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Krog, Jesper Schak; Larsen, Lars Erik; Schultz, Anna Charlotte

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Short communication

Enteric porcine viruses in farmed shellfish in Denmark

J.S. Krog [a,b,*], L.E. Larsen [a], A.C. Schultz [b]

[a] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870, Frederiksberg C, Denmark
[b] National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860, Søborg Denmark

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ABSTRACT

Bivalve shellfish are at constant risk of being exposed to pathogens as a consequence of contamination of the shellfish beds with human or animal waste originating from sewage treatment plants or slurry fertilized fields. Consumption of contaminated oysters and mussels are frequently reported as causes of disease outbreaks caused by norovirus or hepatitis A virus. Other zoonotic pathogens such as hepatitis E virus (HEV), rotavirus (RV) and *Salmonella* from livestock may also be transmitted to shellfish via this route. In this study, 29 pooled samples from commercial Danish blue mussels were tested for porcine pathogens and indicator bacteria *Escherichia coli* (*E. coli*). All samples tested negative for HEV, RV and *Salmonella*, whereas *E. coli* and the highly stable porcine circovirus type 2 (PCV2) were detected in eight and 12 samples, respectively. This is the first study to report the detection of PCV2 in commercial mussels. Based on the detection of PCV2 in clean areas with low prevalence of the normally applied fecal indicator *E. coli*, testing for PCV2 may be a more sensitive and robust specific porcine waste indicator in shellfish harvesting areas.

1. Introduction

Bivalve shellfish such as oysters, clams and mussels are recognized as important sources of foodborne pathogens. Bivalves take up nutrition from the surrounding water by filtering up to 4.8 L/h (Carver and Mallet, 1990; Winter, 1973) and will simultaneously concentrate microorganisms if present (Burkhardt and Calci, 2000). Human pathogens can enter the shellfish beds in case of waste water managing system failures or in connection to flooding. Animal pathogens can contaminate the beds via runoff from fields applied with animal waste. A high number of food related outbreaks caused by contaminated bivalves has indeed been related to consumption of raw or lightly cooked oysters or mussels contaminated with noroviruses (NoV) (Westrell et al., 2010) or hepatitis A virus (HAV) (Pintó et al., 2009). Both NoV and HAV are shed in high amounts from infected humans, they are stable in the environment, and the infectious dose is very low (Koopmans and Duizer, 2004; Teunis et al., 2008). Other viruses with zoonotic potential, such as hepatitis E virus (HEV) and rotavirus (RV), are also prevalent in production animals and are shed in large amounts in feces. HEV causes acute self-limiting hepatitis in humans similar to HAV (Cacopardo et al., 1997; Koizumi et al., 2004; Renou et al., 2008; Said et al., 2009) and is highly prevalent among Danish pig herds where 92% of herds have animals with antibodies against the virus (Breum et al., 2010). Group A rotavirus (RV-A) is excreted in feces from a range of production animals including bovines and pigs and may also have zoonotic potential (Fischer et al., 2005; Martella et al., 2010; Midgley et al., 2012). Thus, shellfish produced close to land, where spillover with porcine waste can occur, may accumulate zoonotic enteric viruses and by that act as a vehicle for human exposures and subsequent diseases.

Currently, the application of slurry to farmland is tightly regulated in most countries, but failure to follow regulations or extreme weather conditions may nevertheless cause release of virus contaminated slurry into the surrounding water environment.

The hygienic control of fecal contamination in shellfish beds is based solely on the levels of the indicator bacteria *Escherichia coli* (*E. coli*) in shellfish meat according to the European directive 91/492/EC (Anonymous, 1991) and by fecal coliform in waters used for shellfish harvesting areas in the US according to the National Shellfish Sanitation Program issued by the FDA (FDA, 2009). These regulations have successfully reduced the number of clinical cases associated with bacterial infections caused by ingestion of seafood (Lees, 2000). However, the presence of bacterial indicators has been shown to be insufficiently correlated to the presence of enteric viruses (Lees, 2000). Additionally, these indicators do not provide information of the source (human or animal) of contamination. Furthermore, the commercially applied "depuration", a process where shellfish is placed in a tank of clean water to clear out pathogens, efficiently clears bacteria, but this process has limited impact on the clearance of viruses (Loisy et al., 2005; Love et al., 2010; Schwab et al., 1998). Consequently, half of the clinical cases caused by seafood consumption in i.e. New York are now caused by viruses (Butt et al., 2004; Wallace et al., 1999).

* Corresponding author at: National Veterinary Institute, Technical University of Denmark, Bülowsvej 24, DK-1870 Frederiksberg C, Denmark. Tel.: +299 4535886606; Fax: +299 4535886340.
E-mail address: jsck@vet.dtu.dk (J.S. Krog).

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Denmark has a substantial pig as well as shellfish production and is surrounded by water and therefore there is a risk of shellfish being contaminated by viruses present in pig slurry. The primary aim of this study was to investigate the presence of the viral pathogens HEV and RV-A, in blue mussels produced near the coast of Denmark. Secondarily, the aim was to evaluate the potential of the highly stable and pig specific porcine circovirus type 2 (PCV2), which is considered ubiquitous in swine herds (Kristensen et al., 2013), to serve as an indicator of porcine waste and determine the correlation between the porcine viruses and the presence of *E. coli* and *Salmonella* in mussels.

2. Material and methods

2.1. Samples

Twenty nine samples of blue mussels (*Mytilus edulis*), from 19 different Danish commercial harvesting areas (see Table 1) were collected by the Danish Veterinary and Food Administration (DVFA) during the official national control program of the fishermen's own control program in 2008 and 2009.

2.2. Bacterial analysis

The mussels were tested for *E. coli* and *Salmonella* according the EU reference methods (Anonymous, 2002, 2005) within 48 hours post-harvest by the DVFA Regional Control Laboratory, North.

2.3. Viral analyses

To extract viral nucleic acid from mussels, digestive tissues (DT), defined here as the digestive glands which surround the entire stomach and part of the intestine (Gosling, 2003), from at least 10 animals originating from one or two neighbor harvesting areas were excised, pooled and comminuted by razor blades. Viral nucleic acid was extracted from 2.0 g sub-samples of DT according to the method included in the newly developed ISO TS 15216 standard (Anonymous, 2013), except that the entire amount of homogenized DT using three ml of lysis buffer and 140 μl magnetic beads was processed as described by Uhrbrand et al. (2010).

To evaluate the extraction efficiency of viral nucleic acids from the mussel tissue, approximately 10^4 plaque forming units of mengovirus (MC_0), was added as internal process control to all portions of homogenized DT prior to proteinase K (>30 units/mg, FinnZymes, Finland) treatment. The relative recovery efficiencies and the inhibition during detection of HEV, RV-A and PCV2 in mussel DT, were determined in two independent runs using mussel DT from a confirmed negative sample and pig slurry previously shown to contain 1.2×10^4, 5.4×10^4 and 3.8×10^5 PCR units ml^{-1} of HEV, PCV2 or RV-A particles, respectively. One PCR unit was defined as the highest dilution that tested positive by the assay. The recovery efficiencies were calculated as the differences in average Ct (ΔCt) values obtained from nucleic acid extracts of 140 μl pig slurry alone and 2 g DT spiked with 140 μl slurry prior to PK treatment. To determine the effect of PK treatment on the virus recoveries during nucleic acid extraction, Ct values obtained from virus detection in pig slurry by the inclusion and exclusion of PK prior to the nucleic acid extraction were compared.

The inhibitory effect of the mussel extract was calculated as the differences in Ct values obtained from testing 1 μl of slurry extracts alone, and spiked with undiluted and 10-fold diluted mussel extracts (5 μl).

Detection of viruses was carried out by real time RT-PCR on a RotorGene Q (QIAGEN, Hilden, Germany) using the RotorGene Q Series software 2.0.2. All samples were assayed in duplicates of undiluted and 10-fold diluted nucleic acid extracts. HEV was detected using the assay by Breum et al. (Breum et al., 2010) applying modified primer and probe concentrations, HEV2-R and HEV2-P (500 nM) and HEV-F (100 nM), and reaction conditions, denaturation (15 s), annealing (15 s) and elongation (20 s). From the 10-fold dilution series of a plasmid containing the target HEV region, an amplification efficiency of 88% and a slope of −3.64 were calculated. RV-A and MC_0 were detected using the RNA Ultrasense One-Step qRT-PCR System (Invitrogen, cat number 11732-

Table 1
Summarized data for all samples.

ID	RV-A	HEV	PCV2 copies/g DT	*E. coli*[a]	Salmonella	Harvest date	Area	Production site
1	-	-	9.23E + 03	<20	-	03-04-2008	a	Fjord
2	-	-	3.85E + 03	<20	-	03-04-2008	a	Fjord
3	-	-	7.89E + 02	20	-	07-05-2008	b	Fjord
4	-	-	3.42E + 02	<20	-	07-05-2008	c	Fjord
5	-	-	2.32E + 02	<20	-	07-05-2008	b	Fjord
6	-	-	1.02E + 02	<20	-	07-05-2008	d	Fjord
7	-	-		<20	-	07-05-2008	d	Fjord
8	-	-	3.82E + 02	<20	-	07-05-2008	b	Fjord
9	-	-		40	-	11-06-2008	e	Fjord
10	-	-		<20	-	11-06-2008	f	Fjord
11	-	-	1.04E + 02	<20	-	25-06-2008	g	Fjord
12	-	-		20	-	26-08-2008	h	Fjord
13	-	-	1.83E + 02	40	-	08-10-2008	g	Fjord
14	-	-	7.80E + 03	310	-	29-10-2008	i	Bay area
15	-	-	4.00E + 02	<20	-	29-04-2009	j	Fjord
16	-	-		<20	-	29-04-2009	j	Fjord
17	-	-		<20	-	17-08-2009	k	Bay area
18	-	-		40	-	17-08-2009	k	Bay area
19		-		40	-	17-08-2009	k	Bay area
20	-	-		<20	-	17-08-2009	l	Ocean
21	-	-		<20	-	17-08-2009	m	Ocean
22	-	-		<20	-	18-08-2009	n	Ocean
23	-	-		<20	-	19-08-2009	o	Ocean
24	-	-		<20	-	19-08-2009	p	Ocean
25	-	-		20	-	19-08-2009	q	Ocean
26	-	-	1,48E + 02	<20	-	11-11-2009	r	Fjord
27	-	-		<20	-	11-11-2009	d	Fjord
28	-	-		<20	-	11-11-2009	s	Fjord
29	-	-		N.T	-	04-01-2010	?	Fjord

[a] MPN per 100 g mussel flesh and liquid.

927) and the primers, probe and reaction conditions described by Pang et al. (Pang et al., 2004) for RV-A and by Pintó et al. for MC_0 (Pintó et al., 2009). The standard curve for RV-A was made from a serial dilution of RNA extracted from RV-A cultivated in a MA104 cell line. The amplification efficiency of the assay was 92% and the slope −3.54. The detection of PCV2 by the real time PCR and production of standard curves was performed as described by Hjulsager et al. (2009). The amplification efficiency of the assay was 100% and the slope −3.34. Pearson's product–moment correlation coefficients between bacteria positive values and viral positive values were calculated to determine the correlation between bacterial and viral contamination.

3. Results

3.1. Detection of pathogens and fecal indicators

All 29 mussel samples tested negative for HEV, RV-A and *Salmonella*, 12 samples (41 %) tested positive for PCV2 in the range of 1.02–92.3 × 10^2 genome copies (GC)/g of DT, and eight samples (28 %) had detectable levels of *E. coli* with 20–310 MPN/100 g flesh and liquid (Table 1). The process control, MC_0, was consistently recovered in all mussel extracts, indicating robust extraction performance, although with a low mean extraction efficiency when testing undiluted (1.16% ± 1.78) and 10 × diluted (1.58% ± 1.96) RNA extracts corresponding to approximately 2 log reduction. To estimate the inhibition during detection of MC_0, the extraction efficiency in undiluted and 10 × diluted RNA extract obtained for each sample were compared, and the mean difference of Δ% (recovery efficiency) was calculated to be 0.68% ± 1.14, indicating very low inhibition from the extracts. For RV-A, HEV and PCV2, the recovery efficiency in the nucleic acid extracts of mussels varied according to the type of virus (Table 2) with a net reduction in viral genome detection corresponding to a 2.1, 0.5 and 0 log reduction, respectively. Based on the Ct values obtained from virus detection in slurry extracts compared to extracts of mussel homogenates spiked with slurry (Table 2), the efficiency in purification of HEV and PCV2 were consistently similar in the two extracts with maximum ΔCt of 1.61 in the undiluted extracts. In contrast, the recovery efficiency of RV-A decreased considerably when purified from pig slurry alone, compared to mussel extracts spiked with pig slurry with a ΔCt of 7.51 in undiluted extracts. To identify the cause of poor RV-A assay performance, loss of viral particles during PK treatment and inhibition during detection were studied. Unlike for PCV2, the PK treatment of pig slurry during nucleic acids extraction had a reducing effect of approximately 1 log on the detection of HEV and RV-A. However, as negligible ΔCt-values between extracts of slurry and PK treated slurry were observed when testing 10-fold diluted extracts, the reduction in slurry extracts must be attributed to inhibition arising from PK treatment rather than loss of viral particles. To further test for inhibition, Ct-values obtained from virus detection in pig slurry extracts alone and pig slurry extracts spiked in mussel extracts were compared (Table 2). This corresponded to 1.1, 1.6 and 0.4 log inhibition of the RV-A, HEV and PCV2 assays, respectively, in undiluted extracts which could be reduced to 0.3, 0.8 and 0.3 log by testing for inhibitors in 10-fold diluted mussel extracts.

3.2. Correlation between pathogens and fecal indicators

The correlation between mussel samples positive for both PCV2 and the bacteria, *E. coli* and/or *Salmonella* were determined (Table 1). The bacterial content were in general very low (≤40 MPN *E. coli*/100 g flesh and liquid), except for sample 14 which contained a high amount of both *E. coli* (310 MPN/100 g) and PCV2 (7.80 E + 03 copies/g DT). However, as only three of the 12 PCV2 positive samples were found positive for *E. coli*, there was no statistical significant correlation between the presence of PCV2 and bacteria in shellfish ($P = 0.43$). The geographic distribution of the PCV2 positive shellfish samples revealed that positive samples were localized to the Limfjord in the northern part of Jutland and a bay area in the south-western part of Jutland, Denmark.

4. Discussion

Here we demonstrate that genome material from PCV2 could be detected in a high fraction of mussels in Denmark. PCV2 is a pathogen that has only been detected in pigs and is not considered a threat to public health. The presence of a porcine virus in shellfish does, however, document that virus present in pig slurry has the potential to be accumulated in shellfish which is often consumed raw or lightly cooked. Interestingly, the zoonotic viruses RV-A and HEV were not detected in shellfish in the present study despite that these viruses indeed are present in pig slurry in positive herds and the HEV prevalence in Denmark is more than 90% (Breum et al., 2010). Even though RV-A and HEV are considered stable in the environment, PCV2 is one of the most resilient viruses known (Ansari et al., 1991; Balayan, 1997; Kim et al., 2009; Parashar et al., 2011; Welch et al., 2006). Nevertheless, RV has previously been detected in shellfish, and experiments with virus-like particles have shown that RV has the potential to persist in shellfish (Abad et al., 1997; Hansman et al., 2008; Le Guyader et al., 2008). The persistence of HEV in soil has been compared to that of HAV. HEV survived for nine weeks at outdoor temperatures and for ten weeks at 37°C whereas HAV persisted for 8 and 13 weeks, respectively (Parashar et al., 2011).

The validation of the assays showed different performance against virus stability towards the rigorous treatment with PK, during viral nucleic acid extraction of mussels and inhibitors during detection, which could also explain why HEV and RV-A were not detected. The RV-A assay was pronouncedly affected by inhibition due to both the PK treatment and constituents left over from the mussel homogenate. Unlike RV-A, the PCV2 assay was not affected by inhibition and losses during nucleic acid extraction, whereas the HEV assay showed mild inhibition compared to RV-A. Especially the two log reduction of RV-A would indeed exclude weak positive samples from being detected. The low recovery of the process control MC_0 (~1%) could also be attributed to this rigorous PK treatment although routinely the purification efficiency tends to be somewhat higher.

Table 2
Method performance.

	Virus recovery, Ct average (±SD)						Effect of PK ΔCt[a]		Inhibition ΔCt[b]	
	Slurry		Mussels		ΔCt (mussel-slurry)					
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10
PCV2	33.85 ± 1.30	36.33 ± 1.72	33.61 ± 1.65	36.29 ± 2,11	−0.25	−0.04	0.89	−1.70	1.69	1.32
HEV	32.32 ± 0.51	35.63 ± 1.71	33.92 ± 0.63	36.87 ± 1,40	1.61	1.24	3.38	−0.39	5.84	3.11
RV-A	26.22 ± 1.06	27.97 ± 2.12	33.73 ± 1.61	37.65 ± 2,12	7.51	9.68	3.52	0.06	3.91	1.14

Viral recovery during nucleic acid extraction and inhibition during detection of viral genomes.
[a] The effect of PK treatment on virus detection in slurry extracts, ΔCt of pig slurry treated with PK vs. untreated.
[b] Inhibition in extracts during detection, ΔCt of mussel RNA spiked with slurry RNA vs. slurry RNA alone. 1:10 indicates dilution of the mussel RNA prior to spiking.

According to the *E. coli* monitoring of Danish shellfish harvesting areas, a generally low rate and level of fecal pollution have been demonstrated in connection to the national classification system with more than 95% of samples complying to class A-status. Our data support that the microbial contamination of Danish shellfish is low, but the identification of a porcine specific virus in some of the shellfish pools strongly indicate that Danish shellfish waters are indeed exposed to constituents originating from pigs waste. The impact of this finding in relation to food safety is not clear.

The standard practice of using indicator bacteria enumeration to assess risk of pollution with enteric viruses from human and animal waste is of particular concern. This indicator may greatly underestimate the content of viruses because some viruses are; highly stabile in the environment, infectious at low doses and have a broad host-range. Indeed acute and chronic viral disease in humans have frequently been shown to be implicated in human disease related to consumption of food and drinking water as well as to swimmer-associated illnesses (Fong and Lipp, 2005; Ethelberg et al., 2010; Westrell et al., 2010).

To better identify fecal contamination and sources, a suite of microbial source tracking (MST) genetic markers targeting host-associated bacteria and viruses have been identified (Roslev and Bukh, 2011). Host specific viruses frequently found in fecal samples of specific origin and which are highly stable in the environment have been suggested as fecal indicators and source tracking tools to specify fecal contamination of human or animal origin in water and in shellfish. Among these are the porcine DNA adenoviruses (PAdV) and RNA teschoviruses (PTV) which both can be detected and quantified using culture- and library-independent realtime PCR based methods (Hundesa et al., 2006, 2009; Maluquer de Motes et al., 2004; Mahnel et al., 1977; Jimenez-Clavero et al., 2003; Cano-Gomez et al., 2011). As the present and previous studies have shown, DNA viruses can be PCR quantified with greater accuracy and with improved robustness than RNA viruses (Bofill-Mas et al., 2012).

PCV2 is a porcine specific, highly stable DNA virus considered ubiquitous in swine herds globally (Kristensen et al., 2013). We have shown in this study, that PCV2 can be easily detected in mussels by library- and culture independent quantitative realtime PCR assay without interferences of inhibitors. We therefore believe that PCV2 fulfills the requirements for a sensitive and host specific marker to be used for source identification of porcine waste and suggest that further studies are initiated to confirm this.

The lack of HEV detection in Danish mussel samples is in accordance with a surveillance of HEV in mussels (213 samples) from Thailand (Namsai et al., 2011), but in contrast to data obtained in United States and United Kingdom where two of 46 clam samples and 41 of 48 mussel samples, respectively, were found positive for HEV (Crossan et al., 2012; Hansman et al., 2008). In the study from United Kingdom, 26 samples were collected near an outlet of wastewater from a pig processing plant possibly supplying a constant outlet of HEV. The possible continuous delivery of this wastewater, could explain the high prevalence even if HEV does not persists for long in shellfish.

The fact that PCV2, but not HEV, was detected in the Danish mussels could be due to a potential selective accumulation of PCV2 (Burkhardt and Calci, 2000). In a study by Sobsey et al. (1987) the depuration of HAV and poliovirus was significantly different with poliovirus being removed from the shellfish at a much higher rate than HAV. Furthermore, different species of oysters have shown different ability to accumulate and depurate viruses. As an example, the oyster *Crassostrea ariakensis* was shown to more efficiently accumulate NoV, murine norovirus 1 and HAV than *Crassostrea virginica* (Nappier et al., 2008). Moreover, a difference in accumulation and persistence between different strains of NoV in a single species of shellfish has been shown (Le Guyader et al., 2012). This can be explained by a difference in ligand/receptor affinity, meaning that certain shellfish species have receptors that bind certain strains of viruses. This is relatable to RV-A that has 14 different G-types referring to its VP-7 viral particle component and 22 P-Types referring to its VP-4 protein of the viral particle, making it possible that certain G and P types of RV-A is preferentially accumulated. In one study, the G and P type detected in oysters were not genotypes typically associated with pigs (Le Guyader et al., 2008). In a study on Japanese clams (*Corbicula japonica*) G3 and G4 were found (Hansman et al., 2008), which are two of the four primary genotypes found in pigs (Midgley et al., 2012). These are however also prevalent in humans (Santos and Hoshino, 2005). Similarly the high prevalence of PCV2 in the mussels assayed in this study could be due to preferential binding in the digestive tissue of the mussel as well as high stability of the virus. More investigations of the interaction between PCV2 and mussels are needed to determine if PCV2 simply are extremely well bound to the mussel tissue or if the observed prevalence is caused by more frequent introductions or introductions at higher loads compared to other porcine enteric viruses.

A number of factors can contribute to the contamination of PCV2. The positive samples were exclusively found in confined water bodies such as fjords and bay area. Both serve as an outlet of streams making the contact area of agricultural farmland vast. The pollution from mainly agriculture (water drained from fields and spillover) and wastewater to Danish streams and fjords have been a concern for decades because pesticides and nitrate has been shown to enter these reservoirs (Kronvang et al., 2003). Therefore it was not surprising to find PCV2 positive samples in these areas. Another possible factor is season. Positive samples were mostly detected in spring/early summer and in early fall, which is consistent with periods of the delivery of slurry to farmland in Denmark.

To our knowledge this is the first report of PCV2 in shellfish. More information is needed regarding accumulation and persistence of this type of viruses in blue mussels and other species of shellfish. However, based on the detection of high number of PCV2 positive samples in hygienically clean shellfish, PCV2 should be considered as a new host specific and sensitive marker, to source identify porcine waste contamination of shellfish. This information can be used to evaluate the hygienic quality of shellfish, but also to rectify problems with spill over from porcine industry and agriculture. PCV2 is not a virus which is of public health concern, but other single stranded circular DNA viruses such as Torque Teno-like viruses have been found in pigs and in humans, so the interspecies transmission and possible reservoirs of this type of viruses should be monitored more closely (Kekarainen and Segalés, 2012; Li et al., 2010).

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