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Lack of evidence so far for Noroviruses in food samples collected on cruise ships sailing the Mediterranean waters

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Abstract

Closed living spaces like cruise ship settings create an environment in which enteric viruses including Noroviruses (NoVs) can easily spread and outbreaks readily occur. The contribution of sources other than common confirmed vehicles to foodborne illness in cruise ships was investigated. None of the 189 collected samples (including meat, fish, buffet meals, fruits, vegetables etc.) tested by real-time RT-PCR (ISO/TS 15216-2:2013) reveled NoVs genome. Naturally contaminated food items other than shellfish have been demonstrated to be cause of NoVs infection thus, our results could neither indicate nor exclude food items as possible vehicle of infection. Reducing risk factors associated with NoVs outbreaks (contaminated raw ingredients, cross contamination, infected food handlers, inadequate temperature controls, heat treatments) is always strongly recommended.

Introduction

Human food-borne viruses (FBV) including norovirus (NoV) and hepatitis A virus (HAV) have been responsible all together for the 14.1 % of all EU outbreaks in 2012 (EFSA/ECDC, 2014). These viruses are highly contagious because of the small dose needed for infection, their stability outside the host and the short-term immunity (Green, 2007; Rockx *et al*., 2002; Koopmans and Duizer, 2004). They are transmitted mainly by fecal-oral route, either by person-to-person transmission or by ingestion of contaminated foods and environment (Fretz *et al*., 2005; Karsten *et al*., 2009).

Closed living spaces such as cruise ship settings create an environment in which NoVs can easily spread and outbreaks readily occur. Outbreaks may be of serious public health concern and result in high economic losses to a growing shipping industry (ECDC, 2010). While consumption of shellfish and berries remain the major risk factor for food-borne outbreaks (Doyle *et al*., 2004; Ponka *et al*., 1999), many types of foodstuffs have been confirmed or suspected to be vehicles of these enteric viruses in cruise ships (Morillo *et al*., 2012). Moreover, the common contributory factors associated with the spreading of the infection on-board include inadequate food temperature control, cross-contamination, unprocessed contaminated ingredients and infected food handlers (EFSA/ECDC, 2014). NoVs belong to the genus Norovirus in the Caliciviridae family (Green *et al.*, 2000). The genome is a positive sense, single-stranded RNA of about 7.5 Kb. NoVs are classified into 5 different genogroups GI-GV. Within the genogroup, they are further categorized into clusters, and within the clusters, viruses assigned to an outbreak are referred to as a strain (Zheng *et a*l., 2006). Strains of GI, GII and GIV are known to infect humans. GII viruses have additionally been detected in pigs, and GIV viruses have been detected in a lion cub and a dog. GIII viruses infect cattle and sheep and GV viruses infect mice (EFSA, 2011).The aim of the present study was to investigate the possible contribution of different sources (fresh produces, food environments etc.) to foodborne illness in cruise ships sailing the Mediterranean waters.

Materials and Methods

Sampling

This study was based on investigations carried out within three months (June to February 2013) in two cruise ships sailing the Mediterranean waters with a capacity of 6000 people among vacationers and crew

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Table 1. Collection of food and environmental samples from two cruise ships sailing the Mediterranean waters

Sample typology	No. Samples					No. (%) positive samples
		Ship cruise A	Period	Ship cruise B	Period	
Meat	**157**	**148**		**9**		**0**
Poultry	30	29	June	1	July	0
Bovine	105	100	June	5	July	0
Swine	7	4	June	3	July	0
Sheep	1	1	June			0
Mix meat (Bovine and Swine)	14	14	June			0
Vegetable	**9**	**7**		**2**		**0**
Celery	1	1	June			0
Red chicory leaves	1	1	June		July	0
Tomatoes	3	2		1	July	0
Asparagus	1			1	July	0
Salads	3	3				0
Fruit	**6**	**5**		**1**		**0**
Blackberry	1	1	June			0
Red Currant	1	1	June			0
Raspberry	1	1	June	0	July	0
Blueberry	1	1	June	0	July	0
Pineapple	1			1	July	0
Fruit mix	1	1	June			0
Fish	**16**	**12**		**4**		**0**
Salmon	2	1	June	1	July	0
Shrimp	5	4	June	1	July	0
Cod	2	1	June	1	July	0
Fillets	1	1	June			0
Squid	1	1	June			0
Swai fillets	1			1	July	0
Mussels	4	4				0
Environment	**1**	**1**				**0**
Handwash	1	1	June			0
Total	**189**					**0**

members. Food borne viruses causing human illness can easily spread in this kind of settings leading to a severe public health issue involving a large amount of people. In order to investigate the occurrence of NoVs, a total of 189 food samples (Table 1) were collected. Considering the ability of viruses to survive for long out of the host, an additional environmental sample (Table 1) was collected from food preparation areas. Collection (in three sampling days) occurred every time the ships docked at two seaports in Southern Italy (approximate distance 55 km) and following the food manager crew availability. On board, foods were stored separately according to their risk profiles in chilling (≤ 5°C) (vegetables and fruits) or freezing (≤ -18 °C) (meats and poultry, fish, shellfish) rooms. Before processing, thawing was carried out in containers kept at refrigeration temperature (≤ 5°C). In these chilling rooms, thawed perishable foods (raw meats with run off liquid and poultry, raw fish and shellfish) were stored at the bottom, unprocessed vegetables and fruits in the middle, and ready-to-eat foods on the top of the shelves. Both food samples and environmental sample were put in sterile stomacher bags, transported cooled to the laboratory and kept at -80°C. Further analyses were conformed to ISO/TS 15216-2:2013.

Samples preparation and nucleic acid extraction

Two grams of defrost sample were transferred to a centrifugation tube and added of 2 ml of proteinase K (30U/mg) and 10 µl of Mengovirus suspension (108 TCID50/ml) as process control. Following incubation in a shaking incubator (37°C per 60 min) to allow digestion, tubes were then transferred in a water bath (60°C per 15 min) to inactivate the enzyme. Samples were then centrifuged at 3000 g per 5 min, and the supernatant (approximately 3 ml) was transferred into a clean tube for RNA extraction.

RNA extraction was performed on 500 µl of supernatant using bioMérieux NucliSens® system (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Briefly, the supernatant was added of 2 µl of NucliSens® lysis buffer and shaken. Samples were incubated at room temperature per 10 min. Subsequently, 50 µl of magnetic silica solution were added to each sample in order to allow the viral capsid disruption with a chaotropic reagent (guanidine thiocyanate) followed by adsorption to silica particles. After incubation at room temperature per 10 min, centrifugation was done at 1500 x g per 2 min. The pellet was resuspended in 400 µl of wash buffer. Washing was done repeatedly using the magnet of NucliSENS® easyMAG® extraction system to allow silica particles sedimentation. Nucleic acids

Table 2. Primers and probes used for real-time RT-PCR (ISO/TS 15216-2:2013) in food and environmental samples collected from cruise ships sailing the Mediterranean waters

PRIMERS AND PROBES	SEQUENCE	REFERENCE
NOROVIRUS GI		
QNIF4 (FW)	CGC TGG ATG CGN TTC CAT	da Silva *et al.*, 2007
NV1LCR (REV)	CCT TAG ACG CCA TCA TCA TTT AC	Svraka *et al.*, 2007
NVGG1p (PROBE)	TGG ACA GGA GAY CGC RAT CT	Svraka *et al.*, 2007
NOROVIRUS GII		
QNIF2 (FW)	ATG TTC AGR TGG ATG AGR TTC TCW GA	Loisy *et al.*, 2005
COG2R (REV)	TCG ACG CCA TCT TCA TTC ACA	Kageyama *et al.*, 2003
QNIFS (PROBE)	AGC ACG TGG GAG GGC GAT CG	Loisy *et al.*, 2005
MENGO VIRUS		
MENGO 110 (FW)	GCG GGT CCT GCC GAA AGT	Pintó *et al.*, 2009
MENGO 209 (REV):	GAA GTA ACA TAT AGA CAG ACG CAC AC	Pintó *et al.*, 2009
MENGO 147 (PROBE)	ATC ACA TTA CTG GCC GAA GC	Pintó *et al.*, 2009

** The two probes for NoVs GI and GII were labeled with 6-carboxyfluorescein (FAM) at the 5' end and with 6-Carboxytetramethylrhodamine (6-TAMRA). The probe for Mengo virus was labeled with FAM at 5' end and minor groove binder (MGB) at 3'end.

were finally eluted in 100 µl of elution buffer and retained at -80°C.

Recovery of Mengovirus was determined with real-time RT-PCR assay by comparing C*t* values obtained for the sample extracts with those for the virus control. Recovery was considered acceptable when ≥1%. Samples failing to meet these criteria were re-extracted. Moreover, to determine the level of RT-PCR inhibition in each sample an external control (EC) RNA was added.

Real-time RT-PCR

Real-time RT-PCR was performed using the Invitrogen RNA UltraSenseTM one-step qRT-PCR system (Invitrogen, Saint Aubin, France) following the manufacturer's instructions. Primers and probes are shown in table 2. Reactions were performed in a 25 µl reaction mixture containing 5 µl of RNA extract, 1 x UltraSenseTM master mix, 1.25 µl of RNA UltraSenseTM enzyme mix, 500 nM forward primer, 900 mnM reverse primer, 250 nM Taqman probe, 1 x ROX reference dye and water. The cycling parameters consisted of reverse transcription at 55°C per 60 min, preheating at 95°C per 5 min, and amplification for 45°C cycles at 95°C per 15 s (denaturation), at 60°C per 1 min (annealing) and at 65°C per 1 min (extension). Reverse transcription and PCR were performed in one step using CFX96 Touch Deep Well™ (Bio-Rad Laboratories, Hercules, CA, USA). Extraction and real-time PCR efficiency results were interpreted as described elsewhere (ISO/TS 15216-2:2013).

Results and Discussion

Outbreak involving cruise ship environment are of particular public health importance because of the recently shipping industry growth and because people confined in close spaces can represent susceptible cohorts. In the present study, none of the food samples tested by real-time RT-PCR reveled NoVs genome. All of them were found to be conformed to the external control as described in the method ISO/TS 15216-2:2013.

Previous works reported in literature mainly focused on verified cruise ship outbreaks (Verhoef *et al*., 2008) whereas this paper outlines whether raw foods might represent source of infection for passengers. Samples were collected considering that the distribution of food vehicles of outbreaks caused by Caliciviruses including NoVs in EU 2011, is split between mixed food (20.7%), seafood (19.5%), buffet meals (18.4%) (EFSA/ECDC, 2013).

Infected food handlers (experience, understanding of safe handling procedures) together with unprocessed contaminated ingredients and cross contamination in working areas can act as contributory factors, and might influence food safety standards and the likelihood of illness on board. To enhance probability of detection, samples were taken mainly during food processing and handling by people working in the kitchen.

In Europe, NoVs outbreaks are highly seasonal with most outbreak reported from October through April (Lopman *et al*., 2003), our samples were collected during the off-seasonal period (May through September) following the company

availability. Nevertheless, a correlation between the number of off-season outbreaks on cruise ship and higher NoVs activity in the subsequent winter season was hypothesized implying that cruises might be considered as possible "early indicator" for NoVs circulating (Verhoef *et al*., 2008).

After the first infection is introduced in a closed setting, an outbreak is likely to occur through person-to-person contact and only a human short-term acquired immunity is possible. In our study, no previous stories of infection were orally reported from the cruise food manager, reducing the likelihood of detection. Detailed information on food supply was not obtainable thus, our results could neither indicate nor exclude food items as possible vehicle of infection.

Conclusion

To sum up, none of the collected samples showed to be a vehicle of the viruses. Nevertheless, naturally contaminated food items other than shellfish have been demonstrated to be cause of NoVs infection in Brazil (Morillo *et al.*, 2012); and contaminated raw ingredients, cross contamination, infected food handlers together with inadequate temperature controls and heat treatments still represent risk factors associated with outbreak.

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