

Separate norovirus outbreaks linked to one source of imported frozen raspberries by molecular analysis, Denmark, 2010–2011

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SUMMARY

Norovirus outbreaks occur frequently in Denmark and it can be difficult to establish whether apparently independent outbreaks have the same origin. Here we report on six outbreaks linked to frozen raspberries, investigated separately over a period of 3 months. Norovirus from stools were sequence-typed; including extended sequencing of 1138 bp encompassing the hypervariable P2 region of the capsid gene. Norovirus was detected in 27 stool samples. Genotyping showed genotype GI.Pb_GI.6 (polymerase/capsid) with 100% identical sequences. Samples from five outbreaks were furthermore identical over the variable capsid P2 region. In one outbreak at a hospital canteen, frozen raspberries was associated with illness by cohort investigation (relative risk 6·1, 95% confidence interval 3·2–11). Bags of raspberries suspected to be the source were positive for genogroup I and II noroviruses, one typable virus was genotype GI.6 (capsid). These molecular investigations showed that the apparently independent outbreaks were the result of one contamination event of frozen raspberries. The contaminated raspberries originated from a single producer in Serbia and were originally not considered to belong to the same batch. The outbreaks led to consultations and mutual visits between producers, investigators and authorities. Further, Danish legislation was changed to make heat-treatment of frozen raspberries compulsory in professional catering establishments.

Key words: Foodborne infections, gastrointestinal infections, molecular epidemiology, Norwalk agent and related viruses, outbreaks.

INTRODUCTION

Norovirus is a highly infectious non-enveloped RNA virus, which is a frequent cause of gastroenteritis.

It accounts for a large proportion of foodborne outbreaks in the European Union (EU) [1], including in Denmark, where norovirus for several years has been the major causative agent of registered foodborne outbreaks [2]. In Denmark, and indeed most Western countries, sporadic infections are rarely diagnosed and no laboratory surveillance for the disease exists. Thus, outbreaks are primarily discovered by

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the occurrence of an excess number of symptomatic persons, often in a closed setting (hotel, hospital, restaurant, etc.). Microbiological analysis of stools is generally only performed in situations where an outbreak is suspected.

In Denmark, the majority of foodborne norovirus outbreaks occur because food is contaminated when handled in catering kitchens by infected food handlers [3]. However, foods contaminated at the source of production may also cause outbreaks such as observed with oysters [4], lettuce [5] and frozen raspberries [6]. Frozen raspberries were recognized as the source of norovirus outbreaks for the first time in Denmark in 2005 when a batch of imported frozen raspberries from Poland caused more than 1000 cases in five separate outbreaks in institutions and customers of catering companies [6]. In recent years, frozen raspberries have been established as a well-known source of norovirus illness internationally [7–12]. In 2011, 525 calicivirus outbreaks were reported in the EU as a whole and where a food vehicle was found, 17% were caused by ‘fruit, berries and juices’ [1]. Far less frequently, other types of berries have also been found as sources of norovirus outbreaks; a somewhat spectacular example being a very large outbreak in German school pupils caused by imported frozen strawberries [13]. A recently published expert opinion from the European Food Safety Authority highlighted the subject of frozen berries in connection with the risk of norovirus infections [14].

Batches of contaminated frozen raspberries or other red berries are often large (tonnes), packed in smaller bags, widely distributed and typically have a shelf life of around 2 years. They therefore have the potential to cause repeated outbreaks over extended time periods and to cause a considerable disease burden. It is thus important that series of outbreaks caused by contaminated frozen raspberries are identified and linked in order to withdraw the incriminated batches from the market. This, however, is only possible if trace-back can be performed in a timely manner and when epidemiological investigations of norovirus outbreaks are fully conclusive. In situations where the former is not feasible, sequence analysis of viral genomic elements may provide the evidence needed to determine whether separate outbreaks have the same origin.

From October 2010 to January 2011, a series of norovirus outbreaks occurred in Denmark. Through epidemiological investigations and viral sequence analysis, these outbreaks were linked to consumption of frozen raspberries. Characterization of isolates

from outbreak cases indicated that they were identical and this was further corroborated by development of a method involving sequencing of a 1 kb genetically variable part of the viral genome. In this way seemingly distinct batches of frozen raspberries were shown in fact to be part of the same large batch. Here we describe these outbreaks, their investigation, present the molecular method that was developed and discuss the public health implications of the investigations.

METHODS

Microbiological investigations

Stool samples submitted to Statens Serum Institut (SSI) for virological analysis were analysed for norovirus, sapovirus, rotavirus, adenovirus and astrovirus by real-time polymerase chain reaction (PCR) as described elsewhere [15, 16]. Samples positive for norovirus were genotyped by reverse transcriptase (RT)–PCR and sequence analysis of partial capsid and/or polymerase genes [15, 16].

A PCR was developed to amplify a longer region (1138 bp) of the capsid gene, including the variable P2 region [17]. The specific primers used were GI.6 F (forward): AYKTVATTGCTGATGTTAGGAC and GI.6R (reverse): CACAGGCTTDARYTGATAAA ATC. RT–PCR was performed using the Qiagen One-Step RT–PCR kit (cat. no. 210212) and AmpliTaq[®] 360 DNA polymerase (Applied Biosystems, USA), respectively according to the manufacturer’s instructions. RT–PCR cycling conditions were: 50 °C for 30 min, 95 °C for 15 min and 40 amplification cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min and a final elongation step at 72 °C for 10 min. If a PCR product was not obtained a nested PCR was employed, which was performed with primers GI6Fnest (TCAAATTCTCGTGTCCTCC TGTGY) and GI6Rnest (GTTCTTRCAGAAGT GGGTRAT) with the following cycling conditions: 95 °C for 15 min and 45 amplification cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min and a final elongation step at 72 °C for 10 min. Purification of PCR products, sequencing and sequence analysis was performed as described elsewhere [16], except that sequencing was performed using either an ABI PRISM[®] 3100 Genetic Analyzer or an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) and sequence assembly was performed using BioNumerics v. 6.10 (Applied Maths, Belgium) which is integrated into the laboratory IT infrastructure at SSI.

Norovirus capsid and/or polymerase sequences, representative for each of the analysed outbreaks, were deposited in Noronet with outbreak numbers: Denmark: 2010–12, 2010–13, 2011–1, 2011–3, 2011–6; for outbreaks 1–5 respectively. One representative long capsid P2 sequence was deposited in GenBank with the accession number JN222366.1.

For detecting norovirus in raspberries, berries that originated from the implicated batches were collected at the outbreak setting and/or from the supplier and analysed. From each raspberry bag, viral RNA was extracted from 2–4 25-g portions of raspberries and tested by TaqMan realtime RT–PCR for the presence of GI and GII noroviruses. Recovery, extraction and detection of viral RNA was performed according to the newly developed Technical Specification, ISO TS 15216 [18], using minor modifications [19]. In short, virus was eluted from the berries during agitation with alkaline buffer, separated by low-speed centrifugation, precipitated from the resulting supernatant, first treated with pectinase, using polyethylene glycol and pelleted by low-speed centrifugation. The PBS suspended pellet was clarified by chloroform-butanol extraction followed by centrifugation and the resulting virus containing water phase was treated with Plant RNA Isolation Aid. Nucleic acid extraction was conducted using the NucliSENS[®] magnetic extraction reagents and miniMag platform (bioMérieux, Denmark) according to the manufacturer's instructions, except for scaling up the use of lysis buffer and magnetic beads (bioMérieux). Appropriate quality controls were applied and checked for performances [18]; during detection of norovirus GI and GII, RNA transcripts of GI.3b and GII.1 [20] served as positive RT–PCR controls (5–50 genome copies) and inhibition controls (10²–10³ genome copies) and nucleic acid-free water as non-template control.

For genotype characterization, part of the norovirus GI and GII capsid region was amplified by single and nested conventional RT–PCR [21, 22] and visible amplicons of expected size for norovirus GI (381 bp) were purified using GFX columns (Amersham Biosciences, Denmark) and sequenced by the Sanger method in both directions using the primers for the single PCR at MacroGen (MacroGen Inc., Korea). Sequences were then aligned with those obtained from human samples.

Epidemiological investigations

The outbreaks were all point-source outbreaks and investigated separately, as there was no initial

suspicion of links between the outbreaks. In all outbreaks, a case was defined as a person belonging to the relevant cohort and suffering from vomiting or diarrhoea (≥ 3 loose stools within 24 h) within 4 days of the time of suspected exposure. The local Food Control Offices inspected kitchens, took samples from relevant foods for analysis and made inquiries about kitchen practices, origin of foods used and relevant self-check procedures.

For three larger outbreaks (nos. 1–3) cohort investigations were performed on the persons eating in the canteens/restaurants. For these individuals, paper questionnaires were distributed including questions about symptoms, time of onset and consumed items from a menu list. Data were entered using EpiData (EpiData.dk) or Epi Info7 (CDC, USA) and canteen use and food specific attack rates calculated using Stata v. 12 (Statacorp, USA). The Danish Veterinary and Food Administration undertook trace-back of the raspberries and recall of products and sent notifications through the EU Rapid Alert System for Food and Feed (RASFF). In Denmark, foodborne outbreaks are registered in the national Database for Food- and Waterborne Outbreaks [2]. Data on outbreaks in 2010 and 2011 where norovirus was indicated as the aetiological agent, based on microbiological analysis or the Kaplan criteria [23], were extracted from this database.

RESULTS

During 2010–2011, a total of 93 norovirus outbreaks were reported and investigated in Denmark (Fig. 1). Based on genotyping, six of these outbreaks occurring in the period between 14 October 2010 and 25 January 2011 could be linked (Fig. 2). Additionally, two further small outbreaks occurring in September 2011 were later also linked to this cluster of outbreaks (see Fig. 1).

Microbiological investigations

A total of 52 samples from 28 patients originating from the six linked outbreaks were tested for viruses. All samples tested negative for norovirus GII, sapovirus, adenovirus, astrovirus and rotavirus. Fifty samples, representing 27 patients, were positive for norovirus GI. Norovirus from 17 patients, representing five of the outbreaks, were successfully typed by partial sequencing of the polymerase and/or capsid genes and were all identified as genotype, GI.Pb_GI.6 (polymerase/capsid). In fact, the norovirus capsid and

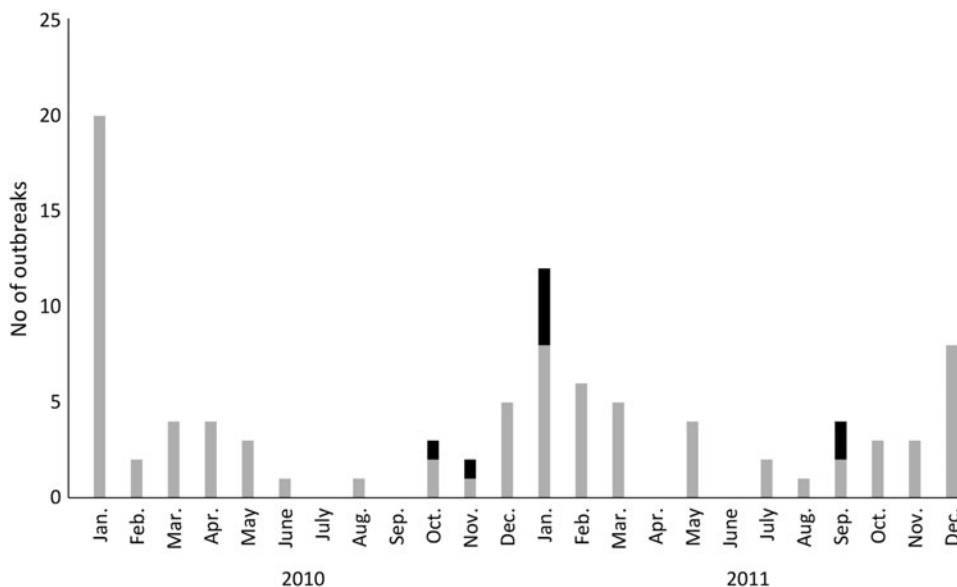


Fig. 1. Outbreaks with norovirus reported to the Danish Database for Food- and Waterborne Outbreaks, by month for 2010 and 2011. The eight outbreaks indicated in black colour could be linked by molecular typing and epidemiology.

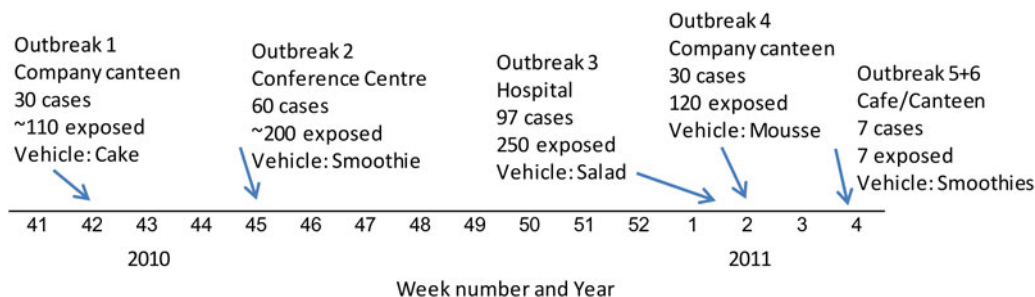


Fig. 2. Timeline for six related norovirus outbreaks, Denmark 2010–2011.

polymerase sequences from these 17 patients were 100% identical.

To further substantiate the genetic relationship, an additional typing procedure was developed and applied, consisting of sequencing of an ~1 kb area of the capsid gene including the hypervariable domain. Six selected patient samples representing the five first outbreaks were characterized this way and the sequences were found to be 100% identical in all samples, with the exception of a single nucleotide polymorphism in viral sequences from a sample obtained from outbreak 2. An overview of the six outbreaks and the test results is presented in Table 1.

To put this in perspective; before 2010 genotype GI.Pb_GI.6 was only sporadically observed in genotyped routine samples received at SSI from all over Denmark, but it appeared in two series of outbreaks in 2010 (those described here and outbreaks with imported lettuce as the source [5]). However, these

two series of outbreaks were characterized by distinctly different partial polymerase/capsid sequences: 2.4% (6/251 different nucleotides) and 2.7% (8/298 different nucleotides), respectively, while the difference was 4.6% (46/1000 different nucleotides) for the capsid gene including the P2 region (only one 1-kb area sequence was available from the lettuce outbreak series).

Bags of raspberries obtained from the outbreak settings were examined for the presence of norovirus. Virus was detected both from contents of bags that had been opened (partly used) and from bags that were still sealed. Norovirus of both GI and GII were detected (Table 2). In one instance, a PCR product could be obtained for sequencing. This was a 305 bp GI capsid gene fragment obtained from outbreak no. 3 which upon sequencing was found to be GI.6 (capsid) with an identical nucleotide sequence compared to the patient samples.

Table 1. *Norovirus outbreaks associated with consumption of frozen raspberries, Denmark, October 2010–January 2011*

Outbreak number	Date of event (first exposure)	Setting	Geographical region	No. of cases	No. of exposed	Vehicle	No. positive patient samples/no. tested	Genotype (polymerase/capsid)
1	14 Oct. 2010	Company canteen	Central Denmark	30	110	Raspberry cake	3/3	GI.Pb_GI.6
2	9 Nov. 2010	Conference centre	Central Denmark	60	200	Raspberry smoothie	4/4	GI.Pb_GI.6
3	10 Jan. 2011	Hospital canteen	Zealand	97	250	Red cabbage salad	14/15	GI.Pb_GI.6
4	14 Jan. 2011	Company canteen	Capital	30	120	Raspberry mousse	2/2	GI.Pb_GI.6
5	24 Jan. 2011	Café	Capital	5	5	Raspberry smoothie	4/4	GI.Pb_GI.6
6	25 Jan. 2011	Company canteen	Capital	2	2	Raw raspberries	0	n.d.

n.d., Not determined.

Table 2. *Norovirus analysis, trace-back, and control of raspberries implicated in six norovirus outbreaks, Denmark 2010–2011*

Outbreak number	Date batch was recalled	RASSF reference	Condition of bag of raspberries examined	Batch ID*	Raspberry farm area†	Result of analysis for norovirus
1	30 Oct. 2010	2010-1492	Opened	A	A	Negative
			Unopened	B	B	GII
2	12 Nov. 2010	2010-1563	Opened	C	B	Negative
			Unopened	C	B	Negative
3	17 Jan. 2011	2011-0068	Opened	D	B	GI
			Unopened	D	B	GI and GII
4	17 Jan. 2011	2011-0068	Opened	D	B	Negative
			Unopened	D	B	GI
5	02 Feb. 2011	2011-0136	Opened	E	A	Negative
			Unopened	E	A	GII
6	02 Feb. 2011	2011-0136	Opened	E	A	GI
			Unopened	E	A	GII

RASSF, Rapid Alert System for Food and Feed.

* All batches involved originated from the same main importer and producer. Lot numbers were as follows: A, 0301–2207; B, 0302–1208; C, 0302–1408; D, 0302–2809; E, 0301–021.

† The different batches of raspberries constituted berries delivered from two different areas of raspberry farms, A and B.

Epidemiological investigations

Trace-back investigation showed that raspberries from each outbreak originated from Serbia and were delivered by the same producer. The berries were grown in two adjacent areas near the freezing/packing facility (Table 2).

Outbreak 1 occurred in October 2010 in a company with about 110 employees in the city of Viborg. Patients were based in different company buildings and

the investigation showed no association between illness, building or other facilities. Further, there were no reports of cases having had contact to persons with gastrointestinal infection in the days prior to the outbreak. A cohort investigation was undertaken in which 70 employees responded; 30 fulfilled the case definition. There was a significant association between illness and having eaten in the canteen on 10 October 2010 [relative risk (RR) 11.6, 95% confidence interval (95% CI) 1.6–80]. No particular foods were associated with illness in the analysis.

However, a cake served containing non-heat-treated raspberries was considered as a possible source. The attack rate in those that had consumed cake was 61%; 6/9 persons who reported consuming only cake and nothing else from the canteen fell ill; the RR for consuming cake was 1.8 (95% CI 0.4–9). Stool samples from three persons were tested and norovirus detected in all. Raspberries seized from the canteen were sent for analysis and norovirus GII found in an unopened bag of one of the two batches used to make the cakes. Subsequently, on 30 October 2010, all bags of raspberries from the same producer and with the expiry date similar to those seized from the canteen were withdrawn from the Danish market by the Food Authority.

Outbreak 2 occurred 3 weeks later at a conference centre in the city of Aarhus. Approximately 60/200 guests were reportedly ill. Stool samples from four cases were submitted for laboratory analysis and they were all norovirus positive. A cohort study was performed with an emailed questionnaire among 59 guests with known email addresses. The response rate was 36%. No foods were statistically significantly associated with illness. Intake of smoothies made with raw frozen raspberries showed an RR of 2.2 (95% CI 0.7–7). Several bags of raspberries were tested and found to be negative for norovirus. The conclusion from the epidemiological investigation was that the frozen raspberries could not be confirmed as the vehicle of the infections. Nevertheless, as a measure of precaution the raspberries with the relevant expiry date were withdrawn from the market solely on suspicion on 12 November 2010.

Outbreak 3 began on 12 January 2011, when a number of employees at the hospital in the city of Køge fell ill with gastroenteritis. Infection with norovirus seemed likely and suspicion centred on the employees' canteen as the source of the illnesses. Before conclusive evidence of the source had been obtained, the kitchen was temporarily closed for cleaning and disinfection and 22 tonnes of food was discarded. Information on proper hygiene practices was distributed among the employees. No transmission to patients in the hospital was identified. A questionnaire was distributed by email on 13 January to all hospital employees (around 1500 persons) inquiring about use of the canteen on Monday 10 and Tuesday 11 January; 241 questionnaires were returned. Of the 150 persons that reported having used the canteen on the 2 days in question, 65% met the case definition and the RR for use of the canteen was 14.7 (95% CI 5.6–39). Raw raspberries were served as part of a

red cabbage salad in the canteen on both days. In an analysis excluding persons not using the canteen, a statistically significant relationship between illness and an individual food item was observed for this salad only. The attack rate in those who had consumed the red cabbage salad on the Monday was 97% (70/72) and the RR for consumption was 6.1 (95% CI 3.2–11). The RR for consumption of the salad on the Tuesday was 2.4 (95% CI 1.3–3.8); the combined RR for consuming salad on either day was 9.1 (95% CI 4.0–21). Norovirus GI was detected in stool samples from 14/15 persons tested. Norovirus GI was furthermore found in raspberries from an open bag and GI and GII also from an unopened bag, both collected from the kitchen. Raspberries with the relevant expiry date were withdrawn from the market on 17 January.

Outbreak 4 occurred during the same week as outbreak 3 in employees in a company in the city of Herlev. About 30 persons fell ill, out of about 120 persons who had used the company canteen. A mousse containing raspberries from the same batch as used in outbreak 3 was served at the company canteen. A cohort study was not performed. Stool samples from two persons were tested and both were found positive for norovirus. Norovirus GI was found in an unopened bag of raspberries collected from the canteen kitchen.

Outbreaks 5 and 6 occurred towards the end of January. Both were small outbreaks and involved consumption of raspberries again originating from the same batch as in outbreaks 3 and 4. The setting of the first outbreak was a café in Copenhagen where five guests from the same group of friends had consumed raspberry smoothies after which they all fell ill. Four patients submitted stool samples for examination and all samples were found positive for norovirus. Norovirus GII was detected in raspberries from unopened bags collected at the café and from bags collected at the supplier. In the second outbreak two kitchen employees at a company canteen fell ill after tasting non-heat-treated raspberries defrosted in the kitchen. These raspberries were never served raw to canteen guests but used as an ingredient in an oven-baked cake. Norovirus GI was detected in raspberries from an open bag collected at the company canteen.

Outbreaks 7 and 8 were two additional outbreaks that occurred in September 2011, at a time when the

investigation, including full trace-back, of the above-mentioned six outbreaks had been concluded. At this time all raspberries from the incriminated batches had been identified and were believed to have been destroyed. However, in September the exact same viral sequence was again found in patient samples in Denmark on two different occasions. Five positive samples were obtained from five persons in two different unrelated groups of friends that privately had consumed raspberries. This led to a renewed trace-back investigation. In the first of these outbreaks, the raspberries were part of bags with mixed frozen berries (raspberries, strawberries, redcurrants) packaged in Belgium using raw materials from, among other countries, Serbia. In consultation with the Serbian Food Authorities, it was concluded that the raspberries in these mixed bags came from the same original batch of contaminated raspberries as the six previous outbreaks. The second incident was caused by whole frozen raspberries. These were found to originate from one of the same farming areas where the previous batches had been grown but to have been handled by a different packaging establishment within Serbia and thus had escaped the recall.

DISCUSSION

The results of the epidemiological, microbiological and trace-back investigations presented here, showed that the outbreaks were caused by frozen raspberries from the same facility. However, the key to understanding this came from the genetic analyses of viruses linking the outbreaks together. As this report shows, the individual outbreak investigations, performed routinely by regional investigators, provided varying degrees of evidence. Although they generally involved the use of cohort studies, food tracing and stool examinations, the results obtained were influenced by the circumstances of the actual outbreaks. Whereas the large hospital outbreak provided for an investigation with strong evidence, the results of another investigation (outbreak 2) were inconclusive. In our experience, providing firm evidence for a particular food vehicle in norovirus outbreaks is often challenging, highlighting the importance of centrally performed sequence analysis. The investigation also demonstrates how the complexities of trace-back analyses may limit timely intervention. Recalls were done in several stages based on the epidemiological findings of each outbreak. This, however, was clearly not sufficient to

prevent further outbreaks. Sufficient evidence to recall all potentially contaminated batches was only obtained after performing a full trace-back analysis.

Because norovirus is a single-stranded RNA virus with a high mutation rate resulting in a high variety of virus variants, sequencing and comparative analysis of the capsid and/or P2 regions is often used as a tool in epidemiological investigations of norovirus GI and GII [24, 25]. Norovirus GI.6 (and other GI) sequences from the same outbreak had earlier been reported as 100% identical [26]. We consider it very unlikely to find identical GI.6 sequences unless they are epidemiologically linked. Thus, the combination of supporting epidemiological evidence and additional sequencing of a larger and more variable part of the genome (in comparison to routine norovirus typing purposes) is a strong tool in assessing if different outbreaks share a common source. In the present case, analysis of faecal samples revealed only norovirus GI of the same genotype and with identical RNA sequences – even in the instances where a large and variable area of more than 1 kb was sequenced. These molecular results imply that the source of infection is the same. As such, the individual outbreaks could be seen as manifestations of the same overall contaminated source. The power of the molecular method became even more clear when in September 2011, 6 months after the supposed end of the outbreaks, typing of viruses from patient samples were used to trace as yet unidentified parts of the contaminated batches which had again been introduced to the Danish market.

The outbreaks led to a collaboration between the Danish and Serbian food authorities with the aim of establishing how the raspberries may have become contaminated and how similar future incidents can be prevented. Further investigation by the Serbian competent authority, the Serbian packer, the Danish branch of the Swedish company that imported the berries and the Danish Veterinary and Food Administration and local Food Control Office in Odense to establish where the raspberry production chain was contaminated were not conclusive. According to the Serbian competent authority, additional samples taken at the freezing and packing establishment tested negative for norovirus.

Mutual visits and consultations between Denmark and Serbia followed and helped to address specific aspects of the production that are particularly vulnerable to contamination. Issues that were addressed involved: providing winter-training programmes to

the berry pickers about hygiene (hand hygiene in particular) and the risk of contaminating berries, establishment of increased hygiene procedures at the packing sites, implementing risk-based traceability and, at governmental level, enforcing measures through an independent control authority.

In Denmark, new measures have been implemented as a direct result of these outbreaks. Frozen raspberries are still considered a ready-to-eat product that should be sold free of pathogens. However, the Danish legislation has now been changed, and since 1 October 2012 all food business operators (restaurants, cafés, canteens, etc.) must heat-treat frozen raspberries at 100 °C for 1 min before serving. Private persons/households are also advised to follow this procedure and this advice is now printed on the bags of commercial frozen raspberries.

Detection of norovirus in foods remains technically difficult and to date norovirus has only successfully been detected in a limited number of products as part of outbreak investigations; e.g. in oysters [4], berries [10], lettuce [5] and drinking water [15]. For the outbreaks described here, norovirus of different types were detected in raspberries from bags related to five of the six initial outbreaks, showing that the berries were contaminated. The only viral product that could be typed, matched the viruses detected in patient samples, showing that the strain causing the outbreak was present in the raspberries. However, norovirus GII was also detected in berries originating from unopened bags related to four of the outbreaks, while GII viruses were not detected in any of the patient samples. We cannot readily explain why several genotypes of viral strains were detected in the raspberries when only one type was found in patient samples, but one possibility is that the GII virus(es) were present in a form that had reduced or no infectivity. The PCR test strictly speaking only provides evidence of the presence of viral RNA, not infectious particles.

Frozen raspberries may constitute a particular risk of transmission of norovirus infection for a number of reasons. Raspberries have a fragile texture, are handled manually and do not undergo rinsing in the process of production (unlike other berries which can be mechanically picked, rinsed, frozen and packaged). Since a practically applicable test for norovirus in berries is not available and since the virus may often not be evenly distributed in a batch and or present in low amounts only, testing of batches will most likely not be instrumental for prevention of outbreaks such

as those described here. In outbreaks 5 and 6, the batch involved had already been analysed as part of the importers own check scheme, but norovirus had not been detected. Therefore, the production should be organized so as to minimize the risk of contamination.

To summarize, we have here described a series of outbreaks which collectively caused 242 known cases of norovirus infections of which 32 were laboratory confirmed. One outbreak in particular, outbreak no. 3 at the hospital canteen, was seen as severe as it had the potential to spread to particularly vulnerable patients, and it resulted in discarding large quantities of food [27]. The cases were part of eight independent outbreak incidents, linked together by detailed molecular characterization of viruses from patient material. Virus was also detected directly in the berries and one material from one sample that was typable had the exact same RNA sequence as in the patient samples. Because norovirus outbreaks are often not reported, it is reasonable to expect that the burden of illness was considerably larger than uncovered here, as many more small-sized outbreaks caused by frozen contaminated raspberries could have occurred in Denmark during this period of time.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **European Food Safety Authority & European Centre for Disease Prevention and Control.** The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA Journal* 2013; **11**: 3129.
2. **Anonymous.** Annual Report on Zoonoses in Denmark 2013. National Food Institute, Technical University of Denmark. 2014.
3. **Franck K, et al.** Sources of calicivirus contamination in foodborne outbreaks, Denmark, 2005–2011 – the role of the asymptomatic food handler. *Journal of Infectious Diseases*. Published online: 25 August 2014. doi:10.1093/infdis/jiu479.

4. **Westrell T, et al.** Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010. *Eurosurveillance* 2010; **15**: 12.
5. **Ethelberg S, et al.** Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. *Eurosurveillance* 2010; **15**: 6.
6. **Falkenhorst G, et al.** Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. *Eurosurveillance* 2005; **10**: 9.
7. **Baert L, et al.** Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context. *Epidemiology and Infection* 2009; **137**: 316–325.
8. **Cotterelle B, et al.** Outbreak of norovirus infection associated with the consumption of frozen raspberries, France, March 2005. *Eurosurveillance* 2005; **10**: 4.
9. **Hjertqvist M, et al.** Four outbreaks of norovirus gastroenteritis after consuming raspberries, Sweden, June–August 2006. *Eurosurveillance* 2006; **11**: 9.
10. **Le Guyader FS, et al.** Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *International Journal of Food Microbiology* 2004; **97**: 179–186.
11. **Maunula L, et al.** Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks. *Eurosurveillance*. 2009; **14**: 49.
12. **Sarvikivi E, et al.** Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiology and Infection* 2012; **140**: 260–267.
13. **Bernard H, et al.** Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012. *Eurosurveillance* 2014; **19**: 8.
14. **Anon.** Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (Salmonella and Norovirus in berries). *EFSA Journal* 2014; **12**: 3706–3801.
15. **Van Alphen L, et al.** The application of new molecular methods in the investigation of a waterborne outbreak of norovirus in Denmark, 2012. *PLoS ONE*. Published online: 15 September 2014. doi:10.1371/journal.pone.0105053.
16. **Franck KT, et al.** Norovirus epidemiology in community and health care settings and association with patient age, Denmark. *Emerging Infectious Diseases* 2014; **20**: 1123–1131.
17. **Donaldson EF, et al.** Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. *Immunological Reviews* 2008; **225**: 190–211.
18. **International Organization for Standardization.** ISO/TS 15216–2. Microbiology of food and animal feed – Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR – Part 2: Method for qualitative detection. 2013; Geneva, Switzerland.
19. **Schultz AC, Detection and Inactivation of Noroviruses in Raspberries.** Ph.D. thesis. Faculty of Health and Medical Sciences, University of Copenhagen and National Food Institute, Technical University of Denmark, 2012. [Can be requested from the author or through library: <https://bibliotek.dk/eng/work/870970-basis%3A29480869>.] Accessed 10 October 2014.
20. **Gentry J, Vinje J, Lipp EK.** A rapid and efficient method for quantitation of genogroups I and II norovirus from oysters and application in other complex environmental samples. *Journal of Virological Methods* 2009; **156**: 59–65.
21. **Kojima S, et al.** Genogroup-specific PCR primers for detection of Norwalk-like viruses. *Journal of Virological Methods* 2002; **100**: 107–114.
22. **Nishida T, et al.** Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. *Applied Environmental Microbiology* 2003; **69**: 5782–5786.
23. **Kaplan JE, et al.** Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. *Annals of Internal Medicine* 1982; **96**: 756–761.
24. **Sukhrie FHA, et al.** Using molecular epidemiology to trace transmission of nosocomial norovirus infection. *Journal of Clinical Microbiology* 2011; **49**: 602–606.
25. **Xerry J, et al.** Transmission events within outbreaks of gastroenteritis determined through analysis of nucleotide sequences of the P2 domain of genogroup II noroviruses. *Journal of Clinical Microbiology* 2008; **46**: 947–953.
26. **Xerry J, et al.** Genetic characterization of genogroup I norovirus in outbreaks of gastroenteritis. *Journal of Clinical Microbiology* 2010; **48**: 2560–2562.
27. **Statens Serum Institut.** Norovirus outbreak (raspberries) in the staff canteen at Køge Hospital. *EPI-NEWS* 2011; **5**.