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Estimating risk associated with human norovirus and hepatitis A virus in fresh Australian leafy greens and berries at retail

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ABSTRACT

The apparent international rise in foodborne virus outbreaks attributed to fresh produce and the increasing importance of fresh produce in the Australian diet has led to the requirement to gather information to inform the development of risk management strategies. A prevalence survey for norovirus (NoV) and hepatitis A virus (HAV) in fresh Australian produce (leafy greens, strawberries and blueberries) at retail was undertaken during 2013–2014 and data used to develop a risk profile. The prevalence of HAV in berries and leafy greens was estimated to be < 2%, with no virus detected in produce during the yearlong survey. The prevalence of NoV in fresh strawberries and blueberries was also estimated to be < 2% with no virus detected in berries, whilst for leafy greens the NoV prevalence was 2.2%. Prevalence of a bacterial hygiene indicator, *Escherichia coli*, was also investigated and found to range from < 1% in berries to 10.7% in leafy greens. None of the NoV positive leafy green samples tested positive for *E. coli*, indicating it is a poor indicator for viral risk. The risk was evaluated using standard codex procedures and the Risk Ranger tool. Taking all data into account, including the hazard dose and severity, probability of exposure, probability of infective dose and available epidemiological data, the risk of HAV and NoV foodborne illness associated with fresh Australian berries (strawberries and blueberries) sold as packaged product was deemed to be low. The risk of foodborne illness from HAV associated with leafy greens was also deemed to be low, but higher than that for fresh berries, due mainly to the potential for re-contamination post-processing if sold loose. The risk of foodborne illness from NoV associated with leafy greens was deemed to be low/moderate. Despite the prevalence of NoV in leafy greens being low and the inability to discriminate between infective and non-infective virus using PCR based methodologies, the fact that NoV was detected resulted in a higher risk associated with this pathogen-product pairing; compounded by the higher prevalence of NoV within the community compared to HAV, and the potential for leafy greens to become contaminated following processing if sold loose.

1. Introduction

Foodborne viruses are a significant food safety hazard and represent the most reported cause of illness outbreaks worldwide (WHO, 2018). Norovirus (NoV) and Hepatitis A virus (HAV) are the viruses of most concern based on the incidence of reported foodborne disease, morbidity, mortality and the potential for transmission via foods (FAO/WHO, 2012). These viruses have been mainly associated with shellfish, water, berries, green onions, leafy greens, and semi-dried tomatoes (Bidawid et al., 2000; Bosch and Le Guyader, 2010; Butot et al., 2007; Cheong et al., 2009; Dentinger et al., 2001; FAO/WHO, 2011; OzFoodNet, 2012; Petrignani et al., 2010; Wheeler et al., 2005). Estimates of the proportion of viral illness attributed to food are in the range of 5% for HAV and 12–47% for NoV (FAO/WHO, 2008). Norovirus has been responsible for most of the produce related foodborne outbreaks between 2004 and 2012, and mainly associated with consumption of salad in the United States and berries in the European Union (Callejo et al., 2015). A number of significant outbreaks of illness attributed to foodborne viruses associated with produce have occurred in recent years, including an outbreak of HAV associated with semi-dried tomatoes in Australia in 2009–2010 (Donnan et al., 2012; OzFoodNet, 2015a), a large multistate German outbreak (> 11,000 cases) of NoV related to the consumption of imported frozen strawberries in 2012 (Bernard et al., 2014), a multinational European HAV outbreak associated with consumption of berries in 2013 (EFSA, 2014c) and multistate USA outbreak of HAV associated with consumption of pomegranate arils (Collier et al., 2014). Epidemiological investigation of outbreaks of HAV and NoV in the European Union have highlighted

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frozen berries as a vehicle of infection with NoV most commonly implicated; 27 out of 32 berry related foodborne outbreaks identified between 1983 and 2013, accounted for 15,000 cases (Tavoschi et al., 2015). Investigations of Australian foodborne outbreaks of HAV have implicated frozen imported berries as the vehicle in 2009 (n = 6) (OzFoodNet, 2015a) and between October 2014 and May 2015 (n = 35) involving multiple jurisdictions (OzFoodNet, 2017).

The consumption of leafy salads and berries has increased internationally in recent years (Callejo et al., 2015). These foods are generally consumed fresh and are not subject to further processing to eliminate pathogenic microorganisms such as viruses. The focus of risk control is good agricultural practice during growing and good hygienic practice during processing. Food Standards Australia New Zealand (FSANZ) reviewed data on outbreaks of foodborne illness related to horticultural products and reported that "*Salmonella* and noroviruses were suspected or identified as being responsible for the majority of recorded outbreaks. These pathogens were associated with salads and fresh fruits and vegetables" (FSANZ, 2010). Internationally there has been limited monitoring of leafy greens or berry fruits for the presence of foodborne viruses. There is very limited prevalence data on the rates of NoV and HAV contamination of berries not involved in foodborne outbreaks in the peer-reviewed literature (EFSA, 2014b). The few research surveys conducted on foodborne viral contamination of berries have been largely limited to strawberries and raspberries (Baert et al., 2011; Loutreul et al., 2014; Maunula et al., 2013; Stals et al., 2011). The prevalence of NoV and contamination levels of fresh produce (leafy greens and soft red fruits) were investigated by compilation of survey data collected in Belgium, Canada and France (Baert et al., 2011). Prevalence of enteric viral pathogens in leafy greens at various points in the supply chain have been investigated in Greece, Serbia, Poland, Mexico, Canada, Italy, Spain, Belgium, France and Tunisia (Felix-Valenzuela et al., 2012; Kokkinos et al., 2012; Loutreul et al., 2014; Mattison et al., 2010; Terio et al., 2017). Differences in the sensitivities of the various detection methodologies employed complicate analysis and comparison of the data (Baert et al., 2011). It was also noted that NoV genomic copies detected in fresh produce rarely related to infection or outbreaks and recommended further investigation of the probability of infection related to the presence or levels of NoV genomic copies. The standard method for quantitative detection of NoV and HAV from foods was published in 2013 (ISO/CEN, 2013) which allowed direct comparisons of prevalence rates to be made over time and between regions.

The European Food Safety Authority (EFSA) Panel on Biological Hazards has prepared several scientific assessments including the "Opinion on the public health risk posed by pathogens that may contaminate food of non-animal origin" (EFSA, 2014a, 2014b, 2014d, 2014e). These reports note that NoV prevalence and infectivity data on leafy greens and berries are limited, as is quantitative data on viral load. Consequently, establishing microbiological criteria for NoV on leafy greens and berries is difficult. Due to the emergence of NoV outbreaks associated with frozen raspberries and strawberries in Europe, it was recommended that appropriate data be collected as a priority for the development of microbiological criteria to support improved control of NoV in frozen berries.

Despite the apparent international rise in foodborne virus outbreaks related to fresh produce and the increasing importance of fresh produce in the diet, no surveys for foodborne viruses had been undertaken in fresh leafy greens or berries produced in Australia. To inform the development of future risk management strategies to minimise the risk of foodborne virus illness to consumers, a survey to determine the prevalence of NoV and HAV in fresh Australian produce (leafy greens, strawberries and blueberries) at retail was undertaken. Detection of *E. coli* was also used as a hygiene indicator for the samples. Positive results would indicate faecal contamination from warm-blooded mammals. The presence of faecal contaminants could indicate the potential for contamination with foodborne enteric viral pathogens, such as NoV or HAV. Results of this survey were used to develop a risk estimate of public health impact of NoV and HAV associated with Australian produce using Risk Ranger, a semi-quantitative food safety risk assessment tool (Ross and Sumner, 2002). The underlying model in Risk Ranger is a simplification of the harvest to consumption pathway. The Risk Ranger tool allows a comparative risk with other hazard food combinations to be undertaken and assists with decision making on the scale of risk management required. It also aids with the identification of knowledge gaps and which of these have the greatest impact on the risk estimate, hence having higher priority for further investigation.

2. Materials and methods

2.1. Prevalence survey for NoV and HAV in fresh Australian produce at retail

2.1.1. Sampling plan

The sampling plan focussed on the major produce commodities and supply pathways. The majority of fresh produce consumed within Australia is produced domestically and strawberries and blueberries are the most significant berry types produced. Furthermore, the majority of fresh produce sold within Australia is through retail outlets, of which supermarkets dominate. The survey sampled and tested a total of 302 samples comprising 152 fresh berries and 150 fresh leafy green vegetables produced in Australia and sold through supermarkets and greengrocers or farmers markets (GFMs). The survey was conducted over a year with sampling beginning in July 2013 and finishing in June 2014. Sampling was done weekly to fortnightly.

A significant proportion of the total national production of leafy salad vegetables and berries is produced in Victoria (69% and 71% respectively), and a substantial proportion of this produce is transported interstate for sale according to the Australian Bureau of Statistics (ABS) data (ABS, 2010). The quantities of berries and leafy salad vegetables consumed in each capital city was unknown, largely due to information gaps regarding meal sizes consumed and frequency of consumption of these product types. Therefore, it was assumed that the amount of berries and leafy salad vegetables consumed in each city per annum was proportional to the population size. Hence, the samples were proportionally allocated to Melbourne and Adelaide based on their relative populations reported in ABS data from 2012 (ABS, 2012).

Blueberries and strawberries are the most significant berry fruit produced in Australia, representing 8% and 88% of total berry fruit production respectively (ABS, 2010). All other berries account for approximately 3%, with raspberries representing 2% of the total berry production in Australia. This survey included only strawberries and blueberries as the major berry produce commodities, assigning the 152 berry samples proportionally to blueberries (n = 14) and strawberries (n = 138).

Approximately 75% of fresh salad greens sold in supermarkets were pre-packaged and 25% were loose (e.g. heads of iceberg, cos and other similar lettuces and mixed salad greens), an observation supported by the consumer purchasing study of Welch (2013). These proportions were used to determine the number of samples of pre-packaged and loose salad greens collected in supermarkets. Only loose greens, and not bagged leafy greens, were sampled from GFMs. Of the total 150 samples, 76 were loose salad vegetables; 74 were pre-packaged salad vegetables. Loose and packed leafy green products sampled represented spinach, rocket, kale, various mixed lettuces, cos lettuce, butterhead lettuce, lamb's lettuce and mesclun. The berries and leafy greens sampled were sourced from various producers and suppliers.

Two major supermarkets dominated the Australian retail sector at the time of the survey with 80% of the market share (43%; Supermarket 1 and 37%; Supermarket 2, respectively). Therefore, samples were collected proportionally from these two supermarkets as well as GFMs. Table 1 shows the number of samples per commodity collected from various retail outlets in Melbourne and Adelaide metropolitan areas

Table 1
Number of berry and leafy green samples collected from retail outlets in Melbourne and Adelaide.

Retail setting	Blueberry	Strawberry	Loose greens	Packed greens	Total
Melbourne - population 4.14 million (77% of total)					
Supermarket 1	4	37	11	31	83
Supermarket 2	3	33	8	26	70
GFMs	4	37	40	0	81
Sub-total	11	107	59	57	234
Adelaide - population 1.21 million (23% of total)					
Supermarket 1	1	11	3	9	24
Supermarket 2	1	9	2	8	20
GFMs	1	11	12	0	24
Sub-total	3	31	17	17	68
Total	14	138	76	74	302

during the survey period.

Each sample comprised at least one full commercial unit of each product and a minimum of 300 g. When one commercial unit was < 300 g, several units were combined to ensure a minimum of 300 g. Microbiological testing of samples usually commenced on the day of receipt or as soon as possible with samples held at 4 °C until tested. Samples collected in Adelaide were transported in a refrigerated van to the SARDI Food Science Laboratories on the same day as collection. Samples collected in Melbourne were transported in polystyrofoam boxes containing cold packs via air freight to the SARDI Food Science Laboratories. Samples were received within 24–48 h of collection.

2.2. Microbiological analysis

2.2.1. Escherichia coli enumeration

The *E. coli* levels were determined by the most probable number (MPN) method as outlined in AS 5013.15 - 2006: Microbiology of food and animal feeding stuff – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique (Standards Australia, 2006). The limit of detection of this test is 3 organisms/g of food matrix.

2.2.2. Horizontal method for detecting of NoV and HAV

Testing for NoV and HAV in berries and leafy greens was done from 25 g of product using the ISO/TS 15216 – 2013 protocol: Microbiology of food and animal feed - Horizontal method for determination of Hepatitis A virus and Norovirus in food using real-time RT-PCR (ISO/CEN, 2013). A variation on the ISO/TS 15216 method was that murine norovirus (MNV) was used as the process control virus instead of mengovirus. The strain of MNV used was MNV-1 (ATCC PTA-5935). Extraction and purification of viral RNA was done using the bioMerieux NucliSENS® Minimag system (bioMerieux Pty. Ltd. Baulkham Hills, Australia), following the manufacturer's recommendation. Real-time RT-PCR for HAV, NoV GI and NoV GII was done using primers and probes as specified in ISO/TS 15216-1. Primers and probes for real-time RT-PCR of the process control virus (MNV) were those specified by Hewitt et al. (2009).

The RT-PCR master mix used for all assays was the RNA Ultrasense™ one-step qRT-PCR system (Invitrogen, Scoresby, Australia), prepared following the manufacturer's recommendations. Real-time RT-PCR cycling parameters were as specified in ISO/TS 15216-1 and included an initial incubation at 55 °C for 1 h followed by denaturation at 95 °C for 5 min and 45 cycles of 95 °C for 15 s, 60 °C for 1 min and 65 °C for 1 min. Real-time RT-PCR was run in a 384-well format (ViiA™ 7 system, Applied Biosystems, Scoresby, Australia), with mastermix and template being dispensed using a Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, USA). The ISO/TS 15216 protocol outlines a number of controls and standards including the use of a process control virus (MNV) to determine virus extraction efficiency and external control (EC) RNA to determine amplification efficiency. Plasmid standards for NoV GI, NoV GII and HAV (provided by James Lowther, Centre for Environment, Fisheries and Aquaculture Science, United Kingdom) were used for quantification of virus detected within a sample and generation of EC RNA (ISO/CEN, 2013). The theoretical limit of detection (tLOD) and theoretical limit of quantification (tLOQ) were determined for NoV GI, NoV GII and HAV using the modified method of Armbruster and Pry (2008) and Bustin et al. (2009). For each assay, fourteen replicates of five dilutions of the standard were analysed. The lowest dilution with a standard deviation (σ_{CtLoD}) of < 1 and detection > 95% was used to calculate the average Ct_{LoD}. The Ct_{LOQ} was calculated as the Ct_{LOD} minus 2(σ_{CtLOD}). The tLOD and tLOQ were calculated as follows:

$$\text{Log}_{10}\,\text{concentration(tLOD)} = (\text{Ct}_{\text{LOD}} - \text{y intercept})/\text{slope reaction}$$

$$\text{Log}_{10}\,\text{concentrtion(tLOQ)} = (\text{Ct}_{\text{LOQ}} - \text{y intercept})/\text{slope reaction}$$

2.3. Statistical analysis

It was determined that a sample size of 150 for each of the two commodities (berries and leafy greens) would provide a statistical probability of 0.95 of detecting at least one sample with detectable levels of viruses if ≥2% of the samples were contaminated. The sample size calculation was based on the binomial distribution:

$$P(X = x) = \binom{n}{x} p^X (1 - p)^{n-x}$$

where X is the discrete random variable representing the number of samples with detected virus out of the total number of samples, x = 0, p = 0.02 (assumed prevalence) and n, the total sample size, is the variable of interest. In addition, the largest margin of error for a prevalence estimate with this sample size is ± 8% for a 95% confidence interval.

The R software (R Core Development Team, version 3.1.3) was used to perform statistical analysis and generate the prevalence estimates and associated 95% confidence intervals (CI) for *E. coli*, NoV GI, NoV GII and HAV. The estimate of prevalence is the number of samples with detected levels of virus expressed as a proportion of the total number of samples. The upper bound for the prevalence estimate was calculated based on the sample size and a 95% probability of detecting at least one sample with detectable levels of viruses.

2.4. Risk profile for NoV and HAV in fresh Australian berries and leafy greens at retail

The current prevalence survey results were used to develop a risk profile for NoV and HAV in fresh Australian berries and leafy greens at retail according to standard Codex principles (FAO/WHO, 2007), including hazard identification, hazard characterisation, exposure assessment and risk characterisation. The purpose of the risk profile was to provide contextual and background information relevant to these food/hazard combinations, hence enabling risk managers to make decisions and, if necessary, take further action. A further aim of the risk profile was to identify any knowledge gaps preventing a full risk assessment.

Risk Ranger, a semi-quantitative food safety risk assessment tool, was used to evaluate the risks posed by NoV and HAV in fresh Australian berries and leafy greens at retail (Ross and Sumner, 2002). The Risk Ranger tool is in spreadsheet software format. Data on probability of exposure to a foodborne hazard (frequency of consumption, proportion of population consuming), the concentration of the hazard in a food when present, and the probability and severity of outcomes that might arise from the level and frequency of exposure is input. Steps from harvest to consumption are taken into consideration in the evaluation, such as, initial levels of pathogen contamination, effect of processing, potential for recontamination, effectiveness of post-processing control systems and the impact of meal preparation steps. The tool calculates a risk estimate which can be used to rank risk of various pathogen (bacteria, virus and toxin) and product (red meat and seafood) combinations (Sumner and Ross, 2002; Sumner et al., 2005). The software produces a probability of illness through the product of four estimates: contamination level of the consumed meal relative to the level required to produce infection (taking into consideration the prevalence of contamination, the increase or decrease due to processing, cooking steps and estimates of the increase of contamination required to produce an infective dose); the proportion of the population susceptible to illness; and consumption frequency. It converts this probability into a comparative risk estimate which is the product of the probability of illness, the proportion of population consuming the food, the hazard severity and the size of the affected population. The comparative risk estimate is then converted to a risk ranking value scaled from 0 and 100, where 0 represents no risk and 100 represents all meals containing a lethal dose of the hazard. The tool aids in focussing attention on factors that most affect food safety risk. An assessment of the potential impacts of the identified knowledge gaps on the risk evaluations was also done by inputting various parameters into Risk Ranger and evaluating impact on the overall risk estimate.

3. Results

3.1. Microbiological analysis of bacterial hygiene indicator and foodborne viruses

3.1.1. Prevalence of E. coli

E. coli was detected in seventeen samples during the survey, as detailed in Table 2. *E. coli* was detected in one strawberry sample out of a total of 152 berry samples, giving an estimated prevalence of 0.7% (95% CI of 0.02 to 3.6%) for berries (Table 3). *E. coli* was detected in sixteen leafy green samples (ten loose and six packed) out of a total of 150, giving an estimated prevalence of 10.7% (95% CI of 6.2 to 16.7%) for greens (Table 3). The counts of *E. coli* in these samples ranged from 3 to 1100 CFU per gram of product (Table 2). Also, of note is that 94% of these *E. coli* positive samples were collected in the warmer months of the year.

Table 3
Prevalence of *E. coli*, NoV and HAV in fresh Australian berries and leafy greens at retail.

Produce	Samples analysed	Valid test results	Positive samples	Prevalence estimate (95% CI)
E. coli				
Leafy greens	150	150	16	10.7% (6.2–16.7%)
Berries	152	152	1	0.7% (0.02–3.6%)
NoV GI				
Leafy greens	150	137	3	2.2% (0.5–6.3%)
Berries	152	143	0	< 2% (0–2.5%)
NoV GII				
Leafy greens	150	126	1	0.8% (0.02–4.3%)
Berries	152	137	0	< 2% (0–2.7%)
HAV				
Leafy greens	150	140	0	< 2% (0–2.6%)
Berries	152	141	0	< 2% (0–2.6%)

3.1.2. Prevalence of NoV and HAV

Each sample was analysed for NoV GI, NoV GII, HAV and the process control virus (MNV). The process control virus recovery was calculated by reference to the process control virus RNA standard curve. The results of samples with virus extraction efficiencies < 1% or an amplification efficiency ≤25% were deemed not valid, as specified by the ISO/TS15216-1:2013 method and therefore not included in the prevalence estimate calculation (Table 3). The tLOD for NoV GI, NoV GII and HAV was 6.2, 174.7 and 6.6 copies/reaction respectively. If this was from berries/lettuce samples this would be equivalent to 24.8, 698.8 and 26.4 copies/25 g for NoV GI, NoV GII and HAV, respectively. The tLOQ was 17.3, 615.7, 14.3 copies/reaction for NoV GI, NoV GII and HAV, respectively.

All 152 berry samples tested resulted in a valid virus extraction efficiency ranging from 1 to 100% (median 28.0% and mean 37.9%). The virus extraction efficiency from blueberry samples was higher than from strawberry samples; range 5–100% (median 83.5% and mean 93.4%) versus 1–100% (median 27.0% and mean 32.3%). Ninety-three percent of the leafy greens tested (n = 140/150) resulted in a valid virus extraction efficiency. Of these valid tests the virus extraction efficiency ranged from 1 to 100% (median 10.9% and mean 22.5%) and

Table 2
E. coli and Norovirus detections and quantification.

Produce	*E. coli* (CFU/g)	NoV (genome copies/25 g)	Product type	Retail setting	Metropolitan area
Berries	23	ND	Strawberry	GFM	Melbourne
Loose greens	3.6	ND	Mixed lettuce	Supermarket 1	Melbourne
	14	ND	Gourmet lettuce mix	Supermarket 2	Melbourne
	ND	GI[a] (54) GII[a] (1080)	Baby spinach	Supermarket 2	Melbourne
	1100	ND	Mixed lettuce	GFM	Melbourne
	43	ND	Baby spinach	GFM	Melbourne
	3.6	ND	Mixed lettuce	GFM	Melbourne
	3.6	ND	Rocket mix	GFM	Melbourne
	240	ND	Mixed lettuce	GFM	Melbourne
	23	ND	Baby spinach	GFM	Melbourne
	3.6	ND	Baby spinach	GFM	Melbourne
	ND	GI[a] (36)	Rocket	GFM	Melbourne
	ND	GI[b]	Mixed lettuce	GFM	Melbourne
	3	ND	Mixed salad	GFM	Adelaide
Packed greens	3.6	ND	Leafy mix	Supermarket 1	Melbourne
	15	ND	Rocket salad mix	Supermarket 1	Melbourne
	3.6	ND	Rocket salad mix	Supermarket 1	Melbourne
	7.2	ND	Baby spinach & rocket	Supermarket 2	Melbourne
	3	ND	4 Leaf blend	Supermarket 2	Melbourne
	9.2	ND	4 Leaf blend	Supermarket 2	Melbourne

ND = not detected.
[a] Detected in both of the test duplicates.
[b] Detected in one of the test duplicates at low levels (Ct = 41), hence not quantified.

Table 4
Inputs used in the assessment of risk associated with foodborne virus contamination of fresh Australian berries and greens at retail.

Risk criteria	HAV		NoV	
	Berries	Leafy greens	Berries	Leafy greens
Dose and severity				
Hazard severity	Mild – sometimes requires medical attention		Minor – patients rarely seek medical attention	
Susceptibility	General – all members of the population			
Probability of exposure				
Frequency of consumption[a]	Daily			
Proportion consuming[a]	6.1%	16.5%	6.1%	16.5%
Size of population[b]	23,490,700			
Probability of infective dose				
Probability of contamination[c]	< 2% (range 0–2.6%)		< 2% (range 0–2.7%)	2.2% (range 0.5–6.3%)
Effect of processing[d]	No effect			
Possibility of recontamination[d]	No			
Post process control[d]	Not relevant			
Increase to infective dose[e]	None			
Effect of preparation before eating[f]	No effect			
Probability of illness per day per consumer of interest	$0–2.60 \times 10^{-2}$	$0–2.60 \times 10^{-2}$	$0–2.70 \times 10^{-2}$	$5.00 \times 10^{-3}–6.30 \times 10^{-2}$
Total predicted illnesses/annum in population of interest	$0–1.36 \times 10^{7}$	$0–3.68 \times 10^{7}$	$0–1.41 \times 10^{7}$	$7.07 \times 10^{6}–8.91 \times 10^{7}$
Risk ranking (0 − 100)	0–67	0–69	0–61	60–66

[a] From the ABS (2012) data, 6.1% of the Australian population reported consumption of berry fruit and 16.5% reported consumption of leaf vegetables in a 24 hour recall period (ABS, 2014b).
[b] Australian population size as of 30 June 2014 (ABS, 2014a).
[c] Probability of contamination with NoV for berries and HAV for berries and leafy greens is likely to be an over-estimate, but is based on the fact that the viruses were not detected in these food products at retail during the survey resulting in a prevalence estimate of < 2%. NoV was detected in leafy greens at retail with a prevalence of 2.2% (95% CI 0.5–6.3%). A range cannot be entered into Risk Ranger, therefore, the upper and lower CI limits were used from the respective prevalence estimates to generate an upper and lower risk estimate.
[d] No effect of processing, no possibility of recontamination and non-relevance of post process controls were selected as sampling and prevalence estimates were determined at retail and captured recontamination by handlers or consumers in the case of leafy greens.
[e] Increase to infective dose is only relevant for organisms which can multiple in the food. Viruses do not grow in food matrices.
[5] It was assumed that leafy greens and fresh berries would be eaten raw, hence no deactivation of viable virus.

was lower than for the berries.

HAV was not detected in any of the greens or berries sampled, indicating a prevalence estimate of < 2%. NoV GI or GII was not detected in any of the berry samples, also giving a prevalence estimate of < 2%. However, NoV GI was detected (≤54 copies/25 g) in three loose leafy green (baby spinach, rocket and mixed lettuce) samples (Table 2) with an estimated prevalence of 2.2% (95% CI of 0.5–6.3%) (Table 3). Two of the loose leafy green positive NoV GI samples were collected from the same retail outlet on the same day, although the product types differed. NoV GII was detected (1080 copies/25 g) in one loose leafy green (baby spinach) sample which had also tested positive for NoV GI giving an estimated prevalence of NoV GII in greens of 0.8% (95% CI of 0.02–4.3%). Of the leafy green samples which tested positive for NoV, the virus extraction efficiency varied; 5.4% (baby spinach), 17.3% (mixed lettuce) and 100% (rocket). *E. coli* was not detected in any of these samples.

3.2. Risk estimate for foodborne viruses in fresh Australian produce at retail

Inputs and assumptions used in Risk Ranger in the evaluation of the risk posed by NoV and HAV in fresh Australian produce are shown in Table 4, along with the resulting estimates for probability of illness and predicted illnesses per annum in Australia. Risk Ranger ranked the risk of NoV in berries as risk ranking (RR) = 0–61, lower than that of NoV in leafy greens where RR = 60–66. The risk of HAV in berries and leafy greens was equivalent; RR = 0–67 and RR = 0–69, respectively.

The impact of assumptions made, on the final risk estimate can be both positive (uncertainty with potential to cause over-estimation of exposure/risk) or negative (uncertainty with potential to cause under-estimation of exposure/risk). Variation in the probability of infective dose estimate had the greatest potential to impact on the risk ranking as demonstrated by the range in risk estimates obtained when the upper and lower confidence intervals obtained from the NoV and HAV virus prevalence estimates were input into the Risk Ranger tool (Table 4). For example, a foodborne virus prevalence of 0% would result in RR = 0, whilst a foodborne virus prevalence of 2.6–6.3% would result in RR = 61–69; an increase of 10 to 11 orders of magnitude. Probability of exposure (proportion consuming) also had an effect on the risk ranking estimate, although much smaller in magnitude. A 10% increase in consumption would result in a RR = 69 for HAV in berries, an increase of less than one order of magnitude. Furthermore, potential for recontamination after processing also had an impact on the risk ranking estimate. We made the assumption that no recontamination post processing occurred, as the survey was done at retail where recontamination may have already occurred, particularly in the case of loose leafy greens. However, if recontamination was assumed to occur at 1% and 5% for NoV in leafy greens, then the risk ranking estimate would only change from RR = 60 (0.5% foodborne viral prevalence) to RR = 61 and RR = 65, respectively. Alternatively, if a prevalence estimate of 6.3% was assumed for NoV in leafy greens, then a potential recontamination of 5% post processing did not alter the risk ranking estimate.

4. Discussion

A survey of NoV and HAV in fresh Australian produce (leafy greens and berries) during 2013–2014 has provided prevalence data for these foodborne viruses within the Australian context. This data has enabled the development of a risk estimate for these pathogen-food pairings to determine public health impact and identify significant knowledge gaps. The survey results indicate that the estimated prevalence of foodborne viruses in fresh leafy greens and berries at retail was low. During the survey period no HAV was detected in any product tested and NoV was only detected in loose leafy greens, with potential contamination post production. These survey results were consistent with available epidemiological data during the survey period; with no HAV outbreaks reported and of the twelve NoV outbreaks reported in Australia only two were potentially associated with consumption of salad (OzFoodNet, 2015b, 2015c, 2015d, 2016). In the case of possible NoV illness linked to leafy greens, these were identified in commercial settings (i.e. restaurants), where contaminated food handlers may also have been the cause. Despite a low prevalence of NoV, loose leafy greens may pose a higher risk of NoV related foodborne illness.

Although, no definitive cases of HAV or NoV have been associated with consumption of fresh berries in Australia (OzFoodNet, 2015a), there have been several implicated HAV foodborne cases associated with frozen imported berries in Australia between 2009 and 2017 (OzFoodNet, 2015a, 2017). Frozen berries/fruit have also been implicated in several large international outbreaks of NoV and HAV (Bernard et al., 2014; EFSA, 2014c; Tavoschi et al., 2015). Frozen imported berries have not been considered in this risk profile, as it was beyond the scope of the study. From recent industry information it can be estimated that approximately 26%, 30% and 85% of strawberries, blueberries and raspberries consumed annually per capita are processed product (including frozen berries), which are largely imported (DPIPWE, 2014a, 2014b, 2014c). Frozen berries have a long shelf life, are distributed globally and are not required to undergo viral testing. In Australia they are required to undergo microbiological testing for *E. coli* as a hygiene indicator (DAWR, 2017). It should be noted that whilst detection of *E. coli* in produce implies a potential viral risk, a lack of *E. coli* does not imply a lack of viral risk, as confirmed in our current study. Although NoV and HAV do not replicate in the environment, they are more resilient than bacteria under certain conditions e.g. freezing. Investigations into the effect of freeze drying on the inactivation of enteric viruses on surfaces of berries and herbs have shown $< 2 \log_{10}$ reduction in infective HAV (Butot et al., 2009). NoV is resistant to refrigeration, freezing and low pH (FAO/WHO, 2003).

The prevalence of *E. coli* in fresh berries and leafy greens was also investigated, as it is commonly used as a hygiene indicator. *E. coli* was detected at < 10 CFU/g in 10 leafy green samples and at > 10 CFU/g in one strawberry and 6 leafy green samples. Only two samples of loose leafy greens taken from GFMs showed high levels of *E. coli* (> 240 CFU/g) indicating significant contamination with mammalian faeces at some point in the supply chain. Food safety accreditation systems set by the two major supermarket chains sampled during this survey specify that produce should comply with < 10 CFU/g for *E. coli* in either all fruit and vegetables or high risk product (deemed mushrooms, sprouts, berries and herbs) (FSANZ, 2011a).

The point of contamination of loose leafy greens with NoV was not determined in this study, but may have been associated with handling in the retail setting; either directly by staff or customers or through cross contamination of product via the use of contaminated serving utensils. None of the virus positive samples were positive for *E. coli*, suggesting it is a poor indicator for the risk of enteric virus contamination. No NoV or HAV was detected in packaged product at retail, which is indicative of good sanitary and process controls throughout the Australian berry and leafy green production and post-harvest chains. The most effective risk management strategy for NoV and HAV in ready-to-eat fresh produce is to prevent contamination, as no effective control measures are available to eliminate these viruses without changing the characteristics of the food. The Codex Alimentarius Commission released guidelines on general principles of food hygiene to control viruses in food in 2012 (FAO/WHO, 2012). Annex II of the guidelines covers recommendations for handling practices to minimise the likelihood of illness arising from the presence of NoV and HAV in fresh produce. The control measures emphasised relate to the main sources of contamination: sewage treatment plant effluent, the use of human excreta as fertiliser, agricultural workers, personnel hygiene and toilet and hand-washing facilities on primary production sites, and the use of clean water during processing. In Australia, these measures are covered by Good Hygienic Practices and Hazard Analysis and Critical Control Point procedures (FSANZ, 2011b). Berries are hand or mechanically picked and packed in the field or at a processing facility and are not washed prior to sale.

The Risk Ranger estimates for NoV (risk ranking = 0–61) and HAV (risk ranking = 0–67) in berries and HAV (risk ranking = 0–69) in leafy greens generated a broad range for risk. The breadth of the range was driven by uncertainty in the prevalence estimate for these viruses in fresh Australian produce. As we did not detect these viruses in these particular products during our survey we can only estimate the prevalence of HAV and NoV. To achieve more accurate prevalence estimates for these viruses in fresh produce, much larger sample sizes are required. The range in Risk Ranger estimate for NoV (RR = 60–66) in leafy greens was narrower, representing only one order of magnitude in difference. The survey was not designed to give separate prevalence estimates for packaged and loose leafy greens; double the samples per category would have been required to give individual prevalence estimates with the same confidence. Risk Ranger has been used to assess 10 seafood hazard/product combination (Sumner and Ross, 2002). Viruses in shellfish from uncontaminated waters with no documented cases of food-borne illness resulted in a RR = 31, whilst viruses in shellfish from contaminated waters and foodborne illness resulted in a RR = 67.

Risk Ranger gave estimates of predicted illnesses from NoV and HAV in fresh Australian berries and leafy greens which were several orders of magnitude greater than what is estimated for all food attributed illness based on epidemiological data. 2010 Australian data has estimated that 18% of NoV illness is acquired via contaminated food accounting for 276,000 cases per annum (90% Crl: 78,100-563,000) and 12% of HAV illness is acquired via contaminated food accounting for 40 cases per annum (90% Crl: 10–100) (Kirk et al., 2014). Furthermore, between 2000 and 2013 no foodborne HAV outbreaks have been attributed to consumption of fresh Australian berries or leafy greens (OzFoodNet, 2015a). Possible NoV cases implicating leafy greens as the source have only been reported from commercial settings, where food handlers may have been the cause of contamination. Sumner and Ross (2002) found that a risk ranking of < 32 was associated with no documented cases of foodborne illness whilst a risk ranking > 48 was in the order of, or higher than, those estimated for total foodborne illness/annum. Although, Risk Ranger is not definitive and the model underpinning the tool is a simplification, it does offer a rapid and simple way of comparing foodborne risks and provides a means of ranking and prioritising risks. The relative risk rankings derived from Risk Ranger reflect the higher severity of illness due to HAV compared to NoV and the higher prevalence of NoV in leafy greens compared to berries.

Assumptions used in defining risk criteria within Risk Ranger impact on the risk ranking value attributed to that hazard/commodity pairing. Although some inputs were based on known information, such as severity of the hazards, others were based on informed assumptions as no precise data was available. Knowledge gaps identified in the development of the risk profile were frequency of consumption of a particular food and the proportion of the population consuming that food. Detailed Australian consumption data, broken down to various types of leafy greens and uncooked berries were not available. Information on frequency of consumption and portion size per serving was also lacking (ABS, 2014b). This study only considered fresh Australian produce, yet information on the breakdown of berries consumed as fresh or frozen raw product within Australia was lacking (ABS, 2014b). Furthermore, different berry and lettuce types may have different risks associated with them due to different production practices or persistence/affinity of various enteric viruses to particular products (Butot et al., 2009).

The probability of an infective dose and more precise data around the foodborne virus prevalence estimates were also significant knowledge gaps. Compounding this were the limitations of the testing methodology, which determines total viral genome copies present but does not differentiate between infective and non-infective virus particles. Furthermore, human susceptibility to NoV infections is determined by the individual's ABH and Lewis histoblood group antigens; hence most NoV strains only ever infect a subset of the population (Le Pendu et al., 2006; Maalouf et al., 2010). As viruses do not grow in food products it was assumed that there was no change in the probability of an infective dose. If the virus was detected we assumed that it would be infective which is an over simplification. The median infectious dose (ID_{50}) of NoV is low and has been estimated to be between 18 and 1015 genome copies (Teunis et al., 2008). More recently, the ID_{50} has been reported to be higher (1320 to 2800 genome equivalent copies) than previously estimated and similar to that of other RNA viruses (Atmar et al., 2014). In Human NoV challenge experiments, susceptible subjects demonstrated a dose-dependent probability of becoming ill, ranging from 0.1 (at a dose of 10^3 genomes) to 0.7 (at 10^8 virus genomes) (Teunis et al., 2008). As the number of genome copies detected by qRT-PCR are not directly related to infectious virus particles, these methods can only be used to provide an indirect measure of risk and infection risk associated with low level contaminated foods as determined by qRT-PCR may be overestimated (EFSA, 2011).

The establishment of the ISO/TS 15216 method for detection of HAV and NoV in foods has enabled benchmarking and comparison of test results among laboratories and studies. However, the revised 2017 method still lacks clear direction on sub-sampling and reporting of LOD and LOQ. Between 2009 and 2016 a total of 2015 berry samples were analysed for HAV, NoV GI and GII by 11 service laboratories using the ISO/TS 15216 method (Li et al., 2018). Of the 11 service laboratories six did not report an LOD and the others were inconsistent in what and how they reported the LOD or delineating whether the LOD reported was in a specific food matrix or the PCR assay alone. This emphasizes the need to agree on and include in the ISO standard method a more uniform expression of the LOD that can be used by diagnostic laboratories. The study by Li et al. (2018) demonstrated that monitoring programs were a useful means of providing baseline data for more comprehensive risk assessments related to foodborne viruses in berries but not useful for end product control. Our study determined the tLOD but not LOD in the individual food matrices investigated. Lowther et al. (2017) determined the LOD of NoV GI, NoV GII and HAV using the ISO 15216-1 method in raspberries (16.3, 19.8 and 99.3 copies/25 g, respectively) and lettuce (11.5, 22.0 and 79.5 copies/25 g, respectively) (Lowther et al., 2017).

Of the assumptions used in defining risk criteria in Risk Ranger, the uncertainty associated with the confidence interval of the prevalence estimate had the largest impact on the risk ranger estimate and further research in improving this estimate for particular berries (strawberries, blueberries and raspberries) and leafy greens (packaged versus loose) would be of value. Knowledge gaps identified in the development of the risk profile were frequency of consumption of a particular food, the proportion of the population consuming that food and probability of an infective dose.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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