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Occurrence of Human Enteric Adenoviruses in Fresh Tropical Seafood from Retail Markets and Landing Centers

Subal Kumar Ghosh, Manjusha Lekshmi, Oishi Das, Sanath Kumar, and Binaya Bhusan Nayak

Abstract: Human adenoviruses (HAdVs) are the foodborne enteric pathogens transmitted by the consumption of contaminated shellfish. In this study, the occurrence of enteric adenoviruses in finfish and shellfish was investigated by virus concentration and polymerase chain reaction (PCR). Total plate count, total coliform, and fecal coliform levels were determined and correlated with the presence of adenovirus. Samples of fish, bivalve mollusks, crustaceans, and cephalopods were collected from supermarkets, landing centers, and retail fish markets of Mumbai, India for the study. Overall, the adenovirus DNA was detected in 21.27% of all the samples analyzed. The highest incidence was detected in clams (14.89%), followed by oysters, shrimps, and finfish (2.13% each). High prevalence of enteric adenovirus in filter-feeding bivalves, such as clams and oysters, as well as in fish suggests persistent fecal contamination of coastal waters in the region of study. The occurrence of adenoviruses in samples showed a positive correlation with the bacteriological indicators of fecal contamination, suggesting that fecal indicator bacteria may be used to monitor the presence of adenoviruses in seafood.

Keywords: adenovirus, fecal coliforms, finfish, PCR, shellfish

Practical Application: This research demonstrates the occurrence of human adenoviruse (HAdV) in fresh seafood and the utility of fecal coliforms as indicators of HAdV presence in seafood. The study emphasizes the need to identify HAdV in seafood as a human health hazard and implement measures to prevent sewage pollution of fish and shellfish harvesting areas in India.

Introduction

Human adenoviruses (HAdVs) are an important group of enteric DNA viruses responsible for human infections (Ghebremedhin, 2014). They are 90 to 100 nm nonenveloped viruses containing a double-stranded DNA of 26 to 45 kb enclosed within an icosahedral capsid (Fong & Lipp, 2005; Greening, 2006). There are 52 HAdV serotypes based on their nucleic acid content, hexon and fiber protein characteristics, and biological properties that are further divided into six species, human adenovirus A to G (Jones et al., 2007). Among these, subgenera "F" with AdV types 40 and 41 and "A" with AdV types 12, 18, and 31 are associated with acute gastroenteritis (Shimizu et al., 2007; Wilhelmi, Roman, & Sánchez-Fauquier, 2003). Symptoms occur after 8 to 10 days of infection that include diarrhea, dehydration, vomiting, and fever that lasts for 7 to 8 days, and viral shedding can continue to occur for 7 to 14 days postinfection (Wood, 1988). Several adenoviruses can cause upper respiratory tract infections and conjunctival diseases, but these infections are usually self-limiting. Transmission of adenovirus can occur via aerosol droplets, the fecal–oral route, and by contact with contaminated fomites. HAdv are widely distributed in the environment and are shed in large numbers in the feces of infected individuals. Adenoviruses can survive for long periods on environmental surfaces owing to their resistance to disinfectants due to their nonenveloped nature but are inactivated by heat, formaldehyde, or bleach (Bosshard, Armand, Hamelin, & Kohn, 2013; Liu, 1991).

Fish and shellfish harvested from fecally contaminated coastal waters can act as important vehicles for the transmission of adenovirus in humans. Molluscan shellfish, being filter feeders, filter large volumes of water and concentrate different types of pathogens from polluted waters including viruses (Bosch, Pinto, & Le Guyader, 2009). From the stomach and digestive diverticula, enteric viruses may be transported through the walls of the digestive tract into interior portions of the shellfish (Le Guyader et al., 2000). Crustaceans such as crabs and shrimps acquire enteric viruses when they feed on contaminated oysters or other organisms and thus act as sources of further transmission to humans (Umesha et al., 2008). HAdVs are resistant to the conventional sewage treatment processes and can withstand environmental conditions to survive longer periods of time in water and shellfish (Haramoto, Katayama, Oguma, & Ohgaki, 2007; Thurston-Enriquez, Haas, Jacangelo, Riley, & Gerba, 2003). In developing countries, coastal waters are often contaminated by the release of inadequately treated sewage (Anbazhagi & Kamatchiammal, 2010). Besides, poor hygiene and sanitation in landing centers and retail fish markets lead to seafood contamination with enteric viruses at various stages of their handling and storage (Lekshmi, Das, Kumar, & Nayak, 2018). In the context of seafood increasingly being recognized as important carriers of enteric viruses, the present study was carried out to determine the prevalence of adenovirus in seafood from different locations in Mumbai, India

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and also to analyze the relationship between the occurrence of adenovirus and fecal coliforms in these samples.

Material and Methods

Samples

Seafood samples ($n = 47$) comprising of fish and shellfish were collected between September 2016 and May 2017 from two retail fish markets, a retail supermarket, a landing center, and a mud flat (coastal lagoon) at Juhu along the coast of Mumbai, India. The mudflat is an important source of edible bivalve mollusks in the region. The samples were collected in sterile sampling bags (Nasco Whirl-Pak, Hi-Media, India), brought to the laboratory in ice and processed immediately.

Virus concentration and DNA extraction

Concentration of viruses from bivalve samples was done according to the method of Sdiri, Khelifi, Belghith, and Aouni, (2006) with some modifications. Digestive glands (~50 g) were dissected carefully from live clams and oyster samples along with the valvular fluid, pooled in a stomacher bag (Seward Medical, London, UK) and homogenized for 60 s in a stomacher (Seward Stomacher 80, Lab system, London, UK). In the case of fish, crab, squid, and shrimp, muscle samples were collected from at least three specimens, pooled to 50 g and homogenized. The homogenate was transferred into a conical flask and diluted with an equal volume of glycine buffer (0.5 M glycine, 0.15 M NaCl, pH 9.5). The suspension was mixed using a magnetic stirrer for 15 min at room temperature to release viruses from the tissues. The suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C to precipitate the tissue. The supernatant was recovered and an equal volume of meat extract was added to adsorb and precipitate the viruses. After adjusting the pH to 3.5, the supernatant was agitated at room temperature for 30 min followed by centrifugation at 10,000 × *g* for 15 min at 4 °C. The pellet was resuspended in 5 mL of phosphate buffered saline (PBS; pH 7). The extract was precipitated with 20% polyethylene glycol (PEG) 8000 added in a ratio of 1:4 (v / v). The pH was then adjusted to 7.2, and the solution was incubated overnight at 4 °C. The precipitate was clarified by centrifugation at 10, 000 × *g* at 4 °C for 45 min. The pellet was finally resuspended in 5 mL of PBS (pH 7), aliquoted and stored at –20°C. DNA was extracted from the concentrated samples using NucleoSpin® Virus kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Briefly, 200 µL of the virus concentrate was mixed with proteinase K and a carrier RNA, followed by the precipitation of viral DNA using ethanol. A silica membrane-spin column was used to bind the viral DNA, which was finally eluted with 30 µL of elution buffer.

Polymerase chain reaction detection of HAdV

Polymerase chain reaction (PCR) was performed using previously described oligonucleotide primers and thermocycling conditions (Table 1). Primers ADV-F/ADV-R target the capsid protein gene of adenoviruses (Allard, Albinsson, & Wadell, 1992), while the nested-PCR amplifies an open reading frame of the hexon gene (Pina, Puig, Lucena, Jofre, & Girones, 1998). The thermocycling conditions used in this study consisted of an initial DNA denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 92 °C for 30 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 1 min. The products of PCR were separated on 1.5% agarose gel, stained with ethidium bromide (0.5 µg/mL), and photographed in a gel documentation system (Bio-Rad, Hercules, CA, USA). Representative PCR products from each sample were purified using Gene JET gel extraction kit (Thermo Fisher, Waltham, MA, USA) and sequenced to confirm the presence of HAdV genome sequences in the amplified products.

Bacteriological analysis

Bacteriological study was done to evaluate the sanitary conditions of seafood and to understand the fecal contamination status of seafood samples. The parameters studied included total aerobic plate count (TPC) by spread plate method, total coliforms, and fecal coliforms by the most probable number (MPN) method (Food and Drug Administration, 2011).

Statistical analysis

The samples were grouped under different ranges of TPCs, total coliform counts, and fecal coliform counts based on the lowest and highest values obtained for each of these parameters. The values of TPCs were categorized into four groups 10^4, 10^5, 10^6, and 10^7 CFU/g. The total coliform counts were categorized into three groups viz., ≥1,600 MPN/100 g, 100 to 1,500 MPN/100 g, and <100 MPN/100 g, whereas the fecal coliform counts were categorized into three groups of >200 MPN/100 g, 20 to 200 MPN/100 g, and <20 MPN/100 g. The number of samples in each group and the number of samples positive for adenovirus were used for correlation analysis. The correlations between the presence of adenovirus and the TPCs, total coliform counts, and fecal coliform counts were determined using Spearman's rank-order correlation method to understand the relationship between the bacteriological parameters and the occurrence of HAdV in the samples.

Results

Prevalence of adenoviruses in seafood

The details of seafood samples analyzed, their sources, and the results of PCR are shown in Table 2. Of 47 samples analyzed, 10 (21.3%) were positive for adenoviruses, which included seven samples of clams (14.89%) and one each of oyster (2.13%), fish (2.13%), and shrimp (2.13%). Three PCR protocols that included a nested PCR were used for detection of adenoviruses. Overall, the incidence of HAdV was highest in Asiatic hard clam *Meritrix meritrix* with six out of nine samples being positive by PCR. One sample each of cupped oyster *Crassostrea gryphoides*, paste shrimp *Acetes indicus*, and the Indian anchovy *Stolephorus indicus* were also positive for HAdV. Location-wise analysis of HAdV-positive samples showed that three samples were from retail fish market-1, two samples from the mudflat, and one each from retail fish market-2, retail super market, and landing center harbored HAdV (Table 2).

Bacteriological analysis

The bacteriological quality of all seafood samples ($n = 47$) was determined, and the association between these and the presence of adenovirus in seafood was statistically analyzed. Table 3 shows the bacteriological parameters of samples positive for HAdV. The total coliform counts of HAdV-positive samples ranged from 130 to >1,600 MPN/100g, whereas the fecal coliform counts were in the range of 94 to 1,600 MPN/100 g (Table 3). TPCs of 13 (27.65%) samples were more than 10^7 CFU/g, followed by 10^6 CFU/g in 23 (48.93%) samples, 10^5 CFU/g in 10 (21.27%) samples, and 10^4 CFU/g in one (2.12%) sample (Table 4).The total coliform counts varied among samples with four samples showing

Table 1–Adenovirus-specific oligonucleotide primers used in this study.

Primer name	Nucleotide sequence (5'-3')	Product size (bp)	Reference
ADV-F/ADV-R	gccgcagtggtcttacatgcaca cagcacgccgcggatgtcaaagt	301	Allard et al. (1992)
AD1-F/AD2-R	tacgccaactccgcccacgcgctgccgagaagggcgtgcgcaggta	161	Hierholzer et al. (1993)
AV-A1/AV-A2	gccgcagtggtcttacatgcacatccagcacgccgcggatgtcaaagt	300	Allard et al. (1992)
AV-B1 /AV-B2	gccaccgagacgtacttcagcctgttgtacgagtacgcggtatcctcgcggtc	143	Allard et al. (1992)

Table 2–Details of seafood samples analyzed for adenovirus.

Sample source	Common name	Scientific name	Number of samples	Number positive by PCR
Retail fish market-1 ($n = 20$)	Asiatic hard clam	*Meritrix meritrix*	9	4
	Bombay duck	*Harpadon nehereus*	1	–
	Threadfin bream	*Nemipterus japonicus*	1	–
	Jinga shrimp	*Metapenaeus affinis*	2	–
	Double lined tongue sole	*Paraplagusia bilineata*	1	–
	Paste shrimp	*Acetes indicus*	1	–
	Indian anchovy	*Stolephorus indicus*	1	–
	Indian white shrimp	*Penaeus indicus*	1	–
	Gold spotted grenadier anchovy	*Coilia dussumieri*	2	–
	Indian oil sardine	*Sardinella longiceps*	1	–
Retail fish market-2 ($n = 6$)	Asian green mussel	*Perna viridis*	2	–
	Asiatic hard clam	*Meritrix meritrix*	2	1
	Paste shrimp	*Acetes indicus*	1	–
	Indian white shrimp	*Penaeus indicus*	1	–
Retail fish market-3 ($n = 2$)	Asiatic hard clam	*Meritrix meritrix*	1	–
	Indian mackerel	*Rastrelliger kanagurta*	1	–
Retail super market ($n = 4$)	Asiatic hard clam	*Meritrix meritrix*	2	1
	Indian mackerel	*Rastrelliger kanagurta*	1	–
	Tigertooth croaker	*Otolithes ruber*	1	–
Fish landing center ($n = 8$)	Paste shrimp	*Acetes indicus*	2	1
	Indian Squid	*Uroteuthis duvaucelii*	1	–
	Indian White shrimp	*Penaeus indicus*	2	–
	Indian anchovy	*Stolephorus indicus*	1	1
	Three-spot swimming crab	*Portunus sanguinolentus*	1	–
	Banana shrimp	*Fenneropenaeus merguiensis*	1	–
Mudflat ($n = 7$)	Hooded oyster	*Saccostrea cucullata*	2	–
	Common periwinkle	*Littorina littorea*	1	–
	Cupped oyster	*Crassostrea gryphoides*	2	1
	Asiatic hard clam	*Meritrix meritrix*	2	1
	Total		47	10

Table 3–Bacteriological qualities of adenovirus PCR-positive seafood samples.

Location of samples	Sample	Total plate count (CFU/g)	Total coliform count (MPN/100 g)	Fecal coliform count (MPN/100 g)
Retail fish market-1	Asiatic hard clam	4.90×10^6	540	350
Retail fish market-1	Asiatic hard clam	4.06×10^7	430	430
Retail fish market-1	Asiatic hard clam	4.21×10^6	1,600	1,600
Retail fish market-1	Asiatic hard clam	8.56×10^6	>1,600	1,600
Retail fish market-2	Asiatic hard clam	5.80×10^5	210	140
Retail supermarket	Asiatic hard clam	6.23×10^6	180	94
Fish landing center	Indian anchovy	2.85×10^5	130	130
Fish landing center	Paste shrimp	7.23×10^5	280	180
Mudflat	Asiatic hard clam	2.43×10^6	180	110
Mudflat	Cupped oyster	2.03×10^6	280	350

a count of <20 MPN/100 g, 13 samples showing a count between 20 and 100 MPN/100 g, and 21 samples showing a count of 100 to 300 MPN/100 g (data not shown).

Association of adenovirus-positive samples with the bacteriological quality of samples

All the samples were fecally contaminated as indicated by the MPN for fecal coliforms. TPCs of all the samples were more than the limit of 5×10^5 CFU/g for fish and fishery products established for India (FSSAI, 2017). TPCs of 13 samples (27.66%) were more than 10^7 CFU/g. Majority of the samples (48.93%, $n = 23$) gave a TPC value at 10^6 CFU/g, whereas 11 samples (23.40%) were found to have a TPC of $\leq 10^5$ CFU/g (data not shown). The total coliform counts of 34 samples were between 100 and 1,500 MPN/100 g and four samples had counts less than 100 MPN/100 g. The highest total coliform counts of ≥1,600 MPN/

Table 4–Relationship between bacterial load and the incidence of adenovirus in samples.

Bacterial count	Number of samples	Number positive for adenovirus[a]	Spearman's correlation coefficient (rho)
Total plate count (CFU/g)			
10^7	13	1	0.80
10^6	23	6	
10^5	10	3	
10^4	1	0	
Total coliform count (MPN/100 g)			
≥1,600	9	2	0.94
100 to 1,500	34	8	
<100	4	0	
Fecal coliform count (MPN/100 g)			
>200	15	5	0.86
20 to 200	29	5	
<20	3	0	

[a]Based on the detection of viral nucleic acid.

100 g were observed in nine bivalve samples comprising of clams and oysters (Table 4). The fecal coliform count was more than 200 MPN/100 g in 15 samples, between 20 and 200 MPN/100 g in 29 samples, and less than 20 MPN/100 g in three samples (Table 4). The highest fecal coliform counts of 1,600 MPN/100 g were recorded in clams. *Escherichia coli* was isolated from 41 (87%) of 47 fecal coliform-positive samples.

The association of adenovirus with the bacteriological indicators was determined based on the number of adenovirus-positive samples and bacteriological data of all the samples (Table 4). All the bacteriological parameters were quite high in all the samples that were PCR positive for adenovirus, indicating significant levels of fecal contamination. Statistical analysis revealed a significant ($P < 0.05$) positive correlation of the presence of adenovirus and the bacteriological parameters. The highest correlation was observed for total coliforms ($\rho = 0.94$), followed by fecal coliforms ($\rho = 0.86$) and TPC ($\rho = 0.8$) (Table 4).

Discussion

HAdVs are enteric viral pathogens posing significant health risks to humans. The transmission of HAdVs occurs through the fecal–oral route following consumption of contaminated shellfish. HAdVs are present in large numbers in sewage as a consequence of fecal shedding by infected individuals. Sewage treatment may not effectively remove the enteric viruses, and coastal discharge of sewage results in the contamination of fish and shellfish with enteric viruses (Formiga-Cruz et al., 2002). Enteric viral disease outbreaks linked to the consumption of contaminated bivalve shellfish have been reported from many countries (Lees, 2000). However, literature reporting the occurrence of enteric viruses in shellfish from India is sparse. Studies from other countries suggest the contamination of shellfish with HAdV. A study on packaged clams in the western states of Japan detected HAdV in 52% of the packages (Hansman et al., 2008). Analysis of 162 shellfish samples collected from Chinese coast revealed the presence of adenoviruses in 9% of the samples (Ming et al., 2013). Studies from Morocco (Karamoko, Ibenyassine, Aitmhand, Idaomar, & Ennaji, 2005), Norway (Myrmel, Berg, Rimstad, & Grinde, 2004), Brazil (Rigotto et al., 2010), and Spain (Rodriguez-Manzano et al., 2014) have reported the contamination of shellfish with HAdV primarily due to sewage contamination of shellfish harvesting areas. The present study was aimed at determining the occurrence of adenovirus in seafood from Mumbai region, India, as well as evaluating the association of prevalence of adenoviruses with bacteriological indicators of quality of seafood.

In our study, the incidence of adenovirus in seafood from different landing centers and markets in Mumbai region was 21.3%. The highest incidence was in clam samples (14.89%). Of 12 samples analyzed, one sample of paste shrimp (*Acetes indicus*) was detected with adenovirus, whereas one (Indian anchovy) of 11 finfish samples harbored HAdV. The only available study on seafood from India (Umesha et al., 2008) reported HAdV incidence in 27% of the clam and 17% of the oyster samples out of 60 samples analyzed from the south-west coast of India. Unlike bivalve molluscs, finfish do not accumulate enteric viruses (Lees, 2000). Therefore, the occurrence of HAdV in finfish observed in the study could be attributed to persistent as well as recent fecal contamination of coastal waters. High anthropogenic activities in coastal areas and release of untreated sewage directly into the creeks and oyster harvesting areas are responsible for the presence of HAdV in fresh seafood (Lekshmi, Das, Kumar & Nayak, 2018).

We correlated the incidence of HAdV with the bacteriological indicators to establish the role of fecal contamination in introducing HAdV into seafood. The results showed a positive correlation between the bacteriological qualities of the samples and presence of adenovirus in them. The adenovirus detection in samples positively correlated with the fecal coliform counts, total coliform counts, and the total bacterial counts ($P < 0.05$). This indicates that there exists a relationship between the presence of enteric adenovirus and the bacteriological indicators of fecal contamination in the samples. *Escherichia coli* is generally considered as not a good indicator of enteric viruses in shellfish due to the fact that enteric viruses survive longer in the environment and shellfish than their bacterial indicators (Abad, Pintó, Gajardo, & Bosch, 1997; Lees, 2000; Romalde et al., 2002). However, in our study, all samples with high levels of fecal coliform counts also harbored adenovirus indicating a strong relationship between them. The accumulation of fecal coliforms in high numbers in filter feeding shellfish might occur when contamination of harvesting areas occurs continuously for prolonged periods of time. Further studies are necessary to understand the survival dynamics of fecal coliforms and HAdV in tropical shellfish to prove the utility of fecal coliforms as indicators of enteric viruses.

Conclusion

This study reports the incidence of HAdV in finfish for the first time from India. Although there are no epidemiological studies linking seafood-borne human enteric infections with HAdVs, their occurrence in seafood is a health concern. Since fecal contamination is a major route of contamination of seafood, the presence of HAdVs in seafood highlights contamination of coastal waters, poor sanitation and lack of hygiene in landing centers and retail markets. Although there are no statutory restrictions on HAdVs in seafood owing to unknown human health impacts, their incidence certainly necessitates further studies on the genotypes, persistence in seafood and the coastal water, as well as their human health implications.

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Author Contributions

S.K.G. and M.L. conducted the study. O.D. helped in sample analysis and molecular studies. M.L., S.K., and B.B.N. planned the research and wrote and edited the manuscript. All authors proof read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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