Canadian Food Inspection Agency
Agence canadienne d'inspection des aliments

Targeted Surveys

REPORT

2012/13 – 2013/14

Targeted Survey Investigating Bacterial Pathogens and Generic *E. coli* in Green Onions

RDIMS # 6335653

Canada

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Executive Summary

Targeted surveys are used by the Canadian Food Inspection Agency (CFIA) to focus its surveillance activities on areas of highest health risk. The information gained from these surveys provides both support for the prioritization of the Agency's activities to areas of greater concern, and scientific evidence to address areas of lesser concern. Originally started under the Food Safety Action Plan (FSAP), targeted surveys have been incorporated into the CFIA's regular surveillance activities as a valuable tool for generating information on certain hazards in foods, identifying/characterizing new and emerging hazards, informing trend analysis, prompting/refining health risk assessments, highlighting potential contamination issues, as well as assessing and promoting compliance with Canadian regulations.

Green onions have been reported to be responsible for several outbreaks of foodborne illness in North America. Often eaten raw, green onions are subject to extensive handling during and after harvest, where pathogens can be introduced at any step of production. Furthermore, their hollow structure provides favorable conditions for pathogen growth and protection from washing. The Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) has ranked green onions in the second highest priority group of concern for microbiological hazards in fresh fruits and vegetables.

Considering these factors and their relevance to Canadians, green onions have been selected as one of the priority commodity groups of fresh fruits and vegetables for enhanced surveillance. Over the course of a four-year baseline study (2010/11 to 2013/14), approximately 4,500 green onion samples were collected from retail locations and tested for the presence of various pathogens of concern.

The main objectives of these targeted surveys (2012/13 – 2013/14) were to generate baseline surveillance data on the bacterial pathogens *Salmonella*, *Shigella*, and *Escherichia coli (E. coli)* O157:H7/NM (non-motile), as well as on generic *E. coli*, an indicator of fecal contamination, in green onions available in the Canadian market. In total, 2,903 samples of green onions (imported and domestic, conventionally and organically grown) were collected and analyzed. Most samples (99.7%) were assessed as satisfactory. Eight samples were assessed as unsatisfactory: one for the presence of *Salmonella* and seven for high levels of generic *E. coli* (>1,000 MPN/g). Subsequent food safety investigations resulted in one product recall. In addition, two samples were assessed as investigative for elevated, yet marginally acceptable levels of generic *E. coli* (100 – 1,000 MPN/g). Further evaluation of these samples resulted in no immediate follow-up action. These findings suggest that the green onions sampled from the Canadian market over the course of this survey were predominantly produced under Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs).

The CFIA regulates and provides oversight to the industry, works with provinces and territories, and promotes the safe handling of foods throughout the food production chain. However, it is important to note that the food industry and retail sectors in Canada are ultimately responsible for the food they produce and sell, while individual consumers are responsible for the safe handling of the food they have in their possession. Moreover, general advice for the consumer on the safe handling of foods is widely available. The CFIA will continue its surveillance activities and will inform stakeholders of its findings.

1 Introduction

1.1 Targeted Surveys

The Canadian Food Inspection Agency (CFIA) monitors both domestic and imported foods for the presence of allergenic, microbiological, chemical, and physical hazards. One of the tools used to maintain this oversight is targeted surveys, which are a means to establish baseline information on specific hazards in food commodities and to investigate emerging risks. Targeted surveys are one of the Agency's core surveillance strategies, which also include the National Chemical Residue Monitoring Program (NCRMP), the National Microbiological Monitoring Program (NMMP), and the Children's Food Project (CFP). The targeted surveys complement the CFIA's surveillance activities by examining foods and hazards that may not be routinely assessed in these national monitoring programs.

Targeted surveys are used to gather information on the possible occurrence or prevalence of hazards in defined food commodities. These surveys generate essential information on certain hazards in foods, identify or characterize new and emerging hazards, inform trend analysis, prompt or refine human health risk assessments, assess compliance with Canadian regulations, highlight potential contamination issues, and/or influence the development of risk management strategies, as appropriate.

Due to the vast number of possible food-hazard combinations, it is neither feasible, nor should it be necessary, to use targeted surveys to identify and quantify all hazards in all food commodities. To identify the food-hazard combinations of greatest potential health risk, the CFIA uses a combination of scientific literature, the media, and/or a risk-based model developed by the Food Safety Science Committee, a group of federal, provincial and territorial subject matter experts in the area of food safety.

These targeted surveys (2012/13 – 2013/14) represents part of the collection of approximately 4,500 green onion samples over four years (2010/11 – 2013/14), and were designed to gather baseline information on the prevalence of *Shigella*, *Salmonella*, *Escherichia coli* (*E. coli*) O157:H7/NM, and generic *E. coli* in green onions available to Canadians at retail.

1.2 Codes of Practice, Acts, and Regulations

International food safety standards, codes of practice, and guidelines relating to food, food production, and food safety are developed under the joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Codex Alimentarius Commission. Producers of fresh fruits and vegetables are encouraged to follow these international codes of practice. Of relevance for this survey are the *Code of Hygienic Practice for Fresh Fruits and Vegetables* (CAC/RCP 53-2003) (1) and the *Recommended International Code of Practice - General Principles of Food Hygiene* (CAC/RCP 1-1969) (2). These codes address Good

Agricultural Practices (GAP) and Good Manufacturing Practices (GMP), which, when applied, help to control and reduce the potential for contamination with microbial, chemical, and/or physical hazards, at all stages of the production of fresh fruits and vegetables, from primary production to packaging.

Fresh fruits and vegetables available in the Canadian market must comply with the *Food and Drugs Act* (FDA) (3) and the *Food and Drug Regulations* (FDR) (4), which prescribe certain restrictions on the production, importation, sale, composition and content of foods and food products. Section 4(1)a of the FDA prohibits the sale of foods contaminated with foodborne pathogens, while sections 4(1)e and 7 prohibit the sale of unsafe food and food produced under unsanitary conditions.

Fresh fruits and vegetables that are imported or domestically produced and marketed inter-provincially must also comply with the safety requirements of the *Fresh Fruit and Vegetable Regulations* (5) under the *Canada Agricultural Products Act* (6). These regulations are intended to ensure that fresh fruits and vegetables sold to Canadian consumers are safe, wholesome, and properly graded, packaged and labelled.

The *Fresh Fruit and Vegetable Regulations* and the food-related sections of the FDA and FDR are enforced by the CFIA.

Targeted surveys are primarily conducted for surveillance and not for regulatory compliance purposes. However, results indicating a potential risk to public health for any samples tested under these surveys will trigger food safety investigations, including activities such as follow-up sampling, inspections of facilities, and consultations with Health Canada for health risk assessments. Depending on the findings, a recall of the affected product may be warranted.

2 Survey on Green Onions

2.1 Rationale

Green onions have been reported to be responsible for several outbreaks of foodborne illness in North America. From 1994 to March 2014, there were eight documented outbreaks associated with green onions contaminated with microbial pathogens (Appendix B). Some of these outbreaks were associated with the bacterial pathogens *Shigella*, *Salmonella*, and *E. coli* O157:H7/NM. Surveys of fresh produce conducted in 1999 and 2000/2001 by the United States Food and Drug Administration (US FDA) have also identified the presence of *Shigella* or *Salmonella* in 3.2% of domestic and 1.7 % of imported samples of scallions/green onions, suggesting that these were generally more likely to be contaminated than many other vegetables analyzed in those surveys (7, 8).

Green onions were also classified in the second highest priority group of concern for microbiological hazards in fresh fruits and vegetables during a joint FAO/WHO Expert Meeting in 2007 (9). This was based on multiple factors, including historical outbreaks and the potential for contamination with pathogens.

Green onions are grown at ground level and can therefore be contaminated in the field through the use of improperly composted manure, wildlife feces, and/or untreated irrigation water. During processing, the use of contaminated water for rinsing, cooling and icing also presents a potential source of pathogens. In addition, green onions require extensive handling during harvesting and packaging and can therefore be contaminated by infected handlers. The unique structure of green onions consists of moist and hollow tubular leaves which provide microbial pathogens with ideal growth conditions as well as protection from washing. Even though pathogens associated with green onions can be destroyed with cooking, their presence creates a potential risk for foodborne illness as green onions are often consumed raw.

Based on the above information and recommendations from the Food Safety Science Committee, green onions have been selected for targeted surveillance. The overall objective of this surveillance is to gather baseline information on the occurrence of pathogens of concern and on the presence and levels of *generic E. coli*, an indicator of fecal contamination, in green onions available to Canadians at retail.

These targeted surveys (2012/13 – 2013/14) constitute part of this enhanced surveillance and focuses on investigating the prevalence of *Shigella*, *E. coli* O157:H7/NM, *Salmonella*, and generic *E. coli*, in imported and domestic, as well as conventionally and organically grown green onions.

2.2 Targeted Microorganisms

2.2.1 Bacterial Pathogens *Salmonella*, *E. coli* O157:H7/NM and *Shigella*

The bacterial pathogens *Salmonella* and *E. coli* O157:H7/NM are found naturally in the intestines of animals such as poultry and cattle, respectively (10). Most outbreaks associated with these bacterial pathogens are linked to the consumption of contaminated food of animal origin (e.g., chicken and beef). However, in the last decade, fresh fruits and vegetables have emerged as significant sources of illnesses related to these bacteria (11). Fruits and vegetables can become contaminated with *Salmonella* and *E. coli* O157:H7/NM in the field, through improperly composted manure, contaminated water, and/or wildlife feces (12).

Humans are the only host of *Shigella*. Food contaminated by infected food handlers and water contaminated with human feces are the most common causes of shigellosis (infection with

Shigella). Shigellosis illnesses have been known to be associated with the consumption of contaminated fruits, vegetables, shellfish, and chicken (10).

2.2.2 Generic *E. coli* - an Indicator of Fecal Contamination

Typically, the *E. coli* bacteria that inhabit the large intestines of humans and animals are harmless. Due to their regular presence in the stools of humans and animals, the occurrence of *E. coli* in foods is an indication of direct or indirect contamination with fecal matter (13). The presence of generic *E. coli* in foods can also indicate potential contamination with pathogenic enteric microorganisms such as *Salmonella* or *E. coli* O157:H7/NM, which also live in the intestines of infectious humans and animals. It is important to note that the presence of generic *E. coli* in foods only implies an increased risk of contamination with pathogenic microorganisms, and does not conclusively indicate that these pathogens are present. High levels of generic *E. coli* in fresh produce sold at retail are an indication that contamination has occurred at some point between primary production and point of sale.

2.3 Sample Collection

All samples were collected from national retail chains, local/regional grocery stores, other conventional retail and natural food stores, as well as farmers' markets located in several cities across Canada. The number of samples collected in the various regions was based on the relative proportion of the Canadian population in each of these respective regions. Samples were collected between April 2012 and March 2014. Domestic samples were generally collected during the summer months while imported samples were collected primarily during the rest of the year, throughout the fall, winter, and spring. Samples that were labelled as organic at retail were identified as "organic" in this survey. All other samples were identified as "conventional".

For this survey, a sample consisted of a single sample unit (e.g., individual consumer-sized bundle(s) from a single lot) with a total weight of at least 200 g. This sampling approach is common for surveys conducted at retail, and is also used by other federal partners such as the Public Health Agency of Canada (PHAC) for the retail component of their FoodNet Surveys (14). Collected samples were required to be shipped under conditions that limited the growth of microorganisms during transit. Samples were declared "unfit" for analysis if there were issues regarding the conditions in which they were handled or transported.

2.4 Analytical Methods and Assessment Guidelines

The samples were analyzed using the analytical methods published in Health Canada's *Compendium of Analytical Methods for the Microbiological Analysis of Foods* (15) (Appendix C). These methods are used for regulatory testing by the CFIA and are fully validated for the analysis of fresh fruits and vegetables, including green onions.

The assessment criteria presented below (Tables 1 and 2) are based on the principles of the *Health Products and Food Branch Standards and Guidelines for Microbiological Safety of Foods* (16) and associated methods published in Health Canada's *Compendium of Analytical Methods* (15).

Table 1. Assessment Guidelines for Bacterial Pathogens in Green Onions

Bacterial Analysis* (Method Identification Number)	Assessment Criteria	
	Satisfactory	**Unsatisfactory**
***E. coli* O157:H7/NM** (MFLP-30, and MFLP-80)	Absent in 25 g	Present in 25 g
Salmonella* spp.* (MFLP-29 modified and MFHPB-20)	Absent in 25 g	Present in 25 g
Shigella* spp.* (MFLP-26 and MFLP-25)	Absent in 25 g	Present in 25 g

* *Compendium of Analytical Methods* (15).
**At this time, no criteria have been established by Health Canada for these bacterial pathogens in fresh fruits and vegetables. However, in the absence of a specified criteria, presence in foods is considered to be a violation of FDA Section 4(1)a and is therefore assessed by the CFIA as unsatisfactory.

Table 2. Assessment Guidelines for Generic *E. coli* in Green Onions

Bacterial Analysis* (Method Identification Number)	Assessment Criteria		
	Satisfactory	**Investigative**	**Unsatisfactory**
Generic *E. coli* (MFHPB-19 or MFHPB-27)**	≤ 100	100 < x ≤ 1,000	> 1,000

* *Compendium of Analytical Methods* (15).
** Concentration unit depends on method used. For MFHPB-19 method: MPN/g, for MFHPB-27 method: CFU/g.

Unsatisfactory sample assessments are subject to follow-up actions, such as directed follow-up sampling, inspection of establishments, health risk assessments, and/or product action (e.g., product recall).

Samples assessed as investigative require some follow-up activity. This could include, for example, further sampling to verify the levels of generic *E. coli* in the product in question.

2.5 Limitations

Samples tested during this survey were collected at retail locations across Canada, in contrast to monitoring samples that are picked up at distribution points and warehouses. As such, products sampled at retail could be mixed and originate from different shipments and/or suppliers.

Although this represents what the Canadian consumer experiences, this imposes certain limitations with respects to the traceability of the products and the identification of the source of contamination in the case of positive results.

Results obtained for a targeted survey sample are from the analysis of a single sample unit. This sampling and testing strategy generally precludes the extrapolation of the laboratory result to the whole production lot as it is not statistically representative. Therefore, in the absence of additional information, this imposes certain limitations on the interpretation of results as they relate to the specific lot.

Finally, given the seasonality as well as the varying channels of commerce, the source of products can change dramatically from one season to the next. As such, there are an insufficient number of samples in this survey to carry out a detailed analysis of the results based on the products' country of origin. In the case of positive results, unsatisfactory rates between countries are not considered to be statistically comparable.

3 Results

3.1 Sample Distribution

A total of 2,903 samples were collected, consisting of green onions that were imported (71.1%) and domestically produced (28.9%), as well as conventionally (76.3%) and organically (23.7%) grown (Table 3). The majority of the imported samples originated from Mexico (87.3%) followed by the USA (11.1%). One imported sample came from Jamaica, and 32 samples were from unidentified foreign countries.

Table 3. Distribution of Green Onion Samples
(Percentage of the total number of samples shown in brackets)

Product Origin	Production Practice		Total
	Conventional	Organic	
Canada	812 (28.0%)	28 (1.0%)	**840 (28.9%)**
Subtotal - Domestic	*812 (28.0%)*	*28 (1.0%)*	***840 (28.9%)***
Jamaica	1 (0.03%)	0 (0%)	**1 (0.03%)**
Mexico	1,254 (43.2%)	547 (18.8%)	**1,801 (62.0%)**
United States	121 (4.2%)	108 (3.7%)	**229 (7.9%)**
Unknown	27 (0.9%)	5 (0.2%)	**32 (1.1%)**
Subtotal - Imported	*1,403 (48.3%)*	*660 (22.7%)*	***2,063 (71.1%)***
Total	**2,215 (76.3%)**	**688 (23.7%)**	**2,903 (100%)**

3.2 Assessment Results

A total of 2,903 green onion samples were analyzed for the pathogenic bacteria *Salmonella*, *Shigella*, and *E. coli* O157:H7/NM, as well as for the indicator of fecal contamination, generic *E. coli*.

E. coli O157:H7/NM and *Shigella* were not detected in any of the green onion samples that were collected. *Salmonella* and generic *E. coli* (> 100 MPN/g) were not found in most samples (99.7%), which were assessed as satisfactory (Table 4).

Table 4. Summary of Results for Green Onion Samples Tested for *E. coli* O157:H7/NM, *Salmonella, Shigella* and Generic *E. coli*

Production Practice	Product Origin	Sample Assessment			Total
		Satisfactory	Investigative	Unsatisfactory	
Conventional	Imported	1,398	1	4	1,403
	Domestic	811	1	0	812
	Subtotal	***2,209***	***2***	***4***	***2215***
Organic	Imported	656	0	4	660
	Domestic	28	0	0	28
	Subtotal	***684***	***0***	***4***	***688***
Total		**2,893 (99.7%)**	**2 (0.07%)**	**8 (0.3%)**	**2,903 (100%)**

Eight samples were found to be unsatisfactory (Table 5). One sample was unsatisfactory due to the presence of *Salmonella* Typhimurium and the seven other samples had high levels (> 1,000 MPN/g) of generic *E.coli*. Follow-up investigations of these samples resulted in one product recall. It is important to note that there were no reported illnesses associated with the consumption of the *Salmonella* contaminated product sampled during this survey.

Table 5. Summary of Unsatisfactory Samples

Production Practice / Country of Origin	Reason for Unsatisfactory Assessment
Organic / Mexico	Generic *E. coli* counts > 1,600 MPN/g
Organic / Mexico	Generic *E. coli* counts > 1,600 MPN/g
Organic / Mexico	Generic *E. coli* counts > 1,600 MPN/g
Organic / Unidentified	Generic *E. coli* counts > 1,600 MPN/g
Conventional / Mexico	Generic *E. coli* counts > 1,600 MPN/g
Conventional / Mexico	Generic *E. coli* counts > 1,600 MPN/g
Conventional / Mexico	Generic *E. coli* counts > 1,600 MPN/g
Conventional / Mexico	*Salmonella* Typhimurium detected, var. Copenhagen

In addition, elevated levels of generic *E. coli* (counts between 100 and 1,000 MPN/g) were detected in two samples, which were assessed as investigative (Table 6). After further evaluation of these results, no immediate follow-up actions were deemed necessary.

Table 6. Summary of Investigative Samples

Production Practice / Country of Origin	Reason for Unsatisfactory Assessment
Conventional / Mexico	Generic *E. coli* counts = 120 MPN/g
Conventional / Canada	Generic *E. coli* counts = 400 MPN/g

4 Discussion and Conclusion

The majority (99.7%) of green onion samples collected over the course of these surveys (2012/13 – 2013/14) were assessed as satisfactory. The results indicate that the bacterial pathogens *E. coli* O157:H7/NM and *Shigella* were not detected in any of the 2,903 samples that were tested. *Salmonella* was detected in one sample, and high levels of generic *E. coli* (>1,000 MPN/g) were found in seven samples. These samples were assessed as unsatisfactory. Two samples were assessed as investigative for having elevated levels of generic *E. coli* (100 – 1,000 MPN/g).

As a result of these findings, the CFIA initiated appropriate food safety investigations, including directed sampling, inspection of facilities, review of importation procedures, and health risk assessments conducted by Health Canada. One product recall resulted from the subsequent food safety investigations. It is important to note that there were no reported illnesses associated with the unsatisfactory green onions sampled during this survey. After further evaluation of the investigative results, no further actions were deemed necessary.

Surveys conducted by the US FDA on bacterial pathogens in fresh produce in 1999 and 2000/2001 found that 1.7% of imported and 3.2% of domestic samples of green onions were contaminated with either *Shigella* or *Salmonella* (7, 8). More recent studies from other jurisdictions in Ontario and Alberta (17, 18), and testing from the United States Department of Agriculture (USDA) Microbiological Data Program on fresh produce sold in the USA (19) have shown results similar to those obtained in this survey, with levels of bacterial pathogens in green onions ranging from 0 to 0.8%.

The overall findings of these surveys suggest that green onions available in the Canadian market are generally produced and handled under acceptable GAPs and GMPs.

While the food industry and retail sectors in Canada are ultimately responsible for the food they produce and sell, and individual consumers are responsible for the safe handling of the food they have in their possession, the CFIA regulates the industry, provides oversight, and promotes the safe handling of foods throughout the food production chain. The CFIA will continue its surveillance activities and will inform stakeholders of its findings.

5 Acknowledgments

We would like to express our sincere thanks to Judy D. Greig, Public Health Agency of Canada for providing the summary of outbreaks (Appendix B).

6 References

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Appendix A: List of Acronyms and Abbreviations

CFIA: Canadian Food Inspection Agency
CDC: Centres for Disease Control and Prevention
CFP: Children's Food Project
CFU/g: colony forming units per gram
E. coli: *Escherichia coli*
FAO: Food and Agriculture Organization of the United Nations
FDA: *Food and Drugs Act*
FDR: *Food and Drug Regulations*
GAP: Good Agricultural Practices
GMP: Good Manufacturing Practices
MFHPB: Microbiology Food Health Protection Branch
MFLP: Microbiology Food Laboratory Procedures
MPN: Most Probable Number
NCRMP: National Chemical Residue Monitoring Program
NM: non-motile
NMMP: National Microbiological Monitoring Program
PCR: Polymerase Chain Reaction
PHAC: Public Health Agency of Canada
spp.: species
USDA: United States Department of Agriculture
US FDA: United States Food and Drug Administration
WHO: World Health Organization
g: gram

Appendix B: Global Foodborne Disease Outbreaks Associated With Green Onions Contaminated with Microbial Pathogens (1994 – March 2014)*

Year	Micro-organisms	Vehicle	Country	Cases	Source
1994	*Shigella flexineri*	Onion, green	USA, Multi-state	97	Outbreak alert database, Center for Science in the Public Interest
1996	Hepatitis A Virus	Onion, green	USA	60	CDC line list 1996**
1997	*Cryptosporidium parvum* (Protozoan parasite)	Onion, green (suspected)	USA	54	US FDA: Analysis and Evaluation of Preventive Control Measures for the Control and Reduction/Elimination of Microbial Hazards on Fresh-cut Produce, Chapter IV**
1998	Hepatitis A Virus	Onion, green	USA	43	J Infect Dis 2001 18398):1273-6**
2000	Hepatitis A Virus	Onion, green / scallions	USA, Multi-state	32	Outbreak alert database, Center for Science in the Public Interest
2003	Hepatitis A Virus	Onion, green	USA	742	MMWR November 28, 2003. 52(47);1155-1157**
2006	*Escherichia coli* O157:H7	Onion, green / scallions (suspected)	USA	300	CDC**
2010	*Salmonella* Oranienberg	Onion, green (suspected)	Canada	25	CFIA Health Hazard Alert; Ontario Ministry of Health and Long-Term Care; Foodborne Illness Outbreaks; Food Safety News

* The data presented were collected from several sources of information, such as peer-reviewed journals, newspapers, press releases, health units, national laboratory and government websites.

** Information provided by Judy D. Greig, Laboratory for Foodborne Zoonoses, PHAC

Appendix C: Analytical Methods Used for Microbial Analysis

Bacterial Analysis	Method Identification Number (Date Issued)*	Title of Method
***Shigella* spp.**	MFLP-26 (February 2006)	Detection of *Shigella* spp. in Foods by the Polymerase Chain Reaction (PCR)
	MFLP-25 (March 2006)	Isolation and Identification of *Shigell*a spp. from Foods
***E. coli* O157:H7/NM**	MFLP-30 (November 2012)	Detection of Escherichia coli O157:H7 in select foods using the BAX® System E. coli O157:H7 MP
	MFLP-80 (March 2008)	Isolation of *E. coli* O157:H7 or NM in Foods
***Salmonella* spp.**	MFLP-29** (June 2012, modified)	The Qualicon Bax® System Method for the Detection of Salmonella in a Variety of Food and Environmental Samples
	MFHPB-20 (March 2009)	Methods for the Isolation and Identification of *Salmonella* from Foods and Environmental Samples
Generic *E. coli*	MFHPB-19 (April 2002)	Enumeration of Coliforms, Faecal Coliforms and of *E. coli* in Foods
	MFHPB-27 (October 2012)	Enumeration of *Escherichia coli* in Foods by a Direct Plating (DP) Method

* Published in the *Compendium of Analytical Methods* (22)

** MFLP-29 was performed as written with the following modification: Secondary enrichment was performed as outlined for cantaloupes, i.e., transferred from buffered peptone broth as specified to RVS and TBG broths (Rappaport-Vassiliadis Soya Peptone broth and Tetrathionate Brilliant Green broth) and incubated for 24 ± 2 h at 42.5°C. After incubation 2 ml from each of RVS and TBG are combined to one sample and proceed with step 7.3.1.4 of the method.