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Rapid and sensitive method to assess human viral pollution in shellfish using infectious F-specific RNA bacteriophages: Application to marketed products

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ABSTRACT

F-specific RNA bacteriophages (FRNAPH) have been used as indicators of environmental fecal pollution for many years. While FRNAPH subgroup I (FRNAPH-I) are not host specific, some FRNAPH-II and -III strains appear specific to human pollution. Because a close relationship has been observed between FRNAPH-II genome and human norovirus (NoV) in shellfish, and because FRNAPH infectivity can easily be investigated unlike that of NoV, the detection of human infectious FRNAPH could therefore provide a valuable tool for assessing viral risk. In this study, an integrated cell culture real-time RT-PCR method has been developed to investigate infectious FRNAPH subgroup prevalence in oysters. This rapid screening method appears more sensitive than *E. coli* or NoV genome detection, and allows an FRNAPH subgroup present in low concentrations (0.05 PFU/g of oyster) to be detected in the presence of another 1000 times more concentrated, without any dissection step. Its application to marketed oysters (n = 135) over a 1-year period has allowed to identify the winter peak classically described for NoV or FRNAPH accumulation. Infectious FRNAPH were detected in 34% of batches, and 7% were suspected of having a human origin. This approach may be helpful to evaluate oyster's depuration processes, based on an infectious viral parameter.

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1. Introduction

Bivalve mollusks have long been recognized to be involved in viral outbreaks, due to their ability to accumulate and concentrate microorganisms while filtering large volumes of water. Virus transmission through the consumption of shellfish is generally responsible for gastroenteritis (e.g. norovirus [NoV], Adenovirus) or hepatitis (e.g. hepatitis A virus) (Bellou et al., 2013; Koopmans and Duizer, 2004; Olalemi et al., 2016). Shellfish in contact with waters impacted by fecal contamination (e.g. urban wastewater treatment plant effluents), even during a short period of time (Humphrey and Martin, 1993), are thus mainly at risk (Le Guyader et al., 2000). Among them, oysters are most frequently involved because they are usually consumed raw. Actually, two major problems are repeatedly faced when evaluating the microbiological quality of shellfish.

First of all, the microbiological quality of shellfish harvesting areas in Europe currently depend on their levels of fecal pollution, estimated by *Escherichia coli* (*E. coli*) monitoring (European Parliament, 2004). However, this indicator have long been described as having limitations (de Mesquita et al., 1991; Richards, 1988), and now, it is widely accepted that its rapid depuration does not reflect the behavior of pathogenic viruses (Formiga-Cruz et al., 2002; Lees, 2000). Thereby, despite these controls, outbreaks associated with the consumption of oysters free from *E. coli* have been reported to occur, even after depuration process during an appropriate residence time (Le Guyader et al., 2008).

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Secondly, many waterborne viruses, especially NoV, are non-cultivable routinely. They are thereby generally targeted by genome detection using RT-PCR (Fuentes et al., 2014; International Organization for Standardization, 2013). It is now commonly admitted that negative or positive results are difficult to interpret regarding virus risk. On the one hand, the main part of the genome detected may correspond to non-infectious viruses because of its higher persistence (Gassilloud et al., 2003; Hartard et al., 2015; Ogorzaly et al., 2010). This may partly explain why NoV genome is often detected in oysters (Costantini et al., 2006; European Food Safety Authority, 2012; Lowther et al., 2012a, 2012b; Mesquita et al., 2011; Terio et al., 2010), whereas the number of reported cases of shellfish–associated NoV outbreaks is conflictingly low. On the other hand, taking into account the sample volume analyzed, the recovery rate, and the presence of RT-PCR inhibitors, the NoV detection threshold using the ISO/TS standard 15216-1 is around 1000 genome copies (gc)/2 g of shellfish hepatopancreas (International Organization for Standardization, 2013), while the estimated 50% infectious dose is approximately 18 infectious particles for NoV (Teunis et al., 2008). This may explain why RT-PCR sometimes failed to detect any genomes in specimens involved in NoV gastroenteritis outbreaks (European Food Safety Authority, 2012). In this context, other indicators must be investigated to assess the risk of infection in case of NoV genome positive results and may also be highly sensitive to overcome the false negative results.

Unlike *E. coli*, F-specific RNA bacteriophages (FRNAPH) are proposed as relevant indicators of viral contamination because of their structural similarity with the main waterborne pathogenic viruses. They are found in high levels in sewage, and may be easily detected by culture, giving information about infectivity. Several arguments justify particularly their detection in shellfish to evaluate virus fate (Doré et al., 2003; Doré et al., 2000; Flannery et al., 2009; Mieszkin et al., 2013). Indeed, these indicators are removed from shellfish more slowly than *E. coli* (Doré et al., 1998; Doré and Lees, 1995). Furthermore, while the presence of ligands promoting NoV accumulation in oysters has been described (Le Guyader et al., 2006; Tian et al., 2007), with a higher rate during the winter season (Maalouf et al., 2010), high accumulation of infectious FRNAPH has also been observed during this period (Burkhardt and Calci, 2000; Doré et al., 2003; Myrmel et al., 2004).

Detection of all infectious FRNAPH is however not recommended because only a few are of human origin (i.e. FRNAPH from subgroup II [FRNAPH-II] and FRNAPH-III) (Cole et al., 2003; Hartard et al., 2015; Schaper et al., 2002). Recent studies have particularly highlighted a close relationship between FRNAPH-II and NoV genome in shellfish (Flannery et al., 2013; Hartard et al., 2016) or in water (Vergara et al., 2015). If considering that FRNAPH-II and NoV have similar behavior, infectious FRNAPH-II can be seen as having strong potential for evaluating viral infection risk. Nevertheless, no rapid method allows the specific detection of infectious FRNAPH-II in shellfish, which are often hidden because of the high prevalence of non-human specific FRNAPH-I (Hartard et al., 2016; Hata et al., 2016). An easy-to-use method able to detect such phages would be very useful if applied to oysters free from *E. coli*, to indirectly assess the viral hazard. Their detection may also be helpful as viral surrogate during shellfish depuration processes.

The objective of this work was to develop a rapid screening method allowing the sensitive detection of infectious FRNAPH-II in oysters. One of the requirements of the method was the possibility to analyze a large sample volume with sufficient sensitivity, in order to lower the detection limit as much as possible. Another requirement was the capacity to detect a minority FRNAPH subgroup (e.g. FRNAPH-II), potentially hidden by the presence of a large number of phages from another subgroup (e.g. FRNAPH-I). Finally, the results had to be done within a working day. An Integrated cell culture real-time RT-PCR method (ICC-RTqPCR) was thus developed and has been used to evaluate the prevalence of infectious FRNAPH in fresh marketed oysters during a 1-year study period.

2. Materials and methods

2.1. Oyster samples

Oysters (*Crassostrea gigas*) were collected from several markets over a 1-year period (n = 135). Between 6 and 14 shellfish batches were collected monthly, depending on the season and market availability. Samples were kept at 4 °C for less than 3 h before shucking. Depending on their sizes, 3 to 5 specimens of the same batch were used for analysis. After shucking, the flesh and intravalvular liquid (FIL) of oysters was mixed for 3 min in a DT-50 tube with Ultra-Turrax® Tube Drive (IKA-Werke GmbH & Co. KG, Staufen, Germany). The oyster homogenate was then stored at −20 °C before analysis.

2.2. Infectious FRNAPH

Mixtures of environmental FRNAPH-I and/or FRNAPH-II strains isolated from a previous study (Hartard et al., 2015) were used to artificially contaminate the oyster samples used for the method development. Quantities used for the contamination were systematically checked by a double agar layer plaque assay method according to ISO standard 10705-1 (International Organization for Standardization, 2001).

2.3. Method development

Development of the method was performed using oyster batches collected in summer (considered '*a priori*' free from infectious FRNAPH). The results obtained for the detection limit allowed samples to be confirmed '*a posteriori*' as being effectively free from infectious FRNAPH.

Culture was performed in 100 mL Erlenmeyer flasks, in a 20 mL final reaction volume. The growing medium used was composed of 2 mL of a 10X tryptone-yeast extract-glucose broth (TYGB), with addition of 200 µL of a calcium-glucose solution prepared as described in ISO standard 10705-1 (International Organization for Standardization, 2001) and 80 µL of a 25 mg/mL kanamycin and nalidixic acid solution. *Salmonella enterica* serovar Typhimurium WG49 (NCTC12484) was used as the host strain (Havelaar and Hogeboom, 1984). A 2 mL aliquot of the bacteria suspension was added to the growing medium after a preliminary preculture performed as follows: inoculation was performed as described in ISO standard 10705-1 (International Organization for Standardization, 2001) but the 37 °C incubation was extended to 16–18 h to obtain stationary-phase bacteria, with a concentration of approximately 10^9 CFU/mL. Finally, 10 mL of the oyster homogenate was added to the flask and the volume was adjusted to 20 mL with PBS. After artificial contamination with different quantities of infectious FRNAPH, samples were incubated at 37 °C, under agitation (110 rpm), for 2, 4, 8, or 24 h in order to determine the optimal duration for biological amplification and the sensitivity of the technique.

2.4. Genome extraction and real-time RT-PCR

FRNAPH genome extraction was performed after biological amplification from 1 mL of the suspension. After centrifugation (18,000 × *g*, 3 min), 500 µL of the supernatant was collected and