Monitoring of selected parasites and other foodborne microorganisms in vegetable products at point of sale

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Abstract

It is supposed that epidemiological data linked to cases of alimentary infection from fresh produce are scarce and usually underestimated. The present study reveals data concerning the occurrence of less studied foodborne pathogens such as parasites (*Giardia intestinalis* , *Cryptosporidium parvum/hominis*) and viruses (norovirus, hepatitis A virus) in 175 samples of fresh-cut vegetables, frozen vegetables and sprouts. In addition, samples were also analyzed for the presence of bacterial indicators of production hygiene such as *Escherichia coli* and *Staphylococcus aureus* . Our results revealed that DNA of *G. intestinalis* was found in two samples (1.1%) of mixed fresh vegetable salads and mixed frozen vegetables at a level of 100 CFU/g. Norovirus was detected in two samples (1.1%) of rucola and frozen mixed vegetables. No sample was positive for *C. parvum/hominis* or hepatitis A virus. Our results highlight the need for microbial analysis of food of non-animal origin for specific parasitic and viral agents not generally monitored in these food category types, but their presence poses risks for alimentary infection.

Background

According to epidemiological statistics it has been demonstrated that outbreaks of foodborne infections are mostly associated with foods of animal origin. However, a higher number of announced outbreaks and cases associated with food of non-animal origin has been documented recently [1]. Generally, the most commonly associated pathogens with fresh and minimally processed produce are norovirus (NoV), *Salmonella* spp. and pathogenic *Escherichia coli*. Regardless, other foodborne pathogens linked with fresh produce may be recorded. Except for other well known bacterial agents (*Shigella* sp., *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Staphylococcus aureus* and *Listeria monocytogenes*), viral agents such as hepatitis A virus (HAV) and protozoan agents such as *Cryptosporidium* spp., *Giardia intestinalis* (syn. *G. lamblia* or *G. duodenalis*), and *Cyclospora* sp. have been highlighted in recent years [1, 2, 3]. Nevertheless, it is supposed that epidemiological data linked to cases of alimentary infections originating from fresh produce are scarce and usually underestimated. For example, underestimation of parasitical infections is caused by the fact that the diseases manifest after long incubation period (average of 7 days) and shelf life of fresh vegetable products is too short to confirm the parasitic agents in contaminated products. In comparison, infection caused by viral agents such as NoV have short incubation time and last just a few days (e.g. 24–48 hours for NoV) and people usually heal without seeing a physician. Additionally, physicians do not carry out any identification of the pathogen in majority of cases of intestinal infection. Furthermore, notification to public health authorities is not compulsory for most parasitic and viral diseases, and therefore official reports do not reflect the true prevalence or incidence of disease occurrences [2].

In our survey, we focused on minimally processed vegetables such as cut vegetables, ready to eat vegetable salads and sprouted seeds that pose a higher risk of foodborne infection as they are consumed without heat treatment. Further, traditional washing with authorized disinfection methods cannot properly eliminate foodborne pathogens from these matrices. A failure in decontamination may be attributed to biofilm formation and incorporation of pathogens on the inner side of plant tissue or higher resistance of some strains to disinfection.

Although information on the prevalence, survival and multiplication of bacterial pathogens on fresh produce is common [4, 5, 6], there is still insufficient information on the prevalence of foodborne parasites and viruses.

Generally, it is supposed that parasitic diseases are the main issue in developing countries, but due to the cosmopolitan markets with food, changes in climate and agriculture, the increasing number of immunocompromised humans, they may pose a threat to human health in high-income countries as well. In the present work we focused on medically important protozoa *Cryptosporidium* sp. and *G. intestinalis*. Both agents are considered the most important diarrheal pathogens affecting people worldwide. The most commonly affected patients are young children and immunocompromised patients who experience clinical disease more frequently and with higher severity. According to the Striepen [7], cryptosporidiosis is the second most common diarrheal infection in infants under two years in developing countries. In industrialized countries, reported cases of cryptosporidiosis are not so prevalent, however the number of diagnosed cases are increasing. In the EU, 10 915 cases of cryptosporidiosis were confirmed in 2015, which was 41% more than reported in 2014 [8]. In the same year, giardiasis was confirmed in 18 031 cases with an increase of 4.4% from 2014 [9]. Both infections are transmitted *via* the fecal-oral route and result from ingestion of cysts/oocysts through the consumption of contaminated food or water or through direct contact with infected humans. Genotyping and subtyping data suggest that zoonotic transmission is more prevalent in the epidemiology of cryptosporidiosis than of giardiasis.

Norovirus is the most common worldwide cause of non-bacterial gastroenteritis. This virus is highly infectious with an incubation period of one to three days and an infectious dose requires only a few copies of viral particles. In comparison, a long incubation period lasting three to six weeks is observed in HAV infection, which complicates the tracing of contaminated food. Generally, viruses transmitted via the fecal-oral route are highly resistant to cooling, freezing, a wide range of pH and various disinfectants that are effective for bacterial agents. The World Health Organization identified both NoV and HAV in fresh produce including vegetables as a priority virus/commodity combination for which control measures should be considered [10].

With regard to statistics of the most common foodborne infections and to the limited information about the presence of parasitic and viral foodborne pathogens in fresh produce, the present study focused on the determination of parasitic agents *G. intestinalis* and *Cryptosporidium parvum/hominis* and viral agents NoV and HAV in processed vegetables (fresh-cut vegetables, vegetable salads and frozen vegetables) and sprout seeds manufactured in different countries in the European Union and purchased from supermarkets in the Czech. To picture a complex characterization of the fresh produce indicators of fecal contaminations and production, hygiene quality from bacterial strains such as *E. coli* and *S. aureus* were assessed by conventional standard culture methods.

Results

The results obtained from the examination of fresh pre-cut produce, frozen vegetables and sprout seeds are summarised in Table 1. Parasites and viruses were each detected in 1.14% of studied samples, separately. *G. intestinalis* was recovered from one sample of fresh leafy green mixed salads and from one sample of frozen vegetables. No sample was positive for *C. parvum/hominis*. Out of studied viruses, only NoV was detected in one sample of rucola and one sample of mixed frozen vegetables. Both NoVs were classified as NoV GI.

S. aureus was isolated from 1.7% of samples (one fresh mixed salad and two frozen peas) at a level less than 50 CFU/g. Based on PCR analysis, genes for production of classical enterotoxins (*sea-see*) were not confirmed in these isolates. *E. coli* was isolated from 43.4% of samples, when sprouts seed were the most commonly contaminated category with the highest level of contamination. Fifty-one percent of sprout seeds samples exceeded the level of 10^3 CFU/g and 20.0% of samples exceeded the level of 10^5 CFU/g. In comparison, the

contamination level of fresh pre-cut produce by *E. coli* was only 3.1% with the maximum level of *E. coli* 3×10^3 CFU/g. Surprisingly, *E. coli* was not present in both NoV positive samples and both *G. intestinalis* positive samples contained *E. coli* but only at a level of less than 10^2 CFU/g.

Discussion

Out of 175 examined samples, four samples (2.2%) were positive for parasitic or viral pathogens. Although parasites such as *Cryptosporidium* spp. and *G. intestinalis* may be present in produce and can be identified as causes of foodborne diseases [11, 2], there are only a few reports concerning their prevalence on produce. Most studies are from developing countries where poor hygiene is prevalent [12, 13, 14] and from north European countries where outbreaks connected with parasites were described in recent years [11, 15, 16].

In the present study, the occurrence of parasites in fresh-cut vegetables, frozen vegetables and sprouts was low (1.1%). *G. intestinalis* was detected in two samples (mixed fresh vegetable salads and mixed frozen vegetables) at a level of 100 CFU/g. No sample was positive for *C. parvum/hominis*. Dixon et al. [17] revealed a similar rate of contamination of *G. intestinalis* (1.8%) in packed pre-cut salads and leafy greens retailed in Canada. On the other hand, the occurrence of *Cryptosporidium* spp. in Canada was higher, and reached 5.9%. In comparison in Egypt, *Gardia* spp. was found in 8.8% and 8.2% of vegetables by Eraky et al. [13] and Hassan et al. [18], respectively. Duedu et al. [12] revealed 6.0% of *G. intestinalis* and 17.0% of *Cryptosporidium* spp. in fresh vegetables sold in Ghana. The waterborne route and manure application to vegetable fields is the most important means of transmission of these parasites [16]. In some cases, transmission of these pathogens to vegetables were attributed to infected food-handlers [19].

NoV (NoV GI) was demonstrated in both raw minimally processed (rucola) and frozen vegetables (frozen mixed vegetables) in 1.1% of studied samples. This relatively low rate of NoV contamination of fresh and frozen vegetables is in general agreement with data reported in previous European studies in which NoV was detected in less than 0.1% (1/1372) in fresh leafy samples and in no samples of 1 160 tested ready to eat vegetables [20]. Despite the generally low rate of NoV contamination of food, an increase of foodborne outbreaks caused by NoV has been noted and many outbreaks were traced to food that was handled by infected workers during food preparation. The impact of vegetable contaminated by NoV has been outlined e.g. by annual EFSA reports [21,22].

S. aureus is part of the natural microflora of humans and therefore the most common way for produce contamination is through direct contact with handlers. Bacteriological examination of food handlers carried out by Saeed and Hamid [19] revealed *S. aureus* as the most abundant pathogen. Illness results from an enterotoxin, which is produced by some strains on the food. The true incidence of staphylococcal food poisoning is unknown for a number of reasons, including the short period of illness usually not requiring seeing a physician. In the present study, *S. aureus* was isolated from 1.7% of samples (one mixed salad and two frozen peas) at a low level less than 50 CFU/g. Genes for production of classical enterotoxins (*sea-see*) were not confirmed. This result shows a lower prevalence of *S. aureus* than revealed in Thailand and China [23,24]. Ananchaipattana et al. [23] revealed *S. aureus* in 67% of leafy vegetable samples from open markets and 25% from supermarkets in Thailand. Xing et al. [24] detected *S. aureus* in 27.6% salad vegetable samples. On the other hand, Cardamone et al. [5] did not isolate any *S. aureus* in fresh produce from Italian supermarkets and greengrocer's shops.

E. coli is frequently used as an indicator of fecal contamination and thus has been included in European Union regulations 2073/2005 for fresh pre-cut ready to eat vegetables as an indicator of microbial processing quality

[25]. Nevertheless, these microbial regulations do not assign maximum levels for *E. coli* in fresh produce during the expiration date (sampled at point of sale). In the present study, 43.4% of samples were *E. coli* positive, 14.3% of samples exceeded the level of 10^2 CFU/g and 12.0% of samples exceeded the level of 10^3 CFU/g (Table 1). As expected, the sprouts were the most commonly contaminated samples with the highest level of contamination. Fifty-one percent of sprout samples exceeded the level of 10^3 CFU/g and (20.0%) of samples exceeded the level of 10^5 CFU/g. The high number of *E. coli* positive sprouts samples with high counts of these bacteria suggests insufficient decontamination procedures of seeds before sprouting. Due to sprout manufacturing providing excellent conditions for bacterial growth, any pathogen contamination at the beginning may produce high counts in the final product and pose a health risk to humans [1, 26]. In cut green leaves and salads the counts of *E. coli* were low, only in 3 (3.0%) samples the counts exceeded the level of 10^3 CFU/g.

The *E. coli* indicator is used to assess possible contamination by enteric pathogens. However, according to our results and results of others [27], the occurrence of enteric pathogens do not often correlate with *E. coli* presence. Both NoV positive samples were negative for the presence of *E. coli* and both *G. intestinalis* positive samples harbored *E. coli* but only at a level of less than 10^2 CFU/g.

The economic implication of outbreaks is very serious and may cause the closure of individual companies and long lasting damage to particular sectors of the food industry. An *E. coli* outbreak in Germany caused US\$ 1.3 billion in losses for farmers and industries and US\$ 236 million in emergency aid payments to 22 European Union Member States [28]. Therefore, surveillance and preventive steps to avoid microbiological contamination of fresh produce are crucial in food safety. Our study contributes particularly to the gap of knowledge in the occurrence of parasitic and viral agents on produce originating from various countries of the European Union sold in the Czech Republic. In addition, we also examined bacterial indicators of microbial processing quality *E. coli* and *S. aureus*.

Conclusion

Our results revealed that parasitic and viral pathogens such as *G. lamblia* and NoV were present in processed vegetables and may constitute potential health risks in these food categories. Noticeably, detection of high levels of the indicator microorganism *E. coli* was seen in sprouted seeds. These findings indicate that there is a need for better prevention strategies and knowledge about foodborne parasitic and viral pathogens.

Methods

Food samples

A total of 175 samples from 32 manufacturers were collected over a period of one year from different supermarkets in the Czech Republic. Samples included minimally processed vegetables (97 samples) such as vegetable salads and cut green leaves (e.g. rucola, spinach, Lamb's lettuce), frozen vegetables (43 samples; e.g. peas, carrot, maize and mixed, packed or not packed) and sprouted seeds (35 samples). Seventy-six samples originated from the Czech Republic, 77 samples originated from other countries in the European Union, specifically Austria, Belgium, France, Italy, Germany, Great Britain, Netherlands, Poland and Slovak Republic, six samples from Israel and in 16 samples no country of origin was recorded. All samples were transported to the laboratory in a chilled state, stored in a refrigerator (frozen vegetables in a freezer) and processed within 24 hours.

Preparation of samples for parasitological and virological analyses

One hundred grams of each sample were placed in a stomacher bag and artificially contaminated with 5 μL of process control virus (PCV, 106 particles/ μL) to verify analysis for the presence of viral pathogens [29]. Subsequently, 230 mL of Trisma base-glycine beef extract (TGBE washing buffer, pH 9.5, in house) was added and homogenized for 2 min in order to remove microorganisms from vegetable surfaces. The liquid was decanted into tubes and centrifuged for 20 min at $8\,000 \times g$ at 4°C . The sediment (pellet) was used for DNA isolation and subsequent detection of parasitic agents by real-time PCR (qPCR) and the supernatant was used for further analyses focusing on the presence of foodborne viruses.

Isolation of parasitic DNA and detection of *G. intestinalis* and *C. parvum/hominis* by qPCR

The pellet was immediately resuspended with 10 mL of BPW; two ml of the resuspended pellet was centrifuged at $14\,000 \times g$ for five min. DNA was isolated from the prepared pellet using the PowerSoil DNA isolation kit (MoBio Laboratories, U.S.A) following the manufacturer's instructions. The protocol was slightly modified to include mechanical homogenization with zirconia/silica beads (0.1 mm) in a MagNA Lyser instrument (Roche, Mannheim, Germany) at 6 400 rpm for 60 s and each sample was supplemented with Carrier DNA solution (salmon sperm DNA, 50 ng/ μL ; Serva, Heidelberg, Germany). Isolated DNA was stored at -80°C or directly applied for qPCR. The detection of *G. intestinalis* and *C. parvum/hominis* via species specific loci (β - giardin and hsp70) was adopted from Helmi et al. [30] and performed on the LightCycler 480 Instrument (Roche Molecular Diagnostics, Germany). The quantity of protozoal oocyst was subsequently recalculated according to the plasmid gradient and according to the parameters of DNA isolation to grams of the initial sample amount.

Analyses of samples for presence of norovirus and hepatitis A virus by RT-qPCR

The samples analyzed for presence of viral agents was performed according to ISO (ISO/TS 15216–2) technical specifications [31] with small modifications. Briefly, 200 ml of the aforementioned supernatant were transferred into clean tubes and pH was adjusted to 7.2. Subsequently, 50 mL (0.25 volumes) of 50% (w/v) polyethylene glycol 8000/1.5 M NaCl (both Sigma-Aldrich) were added. The suspension was homogenized by shaking and incubated with gentle rocking at 4°C for 60 min. Centrifugation at $10\,000 \times g$ for 30 min at 4°C followed. The supernatant was discarded. A further centrifugation at $10\,000 \times g$ for 5 min at 4°C was performed and the pellet resuspended in 1 ml of phosphate buffered saline (PBS), transferred into a clean tube and 1 mL of chloroform: butanol (1:1) was added. The mixture was vortexed, incubated at room temperature for 5 min and centrifuged at $10\,000 \times g$ for 20 min at 4°C . The aqueous phase was transferred into a clean tube and retained for nucleic acid isolation, which was done by the NucliSENS Magnetic Extraction Reagents (BioMerieux, Durham, NC, U.S.A) according to the manufacturer's instructions with a final repeated elution resulting in 100 μL of nucleic acid solution. The isolated nucleic acids were assayed immediately.

The presence of NoV (NoV GI and NoV GII) and HAV genomes was determined by one step duplex RT-qPCR as described by ISO/TS 15216–2 [31], however an internal amplification control (IAC) was included according to Vasickova et al. [32]. Detection was performed on the LightCycler 480 Instrument (Roche Molecular Diagnostics), the subsequent analysis of results was carried out using the “Fitpoint analysis” option of the LightCycler 480 Software release 1.5.0 (version 1.5.0.39). Quantification of PCV as well as calculation of processing efficiency (estimate of PCV recovery) was done as defined by Mikel et al. [29]. The samples where processing efficiency was lower than 1% were not considered as valid results and not included in further analyses.

Detection and enumeration of *S. aureus*

After pre-enrichment of 25 grams of sample in buffered peptone water (BPW), *S. aureus* was detected on Baird-Parker medium (BioRad, France) and incubated at 37°C for 24 h. For enumeration of *S. aureus*, 200 µL of homogenized sample was streaked on Baird-Parker medium (BioRad, France) in triplicate and incubated at 37°C for 24 h. Identification of *S. aureus* was confirmed using MALDI-TOF MS and the detection of genes for the production of classical enterotoxins (*sea-see*) was performed using PCR [33].

Detection and enumeration of generic *E. coli*

Generic *E. coli* was used as an indicator of fecal contamination. Detection of *E. coli* was performed after enrichment in BPW at 37°C overnight, followed by culture on TBX (triptone bile glucuronic, BioRad, France) agars simultaneously at 44°C and 37°C. For enumeration, samples were decimally diluted and streaked in triplicate on TBX agar plates and incubated at 44°C and 37°C for 24 h. Characteristic colonies were subcultured on MacConkey medium (BioRad, France). *E. coli* was confirmed by detection of the *uidA* gene by qPCR [34].

Abbreviations

G. intestinalis: *Giardia intestinalis*; NoV: norovirus; spp.: species; HAV: hepatitis A virus; EU: European Union; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; CFU/g: colony forming units per gram; PCR: polymerase chain reaction; EFSA: European Food Safety Authority; e.g.: for example; PCV: process control virus; µL: mikrolitr, mL: mililitr; TGBE: Trisma base-glycine beef extract; DNA: deoxyribonucleic acid; min: minutes; g: gram; qPCR: Quantitative PCR (real-time PCR); s: seconads; RT-qPCR: Quantitative reverse transcription PCR; ISO: International Organization for Standardization; PBS: phosphate buffered saline; BPW:buffered peptone water; TBX: triptone bile glucuronic; MALDI-TOF MS: Matrix-Assisted Laser Desorption - Ionisation-Time of Flight Mass Spectrometry

Declarations

Ethics approval and consent to participate

Not applicable. Samples and bacterial isolates used in the present study do not require ethical approval or owner consent.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interest

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Author contributions

M. Moravkova contributed to the design of the study, execution and interpretation of experiments, and to drafting the manuscript; PV and PK contributed to design of the study, data interpretation and writing the manuscript; PM contributed to carrying out the experiments; MS contributed to execution and interpreting results, and writing the manuscript, IK and RK contributed to execution and interpreting the results. All authors read and approved the final manuscript.

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Table

Table 1 Prevalence of the studied parasitic, viral and bacterial agents in minimally processed and refrigerated produce, and sprout seeds

Produce type	Total No. of samples	Parasites		Viruses		Bacteria			
		<i>C. parv/homin</i>	<i>G. intest</i>	HAV	NoV	<i>S. aureus</i>	<i>E. coli</i>	<i>E. coli</i> (≥ 100 CFU/g)	<i>E. coli</i> (≥ 1000 CFU/g)
Fresh pre-cut produce ^a	97	0 (0,0%)	1 (1,0%) ^b	0 (0,0%)	1 (1,1%) ^c	1 (1,0%) ^b	33 (34,0%)	6 (6,2%) ^c	3 (3,1%) ^c
Frozen vegetables ^d	43	0 (0,0%)	1 (2,3%) ^e	0 (0,0%)	1 (2,3%) ^e	2 (4,7%) ^f	17 (39,5%)	0 (0,0%)	0 (0,0%)
Sprout seeds	35	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	26 (74,3%)	19 (54,3%)	18 (51,4%)
Total	175	0 (0,0%)	2 (1,1%)	0 (0,0%)	2 (1,1%)	3 (1,7%)	76 (43,4%)	25 (14,3%)	21 (12,0%)

HAV - Hepatitis A virus; **NoV** – norovirus (NoV GI); ***C. parv/homin*** - *Cryptosporidium parvum* and *C. hominis*, primers and probe used in qPCR analysis allowed for the detection of *Cryptosporidium parvum* and *C. hominis*, previously known as the two genotypes (bovine and human) of *Cryptosporidium parvum*; ***G. intest*** – *Giardia intestinalis*; ***S. aureus*** – *Staphylococcus aureus*

^a Ready to eat product such as fresh leafy green salads, spinach, rucola, fresh leafy green mixed salads and lamb's lettuce

^b Fresh leafy green mixed salads

^c Fresh leafy green salads, spinach, rucola and lamb's lettuce

^d Mixed frozen vegetables, frozen peas, frozen maize, frozen spinach, beans with pods

^e Mixed frozen vegetables

^f Frozen peas