An outbreak of norovirus genogroup II associated with New South Wales oysters

Tove-Lysa L Fitzgerald, Anthony Zammit, Tony D Merritt, Catherine McLeod, Lina M Landinez, Peter A White, Sally A Munnoch, David N Durrheim

Abstract

Introduction: Currently available antigen tests for norovirus (NoV) have excellent specificity but negative results do not always rule out infection. Real-time reverse transcription polymerase chain reaction (RT-PCR) is a useful method for detecting and genotyping NoV in humans and oysters. An outbreak of NoV associated with oyster consumption in northern New South Wales confirmed the value of real-time RT-PCR where immunochromatography (ICT) tests were negative.

Methods: Eight cases of gastrointestinal illness in northern NSW, clinically suggestive of NoV infection, were associated with consumption of oysters. A joint environmental investigation was conducted by the New South Wales Food Authority and local council. One human sample was collected and tested for NoV using ICT and real-time RT-PCR. Oyster samples were tested for NoV utilising real-time RT-PCR.

Results: The patient with a stool sample had NoV genogroup II (GII) confirmed by real-time RT-PCR after testing negative by ICT. Illness in all cases was consistent with NoV with median incubation and duration of 36 and 50.5 hours respectively. All cases consumed oysters that were harvested from the same area. Three oyster samples from the harvest area were also positive for NoV GII. A nearby leaking sewer line was identified as the likely source of the contamination with hydrological studies confirming its potential to contaminate implicated oyster leases.

Conclusion: This investigation confirmed the value of real-time RT-PCR testing of human specimens where ICT tests are negative and clinical illness is suggestive of NoV infection. NoV real-time RT-PCR and epidemiological evidence effectively linked human infection with oyster contamination to motivate a thorough environmental investigation and appropriate action to mitigate further public health risk. *Commun Dis Intell* 2014;38(1):E9–E15.

Keywords: oyster, outbreak, norovirus, RT-PCR, genotyping, epidemiology

Introduction

Norovirus (NoV) is a highly infectious pathogen that causes acute gastroenteritis in humans.[1] It is the most frequently identified cause of gastroenteritis in the community and institutional settings in Australia.[2,3] NoV is robust and may survive in marine environments in high concentrations if sewage is released, thus posing a contamination threat to shellfish.[1] Oysters have previously been identified as a transmission vehicle in NoV outbreaks.[4–6]

During an outbreak investigation, the detection of the same pathogen in human cases and epidemiologically implicated food assists investigators to implement appropriate public health action. This is particularly useful when the pathogen is indistinguishable in food and clinical samples using a discriminating sub-typing method. Australia has only recently developed the capacity for NoV detection and sub-typing in oyster tissue. Previously, oyster samples were processed in New Zealand laboratories with resultant delays in withdrawal of product contaminated with norovirus.[6]

The methods available for the detection of NoV in human faeces include electron microscopy, real-time reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assays (ELISA) and immunochromatographic tests (ICT).[7] ELISA and ICT methods are relatively quick and inexpensive to use but the sensitivity of ELISA varies from 36%–90% while that of ICT varies from 57%–90%.[7,8] They are useful when screening large numbers of faecal samples where a single false negative is less critical. Real-time RT-PCR is resource intensive but is more sensitive (91%–98%)[9], and is useful for assessing critical samples that are clinically suspicious but negative using ELISA or ICT. In oysters, real-time RT-PCR is the preferred method for detecting NoV, as viral concentrations are generally much lower than those found in clinical specimens.[1]

There are currently 6 recognised NoV genogroups (G), three of which cause human infection (GI, GII and GIV).[10] The NoV GII currently accounts for most (>80%) human infections.[1] Both NoV GI and GII have previously been implicated in outbreaks associated with oyster consumption. It is not uncommon to find both genogroups in oyster samples collected during outbreak investigations.[5,6]

We report a NoV outbreak associated with New South Wales oysters where the value of real-time RT-PCR and genotyping was proven; real-time RT-PCR allowed confirmation of human infection where ICT tests were negative, and genotyping of both the human and oyster samples supported the epidemiological link between NoV infection and oyster consumption.

Methods

Ethical approval for this investigation was not required under the *NSW Public Health Act, 2010*.

Epidemiological investigation

On 30 October 2012, the Hunter New England (HNE) OzFoodNet site was notified by the New South Wales Food Authority (NSWFA) of gastrointestinal illness in 6 people from a cohort of 30 that had attended a social event at a caravan park between 22 and 26 October 2012. The HNE OzFoodNet site is part of the national OzFoodNet network that is responsible for the investigation and management of foodborne illness in Australia. An additional 2 cases with similar symptoms, but with no links with the social event, were also notified to HNE OzFoodNet on 1 November 2012, by the NSWFA. All 8 cases were interviewed using a standardised questionnaire for suspected foodborne illness. The interviewers were trained in the use of the questionnaire. Details on demographics, symptom profile, onset, duration, contact with ill persons, social activities, accommodation, travel and a 3 day food history were collected. Stool specimens were requested.

A suspected case was defined as a person from the region of interest who reported vomiting and/or diarrhoea plus one or more of headache, fever, abdominal cramping, lethargy or joint/muscle pain with onset between 25 and 27 October 2012. A confirmed case was a person who met the suspected case definition, and who had NoV GII detected by real-time RT-PCR in a stool sample.

The HNE OzFoodNet team initiated active case finding utilising the Public Health Real-Time Emergency Department Surveillance System (PHREDSS) to identify gastroenteritis presentations at emergency departments in this health district and two adjoining health districts.[11]

Laboratory investigation

Human sample

Only 1 stool sample was collected. The sample was initially tested for NoV by ICT (SD-Bioline) at a local laboratory. The sample was then sent to the University of New South Wales (UNSW) school of Biotechnology and Biological Sciences (BABS) for real-time RT-PCR testing and genotyping as UNSW is the only place in New South Wales that has the capacity and the validated methods to perform NoV genotyping.[12] RNA was extracted directly from stool samples using QIAamp Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and suspended in 200 μL of elution buffer. RNA (10 μL) was converted to cDNA using the High Capacity Reverse Transcription Kit with RNase inhibitors (Applied Biosystem, Foster City, CA) according to the manufacturer's instructions. NoV GI or GII RNA was detected by real-time RT-PCR, and the products were purified, sequenced and genotyped as described previously.[12]

Oyster samples

Eleven samples collected on 5 November 2012 were analysed for the presence of NoV (GI and GII RNA), and for *Escherichia coli* as a recognised indicator of faecal contamination.[13] A further 8 samples collected on 14 January 2013 were analysed for NoV GI and GII RNA only. Samples collected were representative of the harvest area where the implicated lease was located. Not all leases in the harvest area could be sampled due to operational and resource constraints. Leases that were not sampled were either empty or contained immature stock. More samples were collected from leases with large volumes of saleable stock.

Samples were labelled and cold stored at 4°C for shipping to the laboratory. Once at the laboratory, the samples were assigned a laboratory number and stored at 4°C until processed.

The protocol used for NoV testing of the oysters was based on the International Standard horizontal method for detection of hepatitis A virus and norovirus in food matrices (European Committee for Standardization in collaboration with ISO, committee 34 – ISO/TC34).[14–16]

Briefly, the method consists of 5 steps: sample preparation, virus extraction, nucleic acid extraction, molecular detection using real-time RT-PCR, and result analysis. Samples were prepared by dissecting and finely chopping the digestive glands from the oysters. Proteinase K was then used to extract the virus from 2 g of the chopped digestive gland and the virus control (murine NoV) was added to determine the efficiency of the extraction. The Minimag® System (NucliSENS®) was used for RNA extraction using guanidine thiocyanate to disrupt the virus capsid and silica particles to adsorb RNA and to assist purification.[14–16]

Real-time RT-PCR was carried out in a 1 step process with a different set of primers/probes for NoV GI and GII (as specified in the ISO standard method). Each sample was tested in triplicate, and 2 serial dilutions were included. Positive and negative controls for each step of the method were included as per the ISO technical specification.

Environmental investigation

On 1 November 2012, the harvest area (Figure) was closed and a joint environmental investigation by the NSWFA and local council officers into the source of the contamination was initiated. All oyster farmers in the harvest area were contacted and advised to hold oyster sales at this time. A trade level product retrieval was commenced.

Amongst the 11 oyster samples collected for NoV analysis on 5 November 2012, sample size varied from 15 to 30 oysters, with 250 individual oysters collected in total.

Two samples were obtained from embargoed product held in an oyster farmer's cool-room on 2 November 2012 and 9 samples were obtained directly from the implicated oyster harvest area on 5 November 2012. The samples from the cool-room were harvested from the implicated area on 29 October 2012 and had been depurated for 36 hours.

Inspections of potential pollution sources were undertaken on 6 and 7 November 2012. All sewage pumping stations, manholes and on-site sewage management systems in the towns surrounding the implicated oyster leases were visually inspected for signs of recent discharge. A number of stormwater drains were sampled for faecal coliforms.

A local caravan park's reticulation system was targeted for further investigation due to the size of the system and its close proximity to the harvest area. On 27 November 2012 the NSWFA, in conjunction with local council officers, undertook dye testing of the system to check for leaks in the system.

On 20 December 2012, NSWFA officers conducted a basic hydrological study in relation to a subsequently identified sewage spill site. The study used marked oranges to provide a visual indication of river flow. The marked oranges were deployed at 8 am on 20 December (approximately 10 minutes after the turn of the tide) and observed for 6 hours.

On 14 January 2013, 8 samples of oysters were collected from leases located throughout the oyster harvest area for follow-up testing including 3 samples from the implicated leases. Each sample was analysed by real-time RT-PCR for NoV GI and GII RNA.

Results

Epidemiological investigation

No additional cases were detected through the PHREDSS review. A total of 8 cases were interviewed, with a median age of 69.5 years (range 64 to 77 years) and four were female. The median incubation period from oyster consumption to illness onset was 36 hours, with median illness duration of 50.5 hours. Symptoms included vomiting (6/8) and diarrhoea (8/8).

Case summaries

The 1st cases were a married couple and another adult who had stayed in separate self-contained motorhomes in the caravan park where the social event was hosted. Their only shared meal was 2 dozen oysters consumed at 2 pm on 24 October 2012. These oysters had been purchased directly from a local oyster supplier 2 hours earlier on the same day. Although the group had other social contact in the 4 days prior to onset, none had symptoms of gastroenteritis prior to, or during the shared meal. The onset of illness for this group was between 3 am and 9 am on 26 October 2012.

A further affected individual purchased and consumed 1 dozen oysters on 25 October 2012 at midday. These oysters were purchased from the same supplier as the first sub-group. He had not previously been ill and had no recollection of contact with ill persons prior to consuming the oysters. Although he was a participant in the social event, the only common link between this case and the first sub-group was consumption of oysters from the same supplier. His onset of illness was 3 am on 27 October 2012.

Two cases consumed oysters at a restaurant located in close proximity to the caravan park on 25 October 2012 at the same dinner service but at separate tables. The dinner service was at 7 pm. These 2 people had resided in two separate accommodation sites in the same caravan park during the social event. They had not had prior contact with each other nor with other cases attending the social event. This was the only meal that was common to both these people. The onset of illness in this group was between 7 am and midday on 27 October 2012.

Two further cases were a married couple who did not reside at the caravan park and were not associated with the social event at the caravan park. They purchased and consumed oysters from the same oyster supplier as the first 3 sub-groups on 25 October 2012 at 7.30 pm but also shared a number of additional food exposures prior to illness

onset. Their illness onset was identical at midnight on 26 October 2012. They did not report any contact with ill persons prior to their illness.

All cases reported oyster consumption with six of them purchasing oysters directly from the same supplier, while the trace back investigation identified that the oysters that the remaining 2 cases had purchased from a local restaurant were also sourced from that supplier. One stool sample was collected from a case in the first sub-group, who had recovered.

Laboratory investigation

Human specimen

The single human sample was negative for NoV by ICT, however, tested positive for NoV GII RNA by real-time RT-PCR. Sequencing and phylogenetics revealed the NoV was a GII.4 New Orleans 2009 variant.

Oyster specimens

A total of 19 samples of oysters were analysed for NoV GI and GII RNA. The results of the NoV analysis for 11 samples collected on 5 November are shown in the Table. None were positive for NoV GI by real-time RT-PCR. Three samples, collected from the western upstream section of the oyster harvest area were positive for NoV GII. It was not possible to genotype these samples due to the low levels of viral genetic material. *E. coli* was detected at 70 most probable number (MPN)/100 gram or less for the samples positive for NoV GII. There was no correlation between *E. coli* and NoV positive results. All samples collected on 14 January 2013 were negative for NoV GI and GII.

Environmental investigation

Of the 3 oyster samples that were positive for NoV GII, one was from the oyster farmer's coolroom (harvested on 29 October 2012, from the upstream section of the western side of the river) and the other 2 positive samples were collected on 5 November 2012 with one taken from each side of the river (Figure).

Follow up investigations revealed that the NoV positive oyster sample from the eastern side of the river had only recently been moved there from the western side of the river. All 3 positives samples were traced back to the western upstream section of the oyster harvest area.

On 6 and 7 November 2012, all sewerage pumping stations and on-site sewerage management systems in the proximity of the harvest area were found to be well maintained, with no signs of overflow, leakage or discharge to the environment. The results of the stormwater sampling were generally low (in the range of < 2–130 coliforms/100 ml) with one sample result of >200 faecal coliforms/100 ml from a stagnant pond attributed to contamination by duck faeces. None were suggestive of sewage ingress into the storm water system. The dye study conducted on 27 November 2012 indicated that there were no leaks in the caravan park reticulation system.

On 2 December 2012, a sewage leak was detected in a main sewerage line immediately east of the road bridge after flow from the leak caused subsidence to a section of road adjacent to the bridge (Figure). The leak was just upstream of where the caravan park line enters the system preventing its detection in the dye tracing study. The leaking sewerage line was promptly repaired by council on the same day.

The marked oranges released near the discharge point of the leaking sewerage line on the eastern side of the bridge drifted towards the centre of the river and proceeded down the main channel (Figure).The oranges by-passed the leases on the eastern side of the river, keeping to the main channel in the centre of the river. About 1.8 km downstream of the bridge the oranges started to drift apart. By 2 pm oranges were observed on both sides of the river in line with the leases implicated in the initial illness outbreak. The prevailing wind during the study was generally westerly at about 7 km per hour, which would have influenced the drift of the oranges in an easterly direction.

Applying a precautionary approach, all leases in the implicated area were initially closed with product that had been released to the market recalled on

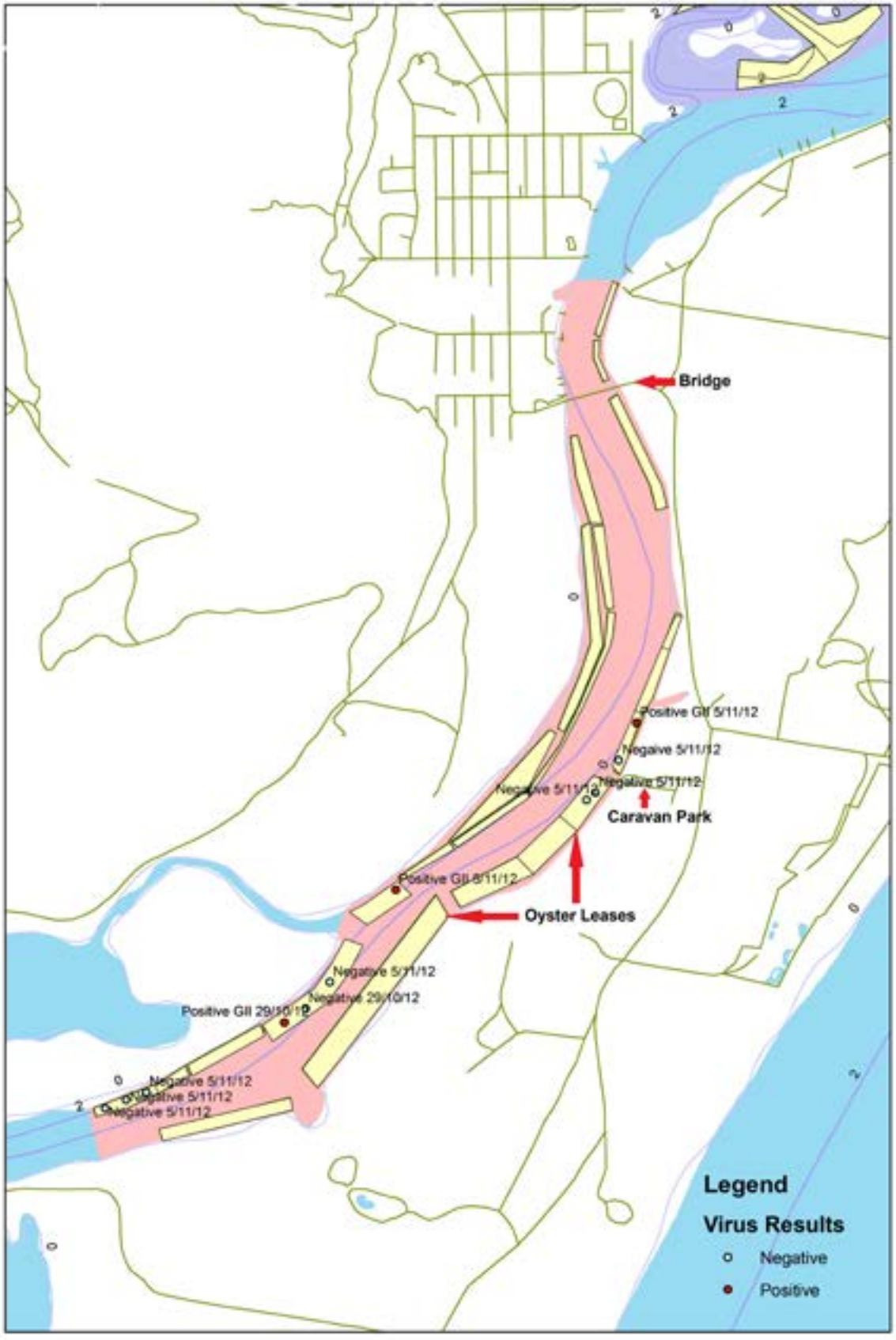
Table: Oyster sampling results, November 2012, implicated harvest area

ID	*E. coli* MPN/100 g	NoV GII	NoV GI
1	<20	Positive*	<LoD
2	<20	<LoD	<LoD
3	40	Positive*	<LoD
4	<20	<LoD	<LoD
5	<20	<LoD	<LoD
6	70	Positive*	<LoD
7	310	<LoD	<LoD
8	70	<LoD	<LoD
9	40	<LoD	<LoD
10	160	<LoD	<LoD
11	110	<LoD	<LoD

* Cyclic threshold values over 39 were considered positive values. In this case, cyclic threshold values ranged from 37 to 39.

LoD Level of detection

Figure. Map of the oyster harvest area



1 November 2012. Only a small amount of product was recalled as there was a limited amount of product in the market at the time of recall. The oyster leases with confirmed contamination were closed for 3 months. Surrounding oyster leases that were not implicated were cleared to resume operation by the NSWFA once the initial environmental investigation was completed.

Discussion

NoV GII RNA was detected in the human and oyster samples, supporting the epidemiological link between cases and oyster consumption. Further genotype discrimination beyond GII was only conducted on the human sample. It is possible that the genotype in the oyster tissue may have differed to that of the human sample, which was GII.[4]

Other possible sources of contamination included pollution events from passing marine vehicles, stormwater and the caravan park sewerage system. However these sources were investigated and deemed unlikely to be the cause of the oyster contamination. The main sewer line had been leaking for some time as evidenced by the road subsidence and the hydrological study was consistent with tidal flows over the implicated oyster lease.

Closure of the harvest area based on epidemiological and trace back evidence occurred on 1 November 2012 in accordance with the requirements of the Australian Shellfish Quality assurance Program (ASQAP).[17] A trade level product retrieval was conducted at this time and oyster farmers who were operating in the same river ceased trading voluntarily until environmental and laboratory investigations were complete. The real-time RT-PCR results identified which oyster leases were affected and provided further evidence to support and maintain the closure of these leases. This was important to prevent further cases and ensure that actions comply with the ASQAP requirements.[17] It was also important as the public health action had trade and financial implications for all of the oyster producers in the harvest area.

Although real-time RT-PCR successfully detected NoV GII in the oyster samples, further subtyping was not possible due to low viral levels. Factors affecting NoV detection in oysters include low viral levels, variability in the NoV genome and the complex extraction process, as well as inhibitory substances that interfere with real-time RT-PCR detection.[1,18] Despite these factors, real-time RT-PCR remains an internationally recognised and validated method of norovirus detection in oysters.[13–16] Further genotyping of the oyster samples to identify the strain beyond the genogroup level would have been useful.

In this outbreak only 1 human sample was collected. It is possible that this may have introduced a selection bias, however, all cases had consumed oysters from the same supplier and the onset of illness in cases reflected the recognised incubation period of norovirus. The sample was positive for NoV RNA by real-time RT-PCR but negative for NoV antigen by ICT (SD-Bioline). This highlights the limitations of ICT for detecting NoV in sporadic cases. Studies that have examined the sensitivity of antigen methods recommend collecting multiple samples to increase the probability of NoV detection, however, this can be difficult in practice.[8,19]

The environmental investigation quickly eliminated a number of potential pollution sources, including overflows from sewerage pumping stations, manholes and domestic on-site sewage management systems.

The results of the basic hydrological survey, coupled with the information from the laboratory analysis and the epidemiological data provided evidence that the leaking sewer line at the bridge was the most likely source of the viral contamination that caused the outbreak. The distance between the impacted leases and the source of the sewage discharge (2.5 km) and the low volume of the sewage discharge (~20 litres per hour) demonstrates the sensitivity of shellfish harvest areas to sewage contamination.

The negative virus results from the follow-up samples collected on 14 January 2013, verified the most likely source of contamination had been successfully remediated following the repair of the sewerage pipe. Subsequently, the affected leases were cleared to resume operation under standard quality assurance operating protocols.[17]

The risk of NoV contamination of oysters is best mitigated by controlling pollution sources. Australia currently has limited laboratory capacity to test oysters for NoV using real-time RT-PCR, which may lead to delayed results in the context of a large outbreak. In the future, cost effective molecular testing for human viruses could be considered for inclusion in oyster quality assurance programs as faecal coliforms and *E. coli* are poor predictors of viral contamination and depuration does not reliably clear viral pathogens, as demonstrated in this investigation.[13,20,21]

Conclusion

The findings of this investigation suggest that real-time RT-PCR testing of human specimens may be valuable where ICT tests are negative, but clinical illness is suggestive of NoV infection. NoV real-

time RT-PCR assisted in linking human infection with oyster contamination and this, together with good descriptive epidemiology led to a thorough environmental investigation and appropriate action to mitigate further public health risk.

Author details

Mrs Tove L Fitzgerald, Epidemiologist,[1,2]
Mr Anthony Zammit, Manager[3]
Dr Tony D Merritt, Public Health Physician[1]
Ms Catherine McLeod, Sub Program Leader, Seafood[4]
Ms Lina M Landinez, Research Officer, Food Safety[4]
Professor Peter A White, Professor[5]
Mrs Sally A Munnoch[1]
Professor David N Durrheim, Service Director, Health Protection[6]

1. OzFoodNet, Hunter New England Population Health, Wallsend, New South Wales
2. National Centre for Epidemiology and Population Health, Australian National University, Acton, Australian Capital Territory
3. NSW Shellfish Program, New South Wales Food Authority, New South Wales
4. South Australian Research and Development Institute, South Australia
5. School of Biotechnology and Biomolecular Sciences
6. Hunter Medical Research Institute, Newcastle, New South Wales

Corresponding author: Mrs Tove Fitzgerald, Epidemiologist, HNE OzFoodNet, Hunter New England Population Health, Locked Bag 10, Wallsend, NSW, Australia, 2287. Telephone: +61 2 4924 6477. Email: ToveLysa.Fitzgerald@hnehealth.nsw.gov.au

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