DETECTION OF HUMAN VIRUSES IN SHELLFISH AND UPDATE ON REDRISK RESEARCH PROJECT, CLEW BAY, CO. MAYO.

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Introduction

Bivalve shellfish are filter feeders and can accumulate human pathogens when grown in sewage-contaminated waters. This, allied to the fact that they may be consumed raw, means that they can present a public health risk. Current control measures rely on classification of harvesting areas based on their sanitary quality using *E. coli* as an indicator of sewage contamination. However, *E. coli* may underestimate the risk of viral contamination and outbreaks of viral illness can still occur.

In northern Europe gastroenteritis caused by norovirus (NV) is the most prevalent viral infection associated with shellfish consumption. As NV cannot be cultured, detection methods in shellfish have relied on the use of molecular techniques. In particular the use of the polymerase chain reaction (PCR) is common. Conventional PCR methods for detection of viruses in shellfish have a number of technical limitations, which have acted as a barrier to their application in monitoring shellfisheries for viral quality. However, more recently, the development of real-time PCR methods, has addressed some of these technical deficiencies and provides a more robust and reliable assay for detection of noroviruses in shellfish.

Given the continued public health risks associated with shellfish consumption, improved risk management procedures for controlling viral hazards are required. To address this, the Irish National Reference Laboratory (NRL) is involved in a European-wide project called Reduction of risk in shellfish harvesting areas (REDRISK). The REDRISK project aims to identify the main environmental risk factors causing viral contamination of shellfish to allow the development of improved risk management approaches during primary production of shellfish. The ultimate aim is to identify environmental conditions that can be monitored in real-time to determine when viral contamination may occur.

This paper describe the progress in norovirus detection methods and initial results from the REDRISK study.

Detection of norovirus in shellfish

Detection of NV in shellfish using molecular techniques is complicated compared with detection in clinical samples. This is because of the complex non-homogenous nature of the sample matrix which contains PCR inhibitors and the low levels of virus present. In addition, the existence of two virus genogroups further complicates the situation. A number of strategies have been developed to overcome these problems.

Virus and RNA extraction

All human viruses present in shellfish are restricted to the digestive tract, primarily the hepataopancreas, of the animal. In the procedure used the hepatopancreas of at least 6 animals (minimum weight 3 g) are isolated, finely chopped and treated with the enzyme proteinase K. The procedure allows extraction of the virus from the hepatopancreas, without homogenisation of the animal tissue and substantially reduces the amount of inhibitory material introduced into the assay.

The nucleic acid material in NV is RNA. Unlike DNA, RNA is not stable as it is prone to digestion by cellular enzymes. Therefore careful RNA isolation is a must for the preparation of template for the PCR assays. The application of commercially available kits used with clinical samples is limited due to the shellfish matrix. The method used by the Irish NRL is commonly referred to as the Boom method (Boom *et al.,* 1990). This facilitates extraction and purification of nucleic acid from a variety of sample types using guanidine isothiocyanate to denature cells releasing the nucleic acid. The RNA released from the virus particles is adsorbed onto silica particles to facilitate purification through several washing steps. RNA itself cannot be used as a template in PCR assays and therefore must be converted back or "reverse transcribed" to its complementary DNA (cDNA) copy using the enzyme reverse transcriptase.

DNA amplification and Real-time PCR

Once the cDNA is isolated it is necessary to amplify the DNA before it can be detected. The PCR amplifies a specific sequence of DNA using two short DNA sequences (primers) each of which is complementary to either end of the DNA target sequence. Figure 1 demonstrates the principle of PCR.

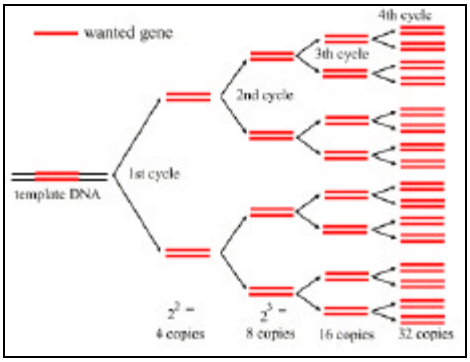


Figure 1. The polymerase chain reaction (PCR). This diagram represents the first four cycles of a PCR.

Real-time PCR makes use of sophisticated instrumentation to detect amplified PCR product in real time. In the TaqMan® PCR format an additional short DNA sequence (the probe) binds internally of the two primers. Two fluorescent labels are attached to either end of the probe sequence. The chemistry of the primer/probe arrangement is such that as the quantity of amplified product increases, the fluorescent signal also increases proportionately, therefore allowing the early stages of the PCR reaction to be detected. This allows the quantification of the initial starting material i.e. virus level. Separate assays have been developed for genogroup I and II NVs. The use of NV GI or GII specific probes allows for "in built" confirmation of a NV positive result without the need to sequence the PCR product.

The units of quantification in the real-time PCR are known as cycle threshold (Ct) values. The Ct value is the cycle number at which the fluorescence generated from the amplification of the target sequence crosses the threshold (Figure 2). **The lower the Ct value the more virus is present in the sample.** The quantity of target sequence i.e. virus copies, in the samples can be determined by extrapolation from the standard curve. Appropriate standards for the absolute quantification of NV are not readily available. However, close observation of the Ct values allows relative

quantification of the virus copy number. Although absolute quantification is not currently possible, results from trials within the Irish NRL indicate that an increase in the Ct value of 3 approximately equates to 10 fold increase in virus levels (Table 1). To date experience both in Ireland and in other European countries indicates that in general the level of virus detected in category B shellfish is at the limit of detection of the assay (Ct values of approximately 35-37). Ct values higher than this often results in NV being detected in only 1 or 2 of the 3 replicates as observed in Figure 3. It remains unclear as to whether these low levels of virus present a public health problem.

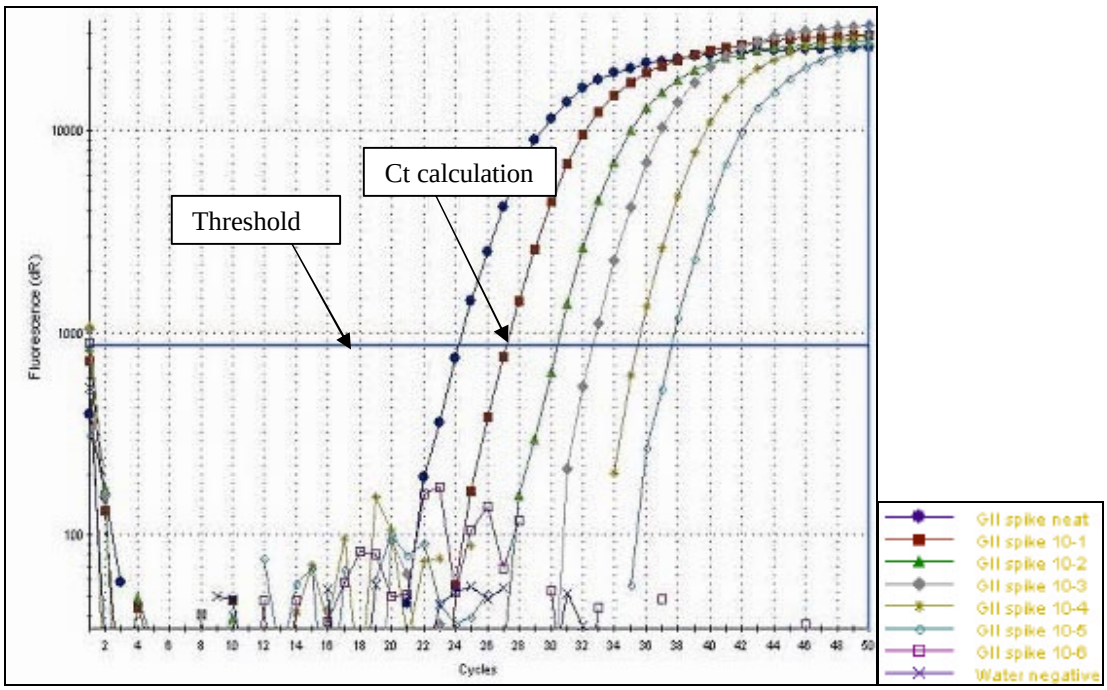


Figure 2. Amplification plots for NV GII assay demonstrating the threshold and calculation of the cycle threshold (Ct)

Table 1. Ct values for NV GI and GII detected in an oyster sample spiked with GI and GII positive stool sample

Spiked oyster sample	**Ct NV GI assay**	**Ct NV GII assay (Fig 4)**
Neat	23.7	24.2
10^{-1}	26.7	27.2
10^{-2}	29.3	30.3
10^{-3}	33.3	32.6
10^{-4}	35.8	35.3
10^{-5}	44.3	37.5
10^{-6}	No Ct	No Ct

NV negative oyster samples were spiked separately with NV GI and GII positive faecal material. Viral RNA was isolated and reverse transcribed to cDNA. The cDNA was serially diluted to 10-6 and all dilutions were run on the respective assays

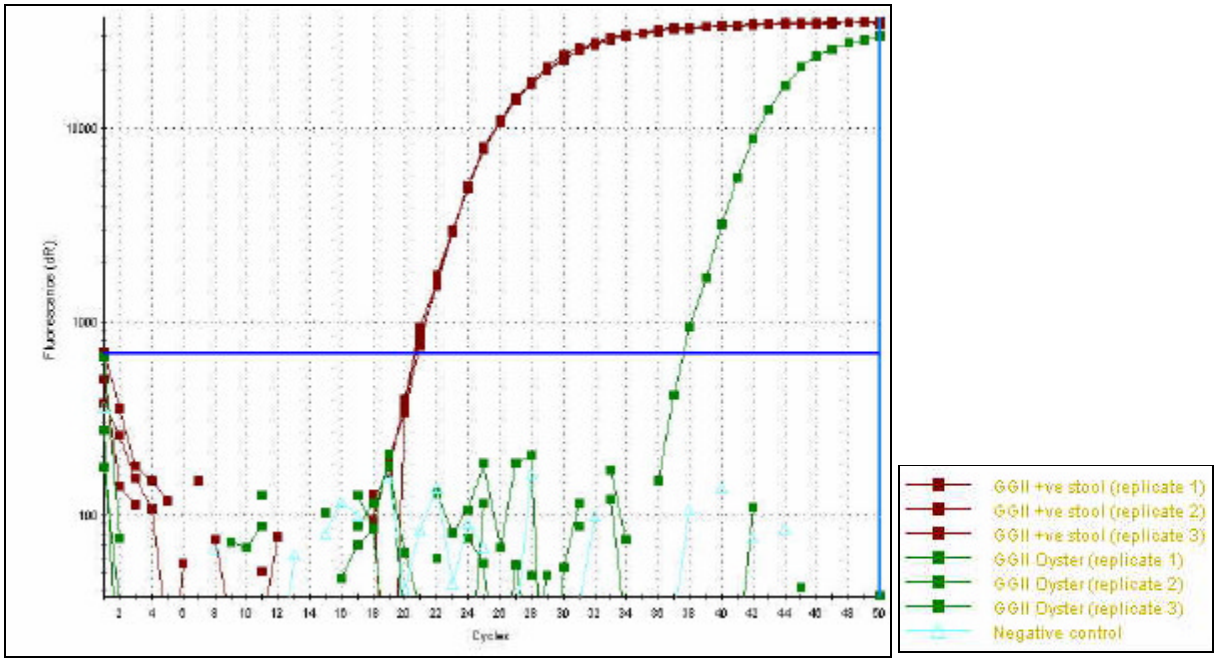


Figure 3. Amplification plots for NV GII identified in a stool sample (Ct average of 20.7) and an oyster sample. Note only 1 of the 3 replicates tested for the oyster sample was positive for NV GII with a Ct value of 37.5.

Real-time PCR Controls

The quantitative nature of the real-time PCR assay allows the use of accurate controls within the assay allowing greater standardisation and reliability. This is a major improvement over conventional PCR systems. A number of internal process controls have been introduced into the real time PCR assay.

Controls for the RNA extraction step ensure the successful isolation of viral RNA from the shellfish matrix and its subsequent reverse transcription into cDNA. The control used is another virus of the calicivirus family called feline calicivirus (FCV). A known amount of FCV is spiked into the sample homogenate. If the extraction step is successful, the level of FCV detected (as judged by the Ct value) is consistent with the previously determined Ct value for the amount of FCV added. If Ct values are higher than expected this indicates poor extraction of the RNA and requires repeat testing of the sample.

To control for inhibitors of the real-time PCR reaction an "internal exogenous control (IPC)" is used. This IPC kit contains a piece of DNA that is totally unrelated to the target DNA i.e. NV or FCV, and primers and probe designed to amplify this DNA only. The reagents for this assay are included when each sample is analysed for NV. If this IPC DNA target is not amplified in the reaction, this indicates the presence of inhibitors from the shellfish extract and if the IPC reaction has been inhibited it can be concluded that the NV reaction is inhibited also. These control measures provide confidence in the accurate detection of NV GI and GII from shellfish samples.

Norovirus negative and positive controls are also included in each assay run.

REDRISK project

The REDRISK project is part of the wider SEAFOODplus EU Framework six integrated research project. It is being carried out in four European countries, England, Spain, France and Ireland. In Ireland Clew Bay was selected as the study site because it has generally good water quality but within the bay there are also a variety of pollution sources, which may impact on its quality. There are areas classified as both Category A and B for shellfish production. The Clew Bay Marine Forum provides logistical support and local advice on the REDRISK project. Essentially the project is divided into two parts:

- A data collection programme to investigate possible sources of pollution and collect environmental data
- A sampling programme collecting shellfish for microbiological analysis

Environmental data collection and site selection:

Clew Bay is a westerly-facing bay made up of a complex series of islands and interlocking bays. The two main towns are Westport and Newport (Figure 4). Westport has a population of 6000 serviced by a new wastewater treatment plant (WWTP). Secondary treatment involving aeration and settlement is provided for a population equivalent of 15,000. Newport has a population of 600 but has only a very basic wastewater collection and treatment system and septic tanks.

Background data were collected to characterise the bay. This data comprised of human and animal population numbers (Central statistics office, CSO), *E. coli* results both historical and from ongoing monitoring (DCMNR), wastewater treatment details and licensed discharges (Mayo County Council), integrated pollution control licenses and river flow volumes (EPA), aquaculture production (BIM), hydrographic model details (MI) and health data from pharmacies. From a survey of this background data four sites were selected for further investigation. The characteristics of each of these sites are outlined in Table 2.

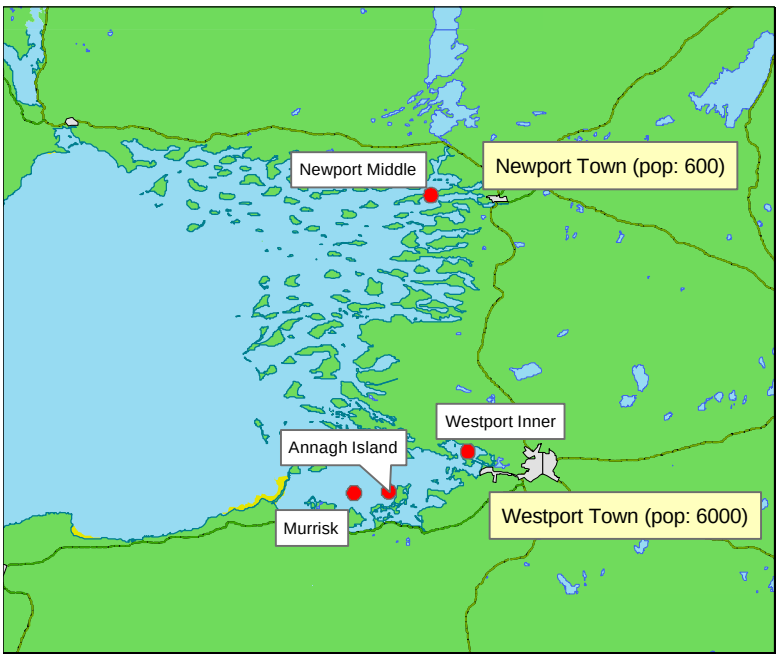


Figure 4. Clew Bay with major towns impacting the bay and sampling REDRISK points .

Table 2. Comparison of REDRISK sampling sites.

Westport Inner	**Annagh Island**	**Murrisk**	**Newport Middle**
Native and Pacific oysters	Native and Pacific oysters	Pacific oysters	Pacific oysters
Class B area	Class B area	Class A area	Class B area
300 m from WWTP outfall	3500 m from Westport town	4500 m from Westport town	1600 m from Newport sewage outfall (minimum treatment)
Population 6000	Local population 0	Local population low (some septic tanks)	Population approx. 600
Close to mouth of Carrowbeg river (average flow 1 m^3/s)	Some distance from two rivers of influence	Very little freshwater input	High freshwater input, Newport river (average flow 6 m^3/s)
Some agricultural input	Some animal farming nearby	High numbers of animals	Some agricultural input

WWTP = Waste water treatment plant

Oysters were placed at each site in conditions as close to natural conditions as possible.Pacific oysters (*Crassostrea gigas*) were placed at all four sites in growing bags, on tressles (Figures 5 and 6). At 2 sites, Westport and Annagh Island native oysters (*Ostrea edulis*) were placed in boxes on the bottom.

Figure 5 and 6. Fergal Guilfoyle (MI) and Mike Struth (CBMF) sampling Pacific oysters from tressle and sampling native oysters from "Ortek" box. Photos courtesy of Niall O'Boyle, CBMF.

Microbiological monitoring

Weekly sampling of oysters from the four sites began in August 2005 and 15 samples have been collected in 2005. Each oyster sample was tested for *E. coli*, FRNA bacteriophage and norovirus.

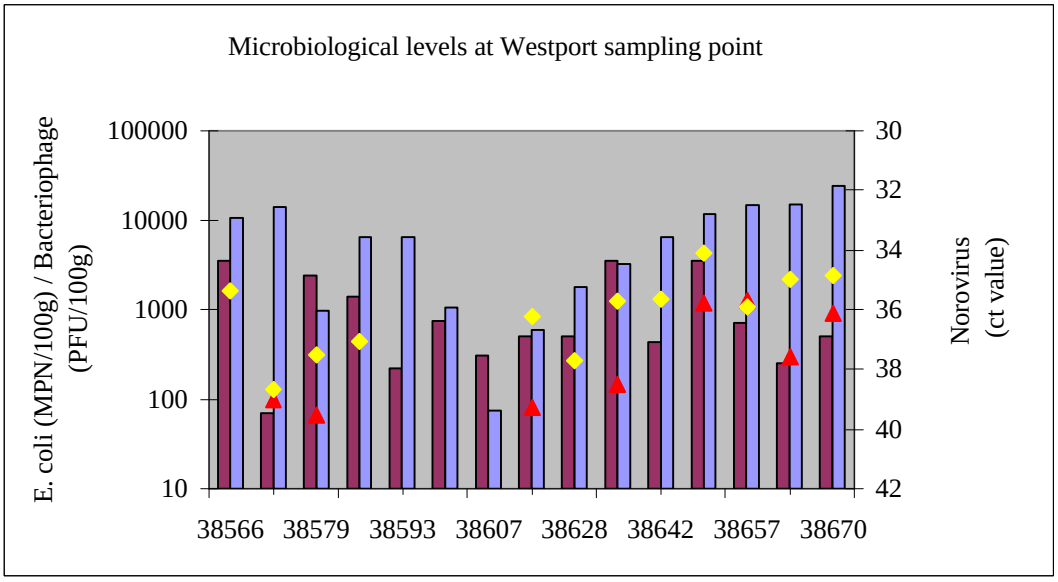
FRNA bacteriophage form a group of viruses which infect a group of bacteria and are found in high numbers in sewage, they are not pathogenic to humans and are considered to be a good indicator of viral contamination as they exhibits similar survival characteristics to pathogenic viruses i.e. norovirus.

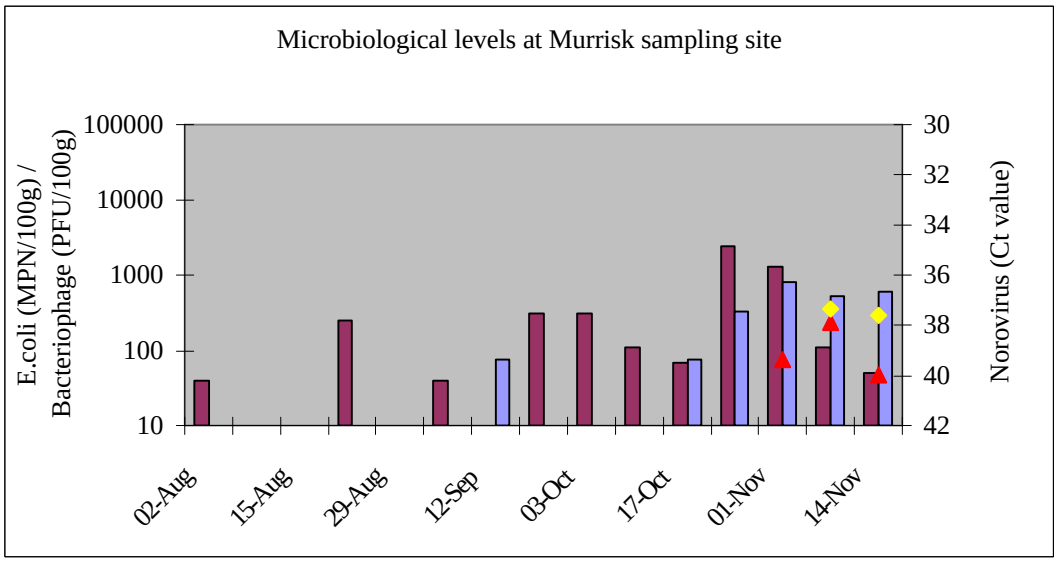
Environmental monitoring

Environmental data were also collected at each site, temperature is logged continuously at each site, salinity is collected at each site during sampling and turbidity is recorded during sampling. Riverflows are automatically recorded by the EPA, weather data was collected by MI/Met Eireann in Newport, wastewater treatment plant output volume is recorded by Mayo County Council and all the pharmacies in the area recorded sales of diarrhoea medicines.

REDRISK results (August – November, 2005)

The REDRISK project is ongoing and therefore only initial results are available. The preliminary results reported here are for two sampling sites (Murrisk and Westport) from August – November 2005. Figures 7 and 8 show levels of the different microbiological parameters from pacific oysters from these sites.





Figures 7 and 8. Levels of *E. coli*, (purple bar), FRNA bacteriophage (blue bar) and GI (red triangle) and GII (yellow diamond) norovirus in Pacific oysters (C. gigas) from Westport Inner and Murrisk. Ct values for norovirus have been inverted.

The initial results indicate;

1. Clear differences were observed in levels of both *E. coli* and FRNA bacteriophage in oysters collected from the Westport site and in oysters collected from the Murrisk site. This was consistent with the proximity of sources of sewage pollution to each site.
2. Norovirus (GI and GII) has been detected in oysters from the Westport Inner sampling site on a regular basis correlating with the levels of indicator organisms and demonstrating the potential risk associate with shellfish from this site.
3. The Ct values obtained for norovirus from August 2nd to October 17th indicates low level of virus contamination (average Ct value of 38). However, a decrease in Ct values (average Ct value of 35) from November 1st, may indicate a ten-fold increase in virus level. This is consistent with the known winter seasonality of norovirus infection in the community.
4. Samples collected from Murrisk contained low levels of *E. coli* and FRNA bacteriophage during the sampling period.
5. Norovirus was absent from shellfish in this area except for a contamination event in November. The Ct values at this time were high (near limit of detection) indicating a very low number of virus present.
6. Similar results have been observed by project partners in the contributing to the REDRISK project.

The next step is to further analyse the microbiological results with the environmental information gathered to establish if there is a relationship with viral contamination and environmental factors. Work to date has shown that there is possible link between river flow, wastewater treatment plant outfall volumes and levels of norovirus and microbiological indicators. However, further sampling and data analysis is required to establish this link. The REDRISK project will continue to collect data and test shellfish in 2006

Conclusions

The new real-time PCR method being deployed by the Irish NRL allows relative quantifcation of norovirus in shellfish samples for the first time. In addition, the introduction of quantitative controls within the assay allows better standardisation and increased degree of certainty of the result over conventional PCR procedures. The application of the real-time PCR procedure to shellfish samples is giving an increased understanding of the viral risk associated with shellfish.

Using the real-time PCR procedures to determine the relative levels of norovirus in shellfish during the REDRISK project will allow the possibility of identifying environmental factors responsible for viral contamination of shellfisheries. This may provide the framework for introducing improved risk-management procedures for controlling the risk associated with viral contamination in shellfisheries.

The authors would like to acknowledge the assistance of the following: Terence O'Carroll (BIM), Niall O'Boyle, Sean O'Grady, Mike Struth (CBMF), National Diagnostics Centre (NUIG), Hugh McGinley (EPA), Jimmy Carney (DCMNR) and Mayo County Council.

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