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Detection of viral agents in batches of *Mytilus galloprovincialis* placed on the market in Italy

Ricerca di agenti virali in lotti di *Mytilus galloprovincialis* commercializzati in Italia

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Abstract

Hepatitis A Virus (HAV) and Human Norovirus (HNoV) gene groups I and II are the most common causes of gastrointestinal viral infection in Europe. Mediterranean molluscs, or *Mytilus galloprovincialis*, are posing as a source and are capable of infecting humans via food-borne transmission through the consumption of raw or undercooked molluscs. Due to increased demand and availability, there has been an increase in cases of food poisoning caused by consumption of molluscs in recent years. Because mussels are filter feeders, the virus's main accumulation site is at the hepatopancreatic gland, which is indistinguishable from the rest of the organism at the time of consumption. Therefore, only a proper cooking temperature can reduce the risk for the consumer and prevent the infection. The primary goal of this thesis is to collect data on Norovirus GI, GII, and HAV from batches of *Mytilus galloprovincialis* placed on the market in Italy, to determine their prevalence and assess a correlation between geographical origin of the mussels and seasonality of viral contamination. Overall, only NoV GII was detected in 53 batches of *M. galloprovincialis* tested (11.3% prevalence) and mostly during cold months (November and March). At last, another important goal of this dissertation is to raise the consumer's awareness about the risk associated with consumption of raw or undercooked mussels.

Riassunto

Il virus dell'epatite A (Hepatits A Virus; HAV) ed il Norovirus umano di geno gruppo I e II (HNoV) sono le cause più comuni di infezione virale a sintomatologia gastroenterica in Europa. Le cozze o mitili (nome scientifico: *Mytilus galloprovincialis*) rappresentano un'importante fonte di contaminazione alimentare per l'uomo, in particolare per quanto riguarda i consumatori di molluschi crudi o poco cotti. A causa dell'aumento della domanda e della disponibilità di mitili, negli ultimi anni si è registrato un aumento dei casi di infezione. Essendo organismi filtratori, nei mitili il principale sito di accumulo dei virus è rappresentato dalla ghiandola epatopancreatica, che è indistinguibile dal resto del mollusco al momento del consumo. Pertanto, solo adeguate temperature di cottura possono garantire la distruzione degli agenti virali e prevenire l'infezione nel consumatore. L'obiettivo primario della presente tesi di laurea è raccogliere dati sulla contaminazione da Norovirus GI, GII e HAV in campioni di *Mytilus galloprovincialis* messi in commercio in Italia, per valutarne la prevalenza e determinare una eventuale correlazione tra la loro origine geografica e la stagione di raccolta. Su 53 lotti di *M. galloprovincialis* testati, si è identificato solo NoV GII con una prevalenza dell'11,3%. La contaminazione ha interessato prevalentemente i mesi freddi (Novembre- Marzo). In ultima analisi, lo studio ha la finalità di far acquisire consapevolezza al consumatore sul rischio associato al consumo di mitili crudi o poco cotti.

1. Introduction

Viruses have accompanied humans since prehistory. For most of human history and prehistory, it has been difficult to understand not only the origins of such organisms but also how they behaved and spread among humans. A perfect example of such viral agents is given by those that cause gastrointestinal symptoms, which for a long time have been thought to pass only through the consumption of "food that has gone bad or rotten".

For many years now, it has been known that such viral agents not only pass in such ways as foodborne but also have multiple modes of transmission, including person-to-person and aerosol, and depend on multiple factors, whether they are environmental factors such as temperature, humidity, or pH, in which these agents can survive, but also the population and its sub-populations (such as pediatrics, children, adults, and geriatrics), and are able to infect successfully to continue spreading.

The common symptoms of such viral agents are usually nausea, vomiting, stomach aches, and diarrhea which, at first glance, may not be taken very seriously by the average healthy adult. However, not only could such symptoms have fatal consequences for some high risk people (for example, paediatric or geriatric populations), but there are also some atypical forms that can develop into haemorrhagic lesions, relapsing hepatitis, protracted cholestasis, difficult instances with acute renal damage, and a rare autoimmune hepatitis, anorexia and fever are proposed pathways of renal injury, as are the nephrotoxic consequences of hyperbilirubinemia, immune complex-mediated nephritis, interstitial nephritis, and (rarely) extensive intravascular hemolysis.

In this study, the presence of two of these viral agents, Norovirus and Hepatitis A virus, was assessed, in samples of Mediterranean mussels (*Mytillus galloprovincialis*) places on the market in northern Italy.

Norovirus is an RNA virus of the family *Caliciviridae*, which are non-enveloped, positive-sense single-stranded RNA viruses with five genera, two of which are notable human diseases, Norovirus and Sapovirus. The genus Norovirus is classified into seven genogroups. Norovirus is responsible for a human intestinal infection that produces significant morbidity in both medical and community settings. Several variables contribute to Norovirus

transmission, including the minimal inoculum necessary to cause infection, extended viral shedding, and the virus's capacity to survive in the environment[5].

Hepatitis A virus is a single-stranded, non-enveloped RNA virus that belongs to the *Picornaviridae* family and the Hepatovirus genus. It can persist in the environment because it is stable at low pH and resistant to mild heat, and it can be transferred via the fecal-oral route. Virions enter the liver via the portal circulation and are taken up by hepatocytes after intake. Virus particles proliferate, agglomerate, and are released into the biliary canaliculus in hepatocytes, where they enter the bile duct and small intestine. Following infection, many virions are excreted in the feces and survive on human hands and inanimate items[6].

Contact with a hepatitis A infected individual is the most common identified route of infection, however transmission has also been demonstrated by foreign travel, injectable drug use, men having sex with men, and food or waterborne outbreaks. The greatest known outbreak of hepatitis A was caused by the intake of infected seafood, which was caused by eating raw mussels[6].

The history of *Mytilus galloprovinciallis* as a part of the human diet can be traced back to the 4th century B.C., when the locals of the Galician Region (northwest Spain) left behind significant bivalve mollusc shell deposits, including mussel shells. These "concheiros" deposits, which were discovered close to Roman settlements from the first century A.D., meaning that even in those times mussels were a part of the human diet[2].

Even today, we have a great variety of dishes in a number of cultures in different countries that include on their menus either raw or cooked mussels in varying forms, such as mussels in tomato sauce in Italy, Paella in Spain and many others.

For epidemiological purposes, it is crucial to establish the connections between the geographic origin and the viral agents that may pollute waterways of different areas and, ultimately, constitute a serious hazard to the mussels' consumers.

The goal of this study is to assess the contamination rate by Norovirus and Hepatitis A virus in *M. galloprovincialis* batches placed on the market in northern Italy and provide additional information about how product safety varies depending on where it comes from.

2. Characteristics and production cycle of Mytilus galloprovincialis

Mytilus galloprovincialis elongs to the Order *Mitiloidea*, Family *Mytilidae*. The principal characteristics of the *Mytilidae* family are the following: Lamellibranch mussels with equivalve shell, markedly inequilateral, mostly ovalifolium elongated transversely. A small byssal fissure is frequently present centrally. Umbo is directed anteriorly, located at the anterior end of the valve or a little indirectly of it. Presence on the inner face of the two valves of two muscular imprints (of the adductor muscles) of different sizes: the front is the smallest (may be absent in adult subjects of the genus *Perna*), while the rear is more developed and somewhat confluent with the imprint of the posterior retractor muscle. Hinge without teeth or with some small teeth. Entire margin smooth or crenulated. Periostracum well developed, smooth in some species, recanted lamellae or filaments. Pallial line without a sinus. Presence of short siphons or their absence. Elongated foot, which is fixed to the substrate by means of well-developed elastic filaments (byssus) secreted by a special gland (Figure 1)[1].

It is a family of bivalve mussels to which numerous species that live in marine waters around the world belong. They are sedentary species which attach themselves to the substrate by means of their byssus, made up of filaments which are secreted by a special gland (byssus gland). Some of them live nestled in the rock or in the coral that they drill, obtaining a niche. The sexes are separate[1].

In fishing and aquaculture, there are numerous species of this family and they are of primary commercial and alimentary interest in various parts of the world. Some are bred in very large quantities to face the simple growing demand of the markets where they are regularly present in practically every continent. They are fished above all with trawl objects with trawl nets, rakes, dredgers and by hand[1].

The commercial English name of the *Mytilus galloprovincialis* is Mediterranean mussel. It reaches a maximum of length of 15 cm. Distinctive characters: Shell equivalve, inequilateral, sub-quadrangular to roughly triangular, elongated, somewhat enlarged in the central part. Upper margin more or less curved and not forming an angle with the hinge. Lower margin straight, posterior border rounded, anterior border much reduced, lunula-shaped. Umbo in terminal position, pointed and curved forward. The postero-dorsal area of the valves is characterized by a flattened expansion which makes the edge of the ligament somewhat

protruding. Presence on the inner face of the valves of an imprint of the posterior adductor muscle (confluent with the imprint of the posterior retractor muscle) and of one of the anterior adductor muscles, which is small but evident. Lateral face of the valve flattened in the area behind the umbo. External face of the valves covered by a thin and shiny periostracum, and bearing concentric growth striae[1].

Hinge with only 3-4 teeth. External coloration blackish, purplish-black. Internal nacreous coloring. Edge of coat usually violet or violet-purple. It is a common species in the Mediterranean Sea, but is not frequent on the northern coasts of Africa. In the Atlantic Ocean it is present from the Western Channel to Gibraltar[1].

It has great commercial value, being an intensively farmed species of notable commercial interest, daily present on most markets in the Mediterranean area. Marketed alive, frozen, shelled, and prepared in preserves. Its meats are good, tasty, eaten both cooked and raw[1].

2.1 History and farming of *M. galloprovincialis*

The history of *M. galloprovinciallis* can be traced back to the 4th century B.C., when the locals of the Galician Region (northwest Spain) left behind significant bivalve mollusc shell deposits, including mussel shells. These "concheiros" deposits, which were discovered close to Roman settlements from the first century A.D., have been named from their ancestor consumers. People from Portugal traveled to flooded river valleys, also called “Rias”, such as the Ria of Arosa in Cambados in the 16th century to collect mussels, clams, and cockles. When farmers started raising mussels at the beginning of the 20th century, they started to become significant in Spain. Using poles resembling those used in France, the first mussel cultivations were conducted at Tarragona and Barcelona (both in the northeast of the Iberian Peninsula), in 1901 and 1909, respectively. After the initial tests, this technique was given up, and floating structures were used instead. At the time, manure form farms and, to a lesser extent, a supply of mussel seed for culture were the principal uses for the wild populations of mussels in the Galician rias. Farmers cultivated mussels in bottom culture in a few parks; the mussels were then marketed around the Mediterranean coast. Mussels were first grown on rafts in the Galician region in 1946, and within a few years, production rose significantly[2].

Old ships that were repaired to hold wooden frameworks from which farmers attached ropes made of esparto grass (*Stipa tenacissima*) made up the earliest rafts. These rafts were square and had a central float. Farmers tied the mussel seed to the ropes, and once the seed grew to a size suitable for harvesting, they removed it by hand or with a specialized pin wheel. In their place, modest wooden dwellings supported by square or rectangular frames were built in place of the old ships. With order to float, wooden floats were covered in concrete and encased in wire mesh. A few older rafts are still around today, but the majority of the new ones are built with a eucalyptus wood framework[2].

Nowadays, China is the largest producer of *M. galloprovincialis* worldwide, however little information is known about it. In Europe, it is mostly raised in coastal waters from Galicia (northwest Spain) to the Mediterranean Sea's northern coastlines. However, there have also been reports of production from South Africa, the Russian Federation, Ukraine, and several nations in the southern Mediterranean[2].

The environments where culture is present are quite similar. The flooded river valleys, or "rias," that line the Galician coast are notable for being used by farmers to cultivate mussels. Rias can be up to 25 km long, 2–25 km broad, and 40–60 m deep; they are surrounded by hills and have muddy bottoms. The salinity is about 34%, the temperature is between 10 and 20 °C, and the tidal range is typically 4 m. Strong tidal currents exist. Continuous upwelling of cold, nutrient-rich water likely encourages an abundance of phytoplankton, along with nutrients that wash down the hills after heavy rains (mean annual precipitation is 1,250 mm). This in turn encourages the development of mussels[2].

These protected rias offer the perfect setting for growing mussels on ropes suspended from moving rafts. The Ria de Arosa, which produces 60% of the Spanish mussels, is the most significant culture region, followed by the Ria de Vigo and the Ria de Pontevedra. The mouths of rivers and islands with intertidal rocky beaches are home to abundant natural populations of mussels, with the mean density in the densest beds hovering about 24,000 mussels/square m^2.

They generally develop on rocky terrain, cliffs, and boulders along the rias as well. These locations are where farmers gather mussel seed that they then suspend from their rafts. The

main settlement season is from May to September; however, seed mussel recruitment continues all year long[2].

Mussels may move by using their “foot”. Byssal threads are secreted by the byssus gland and enable for their adhesion to the substrate. The gills, which filter food particles from the water, are two pairs of wide plates made up of numerous parallel filaments. A 5 cm long mussel can filter 5 liters per hour. The digestion gland, which is brownish-green in colour and located in the body's center, is where digestion occurs. Mussels consume organic debris and phytoplankton for food[2].

The coat secretes the shell and holds the gametes when it comes into direct touch with the shell's interior (eggs or sperm). Reproduction is possible throughout the year in Galicia. Mussels lose a lot of their stored glycogen during the millions of eggs they produce. Mussels that have just hatched are too thin to be sold. External factors fertilize a crop. Eggs that have been fertilized become trochophore larvae, which eventually transform into “veliger” forms that are carried by the tide and currents. The pediveligers connect to filamentous substrates with their byssus threads once their shell length reaches 0.25 mm. They have the capacity to separate from one substrate and reconnect to another[2].

Figure 1: *Mytilus galloprovincialis* (https://www.researchgate.net/figure/Three-perspectives-of-*Mytilus-galloprovincialis*-a-shell-appearance-b-side-view-of_fig4_263550724)

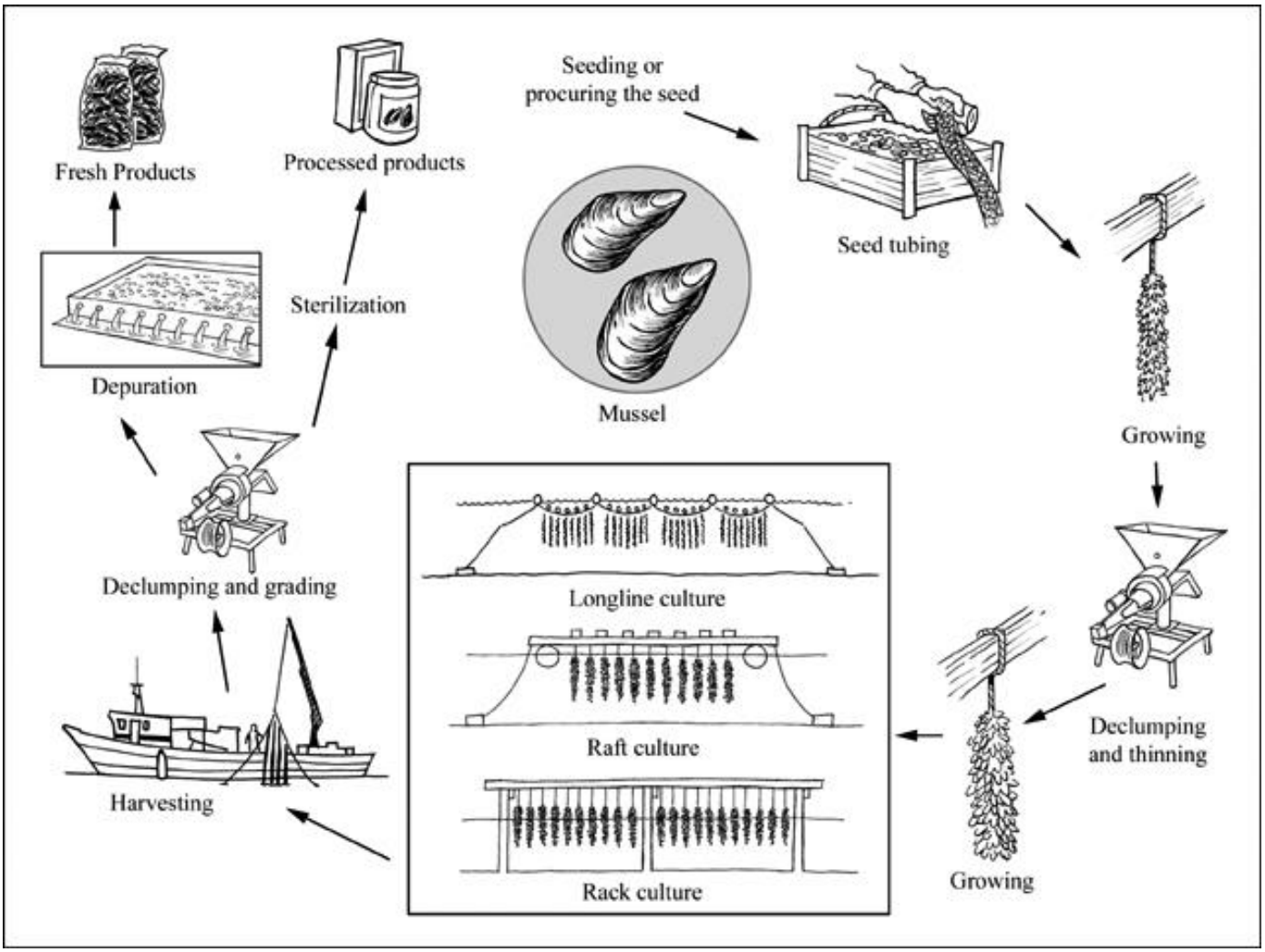

Figure 2: Production cycle of *Mytilus galloprovincialis*. (© FAO 2023. *Mytilus galloprovincialis*. Cultured Aquatic Species Information Programme. Text by Figueras, A.. Fisheries and Aquaculture Division [https://www.fao.org/fishery/en/culturedspecies/*mytilus_galloprovincialis*/en].

The rearing and production process for *M. galloprovincialis* is always labor-intensive (Figure 2). Young mussels are harvested from the sea and can be cultured on ropes suspended from rafts, wooden frames, or longlines of swaying plastic buoys. These ropes are covered in mussel seeds held in place by nylon nets. A significant amount of EU production is grown on suspended ropes, a method that, despite being extremely vulnerable to plankton blooms, can be used further offshore and is the only one that may enhance output[2].

Farmers start culturing by gathering mussel seed, primarily from natural beds (60–70%); the remaining amount is obtained using collector ropes hung from their rafts. Farmers are able to harvest up to 1,500 kg of seed every low tide from the exposed rocky coasts on the ocean side of rias and islands in around 4 hours. They employ a specialized shovel made of steel called a "rasqueta," which has a blade measuring roughly 10 square cm and a wooden handle. Farms either sell the mussels to other farmers or suspend them from their own rafts. Every culture cycle, farmers collect about 4,500 tons of mussel seed (mean length = 2 cm) from these regions[2].

M. galloprovincialis is raised in rafts in Galicia. Raft sizes range widely from less than 100 square m to more than 500 square m. These buildings are supported by one to six floats that are made of steel or wood and are covered in expanded polyester, fiberglass, or both. The useable culture area can range from 80% when a central float is utilized to 90% when four to six floats are employed, depending on the number of floats. Farmers use one or two iron chains and a 20-ton concrete anchor to secure the rafts. They only utilize one mooring chain in protected locations with low boat traffic. In exposed regions, when the rafts are close to the beach, or when there is a lot of boat traffic, two chains are preferred[2].

The rafts are grouped together in areas called parks, but are separated from one another by 80 to 100 meters. These parks have varying numbers of rafts, and marine authorities control where they can be found. The number of rafts increased gradually from the start of mussel production in 1946 reaching 400 units in 1956, but thereafter they rose quickly. Between 1977 and 1984, the rafts' average size rose from 297 square m to 369 m^2. [2]

Farmers currently operate shallow-draft, wide-beam boats (9 tones in weight) with diesel engines that have roughly 24 horsepower. Both have a crane to lift the ropes and a basket, as well as equipment to separate and thin the mussels[2].

To assist with the numerous farming methods, in particular the grading and wrapping of spat to ropes, specific equipment has been designed[2].

Farmers either manually or mechanically fasten the seed to the ropes using a specific cotton or rayon mesh that falls apart after a few days. The mussels have by that time adhered themselves to the ropes and secreted fresh byssus. The average weight of seed for each rope is 14 kg, and farmers attach 1.5–1.75 kg of seed every meter of rope. The ropes range in length from 6 to 10 meters, are typically 3 cm thick, and are constructed of nylon, polyethylene, or esparto grass. Their rough surfaces make it easier for the mussels to connect[2].

Each mussel-adorned rope includes a loop at one end that is tied to a 13 mm thick, thinner polyester rope known as a "rabiza" which is then fastened to the rafts' girders. Due to exposure to air and sunshine, the rabiza often only lasts 3 to 4 years, but the larger ropes last 5.8 years on average. There are 200 to 700 ropes on each raft. To stop the mussel clumps from slipping down, 20–30 cm long wooden or plastic pegs are put every 30–40 cm between the rope's strands. Farmers attach 1-3 ropes every square meter of raft. This distribution keeps the mussel ropes from touching each other and permits a sufficient flow of water that is rich in food for the mussels. Farmers install the ropes mainly from November to March[2].

After receiving the seed and securing it, the mussels must be thinned in order to avoid them from slipping off during inclement weather. Thinning also promotes quick and uniform growth. This is done by farmers when the mussels have grown for 5–6 months, often from June to October, and are halfway developed (shell length 4-5 cm). They use a crane to hoist the ropes into their boats, then manually rub the mussel clusters onto a steel screen to sort them into various sizes. Another option is a mechanical cylindrical screen. Using cotton or rayon netting, the mussels from each original rope are fastened to two to four new ropes[2].

The ropes weigh 46 kg on average. For 500 ropes of 10 m length each, the farmers, who automate this process, spend up to 5–15 seconds collecting each rope, resulting less than 14 hours in total to collect every time. If the mussels grow quickly, this work is repeated once more prior to harvest (in which case their weight and density increase the risk that the mussel clusters will fall off). To make sure that all mussels are the same size when harvested, this procedure must also be repeated[2].

The fourth step in mussel farming is mussel rearing. Mussels can reach market size (8–10 cm) in the Galician region, where growth needs only 8–9 months, particularly in the areas that are closest to the ocean side of the rias. Typically, some bays take about 13 months to complete. High raft densities, however, can impede mussel growth. Growth is lower during the summer and higher during winter. The relative availability and abundance of food (phytoplankton) at that time in the water column is related to the slow summer growth; this factor is more significant than high temperatures and causes the seed strung on ropes in the spring and the fall to reach the same size at the end of the first winter[2].

In order to sustain continuous production, each raft often carries three different types of ropes: one for gathering seed, one with growing mussels, and one with marketable mussels. Some producers occasionally invert the ropes to produce mussels that are around the same size because the mussels grow more quickly at the water's surface. Farmers who use rafts with just one central float must place water-filled containers in the proper location on the framework to prevent the rafts from being tilted when they hoist ropes for harvesting or thinning. Farmers must occasionally clean the floats since a lot of mussel seed and fouling creatures stick to them and proliferate, increasing the weight of the raft[2].

The raft floats higher when it is almost empty, which makes this procedure simpler because it exposes more mussels and other fouling creatures to the air, where they die and are simple to remove. Farmers transport the rafts to shipyards or companies for significant repairs to the structure or floats. Modern fiberglass rafts survive much longer than a medium-sized timber raft, which lasts about 10 to 15 years. Rafts can be up to 30 years old, with 8 years average age[2].

Commercial-sized mussels are accessible throughout the year and can be picked at any time, although the major harvest period is from October to March in Spain, when the market demand is the highest and the mussels are in the best condition. On the contrary, in Italy, it ranges on the opposite time period (from April to September), where the demand for national product is the highest. When the mussels are in prime condition, the meat weights can be close to 50% of the total wet weight. It is advisable to wait until mussels are in better condition when a significant proportion of them have just finished or are close to spawning. A full raft’s average production is 20–100 tons, with a mean value of roughly 47 tons, or 130 kg/m2 of raft area. These numbers vary greatly and are based on the size of the rafts[2].

Another way to describe production is as roughly 10 kg of mussels every meter of rope. It has been calculated that handling and natural mortality losses account for 15% of annual losses. Recent experimental findings indicate that about 5% of mussels die naturally. When it's time to harvest, farmers use a crane to hoist the ropes to their boat, where they separate and sort the mussels by rubbing them against an iron grid. After that, they are cleaned to remove ascidians, silt, small mussels, and other undesired creatures. Mussels that are too little to be sold are wrapped onto fresh ropes to continue growing[2].

2.2 Marketing of *M.galloprovincialis*

Marketable mussels are placed in nylon bags and transported by boat directly to processing facilities or canneries. Every eight hours, each person handles around 200 kg of mussels. To minimize mussel shell damage during handling and prolong the mussels' shelf life during transportation, little mechanization is used. Refrigerated trucks are utilized to carry the mussels during the summer months. Mussels are sometimes shipped by train to the market. The mussels with the worst quality and size are those that are sent directly to the canneries. They can be served in a variety of ways after being fried or boiled and then dusted with various sauces[2].

Concerning *M. galloprovincialis* supply data, more than 663,000 tons of mussels were produced in China in 2002, according to statistics categorized as "sea mussels". The majority of these mussels were *M. galloprovincialis*, with minor numbers of *Mytilus coruscus, Musculus senhouse*, and *Perna viridis*. However, almost all of Spain's significant mussel production, which accounted for 201,025 tons in 2002, is actually *M. galloprovincialis*[2].

All Western European mussels were thought to be *Mytilus edulis* until the early 1990s. Now that we know, *M. galloprovincialis* mussels are found from southern Brittany (France) to the Mediterranean Sea. This fact is not taken into consideration in the statistics data from the Food and Agriculture of the United Nations FAO and other international organizations, which causes a misconception. The analysis is further complicated by the paucity of information about Chinese mussel culture technology and practices in Western countries[2].

There are two mussel markets: one for fresh mussels before depuration and one for processed mussels. Although fresh mussels are sold in greater quantities, the market for processed

products has grown since 1984[2]. Mussels from depuration facilities are sold to central markets, where they are then bought by customers and restaurants. Markets outside of Spain receive the mussels that are left over. However, according to European legislation, mussels could not be sold before depuration unless they are farmed in class A water (Regulation EC 853/2004)[2].

The processed mussel business also involves the sale of preserved (boiling and preserving in sterile brine) and frozen goods. The transformed goods are packaged in shipping cases after being labeled, heated (sterilized) in a retort, and sealed. From 1984 to 1989, the sale of transformed mussels more than doubled[2].

Markets for mussels have changed. Spanish mussel farming began with a modest national consumption, but today it exceeds 100,000 tons per year, mostly as not preserved mussels. The distribution of mussels was roughly 40% for the market (with 76 percent going to the local market and 24 percent going to export), 50% for canning (with 89 percent of the canned mussels being consumed domestically and the remaining 11 percent going to export), and 10% for freezing. The amount of frozen food has been growing and is now projected to be 15,000 tons annually. Most exports go to Italy, France, and Germany. France imported 3,800 tons of mussels from Spain in 2001 and 4,608 tons in 2002, while Italy imported 1,100 tons in 2001 and 1,400 tons in 2002[2].

2.3 *M. galloprovincialis* Italian production

Concerning Italian manufacturing, there were 245 farms in 2016, with six areas accounting for more than 90% of the national production. In terms of volume, Emilia-Romagna, Veneto, and Puglia regions produce most (72%) of the mussels in Italy, followed by Friuli-Venezia-Giulia, Sardinia, and Liguria regions. With 21,601 tons produced in 2016, Emilia-Romagna is the region with the highest output (34% of the overall amount) [10].

Sardinia is both a significant exporter and importer of mussels from other Italian areas and other EU Member States. In Sardinia, imported or locally produced mussels are primarily marketed to other Italian areas. Sardinia sells over 15,000 tons of mussels annually (both locally and abroad) but the regional production is much lower; for example, it-was 4,100 tons in 2016 [10].

In Sardinia, the high level of sales is due to favorable environmental factors for the growth and maturation of mussels, high levels of local consumption resulting from tourism, and the expansion of marketing initiatives through commercial brands. In addition, organic production allows, stakeholders to expand their markets, particularly for exports to France[10].

Italy's mussel output fluctuated between 52,526 tones (the lowest value recorded in 2015) and 79,520 tones (the highest value recorded in 2011) between 2007 and 2016. Production reached 63,700 tons in 2016 but, in 2017, 73,066 tons of mussels were imported into Italy for 61 million euros. Alive mussels make up 52% of the total volume, mostly from Spain and to a lesser extent, Greece. A proportion of 37% of the total are prepared or preserved mussels, mostly from Chile, and 11% are frozen mussels, mostly from Spain[10].

Italian export of alive mussels reached 7,435 tones and 13.4 million euros in 2017, thus showing an increase of 9% since 2007). In 2017 Italy exported mussels especially to Spain (26% of exports) and France (46% of exports), but mussels exported to France cost 0.89 euros/kg, while mussels sent to Spain costed 0.66 euros/kg[10].

In 2017 the amount of mussels consumed by the Italian families was 42,750 tones, which corresponded to a value of 102 million euros. The volume comprises alive and processed mussels, and cannot be compared with the apparent consumption, which is given in live weight). Between 2009 and 2013, household consumption dropped slightly, but it is now rising[10].

The highest consumption amount was 45,189 tons (corresponding to 122 million euros) in 2009, while the lowest was 37,594 tons (corresponding 93 million euros) in 2013. Consumption is most seasonal, with peaks during the summer (June to September), when 4,000 to 5,000 tons are consumed monthly because demand rises owing both to tourism and accessibility of domestic produce[10].

Meanwhile, national mussels are not accessible in December, when the consumption is high (about 4,000 tons) and they are mainly imported from Spain. The average household uses between 2,000 and 3,500 tons per month[10].

3. Legislation in force in the European Union

Nowadays, Food Business Operators (FBOs) should take full responsibility for the food they place on the market. Unsafe food cannot be placed on the market, as assessed by the General Food Law (Regulation (CE) No 178/2002). In addition, the so called "Hygiene Package", which became applicable on 2006, includes Regulation (EC) No 853/2004 on specific hygiene rules for food of animal origin, including live mussels. In this chapter, a description of the articles and definitions related to live mussels will be given, together with the duties and responsibilities of FBOs.

3.1 Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin (OJ L 139, 30.4.2004, p. 55)

Article 6 : products of live animal origin outside the European community : in the case of live bivalve molluscs, echinoderms, tunicates and marine gastropods, the production area appears on a list drawn up in accordance with Article 13 of that Regulation, when applicable;

Article 11: specific decisions: to lay down other treatments that may be applied in a processing establishment to live bivalve molluscs from class B or C production areas that have not been submitted to purification or relaying;

3.2 Anex 1: definitions:

LIVE BIVALVE MOLLUSCS:

- 'Bivalve molluscs' means filter-feeding lamellibranch molluscs.
- 'Marine biotoxins' means poisonous substances accumulated by bivalve molluscs, in particular as a result of feeding on plankton containing toxins.
- 'Conditioning' means the storage of live bivalve molluscs coming from class A production areas, purification centres or dispatch centres in tanks or any other installation containing clean seawater, or in natural sites, to remove sand, mud or slime, to preserve or to improve organoleptic qualities and to ensure that they are in a good state of vitality before wrapping or packaging.
- 'Gatherer' means any natural or legal person who collects live bivalve molluscs by any means from a harvesting area for the purpose of handling and placing on the market.

- ‘Production area’ means any sea, estuarine or lagoon area, containing either natural beds of bivalve molluscs or sites used for the cultivation of bivalve molluscs, and from which live bivalve molluscs are taken.
- ‘Relaying area’ means any sea, estuarine or lagoon area with boundaries clearly marked and indicated by buoys, posts or any other fixed means, and used exclusively for the natural purification of live bivalve molluscs.
- ‘Dispatch centre’ means any on-shore or off-shore establishment for the reception, conditioning, washing, cleaning, grading, wrapping and packaging of live bivalve molluscs fit for human consumption.
- ‘Purification centre’ means an establishment with tanks fed by clean seawater in which live bivalve molluscs are placed for the time necessary to reduce contamination to make them fit for human consumption.
- ‘Relaying’ means the transfer of live bivalve molluscs to sea, lagoon or estuarine areas for the time necessary to reduce contamination to make them fit for human consumption. This does not include the specific operation of transferring bivalve molluscs to areas more suitable for further growth or fattening.

OTHER DEFINITIONS

‘Products of animal origin’ means:

- food of animal origin, including honey and blood;
- live bivalve molluscs, live echinoderms, live tunicates and live marine gastropods intended for human consumption; and — other animals destined to be prepared with a view to being supplied live to the final consumer.

SECTION VII: LIVE BIVALVE MOLLUSCS

1. This Section applies to live bivalve molluscs. With the exception of the provisions on purification, it also applies to live echinoderms, live tunicates and live marine gastropods. The provisions on the classification of production areas set out in Chapter II, Part A, of that Section do not apply to marine gastropods and to echinoderms which are not filter feeders.

a. For the purpose of this Section, 'intermediary operator' means a food business operator, including traders, other than the first supplier, with or without premises, who carries out its activities between production areas, relaying areas or any establishments.

2. Chapters I to VIII apply to animals harvested from production areas that the competent authority has classified in accordance with Regulation (EC) No 854/2004. Chapter IX applies to *pectinidae* harvested outside those areas.
3. Chapters V, VI, VIII and IX, and point 3 of Chapter VII, apply to retail.
4. The requirements of this Section supplement those laid down in Regulation (EC) No 852/2004:
   a. In the case of operations that take place before live bivalve molluscs arrive at a dispatch or purification centre, they supplement the requirements of Annex I to that Regulation.
   b. In the case of other operations, they supplement the requirements of Annex II to that Regulation.

CHAPTER I: GENERAL REQUIREMENTS FOR THE PLACING ON THE MARKET OF LIVE BIVALVE MOLLUSCS

1. Live bivalve molluscs may not be placed on the market for retail sale otherwise than via a dispatch centre, where an identification mark must be applied in accordance with Chapter VII.
2. Food business operators may accept batches of live bivalve molluscs only if the documentary requirements set out in points 3 to 7 have been complied with.
3. Whenever a food business operator moves a batch of live bivalve molluscs between production areas, relaying areas or any establishments, a registration document must accompany the batch.
4. The registration document must be in at least one official language of the Member State in which the receiving establishment is located and contain at least the information specified below.
   a. In the case of a batch of live bivalve molluscs sent from a production area, the registration document must contain at least the following information:

i. the gatherer's identity and address;

ii. the date of harvesting;

iii. the location of the production area described in as precise detail as is practicable or by a code number;

iv. the health status of the production area;

v. the shellfish species and quantity; and

vi. the destination of the batch.

b. In the case of a batch of live bivalve molluscs sent from a relaying area, the registration document must contain at least the information referred to in (a) and the following information:

i. the location of the relaying area; and

ii. the duration of relaying.

c. In the case of a batch of live bivalve molluscs sent from a purification centre, the registration document must contain at least the information referred to in (a) and the following information:

i. the address of the purification centre;

ii. the duration of purification; and

iii. the dates on which the batch entered and left the purification centre.

d. Where a batch of live bivalve molluscs is sent by an intermediary operator, a new registration document, filled-in by the intermediary operator, must accompany the batch. The registration document must contain at least the information referred to in points (a), (b) and (c) and the following information:

i. the name and address of the intermediary operator;

ii. in the case of conditioning or in the case of re-immersion for storing purpose, the date of starting, the date of end and the place of the conditioning or the re-immersion;

iii. if a conditioning in a natural site was carried out, the intermediary operator must confirm that the natural site where the conditioning took place was classified at the time of conditioning as an class A production area open for harvest;

iv. if a re-immersion in natural site was carried out, the intermediary operator must confirm that the natural site where the re-immersion took place was

classified at the time of re-immersion with the same classification of the production area where the live bivalve molluscs were harvested.

v. if a re-immersion was carried out in an establishment, the intermediary operator must confirm that the establishment was approved at the time of the re-immersion. The re-immersion shall not cause additional contamination to the live bivalve molluscs.

vi. in the case of grouping, the species, the date when the grouping started, the date of the end of the grouping, the status of the area where the live bivalve molluscs were harvested, and the batch of the grouping, that always consists of the same species, captured on the same date, and in the same production area.

5. Food business operators sending batches of live bivalve molluscs must complete the relevant sections of the registration document so that they are easy to read and cannot be altered. Food business operators receiving batches must date-stamp the document on receipt of the batch or record the date of receipt in another manner.
6. Food business operators must keep a copy of the registration document relating to each batch sent and received for at least twelve months after its dispatch or receipt (or such longer period as the competent authority may specify).
7. However, if:
   a. the staff gathering live bivalve molluscs also operate the dispatch centre, purification centre, relaying area or processing establishment receiving the live bivalve molluscs; and
   b. a single competent authority supervises all the establishments concerned, registration documents are not necessary if that competent authority so permits.
8. Intermediary operators must be:
   a. registered with the competent authority as a food business carrying out primary production as referred to in Article 4(2)(a) if they do not have premises or if they have premises where they only handle, wash and store at ambient temperature live bivalve molluscs, without grouping nor conditioning, or
   b. approved by the competent authority as a food business operator in accordance with Article 4(2) if, in addition to carrying out the activities referred to in point (a),

they have a cold store or they group or split batches of live bivalve molluscs or they carry out conditioning or reimmersion.

9. Intermediary operators may receive live bivalve molluscs from production areas classified as A, B or C, from relaying areas or from other intermediary operators. Intermediary operators can send live bivalve molluscs:
   a. from class A production areas to dispatch centres or another intermediary operator;
   b. from class B production areas only to purification centres, processing establishments or to another intermediary operator;
   c. from class C production areas to processing establishments or to another intermediary operator with premises.

CHAPTER II: HYGIENE REQUIREMENTS FOR THE PRODUCTION AND HARVESTING OF LIVE BIVALVE MOLLUSCS

A. REQUIREMENTS FOR PRODUCTION AREAS

1. Gatherers may only harvest live bivalve molluscs from production areas with fixed locations and boundaries that the competent authority has classified — where appropriate, in cooperation with food business operators — as being of class A, B or C in accordance with Regulation (EC) No 854/2004.
2. Food business operators may place live bivalve molluscs collected from class A production areas on the market for direct human consumption only if they meet the requirements of Chapter V.
3. Food business operators may place live bivalve molluscs collected from class B production areas on the market for human consumption only after treatment in a purification centre or after relaying.
4. Food business operators may place live bivalve molluscs collected from class C production areas on the market for human consumption only after relaying over a long period in accordance with Part C of this Chapter.
5. After purification or relaying, live bivalve molluscs from class B or C production areas must meet all of the requirements of Chapter V. However, live bivalve molluscs from such

areas that have not been submitted for purification or relaying may be sent to a processing establishment, where they must undergo treatment to eliminate pathogenic micro-organisms (where appropriate, after removal of sand, mud or slime in the same or another establishment). The permitted treatment methods are:

a. sterilisation in hermetically sealed containers; and
b. heat treatments involving:
    i. immersion in boiling water for the period required to raise the internal temperature of the mollusc flesh to not less than 90 °C and maintenance of this minimum temperature for a period of not less than 90 seconds;
    ii. cooking for three to five minutes in an enclosed space where the temperature is between 120 and 160 °C and the pressure is between 2 and 5 kg/cm2 , followed by shelling and freezing of the flesh to a core temperature of – 20 °C; and
    iii. steaming under pressure in an enclosed space satisfying the requirements relating to cooking time and the internal temperature of the mollusc flesh mentioned under (i). A validated methodology must be used. Procedures based on the HACCP principles must be in place to verify the uniform distribution of heat.

6. Food business operators must not produce live bivalve molluscs in, or harvest them from, areas that the competent authority has not classified, or which are unsuitable for health reasons. Food business operators must take account of any relevant information concerning areas' suitability for production and harvesting, including information obtained from ownchecks and the competent authority. They must use this information, particularly information on environmental and weather conditions, to determine the appropriate treatment to apply to harvested batches.

B. REQUIREMENTS FOR HARVESTING AND HANDLING FOLLOWING HARVESTING

Food business operators harvesting live bivalve molluscs, or handling them immediately after harvesting, must ensure compliance with the following requirements.

1. Harvesting techniques and further handling must not cause additional contamination or excessive damage to the shells or tissues of the live bivalve molluscs or result in changes significantly affecting their suitability for treatment by purification, processing or relaying. Food business operators must in particular:
    a. adequately protect live bivalve molluscs from crushing, abrasion or vibration;
    b. not expose live bivalve molluscs to extreme temperatures;
    c. not re-immerse live bivalve molluscs in water that could cause additional contamination; and
    d. if carrying out conditioning in natural sites, use only areas that the competent authority has classified as being of class A.
2. Means of transport must permit adequate drainage, be equipped to ensure the best survival conditions possible and provide efficient protection against contamination.

C. REQUIREMENTS FOR RELAYING LIVE BIVALVE MOLLUSCS

Food business operators relaying live bivalve molluscs must ensure compliance with the following requirements.

1. Food business operators may use only those areas that the competent authority has approved for relaying live bivalve molluscs. Buoys, poles or other fixed means must clearly identify the boundaries of the sites. There must be a minimum distance between relaying areas, and also between relaying areas and production areas, so as to minimise any risk of the spread of contamination.
2. Conditions for relaying must ensure optimal conditions for purification. In particular, food business operators must:
    a. use techniques for handling live bivalve molluscs intended for relaying that permit the resumption of filter-feeding activity after immersion in natural waters;
    b. not relay live bivalve molluscs at a density that prevents purification;
    c. immerse live bivalve molluscs in seawater at the relaying area for an appropriate period, fixed depending on the water temperature, which period must be of at least two months' duration unless the competent authority agrees to a shorter period on the basis of the food business operator's risk analysis; and

d. ensure sufficient separation of sites within a relaying area to prevent mixing of batches; the 'all in, all out' system must be used, so that a new batch cannot be brought in before the whole of the previous batch has been removed.

3. Food business operators managing relaying areas must keep permanent records of the source of live bivalve molluscs, relaying periods, relaying areas used and the subsequent destination of the batch after relaying, for inspection by the competent authority.

CHAPTER III: STRUCTURAL REQUIREMENTS FOR DISPATCH AND PURIFICATION CENTRES

1. The location of premises on land must not be subject to flooding by ordinary high tides or run-off from surrounding areas.
2. Tanks and water storage containers must meet the following requirements:
   a. Internal surfaces must be smooth, durable, impermeable and easy to clean.
   b. They must be constructed so as to allow complete draining of water.
   c. Any water intake must be situated in a position that avoids contamination of the water supply.
3. In addition, in purification centres, purification tanks must be suitable for the volume and type of products to be purified.

CHAPTER IV: HYGIENE REQUIREMENTS FOR PURIFICATION AND DISPATCH CENTRES

A. REQUIREMENTS FOR PURIFICATION CENTRES

Food business operators purifying live bivalve molluscs must ensure compliance with the following requirements.

1. Before purification commences, live bivalve molluscs must be free of mud and accumulated debris and washed if necessary, using clean water.
2. Operation of the purification system must allow live bivalve molluscs rapidly to resume and to maintain filter-feeding activity, to eliminate sewage contamination, not to become re-contaminated and to be able to remain alive in a suitable condition after purification for wrapping, storage and transport before being placed on the market.

3. The quantity of live bivalve molluscs to be purified must not exceed the capacity of the purification centre. The live bivalve molluscs must be continuously purified for a period sufficient to achieve compliance with allow the health standards of Chapter V and microbiological criteria adopted in accordance with Regulation (EC) No 852/2004.
4. Should a purification tank contain several batches of live bivalve molluscs, they must be of the same species and the length of the treatment must be based on the time required by the batch needing the longest period of purification.
5. Containers used to hold live bivalve molluscs in purification systems must have a construction that allows clean seawater to flow through. The depth of layers of live bivalve molluscs must not impede the opening of shells during purification.
6. No crustaceans, fish or other marine species may be kept in a purification tank in which live bivalve molluscs are undergoing purification.
7. Every package containing purified live bivalve molluscs sent to a dispatch centre must be provided with a label certifying that all molluscs have been purified.

B. REQUIREMENTS FOR DISPATCH CENTRES

Food business operators operating dispatch centres must ensure compliance with the following requirements.

1. Handling of live bivalve molluscs, particularly conditioning, calibration, wrapping and packing, must not cause contamination of the product or affect the viability of the molluscs.
2. Before dispatch, the shells of live bivalve molluscs must be washed thoroughly with clean water.
3. Live bivalve molluscs must come from:
    a. a class A production area;
    b. a relaying area;
    c. a purification centre; or
    d. another dispatch centre.

4. The requirements laid down in points 1 and 2 also apply to dispatch centres situated on board vessels. Molluscs handled in such centres must come from a class A production area or a relaying area.

CHAPTER V: HEALTH STANDARDS FOR LIVE BIVALVE MOLLUSCS

In addition to ensuring compliance with microbiological criteria adopted in accordance with Regulation (EC) No 852/2004, food business operators must ensure that live bivalve molluscs placed on the market for human consumption meet the standards laid down in this Chapter.

1. They must have organoleptic characteristics associated with freshness and viability, including shells free of dirt, an adequate response to percussion and normal amounts of intravalvular liquid.
2. They must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:
    a. for paralytic shellfish poison (PSP), 800 micrograms of saxitoxin equivalents diHCl per kilogram;
    b. for amnesic shellfish poison (ASP), 20 milligrams of domoic acid per kilogram;
    c. for okadaic acid and dinophysistoxins together 160 micrograms of okadaic acid equivalents per kilogram;
    d. for yessotoxins, 3,75 milligrams of yessotoxin equivalent per kilogram;
    e. for azaspiracids, 160 micrograms of azaspiracid equivalents per kilogram.

CHAPTER VI: WRAPPING AND PACKAGING OF LIVE BIVALVE MOLLUSCS

1. Oysters must be wrapped or packaged with the concave shell downwards.
2. All packages of live bivalve molluscs leaving dispatch centres or destined for another dispatch centre, must be closed. Packages of live bivalve molluscs, intended for direct retail sale, must remain closed until they are presented for sale to the final consumer.

CHAPTER VII: IDENTIFICATION MARKING AND LABELLING

1. The label, including the identification mark, must be waterproof.
2. In addition to the general requirements for identification marks contained in Annex II, Section I, the following information must be present on the label:
   a. the species of bivalve mollusc (common name and scientific name); and
   b. the date of packaging, comprising at least the day and the month. By way of derogation from Directive 2000/13/EC, the date of minimum durability may be replaced by the entry 'these animals must be alive when sold'.
3. The retailer must keep the label attached to the packaging of live bivalve molluscs that are not in individual consumer-size packages for at least 60 days after splitting up the contents.

CHAPTER VIII: OTHER REQUIREMENTS

1. Food business operators storing and transporting live bivalve molluscs must ensure that they are kept at a temperature that does not adversely affect food safety or their viability.
2. Live bivalve molluscs must not be re-immersed in, or sprayed with, water after they have been packaged for retail sale and left the dispatch centre.

CHAPTER IX: SPECIFIC REQUIREMENTS FOR PECTINIDAE, MARINE GASTROPODS AND ECHINODERMS WHICH ARE NOT FILTER FEEDERS HARVESTED OUTSIDE CLASSIFIED PRODUCTION AREAS

Food business operators harvesting pectinidae, marine gastropods and echinoderms which are not filter feeders, outside classified production areas or handling such pectinidae, and/or such marine gastropods and/or echinoderms must comply with the following requirements:

1. Pectinidae, marine gastropods and echinoderms which are not filter feeders, must not be placed on the market unless they are harvested and handled in accordance with Part B of Chapter II and meet the standards laid down in Chapter V, as demonstrated by a system

of own checks by the food business operators operating a fish auction, a dispatch centre or a processing establishment;

2. In addition to point 1, where data from official monitoring programmes enable the competent authority to classify fishing grounds – where appropriate, in cooperation with food business operators – the provisions of Part A of Chapter II apply by analogy to pectinidae;
3. Pectinidae, marine gastropods and echinoderms which are not filter feeders, must not be placed on the market for human consumption otherwise than via a fish auction, a dispatch centre or a processing establishment. When they handle pectinidae and/or such marine gastropods, and/or echinoderms food business operators operating such establishments must inform the competent authority and, as regards dispatch centres, comply with the relevant requirements of Chapters III and IV;
4. Food business operators handling pectinidae, marine gastropods and echinoderms which are not filter feeders, must comply with the following requirements:
    a. with the documentary requirements of Chapter I, points 3 to 7, where applicable. In this case, the registration document must clearly indicate the location of the area, indicating the system used to describe the coordinates, where the live pectinidae and/or live marine gastropods and/or live echinoderms were harvested; or
    b. with the requirements of Chapter VI, point 2, concerning the closing of all packages of live pectinidae, live marine gastropods and live echinoderms dispatched for retail sale and Chapter VII concerning identification marking and labelling.

CHAPTER X: MODEL OF REGISTRATION DOCUMENT OF LIVE BIVALVE MOLLUSCS, LIVE ECHINODERMS, LIVE TUNICATES AND LIVE MARINE GASTROPODS

The REGISTRATION DOCUMENT OF LIVE BIVALVE MOLLUSCS, LIVE ECHINODERMS, LIVE TUNICATES AND LIVE MARINE GASTROPODS must contain:

I.1 IMSOC Reference number

I.2 I.2 Internal reference number

I.3 I.3 Supplier Name, Address, Registration or Approval No, Country, ISO Country code, Activity

I.4 I.4 Receiving food business operator, Name, Address, Registration or Approval No, Country, ISO Country code, Activity.

I.5 Description of goods Aquaculture □ Natural Banks □ • CN code or FAO 3-Alpha code | species | quantity | package | batch | date of harvesting| date of starting of conditioning | date of end of conditioning | place of conditioning| date of starting of re-immersion | date of end of re-immersion |place of re-immersion| date of starting of grouping | date of end of grouping | production area | health status including, if applicable, harvested in accordance with Article 62(2) of Implementing Regulation (EU) 2019/627

I.6 From relaying area Yes □ No □ Relaying area Duration of relaying Date of starting Date of end

I.7 From purification/dispatch centre Auction hall Yes □ No □ Purification/dispatch centre/auction hall approval number Date of entry Date of exit Duration of purification

I.8 From Intermediary operator Yes □ No □ Name Address Registration or Approval No Country ISO Country code Activity Date of arrival Date of exit

I.9 Declaration of the supplier I, the undersigned food business operator responsible for dispatching the consignment declare that, to the best of my knowledge and belief, the information provided in Part I of this document is true and complete.

REGISTRATION DOCUMENT OF LIVE BIVALVE MOLLUSCS, LIVE ECHINODERMS, LIVE TUNICATES AND LIVE MARINE GASTROPODS must contain:

Date, name of signatory, signature

II.1 Internal reference number (receiving)

II.2 Declaration of the receiving food business operator I, the undersigned food business operator responsible for receiving the consignment declare that the consignment has arrived on [DATE] in my premises. And name of signatory, signature

Explanatory notes

Description

Part I – Supplier

This part of the document shall be filled by the food business operator dispatching a batch of live bivalve molluscs.

I.1 IMSOC reference number This is the unique alpha-numeric code assigned by the IMSOC

I.2 Internal reference number This box may be used by the dispatching food business operator to indicate an internal reference number.

I.3 Supplier Indicate the name and address (street, city and region/province/state, as appropriate), country and ISO country code of the establishment of origin. In the case of production areas, please indicate the area as authorised by the competent authorities (CAs). In the case of live pectinidae, marine gastropods or echinoderms, indicate the location of the harvesting area. Where applicable, indicate the registration or approval number of the establishment. Indicate the activity (gatherer, purification centre, dispatch centre, auction hall or intermediary activities). Where the batch of live bivalve molluscs is sent from a purification centre/dispatch centre, or, in case of pectinidae, marine gastropods and echinoderms which are not filter feeders harvested outside classified production areas, from a fish auction, indicate the approval number and the address of the purification centre/dispatch centre or fish auction.

I.4 Receiving food business operator Indicate the name and address (street, city and region/province/state, as appropriate), country and ISO country code of the establishment of destination. In the case of production or relaying areas please indicate the area as authorised by the CAs. Where applicable, indicate the registration or approval number of the establishment Indicate the activity (gatherer, purification centre, dispatch centre, processing establishment or intermediary activities).

I.5 Description of goods Indicate as required, the Combined Nomenclature code or FAO 3-Alpha code, species, quantity, type of packaging (bags, bulk, etc.), batch, date of harvesting, date of starting and end of conditioning (when applicable), place of conditioning (indicate the classification of the production area and its location or the approval number of the establishment, when applicable), date of starting and end of re-immersion (when applicable), place of re-immersion (indicate the classification of the production area and its location or the

approval number of the establishment when applicable), date of starting and end of grouping (when applicable), production area and its health status (classification of the production area when applicable). When LBMs have been harvested in accordance with Article 62(2) of the Implementing Regulation 2019/627 then this should be explicitly stated.

Description

When grouping of live bivalve molluscs is performed, the batch must refer to bivalves of the same species, harvested on the same day and coming from the same production area. Delete as appropriate

I.6 From relaying area Where the batch of live bivalve molluscs is sent from a relaying area, indicate the relaying area, as authorised by the CAs, and the duration of the relaying (date of starting and end).

I.7 From purification centre/dispatch centre or fish auction Where the batch of live bivalve molluscs is sent from a purification centre/dispatch centre, or, in case of pectinidae, marine gastropods and echinoderms which are not filter feeders harvested outside classified production areas, from an auction hall, indicate the approval number and the address of the purification centre/dispatch centre or auction hall. If sent from a purification centre, the duration of the purification and the dates on which the batch entered and left the purification centre. Delete as appropriate.

I.8 From intermediary operator Indicate the name and address (street, city and region/province/state, as appropriate), country and ISO country code of the intermediary operator. Where applicable, indicate the registration or approval number and the activity.

I.9 Declaration of the supplier Include the date, name of the signatory and the signature

Part II – Receiving food business operator This part of the document shall be filled by the food business operator receiving a batch of live bivalve molluscs.

I.11 Internal reference number (receiving) This box may be used by the food business operator receiving the batch to indicate an internal reference number.

I.12 Declaration of the receiving food business operator Indicate the date of arrival of the batch of live bivalve molluscs at the premises of the receiving food business operator. In the

case of an intermediary operator without premises indicate the date of purchase of the batch. Include the name of the signatory and the signature.

4. Norovirus

4.1 Human Norovirus background

About 700 million illnesses and 200,000 fatalities are attributed to the human Norovirus (HNoV), the most common cause of acute gastroenteritis worldwide. Due in part to our limited knowledge of human Norovirus biology, there are currently no approved antiviral medications or vaccines. Uncertainty exists regarding the host genes necessary for human Norovirus infection, in vivo cell tropism, and immune evasion and immunity processes. The initial phase of the virus life cycle is viral entrance. It begins with the virus attaching to the cell surface and ends with the release of the viral DNA into the cytoplasm of the host cell[5].

Important implications for host range, cell tropism, and disease result from elucidating the molecular mechanisms by which a virus penetrates its target cell[5].

The human Norovirus, formerly known as the Norwalk virus, was the first viral agent proven to cause gastroenteritis and was initially discovered in stool samples gathered during an outbreak of gastroenteritis in Norwalk, Ohio in 1929. The illness caused by this virus was first referred to as "winter vomiting disease" because to its seasonal inclination and the frequent preponderance of patients who reported vomiting as a primary symptom[6].

When the virus was first discovered in 1968, it mostly showed up as nausea, vomiting, diarrhea, and low-grade fever in 50% of the pupils at a Norwalk elementary school. Ninety-eight percent of those with primary cases reported feeling queasy, and ninety-two percent of them vomited. In addition, sixty-eight percent reported experiencing abdominal cramps, fifty-two percent reported feeling lethargic, thirty-eight percent reported having diarrhea, and thirty-four percent reported having a fever[6].

The assessment of a 48-hour incubation time was made possible by the presence of secondary cases in 32% of family contacts. Each case had a 24-hour sickness that ended with complete recovery[6].

4.2 General characteristics

The human Norovirus (HNoV) belongs to the *Caliciviridae* family of non-enveloped, positive-sense single-stranded RNA (ssRNA) viruses, together with the Sapovirus genera which are responsible for widespread human diseases. There are seven genogroups that make up the genus Norovirus[5]:

- Gengroup (G) I viruses infect humans
- GII infect humans and pigs
- GIII viruses infect cows and sheep
- GIV viruses infect humans, cats and dogs
- GV viruses infect mice
- GVI viruses infect dogs and cats
- GVII viruses infect dogs

4.3 Genome and viral structure

The Norovirus genome is 7.5 kilobases long and encodes two structural proteins, the main capsid protein VP1 (59 kDa), and six non-structural proteins (29 kDa). Although smaller virions with T = 1 icosahedral symmetry have been described, Norovirus particles typically have a diameter of 27 to 30 nm. A flexible linker connects each of the 90 VP1 dimers that make up the viral capsid, each of which has a shell (S) domain and a protrusion (P) domain[5].

The S domain, which makes up the majority of the highly conserved VP1 domain, is all that is necessary to assemble the Norovirus capsid shell, which houses the viral DNA. The P domain, in contrast, is more flexible and contains the P1 and P2 subdomains, which differ in their fundamental amino acid sequence. Six anti-parallel -barrels extend out from the extremely changeable P1 subdomain to form the VP1's outermost structure, which is represented by the P2 subdomain. The P1 domain, the S and P2 domains, plus one helix and eight sheets of connections make up the P1 domain. The known and probable receptor binding sites for MNoV (MNoV) and human Norovirus (HNoV), respectively, are located in the P2 subdomain, which is a target for antibodies that neutralize them[5].

The absence of infectious molecular clones and difficulties with in vitro human Norovirus culture have hampered studies on HNoV entrance. Since there are infectious molecular clones

and highly susceptible cell lines for these viruses, biochemical investigations using human Norovirus-like particles and the related viruses MNoV and feline Calicivirus (FCV) have contributed significantly to our current understanding of Norovirus entrance[5].

Subsequent cryo-electron microscopy research shed light on the molecular mechanism of feline Calicivirus entrance and viral genome delivery, while recent genome-wide CRISPR screens identified CD300lf as the proteinaceous receptor for MNoV, the first receptor characterized of any Norovirus. These studies have significant ramifications for our comprehension of HNoV entrance and pathogenesis[5].

The linear, positive-sense RNA that makes up the HNoV genome is 7.6 kb long. At the 5' end, the genome is covalently joined to the viral protein genome (VPg), and it is polyadenylated at the 3' end. Eight viral proteins are encoded by three open reading frames (ORFs), numbered ORF-1, 2, and 3. Viral protein 1 (VP1) and VP2, the two structural elements of the virion, are encoded by ORF-2 and ORF-3, respectively (Figure 3). 90 VP1 dimers are present in the mature virion, which is built with icosahedral symmetry and organized to produce hollow or cup-like structures on the virus surface. “Calici” is therefore a derivative of the Latin word calyx, which means "cup." [4]

The viral protease and RNA-dependent RNA polymerase are two of the six nonstructural proteins that are produced by proteolytic processing from the polyprotein that ORF-1 encodes.[4]

The Norovirus has been compared to a "shape-shifter," a fantastical being with the ability to change its appearance. This description relates to its diversity, with at least 6 genogroups (genogroup I–VI) and 40 genotypes identified by the VP1 amino acid sequence, as well as its ongoing evolution, maybe in response to the selective pressure applied by the human immune system. In decreasing order of frequency, GII, GI, and, to a very limited extent, GIV (certain genotypes of which also infect pigs) are responsible for HNoV infections. Additionally, antibodies to a bovine Norovirus known as GIII have been found in 22% of people, while antibodies to a canine-only gene group known as GVI have been found in 22.6% of small-animal vets and 5.8% of age-matched controls.[4]

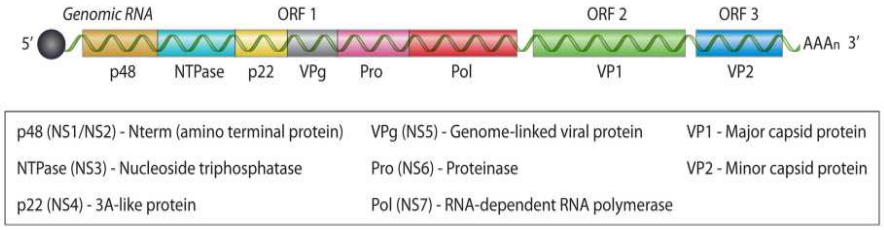

Figure 3: The human Norovirus genome. The genome is comprised of a linear, positive-sense RNA approximately 7.6 kb in length, covalently linked to the viral protein genome (VPg) (solid black circle) at the 5' end and polyadenylated at the 3' end. There are three open reading frames (ORFs), designated ORF-1, ORF-2, and ORF-3, encoding 8 viral proteins. ORF-1 encodes the 6 nonstructural (NS) proteins that are proteolytically processed by the virally encoded cysteine proteinase (Pro). ORF-2 and ORF-3 encode the structural components of the virion, viral protein 1 (VP1) and VP2,

The observation that VP1 amino acid sequences of GII.4 strains differ by 37% to 38% from the prototypical GII.1 Norwalk virus strain and by 5% to 7% within the GII genotype alone, with as much as a 2.8% difference between strains of an individual virus, points to the genetic diversity of human Noroviruses. It has been suggested that the use of sequence difference cutoffs is no longer sufficient to classify Noroviruses and that the addition of a phylogenetic approach would prove more accurate because of the variability, which results from both recombination and mutational events, is of a sufficient magnitude.[4]

4.4 Viral infection of the host cells

As Norovirus is a non-enveloped virus, attachment is a multi-step process that begins with viral attachment to target cells and ends with the transfer of the viral genome into the cytoplasm through the processes of receptor engagement, endocytosis, cell membrane penetration, and uncoating. Notably, viral entry plays a key role in determining host range, pathogenicity, and cell tropism. The binding of a virus to host cells, which is mediated by both host attachment factors and receptors, is the first and frequently rate-limiting phase of viral entry. Although they may not actively promote viral entry, attachment factors are host molecules that concentrate the virus on the cell surface.[5]

While viral infection is made more effective by attachment factors, they are by definition not necessary. Comparatively, viral receptors are necessary host molecules that specifically bind the virus particle, cause a conformational change in the virus, and actively encourage viral

entry. Notably, the viral strain, type of host cell, and culture conditions may all have an impact on how host molecules that are implicated in viral entrance are classified in some situations. It's interesting to note that host molecules, both cell-associated and non-cell-associated, have been described for Noroviruses that increase binding, albeit via different ways. These compounds include sialic acid, bile acids, histo-blood group antigens (HBGAs), and divalent cations (Figure 4).[5]

Viral attachment to the cell surface is the first and frequently rate-limiting step in the viral entrance process. The invasion of the mouse (MNoV) and human (HNoV) Noroviruses can be facilitated by cell-associated host glycans such as terminal sialic acid and histo-blood group antigens (HBGAs), respectively. The virus's ability to adhere to cells can also be enhanced by soluble cofactors such as soluble HBGAs (MNoV and HNoV), bile salts (MNoV and HNoV), and divalent cations (HNoV). These soluble cofactors promote MNoV viral attachment via receptor-dependent mechanisms. Receptor engagement occurs during the second stage of viral entry. The MNoV receptor is an immunoglobulin (Ig) domain-containing membrane proteins, while the feline Calicivirus receptor is the feline junctional adhesion molecule A (fJAM-A), and the HNoV receptor is still unknown[5].

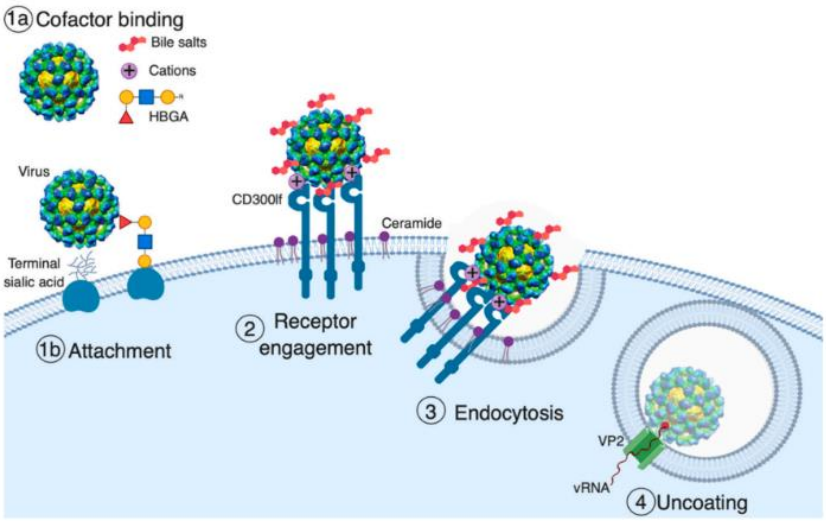

Figure 4. Model of Norovirus entry. The first and often rate-limiting step of viral entry is viral attachment to the cell surface[5].

It's intriguing to note that ceramide modifies CD300lf conformation or clustering, favoring the interaction with MNoV. After receptor activation, the virus is endocytosed, where, at least for

feline Calicivirus, receptor activation causes the minor capsid protein VP2 to generate a membrane portal that may allow viral genome release in the cytosol[5].

Later, it was discovered that long-term resistance was associated with host secretor status, or the capacity to secrete HBGAs into bodily fluids[5].

Mutations in the FUT2 gene, which codes for the enzyme FUT2 ((1,2) fucosyltransferase), affect the secretor status. Carbohydrate H antigen type-1 precursor is transformed by FUT2 into mature H antigen type-1, which can then undergo additional modifications by downstream enzymes to produce a variety of carbohydrate antigens. About 20–30% of individuals have polymorphisms in FUT2 that render the enzyme inactive. The AB0 blood group antigens cannot be secreted into the bodily fluids of these people, known as non-secretors. Importantly, protection against specific HNoV genotypes in people is correlated with the availability of anti-HNoV antibodies that prevent the binding of HBGAs to VP1 in vitro. HNoV genogroups GI.1 and GII.4 are significantly resistant to non-secretors[5].

It's significant to note that HBGAs can be cell-associated or non-cell-associated, and both types may be involved in Norovirus entrance. The details of how GI.1 virus-like particles (VLPs) bind cells from secretor are still unknown. Although they do so with a low affinity (about 400 M) and without significantly altering the structure of VP1, HBGAs bind to the P2 domain of this protein. Various diverse HBGA binding pockets have been described by structural and functional studies across different HNoV strains, which may indicate the strains' capacity to infect non-secretors[5].

While many HNoV strains rely on HBGAs as a key attachment component, more research is necessary to determine whether these glycans actually function as HNoV receptors. In particular, viral entrance is a significant barrier to HNoV replication in cell lines. It is sufficient to create infectious virions by transfecting viral RNA into the cytoplasm of cells, bypassing viral entrance. Noteworthy is the likelihood of one or more post-entry constraints on HNoV replication. It's significant to note that HBGAs are insufficient to give sensitivity to infection in these or other human cell lines, nor do they account for the constrained host range displayed by HNoVes, as other species with comparable HBGAs are not susceptible to HNoV[5].

HBGAs can interact with HNoV since they are present in saliva and linked to cells in the gastrointestinal system. Recent HNoV in vitro experiments have shown that both soluble and

cell-associated HBGAs encourage cellular infection. In vitro HNoV replication in lymphoid cell lines is promoted by cell-free and HBGA-expressing bacteria. Additionally, human intestine enteroids from secretor-negative people were resistant to GII.4 HNoV strains but not GII.3 strains, which is consistent with the epidemiology of these genotypes in the human population. It's interesting to note that successful HNoV replication in the enteroid culture system does not require exogenous HBGAs. The varied vulnerability of non-secretors to HNoV may result from variations in virus genetics, host genetics, host immunology, and/or environmental variables including the microbiota[5].

In addition to sialic acid and heparan sulfate proteoglycans, other host glycans may also be involved in viral attachment. Gangliosides' terminal sialic acid moieties, which are both 2,3- and 2,6-linked, help MNoV adhere to cells while improving feline Calicivirus's ability to bind to target cells. Given that the HBGA binding site and the sialic acid binding site on MNoV VP1 are quite similar, it is likely that other glycans can help different Noroviruses connect. Notably, sialic acid is not necessary for MNoV infection and does not explain the cell tropism or species restriction of MNoV given the widespread expression of sialic acid on several cell types from various species. This is consistent with its function as an attachment factor[5].

Non-glycans play a facilitative role in Norovirus attachment. Bile salts have recently been identified as crucial cofactors for Norovirus infection in vitro. Interestingly, the swine Sapovirus, another member of the Caliciviridae family, had previously been linked to bile salts as critical elements for in vitro reproduction. Bile salts are cholesterol derivatives that the liver produces and secretes into the digestive system, where intestinal bacteria further alter them. Bile salts perform a variety of physiological tasks, such as promoting lipid absorption, controlling cellular metabolism, and preserving intestinal homeostasis. Recently, a HNoV culture system in human intestine enteroids was created by Estes and coworkers[18].

Exogenous bile was necessary in this system for the replication of HNoV GII.3 strains and enhanced the replication of HNoV GII.4 strains. Notably, bile was needed either during or after viral adsorption but not before. Bile salts can similarly intensify MNoV infection in vitro. With roughly 5 M KD, the secondary bile salts glycochenodeoxycholic acid (GCDCA) and lithocholic acid (LCA), but not a number of other bile salts, can bind to a hydrophobic pocket at the dimer interface between the MNoV P1 and P2 subdomains. These secondary bile acids increased MNoV VP1's capacity to bind cells in a CD300lf receptor-dependent way[5].

Notably, additional unidentified non-proteinaceous small compounds discovered in serum could restore virus attachment, showing that bile was capable of increasing MNoV binding and infectivity. Surprisingly, the distance between the CD300lf receptor binding site and the bile salt binding site on MNoV VP1 prevented either from significantly altering the structure of the P domain. More recently, it was discovered that secondary bile salts, such as GCDCA, could also bind the capsid of the HNoV VLPs GII.1, GII.10, and GII.19, but not the VLPs GI.1, GII.3, 4, or 17. Notably, strain-specific differences were seen in the capacity of bile salts of promoting HNoV infection of enteroids. Similar to MNoV, bile salts bind to HNoV with 1:1 stoichiometry and low micromolar affinity and only slightly alter the P domain's structural makeup[5].

Bile salt binding pocket of HNoV, in contrast to MNoV's, was located on the apical side of P2 and overlapped with the HBGA binding site. The finding that comparable bile salts bind the capsid proteins of both MNoV and HNoV points to a shared but unidentified mode of action[5].

Along with glycans and bile salts, phospholipids have most recently been discovered to be important mediators of Norovirus entrance. Sptlcl1 and Sptlc2 were discovered to be proviral genes in MNoV infection by a genome-wide CRISPR screen. These genes produce critical proteins for the serine palmitoyltransferase (SPT) complex, which is a key regulator of membrane fluidity and dynamics by catalyzing the rate-limiting step in the biosynthesis of ceramide and sphingolipids. Noteworthy, ceramide has been mentioned as a potential CD300lf ligand. Although significant, Sptlc1 and Sptlc2 are not required for MNoV binding and infection. Exogenous ceramide can be used to correct this binding problem. CD300lf expression and membrane trafficking are not regulated by ceramide or the SPT complex[5].

Instead, different CD300lf antibody epitopes are variably modified by ceramide, which suggests that it affects conformation and/or clustering of CD300lf on the cell membrane to facilitate viral entry. Ceramide has been linked to the entrance of the porcine enteric Calicivirus, albeit through a different method. Understanding the interactions between HNoV and receptors may benefit from additional research on the function of ceramide in HNoV entrance[5].

In conclusion, substances such as glycans, bile salts, cations, and phospholipids, both soluble and cell-associated, can enhance the binding of Noroviruses to host cells. Ceramide, cations,

and bile salts are examples of receptor-dependent mechanisms for MNoV attachment enhancement, whereas HBGAs and sialic acid are examples of receptor-independent mechanisms[5].

4.5 Norovirus receptors

Although more studies on the receptor that HNoV still not nowhere needed, recent researches involving other Caliciviruses like Feline Calicivirus (FCV) and MNoV has improved our knowledge of Norovirus receptor biology[5].

The first receptor for a member of the *Caliciviridae* family, feline junctional adhesion molecule A (fJAM-A), was discovered to be the Feline Calicivirus receptor. It's interesting to note that human JAM-A has just been identified as the San Miguel sea lion virus' receptor on human cells. JAM-A has two extracellular immunoglobulin (Ig) domains (D1 and D2) and is a type I integral membrane protein. JAM-A modulates tight junction permeability by localizing to cellular tight junctions. Feline Calicivirus infection of feline cells and Feline Calicivirus infection of human cells were both made possible by fJAM-A expression[5].

A conformational shift in the Feline Calicivirus capsid, which is necessary for subsequent viral genome escape, is brought about by the P2 domain of Feline Calicivirus VP1 directly binding the membrane-proximal D1 domain of fJAM-A. CD300lf was most recently discovered to be the MNoV receptor by genome-wide CRISPR screening[5].

Having only one extracellular Ig-like domain, CD300lf is a type I integral membrane protein. As phospholipids are often located on the inner leaflet of cells, CD300lf is a member of a larger family of CD300 molecules that serve as cell death sensors. When various ligands, such as phosphatidylserine and ceramide, bind to CD300lf, the immunoregulatory signals that are produced have a variety of downstream characteristics[5].

Since MNoV infection can be prevented by an anti-CD300lf antibody or genetic CD300lf disruption, CD300lf expression is both essential and sufficient for MNoV infection. Additionally, ectopic murine CD300lf expression on human and other mammalian cell lines can induce cross-species permissivity. This further illustrates that despite their resistance to HNoV, these human cell lines still have the intracellular components required for effective

Norovirus replication. Additionally, the prototypical MNoV clones CW3 (derived from MNV-1) and CR6 do not cause infection in CD300lf-deficient mice. Interestingly to note that overexpression of CD300ld, a similar member of the CD300 family, in non-permissive cells can cause MNoV infection[5].

It is not yet apparent, nevertheless, whether physiological CD300ld expression affects MNoV infection in vitro or in vivo[5].

The molecular processes through which CD300lf interacts with MNoV have been elucidated by a number of mutagenesis and structural investigations. A phospholipid ligand binding pocket and two loop sections (CC' and CDR3) are found in the CD300lf ectodomain on the host side, respectively. An aspartic acid that coordinates a cation and makes it easier for ligands to bind to the binding pocket is located at the bottom of the structure. The buried aspartic acid is not necessary for infection, which is an interesting contrast to the need of CC' and CDR3 loops for MNoV binding. This implies that MNoV partially mimics phospholipids to effectively engage CD300lf. The CD300lf ectodomain of the viral component attaches to the P2 domain's apical surface[5].

Notably, this binding pocket on P2 largely coincides with the equivalent HBGA and bile acid binding sites on HNoV and overlaps with the epitopes of previously discovered neutralizing antibodies. Compared to other documented virus-receptor interactions, affinity of CD300lf for MNoV is quite low. A KD of ~25 µm was reported by binding experiments using soluble CD300lf and MNoV P domains. This shows that CD300lf-mediated entrance is significantly influenced by avidity and that MNoV interacts numerous CD300lf molecules per cell[5].

4.6 Uncoating and endocytosis

Caliciviruses experience endocytosis after they attach to the cell surface. To enter the host cell cytoplasm, the viral genome must first break free from the capsid and pierce the endosomal membrane. The clathrin-dependent, caveolin-dependent, dynamin-dependent, micropinocytosis-dependent, and cholesterol-dependent pathways are only a few of the various endocytic pathways that viruses might utilize to enter cells[5].

The viral genome can be released from the early endosome to the endoplasmic reticulum, depending on where the virus has internalized itself. As a result of the varying pHs in the different endosomal compartments, it has been suggested that other viruses depend on pH[5].

Both Feline Calicivirus and MNoV studies can offer important information. Clathrin is required for Feline Calicivirus entrance, and the acidic environment of the endosome is necessary for uncoating. MNoV entry, on the other hand, is pH independent but dependent on dynamin and cholesterol. It is unknown if the differences between Feline Calicivirus and MNoV in endocytic pathway use and pH dependence are virus-specific, host species-specific, or cell-type-specific. Our knowledge of Norovirus entry, pathophysiology, and immune sensing may be improved by clarifying the mechanism of Norovirus internalization. It's interesting to note that non-enveloped viruses such as Hepatitis A Virus, Poliovirus, Rotavirus, and Noroviruses can be excreted form non-lytically from inside extracellular membrane-bound vesicles. These vesicles, which most likely came from multivesicular bodies, are capable of containing a variety of viral particle counts that can change depending on the viral species[5].

For a certain virus, there can be both enclosed and non-enveloped, or naked, virions, and these various viral forms can bestow various physiologic features[5].

For example, virions in vesicles are more resistant to host antibody neutralization, more virulent per particle, and more environmentally stable. Understanding how non-enveloped viruses leave the vesicle and engage with their cellular receptors is complicated by the fact that vesicles surround what are traditionally thought of as non-enveloped virions. This is because the lipid bilayer inside an extracellular vesicle conceals the viral capsid proteins that control receptor usage and tropism[5].

The Hepatitis A Virus, in which enclosed and non-enveloped virions were recently shown to enter cells by different intracellular trafficking routes, has best illustrated the contrasts between enveloped and naked virion entry[5].

The enveloped form of the Hepatitis A Virus must be transported to the lysosome in order to be destroyed before it interacts with the body's receptors. The naked form of the virus enters the late endosome[5].

HNoV and MNoV both have free and vesicle-bound versions. Regarding the characteristics or effects of vesicle-cloaked HNoV, little is known[5].

Nevertheless, CD300lf is necessary for entrance into both wrapped and naked MNoV. Uncertainty exists regarding whether enclosed and naked MNoV interact differently with CD300lf in terms of molecules, space, and time[5].

4.7 Release of viral genome

The viral genome is released into the host cytoplasm following endosomal escape and viral uncoating, which is the last stage of viral entrance. Numerous methods exist for viruses to escape endosomes[5].

Non-enveloped viruses can release their viral genomes through membrane lysis or by using membrane-piercing structures, whereas enveloped viruses immediately fuse with the plasma or endosomal membrane, releasing their genome into the intracellular milieu. Uncoating and endosomal escape in the *Caliciviridae* were poorly understood until recent studies suggested a potential mechanism for the release of the Feline Calicivirus genome. The capsid protein VP1 undergoes a conformational change upon Feline Calicivirus binding to its receptor fJAM-A, which causes the entire Feline Calicivirus P domain to rotate. The virus capsid shell develops a tiny pore along a single three-fold axis, allowing the minor structural protein VP2 to leave the virion inside. Both the P1 and P2 domains of VP1 are bound by VP2[5].

On the exterior of the viral capsid, twelve VP2 molecules group together and create a sizable funnel-shaped portal-like structure positioned around the pore. Because of its hydrophobic tip, the projecting VP2 protein may be able to pass through the endosomal membrane. This procedure is reliant on receptor binding and an acidic pH, which is consistent with the known biology of Feline Calicivirus entrance[5].

It is known that Norovirus VP2 is not required for virion assembly but is crucial for infection. It will be crucial to see whether Norovirus VP2 experiences a similar conformation in the future[5].

4.8 Clinical traits of HNoV infection

Clinical features of HNoV infection include fecal excretion, which is prevalent in asymptomatic people, particularly in children. Children were found to be excreting Norovirus asymptomatically. Norovirus excretion that is not accompanied by symptoms can affect diagnosis. In an asymptomatic carrier, diarrhea from a different cause could be mistaken for Norovirus infection. Carriage has epidemiological effects as well. The asymptomatic excretion of norovirus has diagnostic implications. Diarrhea from another cause in an asymptomatic carrier may be misinterpreted as norovirus infection. Carriage also has epidemiological effects. A study in South Korea discovered norovirus RNA in the stool samples of 66 of 6,441 (1.02%) asymptomatic food handlers[16]. A smaller study indicated that 26 of 776 (3.4%) asymptomatic food handlers at primary schools in Incheon, South Korea, were excreting the virus[16]. An obvious negative consequence of asymptomatic carrying was the transmission of norovirus by fecal microbiota transplantation utilizing stool samples acquired from asymptomatic donors who had not been tested for the presence of the virus[17].

Infections with symptoms have an incubation time. Most infected people who develop symptoms have an estimated incubation period of 48 hours or less, which is relatively short[4].

Vomiting and diarrhea are the most common HNoV infection symptoms, and they typically last just a short time, as demonstrated by both the index epidemic and experimental passage studies. However, formerly healthy people occasionally develop more serious illnesses. According to a study conducted on 99 military trainees at a military base in the USA, 88% of those with Norovirus infections felt nauseous and 80% of them vomited, while 76% had abdominal discomfort, 67% had diarrhea, 47% had fever or chills, and 22% experienced headaches[4].

In addition, 17% of the individuals had a temperature over 100.4°F, 17% had leukocytosis, and 37% had thrombocytopenia. More strikingly, in an outbreak affecting 29 British service members in Afghanistan some patients not only had fever and gastrointestinal symptoms, but also headache, stiff neck, photophobia, and obtundation4.

In this outbreak, two patients needed ventilator support, and one patient developed diffuse intravascular coagulation. HNoV illness may be more severe and prolonged in individuals with medical comorbidities[4].

Complications include serious illness and death at young and an advanced ages. Infections caused by GII.4 strains are associated with more severe consequences, including death, than infections caused by non-GII.4 strains. Advanced age increases the likelihood of a catastrophic outcome. Thus, HNoV outbreaks in the Netherlands were substantially related with increased mortality in those over the age of 85, coinciding with the development of new virus types[4].

Immunocompromised people may experience Norovirus infection-associated illness that is longer-lasting and more severe, and it may also be accompanied with extraordinarily persistent viral excretion in some people[4].

HNoV diagnosis in hematopoietic stem cell transplant (HSCT) recipients is made difficult by the frequent incidence and many causes of diarrhea in these patients, including gastrointestinal graft-versus-host disease (GVHD)[4].

Supportive care is used to treat HNoV gastroenteritis, focusing mostly on correcting electrolyte imbalances and dehydration. Some people may benefit from antiemetics and antimotility medications[4].

The availability of a particular therapy would be beneficial in patients with persistent symptoms, notably newborns, the elderly, and immunocompromised individuals, however no therapy has been conclusively proven to be helpful[4].

4.9 HNoV routs of transmission

In a variety of geographical areas, HNoV is a well-known cause of epidemic gastroenteritis in both adult and pediatric populations. All age groups of the general population are generally susceptible to disease, but the majority of morbidity and mortality occur at the extremes of age. Even though a number of additional modalities have been described, the fecal-oral route is the most common method of transmission. These modes of transmission include contamination of food, water, and the environment as well as viral aerosolization in vomitus[4].

Testing restrictions on various environmental sources are largely to blame for the difficulties in describing the environmental dispersion of HNoV. As these are thought to concentrate viruses and other microorganisms, bivalves, molluscs, and other shellfish frequently serve as the link between environmental water contamination and foodborne outbreaks[4].

There are two basic models for Norovirus spreading through food. Direct HNoV contamination of food at the point of manufacturing is the first, and food preparation is the second[4].

Due to their raw consumption and potential for Norovirus contamination from water sources, fruits, vegetables, and seafood present a substantial risk for disease transmission. Numerous food items, including oysters, romaine lettuce, raspberries, and other produce, have been shown to contain Norovirus[4].

Additionally, significant outbreaks have been linked to the reconstitution of food goods using polluted water. The burden of Norovirus from shellfish in contaminated waters has been reduced by freezing them and depurating them with pure seawater at higher temperatures (17-20 ºC) for extended periods of three to five days reduces virus levels in mussels significantly[4].

5. Hepatitis A Virus

5. 1 The common history of humans and HAV

The Hepatitis A Virus (HAV) is a leading cause of acute viral hepatitis, which in severe cases can result in acute liver failure and fatality. It spreads by the fecal-oral route and can be linked to consumption of certain types of food, such as mussels. Adults with HAV infection frequently develop symptomatic hepatitis, whereas children typically develop an asymptomatic subclinical illness. The incidence of HAV infection has been declining globally as a result of socioeconomic progress and improvements in public health[7].

Clinical signs and symptoms of HAV infection can range from asymptomatic infection to abrupt liver failure. Some patients also exhibit extrahepatic manifestations and unusual features such relapsing hepatitis or protracted cholestatic hepatitis[7].

The historical record for HAV goes back thousands for years; for example, diseases similar to hepatitis A, both in isolated cases and in epidemics affecting large populations, were reported in Cina centuries ago. Hippocrates described in "De Morbis Internis" about a condition he called benign epidemic yellowing of the skin which was unmistakably similar to hepatitis. In the 17th century, HAV infection was first accurately described and was frequently connected to military operations[11].

More than 40,000 instances of the disease were documented among the Union forces during the American Civil War, and the first outbreak in the UK was in Norfolk in 1812. There were estimated to be 16 million cases of hepatitis among fighters and civilians between World War I and World War II, affecting troops on both sides[11].

5.2 Characteristics of HAV

The non-enveloped, single-stranded RNA virus known as Hepatitis A virus (HAV) belongs to the genus Hepatovirus and family *Picornaviridae*. The virus may persist in the environment and spread via the fecal-oral route because it is stable at low pH and resistant to mild heat treatments[6].

The positive-sense RNA genome of HAV is 7.5 kb long, has a wide-open reading frame, a 5' noncoding region being the internal ribosome entrance site between nucleotides 734–740, and a 3' noncoding region. The primary capsid proteins are encoded by the aminoterminal third of the viral genome, while the remaining nonstructural proteins, which are mostly necessary for viral replication, are encoded by the remaining two thirds. A viral protease translates viral proteins and goes through them proteolytically[6].

The Picornavirus capsid's main job is to shield the genome from immune detection and allow it to be transported into the cellular cytoplasm, where replication can begin[12].

The HAV is particularly stable at room temperature and is resistant to detergents, drying, and low pH values. HAV must be inactivated by heating food (above 85°C) for 1 minute or by sanitizing surfaces with sodium hypochlorite (household bleach) diluted 1:100 for 1 minute. Hepatitis A survives the acidic environment of the stomach after being ingested orally through intestines and eventually reaches the liver[7].

The way the genome is encapsulated, the way interactions with the host cell are coordinated, the manner in which the capsid disassembles to enable genome replication, and how the capsid organization reacts to and evades the antiviral response mounted by the host are some of the aspects of this process that the structure of the capsid can help us to understand it[13].

In contrast to other enteroviruses, the *Hepatovirus* genus, of which the HAV is the type species, has received less attention than the *Picornaviridae* family of viruses[12].

5.3 Capsid structure and transmission

Picornaviruses commonly have a single-stranded RNA genome packaged in a nonenveloped 30 nm diameter capsid. A single lengthy open reading frame and two very brief non-translated RNA segments are both present in the genomic RNA. A viral protease breaks down the one big polyprotein encoded by the open reading frame into three polypeptide intermediates, P1–P3[12].

The three structural proteins VP0, VP3, and VP1 that are produced from P1 later undergo further processing to form a spherical capsid with icosahedral symmetry. After packing the RNA genome, VP0 in many Picornaviruses goes through a final maturation cleavage by a poorly

known process, perhaps catalyzed by RNA. As a result, it gains a VP2 mature capsid protein and VP4, a less complete amino-terminal fragment, are produced[12].

The Picornaviral capsid's general structure has been thoroughly defined. Each of the three biggest capsid proteins, i.e., VP1-3, assumes an eight-stranded antiparallel "barrel" structure in which the carboxy-terminal residues face outward and the amino-terminal amino sequences are positioned on the inner surface[12].

Because it is flexible or because it interacts with a single genomic RNA molecule that lacks strong icosahedral symmetry, the tiny VP4 protein is positioned inside and difficult to see in crystallographic models. The icosahedral fivefold axes forms are surrounded by five copies of the VP1 capsid protein. At the triple axes, three copies of VP2 and VP3 alternate, while at the twofold axes, two copies of VP2 touch[12].

The exterior surface of the formed capsid's interactions with a receptor expressed on the surface of the host cell are crucial to the virus's ability to enter cells. Additionally, the surface of the capsid interacts with immune system antibodies produced by the host, potentially neutralizing infectiousness[12].

Due to their loose structural constraints, the peptide loops on the surface of the capsid can accept deletions, insertions, or amino acid alterations that help the virus elude antibodies. Because of the wide variation in length and structure of these surface loops, several Picornavirus species may have unique forms. Thus, the fivefold vertices of one important genus of viruses, Enterovirus, have a depression pattern that is extremely conserved and typically contains the location of receptor contact[12].

An entryway into a lipidic molecule-binding hydrophobic such as the ones at a cell membrane, is located in the inside of the VP1 -barrel and consists inside its pocket structure. When an Enterovirus binds to its receptor and is concurrently taken up by early endosomes, it seems that the lipid in the pocket is displaced, causing the pocket to collapse. Channels open up on and near the twofold axes as a result of these conformational changes in the capsid, enabling the internal, hydrophobic VP4 and amino terminus of VP1 to externalize and pierce the endosomal membrane[12].

This results in the release of the viral RNA, which then enters the cytoplasm and begins the process of replication by being translated on ribosomes[12].

The VP1-binding pocket is absent in other genera of the *Picornaviridae* family, and the location of receptor binding is variable. Although the specifics are less well understood, it is very plausible that the method of uncoating differs depending on the region of receptor binding[12].

Nearly all other Picornaviruses differ significantly from HAV in terms of their physicochemical characteristics. Being able to resist temperatures of up to 80°C and pH levels as low as 2, it is astonishingly durable genetically and physically. Empty particles also seem to be more resilient than loaded particles. Because of this, cleaning is difficult, and the uncoating procedure is now incomprehensible[12].

Recent research has demonstrated that HAV may reside outside of cells in an enclosed form, perhaps hiding from immune monitoring by acquiring host membrane to create the so-called "quasi-enveloped" HAV particle. This may begin to muddy the line between enclosed from unenveloped viruses, raising the issue of whether this is exclusive to hepatitis A or if it also applies to certain other Picornaviruses and has just gone unnoticed[12].

When complexed with an immunoglobulin A (IgA) antibody, HAV still has the capacity to move across cells through transcytosis. In contrast to other Picornaviruses, VP1's carboxy-terminal extension, a 67-residue polypeptide, remains attached throughout capsid construction[12].

When compared to other Picornaviruses, this would be anticipated to belong to the nonstructural P2 region of the genome (protein 2A in the other viruses); consequently, it is known as VP1-2A or pX. pX is thought to facilitate the connection with the membrane envelope and is eventually broken off by host proteases to release the mature, unenveloped capsid. HAV strains belong all to the same serotype, and the lack of antigenic diversity has been related to the necessity to save uncommon codons[12].

Because of these changes, it was believed that the capsid would have differentiating traits from the structures of other genera, shedding insight on its uncoating process. In fact, the examination of HAV detailed below gave the *Hepatovirus* genus its first structural information[12].

Although naked, nonenveloped virions are shed in the feces of infected humans as well as nonhuman primates, only quasi-enveloped viruses, commonly known as "eHAV", are found in serum. According to the data, HAV is released through the apical plasma membrane of hepatocytes into the proximal biliary canaliculi, where the membrane is torn away from the capsid by the detergent action of bile salts. Most virions seen in infected cell culture supernatant fluids are also quasi-enveloped. In iodixonal, “eHAV” are between 50 and 110 nm in diameter and have a buoyant density of 1.10 gm/cm3, whereas naked virions are 27 nm in diameter and band at a buoyant density of 1.25 gm/cm3[13].

Electron microscopy of negatively stained quasi-enveloped virus preparations reveals membranous vesicles with one, two, or three RNA-containing capsids. As previously stated, these capsids mostly contain unprocessed VP1pX rather than mature VP1 seen in mature naked virions. The encircling membranes completely occlude the capsid, preventing antigen identification and neutralization by antibodies in the absence of detergents. Quasi-enveloped virions resemble exosomes and lack viral glycoproteins (peplomers) on the membrane surface, which separates them from enveloped viruses[13].

5.4 Pathogenesis

The virus's cellular entrance is greatly influenced by quasi-envelopment. Although infection is primarily caused by the ingestion of naked, non-enveloped virions, it is unclear whether cell types are infected initially, or whether there is a main site of replication within the gut. However, once infected, the virus appears to propagate within the host via quasi-enveloped virus, as only quasi-enveloped HAV is found in serum or plasma during acute infection. The method by which quasi-enveloped HAV enters cells must be distinct from that of the naked virion, yet later phases in the entrance process, such as contacts of the capsid with a particular cellular receptor, appear to be shared. Such receptor interactions are essential for other Picornaviruses entry and uncoating, as well as the delivery of their RNA genomes across endosomal membranes. [13].

The initial entry of quasi-enveloped HAV into cells is most likely accomplished through mechanisms similar to those used by cells to internalize exosomes. Regrettably, these are still poorly understood. Endocytosis, macropinocytosis, and phagocytosis mediated by clathrin

have all been implicated. Exosome membrane PtdSer residues and adhesion molecules (integrins and tetraspanins) contribute to exosome uptake, and PtdSer exists on the quasi-enveloped HAV surface and promotes efficient uptake by plasmacytoid dendritic cells. Integrin-3, as well as the tetraspanins CD9 and CD63, are found on the quasi-enveloped HAV membrane[13].

Quasi-enveloped HAV has been demonstrated to enter cells via an endocytic route, with tardy removal of its surrounding membrane, making the capsid susceptible to antibody neutralization in an endosomal compartment, even when antibody is introduced to cell cultures 6-8 h after removing a quasi-enveloped HAV inoculum[13].

The naked virion penetrates more quickly and is not neutralized by postendocytic mechanisms. Chloroquine, a lysosomal toxin, greatly inhibits quasi-enveloped HAV entrance but has no impact on naked HAV virions. Endocytosed quasi-enveloped HAV is most likely transported to a late endosome/lysosomal compartment, where its membranes are dissolved by lysosomal enzymes, allowing the capsid to bind to a cellular receptor[7].

A quasi-enveloped form of quasi-enveloped HAV is detected in the serum and plasma of infected hosts, whereas a non-enveloped, naked form of HAV is shed through feces. Following exposure to high concentrations of bile salts in the biliary canaliculus, HAV is released from hepatocytes and loses its lipid envelope[7].

HAV can take advantage of quasi-enveloped HAV and nonenveloped HAV characteristics for immune evasion and efficient viral transmission, respectively. The quasi-envelope of quasi-enveloped HAV cloaks the capsid within infected hosts, protecting it from neutralizing antibodies that target capsid proteins[7].

The naked, non-enveloped HAV is very stable and is shed in feces via the intestinal tract while retaining its infectivity. Furthermore, because of its high physicochemical stability, nonenveloped, naked HAV is highly transmissible to other hosts in the environment[7].

Peaks of viremia and fecal virus shedding during acute hepatitis A are followed by hepatocellular injury, which is shown by an increase in liver enzymes like ALT in the serum. The duration of viral shedding in feces after the initial rise in serum ALT levels is typically 2 to 3 weeks, though sensitive Reverse Transcription Polymerase Chain Reaction (RT-PCR) methods

may be able to detect it for longer periods of time. Following the resolution of HAV, clinical relapse with fecal viral shedding is possible. Prolonged viremia has also been reported in some adult patients with hepatitis A. Meanwhile, the HLA-DRB1-1301 allele was strongly linked to persistent forms of HAV infection[7].

HAV replication locations outside the liver have been proposed. HAV antigens may be found not only in hepatocytes but also in the spleen, lymph nodes, and kidneys of infected nonhuman primates. This has not been proven in people. Furthermore, HAV has been found in the tonsils and saliva immediately after viremia, despite the fact that the viral titer in saliva is quite low[7].

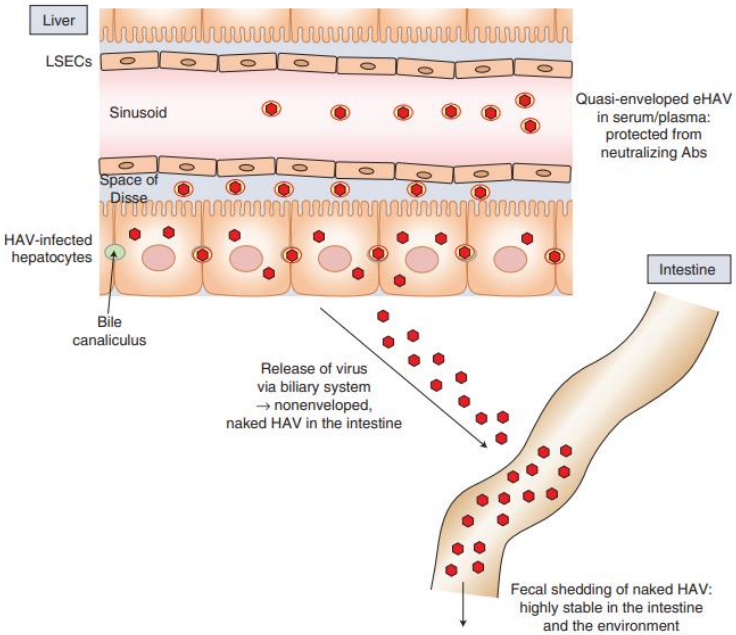

Figure 5. Clinical manifestations and pathogenesis of Hepatitis A (legenda: LSECs, Liver sinusoidal endothelial cells)[7].

The two types of infectious HAV are represented in Figure 5. Quasi-enveloped HAV (eHAV) is found in the infected host's serum and plasma, whereas nonenveloped, naked HAV is found in the feces. Newly reproduced offspriFng virus is discharged from hepatocytes in the quasi-enveloped state and then loses its lipid envelope after being exposed to bile salts in the biliary canaliculus. The viral capsid within the quasi-enveloped HAV virion is immune-protected.

Nonenveloped, bare HAV is extremely stable. It is excreted in feces via the digestive system and maintains infectivity in the environment[7].

Following ingestion, virions travel via the portal circulation and are absorbed by hepatocytes in the liver. The virus replicates, assembles, and is released into the biliary canaliculus among the hepatocytes before moving on to the bile duct and small intestine. Until neutralizing antibodies and other immune mechanisms break the cycle, the enterohepatic cycles of the virus continue[6].

Uncertainty exists over whether HAV initiates replication at a main site within the gastrointestinal tract. Hepatocytes are where HAV replicates before being released into bile and returning to the gastrointestinal tract. Finally, it is eliminated by feces or moved to the liver via the enterohepatic cycle until the virus is neutralized[7].

After infection, a substantial number of virions are lost in the feces and survive on inanimate surfaces and human hands. The most frequent recognized route of infection for HAV is contact with an infected person. Transmission through foreign travel, injectable drug use, males having homosexual activity, and food- or water-borne outbreaks have all been recorded[6].

In Shanghai, China, the largest known outbreak of hepatitis A was recorded and accounted for more than 290,000 cases. It was linked to raw mussels consumption[6].

5.5 Symptoms and clinical manifestations

Patients experience acute hepatitis symptoms and high blood aspartate/alanine aminotransferase levels after an incubation period of 15–50 days following HAV infection. Waves of viremia and massive fecal virus shedding occur prior to symptoms. Because of their high viral load, feces are the main source of HAV transmission[7].

Compared to the feces, serum HAV concentrations are two to three $\log_{10}$ units lower. As a result, the prodromal stage, which comes before symptoms or biochemical signs appear, presents the greatest risk of transmission. At much lower levels, the virus is also shed in the saliva[7].

Anti-HAV immunoglobulin M and then anti-HAV immunoglobulin G emerge in the blood and saliva in accordance with clinical hepatitis symptoms, and these changes are followed by a notable decrease in viremia and fecal viral shedding. Anti-HAV immunoglobulin G endures and provides lifetime protection, but anti-HAV immunoglobulin M is detectable for up to 6 months[7].

As mentioned earlier, the clinical manifestations of HAV infection can range from asymptomatic illness to fulminant hepatitis. The clinical symptoms of hepatitis A vary according to the patient's age. In children under the age of six, around 70% of infections are asymptomatic; in adults, more than 70% of infections are symptomatic, with jaundice and unusually high levels of serum aminotransferases[6].

After a 2–7-week incubation period (4 weeks on average), usual symptoms such as fever, malaise, nausea, vomiting, stomach pain, black urine, and jaundice appear. Myalgia, pruritus, diarrhea, arthralgia, and skin rash are less common symptoms. After acute hepatitis A has clinically come to an end, there is no sign of chronic liver damage or persistent infection[6].

However, some individuals experience persistent or recurrent illness lasting up to 6 months, with extended HAV excretion. Total bilirubin (mean peak 7 mg/dl), alkaline phosphatase (mean peak 319 IU/l), serum aspartate aminotransferase (1,754 IU/l), and alanine aminotransferase (1,952 IU/l) levels were all increased. Clinical disease and test abnormalities resolve within 6 months of illness beginning[6].

5.6 Atypical Hepatitis A manifestations

Atypical Hepatitis A episodes have been observed. Relapsing hepatitis, protracted cholestasis, difficult instances with acute renal damage, and a rare autoimmune hepatitis are among them. Relapsing hepatitis A was distinguished by a biphasic peak of serum aminotransferase increase, with intervals of 4-7 weeks between the first and second peaks. Prolonged cholestatic hepatitis A was distinguished by pruritus, weariness, loose stools, and weight loss in addition to cholestasis. In comparison to typical signs, identification of plasma HAV RNA after 20 days of illness can indicate protracted cholestatic cases, but relapsing hepatitis

remains unexpected based on plasma HAV determination. In 1.5-4.7% of HAV patients, acute renal damage complicated non-fulminant cases[6].

Pre-renal variables linked with anorexia, nausea, vomiting, diarrhea, and fever are proposed pathways of renal injury, as are the nephrotoxic consequences of hyperbilirubinemia, immune complex-mediated nephritis, interstitial nephritis, and (rarely) extensive intravascular hemolysis. According to one study, poor hematocrit and coagulopathy, high CRP concentration, and high peak bilirubin levels were independent predictors of acute kidney damage development in hepatitis A[6].

Extrahepatic manifestations of hepatitis A have been reported in rare cases, including autoimmune hemolytic anemia, aplastic anemia, pure red cell aplasia, pleural or pericardial effusion, acute reactive arthritis, acute pancreatitis, and neurologic complications such as mononeuritis, mononeuritis multiplex, and Guillain-Barré syndrome. Acalculous cholecystitis is frequently complex, as evidenced by an edematous gallbladder on radiological imaging and associated clinical symptoms, many of which appear transiently and resolve spontaneously[6].

Fulminant hepatitis is an uncommon consequence of HAV, with a frequency ranging from 0.015 to 0.5% of cases. Low serum HAV RNA levels and high bilirubin levels were found to be significantly associated with fulminant hepatitis in one study, implying that HAV-related liver failure is caused by a robust host immune response with a marked reduction in viral load, rather than a direct viral effect. Viral strains obtained from cases of fulminant hepatitis A had comparatively few nucleotide alterations in the 5' noncoding region as compared to virus strains derived from cases of non-fulminant hepatitis A. As a result, nucleotide changes in the 5' noncoding region of the HAV may impact the severity of the disease[6].

However, a recent Indian study found no HAV genome variation according to hepatitis severity, suggesting that more research studies are needed to highlight the relationship between HAV genome mutations and illness severity[6].

The risk of fulminant liver failure in elderly people with underlying chronic liver disease is significant. However, HAV-related fulminant hepatitis spontaneously recovers more often than other etiologies of fulminant hepatitis. Consequently, deciding whether or not to transplant is not an easy option. According to the US Acute Liver Failure Study Group, a prognostic index

consisting of four presenting features (serum alanine aminotransferase! 2,600 IU/l, creatinine 1 2.0 mg/dl, intubation, pressors) predicted the likelihood of mortality[6].

6. Experimental study

6.1 Materials

The approach in this study was an experimental one. In order to obtain the varied samples, this study relayed as means of transportation on the monthly visits to the Milan commercial fish market, done by the department of food Inspection of animal origin, during the second semester of the academic year if 2021/22 and the first semester of 2022/23.

A total of 53 samples have been collected during the span of 8 months, all of which came from the Milan commercial fish market. In each visit at the market, passing through the varies fisherman/commercialists were asked for packages of 1 kg of *Mytilus galloprovincialis* of different geographical origin for the same day, meaning that along course of sampling within the 8 months period, samples came from the same geographical origin/commercial marking, but not at the visit at the fish market.

Depending on the season of the year, it has been noticed that the number as well as the origin the samples have varied. For example, the origin of the samples during the winter months came mostly from international importation, mostly Spain. whereas from the start of the spring to the end of Autum all of the samples came from national production from all over Italy, for example Sardinia Golfo di Oristano, La Spezia, Astro, Scadovario, Polignano a Mare, Goro, Chioggia etc.

The number of each sampling varied from one visit to another, varying from a minimum of 5 samples at the start of autumn to a maximum of 11 samples of different origin in the middle of spring.

6.2 Methods

The methods applied concerned the detection of Norovirus and Hepatitis A virus in bivalve mussels using a Real-time Polymerase Chain Reactions (Real-time PCR).

Noroviruses and Hepatitis A virus are significant carriers of foodborne viral illnesses in humans. Currently, there are no standard procedures for cultivating these foodborne viruses. Therefore, their detection is focused on molecular techniques that employ polymerase chain reactions (PCR) following an extraction process that can get rid of any Real-time PCR inhibitors

that may be present in the matrix, generating highly purified RNA. This procedure identifies Noroviruses and/or Hepatitis A virus RNA in the samples tested.

Real-time PCR, which measures the excitation of molecules labeled with fluorophores during PCR cycles to verify the amplification during those cycles, can be used to make the determination. A sequence-specific nucleotide probe with fluorophores attached to it is used in the TaqMan-type Rt-PCR assay to confirm the target template. These adjustments improve the PCR method's sensitivity and specificity and do away with the requirement for additional post-PCR steps to verify the amplified result.

The reference standard methods used are the following:

- ISO-22174, Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food-borne pathogens - general requirements and definitions
- ISO-7218 Microbiology of food and animal feeding stuffs - general rules for microbiological examinations.

The ISO-22174 protocol outlines each stage (release, concentration, extraction, and detection) of a technique for identifying Hepatitis A and Norovirus genotype I (GI) and II (GII) RNA in mollusc matrix. The "one-step Taq-Man Real-time-PCR" is used in this process.

6.3 Terms and definitions

Thurley explained previously, the *Picornaviridae* family of viruses includes the Hepatitis A virus, which causes contagious hepatitis. Six genotypes of Hepatitis A can be distinguished genetically based on the VP1/2A region (genotypes 1, 2, and 3 are found in humans, while genotypes 4, 5, and 6 are found in primates). Only one serotype exists. The fecal-oral route of transmission involves direct human contact, ingestion of contaminated food, and/or contact with contaminated water or food surfaces.

Noroviruses, which belong to the *Caliciviridae* family, are occasionally to blame for acute gastroenteritis epidemics. Noroviruses can be categorized into five genetically different genogroups. Gastroenteritis in humans has been linked to three of them: GI, GII, and GIV. The majority of clinical cases are caused by GI and GII. The fecal-oral route of transmission involves

close personal contact, eating contaminated food, or coming into contact with contaminated food surfaces or water.

A qualitative assessment of Hepatitis A or Norovirus RNA in a predetermined mass or volume of food must be done in line with ISO-22174 protocol to perform the detection of Hepatitis A virus/Norovirus.

The Process control involves adding a virus to the portion of the sample under testing before extraction to ensure that the extraction was successful. The virus used for process control must be different from the target virus. The virus chosen as process control should be a non-enveloped culturable virus with positive single-stranded RNA that is comparable in size to the target virus in order to give a solid genetic, morphological, and physicochemical model. Similar environmental persistence is anticipated for the process control and the target viruses. Hepatitis A is just one example of a virus that can be employed as a process control in this approach; other viruses can also be utilized, and their usage is possible when Hepatitis A itself is not the subject of the investigation. The Mengovirus and the feline calicivirus are usually the subjects chosen for the process control. The virus's RNA is isolated and utilized as a process control, thereafter applicated to calculate the extraction process's efficiency. A water sample is used as negative control for the Real-time PCR process.

The external RNA control is a reference RNA with target function for the Real-time PCR assay, such as the proper dilutions of an in vitro transcript from plasmid containing the target gene sequence or RNA extracted from cultured viruses (Hepatitis A or Feline calicivirus) or fecal samples containing viruses from the same genogroup (Noroviruses). The levels of Real-time PCR inhibition in each sample can be determined by comparing the results obtained for the external control in the presence and absence of the sample.

There is also a theoretical limit of detection, which is the least amount of the target virus that the assay is theoretically capable of detecting. Per volume of RNA evaluated in the assay, it equates to one copy of the genome, albeit it can change depending on the matrix and the quantity of starting material.

Bivalve mussels make up a complex matrix where target viruses may exist in trace amounts. In order to create a sufficient substrate for the following analytical stages, extraction and/or concentration processes must be performed. The chaotropic chemical guanidine thiocyanate

is used to split the viral capsid, and the RNA is adsorbed on silica to undergo, in order to acquire clean RNA and lessen the impact of PCR inhibitors. Purification via a series of washing processes Real-time PCR is performed once purified RNA has been released from silica into a buffer.

In this approach, reverse transcription and PCR amplification are carried out simultaneously in the same reaction tube using a "one-step TaqMan Real-time PCR." The short DNA probe used in TaqMan Real-time PCR has a fluorescent label (reporter) and a fluorescence absorber (quencher) connected to its opposing ends.

6.4 Description of the analytical procedure

The assay's chemistry makes sure that as the amplified product concentration rises, the probe is hydrolyzed and the fluorescence signal of the label rises in proportion. It is possible to monitor fluorescence at every amplification stage. Analysis of fluorescence curves enables estimation of the amount of target sequence in the sample because the first point in the PCR where amplification may be detected is proportional to the amount of beginning template. The choice of reagents appropriate for one-step Real-time PCR and TaqMan PCR primers and probes for target viruses is crucial because to the low levels of viral template frequently found in foods and the genetic variety of target virus strains.

The overall rule for typical laboratory procedures with relation to reagents is outlined in ISO-7218. For the preparation procedure, Proteinase K (30U/mg) is employed with a composition of PK (30U/mg) cal 20+/-0.1 mg and Water for molecular biology 200+/-2 ml.

Proteinase K needs to be dissolved in water in order to be used in Phosphate Buffered Saline. thorough blending. For a maximum of six months, keep in quantities suitable for consumption at -20 +/- 5 C. Use within a week after thawing and storing at 4+/-2C. 8.0+/-0.1g NaCl, 0.2+/-0.01g Potassium Chloride, 1.15+/-0.01g Disodium Hydrogen Phosphate, 0.2+/-0.01g Potassium Dihydrogen Phosphate, and 1000+/-2ml Water for Molecular Biology are all included in the composition. Still, the granules must be dissolved in water for the preparation, with the pH adjusted to 7.3+/-0.2 at 25°C if necessary. Autoclave sterilization is needed.

Viral RNA extraction buffers with silica, lysis, wash, and elution. With the use of guanidine thiocyanate-containing chaotropic lysis buffer and silica as a substrate for RNA binding, these

reagents should make it possible to extract RNA from 500 µl of sample extract. The RNA should be eluted in 100 µl of elution buffer after being washed with washing buffers to get rid of contaminants. The RNA preparation needs to be of a quality and concentration appropriate for the measurement.

The TaqMan's primers and sondes for Hepatitis A and Norovirus GI and GII function as follows: The first TaqMan and the sondes must be published in a peer-reviewed journal and verified for use in relation to a wide variety of target virus strains. The non-codifying region at position 5’ of the genome should be the first area of detection for Hepatitis A. The region between ORFI and ORF2 of the genome should be the first area of detection for the genogroup I and II of Noroviruses.

The primers and probs used in Taq-Man for the detection of Hepatitis A, Noroviruses GI e GII are described below.

Hepatitis A virus testing:

Hepatitis A V68 (FW): TCA CCG CCG TTT GCC TAG

Hepatitis A 240 (REV): GGA GAG CCC TGG AAG AAA G

Hepatitis A 150(PROBE): CCT GAA CCT GCA GGA ATT AA

Probe labeled 5' 6-carboxyfluorescein (FAM), 3' MGB (minor groove binder)

Sequence alignment utilizing every sequence from the target region that is present in GenBank shows that this primer/probe set is sufficient for detecting every genotype. The mutant spectrum has also been subjected to heterogeneous population of virions each of which can differ from the other even just for a point mutation of the genome analysis, which shows that this region is not susceptible to variability and that this assay should be long-term robust. The specificity of the primers was tested using 10 different Picornaviruses, including the Poliovirus (serotype 1 vaccine strain), human Enterovirus B (Echovirus 1), human Enterovirus B (Echovirus 11), human Enterovirus B (Echovirus 30), human Enterovirus B (Coxsackievirus-B5), human Enterovirus C (Coxsackievirus-A24), human Enterovirus D (Enterovirus 70), bovine Enterovirus, porcine Teschovirus (porcine Rnterovirus 1) and

Encephalomyocarditis virus, and other enteric viruses such as Hepatitis E virus, human and porcine Rotavirus (group A), Norovirus, Mamastrovirus (human Astrovirus type 1) and human Adenovirus F (enteric Adenovirus type 40). None of the viruses tested gave positive results either at high concentrations (10^6 – 10^8 TCID50/ml or 10% faecal suspensions) or low concentrations (10^4 TCID50/ml or 1/10 dilutions of 10% faecal suspensions). The LOD of the assay is 10 ssRNA molecules, 1 viral RNA molecule, and 0.05 infectious virus per reaction.

Norovirus GI testing:

QNIF4 (FW): CGC TGG ATG CGN TTC CAT

NVILCR (REV): CCT TAG ACG CCA TCA TCA TTT AC

NVGG1p (PROBE): TGG ACA GGA GAY CGC RAT CT

Probe labelled 5'6-carboxyfluorescein (FAM), 3' 6-carboxy-tetramethylrhodamine (TAMRA)

Norovirus GII testing:

QNIF2 (FW): AYG TTC AGR TTG ATG AGR TTC TCW GA

COG2R (REV): TCG ACG CCA TCT TCA TTC ACA

QNIFS (PROBE): AGC AGC TGG GAG GGC GAT CG

Probe labelled 5'6-carboxyfluorescein (FAM), 3' 6-carboxy-tetramethylrhodamine (TAMRA)

The area selected for the detection of Noroviruses is the highly conserved region of ORF2. Sequence alignment using all sequences available in GenBank for the target region demonstrates that this primer/probe set is adequate for detection of all Noroviruses of Gl and GII, respectively. In addition, the efficacy and sensitivity of primers and probes were verified using 18 Norovirus reference strains: GI.I (Norwalk virus), Gl2 (Whiterose), GI3 (Southampton), G1.4 (Malta), GLS (Musgrove), G1.6 (Mikkeli), GL.7 (Winchester), GL.10 (Boxer), GII.1 (Hawaii), GII.2 (Melksham), GII.3 (Toronto), Gl1.4 (Grismby), GIL.6 (Seacroft). GII.7 (Leeds), GIL.10 (Erfurt), the variants GIIb and Glle and GIV (Alphatron).

The specificity of the primers has been verified against 6 human enteric viruses: Poliovirus (serotype 1 vaccine strain), Hepatitis A virus, Hepatitis E virus, Aichi virus, Astrovirus and Rotavirus. The specificity was also tested on seven bacteria that can be found in mussels. i.e., *Escherichia coli, Shewenella putrefaciens, Chromobacterium violaceum, Aeromonas sobria, Vibrio alginolyticus, Vibrio paraheamolyticus* and *Vibrio cholerae*. None of the tested viruses or bacteria gave positive results. The LOD of the assays is 1 to 10 viral RNA molecules (depending on the 5' end Norovirus strain).

Taq-Man primers and probes (Hepatitis A, Norovirus GI and GII) must be published in confidential peer review and must be verified for use against the virus strain used as a process control. They must also demonstrate no cross-reactivity of target virus comparisons.

The reagents should permit to analyze 5 µl of RNA from the 25 µl of total reaction volume. They need to be TaqMan Real-time PCR one-step reagents sensitive enough to detect viral RNA levels commensurate with common food contamination. To prepare 20 µl of mastermix per reaction in a total of 25 µl volume, the reagents should be added in the amounts recommended by the manufacturers. In accordance with the reagent manufacturer's guidelines, the ideal concentration of primers and probes should be established.

6.5 Master mix composition and Amplification Parameters

Composition of one-step TaqMan real-time RT-PCR mastermixes using the Invitrogen RNA Ultrasense One-step qRT-PCR system kit and amplification parameters

Master mixes

Reagents	The final Concentration in 25µl	Volume per reaction (µl)
5x Ultrasense reaction mix	1x	5.0
FW Primer (12.5 µM)	500 nM	1.0
REV Primer (22.5 µM)	900 nM	1.0
Probe (6.25 µM)	250 nM	1.0
Rox reference dye (50x)	1 X[a]	0.5
RNA Ultrasense enzyme mix	-	1.25
Water for molecular biology	-	10.25
Total volume	-	20±0.2

[a] With Applied Biosystems real-time PCR equipment, ROX should be used at a concentration of 1 x; for Stratagene devices (ie. MX3000), ROX can be used at 0.1x concentration, or it can be omitted from the mastermix. For other manufacturers, consult the appliance instructions.

Amplification parameters

Step	Temperature and time	Number of cycles	Acquisition
Reverse transcription	55°C for 1 h	1	no
Preheating	95°C for 5 mins	1	no
Denaturation	95°C for 15 s		No
Amplification			
Annealing	60 °C for 1 min	45	No
-extension	65°C for 1 min		Yes (single)

The process control virus cell culture supernatant needs to be diluted in an appropriate buffer, like Phosphate Buffered Saline extension, by a factor of 10. This dilution is intended for the unhindered detection of process control virus by TaqMan technology, while retaining a concentration adequate to reproducibly detect the lowest dilution utilized for process control

viral RNA in the standard curve. Divide the diluted cell culture supernatant (process control) into single-use aliquots and store at -80±5°C.

For the external control RNA, distinct single-stranded RNAs purified for each target virus should be used. Divide diluted RNA external control preparations into aliquots for single use and store frozen at ≤-15°C.

The equipment used is standard for microbiological laboratories (ISO-7218) and in particular the following tools are needed:

- Micropipettes and tips with various volume ranges, eg. 1000µl, 200µl, 20µl
- Automatic pipettors and pipettes with various volume ranges, e.g 25ml, 10ml, 5ml
- Vortex
- Microcentrifuge
- Heating block capable of operating at 99+1.0"C
- Technical balance capable of operating with a resolution of +0.1 g
- Incubator with shaking able to operate at 37+1.0°C and 320±20 rpm, or equivalent (i.e. shaker placed inside the incubator)
- Water bath able to operate at 60±1.0°C
- Benchtop centrifuge and rotor capable of operating at 4,000 x g with capacity to accommodate 15ml tubes
- Gloves
- Scissors
- Pliers
- Sterile Petri dishes
- Sterile Falcon tubes
- Sterile Eppendorf tubes
- Apparatus and materials required for RNA extraction using silica and associated reagents
- Real-time PCR instrument capable of supporting a TaqMan reaction chemistry
- Consumables associated with Real-time PCR, e.g. optical plates and caps/films, suitable for use in the adopted PCR apparatus.

Sampling is not part of this procedure and it has been discussed in 6.1 subchapter. Bivalve molluscs should be alive or, if frozen, should be undamaged. Before opening the molluscs, remove any mud that may be present on the shells.

For the extraction of the virus, a minimum of 10 individuals for the largest species must be selected (30 for clams and smaller species) and the valves must be opened with a sterile scalpel or equivalent instrument. Protect the hand holding the shellfish with a safety work glove.

Dissect the digestive tissue (hepatopancreas) using tweezers and small scissors (or equivalent instruments) and transfer the tissue to a clean petri dish. Take a quantity of not less than 2.0±0.2 g. finely mince tissue using a clean scalpel (or equivalent).

Transfer a quantity equal to 2.0±0.2 g of minced hepatopancreas into a centrifuge tube, and to verify the efficiency of the extraction procedure, add 100.1 µl of diluted process control, at the chosen concentration.

Add 2.0±0.2ml of proteinase K solution (0.1mg/ml), mix well.

Incubate the tube at 37.0±1.0 °C with shaking (approx. 320 rpm) for 60± 5 min. Carry out a second incubation by placing the tube in a water bath at 60.0 ± 2.0 °C for 15 ± 1 minute.

Centrifuge at 3000 g for 5 min ±30 sec and transfer the supernatant to a new tube.

Measure and record the volume obtained and bring the sample to the final volume of 3.0 ± 0.3 ml by adding sterile Phosphate Buffered Saline (pH 7.3). Use the suspension for nucleic acid extraction.

The samples thus extracted can be stored at -20°C (or, preferably, -80°C) until use.

6.6 RNA extraction

For the RNA extraction, different commercial extraction kits can be used (e.g. "MiniMag Nuclisens Magnetic Extraction kit" (bioMericux); NucleoSpin® RNA II Macherey-Nagel etc.). Regardless of the kit requirements, concerning quantities of sample to be analyzed it is advisable to use the sample and elution volumes described below:

1. Take 500±10 µl of sample (or all the sample if less than 500 µl) and proceed with the extraction according to the methods defined by the manufacturer of the extraction kit
2. At the end of the procedure, flow the extracted RNA into 100±1 µl of elution buffer
3. Add 100 units of RNase inhibitor and mix by pipetting

Extracted RNA should be stored at 5±3°C for <24 h or ≤15°C for longer periods.

6.7 Real-time PCR setup

Moving on to the Real-time PCR setup, to prepare the reverse transcription and PCR mix (one-step) using the materials in the RNA Ultrasense one-step qRT-PCR kit (Invitrogen-11732927) it is necessary to prepare a mix for the determination of each target (Norovirus GI, GII, Hepatitis A) in sufficient quantity and set up the plate as follows:

1. the samples to be analyzed (5±0.1 µl of sample RNA/well)
2. a negative control (5±0.1 µl molecular biology grade water/well)
3. a positive control (5±0.1µl of water and 1±0.05 µl of external RNA control, i.e., appropriate dilution of RNA extracted from fecal sample in which the presence of Norovirus of the genogroup under examination was detected or RNA extracted from suspension viral of Hepatitis A)
4. an inhibition control (5±0.1 µl of sample and 1±0.05ul of external RNA control, i.e., appropriate dilution of RNA extracted from fecal sample in which the presence of Norovirus of the genogroup under examination was detected or RNA extracted from suspension viral of Hepatitis A)

Add 20±0.5 µl of the relevant TaqMan mastermix to each sample (the mastermix can also be added to the relevant wells prior to template addition).

5. a process control, chosen from those indicated to be tested in parallel by adding a specific mix. This control will be added in case the efficiency of the extraction is verified at each analysis.

To carry out the amplification cycle, close all the wells with optical caps or adhesive film. Subject the plate to a reaction cycle including an initial stage for reverse transcription and at

least 45 cycles of PCR. The duration and temperatures of each step (reverse transcription, Real-time deactivation, denaturation, annealing, extension) will depend on the reagents used, which should be based on the manufacturer's recommendations but can be further optimized.

Proceeding to the analysis of fluorescence data, whose minimum requirements for the analysis of amplification data are defined in ISO 22174. The amplification curves should be analyzed using the approach recommended by the Real-time PCR equipment manufacturer. The threshold value should be set such that it crosses the area where the amplification curves (in logarithmic representation) are parallel (exponential phase).

All amplification curves should be checked for false positives (reactions with Ct values not associated with amplification) caused by high or erratic background signals. The Ct value indicates how many cycles of PCR multiplication must be performed to detect the presence of the viral genetic material. Thus, as a general rule, the higher the Ct value, the less virus is present in the sample.

This should be recorded and the results of any reaction conditioned in this way should be considered negative. In addition, all fluorescence curves should be checked to verify that the Ct values generated by the analysis software correspond to the exponential phase of the amplification (and that it is not distorted by loud or erratic background noise). Where Ct values are biased, the corrected values should be recorded in addition to the software generated value.

In general, for the interpretation of the results, each control (external control to RNA, RNA of the process control) has its own valid expected Ct value or a range of acceptable values, established on the basis of the evaluation tests. If the observed results for the controls differ from those expected, the samples may require retesting.

Negative controls (molecular biology water) should always be negative. If positive results are found in these controls, any specimen showing positive results should be retested. The samples under examination are considered positive if they present a $Ct \leq 44.0$, the results are considered acceptable if the positive control presents a $Ct \leq 44.0$ and the negative extraction and RT-PCR controls present values of $Ct>44.0$.

For result expression, positive results of each target virus should be expressed as "presence of viral genome in 5 µl of molluscs hepatopancreas extract".

If the target virus is not detected (i.e., Ct > 44.0), the results should be expressed as "viral genome not detected in 5ul of molluscum hepatopancreas extract". If no valid result was obtained, the results should be expressed as "no result".

The tests for the evaluation methods are the tests to be performed before applying the method on samples to be analyzed. They allow:

a. The evaluation of the efficiency of the amplification and of the extraction procedure, to be used as quality assurance parameters and not for the calculation of the analytical results.
b. The choice of the EC-RNA concentration to be used in the application of the method. This must provide a Ct value representative of those obtained from naturally contaminated samples.
c. The choice of the process control concentration to be used during the application of the method.
d. The calculation of the Ct values with the relative standard deviations of reference for the controls adopted (EC-RNA, process control) to be considered in the interpretation of the results at each application of the method.

The assays for the evaluation of the efficiencies referred to in point a, i.e., quality assurance parameters and not for the calculation of the analytical results, should be repeated at regular intervals between homogenic determinations (same matrix, same parameter to search for).

6.8 Analysis of the target viruses

In preparation for the analysis of the target virus, 10^{-1}, 10^{-2} and 10^{-3} dilutions in molecular biology grade water of the RNA External Control should be prepared.

For each sample prepare:

- 1 well in the optical plate with 5±0.1 µl of sample RNA
- 1 well with 5±0.1 µl of sample extract and 1±0.05 µl of undiluted RNA external control

For the standard curve of the RNA External Control prepare:

- 1 well with 5±0.1 µl of water for molecular biology and 1±0.05 µl of undiluted RNA external control
- 1 well with 5±0.1 µl of water for molecular biology and 1±0.05 µl of diluted RNA external control 10^{-1}
- 1 well with 5±0.1 µl of water for molecular biology and 1±0.05 µl of diluted RNA external control 10^{-2}

For negative controls prepare:

- 1 well with 5±0.1 µl of water for molecular biology

Add 20±0.5 µl of the relevant TaqMan mastermix to each sample (the mastermix can also be added to the relevant wells prior to template addition).

Process control analysis is immediately prior to processing a batch of samples, pooling together a sufficient number of Process Control aliquots to verify each sample (10ul per sample plus 25ul excess).

Dilute a 20±0.5ul aliquot of process control 1:10 using molecular biology grade water and store at 5±3°C for up to 24 hours or in single-use aliquots at ≤15°C for longer periods. Heat at 99 ± 2°C for 5 min ± 30 see using a heating block or equivalent equipment to release the RNA. Cool the tubes rapidly, centrifuge at ≥3000 x g for 1 min, then transfer the supernatant ("process control RNA") to a new tube.

Prepare 10^{-1}, 10^{-2} and 10^{-3} dilutions of the process control RNA in molecular biology grade water. For each sample, to which process control was added prior to Proteinase K treatment, prepare:

- 1 well with 5±0.1 µl of sample RNA

For the process control RNA standard curve prepare:

- 1 well with 5±0.1 µl of 10^{-1} diluted process control RNA
- 1 well with 5±0.1 µl of 10^{-2} diluted process control RNA
- 1 well with 5±0.1 µl of 10^{-3} diluted process control RNA

For negative controls prepare:

- 1 well with 5±0.1 µl of water for molecular biology

Add 20±0.5µl of the relevant TaqMan mastermix to each sample (the mastermix can also be added to the relevant wells before template addition).

In order to make an evaluation of the reaction efficiencies, a standard curve must be created to control the Ct values of all the standard curve dilution series (process control RNA, external control RNA) for all points that do not fall near the "best fit" line. These values should not be incorporated into the standard curve calculation. Use the remaining values to create the standard curve. Curves with values of r2 <0.98 should not be used for calculations.

In addition, a calculation of the amplification efficiency should be done to check the Ct value for the sample in the wells with external control RNA. Use this value to estimate the amplification efficiency by referring to the standard curve of the RNA External Control. An amplification efficiency >50% is considered acceptable.

The calculation of the extraction efficiency, to verify the Ct value for process control from the wells with sample RNA. Use these values to estimate the process control recovery by referencing the process control RNA standard curve.

For bivalve molluscs calculate the extraction efficiency by dividing the recovery by 0.5 and multiplying by the total volume of homogenate at the end of the extraction (3 ml). An extraction efficiency >1% is considered acceptable.

The theoretical limit of detection is 10 genome copies. For shellfish samples, the limit of detection is obtained by dividing these values by 0.5 and multiplying by the total volume of homogenate measured at the end of the extraction (3 ml).

7. Results

The positive results of the study are shown in Table 1.

Sample/code	Geographical origin	Norovirus		
		GI	GII	GI + GII
C1	Spain	-	+	-
C3	La Spezia - Italy	-	+	-
C6	Farmed in Spain, harvested in Scardovari (RO) - Italy	-	+	-
C14	Scardovari (RO) - Italy	-	+	-
C46	Farmed in Spain, harvested in Goro (FE) - Italy	-	+	-
C48	Farmed in Spain, harvested in Isola di S. Antioco (Sardinia) Italy	-	+	-

Table 1: Origin and Norovirus detection in *M. galloprovincialis* samples.

Six out of 53 (11.3%) *M. galloprovincialis* sample batches collected during an 8-month period (March 2022 - November 2022) were found to be positive for Norovirus gene group II. The positive samples were identified as C1, C3, C6, C14, C46 and C48.

Thirty nine of 53 (73.6%) *M. galloprovincialis* sample batches were of Italian geographical origin. Two samples (2/39; 5.1%) were found positive for NoV GII and were raised in Scardovari (Po River lagoon) (March 2022) and La Spezia (May 2022) (C3 and C14 respectively)..

Fourteen of 53 (26.4%) *M. galloprovincialis* sample batches were of Spanish geographical origin. They were imported from Spain and harvested in Italy. Four batches (4/14) were found to be positive for NoV GII (28.6%). Among the Spanish batches, only C6 (March 2022), C46 (November 2022) and C48 (November 2022) passed through an Italian purification center, but C1 (March 2022) did not.

None of the 53 samples tested positive for more than one viral agent or to the Norovirus gene group I or Hepatitis A virus.

8. Discussion and conclusions

The presence of viral agents such as Hepatitis A virus and Human Norovirus in *Mytilus galloprovincialis* is a real existing threat for the consumers[5]. The ever-growing demand of markets around the world and specifically in Italy, for mussels as a consistent part of the menu, either cooked or raw, brings the farmers, purification centers and marketing centers to expand the domestic production annually. This is the situation commonly seen in the lagoon of the Po River or in Sardinia, as well in *M. galloprovincialis* imported from countries like Spain.

The viral agents described in this degree thesis, are part of larger families of viral agents. The *Caliciviridae* and *Picornaviridae* members are characterized for being non-enveloped, positive-sense single-stranded RNA viruses, which are responsible for widespread human gastroenteric diseases characterized for symptoms like nausea, vomiting, gastroenteric inflammations, loss of corporal liquids, loss of weight and diarrhea. In severe cases they can also lead to long term chronical damage to the gastrointestinal track, coma and even death[5,7].

Although these viral agents pose a threat especially to particular population at risk such as pediatric, geriatric and immunosuppressive individuals, the possibility of healthy adults being affected by them, when mussels are consumed raw cannot be ruled out, as high cooking temperatures do not neutralize these viral agents. Indeed, viral agents are located in the hepatopancreatic gland of mussels, i.e., in their digestive tract. Thus, they need temperatures above 80^{o} C to be destroyed[3].

In order to verify the occurrence and quantity of Noroviruses and Hepatitis A virus in *M. galloprovincialis* batches placed on the market in Italy, in this study the ISO-22174 technique (Polymerase chain reaction (PCR) for the detection of food-borne pathogens) was used.

The findings of the study revealed that the risk of contracting Norovirus gene group II is substantial, especially from Mediterranean molluscs of Spanish geographical origin (28.6%). In comparison, the prevalence of NoV GII positive samples was much lower in *M. galloprovincialis* farmed in Italy (5.1%). Meanwhile, the risk of contracting Norovirus gene group I and Hepatitis A virus seems to be negligible. In fact, only Norovirus GII was detected in 11.3% of the tested samples, while Norovirus GI and HAV were never found.

The reasons for such a difference in prevalence between Spanish and Italian mussels are largely unknown, even though they could be probably attributed to a greater attention posed by the Italian Competent Authorities to the quality of waters they are raised into.

This study has also shown the link between prevalence and seasonality, since the majority of NoV contaminated samples (5/6; 83%.3) were placed on the market in cold months, such as March and November. In fact, only one sample (1/6; 16.7%) was found to be positive during the spring (May 2022). The association between NoV contamination of molluscs and cold months could be due to the good persistence of the viral agents to low temperatures[22].

HNoV and HAV have been identified as a major source of foodborne disease outbreaks. Viruses were responsible for 19% of all foodborne outbreaks in the EU in 2009, causing over 1,000 outbreaks and affecting over 8,700 persons. Since 2007, the total number of viral outbreaks has been increasing. Food can act as a vehicle for the transmission of some viruses to people, some of which are very infectious and can cause extensive epidemics[19].

The EFSA scientific opinion considered Norovirus and Hepatitis A virus in fresh produce, Ready-to-Eat foods, and bivalve molluscs such as oysters, mussels, and scallops, which are recognized as priority hazards by the World Health Organization[19].

Species	No. of analyzed samples	Viral contamination				
		HAV (%)	NoV GI (%)	NoV GII (%)	NoV GI + GII (%)	Total (%)
Mytilus galloprovincialis	181	0	3	26	1	30 (16.6)
Venus gallina	34	0	1	1	0	2 (5.9)
Ostrea spp.	22	0	0	1	0	1 (4.5)
Modiolus barbatus	9	0	0	1	0	1 (11.1)
Acanthocardia tuberculata	6	0	0	1	0	1 (16.7)
Solen marginatus	1	0	0	1	0	1
Total	253	0	4 (1.6)	31 (12.2)	1 (0.4)	36 (14.2)

Table 2: Detection of HAV and NoV from each species of molluscs tested[20]

In table 2 the prevalence of a study conducted in the Apulia region (Southern Italy) from January 2013 to July 2015 is shown. A total of 253 samples of bivalve molluscs collected in harvesting sites over a vast coastline tract (860 km) were tested for HAV and NoV of genogroups GI and GII using real-time reverse transcription qualitative PCR. As shown in Table 2, the prevalence varies between the various species of molluscs as follows:

- *Mytilus galloprovincialis*: 30/181 (16.6%) positive samples (3 for NoV GI, 26 for NoV GII, 1 for NoV GI + GII)
- *Venus gallina*: 2/34 positive (5.9%) samples (1 for NoV GI and 1 for NoV GII)
- *Ostrea spp.* : 1 positive/22 samples (4.5%) (1 for NoV GII)
- *Modiolus barbatus:* 1 positive/9 samples (11.11%) (1 for NoV GII)
- *Acanthocardia tuberculate:* 1 positive/6 samples (16.7%) (1 for NoV GII)
- *Solen marginatus:* 1/1 positive sample (100%) (1 for NoV GII)

HAV RNA was not found in any of the samples tested. In contrast, NoV RNA was found in 14.2% of the samples, with a greater frequency of NoVs from genogroup GII than genogroup GI[20].

Out of 36 NoV-positive samples, 4 (11.1%) were NoV GI, 31 (86.1%) were NoV GII, and only one (2.8%) sample included both genogroups[20].

In the abovementioned study, the contamination by Nov GI and GII was observed in every month, even though it increased in frequency during cold months (December – March)[20], as observed in our study for *M. galloprovincialis* samples. Also, the prevalence of NoV GII contamination was found to be very similar between the two studies, being 14.36% in the study from Apulia region and 11.3% in this graduation study.

From 2013 to 2017, the Netherlands Food and Consumer Product Safety Authority (NVWA) sampled bivalve molluscs to collect data as part of the NVWA monitoring program. Depending on NVWA inspectors' availability and NoV epidemiology, sampling took place from September to March in a variety of places[21].

Bivalve molluscs (mussel and oysters) were collected at Dutch dispatch centers from sample sorting and packaging lines. During this time, packed molluscs were also collected at retail locations in the Netherlands. Additionally, in 2015 and 2016, the NVWA randomly selected

samples from post-harvest batches of unprocessed bivalve molluscs harvested in different countries, such as the United Kingdom, Ireland, Germany, France, or Denmark upon arrival in the Netherlands.

Overall, a total of 490 mussel and 266 oyster samples were collected and tested for the presence of NoV RNA along the entire food chain. The presence of NoV GI and/or NoV GII RNA was found in 53.1% of mussel samples, with 31.6% of samples showing simultaneous presence of both NoV GI and NoV GII. In oysters, NoV RNA was found in 31.6% of samples, with NoV GI and GII RNA found in 10.2% of samples. The percentage of mussel samples that tested positive for NoV GI and/or GII RNA was much higher in post-harvest samples that had not been purified starting one year later, i.e., from 2014 to 2017, a total of 228 oyster and 392 mussel samples were examined for the presence of HAV RNA. Only one mussel sample (0.3%) was positive, while none of the oyster samples were positive. The HAV RNA-containing mussel sample was collected post-harvest in February 2015 from a region near a heavily populated area in Western Europe[21].

It is also worth mentioning the Commission Implementing Regulation (EU) 2019/627 of 15 March 2019, laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls.

In the articles 53 – 55, we find specific requirements for the classification of production and relaying areas for live bivalve molluscs based on the quality of water. The classification is based on the level of E. coli contamination of molluscs and could be related to viral agents contamination, because of their common fecal origin.

<u>Requirements for Class A areas:</u>

1. The competent authorities may classify as Class A areas those from which live bivalve molluscs may be collected for direct human consumption.
2. Live bivalve molluscs placed on the market from such areas shall meet the health standards for live bivalve molluscs set out in Chapter V of Section VII of Annex III to Regulation (EC) No 853/2004.

3. Samples of live bivalve molluscs from Class A areas shall not exceed, in 80 % of samples collected during the review period, 230 *E. coli* MPN per 100 g of flesh and intravalvular liquid.
4. The remaining 20 % of samples shall not exceed 700 *E. coli* MPN per 100 g of flesh and intravalvular liquid.
5. When evaluating the results for the fixed review period for maintenance of a Class A area, the competent authorities may, on the basis of a risk assessment based on an investigation, decide to disregard an anomalous result exceeding the level of 700 *E. coli* MPN per 100 g of flesh and intravalvular liquid.

Requirements for Class B areas

1. The competent authorities may classify as Class B areas those from which live bivalve molluscs may be collected and placed on the market for human consumption only after treatment in a purification centre or after relaying so as to meet the health standards referred to in Article 53.
2. Live bivalve molluscs from Class B areas shall not exceed, in 90 % of the samples, 4, 600 *E. coli* MPN per 100 g of flesh and intravalvular liquid.

Requirements for Class C areas:

1. The competent authorities may classify as Class C areas those from which live bivalve molluscs may be collected
    a. and placed on the market only after relaying over a long period so as to meet the health standards referred to in Article 53.
2. Live bivalve molluscs from Class C areas shall not exceed 46 000 *E. coli* MPN per 100 g of flesh and intravalvular liquid.

Even though the Regulation CE No 853/2004 requires a decontamination step for molluscs raised in class B and C waters, viruses are not guarantee to be absent in the final product. For this reason, the only preventive measure for the consumer is an effective cooking step. This is

much more important if a population at risk is involved in the consumption of bivalve molluscs, such as young, old and immunocompromised people.

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