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Short communication

Enteric porcine viruses in farmed shellfish in 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).

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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.