PRELIMINARY RESULTS FROM A SURVEY OF OYSTER PRODUCTION AREAS IN IRELAND FOR NOROVIRUS

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Abstract

A survey of 18 oyster production areas in Ireland for norovirus (NoV) contamination was initiated in August 2006. The findings presented are the preliminary results from the first seven months of the survey. Prior to the survey commencing, a simple desk bask sanitary survey of each area was undertaken. This provided an assessment enabling each site to be ranked into 3 categories (low, medium and high) on the basis of the risk of NoV contamination. Samples were collected on a monthly basis and tested for the presence of NoV using semi-quantitative real-time PCR allowing relative quantitation of NoV levels. A correlation was observed between occurrence and levels of NoV detected and the risk categories ascribed to each production area. To date NoV was detected in 60.7, 30.0 and 2.5 percent of samples from the high, medium and low risk categorised areas, respectively. A strong seasonal bias towards increased winter contamination was observed with NoV detected in 15.5 and 50 % of samples in August and February, respectively. The preliminary results from this survey indicate that it may be possible to predict the relative risk of NoV contamination in a shellfish harvesting area. This in conjunction with targeted NoV monitoring using real-time PCR could aid the further development of risk management procedures in shellfisheries.

Introduction

There is currently no standard in EU legislation regarding the contamination of bivalve molluscs with human pathogenic viruses, namely norovirus (NoV) and Hepatitis A virus (HAV). However, the introduction of specific virus controls once standardised methods are available is viewed as a high priority. In recognition of this a CEN working group (TC 275/WG6/TAG 4 – Detection of viruses in food) in Europe is currently developing a standardised method based on quantitative real-time PCR for the detection of NoV and HAV in food, including bivalve molluscs. The work of this group represents a concerted effort

towards the technical feasibility required for the inclusion of a virus standard in future EU legislation. However, uncertainty still remains about what such a virus standard should be and the impact on both public health and the shellfish industry.

Previous surveys of oyster production areas in Ireland and elsewhere in Europe have generally been restricted to problematic harvesting areas that have been involved in outbreaks of illness or that are known to be highly polluted. Consequently, high levels of virus positive results have been recorded in these studies. In a study undertaken in Ireland over the winter of 2002/03, shellfish were tested for NoVs from 8 suspected problematic sites. NoV was detected by reverse transcription (RT)-PCR in 59 % of all samples tested during the study period (unpublished data). In the UK, Henshilwood *et al.* (1998) identified NoV by RT-PCR in 56 % of all samples analysed from one category B classified site. These surveys shared a fundamental characteristic in that analysis was biased towards sites impacted by sewage pollution or associated with outbreaks of illness. Therefore, in this study a survey of oyster production areas in Ireland was conducted to provide information on NoV in contamination in oysters from a range of representative sites using a real-time PCR method allowing relative quantitation of NoV levels. An additional feature of this survey was to consider the relative risk of virus contamination in shellfish harvesting areas by taking into account the occurrences and impacts of potential NoV contamination in an area in a desk based study.

The two main objectives from this survey were: (i) to gather information on the relative levels of NoV found in oysters in Irish shellfish harvesting areas using semi-quantitative real-time PCR, and (ii) could a simple risk matrix approach using existing data in a desk based study be used to determine the relative risk of NoV contamination?

Methodology

A desk-based sanitary survey of each of the production areas was undertaken using available local information and data previously collected as part of the implementation of the Shellfish Waters Directive. Prior to the survey commencing each site was ranked according to the expected extent of virus contamination based on factors such as population density, proximity to waste water treatment plants and level of treatment. Subsequently each site was categorised into three levels of risk of NoV contamination, namely low, medium and high (Table 1). Included in the 18 sites were two non-commercial control sites, sites 1 and 2, that were highly impacted by secondary and primary treated waste-water, respectively. The *E. coli* results for

site 1 and 2 complied with category C and B classifications respectively under EU regulations.

Sampling of the 18 oyster sites commenced in August of 2006. Alongside monthly samples of oysters collected routinely for the *E. coli* classification programme an additional 24 oysters were collected. Samples were transported to the laboratory by courier and were received within 48 hours under chilled conditions (< 15ºC). The hepatopancreas from 6 oysters were analysed for NoV using previously published real-time PCR procedures. (Boom *et al.,* 1990; Jothikumar *et al.,* 2005). Three replicates were tested for each sample in the real-time PCR assays for NoV GI and GII. The resulting C_T values were converted to PCR units. This makes use of the weighting provided by the number of positive replicates to yield a more usable value. A PCR unit is converted from the C_T value by assigning a value of 100 PCR units to a C_T value of 37. An increase in C_T value of 1 is considered to correspond to a dilution of 1 in 2 and therefore a C_T value of 38 would be assigned a PCR unit of 50, etc. The three replicates are assigned values in this manner and an average was taken, this was the final PCR unit assigned to that result. In this way a sample with C_T values of 37, 0, 0 is given a PCR unit value of 33.3 whereas a sample with C_T values of 37, 37, 37 would be assigned a PCR unit value of 100, thereby weighting the fact the sample tested with three positive C_T values.

Results

Preliminary results from samples collected during first seven months of the survey (August 2006 – February 2007) are presented. A good correlation between the risk categories ascribed to production areas and the frequency of real-time PCR positive results for NoV was observed from each category. Table 2 demonstrates a decrease in the percentage of NoV positive results as the potential risk of NoV contamination decreases from high to low. Just over 60 % of all samples tested in the high risk category were positive for NoV GII, however, if the two non-commercial control sites are removed from this risk category the level of positives recorded in this group is reduced to 46.1 %. By contrast 33.3% and 2.5% of samples were positive for NoVs in the medium and low risk sites, respectively.

In addition to the frequency of NoV contamination and the relative levels of NoV GII in each site as judged by the PCR units recorded a clear correlation was observed with the risk category ascribed. In the high risk category NoV levels were significantly higher than in the medium and low risk sites, with the highest levels recorded over the winter months

(November 2006 – January 2007) (Table 3). This seasonal trend was also observed in the medium risk category where higher levels of NoV GII were recorded in January 2007 (Table 3). Significantly a high proportion of samples over the study period to date were negative for NoV. In particular in the low risk category areas all sites, apart from one, remained free of NoV contamination to date (Table 3). On the whole these preliminary results demonstrate that 50 % of commercial sites analysed in this study were continuously free from NoV contamination even during the higher risk winter period covered in this report.

Discussion

The first objective of this study was to gather information on the relative levels of NoV found in oysters from Irish shellfish harvesting areas. This study represents the first use of semi-quantitative real-time PCR for the detection of NoV applied to an extensive survey of shellfish harvesting areas in Ireland. Preliminary results demonstrated that this technology provided reliable and robust information on the relative levels of both NoV GI and GII between monthly samples from the same site, as well as between different sites in the study. Previous studies in Ireland investigating sites which were selected because they were suspected of being at a high risk of contamination resulted in 59 % of all samples tested being positive for NoV by non-quantitative nested RT-PCR (unpublished data). In this study preliminary results obtained for NoV indicate that 50 % of the commercial sites were continuously free from NoV contamination even during the higher risk winter period. Previous studies across Europe have demonstrated an association with season and NoV contamination (Le Guyader *et al.,* 2000; Myrmel *et al.,* 2004; Croci *et al.,* 2007). The effect of season was also observed in this survey, where a steady increase in the frequency of NoV positive results recorded was observed as the survey entered the winter (Table 3).

The second objective of this study was to see if a simple risk matrix approach using existing data in a desk based sanitary survey could be used to determine the frequency and level of NoV contamination in shellfisheries. All 18 sites were assessed for the risk of NoV contamination in each area using simple and readily available data. Each site was ranked according to perceived risk and subsequently categorised into low, medium and high risk of NoV contamination sites. Results obtained to date indicate that using this simple approach that it could be possible to predict the relative risk and extent of viral contamination in shellfish harvesting areas based on this simple risk assessment approach. Risk assessment of virus contamination in specific shellfisheries represents an important initial step in developing

site-specific risk management procedures in shellfisheries. The use of a simple desk based risk assessment approach aligned with viral monitoring may represent a relatively cost effective initial route to developing risk management procedures in shellfisheries.

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Table 1. Desk based evaluation of NoV occurrence in each of the survey oyster production areas. Each site was ranked in order of the likely risk of NoV contamination determined by the desk based sanitary survey. Sites were subsequently categorised into high, medium or low risk of NoV contamination. The E. coli classification status of each area is given in brackets beside site number. Factors characterising the areas in each category are also given.

Site	Risk factors
High risk of NoV contamination	
1 (C)	• Close proximity to urban areas • Impacted by largest population numbers • Minimal sewage treatment in area • No sewage treatment in area
2 (B)	
3 (B)	
4 (B)	
5 (B)	
Medium risk of NoV contamination	
6 (B)	• Smaller population numbers • Minimal sewage treatment in area • Level of sewage treatment unknown in area
7 (B)	
8 (B)	
9 (B)	
10 (B)	
Low risk of NoV contamination	
11 (B)	• Low population numbers in area • No immediate population in area • Low risk of sewage contamination (septic tanks) • No immediate risk of sewage contamination
12 (A)	
13 (A)	
14 (A)	
15 (B)	
16 (B)	
17 (A)	
18 (B)	

Table 2. NoV GI and GII levels (PCR units) recorded for each risk category

		NoV GI PCR units				**NoV GII PCR units**			
Risk	**Sites (n)**	**Geo. mean**	**Min**	**Max**	**% Pos.**	**Geo. mean**	**Min**	**Max**	**% Pos.**
HIGH	1 –5 (28)	2.5	<25	259	25.0	46.2	<25	2753	60.7
MED	6 –10 (26)	0.6	<25	42	12.5	1.0	<25	182	30.0
LOW	11 – 18 (43)	0.0	<25	<25	0.0	0.2	<25	79	2.5

Table 3. Preliminary results from NoV survey (August 2006 to February 2007). The frequency of NoV positive results and relative levels (PCR units) are shown for monthly samples from each site in each risk category.

Site	Aug 06	Sep 06	Oct 06	Nov 06	Dec 06	Jan 06	Feb 06
High risk of NoV contamination							
***1 (C)**	**1125**			**133**	**1866**	**2753**	**497**
***2 (B)**	**353**		**41**	**214**	**215**	**2470**	**643**
3 (B)					**53**	**25**	**89**
4 (B)	No sample	No sample	**81**	**1070**		No sample	No sample
5 (B)	No sample	No sample	No sample	No sample		**96**	
Medium risk of NoV contamination							
6 (B)	No sample			No sample	No sample	**182**	**42**
7 (B)				No sample		**42**	
8 (B)	No sample		**28**	No sample	No sample	**29**	**30**
9 (B)					**63**	No sample	**167**
10 (B)			No sample		No sample		No sample
Low risk of NoV contamination							
11 (B)	No sample	No sample			No sample		
12 (A)		No sample		No sample	No sample		No sample
13 (A)				No sample			
14 (A)							**79**
15 (B)		No sample		No sample	No sample		No sample
16 (B)							
17 (A)							
18 (B)							
% Pos	**15.5**	**0.0**	**18.8**	**25.0**	**30.8**	**43.8**	**50.0**

* = *Positive control sites,* ☐ = *<25 PCR units,* ☐ = *25 – 99 PCR units,* ☐ = *100 – 999 PCR units,* ■ = *>1000 PCR units*

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