

Research Article

Study on the Survival of Enteric Virus in Chilled and Frozen Oyster Meat Using MS2 Bacteriophage As a Surrogate

Manjusha Lekshmi^{1*}, Sanath H Kumar¹, Binaya Bhusan Nayak¹

¹Fish Processing Technology Department, ICAR-Central Institute of Fisheries Education, Versova, Mumbai-400061, India

*Correspondence author: Manjusha Lekshmi, Fish Processing Technology Department, ICAR-Central Institute of Fisheries Education, Versova, Mumbai-400061, India; Email: manjusha@cife.edu.in

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Abstract

This study utilized *Escherichia coli* bacteriophage MS2 as a surrogate to evaluate the survival of enteric viruses, particularly the Norovirus in oyster meat. The MS2 phage was propagated in its host, *Escherichia coli*, spiked into oyster meat at a concentration of 8.6×10^{10} PFU/ml (10.93 log CFU/ml) and stored under chilled conditions on ice and at -20°C (frozen storage). Phage titres were measured using the soft agar overlay method, with sampling conducted every second day for ice-stored meat and every fifth day for frozen-stored meat. The survival analysis demonstrated that MS2 phage and by extension enteric viruses, could persist in ice for up to 10 days with counts reaching 4.78 log PFU/g. In frozen storage, the phages survived for up to 35 days, exhibiting 4 log reduction in viral counts. These results highlight a significant reduction in bacteriophage survival in oyster meat during both chilled and frozen storage conditions.

Keywords: Enteric Virus; Norovirus; MS2 Phage; Surrogate; Survival; Oyster

Introduction

Norovirus, formerly known as the Norwalk virus, is an RNA virus of the family Caliciviridae. Based on the differences in the amino acid sequence of viral protein 1, norovirus is classified into 5 genogroups from genogroup I to V, with more than 40 genotypes within these genogroups [1]. Among these, Genogroup II (GII) is reported to cause infections in humans frequently, followed by G I and G IV [2]. A new G II.4 variant called Sydney 2012 emerged in 2012 and has been responsible for increased rates of infections worldwide [3]. The symptoms of norovirus infections start appearing 15-48 hours after exposure and are usually self-limiting. Common symptoms include watery diarrhea, severe vomiting, nausea, abdominal cramps and fever. Individuals across all age groups are susceptible to norovirus, but the elderly and the

immunocompromised are more vulnerable to morbidity and mortality. The infection gets transmitted mainly through the fecal-oral route and also via aerosolized viral particles in vomitus and through food, water and environmental contamination [4]. Epidemiological studies suggest that norovirus is the second major causative agent of viral gastroenteritis worldwide after rotavirus in both developed and developing countries [5]. The annual norovirus-related morbidity and mortality in the USA is estimated to be 20 million illnesses, 56,000-71,000 hospitalizations and 570-800 deaths [6].

Shellfish are known as an important source of food for humans and the diseases caused by the consumption of shellfish containing pathogenic viruses of human origin are also well- documented [7]. Shellfish such as bivalves concentrate viral particles as a consequence of their feeding process, i.e., the filtering of large volumes of water. A large number of shellfish-associated outbreaks have been attributed to enteric viruses, particularly Norovirus (NoV) [8]. Noroviruses may enter the coastal-marine water directly through the discharge of domestic sewage, sewage contaminated rivers and streams, ocean disposal of domestic sewage sludge, malfunctioning boat sewage disposal systems etc [9]. Since shellfish are mostly subjected to minimal cooking, presence of enteric viruses in them can serve as a significant source of food-borne virus infection. Norovirus, hepatitis

A and enterovirus are the most important enteric viruses associated with such shell-fish borne infections.

From the risk assessment point of view, it is important to understand the dynamics of survival and persistence of NoV in bivalve shellfish. Reports from India suggest the prevalence of enteric viruses in fish and shellfish. However, studies on NoV survival in shellfish are sparse owing to difficulties associated with the isolation and detection of enteric viruses. Conventional methods for detection of enteric viruses in foods involve their extraction from the sample through a concentration procedure, followed by viral replication in specific cell lines and visualization of characteristic cytopathic effects. These methods are time-consuming and require expertise in handling cell lines and their maintenance. Some enteric viruses such as noroviruses cannot be propagated in cell cultures. Such viruses can be detected only with the help of molecular techniques such as the Reverse Transcription-PCR (RT-PCR). In this context, the present study investigated the utility of MS2 bacteriophage as a surrogate to understand the survival of NoV in bivalve shellfish preserved on ice and frozen at -20°C.

Material and Methods

Bacterial Strain

Escherichia coli strain C-3000 (ATCC 15597) was used as the host for MS2 bacteriophage. The glycerol stock culture stored at -80°C was grown in Luria Bertani (LB) broth (HiMedia, Mumbai, India) and streaked on LB agar plate. A single colony was streaked on LB agar slope, incubated overnight at 37°C and stored at 4°C in a refrigerator during the study.

Revival and Propagation of Escherichia coli bacteriophage MS2

MS2 bacteriophage (ATCC 15597-B1) was procured from the American Type Culture Collection, USA. To the freeze-dried phage vial, 1 ml of #271 broth (HiMedia, Mumbai, India) was added to rehydrate the phage. The rehydrated phage was serially diluted by adding 100 µl of phage to 900 µl of phage buffer containing 200 mM tris-HCl, 1M NaCl and 100 mM MgSO₄. Double agar overlay method was used for propagation and harvesting of phages [15]. Serial dilutions of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ were made. Bottom agar plates of #271 medium were pre-made and dried adequately. The top soft agar (#271 medium) was melted and maintained at 45°C in a water bath until the start of experiment. To 8 ml of the molten top agar, 250 µl 4-6 h old host *E. coli* culture was added, mixed gently and poured evenly on the surface of bottom agar. The overlaid plates were allowed to harden. One-hundred µl of each dilution was spotted on the surface of set plates, dried and incubated at 37°C for 24 h. After incubation, soft agar surrounding the plaques with visible lysis was scrapped off the surface and centrifuged at 1000 rpm for 15 min to sediment the cellular debris and agar. The supernatant was collected and passed through a 0.22 µm Millipore filter. The phage filtrate stock was stored at 4-8°C for subsequent use.

Estimation of Phage Titre by Double Agar Overlay Method

The estimation of phage titre was done by counting individual plaques formed on plates as previously described [15]. The phage filtrate stock was serially diluted by (10⁻¹ to 10⁻¹²) by mixing 100 µl with 900 µl of phage buffer containing 200 mM tris-HCl, 1M NaCl and 100 mM MgSO₄. Bottom agar plates of #271 medium were pre-made and dried adequately. The top soft agar (#271 medium) was melted and maintained at 45°C in a water bath until the start of experiment. To 8 ml of the molten top agar, 250 µl 4-6 hr old host *E. coli* culture was added, mixed gently and poured evenly on the surface of bottom agar. The overlaid plates were allowed to harden. Ten microlitre of phage dilutions were spotted on the surface of prepared plates and allowed to dry. The plates were incubated at 37°C for 4-5 h. After incubation, individual plaques with visible lysis at higher dilutions were counted. Phage titre was calculated using the number of plaques and dilution factor.

Survival Study of Enteric Virus in Oyster Meat with MS2 Phage as Surrogate Under Ice Storage

The extent of survival of enteric virus in oyster meat under ice storage was studied using MS2 phage as a surrogate inoculated into the oyster meat. Rock oysters (*Saccostrea cucullata*) procured from the intertidal rocky shore region in Mumbai, India were brought live to the laboratory in ice. They were washed under running water, opened using a sterile shucking knife and the meat was separated. Portions of 1 g oyster meat were placed in petri plates and subjected to surface sterilization under UV radiation for 30 min to eliminate the native background flora. Following this, 1 g meat was transferred into 2 ml microfuge tubes and inoculated with 100 µl of MS2 phage with an inoculum titre of 10¹⁰ PFU/ml. The tubes were vortexed gently for proper mixing and stored in ice with regular replacement of ice. Samples were removed every alternate day and the phage titre was determined. For this, phage buffer (900 µl) was added to one gram of ice-stored meat and centrifuged at 1000 rpm for 15 min. The supernatant was collected and passed through a 0.22 µm Millipore filter. The phage filtrate was serially diluted (10⁻¹ to 10⁻¹²) by mixing 100

µl with 900µl of phage buffer. Ten µl of these phage dilutions were spotted on the surface of pre-dried and set double agar overlaid plates and allowed to dry. The plates were incubated at 37°C for 4-5 h. After incubation, individual plaques with visible lysis at higher dilutions were counted. Phage titre was calculated using the number of plaques and dilution factor. Ice storage experiment for phage survival was conducted for a period of 10 days.

Survival Study of Enteric Virus in Oyster Meat with MS2 Phage as Surrogate Under Frozen Storage

The extent of survival of enteric virus in oyster meat under frozen storage was assessed by using MS2 phage as a surrogate inoculated into oyster meat as described in the previous section and stored at -20°C. Samples were removed every fifth day and the phage titre was determined. Frozen storage experiment for phage survival was conducted for a period of 35 days.

Results

Survival of Enteric Virus Surrogate MS2 Phage in Oyster (S. cucullata) Stored in Ice

Aliquots of oyster meat inoculated with enteric virus surrogate MS2 phage were stored in ice and sampled periodically to assess the survival of phages as an indicator of survival of enteric viruses. Samples were drawn at an interval of 2 days and phage survival was determined by estimating the phage titer. The results are shown in Table 1. The survival study indicated that when stored in ice, MS2 phages and hence the enteric viruses, could survive for over 10 days with 6 log reduction in the virus number (Fig. 1). Based on the plaques produced in agar overlay assay, the initial titre of MS2 phage immediately after spiking was determined to be 8.6×10^{10} PFU/g. After two days of storage of phage-spiked oyster meat in ice, there was a negligible reduction in phage number (Table 1). Fig. 2 shows the plaques formed on *E. coli* plates at different dilutions. On day-4 of storage on ice, there was more than 4 log reduction in phage counts. Over 6 log reduction in phage counts was observed on day-8 and day-10 of storage. The oyster meat was rejected by the end of 10 days and thus the survival study was terminated on day-10 of chilled storage.

Survival of Enteric Virus Surrogate MS2 phage in Oyster (S. cucullata) Stored at -20°C

Aliquots of oyster meat inoculated with MS2 phage were stored at -20°C and sampled periodically to assess the survival of phages which is assumed to have similar behaviour as that of enteric viruses. The phage titres in samples collected at 5-day interval are shown in Table 2 and Fig. 3. The initial count immediately after spiking (0-day) was 8.6×10^{10} PFU/g, which declined to 5.0×10^6 PFU/g on day 35 of storage. The survival study indicated that at -20°C, MS2 phage showed a 4 log reduction in number in 35 days.

Day of Storage	Phage Titre (PFU/g)	Log PFU/g
0	8.6×10^{10}	10.93
2	6.1×10^{10}	10.79
4	2.0×10^8	8.30
6	5.1×10^6	6.71
8	7.2×10^4	4.86
10	6.0×10^4	4.78

Table 1: Survival of enteric virus surrogate MS2 phages in oyster (*S. cucullata*) stored in ice.

Day of Storage	Phage Titre (PFU/g)	Log PFU/g
0	8.6×10^{10}	10.93
5	5.2×10^{10}	10.72
10	6.4×10^9	9.81
15	7.3×10^8	8.86
20	4.0×10^7	7.60
25	5.1×10^6	6.71
30	3.0×10^6	6.48
35	5.0×10^6	6.70

Table 2: Survival of enteric virus surrogate MS2 phage in oyster (*S. cucullata*) stored at -20°C.

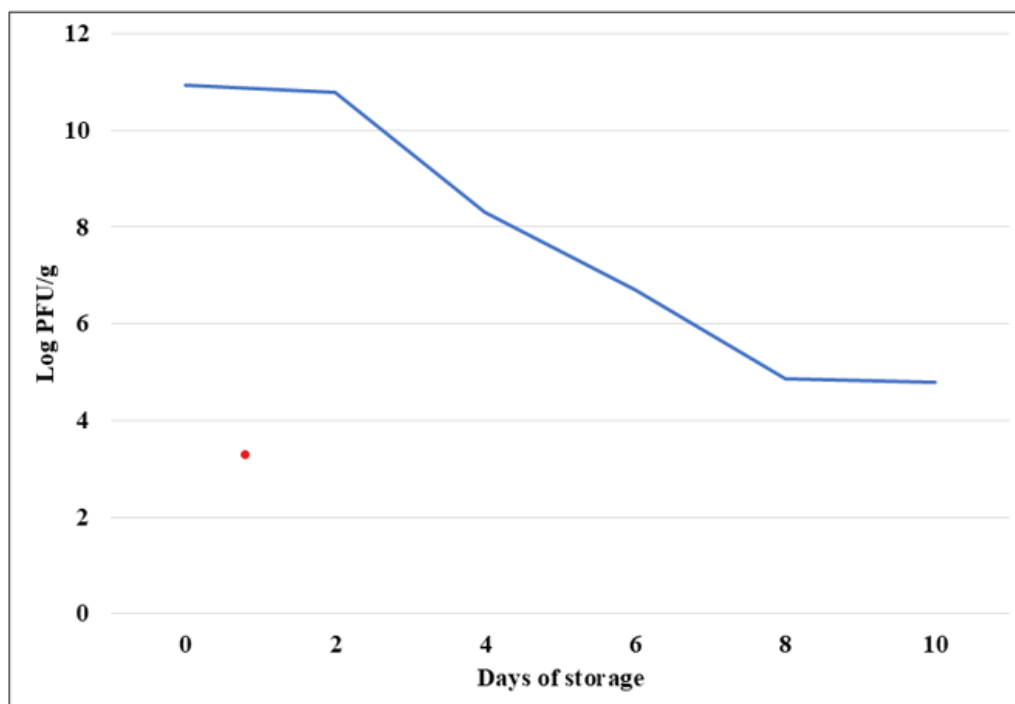


Figure 1: Survival curve of enteric virus surrogate MS2 phage in oyster (*S. cucullata*) meat stored in ice.

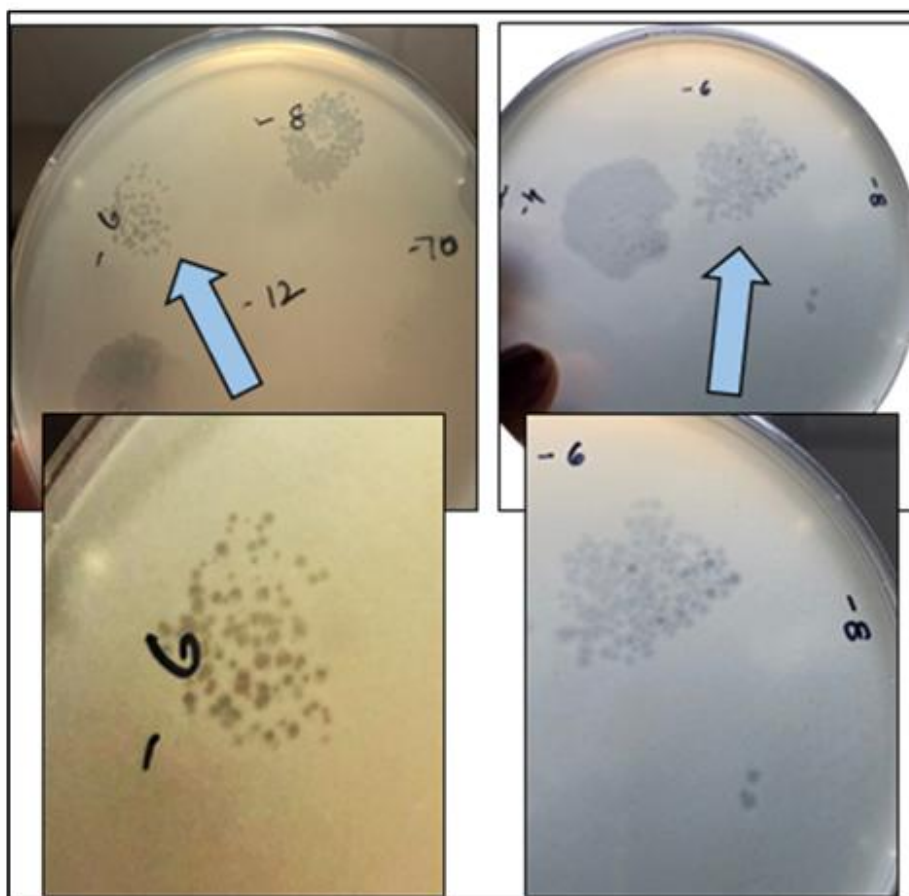


Figure 2: Enumeration of MS2 phage from oyster meat stored in ice.

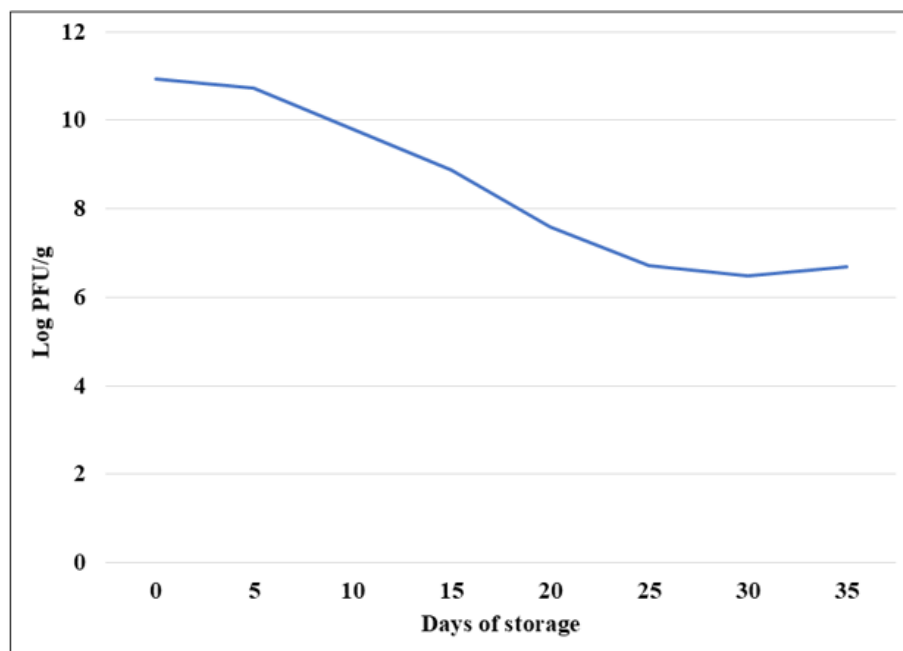


Figure 3: Survival of MS2 phage in artificially inoculated oyster meat stored at -20°C.

Discussion

It is essential to understand the survival of enteric viruses in the environment and in seafood, the factors influencing their survival and in order to devise measures to reduce their loads or inactivate them. The longer a virus can survive outside a host, the greater are the chances of virus getting transmitted. The length of survival is affected by various environmental conditions such as temperature, moisture and the pH [16].

Enteric viruses, especially norovirus, cannot be cultured in the laboratory, making it necessary to use a surrogate. Surrogates are viruses related to the pathogens they have been chosen to represent. When culturing of viruses is not possible or practical, surrogates are used for study of viruses. The selection of a surrogate is based on the ability of the surrogate to be propagated in culture and its genetic, physical and chemical relatedness to the pathogen [17]. Surrogates play a critical role as indicators for the inactivation of enteric pathogens to aid in the design and validation of food-processing methods. MS2 phage belongs to group I of the RNA coliphages within the family Leviviridae. The bacterial host for MS2 phage is *Escherichia coli* and therefore this bacteriophage is found most frequently in sewage and animal faeces. Like noroviruses, MS2 is adapted to the intestinal tract and it can replicate readily in the mammalian gastrointestinal tract [18]. Because of this and its similarity in size, shape and nucleic acid type, MS2 has been suggested as a surrogate for enteric viruses such as hepatitis A virus, enteroviruses and human noroviruses [19].

In the present study, *Escherichia coli* bacteriophage MS2 (ATCC 15597-B1) was used as a surrogate to determine the survival of enteric viruses. The phage was inoculated into oyster to assess its survival under conditions of iced and frozen storage. The survival study indicated that in ice, MS2 phages and hence enteric viruses showed 6 log reductions in the virus number. In frozen storage, phages could survive up to 35 days with 4 log reductions. Earlier reports show that at 4°C, the virus spiked into oyster showed only 1-log inactivation after 29 days for hepatitis A virus and 48 days for MS2, post-inoculation [20]. MS2 bacteriophage stored at 4 and 8°C showed similar survival characteristics with <1 log₁₀ decline in the first 50 days. At 22°C there was almost a 5 log₁₀ reduction within the same period and more than 1log₁₀ decline in the first 9 days [21]. Nevertheless, the results of this study indicated a higher extent of reduction in the number of bacteriophages surviving in oyster meat during storage under ice and frozen storage. This may be attributed to the composition of oyster meat matrix during storage. As the meat and muscle components undergo spoilage and chemical deterioration during post-mortem storage, various kinds of enzymes and other inhibitory substances may be produced. These substances may degrade capsid proteins of virus and damage the nucleic acids, thereby affecting their survival [22]. Also, the spoilage bacterial flora that dominates in the meat during storage may competitively inhibit the growth of bacteriophages not specific to them.

A study investigated the survival of poliovirus in blue crabs by allowing the crabs to accumulate polioviruses from artificial seawater at 25°C for 4 h and then placing the crabs in clean water at 15 and 25 °C for up to 6 days [23]. At 15°C, virus was still detectable up to 6 days in the hemolymph and digestive tract, and up to 3 days in the meat [23]. The loss of titre was 96.4% in the digestive tract, 98.2% in the hemolymph and >98% in the meat after 6 days. At 25 °C, loss of virus proceeded at a greater rate and no virus was detected in the meat or digestive tract after 2 h. Further, the hemolymph had lost 99.7% of its original titre in 20 h and 1 PFU was recovered from the hemolymph of crab after 44 h [23]. This study showed that crabs living in contaminated water can be expected to accumulate some viruses and retain them for several days depending on the water temperature. However, it was also demonstrated that cooking abolished virus infectivity in the crabs within a few minutes. Persistence of poliovirus in whole and shucked oysters (*Crassostrea virginica*) stored at 5°C has been studied [24]. Shellfish were contaminated with virus either through exposure to virus-contaminated seawater or by direct inoculation, before storage for up to 77 days. In all samples, viruses were detectable throughout storage. The authors considered that, as the longest time normally employed between harvesting and consumption is around 28 days, if infectious enteroviruses are present in oysters at the time of harvesting, they will still be present when the shellfish are purchased for processing in the home or food establishment.

A study on the survival of poliovirus in Pacific oyster (*Crassostrea gigas*) and Olympia oyster (*Ostrea lurida*) oysters showed that samples of whole oysters contaminated with 10⁴ pfu per ml of poliovirus and stored 5 °C, the infectious virus in the Olympia oysters was reduced by 60% after 15 days of storage, while after 30 days 13% of the virus was still infectious [25]. In frozen Pacific oysters, the titre of poliovirus was reduced by less than 10% after 4 weeks and after 12 weeks, only 10% of infectious virus remained. The survival of poliovirus in artificially contaminated fresh and frozen green-lipped mussels (*Perna canaliculus*) after 2 days storage at 4 °C and after 7, 14 and 28 days of storage at -20 °C showed that after 2 days at 4°C, 81% infectious virus remained in the mussels. Infectious virus declined to 66%, 53% and 44% of the original number after storage at -20°C for 7, 14 and 28 days, respectively [26]. The higher reduction observed in the number of phages surviving in iced and frozen oysters in the present study may also be due to the lack of specific receptors to which these phages attach, as in the case of live oysters. A separate study showed that hepatitis A virus could survive in marinated mussels at pH 3.75 with 1.7-log reduction, with no reduction in norovirus [27].

Conclusion

In conclusion, this study highlights the effectiveness of MS2 phage as a surrogate model for investigating the survival dynamics of enteric viruses, including norovirus, in fish and shellfish. Additionally, the findings indicate that viral counts in oyster meat decrease more rapidly under chilled storage conditions compared to the frozen storage.

Conflict of Interest

The authors declare that there is no conflict of interest.

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