which were supplied by a fresh produce grower. The suspensions were incubated for 18–24 h at 42 ± 1°C. After incubation, 0.5ml of the incubated sample was added to the sample well on the strip, and then the strip was heated for 5±1 minutes on the VIDAS Heat and Go device. The strip was removed and cooled for 10 minutes subsequently. The SPR and strip was inserted into the mini VIDAS automated platform to conduct the assay. The assay was initiated by following the

operator's manual instructions. After the successful completion of the assay in the mini VIDAS machine, the fluorescence results were measured twice from the strip. The first reading was considered to be the background reading of the substrate cuvette. The second reading occurred during the enzymatic reaction. The Relative Fluorescence Value (RFV) was calculated by subtracting the background reading from the 2nd, enzymatic reaction reading. A test result with a value ≥ of 0.25 indicated the presumptive presence of *Salmonella* species in the sample tested.

3.3.3.2.1 Sensitivity and Specificity calculations

Sensitivity (the probability that a test result will be positive when *Salmonella* was present (true positive rate), and specificity (the probability that a test result will be negative when *Salmonella* was not present in the sample (true negative rate) were calculated for all of the 54 *Salmonella* and 63 non-*Salmonella* bacterial isolates tested with the the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays. Sensitivity was calculated using the formula a/a+b, where 'a' is the number of true positive test results, and 'b' is the number of false-negative test results expected when *Salmonella* was present in the sample. Specificity was calculated using the formula d/c+d, where'd' is the number of true negatives and 'c' is the number of false-positives in samples where *Salmonella* was absent. Additionally, the positive likelihood ratio (defined as the ratio between the probability of a positive test result given the presence of *Salmonella* and the probability of a positive test result given the absence of *Salmonella* was calculated using the formula Sensitivity/100-Specificity, while the negative likelihood ratio (the ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease) was calculated using the formula 100-Sensitivity/Specificity. Additional calculations included the positive predictive value, which is the probability that *Salmonella* is present when the assay reports a positive test

(a/a+c), and the negative predictive value, defined as the probability that *Salmonella* was not present when a test result was negative (d/b+d).

3.4 Results and Discussion

3.4.1 Detection of *Salmonella* and non-*Salmonella* species using VIDAS UP *Salmonella* (SPT) Assay and Reveal 2.0 *Salmonella* assays

The results obtained from VIDAS UP *Salmonella* (SPT) Assay and Reveal 2.0 *Salmonella* assay for the *Salmonella* and non-*Salmonella* strains are shown in **Table 3.1** and **Table 3.2**.

Among the 54 total *Salmonella* isolates, 52 (96.3%) were correctly detected by the VIDAS UP *Salmonella* (SPT) and 43 (79.63%) isolates were correctly detected by the Reveal 2.0 *Salmonella* assay. *Salmonella* Godesberg, *Salmonella* Luciana, *Salmonella* Weston, *Salmonella* Wentworth, *Salmonella* Chingola, *Salmonella* Bergen, *Salmonella* Indikan, *Salmonella* Orientalis and *Salmonella* Luckenwalde were detected by the VIDAS UP *Salmonella* (SPT) assay, but gave negative results with the Reveal 2.0 *Salmonella* assay. Two *Salmonella* serovars (*S.* Duesseldorf and *S.* Hull) tested negative with both assays. With respect to non-*Salmonella* isolates, the VIDAS UP *Salmonella* (SPT) assay detected 3 isolates as *Salmonella*, indicating a false-positive result. These isolates were identified as *Citrobacter koseri/farmeri* by the API 20E biochemical assay, and were isolated from the enrichment cultures that had previously tested positive on the VIDAS UP *Salmonella* (SPT) assay. No false-positive results were detected by the Reveal 2.0 *Salmonella* assay. The sensitivity and specificity results are shown in **Table 3.3**.

ELISA assays have been known to be susceptible to false-negative and false-positive results. For example, various produce matrices have been known to cause false-

negative and false-positive results when ELISAs are used to test samples for *Salmonella*, likely due to differences in extinction values (Isenberg et al., 1987). Since pure isolates were tested in this study, matrix specific interference was not an issue. ELISAs often have issues with cross reactivity, as antibodies used in these tests may cross-react with a small percentage of non-*Salmonella*, resulting in false-positive results (Blackburn et al., 1991; Isenberg et al., 1987). Improvements in enrichment procedures can improve the specificity of ELISAs, as effective enrichment protocols will lead to a favourable ratio of *Salmonella* to competitive bacteria, as well as a decrease in the number of false-negative results, because the number of *Salmonella* cells required for a positive reaction will be reached more easily (Beumer and Brinkman, 1989; Curiale et al., 1990). Another way of achieving better results is improving assay specificity by using highly specific monoclonal antibodies (Permar et al., 1990) and sensitivity by optimizing coating, antigen-antibody and enzyme-substrate reactions (Prusak-Sochaczewski and Luong, 1989). Several studies have investigated the sensitivity and specificity of the VIDAS assay in detecting pathogens. Temelli et al (2012) compared the VIDAS easy *Salmonella* (ESLM) and LightCycler real time PCR (LCPCR), to the International Organization for Standardization Method 6579 (ISO) in detecting *Salmonella* from a total of 105 naturally contaminated samples comprised of poultry meat and poultry meat products. Twelve (33.33%), eleven (30.55%), and eighteen (50.00%) out of 36 poultry meat samples were positive for *Salmonella* by ISO, VIDAS ESLM, and LCPCR, respectively. *Salmonella* detection rates from poultry meat products were 5.80% for ISO and 8.69% for LCPCR, whereas none of these products tested positive by VIDAS ESLM. The authors concluded that the VIDAS ESLM did not seem to be a suitable method for detecting *Salmonella* in poultry meat products.

Bird et al (2013) conducted a multi-laboratory collaborative study to evaluate the VIDAS UP *Salmonella* (SPT) assay in comparison to the U.S. Department of

Agriculture/Food Safety and Inspection Service-(USDA-FSIS) Microbiology Laboratory Guidebook method for isolation and identification of *Salmonella* from Meat, Poultry, Pasteurized Egg and Catfish Products. Each test portion was artificially contaminated with *Salmonella* at three inoculation levels, an uninoculated control level (0 CFU/test portion), a low inoculum level (0.2-2 CFU/test portion), and a high inoculum level (2-5 CFU/test portion). A total of 1656 unpaired replicate samples were analyzed. Of these samples, 476 were presumptive positive by the VIDAS UP *Salmonella* (SPT) method, while 411 were confirmed positive by the USDA/FSIS-MLG reference method. The results indicated that there was no statistically significant difference in the number of positive samples detected by the VIDAS UP *Salmonella* (SPT) method and the USDA/FSIS-MLG method at the 0.05 level. For the 25 g test portions, a statistically significant difference was observed between the VIDAS UP *Salmonella* (SPT) method and the reference method for the low inoculum level, where the VIDAS UP *Salmonella* (SPT) method recovered a higher number of positive results than the reference method. The authors recommended that the VIDAS UP *Salmonella* (SPT) method be adopted for Official First Action status for the detection of *Salmonella* in a variety of foods and environmental samples.

As with the ELISA assays, false-positive results may be observed in lateral flow assays due to denaturation or degradation of the capture antibody. Additionally, the detection antibody may bind non-specifically to denatured capture antibody, producing a false-positive result. In this study, the issue observed with the Reveal 2.0 *Salmonella* assay was not due to false-positive results, but false-negative results. The sensitivity of the assay was only 79.63% as eleven *Salmonella* isolates (from 11 different serovars) were not detected. These isolates belong to serovars that are outside of the top 70 most common serovars that cause illness in Canada each year. Others have reported high false-negative rates with the *Salmonella* Reveal Assay. For example, Peplow et al (1999) compared the

original version of the Reveal *Salmonella* assay, the *Salmonella* BIND assay (a reporter bacteriophage based assay), and a filter monitor method, to the standard *Salmonella* cultural method, for the ability to detect *Salmonella* spp. in environmental samples obtained from poultry houses. Two types of samples were collected; the first set of samples were taken prior to chick placement (preplacement samples). The second set of samples were collected when the birds were approximately 5 weeks old (pre-slaughter samples). The Reveal assay detected the same number of positive results for the preplacement and pre-slaughter fresh samples as the standard method detected. However, there was not 100% agreement between the two methods. With respect to the preplacement samples, the Reveal test yielded ten false-positive results (one was confirmed to be a true positive result) and ten false-negative results. The Reveal assay also yielded 22 false-positive results and 22 false-negative results from the pre-slaughter samples. Hoerner et al (2011) reported that the Reveal 2.0 *Salmonella* test represents an improvement to the original Reveal *Salmonella* test in that an additional polyclonal antibody has been incorporated into the test to improve detection of certain *Salmonella* serovars. Thus, the failure of the Reveal 2.0 *Salmonella* assay to detect isolates from serovars not considered to be clinically important, may be due to the fact that the assay does not contain antibodies capable of detecting targets from isolates within those serovars.

Two *Salmonella* isolates, one each from serovars *S*. Duesseldorf and *S*. Hull tested negative when the VIDAS UP *Salmonella* (SPT) and the Reveal 2.0 *Salmonella* assays were used. In affinity based assays, false-negative results may occur due to decreased or complete lack of expression of the target antigen, due to the target bacterial cell being stressed, or alternatively, a genetic mutation in the target antigen may lead to a change in the secondary or tertiary structure of the antigen, making it no longer able to be recognized by a ligand such as an antibody, or phage binding protein.

3.5 Conclusion

In this study, two rapid *Salmonella* affinity based assays were evaluated for their ability to detect pure isolates of bacteria from common and rare *Salmonella* serovars, as well as for their ability to avoid false-positive detection of closely associated non-*Salmonella* bacteria. It was observed that two *Salmonella* isolates (*S.* Hull and *S.* Duesseldorf) were not detectable by both assays, raising questions regarding the basis for the false-negative test results of these two isolates. More isolates from both serovars should be tested to determine if the false-negative test results are serovar specific, or restricted to just these two isolates.

Additionally, enrichment cultures that produced false-positive results on the VIDAS UP *Salmonella* (SPT) assay were used to identify *Citrobacter* spp., a close relative to *Salmonella*, as the likely candidate for producing false-positive results on VIDAS UP *Salmonella* (SPT). The bacteria causing false-positive test results should be phenotypically and genotypically characterized in order to determine the reasons for the false-positive test results.

Table 3.1. List of *Salmonella* isolates used to assess the specificity of the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays. Additional metadata of the isolates can be found in the SalFoS database at https://salfos.ibis.ulaval.ca/.

Isolate No.	*Salmonella* serovar	Salfos ID	Source or Origin	VIDAS UP *Salmonella* (SPT) Results	Reveal 2.0 *Salmonella* Results
1	*Salmonella enterica* Broughton	S23	Health Canada	Positive	Positive
2	*Salmonella enterica* Tyresoe	S45	Health Canada	Positive	Positive
3	*Salmonella enterica*	S29	Health Canada	Negative*	Negative*

	Duesseldorf				
4	*Salmonella enterica* Weston	S48	Health Canada	Positive	Negative*
5	*Salmonella enterica* Solt	S41	Health Canada	Positive	Positive
6	*Salmonella enterica* Luciana	S37	Health Canada	Positive	Negative*
7	*Salmonella enterica* Banana	S21	Health Canada	Positive	Positive
8	*Salmonella enterica* Canada	S24	Health Canada	Positive	Positive
9	*Salmonella enterica* Amager	S19	Health Canada	Positive	Positive
10	*Salmonella enterica* Pasing	S40	Health Canada	Positive	Positive
11	*Salmonella enterica* Godesberg	S33	Health Canada	Positive	Negative*
12	*Salmonella enterica* Falkensee	S31	Health Canada	Positive	Positive
13	*Salmonella enterica* Westhampton	S47	Health Canada	Positive	Positive
14	*Salmonella enterica* Ball	S20	Health Canada	Positive	Positive
15	*Salmonella enterica* Cremieu	S27	Health Canada	Positive	Positive
16	*Salmonella enterica* Wentworth	S46	Health Canada	Positive	Negative*
17	*Salmonella enterica* Chingola	S26	Health Canada	Positive	Negative*
18	*Salmonella enterica* Hull	S34	Health Canada	Negative*	Negative*
19	*Salmonella enterica* Bergen	S22	Health Canada	Positive	Negative*

20	*Salmonella enterica* Indikan	S35	Health Canada	Positive	Negative*
21	*Salmonella enterica* Daytona	S28	Health Canada	Positive	Positive
22	*Salmonella enterica* Orientalis	S39	Health Canada	Positive	Negative*
23	*Salmonella enterica* Kouka	S36	Health Canada	Positive	Positive
24	*Salmonella enterica* Luckenwalde	S38	Health Canada	Positive	Negative*
25	*Salmonella enterica* Casablanca	S25	Health Canada	Positive	Positive
26	*Salmonella enterica* Elisabethville	S30	Health Canada	Positive	Positive
27	*Salmonella enterica* Enteritidis	S7	Health Canada	Positive	Positive
28	*Salmonella enterica* Enteritidis	S3	Health Canada	Positive	Positive
29	*Salmonella enterica* Enteritidis	S9	Health Canada	Positive	Positive
30	*Salmonella enterica* Enteritidis	S4	Health Canada	Positive	Positive
31	*Salmonella enterica* Typhimuirum	S601	Goodridge Lab Environmental, USA	Positive	Positive
32	*Salmonella enterica* Typhimuirum	S578	Goodridge Lab Environmental, USA	Positive	Positive
33	*Salmonella enterica* Typhimuirum	S597	Goodridge Lab Environmental, USA	Positive	Positive
34	*Salmonella enterica* Bareilly	S603	131685	Positive	Positive

35	*Salmonella enterica* Newport	S620	Goodridge Lab	Positive	Positive
36	*Salmonella enterica* Newport	S621	Goodridge Lab	Positive	Positive
37	*Salmonella enterica* Newport	S622	Goodridge Lab	Positive	Positive
38	*Salmonella enterica* Newport	S623	Goodridge Lab	Positive	Positive
39	*Salmonella enterica* Newport	S624	Goodridge Lab	Positive	Positive
40	*Salmonella enterica* Newport	S625	Goodridge Lab	Positive	Positive
41	*Salmonella enterica* Newport	S626	Goodridge Lab	Positive	Positive
42	*Salmonella enterica* Newport	S627	Goodridge Lab	Positive	Positive
43	*Salmonella enterica* Newport	S628	Goodridge Lab	Positive	Positive
44	*Salmonella enterica* Newport	S629	Goodridge Lab	Positive	Positive
45	*Salmonella enterica* Newport	S630	Goodridge Lab	Positive	Positive
46	*Salmonella enterica* Newport	S631	Goodridge Lab	Positive	Positive
47	*Salmonella enterica* Anatum	S443	Goodridge Lab	Positive	Positive
48	*Salmonella enterica* Anatum	S610	Goodridge Lab	Positive	Positive
49	*Salmonella enterica* Meunster	S608	Goodridge Lab	Positive	Positive
50	*Salmonella enterica* Choloerasuis	S615	Goodridge Lab	Positive	Positive
51	*Salmonella enterica*	S616	Goodridge Lab	Positive	Positive

52	*Salmonella enterica* Choloerasuis	S617	Goodridge Lab	Positive	Positive
53	*Salmonella enterica* Choloerasuis	S618	Goodridge Lab	Positive	Positive
54	*Salmonella enterica* Montevideo	S548	Goodridge Lab	Positive	Positive

*Negative – indicates results that came up negative on VIDAS UP *Salmonella* (SPT) and Neogen

Table 3.2. List of Non-*Salmonella* isolates used to assess the specificity of the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays. Additional metadata of the isolates can be found in the SalFoS database at https://salfos.ibis.ulaval.ca/.

Isolate No.	Non-*Salmonella* species	Salfos ID/ Original ID	Source or Origin	VIDAS UP *Salmonella* (SPT) Results	Reveal 2.0 *Salmonella* Results
1	*Hafnia alvei*	S59	LSPQ[a]	Negative	Negative
2	*Hafnia alvei*	S69	LSPQ	Negative	Negative
3	*Hafnia alvei*	S70	LSPQ	Negative	Negative
4	*Hafnia alvei*	S75	LSPQ	Negative	Negative
5	*Hafnia alvei*	S82	LSPQ	Negative	Negative
6	*Hafnia alvei*	S84	LSPQ	Negative	Negative
7	*Hafnia alvei*	S87	LSPQ	Negative	Negative
8	*Hafnia alvei*	S88	LSPQ	Negative	Negative
9	*Hafnia alvei*	S90	LSPQ	Negative	Negative
10	*Hafnia alvei*	S92	LSPQ	Negative	Negative
11	*Hafnia alvei*	XLD10	Goodridge Lab	Negative	Negative
12	*Proteus mirabilis*	S72	LSPQ	Negative	Negative
13	*Proteus*	S73	LSPQ	Negative	Negative

	mirabilis				
14	*Proteus mirabilis*	S74	LSPQ	Negative	Negative
15	*Proteus mirabilis*	S13	Goodridge Lab	Negative	Negative
16	*Proteus mirabilis*	S14	Goodridge Lab	Negative	Negative
17	*Proteus mirabilis*	S16	Goodridge Lab	Negative	Negative
18	*Proteus mirabilis*	S17	Goodridge Lab	Negative	Negative
19	*Proteus mirabilis*	S18	Goodridge Lab	Negative	Negative
20	*Proteus mirabilis*	S19	Goodridge Lab	Negative	Negative
21	*Proteus mirabilis*	S21	Goodridge Lab	Negative	Negative
22	*Proteus mirabilis*	S22	Goodridge Lab	Negative	Negative
23	*Proteus mirabilis*	S23	Goodridge Lab	Negative	Negative
24	*Proteus mirabilis*	S24	Goodridge Lab	Negative	Negative
25	*Proteus mirabilis*	XLD1	Goodridge Lab	Negative	Negative
26	*Proteus mirabilis*	XLD2	Goodridge Lab	Negative	Negative
27	*Proteus mirabilis*	XLD3	Goodridge Lab	Negative	Negative
28	*Proteus mirabilis*	XLD4	Goodridge Lab	Negative	Negative
29	*Proteus mirabilis*	XLD5	Goodridge Lab	Negative	Negative

30	*Proteus mirabilis*	XLD6	Goodridge Lab	Negative	Negative
31	*Proteus mirabilis*	XLD7	Goodridge Lab	Negative	Negative
32	*Proteus vulgaris*	S76	LSPQ	Negative	Negative
33	*Citrobacter freundii*	S61	LSPQ	Negative	Negative
34	*Citrobacter freundii*	S89	LSPQ	Negative	Negative
35	*Citrobacter freundii*	S66	LSPQ	Negative	Negative
36	*Citrobacter freundii*	S83	LSPQ	Negative	Negative
37	*Citrobacter freundii*	S67	LSPQ	Negative	Negative
38	*Citrobacter freundii*	S15	Goodridge Lab	Negative	Negative
39	*Citrobacter freundii*	S20	Goodridge Lab	Negative	Negative
40	*Citrobacter freundii*	XLD8	Goodridge Lab	Negative	Negative
41	*Citrobacter amalonaticus*	S68	LSPQ	Negative	Negative
42	*Citrobacter amalonaticus*	S63	LSPQ	Negative	Negative
43	*Citrobacter amalonaticus*	S64	LSPQ	Negative	Negative
44	*Citrobacter koseri*	S65	LSPQ	Negative	Negative
45	*Citrobacter koseri*	S79	LSPQ	Negative	Negative
46	*Citrobacter werkmanii*	S78	LSPQ	Negative	Negative

47	*Citrobacter farmeri*	S80	LSPQ	Negative	Negative
48	*Citrobacter braakii*	S62	LSPQ	Negative	Negative
49	*Citrobacter braakii*	S85	LSPQ	Negative	Negative
50	*Citrobacter braakii*	S86	LSPQ	Negative	Negative
51	*Citrobacter braakii*	XLD9	Goodridge Lab	Negative	Negative
52	*Citrobacter* spp.	S81	LSPQ	Negative	Negative
53	*Citrobacter* spp.	S77	LSPQ	Negative	Negative
54	*Citrobacter* spp.	S60	LSPQ	Negative	Negative
55	*Citrobacter* spp.	S91	LSPQ	Negative	Negative
56	*Citrobacter* spp.	S93	LSPQ	Negative	Negative
57	*Citrobacter* spp.	S94	LSPQ	Negative	Negative
58	*Citrobacter* spp.	S95	LSPQ	Negative	Negative
59	*Citrobacter* spp.	S96	LSPQ	Negative	Negative
60	*Citrobacter* spp.	S97	LSPQ	Negative	Negative
61	*Citrobacter amalonaticus*	S646	Fresh Produce Grower	Positive*	Negative
62	*Citrobacter amalonaticus*	S647	Fresh Produce Grower	Positive*	Negative
63	*Citrobacter amalonaticus*	S648	Fresh Produce Grower	Positive*	Negative

[a] Laboratoire de Santé Publique du Québec

*Positive- indicates the results that came up positive on VIDAS UP *Salmonella* (SPT) and Neogen

Table 3.3. Sensitivity and Specificity of the VIDAS UP *Salmonella* (SPT) Assay and the Reveal 2.0 *Salmonella* Assay.

	VIDAS UP *Salmonella* (SPT) [a]		Reveal 2.0 *Salmonella*[b]	
Statistic	**Value**	**95% CI**	**Value**	**95% CI**
Sensitivity[c]	96.3%	87.25-99.55%	79.63%	66.47-89.37%
Specificity[d]	95.24%	86.71-99.01%	100%	94.31-100%
Positive Likelihood Ratio[e]	20.22	6.69-61.09	-	-
Negative Likelihood Ratio[f]	0.04	0.01-0.15	0.20	0.12-0.35
Positive Predictive Value*	94.55%	85.16-98.13%	100%	-
Negative Predictive Value**	96.77%	88.49-99.15%	85.14%	77.17-90.66%

[a]The total number of samples tested using VIDAS UP *Salmonella* (SPT) Assay = 117

[b]The total number of samples tested using Reveal 2.0 *Salmonella* Assay = 117

[c]Sensitivity: probability that a test result will be positive when the isolate is present (true positive rate)

[d]Specificity: probability that a test result will be negative when the isolate is not present (true negative rate)

[e]Positive likelihood ratio: ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease, i.e. = True positive rate / False positive rate = Sensitivity / (1-Specificity)

[f]Negative likelihood ratio: ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease, i.e. = False negative rate / True negative rate = (1-Sensitivity) / Specificity. Confidence intervals for the likelihood ratios are calculated using the "Log method" as given on page 109 of Altman et al., 2000.

[*]Positive predictive value: probability that the disease is present when the test is positive. Confidence intervals for the predictive values are the standard logit confidence intervals given by Mercaldo et al., 2007.

[**]Negative predictive value: probability that the disease is not present when the test is negative. Confidence intervals for the predictive values are the standard logit confidence intervals given by Mercaldo et al., 2007.

CONNECTING TEXT

Detection of *Salmonella* and non-*Salmonella* isolates by the VIDAS UP *Salmonella* (SPT) assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and the Reveal 2.0 *Salmonella* assay (Neogen Corporation, Lansing, Michigan, United States) indicated the potential for false-positives and false-negative test results. Though these methods have advantages of analysing food samples in a short period of time, the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays identified *Salmonella enterica* Hull and *Salmonella enterica* Duesseldorf as false-negatives. In addition, the VIDAS UP *Salmonella* (SPT) assay identified three *Citrobacter* spp. isolates as false-positives. Hence, genomic and bioinformatic approaches were used in this study to identify the reasons for the lack of detection of *Salmonella*, and the cross reactivity observed in the immunoassays.

CHAPTER IV

MOLECULAR CHARACTERIZATION OF BACTERIA THAT CAUSE FALSE-NEGATIVE AND FALSE-POSITIVE TEST RESULTS ON THE VIDAS UP *SALMONELLA* (SPT) AFFINITY ASSAY

4.1 Abstract:

Salmonella is a major foodborne pathogen that causes outbreaks in fresh produce. Rapid immunoassays such as VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* are commercially available for testing and detecting *Salmonella* species in a short period of time in fresh produce samples. Previous studies indicated the potential for these assays to deliver false-negative and false-positive test results. This study focused on the potential reasons for false-negative and false-positive test results obtained from both assays. Whole genome sequencing and bioinformatic analysis was conducted for the bacteria that caused false-positive and false-negative results on the immunoassays. Blast analysis of three false-positive isolates identified them as *Citrobacter amalonaticus* with 92% homology. Two isolates *Salmonella enterica* Hull and *Salmonella enterica* Duesseldorf, that gave false-negative results, and the *C. amalonaticus* isolates were analyzed in order to identify surface exposed components that are used as diagnostic targets in *Salmonella* immunoassays. Comparative analysis of the *fljB* and *fliC* flagellin amino acid sequences in *Salmonella enterica* isolates that tested positive on the VIDAS UP *Salmonella* (SPT) and *C. amalonaticus* isolates that were false-positive showed a high level of homology at amino acid level. On the other hand, analysis of *S.* Duesseldorf and *S.* Hull showed that the *fljB* and *fliC* amino acid sequences differed significantly from other *Salmonella* isolates that tested positive. Analysis of the *S*. Hull genome identified a gene encoding a putative repressor of phase I flagellin, which was located on a cryptic incomplete prophage. Both

of these observations likely indicate, for the lack of complete flagella on the surface of the *S*. Duesseldorf and *S*. Hull isolates, which may be the reason for the false-negative test results. The results of this work have identified the potential basis for false-positive and false-negative test results in rapid *Salmonella* immunoassays.

4.2 Introduction

Rapid diagnostic assays are commonly used to assess the safety of foods destined for human consumption. Sensitivity (the ability to detect the target organism when it is present) and specificity (the ability of the assay to report a negative test result when the target organisms is not present) are important characteristics of any diagnostic assay. In the food industry, a presumptive false-positive test result, in which non-target bacteria produce a positive test result, has serious financial consequences, as the test results must be confirmed which can take a week or more, and often foods must be recalled from retail or destroyed (Potter et al., 2012). False-negative results in which the target organism is present, but fails to lead to a positive test result also has serious financial repercussions, as these test results lead to contaminated products being distributed to the consumer, causing foodborne illness, death and market failure. Although the economic impacts of false-negative test results are difficult to determine, it is clear that such test results significantly impact the economy (Kowitt, 2016).

In a retrospective study, the American Proficiency Institute (API) analyzed the results from 39,500 food proficiency tests conducted between 1999 and 2012 to evaluate the ability of food testing laboratories in the United States to detect four common pathogens (*Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Campylobacter* spp.) in foods. Over the 14-year period, false-negative results ranged from 3.3 percent to 14.0 percent for *E. coli* O157:H7; 1.9 percent to 10.6 percent for *Salmonella* spp; 3.4 percent to 11.0 percent for *L. monocytogenes*; and 0 percent to 19.8 percent for *Campylobacter* spp. (http://www.strategic-consult.com/tag/food-safety-pathogen-testing/). While both false-positive and false-negative rates decreased in the last year of the study, the cumulative false-negative rate for the 14-year period was 6.6 percent (Weschler, 2014).

There are many reasons that false-negative test results can occur during rapid testing of food for foodborne bacterial pathogens. For example, the target organism may be stressed and fail to grow, during enrichment, to a high enough concentration to be detected. Alternatively, background microflora may mask the appearance of the target organism, thereby inhibiting detection. Finally, the presence of mutations in the target microorganism may lead to the diagnostic targets not being expressed or present in the target organism. The presence of cross reactive antigens (affinity tests), or similar genomic regions (molecular test) or PCR-inhibitory substances and too much dilution of samples represents the most like reason for false-positive test results observed when foods are tested for the presence of foodborne pathogens (Josefsen et al., 2004; Mitov et al., 2003)

Previously, we identified two *Salmonella* isolates belong to serovars *Salmonella enterica* Hull and *Salmonella enterica* Dusseldorf, that resulted in false-negative test results when two rapid affinity based assays, the VIDAS UP *Salmonella* (SPT) assay (BioMérieux, Saint-Laurent, Quebec, Canada, Inc.) and the Reveal 2.0 *Salmonella* assay (Neogen Corporation, Lansing, Michigan, United States), were evaluated for detection of *Salmonella* isolates. We also identified three *Citrobacter* isolates that resulted in false-positive test results on the VIDAS UP *Salmonella* (SPT) assay, but not the Reveal 2.0 *Salmonella* assay. The purpose of this study was to employ a comparative genomics approach to characterize the *Salmonella* and *Citrobacter* isolates, in order to investigate potential reasons for the false-negative and false- positive test results that were observed.

4.3 Materials and methods

4.3.1 Bacterial strain isolation

Two *Salmonella* isolates, *S.* Hull S34 and *S.* Duesseldorf S29 previously tested negative using two *Salmonella* affinity based rapid assays, and were characterized in this study.

Additionally, three non-*Salmonella* isolates (S646, S647, S648), identified as *Citrobacter koseri/farmeri* by biochemical methods, caused false-positive test results on the VIDAS UP *Salmonella* (SPT) platform and were included in this study. Information regarding the isolates can be found in **Tables 3.1** and **3.2** in Chapter III. Additional metadata for the isolates is contained within the *Salmonella* Foodborne Syst-OMICS Database (SalFoS), which can be accessed at https://salfos.ibis.ulaval.ca/.

Frozen stock cultures were maintained in 40% glycerol and stored at -80°C until use. Prior to experiments, fresh bacterial cultures were prepared by inoculating the frozen stock cultures onto Tryptic Soy Agar (TSA), followed by incubation overnight at 37°C. Isolated colonies were then inoculated into Tryptic Soy Broth (TSB), and incubated for 24 h at 37°C. Following incubation, 500µL of the culture in TSB was used to prepare frozen 40% glycerol stocks to submit for whole genome sequencing (WGS).

4.3.2 Whole Genome Sequencing

Glycerol stocks were revived and used for WGS at Genomic Analysis Platform (IBIS, Université Laval, Québec, Canada). The genomic library was created for each of the bacterial strains. The Illumina MiSeq platform was used to generate 300-bp paired-end sequences from the genomic DNA library. The resulting raw reads were aligned to achieve a minimum of 30X sequence depth to confirm the sequencing consistency among the bacterial isolates. The raw reads were assembled using an integrated pipeline for *de novo* assembly of microbial genomes based on the A5 pipeline (Tritt et al., 2012). The assembled consensus sequence was used for genome annotation as described below.

4.3.3 Genome annotation

The assembled genomes were annotated using the web user interface automated program, Rapid Annotation using Subsystem Technology (RAST) http://rast.nmpdr.org/. This software was used to identify protein-encoding genes within the whole genome sequences (Aziz et al., 2008). In this study, the WGS files (FASTA) were uploaded to RAST and once the annotation was completed, the annotated files were downloaded for further analysis. AUGUSTUS (http://augustus.gobics.de/) and Blast2GO (https://www.blast2go.com/) bioinformatic tools were also used for individual genomic sequence annotation (Conesa et al., 2005; Stanke and Morgenstern, 2005).

4.3.4 Bioinformatic analysis

Various bioinformatic tools and web user interface software programs were used to identify the gene, genomic, and proteomic variations observed between the false-positive and false-negative bacterial genomic sequences. The genomic and proteomic variations were confirmed based on sequence alignments, and phylogenetic and protein analysis, using various programs including Mauve (http://darlinglab.org/mauve/mauve.html), Multiple Sequence Alignment (MultAlin, INRA) (http://multalin.toulouse.inra.fr/). The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the whole genomic DNA sequence similarities between the false-positive and false-negative species (Madden, 2013). The BLAST analysis was carried out by uploading the bacterial genomic sequences to the NCBI server with the minimum e-value10^5, maximum query coverage and homology (100%). Sequences showing a high percentage of similarity with known bacterial genes within the NCBI database were used for further characterization. The major genes associated with the cell surface structure of the *Salmonella* and

Citrobacter isolates, such as flagellin genes and genes encoding lipopolysaccharide (LPS) were used for sequence comparison and protein prediction analysis using Mauve software (Darling et al., 2004), Phyre^2 (Kelley and Sternberg, 2009) and Multalin (Corpet, 1988) tools.

4.3.4.1 Gene polymorphism analysis

The genes encoding proteins responsible for cross reactivity present on the cell surface of *Salmonella* and *Citrobacter* were analyzed for polymorphisms at the nucleotide and protein levels using MultAlin. Hence, the individual genes sequences were analyzed from both false- positive and false-negative bacterial strains through sequence alignment. Specifically, this program was used to identify single nucleotide polymorphisms (SNPs) within flagellar genes and O-antigen genes.

4.4 Results and discussion

Affinity based methods represent a major class of rapid assays that are used to screen foods for the presence of bacterial pathogens. Advantages of affinity based assays include their ease of use, the diversity of diagnostic formats available, and the lack of labour intensiveness when automated test platforms are employed. A major disadvantage of these assays is their high rates of false-negative and false-positive test results. Thus, the goal of this study was to elucidate the potential reasons for false-negative and false-positive results in affinity based assays, by characterizing two isolates of *Salmonella* that had previously caused false-negative test results with two affinity based assays, as well as three *Citrobacter* isolates that had caused false-positive test results with one affinity based assay.

4.4.1 Identification of *Citrobacter amalonaticus* isolates as the cause of false-positive test results in the VIDAS UP *Salmonella* (SPT) assay

Previously three bacterial isolates (S646-648), were isolated from enrichment cultures that tested presumptive positive on the VIDAS UP *Salmonella* (SPT). The API 20E biochemical assay identified the isolates as *Citrobacter koseri/farmeri* with percentage identifications ranging from 73.7% to 90.9%. Whole genome sequencing of the three isolates was thus employed in order to obtain a more definitive identification of the isolates. Whole genome BLAST analysis identified the isolates as *Citrobacter amalonaticus* with (92%) homology. It was therefore concluded that the isolates were *C. amalonaticus*.

4.4.2 Cross reactivity between *Salmonella* and *Citrobacter amalonaticus* on the VIDAS UP *Salmonella* (SPT) platform

Ibrahim (1986) has defined the necessary characteristics for reliable detection of *Salmonella* by immunoassay. These include the need for the target antigen(s) to be: present in all serotypes, confined to just the *salmonellae*, sufficiently immunogenic to enable production of high titer and specific antisera, isolated from *Salmonella* cells at a high degree of purity, and labeled without impairing immunoreactivity. There is no single antigen on the cell surface of the *salmonellae* that satisfies all of these criteria; as such, several antigens including capsular (K) antigens, pili antigens, somatic (O) antigens, and flagellar (H) antigens have been commonly utilized as targets in *Salmonella* detection. Of these, the O and H antigens appear to be the most common diagnostic targets.

Interference in immunoassays is a serious but underestimated problem (Ismail et al., 2002). Interference is defined as “the effect of a substance present in the sample that alters the correct value of the result, usually expressed as concentration or activity, for an

analyte" (Kroll and Elin, 1994). Cross-reactivity is the most common interference in immunoassays, but mostly in competitive assays. It is a non-specific influence of substances in a sample that structurally resemble the analyte (carry similar or the same epitopes as the analyte) and compete for binding site on antibody, resulting in over or underestimation of analyte concentration. Cross-reaction is a problem in diagnostic immunoassays where endogenous molecules with a similar structure to the measured analyte exist or where metabolites of the analyte have common cross-reacting epitopes (Kroll and Elin, 1994).

Cross-reactivity can be explained by understanding the composition and structure of outer cell membrane of Gram-negative bacteria. The cell wall of Gram-negative bacteria has two distinct parts, the outer membrane (OM) and the inner membrane (IM). The OM is composed of two leaflets: the inner and the outer. The inner leaflet is an asymmetrical lipid bilayer with phospholipids. The outer leaflet is mainly composed of lipopolysaccharides (LPS) (Moran, 2009). LPS is a complex glycolipid that can be categorized into three distinct parts namely the lipid A, the core region and the O polysaccharide chain, also known as the O antigen.

Although, O antigens are known to be very specific and unique to each particular strain of bacteria, it was reported that the outer core of the LPSs of *Salmonella, Escherichia coli, Shigella, Hafnia, Citrobacter,* and *Erwinia* generally consists of a similar six sugar units oligosaccharide linked to heptose sugar (Caroff and Karibian, 2003). Moreover, it was reported that the outer structures seem to be less variable within a genus (Brade, 1999). Accordingly, only one core structure was found for the genus *Salmonella* and five core types for *E. coli*. In the same study, serological relationships have been revealed among the different members of the *Enterobacteriaceae* family. For instance,

Salmonella and *Citrobacter, E. coli* and *Shigella* are related, while *E. coli* also show similarities with *Proteus* strains.

Bacterial flagella are subcellular organelles which impart motility upon the bacterial cell. Flagella are a multicomponent entity composed of 3 main components including the basal structure, the hook and the filament (Ibrahim, 1986). It is well known that the O and H antigens are cross reactive. For example, current serological diagnostic assays for detection of typhoid fever caused by *Salmonella typhi* are based on detecting antibodies against *Salmonella* LPS (O antigen) or flagella (H antigen). These antigens are cross-reactive with antibodies from other *Salmonella* serovars and related Gram-negative bacteria, resulting in a high false-positive rate (Liang et al., 2013). Feng and colleagues (1990) produced a monoclonal antibody against *E. coli* flagella, and demonstrated that it cross reacted with flagella of other members of the *Enterobacteriaceae*, including *Citrobacter freundii*, *Edwardsiella tarda*, *Enterobacter* spp., *Hafnia* spp., *Proteus* spp., *Salmonella* spp., *Serratia* spp., and *Yersinia enterocolitica*.

In this study, we hypothesized that the false-positive results seen in the VIDAS UP *Salmonella* (SPT) assay due to the presence of *C. amalonaticus* were likely due to a surface exposed protein or receptor with high homology to that of *Salmonella* spp. Both the O and H antigens are surface exposed structures that could cause cross-reactivity (Pillay et al., 2013; Ronholm et al., 2011). However, while there is limited information in the scientific literature regarding the cross reactions of *Salmonella* H antigens, many studies have characterized cross reactivity between the *Salmonella* O antigens and other members of the *Enterobacteriaceae* (Ibrahim, 1986). Our efforts therefore focused on analysis of the H antigens, since it is expected that the majority of affinity assays for detection of *Salmonella* target the H antigens (due to supposed decreased cross reactivity).

Similarly, Ibrahim, (1986) also says, little is known regarding the cross reactivity of H antigens among the *Enterobacteriaceae*.

In most isolates of *Salmonella*, two genes encode flagellar antigens. Genes *fljB* and *fliC* encode phase I and phase II flagellar antigens and they are expressed through phase variation (McQuiston et al., 2004). *fliC* is located in one of the flagellar biosynthesis operons, and is present in all *salmonellae*, while *fljB* is located in a region of the genome thought to be unique to *Salmonella*, and is found in four of the six *Salmonella enterica* subspecies. A third flagellar antigen gene*, flpA* was found on a plasmid within a triphasic isolate (Smith and Selander, 1991). Genes that encode bacterial flagellin are typically highly conserved at their 5' and 3' ends while the central region is generally quite variable. The conserved regions encode the flagellar filament backbone and are critical for the assembly of the filament. The central region, corresponding approximately to amino acids 181 to 390, encodes the surface-exposed and antigenically variable portion of the filament (13–15, 29) (McQuiston et al., 2004).

Comparative analysis of the *fljB* and *fliC* amino acid sequences in *S.enterica* isolates that tested positive on the VIDAS UP *Salmonella* (SPT) and the *C. amalonaticus* isolates that caused the false-positive test results revealed a high level of homology at the amino acid level. For example, the 5' and 3' ends of the genes were completely conserved as previously reported. In contrast, the central surface exposed and antigenically variable portion of the *fljB* and *fliC* proteins revealed a number of amino acid substitutions between the *Salmonella* and *Citrobacter* isolates (**Figures 4.1a and 4.1b**). However, it is unclear if these substitutions result in any antigenic differences between the *Salmonella* and *Citrobacter* isolates.

4.4.3 Characterization of *Salmonella* isolates causing false-negative results on the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* platforms

The previous study showed several *Salmonella* isolates were not detected accurately by the VIDAS UP *Salmonella* (SPT) assay and the Reveal 2.0 *Salmonella* assay (Chapter III, section 3.4.1). Two isolates of *Salmonella*, *S.* Hull and *S.* Duesseldorf, tested negative in both assays, raising questions regarding the nature of the false-negative results. There are several reasons for false-negative test results in affinity based rapid assays. Low numbers of *Salmonella* cells present after enrichment due to the presence of natural microflora that impair the growth of *Salmonella* cells to levels necessary for detection may lead to a false-negative test result (Schneid et al., 2006). Additionally, the concentration of the target antigen may be too low to lead to a positive test result. A solution to this problem is to use a combination of antibodies that are specific for several *Salmonella* antigens in order to amplify test results by the higher number of epitopes detected. Another alternative to decreasing false-negative test results is the improvement of enrichment protocols used in affinity based assays test by inhibiting microbial competition and favouring the growth of *Salmonella* to increase the amount of antigen available for detection (Blackburn, 1993; Jasson et al., 2011).

In this work, we tested pure isolates of *Salmonella*, eliminating any concerns with respect to background microflora in the samples that may have inhibited *Salmonella*. We also observed turbid growth of the *Salmonella* in the growth media, indicating that the concentration of *Salmonella* was high enough to enable detection. We therefore focused on alternative reasons for the false-negative results, and hypothesized that the concentration of the target antigen may have been too low for detection in the assays. As with the *C. amalonaticus* isolates, a focus was placed on analysis of genes responsible for flagellar production. When the *fljB* and *fliC* amino acid sequences of *S.* Hull and *S.* Duesseldorf

were compared to *Salmonella* isolates that tested positive on the VIDAS UP *Salmonella* (SPT) assay, we observed significant changes that could explain why these isolates were not detected (assuming that flagellin is the target). While the entire *fljB* protein was conserved in *S*. Hull, and the other *Salmonella* that tested positive on the VIDAS platform, in *S.* Duesseldorf, we observed amino acid substitutions at the 5' end of the protein, and the complete central region and 3' end of the protein (from amino acid residue 117) was completely missing (**Figure 4.2a**). As the 5' and 3' ends of flagellar proteins are responsible encoding the flagellar filament backbone and are critical for the assembly of the filament, it is likely that the *fljB* protein is completely functionless in this isolate of *S.* Duesseldorf (**Figure 4.2a**). A similar situation was observed with respect to *fliC*, which was completely conserved in *S.* Hull, and the other *Salmonella* that tested positive on the VIDAS UP *Salmonella* (SPT) platform, except for the terminal 4 amino acid residues at the 3' end of the protein. In contrast, the terminal 50 amino acid residues at the 3' end of *fliC* were missing in *S.* Duesseldorf (**Figure 4.2b**). When combined with the *fljB* results, it appears that there are no functional flagella expressed on the cell surface.

In *S.* Hull, genome analysis identified the presence of 6 intact and non-intact prophages. The 6th prophage was non-intact and had homology to the P4 prophage (**Figure 4.3a**). Analysis of this region highlighted a gene with homology to phage SSU5 which was identified as a putative repressor of phase I flagellin (**Figure 4.3b**). This may explain the lack of detection in *S.* Hull.

These results provide plausible reasons for the false negative results observed on the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* affinity assays when *S.* Duesseldorf and *S.* Hull were tested, assuming that flagellin is the target antigen in these assays (for proprietary reasons the diagnostic targets in these assays are unknown).

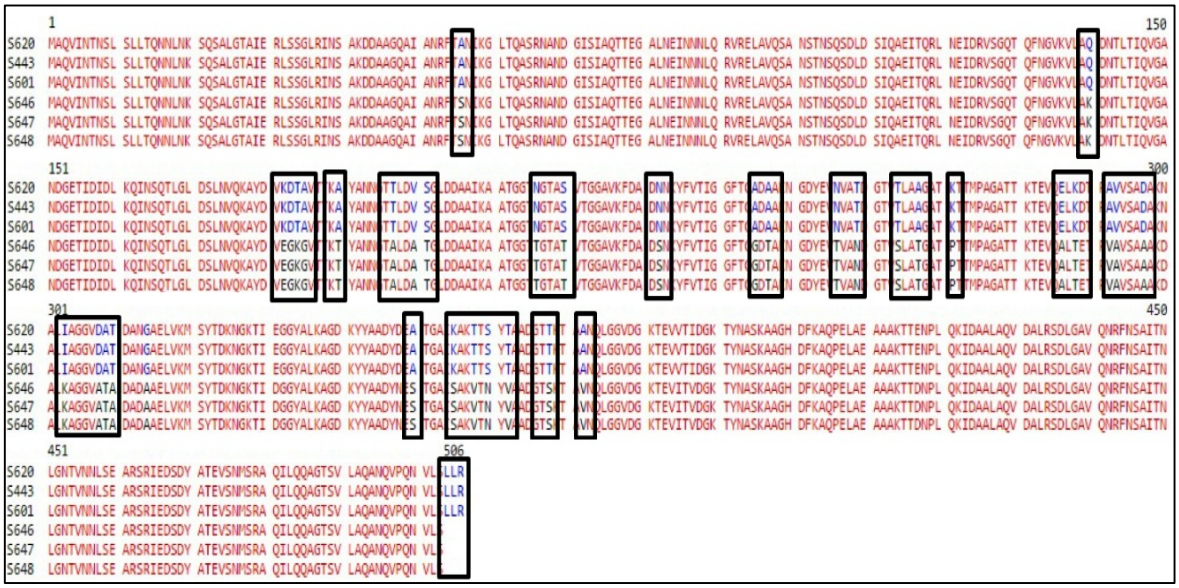

Figure 4.1a. Flagellar protein (*fljB*) comparison between *Salmonella enterica* isolates that were positive on the VIDAS UP *Salmonella* (SPT) assay and *Citrobacter amalonaticus* isolates that caused false-positive results. Key: S620, S443 and S601 are *S. enterica* Newport, *S. enterica* Anatum and *S. enterica* Typhimurium respectively, S646, S647 and S648 are *Citrobacter amalonaticus* isolates. The boxes highlight amino acid residues that are not conserved in all isolates.

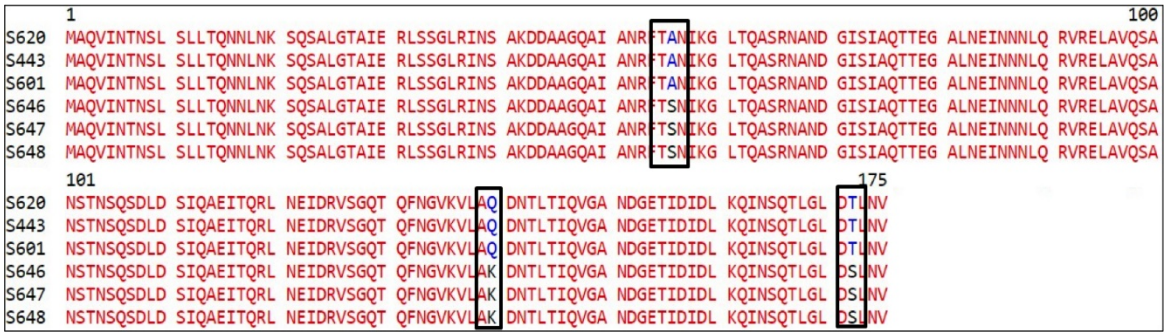

Figure 4.1b. Flagellar protein (*fliC*) comparison between *Salmonella enterica* isolates that were positive on the VIDAS UP *Salmonella* (SPT) assay and *Citrobacter amalonaticus* isolates that caused false-positive results. Key: S620, S443 and S601 are *S. enterica*

Newport, *S. enterica* Anatum and *S. enterica* Typhimurium respectively, S646, S647 and S648 are *Citrobacter amalonaticus* isolates. The boxes highlight amino acid residues that are not conserved in all isolates.

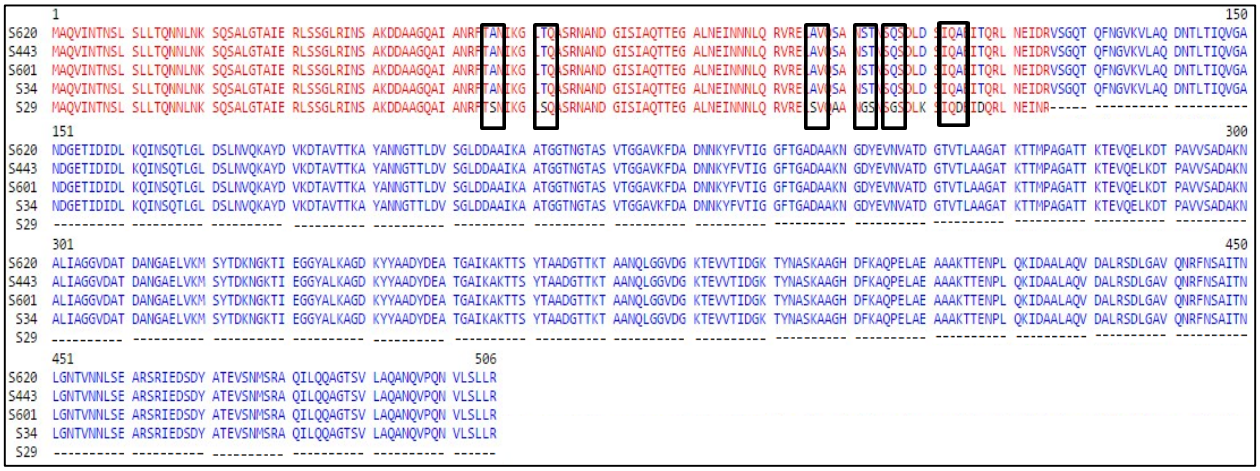

Figure 4.2a. Flagellar protein (*fljB*) comparison between *Salmonella enterica* isolates that were positive on the VIDAS UP *Salmonella* (SPT) assay and *Salmonella enterica* isolates (*S.* Hull and *S.* Duesseldorf) that caused false-negative results on the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays. Key: S620, S443 and S601 are *S. enterica* Newport, *S. enterica* Anatum and *S. enterica* Typhimurium respectively, S34 and S29 are *S.* Hull and *S.* Duesseldorf.

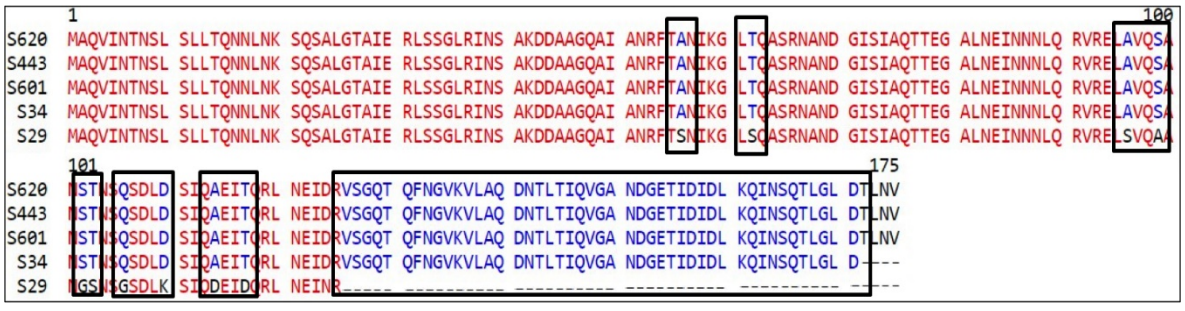

Figure 4.2b. Flagellar protein (*fliC*) comparison between *Salmonella enterica* isolates that were positive on the VIDAS UP *Salmonella* (SPT) assay and *Salmonella enterica* isolates

(*S.* Hull and *S.* Duesseldorf) that caused false-negative results on the VIDAS up *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays. Key: S620, S443 and S601 are *S. enterica* Newport, *S. enterica* Anatum and *S. enterica* Typhimurium respectively, S34 and S29 are *S.* Hull and *S.* Duesseldorf.

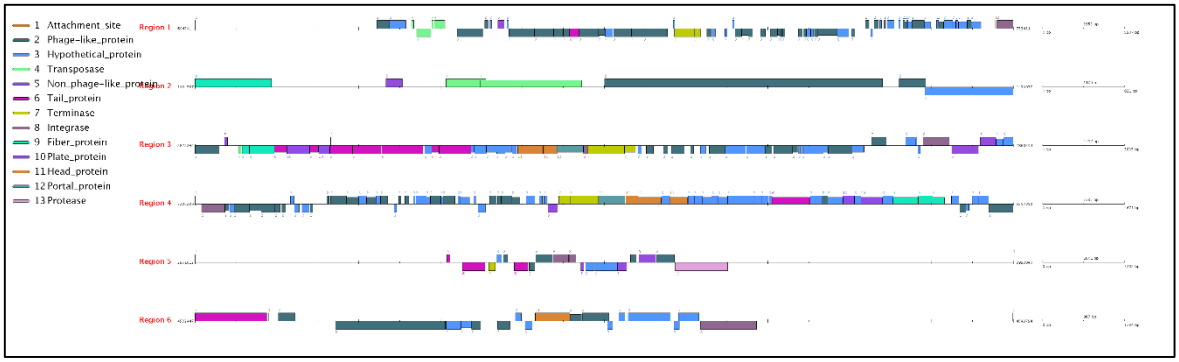

Figure 4.3a. Prophage map showing the presence of six intact and non-intact prophages in *Salmonella* Hull.

Region 6, scaffold23.1, total 15 CDS

#	CDS Position	BLAST Hit	E-Value	Sequence
1	1580..1592	attL	0.0	Show
2	1619..2158	PHAGE_Salmon_SSU5_NC_018843: putative repressor of phase 1 flagellin; PP_04475; phage(gi410491531)	5.57e-17	Show
3	2343..2663	hypothetical; PP_04476	0.0	Show
4	complement(3007..5340)	PHAGE_Entero_P4_NC_001609: DNA primase; PP_04477; phage(gi9627512)	0.0	Show
5	complement(5355..5624)	PHAGE_Entero_P4_NC_001609: hypothetical protein; PP_04478; phage(gi9627513)	2.86e-53	Show
6	complement(5672..5851)	hypothetical; PP_04479	0.0	Show
7	complement(5896..6441)	PHAGE_Entero_P4_NC_001609: putative CI repressor; PP_04480; phage(gi9627516)	3.26e-48	Show
8	complement(6444..6710)	PHAGE_Entero_P4_NC_001609: transcriptional regulator; PP_04481; phage(gi9627517)	2.23e-41	Show
9	7182..7985	PHAGE_Entero_P4_NC_001609: head size determination protein sid; PP_04482; phage(gi9627518)	1.69e-107	Show
10	8036..8227	PHAGE_Erwini_ENT90_NC_019932: transcriptional regulator; PP_04483; phage(gi431810990)	1.85e-07	Show
11	8244..8810	PHAGE_Entero_P4_NC_001609: amber mutation-suppressing protein; PP_04484; phage(gi9627520)	4.84e-70	Show
12	9218..10111	hypothetical; PP_04485	0.0	Show
13	10216..10716	hypothetical; PP_04486	0.0	Show
14	complement(10747..11940)	PHAGE_Entero_P4_NC_001609: integrase; PP_04487; phage(gi9627511)	1.02e-77	Show
15	17366..17378	attR	0.0	Show

Figure 4.3b. Putative repressor of phase 1 flagellin found in region 6, an incomplete prophage with homology to *Enterobacteriaceae* prophage P4.

CHAPTER V

CONCLUSION AND FUTURE WORK

The current study was conducted in order to elucidate the mechanisms by which *Citrobacter amalonaticus* caused false-positive test results on the VIDAS UP *Salmonella* (SPT) assay, as well as the basis for false-negative results observed when *Salmonella enterica* Duesseldorf and *Salmonella enterica* Hull were tested on the VIDAS platform and on the Reveal 2.0 *Salmonella* assay. By focusing efforts on the genes encoding flagella, and their corresponding amino acid sequences, which is a common diagnostic target in affinity based assays, we identified similarities between the *C. amalonaticus* and *Salmonella* flagella that could explain the cross reactivity observed in the VIDAS UP *Salmonella* (SPT) assay. In contrast, we identified significant differences in the amino acid sequences between *Salmonella* that had tested positive and two *Salmonella* isolates (*S.* Duesseldorf and *S.* Hull) that tested negative on the VIDAS UP *Salmonella* (SPT) and Reveal 2.0 *Salmonella* assays.

Several experiments should be conducted to confirm these observations. For example, monoclonal antibodies should be generated against the *fljB* and *fliC* proteins that are used to test the secondary and tertiary structure of the flagella proteins in *S.* Duesseldorf and *S.* Hull in an ELISA format to determine whether functional flagella are present or not on the cell surfaces of these bacteria. Additionally, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis should be conducted to determine if antibodies can recognize the primary amino acid structure of the flagella proteins, to determine if the proteins are being produced in the bacteria and not exported or displayed properly on the cell surface, as opposed to the flagella proteins not being produced properly.

Finally, additional isolates of bacteria from closely associated species that are known to cause false-positive test results on affinity based assays should be tested, on both the VIDAS UP *Salmonella* (SPT) and the Reveal 2.0 *Salmonella* assays. These include *Citrobacter* spp., *Proteus* spp. and *Hafnia* spp. Similarly, additional *Salmonella* isolates should be tested, to better determine the isolates that result in false-negative test results on affinity based platforms. Isolates that cause false-positive and false-negative test results should be characterized as described above, including sequencing the whole genomes of the isolates, followed by characterizing surface associated proteins using bioinformatic and phenotypic methods.

Once the nature of cross reactivity in the closely associated bacteria has been characterized, approaches to solve the issue should be explored. This includes the identification of new antigens that are more specific for *Salmonella*, and that are consistently expressed on the cell surface of all *Salmonella*, to counter challenge associated with false-negative test results. Other approaches would include the development of more selective enrichment methods to eliminate the presence of false-positive bacteria. These methods should also include approaches to increase the expression of surface associated antigens, thereby increasing the sensitivity of affinity based assays.

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