UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO IN

RIPRODUZIONE, PRODUZIONE, BENESSERE ANIMALE E SICUREZZA DEGLI ALIMENTI DI ORIGINE ANIMALE

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INDIRIZZO: **Produzione e Sicurezza degli Alimenti di Origine Animale (XXV CICLO)**

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PREVALENCE, DISTRIBUTION AND PATHOGENIC PROFILE OF *VIBRIO PARAHAEMOLITYCUS* AND *VIBRIO SPP.* CORE IN SHELLFISH (*MYTILUS GALLOPROVINCIALIS* AND *RUDITAPES DECUSSATUS*) COLLECTED IN SARDINIA (ITALY). MOLECULAR FINGERPRINTING USING THREE PCR- BASED METHODS: BOX PCR, REP PCR , AND ERIC PCR.

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ANNO ACCADEMICO 2011 - 2012

Abstract

Regulation (EC) N_o 2073/2005 highlights the need to develop reliable detection methods for pathogenic *Vibrio* strains. The purpose of this study was to investigate the prevalence and concentration of *Vibrio spp.* core and particularly *V. parahaemolyticus* in *Mitylus galloprovincialis* and *Ruditapes decussatus* using conventional biochemical tests and Polymerase Chain Reaction (PCR) method. Detection and enumeration of *Vibrio spp.* core have been carried out in 71 samples (before and after depuration process) of shellfish harvested in three areas located in Sardinia (Italy) during a year (April 2011- May 2012). The prevalence of *Vibrio spp*. was found to be 14% and 16% respectively in *M. galloprovincialis* and *R. decussatus*. Prevalence of *V. parahaemolyticus* was found to be 18% and 27% respectively in *M. galloprovincialis* and *R. decussatus.* Prevalence of *V. vulnificus* was found to be 10% and 6% respectively in *M. galloprovincialis* and *R. decussatus.* Isolates previously positive to biochemical tests were subjected to specie-specific PCR for identification of *Vibrio spp*. core. The analysed samples revealed the presence of 2 strains as *V. vulnificus*, 8 strains as *V. cholera* and 114 as *V. parahaemolyticus. V. parahaemolyticus* samples were tested with conventional PCR for *trh* and *tdh* factors and only 2 samples were *tdh+/trh-* and 2 were *tdh-/trh+.* Not a single sample was *tdh+/trh+*. Seventy-one samples of *V. parahaemolyticus* were subjected to three fingerprint PCR-based method (ERIC-, REP- and BOX-PCR). Two cluster (A and B) were specifically associated with most of strains from clams (clusters B) and the persistence of some genetic clusters over time (in some cases strains showing very similar genetic profiles were isolated for two or three subsequent). These findings confirm the potential human health risk associated with the presence of pathogenic *Vibrio spp.* and especially *V. parahaemolyticus* in shellfish and contribute to implement prevention measures.

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INTRODUCTION

For dietetic, traditional or food availability reasons, consumption of bivalves has been rising dramatically worldwide (Fauconneau. 2002; Johnson & Hayasaka, 1988; Murchie et al., 2005). On the other hand. microbial contamination is chronic and pervasive in growing and harvest areas. By filter-feeding from the surrounding water, bivalves bio-accumulate natural occurring or anthropogenic contaminants. arising this contamination to the consumer (Lees, 2000). Contamination includes pathogenic species capable of producing diseases outbreaks (WHO, 2010; Oliveira et al., 2011; Food and Authority 2011). Recently, there has been observed an increasing concern regarding food safety, particularly in molluscan shellfish products. Extensive efforts have been pursued to assure a safe supply of bivalves, but disease and death due to viruses and naturally occurring bacteria have been observed. This might be a result of underestimated and undermanaged microbial contamination (Oliveira et al. 2011).

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Bivalves, as a food component, are characteristically tender. easily digested. additive-free and minimally processed. These characteristics make them a product that almost completely fulfils the demands of consumers (Murchie et al., 2005). The importance of bivalve shellfish as a food supply increases if we attend to the natural resource that shellfish growing areas may represent (Johnson & Hayasaka. 1988). Dense beds of bivalve shellfish (epifaunal or infaunal species) occur in inshore estuaries with high primary productivity and have been an important source of food since prehistory (Lees. 2000). However, the aquatic environment is becoming over-exploited and as a consequence of over catching the depletion of stocks is leading to the reduction of natural shellfish beds and to the need of human intervention in its production (Pillay & Kutty, 2005). Aquaculture production has been exponentially increasing and becoming one of the fastest-growing food industries. especially in Asia (Defoirdtet al., 2004; FAO, 2006; Sapkota et al., 2008).

In Europe, Italy is the third producing country of shellfish, after Spain and France (Irepa. 2007). In 2005 the selling of molluscs increased by 9.4% growing the total receipts of Italian ichthyic production. In Italy bivalves shellfish species most harvest are *Mytilus* (76%) and *Ruditapes* (24%) (Ismea, 2007).

Contamination of bivalve shellfish occurs mainly because they are suspension feeders that selectively filter small particles of phytoplankton. Zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Burkhardt & Calci, 2000; Defossez & Hawkins, 1997; Dunphy et al., 2006; Lees. 2000).

For the majority of foods, proper refrigeration, storage, handling. cleaning and cooking procedures helps the consumer to control microbial risk and assure product safety. The hazards related to the contamination of bivalves by harmful microorganisms are due to their traditional cooking procedure which may not be enough to ensure consumer's safety. These

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circumstances make them an important vector of foodborne disease (Lees, 2000). The control of the disease risk associated with bivalves requires Hazard Analysis by Critical Control Point (HACCP) procedures together with water environment quality management of growing and harvest areas and post-harvest product processing which might involve depuration and/or heat treatment where appropriate (WHO, 2010).

The true incidence of foodborne disease outbreaks is not known (Oliveira et al., 2011). Foodborne disease is a public health problem which comprises a broad group of illnesses. Among them. gastroenteritis is the most frequent clinical syndrome which can be attributed to a wide range of microorganisms (Molnar, Wels & Adley, 2006). The risk of human infections is related to the ingestion of bivalves contaminated with protozoan parasites, viruses and bacteria.

Microbial contamination is chronic and pervasive in harvest areas. Furthermore, viruses and naturally occurring bacteria are the most often

cited causative agents of disease and death related to shellfish consumption (Croci, 2002; Huss et al., 2000; Lees, 2000; Wittman & Flick, 1995). Shellfish-derived illnesses can have natural causes due to contaminants that are available in the environment and consequently a part of the normal biota (Shumway & Rodrick, 2009), while others can be human-generated before or after shellfish harvesting. Pre-harvest microbial contamination (occurring naturally or as a result of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss et al., 2000; Lees, 2000).

Regardless of the higher abundance of indigenous marine viruses. only viruses derived from anthropogenic contamination are associated with illness in seafood consumers. Noroviruses, hepatitis A viruses, enteroviruses and adenoviruses are the viruses that are more often linked to shellfish contamination (Le Guyader. Atmar. & Albert. 2007; Lees, 2000; Muniain-Mujika et al., 2003). Shellfish may also be contaminated post-

harvesting. Potential hazard due to sub-lethally injured microbiota that may recover and multiply during later storage must be considered (Oliveira et al. 2011).

Among the indigenous microbiota of coastal environments, the family *Vibrionaceae,* particularly *Vibrio parahaemolyticus, V. vulnificus* and *Vibrio cholera,* is targeted as a causative agent of human disease due to the consumption of shellfish (Butt et al., 2004; Hood & Ness, 1982; Normanno et al., 2006; Ripabelli et al., 1999). These natural pathogens remain viable and cultivable in water, even in the absence of organic matter (Croci et al., 2002; Marino et al., 2005; Pruzzo, Gallo & Canesi, 2005). Some species are primarily associated with gastrointestinal illness (*V. cholerae* and *V. parahaemolyticus*) while others can cause non-intestinal illness, such as septicaemia (*V. vulnificus*). In tropical and temperate regions, disease-causing species of *Vibrio* occur naturally in marine, coastal and estuarine (brackish) environments and are most abundant in estuaries. Pathogenic

vibrios, in particular *V. cholera,* can also be recovered from freshwater reaches of estuaries (Desmarchelier, 1997), where it can also be introduced by fecal contamination.

The occurrence of these bacteria does not generally correlate with numbers of fecal coliforms and depuration of shellfish may not reduce their numbers. A positive correlation between water temperature and the numbers of vibrios has also been shown in several parts of the world. Further, according to data from the United States of America and Denmark, there is a positive correlation between water temperature and both the number of human pathogenic vibrios isolated and the number of reported human infections. This correlation is particularly striking for *V. parahaemolyticus* and *V. vulnificus* (Dalsgaard et al., 1996). The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of vibrios (Lipp et al., 2002). Recently, a number of reports have highlighted the pathogenic potential of

vibrios toward humans and marine animals (e.g. corals. Gorgonians, and shrimp) which may be coupled with rising of sea water temperature due to global warming (Makino et al., 2003; Martin. Bonnefont. & Chancerelle, 2002; Rosenberg & Ben-Haim, 2002; Sechi et al., 2000). Seafood was the most frequent vehicle (91%) for outbreaks due to *Vibrio spp.,* a known aquatic genus (Adams et al., 2000; Ray. 2004). The Centers for Disease Control and Prevention report also stated that most cases occur during the summer months and that seafood. e.g., oysters, shrimp and fish had been consumed by the patients. Little attention, however, has been paid to the ability of *V. cholerae* to cause severe wound infections (Johnston et al., 1983;Morris. 1990; Oliver. 2005a; Tsai et al.,2009). During the summer of 2006, such infections – including three fatal cases – were recorded among Swedish patients who had been exposed to Baltic Sea water (Shönning et al.,2008) with another fatal case earlier reported from Finland (Lukinmaa et al., 2006). Several cases of wound infections caused by *V. vulnificus* have

also been described (Melhus et al., 1995; Dalsgaard et al., 1996; Ruppert et al., 2004) but as yet, no clinical case of *V. parahaemolyticus* has been reported from the Baltic Sea.

In the present study was investigated the contamination of *Vibrio spp.* in the seafood products (*Mytilus galloprovincialis* and *Ruditapes decussatus*) harvested in Sardinia (Italy) and evaluate prevalence and quantitative analysis after harvest and depuration. In addition, was made molecular identification of the most important Vibrios (*V. cholera, V. parahaemolyticus* and *V. vulnificus*).

V. parahaemolyticus isolates were subjected to intensive analysis to assess the presence of pathogenic specimens. Moreover, through three different fingerprint PCR-based methods (ERIC-PCR, REP-PCR and BOX-PCR) *V. parahaemolyticus* strains were grouped to understand the variability in environmental investigated sites, seasons and host.

The lack of the official method of detection of *Vibrio* criteria (e.g. EC 2073/2005 etc) is in contrast with this study and with bibliography data that demonstrated the importance of the presence of a standard method in the European rules.

CHAPTER 1

Biology of molluscs

Tassonomy

The phylum Mollusca is one of the largest most diverse and important groups in the animal kingdom. There are over 50000 described species in the phylum and about 30000 of these are found in the sea. Molluscs are soft-bodied animals but most are protected by a hard protective shell. Inside the shell is a heavy fold of tissue called the mantel. The mantle encloses the internal organs of the animal.

There are six major classes of molluscs. The Class Gastropoda is the largest (40000 species), the Class Bivalvia with about 7500 species (includes animals with two shell valves such as mussels, oyster, scallop and clams). Class Cephalopoda with about 650 species. the Classes Polyplacophora (chitons) and Scaphopoda (tusk shell) together contain about 1000 species

and the primitive Class Monoplacophora that contains the single Genus Neopilina (Goslin. 2003).

Freshwater mussels have three basic characteristics which distinguish them from other animals; these include:

1) a two-valved shell,

2) a soft body,

3) a muscular foot.

The "soft" parts consist of respiratory organs, digestive organs and other vital parts. The foot often seen extended from between the two valves, aids the mussel in locomotion, burrowing and positioning in the river bottom.

The mussel's shell contains several diagnostic characteristics which are helpful in separating the species. The shiny layer seen from inside the shell is called the nacre or "mother of pearl". The nacre may be white purple, pink or pale orange. The outer layer is made of protein and serves to

protect the shell. Shells come in a variety of shapes including round, elongate, oval or tear-drop shaped. The beak is the oldest part of the shell and the end closest to the beak is the anterior or front. The outer surface may be smooth or have humps, ridges, depressions, furrows and wings. The outer surface may also be shiny. dull or brightly colored with rays or plain.

Genus *Mytilus*

The marine mussel genus Mytilus includes a complex of 3 sibling species. *M. edulis* L. 1758, *M. trossulus* Gould 1850 and *M. galloprovincialis* Lamarck 1819. All three are now globally widespread and form hybrid zones where they overlap (Hilbish et al. 2000; McDonald et al. 1991; Sarver & Foltz 1993). Mussel in the genus *Mytilus* are a dominant component of rocky shore communities in cooler water of the northern and southern hemispheres.

Mytilus galloprovincialis

The Mediterranean Mussel (*Mytilus galloprovincialis)* is native to the Mediterranean, Black and Adriatic Seas but has spread (mostly via ballast water and ship hull fouling) to many other regions worldwide. *M. galloprovincialis* is characterized by a shell of elongated shape divided into two halves sharpened on one side and rounded on the other. It can move with the aid of the foot. A byssus gland secrets byssal threads that allow their attachment to the substrate. The gills are two pairs of broad plates composed of a large number of parallel filaments that filter food particles from the water. A mussel of 5 cm in length can filter 5 litre/hr. Digestion takes places in the digestion gland (brown-greenish in colour) situated in the centre of the body. Mussels feed on phytoplankton and organic matter. The mantle, immediately in contact with the interior of the shell, secretes the shell and contains the gametes (eggs or sperm). *M. galloprovincialis* produce millions of eggs, losing a considerable amount of their reserve glycogen.

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The habitat of the locations where culture takes place is very similar. Galicia (NW Spain) has been taken as a model in this fact sheet. The Galician coast is characterized by flooded river valleys called 'rias', where farmers culture the mussels. Their bottoms are muddy, and they are bordered by hills. In turn. this favours the growth of mussels. These sheltered rias provide an ideal environment for culturing mussels on ropes suspended from floating rafts. Recruitment of seed mussels occurs throughout the year with the major settlement season from May to September. This type of rearing is most similar to Italian rearing but environmental features are different in the Mediterranean Sea.

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Genus *Ruditapes*

Ruditapes is a genus of marine bivalve molluscs. in the family Veneridae. These marine mussels includes a complex of species and subspecies: *R. aureus.·R. bruguieri.·R. decussata.·R. decussatus.·R. japonica.·R. largillierti*.·*R. philippinarum* (Japanese Littleneck).·*R. phillippinarum.·R. semidecussata. R. variegata and·R. variegatus.*

Ruditapes decussatus

Ruditapes decussatus lives in muddy-sand sediments of tidal flats or shallow coastal areas (Parache, 1982). Clams, like most filter feeders living in the intertidal zone. take advantage of the tidal movement in estuaries: water currents generated by the tides continuously supply a much larger quantity of food than the amount locally available. Moreover clams are very diverse group of bivalves in that is notable variation in the shape, size, thickness, colour and degree of sculpturing of the shell.

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Shell broadly oval to quadrate. umbones distinctly anterior. Posterior hinge line straight, posterior margin truncate, anterior hinge line grading into the down-sloping anterior margin. Prominent posteriorly, where the shell is conspicuously decussate. Sculpture of fine concentric striae and bolder radiating lines growth stages clear, lunule and escutcheon poorly defined. Each valve with three cardinal teeth: centre one in left valve and centre and posterior in right are bifid. Pallial line and adductor scars distinct, pallial sinus U-shaped not extending beyond mid-line of shell but reaching a point below the posterior part of the ligament. Lower limb of sinus distinct from pallial line for the whole of its length. Inner surfaces glossy white, often with yellow or orange tints and with a bluish tinge along dorsal edge, cream, yellowish or light brown, often with darker markings. Its sexes are separate, although hermaphrodites can be found infrequently. Reproduction is external and takes places mainly during summer in the wild and/or on hatcheries. In spring, clams can be artificially conditioned

for hatching with higher temperature water and abundant food. Clams burrow into the sea-bed and consequently shell and body display modifications necessary for this type of existence (Collin & Rehnstam-Holm. 2011).

Bivalve culture

Bivalve molluscs like mussels and oysters are good sources of cheap and high quality protein and their shells are used for ornaments and other industrial products. They are ideal species to culture because of their low position in the food chain. Farming of bivalves can also increase the income of small-scale fishermen faced with dwindling catches, as well as provide livelihood for unemployed people in coastal areas.

In the cultivation of bivalve molluscs a suitable culture site must have the following characteristics:

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(a) sufficient breeding stock to insure adequate spatfall;

(b) protected from prolonged flooding. strong winds and waves;

(c) high natural productivity of the water;

(d) moderate tidal current flow for transport of phytoplankton and oxygen and elimination of wastes;

(e) physio-chemical conditions of the area must be suitable for growth and survival. especially salinity and temperature;

(f) must be free from industrial wastes. sewage and other pollutants (Fujiya. 1970).

Pruduction of *Mytilus galloprovincialis*

The rearing of *Mytilus galloprovincialis* is always extensive in all countries where it is being carried out; the steps that are described in this fact sheet therefore apply to all cases. The young mussels are collected from the sea and can be cultured on suspended ropes; these ropes, which are covered

with mussel seeds kept in place by nylon nets, are suspended either from rafts, or wooden frames, or from long lines of floating plastic buoys. A substantial portion of the EU production is grown on suspended ropes, a technique which can be extended further offshore and which, although quite sensitive to plankton blooms is the only one which could further increase production.

Culture begins when farmers collect mussel seed. mainly from natural beds (60-70 percent); the remainder from the collector ropes hung from their rafts. Farmers can collect up seed from the exposed rocky shores on the ocean side of the rias and islands. They use a special steel shovel which has a blade of about 10 cm^2, attached to a wooden handle. Farmers suspend the mussels from their own rafts or sell them to other farmers. Farmers gather around 4 500 tonnes of mussel seed (mean length=2 cm) from these areas every culture cycle. They take the seed to the rafts, keeping it moist, and attach it to ropes within 24 hours after collection. To collect seed from

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the rafts, farmers use special nets made from old fish nets and suspend them during March and April. Farmers attach the seed to the ropes by hand. or with a machine which secures it with a special cotton or rayon mesh; this mesh disintegrates within a few days. By then, the mussels have secreted new byssus and have attached themselves to the ropes. The ropes, usually 3 cm thick and made of nylon, polyethylene or esparto grass vary in length from 6-10 m. Their rough surfaces facilitate the attachment of the mussels. Each rope with attached mussels has a loop at one end which in turn is lashed to the girders of the rafts. Each raft has from 200 to 700 ropes. Farmers attach from 1-3 ropes/m^2 of raft. This distribution allows an adequate flow of water rich in food for the mussels and prevents the mussel ropes from touching each other. Farmers install the ropes mainly from November to March.

The third step (after obtaining the seed and attaching it) is thinning, which has to be done to prevent the mussels from falling off in rough weather;

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thinning also encourages rapid and uniform growth. Farmers do this when the mussels are half grown (shell length 4-5 cm) after 5-6 months of growth usually from June to October. They lift the ropes into their boats using a crane and rub off the clusters of mussels by hand into a steel screen which separates them into different sizes. A mechanical cylindrical screen may also be used. The mussels from each original rope are attached to two to four new ropes with cotton or rayon netting. The average weight of the ropes is 46 kg. This work is repeated once again before harvest if the mussels grow rapidly (in which case their weight and density increases the risk that the mussel clusters will fall off). It is also necessary to repeat this operation in order to ensure that all mussels reach a similar size at harvest time.

The rearing of mussels constitutes the fourth culture step. Where growth is rapid mussels can attain market size (8-10 cm) in 8-9 months. The usual time required in some bays is around 13 months. However, a high raft

density can retard mussel growth. Growth is minimal in summer and highest in winter. Slow summer growth is related to the relative abundance and availability of food (phytoplankton) in the water column then; this is more important than high temperature and causes the seed placed on the ropes in the spring and the fall to reach the same size at the end of the first winter. Each raft normally holds three types of ropes: those for collecting seed, those with growing mussels and those with marketable mussels; in this way growers maintain continuous production. A large number of mussel seed and fouling organisms attach to the floats and as they grow, the weight of the raft increases; therefore farmers have to clean the floats occasionally. Rafts range in age up to 30 years. with an average of about 8 years.

Mussels of commercial size are available throughout the year and can be harvested at any time but the main harvest is from October to March. when market demand is high and their condition is best.

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For harvesting, farmers use a crane to raise the ropes to their boat, where the mussels are separated and graded by rubbing them over a grid of iron bars. They are then washed clear of small mussels, silt, empty shells, ascidians and other unwanted organisms. Any mussels too small for the market are wrapped onto new ropes for further growing.

The marketable mussels are packed in nylon bags and taken in boats directly to the depurations or to canning factories. The mechanization of handling is minimal to reduce damage to mussel shells and thereby enhance the shelf life of the mussels during transportation. In the warm season, refrigerated trucks are used to transport the mussels.

Pruduction of *Ruditapes decussatus*

Farmers obtain seed from their own parks (protected bottoms) or from the natural clam populations in the spring. They may also dig adult clams from

seaport areas and spread them in their parks. Periodically, they have to clean their parks of predators and mud.

Seed can also be obtained from hatcheries, where breeders, not exceeding 40 mm are maintained for 30-40 days at 20 °C.

Clams can be reared in nurseries within greenhouses, with controlled feeding by using unicellular algae or reared in meshed containers over culture tables. An alternative is to pump environmentally controlled water to inland tanks where clams are placed in cylinders of about 50 cm diameter and 20 cm long, with a bottom made of a rigid mesh.

Culture techniques are simple, consisting of regular maintenance of the substrate, avoiding algae, starfish and other predators; oxygenating the substrate; and maintaining an appropriate clam density and seeding juvenile clams.

Fishermen harvest clams by walking the intertidal areas and using special hand shovels or sometimes by using the rakes that are normally used for

keeping the culture beds clear of seaweed. Clams may also be harvested from boats. Various collection tools are used, including (rake), which are operated from the boats with the help of a long handle. The closed season is from March to October and the minimum size allowed for *Ruditapes decussatus* is 30 mm. In hand (walking) harvesting clams are harvested with the help of different types of small shovels; sometimes the rakes that are usually more employed for cleaning the parks of seaweeds are employed.

Fishermen bring their clams to depuration stations where they are held in tanks for at least 42 hours. The clams are then packed in net bags of 0.5 - 1 and 2 kg and are destined to be canned or eaten fresh. They are transported by refrigerated trucks which maintain their temperature at 3 - 10 °C; the clams have a shelf life of 5 days.

Classification of production and relaying areas

The European Directive 2006/113/CE and the European Directive 2004/41/CE the US interstate agreement set out by the Food and Drug Administration (Anonymous. 1993) or the UK Advisory Committee on Microbiological Safety of Food (Anonymous. 1998) are guidelines based on the levels of microbiological indicators for both shellfish and overlying waters.

The final product is sealed, labelled for traceability and commercialized giving distributors and consumers the confidence of a safe certified product (Jones et al., 1991; Lees, 2000; Shumway & Rodrick, 2009).

Regulation 854/2004 (EC) laying down specific rules for the organization of official controls on products of animal origin intended for human consumption.

The post-harvest treatment required by EC Regulation 854/2004 before bivalve molluscs can be sold for human consumption regard the harvesting

of molluscs that come from natural bed or rearing. Regulation classified in one of three categories production areas according to the level of faecal contamination:

- Class A area: areas from which live bivalve molluscs may be collected for direct human consumption. Live bivalve molluscs taken from these areas must meet the following health standards:

✓ *E. coli:* set at 230 MPN (Most Probable Number) in 100 g of flesh and intra-valvular liquid with MPN test (five-tube, three dilution);

✓ *Salmonella spp.:* assent in 25 g of flesh and intra-valvular liquid;

✓ Mercury: set at 0.5 ppm in flesh ;

✓ Lead: set at 1.5 ppm in flesh ;

✓ Biotoxin: DSP complex (Diarrhetic Shellfish Poison):

  - okadaic acid.diniphysistoxin. pectenotoxin. set at of 160 µg of equivalent okadaic acid/Kg;
  - Yessotoxin set at 1 mg of equivalent yessotoxin/Kg;

- Azaspiracid set at 160 µg of equivalent azaspiracid acid/Kg;

ASP (Amnestic Shellfish Poison) set at 20 mg of domoic acid/kg of edible mollusc part.

PSP (Paralytic Shellfish Poison) set at 800 µg of equivalent saxitoxin/Kg in the edible molluscs part.

Radioactive nuclide: within the limits set by law.

- Class B area: areas from which live bivalve molluscs may be collected. but placed on the market for human consumption only after treatment in a purification centre or after relaying so as to meet the health standards prescribed for the Class A area. Live bivalve molluscs taken from these areas must meet the following health standards:

  - ✓ *E. coli:* set at 4600 MPN (Most Probable Number) in 100 g of flesh and intra-valvular liquid with MPN test (five-tube, three dilution);
  - ✓ Mercury: set at 0.5 ppm in flesh ;
  - ✓ Lead: set at 1.5 ppm in flesh ;

- ✓ Biotoxin: DSP complex (Diarrhetic Shellfish Poison):
  - ➢ okadaic acid.diniphysistoxin. pectenotoxin. set at of 160 µg of equivalent okadaic acid/Kg;
  - ➢ Yessotoxin set at 1 mg of equivalent yessotoxin/Kg;
  - ➢ Azaspiracid set at 160 µg of equivalent azaspiracid acid/Kg;

ASP (Amnestic Shellfish Poison) set at 20 mg of domoic acid/kg of edible mollusc part.

PSP (Paralytic Shellfish Poison) set at 800 µg of equivalent saxitoxin/Kg in the edible molluscs part.

Radioactive nuclide: within the limits set by law.

- Class C area: C areas from which live bivalve molluscs may be collected but placed on the market only after relaying over a long period (not less than two months) as to meet the health standards prescribed for the Calss A area. Live bivalve molluscs taken from these areas must meet the following health standards:

- ✓ *E. coli:* set at 46000 MPN (Most Probable Number) in 100 g of flesh and intra-valvular liquid with MPN test (five-tube. three dilution);
- ✓ Mercury: set at 0.5 ppm in flesh ;
- ✓ Lead: set at 1.5 ppm in flesh;

General consideration

Plants should be constructed in such a way as to prevent stored raw material, the depuration systems, depurated and packaged product and associated processes from contamination from airborne or pest-borne contamination and should not be subject to flooding. The systems themselves and associated processes should be sited within buildings in order to aid control of temperature and contamination.

The product flow should be from dirty to clean going throught the steps below in sequence:

- ✓ Receipt of harvested product

- ✓ Indoor pre-depuration storage;
- ✓ Washing. debussing and culling;
- ✓ Into depuration rank;
- ✓ Depuration;
- ✓ Out of depuration rank;
- ✓ Washing;
- ✓ Culling;
- ✓ Grading and packing;
- ✓ Dispatch of finished product. (FAO. 2008)

Disinfection of water may be not required if the abstraction point is located within an area classified as of a quality whereby shellfish can be marketed directly for human consumption (EU Classs A; US Approved) and the system is of a flow-through design. However, in such circumstance, treatment will provide an extra safeguard against intermittent contamination – it will also provide protection against contamination with

33 Sonia Lamon – "Prevalence, distribution and pathogenic profile of *Vibrio parahaemolitycus* and *Vibrio spp* core in shellfish (*Mytilus Galloprovincialis* and *Ruditapes decussatus*) collected in Sardinia (Italy). Molecular fingerprinting using three PCR- based methods: Box PCR, REP PCR , and Eric PCR" - Tesi di Dottorato di Ricerca in Riproduzione, Produzione, Benessere Animale e Sicurezza degli Alimenti di Origine Alimentare XXV ciclo – Università degli studi di Sassari.

pathogens that may be naturally present in the sea water, such as vibrios. (FAO 2008).

Purification processes

Sanitary regulations rely on bacterial indicators of sewage contamination to classify shellfish harvesting waters and to estimate the efficiency of purification methods (Murchie et al., 2005). Unhealthy harvested bivalves purge contaminants when transferred into clean natural shellfish beds (relaying) or into tanks (depuration) (Richards, 1988; Shumway & Rodrick, 2009).

Depuration

Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores and activated oxygen) or physically (UV

irradiation) disinfected water to allow purification under controlled conditions (Lees, 2000; Richards, 1988; Son & Fleet, 1980). This process usually occurs in 2 days (Lees, 2000).

Purification processes are based on the assumption that if by filtering polluted water shellfish can become contaminated, they may also purge the contaminants by filtering clean water. Thus, microbial depuration decreases the risk for potential infections due to shellfish consumption. Temperature and salinity are two important parameters to consider in the purification process according to the type of shellfish. Variations in environmental requirements among bivalves may reflect shellfish adaptation to in situ conditions. Lowering the temperature may help in keeping bivalves alive longer and maintain lower bacterial concentrations, however, this would also extend the period of time required for effective depuration. Shellfish conditioning, that allows shellfish to acclimate to the temperature and salinity of the water, seems necessary to ensure

maximum depuration (Johnson & Hayasaka, 1988; Richards, 1988). Depuration has a great potential as a means of purging shellfish, at least partially, of microbiological contaminants. Nevertheless, more detailed studies are needed to determine the effect of physiologically parameters, such as food availability, temperature, salinity, dissolved oxygen and shellfish state. This would allow the development of an improved depuration method (Jones et al., 1991).

Relaying

Relaying consists of transferring contaminated harvested bivalves to cleaner areas allowing self-purification in the natural environment for longer periods, at least two months for category C shellfish, according to EU standards (Lees. 2000; Richards, 1988).

In contrast to depuration, where bivalves can only be held for a short period of time (maximum of 48 h), in the relaying method, molluscs can be kept for longer periods (at least two months) (Lees, 2000; Richards, 1988). Moreover, relaying has some drawbacks: lack or availability of acceptable sanitary shellfish growing waters, early harvesting from fishermen and economical considerations namely regarding ownership rights (Lees, 2000; Richards, 1988). In addition, bivalves are more susceptible to environmental disturbances that cannot be controlled such as temperature fluctuations, water movements (tides and storms) and weather (Lees, 2000; Richards, 1988; Son & Fleet, 1980). Smothering and clogging by sediments, physiological stress, shell damage and predation are very likely to occur during the relaying process (Richards, 1988). Furthermore, water quality of relaying areas is difficult to assure. The possibility of recontamination by seasonal variations of naturally occurring bacteria populations or transient pollution (due to heavy rains and associated land

runoff), may contaminate acceptable relay areas, leading to an ineffective microbial reduction (Croci et al., 2002; Ho & Tam, 2000; Lees, 2000; Richards, 1988).

Bivalves contamination and their risk as vehicles of disease

Contamination of bivalve shellfish occurs mainly because they are suspension feeders that selectively filter small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Burkhardt & Calci, 2000; Defossez & Hawkins, 1997; Dunphy et al., 2006; Lees, 2000). The hazards related to the contamination of bivalves by harmful microorganisms are due to their traditional cooking procedure which may not be enough to ensure consumer's safety. These circumstances make them an important vector of foodborne disease (Lees, 2000). The control of the disease risk associated with bivalves, thus, requires Hazard Analysis by Critical Control Point (HACCP) procedures

together with water environment quality management of growing and harvesting areas and post-harvest product processing which might involve depuration and/or heat treatment where appropriate (WHO, 2010), (Oliveira et al, 2011).

The true incidence of foodborne disease outbreaks is not known. Even though there are routine surveillance systems worldwide that compiles the existing information on foodborne diseases, the collected information varies widely between diseases and between countries, not allowing for the numerical comparison of data on foodborne disease (Oliveira et al., 2011). Shellfish were often implicated in disease but did not, as opposed to some other foods, result in death. Since meat (66 outbreaks; 3205 cases) and poultry (52 outbreaks; 1871 cases) are food products that are consumed in a much larger amount, when compared to seafood, the number of cases related to shellfish (47 outbreaks; 1868 cases) is rather alarming (Olsen et al., 2000). When compared to fish (140 outbreaks; 696

cases), molluscan shellfish caused double the number of cases even though being responsible for a much lower number of outbreaks (Huss, Ababouch, & Gram, 2004; Olsen et al., 2000). In the majority of food outbreaks (67.8%) the disease agent was not identified. In 44.7% of the outbreaks caused by shellfish, the etiological agent was identified and viruses were the most frequent causative agent (23.4%) (Olsen et al., 2000). The risk of disease or death due to contaminated shellfish consumption is inherent to all consumers but the risk increases in those that suffer from underlying health disorders and are exposed to the consumption of raw bivalves. Among the high-risk population are individuals with immunosuppressive disorders (cancer patients. AIDS), achlorhydria and epilepsy, patients with diabetes mellitus, liver and chronic kidney disease and steroid dependent patients (for treatment of asthma). Pregnancy, age and alcohol abuse are also factors that may enhance the development of seafood diseases (Butt, Aldridge & Sanders, 2004; Ripabelli et al., 1999). The risk of human

intoxications is linked to the ingestion of bivalves contaminated with chemicals and biotoxins. On the other hand. the risk of human infections is related to the ingestion of bivalves contaminated with protozoan parasites, viruses and bacteria. Chemical hazards (heavy metals. pesticides and drug-residues) are usually associated with aquaculture products or with bivalves caught from polluted waters but. in general, are uncommon in commercially harvested shellfish (Huss, Reilly & Karim Ben Embarek, 2000; Richards, 1988).

Biotoxins, produced by dinoflagellates and diatoms (domoic acid), on the other hand are a serious health problem. These toxins, usually linked to the unpredictable growth of those microalgae (microalgae blooms), are heat resistant which means that even well-cooked bivalves might still present a risk to consumer's safety. Accumulation of toxic marine algae in raw or light cooked shellfish has been associated to Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning

(NSP), Amnesic Shellfish Poisoning (ASP) and Azaspiracid Poisoning (AZP) occurrences (Botana, 2008; FAO, 2004; Hallegraeff, Anderson & Cembella, 2003; Huss et al., 2000). Microbial contamination is chronic and pervasive in harvesting areas. Furthermore. viruses and naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Croci et al.,2002; Huss et al., 2000; Lees, 2000; Wittman & Flick, 1995). Shellfish-derived illnesses can have natural causes due to contaminants that are available in the environment and consequently a part of the normal biota (Shumway & Rodrick, 2009). while others can be human-generated before or after shellfish harvesting. Pre-harvesting microbial contamination (occurring naturally or as a result of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss et al., 2000; Lees, 2000).

Regardless of the higher abundance of indigenous marine viruses, only viruses derived from anthropogenic contamination are associated with

illness in seafood consumers. Noroviruses, hepatitis A viruses, enteroviruses and adenoviruses are the viruses that are more often linked to shellfish contamination (Le Guyader, Atmar & Albert, 2007; Lees, 2000; Muniain-Mujika et al., 2003). Shellfish may also be contaminated post-harvesting. Potential hazard due to sub-lethally injured microbiota that may recover and multiply during later storage must be considered. Contaminant agents may also be introduced through cross contamination. recontamination or faulty handling and processing (Huss et al., 2000; Shumway & Rodrick, 2009). Viruses are frequently the cause of seafood-related infections, but hospitalizations and deaths are especially and generally related with bacteria (Butt et al., 2004).

Among the indigenous microbiota of coastal environments, the family *Vibrionaceae*, particularly *Vibrio parahaemolyticus, V. vulnificus* and *Vibrio cholera,* is targeted as a causative agent of human disease due to the consumption of shellfish (Butt et al., 2004; Hood & Ness, 1982; Normanno

et al., 2006; Ripabelli et al., 1999). These natural pathogens remain viable and cultivable in water, even in the absence of organic matter (Croci et al., 2002; Marino et al., 2005; Pruzzo, Gallo & Canesi, 2005).

Several reports of human disease caused by *Listeria spp*., namely listeriosis, were related to seafood consumption but inconsistent results were observed (probably as a consequence of distinct coast contamination or different efficiencies in the detection and quantification methods). Furthermore, the contamination source (marine environment and processing/handling) and the seasonal fluctuations of the occurrence of these bacteria were not investigated effectively (Butt et al., 2004; Monfort et al., 1998; Rodas-Suárez et al., 2006).

The presence of *Salmonella spp.* in seafood and water may cause salmonellosis, characterized by enteric (or typhoid) fever along with gastroenteritis, abdominal cramps and diarrhea (Brands et al., 2005). Salmonella enterica serovar *Enteritidis* and serovar *Typhimurium* are the

most common salmonella that cause infection and death (Butt et al., 2004). Enterotoxigenic *Escherichia coli, Campylobacter jejuni* and *Staphylococcus aureus* are also among bivalve bacterial contaminants and agents responsible for human disease (Brands et al., 2005; Butt et al., 2004).

In the process of filter-feeding. bivalve shellfish are likely to accumulate a diversity of microbiological contaminants (Burkhardt & Calci. 2000; Croci et al., 2002). Considering that fecal associated pathogens available in the marine environment accumulate in bivalves by filter-feeding. thus sewage contaminants may be recycled into the human community (Hernroth et al., 2002). This gains particular importance due to the fact that bivalves may have been exposed to persistent antibiotic residues and to multi-resistant pathogens as a result of an increased use of antibiotics by humans, in aquaculture and livestock. These multi-resistant pathogens may exist in the environment and may re-enter the food chain (Hektoen et al.,1995; Lees, 2000; Rodas-Suárez et al., 2006; Sapkota et al., 2008). Furthermore, a non-

culturable but viable and latent bacteria species of sanitary importance may be present in water besides the existence of various processes that control the levels of microorganisms in coastal marine environments (Troussellier et al., 1998).

CHAPTER 2

Vibrio *spp.*

Taxonomy

Since the original description of the family *Vibrionaceae* by Veron in 1965, which included a number of morphologic and phenotypically similar gram-negative bacteria primarily residing in acquatic habitats, the genus *Vibrio* has held a preeminent position within this group. Although vibrios were originally defined on the basis of biochemical characteristics, recent systematic studies have supported the taxonomic position of most members initially included in this group (Janda et al. 1988). The genus *Vibrio* has undergone numerous changes in recent years. with a number of less common species described or reclassified. The number of species described in *Vibrionaceae* has been increasing after the establishment of

the genome fingerprint technique (Thompson et al., 2001) and MLSA Thompson et al., 2005; Sawabe Kita-Tsukamoto & Thompson, 2007). Today, over 80 species are recognized in the genus *Vibrio* and its taxonomy is being continuously updated. Currently, there are several pathogenic *Vibrio* species implicated in human infection (Summer et al. 2008).

At present to date, the family *Vibrionaceae* contains seven genera with 128 species; the genera are *Vibrio* (89 spp.), *Photobacterium* (21 spp.), *Aliivibrio* (6 spp), *Salinvibrio* (6 spp.), *Enterovibrio* (4 spp.), *Grimontia* (1 sp.) and *Catenococcus* (1 spp.) (http://www.vibriobiology.net/).

Of particular interest is the so-called core group of *Vibrio* because several of the species are responsible of diseases in humans and marine organism. Members of core group are commonly isolated from tropical waters and rarely from colder environments (<20°C)(Gomez-Gil, Espinoza & Vora, 2011). *Vibrios* are an excellent test model for genomic taxonomy because they are ubiquitous in the marine environment, associated with a wide

range of marine life (some species such as *V. cholerae, V. parah*aemolyticus and *V. vulnificus* cause serious disease in man) and experiencing a variety of environmental conditions and selection forces. leading to high genomic plasticity (Hunt et al., 2008; Thompson et al., 2005) Consequently, differentiation of sister species becomes very difficult. For instance, *V. cholerae* and *V. mimicus* have nearly indistinguishable phenotypes. Among the phenotypic tests used in the Bergey's manual, only sucrose fermentation and lipase activity may discriminate the two species (Thompson et al., 2009). Strains of the same species (including the type strain) share more than 60% mutual AFLP band pattern similarity and more than 95% similarity in MLSA (using the loci *rpoA, recA, pyrH, ftsZ, topA, mreB gyrB* and *gapA*). Several *Vibrio* strains had the genome completely sequenced and are available on the web. Eleven *V. cholera* and two *V. mimicus* genomes formed an ideal test case for taxonomy because of their close relatedness as sister species. These sister species have nearly

identical 16S rRNA sequences and around 70% DDH (DNA-DNA hybridization) (Thompson et al., 2009).

General characteristic

The genus *Vibrio* comprises Gram-negative straight or curved rod-shaped 1.4-2.6 mm wide and motile by means of a single polar flagellum in liquid media. They do not form endospores or microcysts and are facultative anaerobes capable of both fermentative and respiratory metabolism. Most vibrios are oxidase-positive (the only exception is *V. metschnikoviic*). All of them utilize D-glucose as a sole or main source of carbon and energy (Farmer III & Hickman Brenner, 1992). Ammonium salts generally represent their source of nitrogen. Vibrios produce many extracellular enzymes including gelatinase, amylase, chitinase and DNase. The main pathogens, *V. cholera. V. parahaemolyticus and Vibrio vulnificus* decarboxylate lysine and ornithine, with no alkaline reaction from arginine. Most vibrios are

sensitive to the vibriostatic agent 0129 (2.4-diamino-6.7-di-isopropylpteridine) which is used as diagnostic test (Farmer III & Hickman Brenner, 1992). *Vibrios spp.* are mainly halophilic and sodium ions stimulate growth of all species and are an absolute requirement for most. The wide range of salt concentrations necessary for bacterial growth reflects the differing ability of species to live in aquatic environments with various salinities (Tantillo et al., 2004). Vibrios growth well at neutral and alkaline pH values up to pH 9.0; therefore the pH values of both selective and enrichment media are generally 8-8.8 and all species are acid-sensitive. However, since Vibrios are typically marine organisms, most species require 2-3% NaCl or a seawater base for optimal growth. In liquid media they are motile by polar flagella that are enclosed in a sheath continuous with the outer membrane of the cell wall. On solid media they may synthesize numerous lateral flagella which are not sheathed. (Denner et al., 2002; Todar, 2005).

Some species are bioluminescent and live in mutualistic associations with fish and other marine life. Other species are pathogenic for fish, eels and frogs, as well as other vertebrates and invertebrates (Eilers et al., 2000; Todar, 2005). In light of heightened human dependence on marine environments for fisheries, aquaculture, waste disposal and recreation, the potential for pathogen emergence from ocean ecosystems remains a cause for concern (Bahlaoui, Baleux & Trousselier, 1997; Kirn, Jude & Taylor, 2005). A surprising number of *Vibrio spp*. have been reported from marine environments, and the probability of their transmission to humans is correlated with factors that affect their distribution. Both indigenous and introduced pathogens may be a cause of illness acquired from marine environments and their occurrence depends on their ecology, source and survival (Thompson et al., 2004).

Epidemiological observations

To judge the risk from introduced pathogens. level of indicator organism are routinely monitored at coastal sites. However, methods targeting specific pathogens are increasingly being used and are the only way to judge or predict risk associated with the occurrence of indigenous pathogen populations (Igbinosa & Okoh, 2008). Otherwise, in Europe, both vibrios and also liver and enteric viruses were not included in Regulation (EC) n° 2073/2005 or as food safety criteria or as process hygiene criteria. Regulation (EC) n° 2073/2005 (updated by Regulation EC 1441/2007) highlights the need to develop reliable detection methods for pathogenic *Vibrio* strain.

Over the last 25 years epidemiological data for the United States reported that water contamination by micro-organisms of faecal origin were

responsible for only 4% of poisoning due to consumption of bivalve molluscs. while other types of microorganisms naturally present in marine waters, including *Vibrio spp*., were responsible for 20% of pathological episodes (Suffredini & Croci, 2001). Interest in the occurrence of potentially pathogenic vibrios is high from an epidemiological and ecological point of view.

Vibrios able to cause human disease include the cholera toxin-producing strains of *V. Cholera,* responsible for epidemic/pandemic cholera and thermostable direc hemolysin-producing strains of *V. parahaemolyticus* a leading cause of gastroenteritis and *V. Vulnificus,* one of the most invasive and rapidly lethal human pathogens. *V. vulnificus* can infect the bloodstream, causing a severe and life-threatening illness characterized by fever and chills. decreased blood pressure (septic shock) and blistering skin lesions (F. L. Thompson & Klose 2006).

The transmission of *V. cholera* strains from their environmental reservoir to humans through water sources or seafood has been demonstrated. Other non-epidemic *Vibrio* species, including *V. parahaemolyticus* and *V. Vulnificus,* are usually associated with consumption of raw or undercooked shellfish and seafood or exposure of skin wounds to seawater (Todar, 2005).

In Europe, the occurrence of pathogenic vibrios in the marine environment has been well documented by several authors (Harvell et al., 2002; Hervio-Heath et al., 2002).

In Italy, the occurrence of potentially pathogenic vibrios in aquatic environments such as rivers (Cavallo & Stabili. 2002; Stabili et al., 2000). brackish (Maugeri, Caccamo & Gugliandolo, 2000) estuarine (Barbieri et al., 1999) and costal marine sites (Cavallo & Stabili, 2002; Hervio-Heath et al., 2002) has been reported.

In the United States infections (septicaemia, wound infection, gastroenteritis) occurs in the warmer month from May to October and are associated with raw oyster consumption (Hlady & Mullen 1993). In the Gulf of Mexico states there is an annual incidence for infection of at least 0.6 per million population, and a case-fatality rate of about 20% (Levine & Griffin. 1993).

Nonfoodborne *Vibrio* infections (NFVIs) accounted for 1210 (25%) of 4754 reported Vibrio species–related illnesses*. V. vulnificus* was the species most frequently isolated from patients with NFVIs (428 cases), followed by *Vibrio alginolyticus* (356 cases) and *Vibrio parahemolyticus* (232 cases). The remaining 194 NFVIs (16%) were caused by other species, including *V. cholerae* non-01 (58 cases), *Vibrio fluvialis* (37), *Vibrio damsela* (24), *Vibrio furnissii* (1), *Vibrio hollisae* (4), *Vibrio metschnikovii* (1), *Vibrio mimicus* (11), *V. cholerae* 01 (1). *Vibrio* species that were not identified (53), and other *Vibrio* species (4). NFVIs accounted for 45% of *V. vulnificus* infections, 81%

of *V. alginolyticus* infections, 11% of *V. parahaemolyticus* infections, and 16% of infections due to other *Vibrio* species.

Rates of uptake and elimination of bacteria are species and temperature dependent and the kind of microbial population depends on the microflora present in the environment. Molluscs that grow close to human settlements have higher microbial loads than those who grow up in more isolated areas.

Vibrio in bivalves shellfish molluscs

The aquatic environment harbours a rich bacterial flora and bivalves because of their efficient filter-feeding mechanism, can ingest many different kinds of microorganisms. In some cases bivalves can act as passive carriers of human pathogens, without themselves contracting a bacterial disease. Estuarine bacteria of the genus *Vibrio* are also potentially

pathogenic to humans. The most important of these are *V. Cholera, V. parahaemolyticus* and *V. vulnificus*.

V. parahaemolyticus is a normal constituent of the inshore marine flora but its abundance is increased through organic enrichment of coastal and estuarine areas, where bivalve-growing areas are concentrated.

Other important *Vibrio* is *Vibrio alginolyticus*, considered to be a part of normal marine flora (Austin et al., 1995; Vandenberghe et al., 1998; Xie et al., 2005).

CHAPTER 3

V. parahaemolyticus

V. parahaemolyticus is a Gram-negative, halophilic, mesophilic, small rod that may have a single curve to its shape (Su & Liu., 2007; Daniels et al., 2000). It exists as either a swimmer cell with a single polar flagellum, or a swarmer cell covered in lateral flagella (McCarter, 1999). Depending on environmental conditions, *V. parahaemolyticus* can produce a capsule, with over 70 different K antigens detected in various strains (Nair et al., 2007). *V. parahaemolyticus* is found free living in brackish and estuarine waters, and requires salinity for survival (Baffone et al., 2006). In winter

months when water temperatures are unfavorable, *V. parahaemolyticus* may be undetectable. It has been proposed that the organism survives in marine sediment, and is reintroduced to the water column when temperatures rise (Su & Liu., 2007). Infaunal burrows have been demonstrated to contain high levels of *V. Parahaemolyticus*, likely acting as a source for these organisms to be distributed through an estuary environment during favorable conditions (Gamble & Lovell. 2011). In locations where water temperatures do not drop below 15°C. *V. parahaemolyticus* may be detected year round (Su & Liu., 2007) with the number of organisms detected in water, sediment and oysters increasing as water temperatures rise (Johnson et al., 2010). Due to filter feeding, oysters may have a concentration of *V. parahaemolyticus* up to 100-fold higher than surrounding waters. Up to 100% of oysters may be contaminated with *V. parahaemolyticus* and/or *Vibrio vulnificus* during summer months, which increases the chances of infection (Morris., 2003).

V. parahaemolyticus is disseminated throughout the world, and recently, due to warming ocean temperatures, has been detected in coastal waters as far north as the southern coast of Alaska (Daniels., 2003; Vasconcelos, Stang & Laidlaw, 1975; McLaughlin et al., 2005). In Japan. *V. parahaemolyticus* accounts for 20 and 30% of all food poisoning cases and is the leading cause of foodborne illness (Yeung & Boor., 2004). The high rate of infection is attributed to the overall high seafood diet as well as the common practice of eating seafood raw. Gastroenteritis caused by *V. parahaemolyticus* is generally associated with the consumption of raw or undercooked seafood including fish, crab and other crustaceans, and mollusks. Since *V. parahaemolyticus* is sensitive to heat, cooking generally kills the bacterium rendering contaminated food safe to eat. In the United States, the first case of gastroenteritis caused by *V. parahaemolyticus* occurred in 1971 in Maryland and was associated with contaminated crabmeat. *V. parahaemolyticus* is the primary cause of US *Vibrio*-associated

foodborne illness. The majority of these infections are a result of consuming Vibrio-contaminated raw oysters (Yeung & Boor., 2004). An increase in non-cholera *Vibrio* infections that began in the mid-1990s has been associated with the detection of a new clonal group that includes three new serotypes: O3:K6. O4:K68. and O1:K untypeable (Morris, 2003). Since 1996, the most common serotype of *V. parahaemolyticus* has been O3:K6. While non-pandemic variants for this serotype were detected in Japan as early as 1983, the first reported illness caused by this strain was first observed in a Japanese traveler returning from Indonesia in 1995. An outbreak of diarrheal disease then occurred in Calcutta, India in February of 1996 with 50-80% of the isolates confirmed as O3:K6 [7].

V. parahaemolyticus has now been detected in North and South America, Europe, Africa, and Asia with outbreaks of disease occurring worldwide (Nair et al., 2007).

To date, at least 12 pathogenic serotypes of *V. parahaemolyticus* have been identified [14]. Many strains have been sequenced including the O3:K6 serotype strains RimD 2210633 and AQ3810; the O4:K12 strain Vp10329; and the O4:K68 serotype strains AN-5034. K5030 and Peru-466 (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) (Gonzalez-Escalona et al., 2011; Boyd et al., 2008). Numerous clinical and environmental strains without a sequence have been utilized to understand the biology of *V. parahaemolyticus* (Bej et al., 1999; Yeung & Boor, 2004; Zhou, Konkel & Call., 2009). The RimD 2210633 strain was the first fully sequenced and annotated genome (Makino et al., 2003) and is therefore the focus of most studies.

Disease

Infection with *V. parahaemolyticus* can cause three distinct medical conditions: gastroenteritis, wound infections, and septicemia. Acute

gastroenteritis presents with abdominal cramping, diarrhea, nausea, vomiting, low-grade fever, headache and occasional bloody diarrhea different than that seen in other enteric infections. Infection occurs 4-96 h after consumption of contaminated food, and lasts up to three days. The illness is self-resolving in immunocompetent individuals and can be sufficiently treated with oral rehydration alone (Yeung &. Boor, 2004; Nair, 2007). Wound infection is common among fishermen and is generally acquired when small wounds occur in or around seawater (Hlady & Klontz, 1996) This form of *V. parahaemolyticus* infection is sometimes limited to cellulitis, but may progress to necrotizing fasciitis, an uncommon infection of soft tissues characterized by a rapid spread of bacteria with associated inflammation and necrosis of tissues. Septicemia occurs when *V. parahaemolyticus* enters the blood stream of the patient and is disseminated throughout the body. Systemic immune activation leads to inflammation and increased vascular permeability. This can result in

hypovolemic shock, multisystem organ failure and death. The subpopulation of patients most at risk for septicemia includes those with underlying medical conditions including liver disease, diabetes, cancer, and recent gastric surgery (Yeung & Boor, 2004). Immunocompromised individuals and those with liver failure due to liver cirrhosis or hepatitis virus infection seem to be at greatest risk of septicemia (Hlady & Klontz, 1996; Blake et al., 1979).

Cellular factors associated with *V. parahaemolyticus* pathogenicity

Genomic analysis has demonstrated that a common Vibrio progenitor gave rise to *V. parahaemolyticus, Vibrio cholerae,* and the other Vibrio species. The acquisition of a Type III Secretion System (T3SS) similar to that found in Yersinia species and herein referred to as T3SS1, was the basis of a *V. parahaemolyticus* ancestor. The acquisition by some strains of a second Type III Secretion System (T3SS2), and thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH) genes has led to a number of *V.*

parahaemolyticus species with varying degrees of pathogenicity (Broberg. Calde & Orth, 2011). In addition to Type III Secretion and TDH genes, *V. parahaemolyticus* possesses flagella for swimming and swarming, as well as the ability to produce a capsule. These are both factors that likely aid in environmental survival as well as colonization of the human host.

V. parahaemolyticus possesses two different types of flagella with distinct functions. A polar flagellum is constitutively expressed and is used for swimming. The whip of the flagellum is made of six different flagellin proteins and is sheathed, which may aid in attachment. The energy to rotate this flagellum is provided by a sodium motive force, which is advantageous in salt water with an average pH of 8 *V. parahaemolyticus* is capable of swimming at speeds up to 60 mm/s when expressing this single flagellum (McCarter, 1999). A decrease in flagellar rotation speed as a consequence of increased viscosity of the growth environment, or growth under iron-limiting conditions, induces a switch to a swarmer cell type. This

entails the production of a number of nonsheathed peritrichous flagella which allows the bacteria to swarm over solid or semi-solid substrates. These flagella are different than the single polar flagellum in that they are unsheathed, made from a single flagellin protein, and are powered by the proton motive force (McCarter, 1999).

The Type III secretion systems of *V. parahaemolyticus*

The T3SS is a bacterial organelle evolved to deliver proteins, termed effectors, directly into the cytoplasm of a eukaryotic cell (Galan & Wolf-Watz, 2006). Made up of 20-30 proteins, the secretion apparatus consists of a basal body that spans both the inner and outer bacterial membranes, a needle that acts as a conduit between the bacterial and eukaryotic cells, and a translocon pore that is inserted into the eukaryotic cell membrane (Izore, Job & Dessen, 2011). Structurally, the apparatus bears resemblance to a flagellar system from which it may have evolved. Some secretion

apparatus proteins have homology to flagellar export proteins, with core transmembrane proteins showing the highest level of conservation (Marlovits & Stebbins, 2010). The T3SS allows bacteria to translocate effectors from their cytoplasm directly into the host cytoplasm, or to the cytoplasmic face of the host cell membrane without release of the effectors to the extracellular space (Cornelis, 2006). The specific complement of effectors within a pathogen determines not only its lifestyle, but also the disease that it causes. Translocation occurs in an ATP-dependent manner. Effectors. which are generally bound to chaperones in a quiescent, partially folded state. are unfolded and threaded through the needle. The chaperone remains in the bacterial cytoplasm (Galan & Wolf-Watz, 2006; Akeda & Galan, 2005; Arnold, Jehl & Rattei, 2010). Upon entering the host cell cytoplasm, the effectors refold into an active state where they manipulate eukaryotic signaling cascades to facilitate infection and distrupt the host immune response (Galan, 2009).

Adhesion to host cells

During infectionk a variety of bacterial adhesion factors are expressed to provide the contact with the host cell necessary for secretion of effector and toxin proteins. In a recent studyk an outer membrane protein from *V. parahaemolyticus* was described that was necessary and sufficient for initial contact of this pathogen with multiple cultured host cell lines. This multivalent adhesion molecule (MAM) consists of six (MAM6) or seven (MAM7) mammalian cell entry (mce) domains. and has homologs encoded in a number of Gram-negative animal pathogens. MAM7 bound to both fibronectin and phosphatidic acidk and blocking the binding of either of

these substrates could prevent adhesion of MAM7 to host cells. Furthermorek MAM7 was necessary for attachment early in the infection, as well as for T3SS-mediated cell death in some cell types (Broberg et al., 2011).

All *V. parahaemolyticus* strains isolated from clinical samples possess b-hemolytic activity attributed to TDH or TRH. These isolates are able to lyse human erythrocytes when plated on a high-salt media called Wagatsuma agar, a process termed the Kanagawa phenomenon (KP) (Nishibuchi & Kaper, 1995). The KP test is commonly used to identify pathogenic *V. parahaemolyticus* in seafood as well as patient samples. The reproducibility of the KP test is dependent on pH. media salinity, and erythrocyte type. Identification of the *tdh* gene in samples has been shown to more accurately predict virulence, as it is a genetic test rather than a phenotypic test (Hongping et al., 2011) the *tdh* gene is encoded and coregulated with T3SS2 genes (Hongping et al., 2011), The identification of TDH may actually

serve to identify *V. parahaemolyticus* strains with T3SS2, the expression of which may be a significant factor in determining if a reservoir can cause pandemic outbreaks of disease. TDH is a reversible amyloid toxin (Fukui et al., 2005) that has been shown to associate with cholesterol and sphingolipid-enriched lipid rafts. Distruption of these lipid microdomains abrogated cytotoxicity in nucleated cells, but not hemolytic activity against erythrocytes, indicating two potential activities for this toxin [36]. The x-ray crystallographic structure of TDH showed that this protein forms a homotetramer with a central pore 23 A° in diameter. This relatively large pore size helps explain previously observed low ion selectivity, allowing both water and ions to flow through a membrane with little impedance (Matsuda et al., 2010). This alteration in ion flux from affected cells in the intestine may be the mechanism for the diarrhea observed during infection (Honda et al.,1992). TDH was considered to be a major virulence factor for *V. parahaemolyticus* pathogenesis. TRH is predicted to act in a similar

manner to lyse cells based upon high sequence homology (68%) between the *trh* and *tdh* genes.

Another toxin, thermolabile hemolysin (TLH), is found in all strains of *V. parahaemolyticus*, but very little is known about its function. The *tlh* gene was found to be strongly unregulated in a genomic screen performed under conditions meant to mimic the intestinal environment of the human host (Chao et al., 2010). Therefore, this gene may be equally important as the *tdh* and *trh* genes in the process of human infection.

Occurrence of Vibrio parahaemolyticus

Distribution in marine environments

The ubiquitous nature of *Vibrio* species in marine and estuarine environments makes it impossible to obtain seafood free of theses bacteria. *Vibrio* is related to seasonal growth and decline coupled to special

ecologic relationship with higher copepods and other plankton. The vibrios also may be required for a balanced ecosystem in the marine environment and their association with higher organism may provide a beneficial effect on salt retention by copepods and other organism which play vital roles in the food chain (Colwell et al., 1981). When present in any water habitat, vibrios may constitute a major part of the aquatic bacterial flora, *V. parahaemolyticus* is a halophilic vibrios and on the other hand, require Na+ for growth and can reach very high concentrations in waters of 5 to 8‰ salinity.

The distribution of *V. parahaemolyticus* in the marine environments is known to relate also to the water temperatures. Studies have shown that the organism was rarely detected in seawater until water temperatures rose to 15°C or higher. Ecological study of *V. parahaemolyticus* in the Chesapeake Bay of Maryland found that *V. parahaemolyticus* survived in sediment during the winter and was released from sediment into water

column when water temperatures rose to 14°C in late spring or early summer (Kaneko & Colwell. 1973) Another survey of nine U.S. coastal states conducted between 1984 and 1985 reported an average low density of *V. parahaemolyticus* (4 cell/100 ml) in seawater when water temperatures dropped below 16°C (DePaola et al., 1990). However, the densities of *V. parahaemolyticus* in seawater could increase to 1000 cell/100 ml in the water when water temperatures increased to around 25°C (DePaola et al., 1990; Kaneko & Colwell, 1973). A study of occurrence of *V. parahaemolyticus* in Oregon oyster-growing environments between November 2002 and October 2003 also found a positive correlation between *V. parahaemolyticus* in seawater and water temperatures with the highest populations of *V. parahaemolyticus* in water being detected in the summer months (Duan and Su, 2005).

Prevalence in shellfish

The degree of *V. parahaemolyticus* contamination in raw shellfish is also known to relate to the water temperatures. Therefore, it is more likely to detect *V. parahaemolyticus* in molluscs harvested in the spring and the summer than in the winter (Su and Liu, 2007). Although the density of *V. parahaemolyticus* in contaminated oysters is usually lower than 103 cfu/g at harvest (Kaysner and DePaola, 2000). it could exceed 103 cfu/g in oysters harvested from warmer seawater (DePaola et al., 2000) and the organism can multiply rapidly in oysters upon exposure of elevated temperatures. Studies have shown that populations of *V. parahaemolyticus* in unrefrigerated oysters could increase rapidly to 50–790 folds of its original level within 24 h of harvest if oysters were exposed to 26 1C (Gooch et al., 2002). A survey of 370 lots of oysters sampled from restaurants, oyster bars. retail and wholesale seafood markets throughout the US between June 1998 and July 1999 found a seasonal distribution of *V.*

parahaemolyticus in market oysters with high densities (some exceeded 1000MPN/g) being detected in the summer months (Cook et al., 2002).

Incidence of Vibrio parahaemolyticus food poisoning

Incidence in the world

V. parahaemolyticus was first recognized as a cause of food-borne illness in Osaka, Japan in 1951 (Daniels et al., 2000b). It caused a major outbreak of 272 illnesses and 20 deaths associated with consumption of sardines. Sincethen, *V. parahaemolyticus* has been reported to account for 20‑30% of food poisoning cases in Japan (Alam et al., 2002) and identified as a common cause of seafood-borne illness in many Asian countries (Chen et al., 1991; Deepanjali et al., 2005; Wong et al., 2000). *V. parahaemolyticus* was the leading cause of food poisoning (1710 incidents, 24.373 cases) in Japan between 1996 and 1998 (IDSC. 1999) and accounted for 69% (1028 cases) of total bacterial foodborne outbreaks (1495 cases) reported in

Taiwan between 1981 and 2003 and 31.1% of 5770 foodborne outbreaks occurred in China between 1991 and 2001 (Liu et al., 2004).

V. parahaemolyticus was first identified as an etiological agent in the US in 1971 after three outbreaks of 425 cases of gastroenteritis associated with consumption of improperly cooked crabs occurred in Maryland (Molenda et al., 1972). Since then. sporadic outbreaks of *V. parahaemolyticus* infections related to consumption of raw shellfish or cooked seafood were reported throughout the US coastal regions. Between 1973 and 1998, approximately 40 outbreaks of *V. parahaemolyticus* infections were reported to the Centers for Disease Control and Prevention (CDC) (Daniels et al., 2000a). Among them, four major outbreaks involving more than 700 cases of illness associated with raw oyster consumption occurred in the Gulf Coast. Pacific Northwest. and Atlantic Northeast regions between 1997 and 1998. In the summer of 1997, 209 cases (including one death) of *V. parahaemolyticus* infections associated with raw oyster consumption

occurred in the Pacific Northwest (Oregon, Washington, California and British Columbia of Canada) (CDC, 1998). In 1998, two outbreaks occurred in Washington (43 cases) and Texas (416 cases) were associated with consumption of raw oyster (DePaola et al., 2000). In addition, a small outbreak of eight cases of *V. parahaemolyticus* infections was reported in Connecticut, New Jersey, and New York between July and September in 1998 as a result of eating oysters and clams harvested at Long Island Sound of New York (CDC, 1999). Recently, 14 passengers on board a cruise ship in Alaska developed gastroenteritis after eating raw oysters produced in Alaska in the summer of 2004 (McLaughlin et al., 2005). More recently, an outbreak of *V. parahaemolyticus* involving 177 cases occurred in the summer of 2006 was linked to contaminated oysters harvested in Washington and British Columbia (CDC, 2006). The occurrence of these outbreaks indicates that contamination of *V. parahaemolyticus* in oysters is a safety concern in the US.

V. parahaemolyticus infections are rarely reported in European countries. However, sporadic outbreaks have been reported in countries such as Spain and France. Eight cases of *V. parahaemolyticus* gastroenteritis related to fish and shellfish consumption were reported in Spain in 1989 (Molero et al., 1989). An outbreak of 64 illnesses associated with raw oysters consumption occurred in 1999 in Galicia. Spain (Lozano-Leo´ n et al., 2003). A serious outbreak affecting 44 patients associated with consumption of shrimps imported from Asia occurred in France in 1997 (Robert-Pillot et al., 2004). A more recent outbreak involving 80 illnesses of *V. parahaemolyticus* infection among guests attending weddings in one restaurant was reported in Spain in July 2004 (Martinez-Urtaza et al., 2005). Epidemiological investigation associated the outbreak with consumption of boiled crab that had been processed under unhealthy conditions.

Incidence in Italy

In Italy was reported epidemiological information about a pandemic *V. parahaemolyticus* O3:K6 strain isolated from a stool sample of a diarrheal patient hospitalized in central Italy in the summer of 2007 (Ottaviani et al. 2008). The patient had eaten fresh shellfish, bought from a local seller, 24 h before hospitalization.

V. parahaemolyticus infections have been rarely reported in Europe in the past decades (Lemoine et al., 1999; Lozano-Leòn et al. 2003). Based on this, such microorganism is not included in the European Network for Epidemiologic Surveillance and Control of Communicable Diseases and in the Microbiological Surveillance System for Infectious Gastroenteritis. However, since 2001, pandemic *V. parahaemolyticus* O3:K6 has been detected from clinical samples in France, Russia, Spain (Nair et al., 2007) and Italy (Ottaviani et al., 2008). Recently, Ottaviani isolated toxigenic *V. parahaemolyticus* strains, serotyped as O1:K untypeable, from local shellfish in central Italy. Moreover, in May 2007, a *V. parahaemolyticus*

strain with pandemic potential was detected in a seawater sample from the northern Adriatic Sea (Italy) (Caburlotto et al., 2008). All this evidence should prompt official veterinary and health authorities to pay more attention to the epidemiological roles of environmental microorganisms in local food-borne diseases and to increase the microbia surveillance of pathogenic *V. parahaemolyticus* strains isolated from environmental, seafood, and clinical sources (EFSA, 2010).

CHAPTER 4

Methods for detection, isolation and identification of *Vibrio parahaemolyticus*

The laboratory identification procedures

The laboratory identification procedures discussed will be limited to the *Vibrio spp.* which have been implicated as a cause of human disease or have been isolated from clinical specimens. Members of the genus *Vibrio* are fermentative, facultative anaerobic, gram-negative, straight or curved

motile rods whose growth is stimulated by Na^+ which, except for *V. cholerae* and *V. mimicus,* is an absolute requirement (Baumann et al. 1984). They are anaerogenic (exception, *V. furnissii* and some strains of *V. damsela*) and, except for *V. metschnikovii*, oxidase positive, and they reduce nitrate to nitrite. Most are susceptible to the O/129 vibriostatic compound (Baumann et al. 1984). Certain key characteristics that aid in the separation of *Vibrio spp*. from other medically significant bacteria with which they may occasionally be confused (i.e., *Enterobacteriaceae, Pseudomonas, Aeromonas,* and *Plesiomonas spp.*) are the production of oxidase, fermentative metabolism, requirement of NaCl for growth, and susceptibility to 0/129. The positive oxidase reaction differentiated them from members of the *Enterobacteriaceae,* which are oxidase negative. Their fermentative metabolism distinguishes them from the oxidase-positive *Pseudomonas spp*., which are oxidative. The oxidase-positive *Aeromonas* and *Plesiomonas spp*. do not require NaCl for growth.

Aeromonas spp. are resistant to 0/129 and *Plesiomonas* strains vary in their susceptibility to this compound. The techniques and media available routinely in most clinical laboratories can be used to isolate the potentially pathogenic *Vibrio spp.* However, the recognition that an isolate is a vibrio and its identification to species may be more difficult.

Selective Media

Of the many selective media developed for the isolation of vibrios, thiosulfatecitrate-bile salts agar (TCBS) agar is the most convenient and widely used, highly selective medium (Rennels et al., 1980). It is suitable for most of the enteropathogenic vibrios; however, *V. hollisae* may grow poorly or not at all. It is available commercially and easy to prepare. and it differentiates sucrose-fermenting (yellow) from non-sucrose-fermenting (blue-green) colonies; other organisms are almost completely inhibited.

Commercially available dehydrated TCBS agar should be prepared carefully and not overheated. Pathogenic *Vibrio spp*. usually grow on MacConkey agar and will be colorless except for the lactose-fermenting *V. vulnificus.* They generally do not grow on the other enteric selective plating media Moreover, for isolation and detection of *V.parahaemolyticus, V.vulnificus* and *V.cholerae,* recently different study demonstrated efficacy of ChromAgar Vibrio medium (France). Typical appearance of microorganisms colonies is mauve for *V. parahaemolyticus*, green blue to turquoise blu for *V.vulnificus* and *V.cholerae*, while *V.alginolyticus* colonies are colourless. There are several commercial media which may be used for the isolation of vibrios, but tryptone soy agar (Oxoid or Difco) supplemented with 1 to 2% NaCl and marine agar (Difco) generally allow the growth of very healthy colonies after 1 to 2 days of incubation at 28°C.

Vibrios grow well at temperatures between 15 and 30°C, depending on the strain under analysis. Obviously, psychrophilic vibrios, i.e*., V. Logei, V.*

Wodanis, and *V. Salmonicida,* grow poorly at temperatures higher than 20°C. It is recommended to grow strains of these species at 15°C in Luria-Bertani broth (Difco) supplemented with 1 to 3% NaCl.

Modified Colistin-polymyxin B-cellobiose (mCPC) agar was employed for the ability to select and differentiate *Vibrio vulnificus* from background vibrios.

Most vibrios (except *V. Ezurae, V. Gallicus, V. Pectenicida, V.penaeicida, V. salmonicida* and *V. tapetis*) withstand the freeze drying process very well. Coincidentally, these species are also difficult to grow on any culture media. Ampoules containing freeze-dried cultures prepared nearly 30 years ago have yielded viable and healthy colonies on tryptone soy agar. Normally, these ampoules are filled with 0.01 g of bacterial culture previously suspended in 0.5 ml of cryoprotectant mix (horse serum–D-glucose–nutrient broth–MilliQ water. 3:0.3:0.3:1). Alternatively, strains

may be kept viable in Microbank vials, which contain 10% glycerol and porous beads, at -80°C for at least 5 years.

Enrichment Media

Enrichment broth is recommended for isolation of vibrios. Alkaline peptone water with 1% NaCl (pH 8.5) can be used for isolation of vibrios since they grow very rapidly at alkaline pH. The broth should be subcultured to TCBS after 5 to 6 h at 35°C; longer incubation allows for overgrowth of vibrios by other organisms. Longer incubation (18 to 20 h) can be used if the broth is incubated at lower temperatures (18 to 22°C) (Lee et al., 1981).

Gram Stain

All vibrios are short, gram-negative, straight or curved rods. Curvature is not diagnostic, since it is not always obvious in Gram stains, and other

organisms may also show curvature. The microscopic appearance is influenced by the medium on which the organism is grown. Many vibrios are highly pleomorphic and may show involuted forms. particularly when growth conditions are suboptimal.

Biochemical Characteristics

Media commonly used for identification of members of the *Enterobacteriaceae* can be used if the NaCl concentration is increased to 1% (wt/vol). Halophilic vibrios will not grow, or will grow poorly, in Voges-Proskauer, nitrate. 1% peptone, and Moeller decarboxylase broths without added salt. Growth of these species may occur in other media because of the minimal amounts (usually 0.5%) of salt present, but biochemical reactions may not be reliable. The oxidase test must be performed on growth from media containing no fermentable carbohydrates. Susceptibility to the vibriostatic agent should be performed on media low

in salt. It has been shown by Merkel (1972) that both NaCl and $MgCl_2$ block the vibriostatic action of 0/129. The methods used for identification of the clinically significant *Vibrio spp*. have been described in detail by Farmer et al. (1985) and Furniss et al. (1978). The lysine and ornithine decarboxylase and arginine dihydrolase activities of the pathogenic vibrios are useful for separating them into groups, *V. cholerae, V. mimicus, V. parahaemolyticus, V. vulnificus, V. cincinnatiensis.* and *V. alginolyticus* are lysine decarboxylase positive and arginine dihydrolase negative, *V. fluvialis. V. furnissii,* and *V. damsel* are arginine dihydrolase positive. *V. hollisae* is negative for lysine and ornithine decarboxylases and arginine dihydrolase. The lack of oxidase and nitrate activity distinguishes *V. metschnikovii* from the other pathogenic species. *V. cholera* and *V. mimicus* are characterized by their ability to grow in nutrient broth without added NaCl. These two biochemically similar species can be differentiated by sucrose fermentation; *V. cholerae* is positive, while *V. mimicus* is negative. The

classical and El Tor biovars of *V. cholerae* 01 can be distinguished by the Voges-Proskauer reaction, hemolysis of sheep erythrocytes, susceptibility to polymyxin B, agglutination of chicken erythrocytes, and lysis by bacteriophage. Production of gas from carbohydrates by *V. furnissii* (previously designated as an aerogenic biovar of *V. fluvialis*) will distinguish it from *V. fluvialis, Aeromonas sp*., frequently confused with *V. fluvialis,* will grow in nutrient broth without NaCl, while *V. fluvialis* will not. Many of the *V. parahaemolyticus* strains isolated in recent years have been urease positive. Most strains of *V. parahaemolyticus* isolated from humans are Kanagawa positive, while those from the environment are usually negative (Joseph et al., 1982). The Kanagawa test is based on the detection of a heat-stable hemolysin in a special medium (Wagatsuma agar) which contains human erythrocytes (Miyamoto et al., 1969).

The rapid fermentation of lactose and salicin aids in the recognition of *V. vulnificus*. Additional reactions in selected tests that differentiate 11 *Vibrio*

spp. as well *as Aeromonas* and *Plesiomonas spp*. are given inTable 1 (Janda et al., 1988).

The API 20E and 20NE diagnostic strip has been used successfully for identification and confirmation of isolates.

All strains of *V. cholerae,* both 01 and non-O1, share a common flagellar (H) antigen. In 1935, Gardner & Venkatraman divided the cholera vibrios into six subgroups on the basis of their somatic (0) antigens. The cholera strains were assigned to O-subgroup 1, and the noncholeragenic vibrios were placed in groups 2 to 6 (Janda et al.,,1988). Serotyping of suspect *V. cholera* cultures passing the string test using somatic or O antigens gives important epidemiological evidence. Two major serotypes of serogroup O1, Ogawa and Inaba, and serogroup O139 are recognized as human pathogens. The

two serotypes of O1 are seen in both the classical *V. cholera* and the El Tor biotypes. The O139 serogroup resembles only the El Tor biotype.

Although the Classical biotype is rarely encountered. the following are optional tests to differentiate them from the El Tor biotype:

- ✓ Beta-hemolysis. The most common means of differentiating the biotypes of O1 *V. cholerae,* and perhaps the easiest, is to determine β-hemolytic ability on sheep blood agar. El Tor strains are β-hemolytic, while classical strains do not produce a hemolysin. Inoculate a blood agar plate with test cultures by spotting to the surface and incubate 18-24 h at 35° ±2°C. Beta-hemolysin can be determined by a clear zone around the growth of the culture.
- ✓ Polymyxin-B sensitivity. Streak a suspect culture to a dry T1N1 agar plate and place a 50 unit disc of polymyxin-B on the surface. Invert the plate. incubate overnight at 35° ±2°C, and record the result. Classical strains are sensitive (>12 mm zone); El Tor strains are

resistant. If the suspect culture grows on mCPC agar, which contains polymyxin B, it is considered to be of the El Tor biotype (Anon.)

V. parahaemolyticus possesses three antigenic components: H. O, and K. The H antigen is common to all strains of *V. parahaemolyticus* and is of little value in serotyping. The K, or capsular antigen, may be removed from the bacterial body by heating the isolate for 1 or 2 hr at 100°C. This process exposes the O, or somatic, antigen, which is thermostable. Since the K antigen masks the O antigen, it is necessary to remove the former by heating before performing the O agglutination tests. There are 12 O group and over 70 known K antigens (Fujino, Sakazaki & Tamur., 1974). Five of the K antigens have been found to occur with either of two O group antigens; therefore, there are 76 recognized serotypes. Serologic tests by themselves are not used to identify *V. parahaemolyticus* because of cross-reactions with many other marine organisms. However, during

investigations of foodborne outbreaks, serologic tests become a valuable epidemiologic tool (FDA,Anon.).

Except for studies of Shimada and Sakazaki (Shimada & Sakazaki., 1983; Shimada & Sakazaki., 1984) on *V. fluvialis* and *V. vulnificus*, little is known about the antigenic structure of other species of Vibrio.

Genotypic identification

An array of phenotypic and genomic techniques have become available for the identification of vibrios in the last three decades (Dijkshoorn, Towner & Struelens, 2001; Gurtler & Mayall., 2001; Olive & Bean., 1999; Rademaker,Louws &, de Brujin., 1998; Rademaker et al., 2000; Savelkoul et al., 1999; van Belkum et al., 2001; Vandamme et al., 1996). Genotypic detection of the cholera toxin gene by polymerase chain reaction (PCR) provides the search of the CT gene may be present in strains of *V. cholera* and *V. mimicus*, but not expressed under experimental conditions. Thus a genotypic assay such as PCR amplification of the *ctx* gene is recommended.

This procedure offers a more rapid result and is less complicated than phenotypic assays.

The correlation has been well established that *V. parahaemolyticus* strains that cause illness in humans are almost always Kanagawa-positive. The Kanagawa test, or hybridization with the *tdh* gene probe provides reliable information on the presence of pathogenic strains isolated from foods. Due to the difficulty of obtaining fresh blood and the strong correlation between Kanagawa phenomenon and presence of the *tdh* gene, it is recommended to use DNA probe methods to determine potential virulence of *V. parahaemolyticus* isolates instead of the Kanagawa phenomenon.

Alkaline phosphatase- and digoxigenin-labeled DNA probes can be used for the identification of *V. parahaemolyticus*. A thermolabile hemolysin gene, *tlh,* has been found in all strains of *V. parahaemolyticus,* but not in other species and DNA probes have been used for identification. Two DNA probe procedures that have been shown to be equivalent are presented. DNA

probes have also been constructed to detect the thermostable direct hemolysin, *tdh* and thermostable related hemolysin, *trh* genes that are associated with pathogenic strains. Digoxigenin-labeled probes for *tlh* and *trh* were constructed of PCR amplification products using the primer sets reported by Bej et al. (1999). The *tdh* probe was constructed using a primer set based on the oligonucleotide probes of Nishibuchi et al. (1993), using *tdh1* as the forward primer and the reverse compliment of *tdh4 (tdh4c)* as the reverse primer. These amplicons are of the following sizes; 450 bp *tlh*, 424 bp *tdh* and 500 bp *trh* (Anon.). Source of the DNA probe identification of species specific cytolysin gene, *vvhA* for *V. vulnificus* is the same as for *V. parahaemolyticus* (Anon.).

Ribotyping and PCR-based techniques, e.g., amplified fragment length polymorphism (AFLP), fluorescence in situ hybridization (FISH), colony hybridization (CH), microarrays, random amplified polymorphic DNA (RAPD), repetitive extragenic palindromes (REP), enterobacterial repetitive

intergenic consensus (ERIC) sequences, repeated BOX elements (BOX) and restriction fragment length polymorphism (RFLP), along with multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST), have yielded the most valuable information about and new insights into the population structure of some species of the *Vibrionaceae* and have also provided a means of identifying these organisms.

Amplified Fragment Length Polymorphism

The AFLP technique consists of three main steps: (i) digestion of total genomic DNA with two restriction enzymes and subsequent ligation of the restriction half-site-specific adaptors to all restriction fragments; (ii) selective amplification of these fragments with two PCR primers that have corresponding adaptor and restriction site sequences as their target sites; and (iii) electrophoretic separation of the PCR products on polyacrylamide gels with selective detection of fragments which contain the fluorescently labeled primer and computer-assisted numerical analysis of the band

patterns (Huys & Swings., 1999; Jobson., 1996; Vos et al., 1995). AFLP measures the variation in the whole and thus is considered to give useful information about the short- and long-term evolution of bacterial strains (Janssen., 2001).

Janssen et al., (1996) reported that the complexity (i.e., the number and size of the fragments) of the AFLP patterns could be tuned by using different restriction enzymes and selective primers. although in any case the grouping of strains should be very similar. Because each bacterial species had a specific AFLP pattern. they concluded that AFLP could be used as an alternative to bacterial classification and identification. In the following years. AFLP was used to study various vibrios (Thompson et al., 2001), including *V. alginolyticus* (Vandenberghe et al., 1999), *V. cholerae* (Jiang et al., 2000a; Jiang et al., 2000b; Lan & Reeves, 2002), *V. harveyi* (Gomez-Gil et al., 2004; Pedersen et al., 1998), *V. vulnificus* (Arias et al., 1997a; Arias et al., 1997b), *V. wodanis* (Benediktsdottir et al., 2000), and *P.*

damselae (Thyssen et al., 2000), but most of these studies did not include all the recognized Vibrio species. Arias et al. examined 80 *V. vulnificus* strains by several phenotypic (Biolog, API, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, serotyping, enzyme-linked immunosorbent assay) and genotypic (AFLP and ribotyping) methods. With AFLP analysis, the authors were able to discriminate strains with identical ribotypes, and thus they concluded that AFLP is the most suitable and discriminatory tool for epidemiological studies of *V. vulnificus*.

Fluorescence In Situ Hybridization

The application of cultivation-independent techniques such as direct extraction of nucleic acids from environmental samples (e.g., water, gut tissue, and sediment) followed by clone library and 16S rRNA sequencing or alternatively FISH of filter-fixed cells with oligonucleotide probes targeting the 16S rRNA and subsequent visualization by epifluorescence microscopy has provided an efficient means of detecting, identifying, and quantifying

marine bacteria, including vibrios (Eilers et al., 2000a; Eilers et al., 2000b; Giovannoni & Rappe´., 2000; Rappe´ & Giovannoni, 2003). These approaches have shed light on the distribution and ecology of vibrios in the marine environment and have overcome the problem of the great plate count anomaly, i.e., the difference of the order of 102 to 103 between direct cell counts by e.g., epifluorescence microscopy and the CFU counts on. e.g., marine agar plates (Azam, 2001). Vibrios may be in a dormant state (VBNC) or may not be able to grow on the selective media employed (Colwell & Grimes, 2000). Environmental stresses (e.g., nutrient limitation or starvation and variations in pH, salinity, and temperature) could lead to such a state, for which Xu et al. proposed the name “viable but nonculturable.” Some researchers hypothesize that this is a ”genetically programmed physiological response” to enable some bacteria to survive in the environment (McDougald et al., 1998). Changes observed in VBNC bacteria include reduction of cell size, increase of cell wall thickness,

decrease in the amount of RNA and DNA, and biofilm formation, Heidelberg et al. (2002), found that by adding organic substrates (in micromolar concentrations) to the water, vibrios became dominant, reaching up to 65% of the total bacteria in a few hours. Vibrios not only could rapidly respond to nutrient-enrichment experiments but also maintained viability for up to 50 days under starved conditions. These authors concluded the high rRNA content of vibrios provide the potential for such rapid responses, which allow them to grow rapidly, outcompeting other members of the bacterial community. The low fluorescence intensity of marine bacteria is one of the main drawbacks of FISH technology (Eilers et al., 2000a; Eilers et al., 2000b). This is not really a problem for vibrios since these organisms have a high content of ribosomes. On the other hand. because several *Vibrio* species (e.g., *V. harveyi*. *V. campbellii*. *V. rotiferianus*, and other closely phylogenetic neighbours) have very similar

16S rRNA sequences, it may be difficult to perform reliable species identification.

Colony Hybridization by Species-Specific Probes

Detection of vibrios on selective media and subsequent colony hybridization with species-specific probes based on variable regions of the 16S rRNA has also been evaluated as a fast screening alternative tool for *V. anguillarum* (Martı´nez-Picado et al., 1996), *V. halioticoli* (Tanaka et al., 2002), *V. harveyi* (Harris et al., 1996), *V. parahaemolyticus* (Sloan et al., 2003), *V. proteolyticus* (Muniesa-Pe´rez, Jofre & Blanch, 1996), *V. scophthalmi* (Cerda-Cue´llar & Blanch.2002), and *V. vulnificus* (Cerda-Cue´llar et al., 2000; Cerda-Cue´llar et al., 2001). It was demonstrated that different "selective" media were not quite selective, and species-specific media are yet to be formulated. The specificity of certain probes, e.g., for detection of *V. anguillarum,* is not sufficient since they also hybridize with

V. ordallii, V. Diazotrophicus, and *V. navarrensis* (Martı´nez-Picado et al., 1996). The probe for *V. scophthalmi* detection has not been evaluated against *V. ichthyoenteri*. The two species have nearly 100% 16S rRNA similarity, and there is thus a great chance of cross-hybridization. The probe for *V. vulnificus* detection seemed to be very reliable (Cerda-Cue´llar et al., 2001).

Microarrays

Microarrays have been successfully developed since the middle of the last decade (Schena et al., 1995). This method may be considered a refinement of Northern and Southern blot techniques. Microarrays have been successfully used for the detection and quantification of bacteria in the environment (Bodrossy et al., 2003), Dziejman et al. (2002) constructed a microarray based on the genome sequence of *V. cholera* N16961. They spotted about 3.600 open reading frames (ORFs), corresponding to about

93% of the bacterial genome, and showed that nine different representative strains of *V. cholera* have only a 1% difference in gene content.

Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) assay, also referred to as arbitrary primed PCR, was first described by Williams et al. (1990) and Welsh and McClelland (1990). RAPD assays are based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the two primers results. The number and location of these random primer sites vary for different strains of a bacterial species. A number of study have reported success in using

RAPD assay to distinguish vibrios strains among diverse species: genomic diversity within *Vibrio parahaemolyticus* and *V. alginolyticus* (Sudheesh, Jie, & Xu, 2002). to differentiate between pathogenic and nonpathogenic strains *of V. harveyi* (Hernández & Olmos, 2004), to show the existence of different genetic groups strongly correlated to the host origin like *V. tapetis* (José M. Rodríguez et al., 2006).

Repetitive Extragenic Palindromes

Versalovic et al. (1991) described a method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern et al., 1984). REP

sequences have been described for numerous enteric bacteria (Gilson et al., 1984; Hulton et al., 1991; Sharples & Lloyd, 1990; Stern et al., 1984; Yang & Ames, 1988). The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions for these highly conserved. dispersed elements (Gilson et al., 1984; Newbury et al., 1997. Yang & Ames, 1988). Rep-PCR is fast becoming the most widely used method of DNA typing. This technique have been successfully used in the discrimination of strains of *V. cholera* (Rivera et al. 1995) and *V parahaemolytycus* (Wong & Lin, 2001; Khan et al., 2002).

Enterobacterial Repetitive Intergenic Consensus

A DNA-based typing technique, that is frequently used to generate strain-specific fingerprintings (Hulton, Higgins & Sharp, 1991), relies on the polymerase chain reaction (PCR) and primers directed to specific nucleotide sequences, designated ERIC (enterobacterial repetitive intergenic consensus) sequences. ERIC elements have been discovered in

non-coding, intergenic regions of *Escherichia coli* and *Salmonella typhimurium* (Hulton, Higgins & Sharp, 1991). These are small repetitive units of 126 bp containing a conserved central inverted repeat of 40 bp, Versalovic et al. (1991) demonstrated that complex amplification patterns could be generated in *Enterobacteriaceae* isolates by PCR amplification using PCR primers complementary to the inverted repeat. Southern hybridization and PCR amplification indicated ERIC or ERIC-like sequences in a vast variety of eubacterial species (Versalovic et al., 1991). Hence, it is thought that ERIC or ERIC-like sequences might be conserved in the eubacterial kingdom (Versalovic et al., 1991). The ERIC PCR technique is now widely used for typing Gram-negative and Gram-positive isolates, such as the symbiotic rhizobia (de Bruijin, 1992), the pathogenic *Xanthomonas campestris* (Louws et al., 1994), the human pathogen *V. parahaemolyticus* (Marshall et al., 1999) and *Streptococcus pneumoniae* (Hermans et al., 1995).

Repeated BOX Elements

The single BOX PCR test demonstrated excellent resolving powers while maintaining epidemiological linkage (van Belkum et al., 1996). Highly conserved, repeated DNA sequences constitute an important fraction of eukaryotic genomes. The BOX repeat is found to consist of three discriminate regions: boxA, boxB, and boxC, which are 59, 45, and 50 basepairs in length, respectively. Various different combinations of these three elements are found to be present in different BOX loci and limited sequence heterogeneity is encountered among different elements from the same strain or elements sequenced from different strains. The first publication on the BOX repetitive elements also described its intricate secondary structure. supported by compensating base pairing in different loci where the repeat is encountered. Moreover. their location in the vicinity of genes involved in the regulation of various aspects of bacterial competence. genetic transformation and virulence suggest that the

elements might well be involved in coordination of the control of gene expression. Martin et al. (1992). Box elements PCR (BOX-PCR) was performed as described by Martin et al. (1992) by using a single oligonucleotide primer for *V. cholera* (Singh et al. 2001).

Restriction Fragment Length Polymorphism

The PCR-RFLP technique consists of PCR amplification of certain genes, e.g., 16S rRNA, gyrB, and rpoD, and subsequent restriction of the PCR products with endonucleases to obtain band patterns (Chun, Huq, & Colwell., 1999; Chun, Rivera & Colwell, 2002; Le Roux et al., 2002; Tanaka et al., 2001; Urakawa et al., 1997, Urakawa et al., 1998). According to Urakawa et al. (1997), who analyzed the restriction patterns of the 16S rRNA of 35 *Vibrionaceae* species, this technique is useful for the classification and identification of *Vibrionaceae* strains. A closer examination of the data presented by these authors reveals that all the Vibrio core group species (i.e., *V. alginolyticus, V. parahaemolyticus, V. proteolyticus, V. harveyi,* and

V. campbellii) and *V. vulnificus* have the same band pattern and were thus indistinguishable, *P. iliopiscarius, P. leignathi* and *P. phophoreum* showed identical genotypes. This is quite striking since the 16S rRNA sequence similarity between these species is 96.5%, clearly proving the low discriminatory power of PCR-RFLP (F. Thompson & Iida, 2004).

Multilocus Enzyme Electrophoresis and Multilocus Sequenc Typing

MLEE was first applied to bacterial systematics in the 1980s and has become the standard technique for studies of population genetics (Carnahan et al., 1994; Selander & Levin, 1980). MLST was developed recently as an improved adaptation of MLEE and as been advocated as the most reliable molecular tool for epidemiology (Maiden et al., 1998; Urwin & Maiden, 2003)). Both techniques index the variation in housekeeping genes. MLST assigns alleles directly from the nucleotide sequences. while MLEE compares the electrophoretic mobility of the enzymes encoded by the genes (Feil & Spratt, 2001). Obviously, MLST has several advantages

over MLEE, e.g., higher discriminatory power because it detects synonymous and nonsynonymous changes, accuracy and portability of the data, ease of performance, and reproducibility (Maiden et al., 1998). MLEE analysis of 397 *V. cholerae* strains isolated from Mexico and Guatemala suggested that horizontal transfer and recombination are important processes in the evolution of clonal complexes of *V. cholerae* and indicated that successful clonal complexes may persist for decades (Beltra´n et al., 1999). A high genetic diversity. as assessed by MLEE of 15 enzyme loci, was observed among 107 diverse *V. cholerae* isolates (Farfa´n et al., 2000). These authors applied MLST of six housekeeping-enzyme loci, i.e., *asd, cadA, idh, lap, mdh,* and *epd*, on a subset of 31 *V. cholerae* serogroup O139 strains and found four distinct groups of strains (Farfa´n et al., 2002). They concluded that recombination has not occurred among these vibrios. MLST of four loci, i.e*., gyrB, recA, dnaE,* and *gnd,* applied to 81 isolates of *V.*

parahaemolyticus revealed that pandemic strains are clonal (Chowdhury et al., 2004). Clearly, MLST data will be useful to delineate species in vibrios.

Ribotyping

Ribotyping consists of four main steps: (i) restriction of the bacterial chromosome with an endonuclease, (ii) gel electrophoresis of the resulting fragments, (iii) transfer of the fragments to a membrane, and (iv) hybridization of the gel with a labeled probe complementary to the 16S and 23S rRNAs (Grimont & Grimont, 1986). Ribotyping was one of the first fingerprinting techniques to be successfully used in the taxonomy of vibrios, and it has been particularly useful in the study of *V. cholerae* (Grimont & Grimont, 1986; Grimont & Grimont, 2000; Lan &Reeves, 1998; Pourshafie et al., 2000; Pourshafie et al., 2002). A standardized ribotyping scheme was proposed as a reliable tool for epidemiological studies of *V. cholera* (Popovic et al., 1993). Recently, ribotyping has been used to assess

the genomic diversity of environmental *Vibrio* strains associated with fish and oysters (Austin et al., 1997; Macia´n et al., 2000; Pujalte et al., 1999). According to Austin et al. (1997), closely related *Vibrio* species. e.g., *V. anguillarum* and *V. ordalii*, can be differentiated on the basis of ribotyping. Macia´n et al., (2000) analyzed 82 *V. splendidus,* 25 *V. harveyi*, and 10 *V. tubiashii* strains isolated in a 1-year period and found 64, 17, and 9 different ribotypes, respectively. Certain *V. splendidus* ribotypes were typical for isolates found in summer, while others were typical for isolates found winter.

CHAPTER 5

Experimental study

General background and aim of the study

The nutritional and economical value of shellfish is acknowledged worldwide. Similarly, filter-feeding bivalves are well known as efficient transmitters of seafood-born disease (Gosling, 2003). The high-risk nature of these products have been well documented in many investigative reports and international agencies. Preventive measures to enhance the safety of living bivalve shellfish at retail include the monitoring and improvement of the water quality found at the harvesting areas. Several EU Regulations lay down general and specific food hygiene requirements for the living bivalve mollusc production and marketing (Regulations N° 852, 853, 854/2004 and 2073, 2074/2005/EC), included the microbiological

criteria for the production areas and for the products when placed on the market.

Nevertheless, traditional bacterial indicators have considered inadequate predictors of the presence of autochthonous bacteria and human enteric viruses and several authors have confirmed the lack of correlation between the prescriptive microbial indicators (i.e. *E.coli*) and the presence of *Vibrio spp*. (Hood & Ness, 1982; Marino et al., 2005; Normanno et al., 2006; Ripabelli et al., 1999). Fecal indicators provide an inadequate index of microbiological safety for naturally occurring vibrios and underestimate the efficiency of the depuration process. The depuration processes has indeed a different effect on *Vibrio spp*. in comparison on that on *E. coli*.,

The present study is part of a project funded by the Sardinian Region (Regional law 7 of 7 August 2007) entitled: "Development of innovative technologies for molecular epidemiological surveillance of bacterial and viral contaminants for in order to optimize the production of bivalve

molluscs in Sardinia". The main objective of this research project was the study of the occurence of *E.coli, Listeria monocytogenes* and *Salmonella spp*. in molluscs harvesting in three coastal areas of Sardinia and the development of a reliable procedure for the identification of *Vibrio spp.* and viruses (NoV GI. NoV GII HAV) . The project involved 4 Research Units (RUs), which have compared some different PCR methods of isolation and strains characterization. Briefly:

- Unit 1: the National Institute of Health (Rome), that carried out the detection in samples of mussels of some Enteric viruses, Salmonella and *Vibrios.* Furthermore, one of their objectives was the dectection of *V. parahemolyticus* by a Colony Hybridization method;
- Unit 2: the Institute of Biomedical Technologies (National Research Council, Milan), that implemented MacroArray Technologies to detect *V. parahemolyticus*, *V. vulnificus* and *Listeria monocytogenes* isolated by the other units;

- Unit 3: the University of Bologna (Microbiological laboratories located in Cesenatico), that implemented a compared study about the detection of *E.coli*, *Listeria monocytogenes, Salmonella spp* and *Vibrio spp.;*
- Unit 4: the sector of Inspection of Food of Animal Origin of the Department of Veterinary Medicine, of the University of Sassari (Italy), where I have attended my three-year PhD course, that carried out as base-unit, the sampling collection and the detection of total *E.coli,* Verocytotoxin-producing *E.coli, Salmonella*, *Listeria monocytogenes,* in comparison with the other research units.

In my dissertation, the results of a study on potentially pathogenic *Vibrio spp.* and *Vibrio parahaemolyticus* are reported.

The overall objective of this work was to obtain epidemiological data concerning the contamination level of two species of bivalves molluscs (*Mytilus galloprovincialis* and *Rudiapes decussatus*) harvested in three

coastal areas of Sardinia and to investigate the effect of the depuration procedures on the safety of the molluscs. In particular, in this thesis the results of the following objectives will reported:

a) the evaluation of the prevalence and the level of *Vibrio spp.* in Sardinian bivalves molluscs. at harvest level and after depuration;

b) the evaluation of the efficacy of depuration methods on the *Vibrio spp*.;

c) the study of the presence of pathogenic profile of *Vibrio parahaemolyticus;*

d) the comparison of three PCR-based techniques for the characterization of *Vibrio parahaemolyticus.* and the study of the relationship between the presence of this microorganism in the molluscs and some environmental factors.

Stage 1- Detection of Pathogenic *Vibrios* in *Mytilus galloprovincialis* and *Rudiapes decussatus* collected in three coastal areas of Sardinia.

Samples collection

Twelve sampling sessions were carried out from April 2011 to May 2012. Specimens were collected from three bivalve mollusc harvesting. purification and dispatch centres/plantss (A, O, S), associated to three coastal production areas, the main relevant within the Sardinian mollusc production. The characteristics of the production areas were:

- centre/plant A: is an inlet of the Sardinian Sea, located in the central-western cost of Sardinia;
- centre/plant O: is an gulf of the Tyrrhenian Sea, located in the north-eastern coast of Sardinia;
- centre/plant S: is a wide lagoon located in the south of Sardinia, that for extension and relevance of biodiversity is one of the most important wetlands in Europe.

Samples were collected at two levels: at harvesting time (all the centres) and after purification treatment (plants A e O).

Sampling at harvesting time occurred monthly (twelve months). For each centre/plants, during each session 1 kg of *Mytilus galloproncialis* and 1.5 kg of *Ruditapes decussatus* were collected, before handling procedures.

Sampling after depuration occurred with seasonal frequency (four time).

Altogether, A was visited 12 times (12 samples of *M. galloprovincialis* and 11 samples of *R. decussatus*), plant O was visited 12 times (12 samples of *M. galloprovincialis* and 11 samples of *R. decussatus*) and S 4 times (4 samples of *M. galloprovincialis* and 4 samples of *R. decussatus*).

A total of 54 specimens (28 samples of *M. galloprovincialis* and 26 samples of *R. decussatus*) were collected (table 1).

The three centres for growing and harvesting shellfish were mainly conducted by the "Long-Line" system. Then *M. galloprovincialis* samples were taken from ropes.

Some sector of the rows were employed for the oyster and clam. *R. decussatus* samples were collected from these sectors.

After collection all specimens were immediately placed in sterile bags and kept cold (about 10° C). and transported to the laboratory of University o Sassari.

Samples analyzed and their sources				
	Plant A	**Plant O**	**Plant S**	**Total samples**
M. galloprovincialis before depuration	12	12	4	28
M. galloprovincialis after depuration	5	4	0	9
R. decussatus before depuration	11	11	4	26
R. decussatus after depuration	5	3	0	8
Total samples	33	30	8	

Table n.1 – Number of samples collected for each plant.

Detection and enumeration of *Vibrio spp.*

For the preparation of samples for microbiological detection. enumeration and graduated dilutions the ISO 6887-1: 2004 rules are used. All the molluscs were scrubbed free of dirt, washed in hypochlorite solution (0.2

mg/l), rinsed with sterile distilled water. and shucked with scissors. All the samples were submitted to the following microbiological analysis: from a pool of ten-to-twelve animals, 25 g of flesh and intra-valvular liquid were taken; 225 ml of Alcaline Peptone Water (Sigma-Aldrich), charged with NaCl 1%. then homogenized in a stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min. The homogenates were incubated at 35°C for 18-24 h in order to revitalize stressed microorganisms.

For the enumeration of *Vibrio spp.,* 1 ml of each homogenates was spread over the surface of three Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS agar) (Biolife) 90 mm plates (100 µl), using a sterile loop. The plates were incubated at 20°C for 3-5 days. and subsequently were checked for growth of typical colonies of *Vibrio spp..*

The TCBS media allowed differentiation of *V. cholera* and *V. parahaemolyticus*. In addition, TCBS has a very high pH (8.5-9.5) which inhibits the growth of intestinal flora other than *Vibrio spp..*

After incubation, *V. cholerae* grows as yellow-brown colonies, while *V. parahaemolyticus* and *V. vulnificus* show light bluish/green colonies.

Only plates having a colony number between 30 to 300 are counted. The number of colonies of *Vibrio spp.* was determined applying the following formula: a = b / A x C, where b was the number of colonies corresponding to the identification criteria. A was the number of suspect colonies subjected to identification, while C was the number of colonies suspected for *Vibrio spp*..

After incubation, 5 graduated dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) using APW (NaCl 1%) were prepared; each dilution was seeded with a sterile loop onto the surface of both selective medium ChromAgar (Paris, France) plates and Modified Cellobiose-Polymyxin B-Colistin (mCPC) Agar plates. All plates were incubated at 37°C for 48 hours.

On mCPC agar the typical colonies of *V. cholerae* are small, smooth, opaque and green to purple in color. Most of the strains of *V.*

parahaemolyticus not grow on mCPC, while the *V. vulnificus* colonies are round, flat, opaque, yellow and with a 1 to 2 mm diameter.

On ChromAgar the typical colonies of *V.cholerae* and *V.vulnificus* are green blue to turquoise blue, while *V.parahaemolyticus* colonies are colored mauve. Five of presumed to be *Vibrio spp* colonies from the plates of each medium. were streaked on Trypticase soy agar, with 3% of NaCl. (TSA-s) plates. After incubation at 30°C for 24 hours. colonies were selected for typical appearance on TSA-s (convex. light yellow and opaque, with a diameter between 1 to 2 mm) and submitted to the following confirmatory tests for *Vibrio spp*.: gram staining, oxidase test, sensitivity to the vibriostatic agents O/129 test.

- *Confirmatory test for Vibrio spp.*

  - *Gram staining:* one isolated colony was taken and then a microscope slide set up and stained. At the observation by light

microscopy, the suspect colonies of *Vibrio spp*. appeared as curved bacilli, Gram

- *Oxidase test:* an isolated colony was transferred to a filter paper saturated with oxidase reagent (1% N'-tetramethyl-p-phenylenediamine.2HCl). A dark purple color developing within 10 sec indicates a positive test growth.
- *Sensitivity to the vibriostatic agents O/129 test*: susceptibilities of the strains to O/129–2.4-diamino-6.7-diisopropylpteridine-phosphate salt (Sigma) (10 and 150 µg) were determined by disk-diffusion method on TSA-s plates. All clinically significant members of the genus are sensitive to 150 µg of the vibriostatic agent O/129, while *V. cholera* and *V. vulnificus* are sensitive to 10 µg of the vibriostatic agent O/129 too and *V. parahaemolyticus* is resistanced to 0/129. To determine the sensitivities to 0/129 of all the isolates of *V. spp.,* uniform lawns of bacteria. approximately

10' cells per ml, growed in TSA-s were prepared by using a cotton-tipped swab in a dense suspension of saline solution (0.5 Mc Farland). Impregnated discs of both 10 and 150 µg of the 0/129 discs were placed aseptically on the TSA-s plates. After incubation at 30°C for 18 to 24 h a clear zone of inhibition of growth developed around the discs, indicating that the organisms were susceptible to 0/129 at a given concentration. Organisms resistant to 0/129 developed no zone of inhibition.

Sensitivity	*V. cholerae*	*V. vulnificus*	*V. parahemolyticus*
10 µg O/129	Sensitive	Sensitive	Resistant
150 µg O/129	Sensitive	Sensitive	Sensitive

Table n.2 - Susceptibilities of *Vibrio spp.* to O/129–2.4-diamino-6.7-diisopropylpteridine-phosphate salt (10 and 150 µg).

- *Biochemical profile determination:* biochemical characterization of all the isolates was performed using the API 20NE identification system (bioMérieux, Marcy l'Etoile. France). The test consisted of

twenty-one micro tubes containing dehydrated substrates for the detection of enzymatic activities and sugars fermentation. Colonies taken from “fresh” (18-24h) TSA-s plates, were suspended in a vial of API suspension medium (2ml), until reaching an opacity equal to that of McFarland standard 0.5. Subsequently, the bacterial suspension was distributed in tubes. The galleries containing tubes were aerobically incubated at 29°C ± 2°C for24 hours (± 2 hours). All the tests were evaluated. giving a +/- on the appropriate results registration form.

All the isolates having typical morphological and growth characteristics of *Vibrio spp*. have been submitted to biomolecular confirmation and characterization.

RESULTS

Prevalence

The mean prevalence of *V. spp.* in *M. galloprovincialis* was 68% at harvesting time, while after in the same molluscs depuration process was 89%. Prevalence in *R. decussatus* was 78% at harvesting time and 88% after the depuration process.

Prevalence of vibrios in *M. galloprovincialis* and *R. decussatus* harvested in plant A before depuration was 100% and 73% respectively, while after depuration, was 100% .

Molluscs harvested in plant O at harvesting time had a prevalence of Vibrio spp of 92% for *Mytilus* and 82% for *Ruditapes*. After depuration in the same zone the prevalence was 80% and 57%, respectively.

In plant S, the prevalence was 25% and 75%, respectively in *M. galloprovincialis* and *R. decussates*, collected only before depuration.

Vibrio spp Quantitative detection

The count of the *Vibrio spp*. are expressed as $\log_{10}$ colony-forming units per g (Log_{10} CFU/g).

On the whole (Table n.3, Table n.4, Table n.5), *Vibrio spp*. count in *M. galloprovincialis* and *R. decussatus* was (mean±s.d.) 1,65 ± 0,69 (range 0 → 3,26) and 2,03 ± 0,16 (range 0 → 4,52), respectively. In particular:

- in plant A, at harvesting time the average of *Vibrio spp*. in *M. galloprovincialis* was 1,65 ± 1,06 (range 0 → 3,04), while in *R. decussatus* the mean level was 1,96 ± 3,96 (range 0 → 3,94). After the depuration process the mean values in the former was 2,27 ± 0,61 (range 1,60 → 3,00), while in the later was 2,14± 0,70 (range 1,30 → 2,95).

- in plant O, *Vibrio spp.* mean count *in M. galloprovincialis* was 1,63 ± 0,99 (range 0 → 2,70), while in *R. decussatus* was 2,06 ± 1,40 (range 0 → 4,52). *A*fter depuration, in the same two types of molluscs, the *Vibrio spp.* mean count was 1,99 ± 1,26 (range 0 → 3,26) and 2,20 ± 1,98 (range 0 → 3,83) in *M. galloprovincialis* and R. *decussates*, respectively.

- in plant S, where the sampling was carried out only at harvesting time, the *Vibrio spp*. mean count was 0,71± 1,42 (range 0 → 2,84) in *M. galloprovincialis* and 1,79 ± 1,36 (range 0 →2,85) in *R. decussates,* respectively.

Results of identification by Biochemical tests

Within the strains isolated from *M. galloprovincialis* and *R. decussatus*, and showing the typical characteristics of *Vibrio spp*., on the basis of the confirmatory test, a subset of 186, isolated from the two species of molluscs, taking in to account the prevalence, collected from the three areas, before and after depuration, were submitted to the biochemical identification.

Sixty-three strains of *Vibrio spp.* were detected in *M. galloprovincialis*, from the three areas included in our survey, representing a prevalence of 34%. In detail, in these samples the results of identification showed a prevalence of 16%, 13% and 11% of *V.alginolyticus, V. parahaemolyticus* and *V.*

vulnificus respectively. Fifty-three % of strains resulted not *Vibrio spp*., or unidentifiable. These results are referred to the molluscs at harvesting time.

A selection of 27 strains from positives samples of *Mytilus* collected after the depuration process, were also isolated and submitted to biochemical tests. The strains belonged to the following species: 15% *V. spp.*, 26% of *V.alginolyticus,* 15% of *V. parahaemolyticus,* 4% of *V. Vulnificus*. A percentage of 60% of strains were not identified or not *V. spp*..

Furthermore, a selection of 75 strains isolated from *R. decussatus* at harvesting time, resulted, by biochemical identification tests, *Vibrio spp.* 17%, while the prevalence of *V.alginolyticus, V. parahaemolyticus* and *V. vulnificus* were of 7%, 23% and 5%, respectively. The remaining 48% were species not well identifiable or resulted not *V.spp.*. After depuration (selection of 21 strains) the prevalence was of 5% for *V. spp.,* 24% for

V.alginolyticus, 29% for *V. parahaemolyticus., and* 5% for *V. Vulnificus*, while a percentage of 37% were species not well identifiable or not *V.spp.*.

Concerning the results of identification in relation to the three plants, we observed (Table n.7 and n.8):

- in the plant A, a total of 30 strains from *M. galloprovincialis* at harvesting time and of 12 strains after purification process were analyzed. The strains isolated before the depuration belonged to the following species: 20% were *V. spp*., 17% *V. alginolyticus,* 13% *V. parahemolyticus,* 3% *V. vulnificus*; 40% of the strains were not identifiable or resulted not *Vibrio spp*.. Instead, the strains isolated after the depuration belonged to the following species: 25% were V. spp., 17% *V. alginolyticus,* and 25% *V. parahemolyticus*, while a percentage of 33% of the strains were not identifiable or not *Vibrio spp*..

The strains isolated from *R. decussatus* samples collected in the same plant A, before and after the depuration process, were a total of 33 and 12

respectively. Those isolated from samples at harvesting time were, in prevalence order: 12% *V. spp*., 6% *V. alginolyticus,* 21% *V. parahemolyticus,* and 6% *V. vulnificus*; 55% were not identifiable or not *Vibrio spp*.. Instead those isolated from samples after depuration process were identified as the follow: 8% *V. spp*., 8% *V. alginolyticus,* 42% of *V. parahemolyticus,* and 42% of *V. vulnificus*; in this case only 4% were not identifiable or not *Vibrio spp*..

- in the plant O, 26 and 15 strains were isolated from *M. Galloprovincialis*, respectively at harvesting time and post depuration process. The 26 strains were resulted: *V. spp.* 8%, *V. alginolyticus* 19%, *V. parahemolyticus* 12% and *V. vulnificus* 12%. While the strains isolated after the depuration were: 7% of *V. spp*., 33% of *V. alginolyticus*, 7% of *V. vulnificus* and 67% of not identified or not *V. spp* samples. *V. parahemolyticus* were not isolated. From the *R. decussatus* collected in the same plant a total of 41 strains were isolated and submitted to identification test. The strains isolated at harvesting time (32) belonged to the

following species: *V. spp* 16%, *V. alginolyticus* 9%, *V. parahemolyticus* 19%, *V. vulnificus* 3%, and not *V. spp*. + not identifiable were 53%. The strains isolated after the depuration (9) were: *V. alginolyticus* 44%, *V. parahemolyticus* 11%, *V. vulnificus* 11%, while 2 strain resulted not identifiable ..

- in plant S, a total of 17 strains were isolated from *M. galloprovincialis* and *R.decussatus*, only at harvesting time. The strains isolated from the former were identified as:

Discussion

The coastal environment of the three harvesting areas considered in this study were characterized by low sea depth, fine sediments and the contribution of several rivers which transport the residues and sewage of agricultural, rearing, urban and industrial activities. The areas are influenced by the quantity of rivers freshwater and by saline water arriving from the Western Mediterranean Sea. Around the Sardinia island an

intense and very variable circulation current is present; it allows a change of the sea water in almost all the harvesting zones in a very short time. Furthermore the salinity is not so low (~37.8), and it changes in relation of the currents and seasons too (Bouzinac et al., 1999). All these characteristics, together with meteorological and hydrodynamic variability, make this environment very susceptible to sudden changes in chemical–physical properties, that impact on biological changes. It has been demonstrated that environmental changes in recent years were associated with the emergence of new pathogens, the re-emergence of diseases, which had almost been eradicated, and with changing patterns and distribution of numerous infectious agents (Jones et al., 2008; Rose JB et al.,2001, Rosenthal J., 2009). Hence, permanent monitoring of environmental parameters is mandatory to predict and prevent climate-associated diseases and threats. Most of the studies regarding the influence of environmental parameters on vibrios occurrence reported the

effect of individual oceanographic variables on *Vibrio spp.* presence and/or abundance, not always taking into account the interrelations existing among these parameters (Eiler et al., 2007, Gil rt al., 2004, Huq et al., 2005, Ristori et al., 2007).

In the present study, we had considered the presence and the level of the vibrios population in relation with salinity, temperature and pH of the seawater, noticed during the sampling period in the harvesting zones.

Prevalence of Vibrio spp.

These results seem to confirm the considerable diffusion of *Vibrio spp.* in bivalves molluscs and showed interesting epidemiological aspects. The high mean prevalence (68% *Mytilus*, 78% *Ruditapes* before depuration vs 89% *Mytilus*, 88% *Ruditapes* post-depuration) showed the presence of *V.spp.* in aquatic environment; as a consequence *V. spp.* was present in the molluscs both before and after depuration, demonstrating that depuration process

is ineffective against these autoctonous microorganisms, as previous described by different authors.

The prevalence in *R. decussatus* was higher than in *M. galloprovincialis,* probably because they are (*R. decussatus*) filter feeders that live in muddy-sand sediments of shallow coastal areas, while culturing mussels is on ropes suspended from floating rafts. Moreover in this study *Ruditapes* had a lower weight compared to mussels and therefore clams had a capacity to filter the water less than mussels, in according with the allometric relationship between filtration rate (*Fr*; lh^{-1}) and tissue dry weight (*W*; g) described by the equation $Fr=aW^{b}$. The constants *a* and *b* are fitted parameters, *a* represent the filtration rate of an individual of unit body weight and b is the power to which the increase in size raises that rate (Gosling, 2003). Besides, the results confirmed that the post-harvest process (depuration), to which the shellfish of the breeding of "class B areas" were subjected, was not always available systems to reduce in the

shellfish the health risks from vibrios (Barile et al 2009; Croci et al 2002; Meloni et al 2010).

Quantitative detection: the results of quantitative method demonstrate a relationship between culturable vibrios densities in the mussel/clam samples and seasonality. The table 3 shows how the prevalence of *Vibrio spp.* in *Mitylus* and *Ruditapes* samples was not homogeneous during the sampling period and in the different plants.

The highest level on TCBS agar for the *Mytilus* collected in plant *A* was in October 2011, while for *Ruditapes* i April 2012. In October 2011 the highest values of salinity and temperature of the year (salinity 40.1 ppt and temperature 20.79°C), while in April the lowest seawater conditions were found (salinity= 39.5 ppt and T= 16.32°C).

In the plant O, the highest level was found for *Mytilus* in May 2012**,** while for *Ruditapes* in September 2011, but the chemical-physical values of seawater features were not available for these periods.

In the plant S, where only 4 sampling were collected, the high prevalence of *Vibrio spp.* was showed in April 2012 for clam samples, when salinity was 37.31 ppt and temperature was 17°C. The values of pH were always uniform during the year and in all different rearing zones (pH ranged between 8.53 and 8.53).

The results showed, in accordance with other study (Meloni et al., 2010), how the largest number of presuntive vibrios was isolated in areas characterized by a high salinity (~40 ppt). The High salinity decreases the filtration rates of molluscs (Gosling, 2003). Moreover in this areas the value of pH (~8) supports the growth of *Vibrio spp*.. A certain temperature threshold enables an increasing growth of *Vibrio spp*. (Oberbeckmann et al., 2012). All these seawater features, probably explained so high concentration of vibrios in some particular periods of the year.

The high number of Vibrio spp detected in our study was not always related to the temperature, in fact high presence was showed in autumn (T

~23°C) and in spring (T ~16°C). Gosling (2003) explained that, when sea water temperature was low, the mollusc filtration capacity was markedly reduced. For this reason probably we found a high presence of vibrios when temperature was high, as well as in spring, when capacity of filtration decreased and the bacteria were kept inside the molluscs. The results reported in the table n. 4, about the count after the depuration process demonstrate that the treatments used did not have any impact on the*Vibrio spp.* concentration. In fact some species of vibrios are concentrated in the intestines of shellfish, where they can multiply and then therefore ineffective purification systems (Hlady et al, 1997; Desemarchelier et al; 2000). In our study this situation is showed in the table n. 3 where the level of Vibrio spp in *R. decussatus* samples collected after depuration process in May 2011 from plant A was higher than that before depuration.

Biochemical tests

Vibrio spp. was showed in the samples collected from all the areas analyzed, in particular in those from the plant S, and the level were higher in clams (17%) than in mussels (13%).

It is known that a series of environmental factors affects the occurrence and the number of vibrios and that their impact varies in the different geographic areas: for example, in two coastal areas, one in the Atlantic Ocean and one in the Mediterranean Sea (Martinez-Urtaza *et al*., 2008; Caburlotto *et al*., 2012), is the salinity the key factor influencing the occurrence of *Vibrio* and pathogenic *Vibrio* species, while the influence of water temperature is a secondary variable . For these reasons, the incidence and concentration of *Vibrio spp*. in similar or close coastal areas (such as those within the Mediterranean Sea) might be very different. On the base of parameters found in the coastal areas of Sardinia we can

confirmed that salinity is very high respect to other marine zones (average of salinity in harvesting coastal areas was ~ 38 ppt).

Within the *Vibrio* genus, the most frequently isolated species was *V. alginolyticus* (16%) in mussels samples and *V. parahaemolyticus* (23%) in clams samples; other pathogenic species such as *V. vulnificus* was found in 11% and 5% of strains isolated respectively from *Mytilus* and *Ruditapes,* as reported by other authors (Croci et al. 2001). All *Vibrio spp*. core were detected, except for *V. cholera*, in accordance with other Italian studies where the presence of this specie was very restricted (Caburlotto et al. 2011; Serracca et al. 2011; Croci et al. 2001). *V. alginolyticus*, *V. vulnificus* and *V. parahaemolyticus* were isolated in samples from all the three coastal areas included in this study, and during all the sampling period (from April 2011 to May 2012).

	Plant A					Plant O					Plant S				
Date	*M. galloprovincialis*	*R. decussatus*	*T°C*	*ppt*	*pH*	*M. galloprovincialis*	*R. decussatus*	*T°C*	*ppt*	*pH*	*M. galloprovincialis*	*R. decussatus*	*T°C*	*ppt*	*pH*
April 2011	2.19	2.51	15.9	40.30	8.18	1.74	2.72	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
May 2011	1.74	2.51	19.6	40	8.72	1.95	2.88	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
June 2011	1.70	0	21.5	40.12	8.24	2.59	3.30	n.s.	n.s.	n.s.	0	2.85	21.29	33.03	8.37
July 2011	0	0	22.8	40.1	7.93	2.70	2.18	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
September 2011	2.08	0	23.7	40.5	7.92	1.48	4.52	n.s.	n.s.	n.s.	0	0	n.s.	n.s.	n.s.
Oct 2011	3.04	3.00	20.79	40.1	7.92	2.04	1.30	n.s.	n.s.	n.s.	0	1.48	23.41	36.24	8.53
Nov 2011	0.00	1.85	17.8	39.5	7.58	0	0	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
December 2011	2.00	1.74	13.1	38.9	9.53	1.00	1.00	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
January 2012	0.00	2.13	12.15	39.5	8.05	0	2.00	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
March 2012	2.53	3.94	13.66	38.9	8.13	1.98	2.82	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.
April 2012	1.90	3.90	16.4	39.5	8.21	1.00	0	n.s.	n.s.	n.s.	2.84	2.84	18	33	8.32
May 2012	2.56	2.51	18.9	39.5	8.16	3.13	n.e.	n.s.	n.s.	n.s.	-	-	n.s.	n.s.	n.s.

Table n.3 – *Vibrio spp*. count (means log_{10} CFU/g) in molluscs before depuration process in relation with temperature. salinity and pH for each . n.e.: not examined sample. n.s.: not supplied data.

	Plant A					Plant O				
Date	Mytilus galloprovincialis	Ruditapes decussatus	T°C	Ppt	pH	Mytilus galloprovincialis	Ruditapes decussatus	T°C	Ppt	pH
May 2011	1.74	2.95	19.6	40	8.72	1.81	2.79	n.s.	n.s.	n.s.
July 2011	2.23	1.30	23.7	40.5	7.92	2.85	3.83	n.s.	n.s.	n.s.
Nov 2011	1.60	1.90	17.8	39.5	7.58	0	0	n.s.	n.s.	n.s.
Mar 2012	3.00	1.78	13.66	38.9	8.13	2.00	-	n.s.	n.s.	n.s.
May 2012	2.76	2.76	18.9	39.5	8.16	3.26	-	n.s.	n.s.	n.s.

Table n.4 - *Vibrio spp*. count (means log_{10} CFU/g) in molluscs after depuration process in relation with temperature. salinity and pH for each . n.e.: not examined sample. n.s.: not supplied data.

	Plant A		Plant O		Plant S	
	M. galloprovincialis	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*
Before depuration	1.65 ± 1.06	1.96 ± 3.96	1.63 ± 0.99	2.06 ± 1.40	0.71± 1.42	1.79 ± 1.36
After depuration	2.27 ± 0.61	2.14± 0.70	1.99 ± 1.26	2.20 ± 1.98	-	-

Table n.5 – *Vibrio spp*. count (means ± s.d. log_{10} CFU/g) in molluscs before and after depuration process for each .

	Plant A %		Plant O %		Plant S %	
	M. galloprovincialis	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*
Before depuration	100	73	92	82	25	75
After depuration	100	100	80	57	-	-

Table n.6 – *Vibrio spp*. whole prevalence by biochemical tests in molluscs before and after depuration process for each .

	Plant A (%)		*Plant O (%)*		*Plant S (%)*	
	M. galloprovincialis	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*
Vibrio spp	20	12	8	16	14	20
V. alginolyticus	17	6	19	9	0	0
V. parahaemolitycus.	13	21	12	19	29	40
V. vulnificus	3	6	12	3	14	10
No *V. Spp./*not identificable	40	55	0	53	43	30

Table n.7 – *Vibrio spp*. prevalence by biochemical tests (before depuration) process for each plant.

	Plant A (%)		*Plant O (%)*		*Plant S (%)*	
	M. galloprovincialis	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*	*M. galloprovincialis*	*R. decussatus*
Vibrio spp	25	8	7	0	-	-
V. alginolyticus	17		33	44	-	-
V. parahaemolitycus.	25	42	0	11	-	-
V. vulnificus	0	42	7	11	-	-
No *V. Spp./*not identificable	33	4	67	24	-	-

Table n.8 – Prevalence of *V.spp*.d by biochemical identification (after depuration) process for each plant.

145 Sonia Lamon – "Prevalence, distribution and pathogenic profile of *Vibrio parahaemolitycus* and *Vibrio spp* core in shellfish (*Mytilus Galloprovincialis* and *Ruditapes decussatus*) collected in Sardinia (Italy). Molecular fingerprinting using three PCR- based methods: Box PCR, REP PCR , and Eric PCR" - Tesi di Dottorato di Ricerca in Riproduzione, Produzione, Benessere Animale e Sicurezza degli Alimenti di Origine Alimentare XXV ciclo – Università degli studi di Sassari.

Stage 2 –Molecular identification and virulence profile of *Vibrio spp.*

All the isolates having the characteristics of *Vibrio spp.* have been identified by molecular test. considering one or more strains for each positive sample. These strains were selected in relation to the results of the biochemical tests and within the harvest sites. Beside. were analyzed also strains without a specific biochemical identification. to prove the low ability of biochemical identification tests as reported in different studies.

DNA extraction

Vibrio spp. strains were harvested from TSA-s cultures incubated overnight at 37°C. DNA extraction was performed using boiled method. The boiling method is rapid and renders DNA of a minimum purity but enough to use in PCR reactions. From TSA-s plate with a loop were picked up % colonies and were dissolved in a Eppendorf tube where. previously. was added 100 µl of molecular grade water. The suspension was incubated at 100ºC for 15

minutes to obtain cellular lysis. After. the lysate was put on ice for 3-5 minutes and centrifuged (12.000 rpm for 10 min to 4°C). The supernatant was transferred to a sterile Eppendorf tube. The bacterial lysate obtained was stored at -20 C for further analysis.

Multiplex PCR-based

A multiplex PCR assay was set up using primer sets and conditions as described by Rivera et al. (2001). Brasher et al. (1998) and Bej et al.(1999). Detection of the amplicons from all of the pathogenic vibrios in a single assay (mPCR) have significantly increased the speed of detection and thereby improved the microbiological safety of seafood.

This assay allowed to identify the three most pathogenic species of Vibrio (*V. cholera*. *V. parahemolyticus* and *V. vulnificus*). The targeted genes selected and oligonucleotide primer sets used for the detection of each of

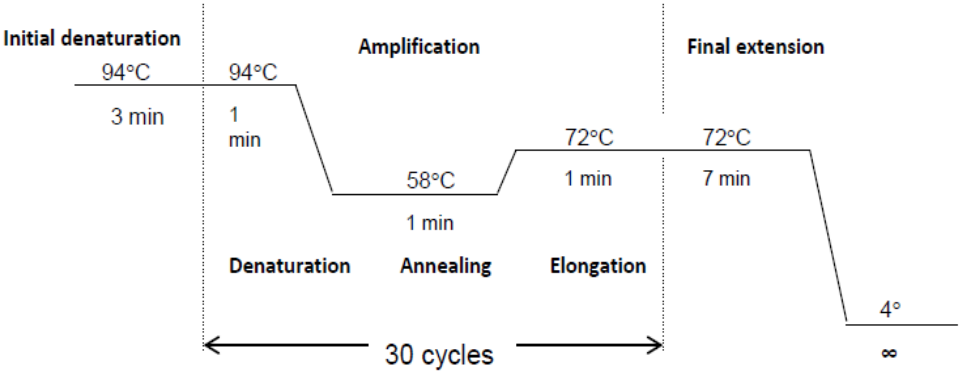
the three pathogens were VC-toxR (779 bp). VP-toxR (368 bp) and VV-vvhA (205 bp). each was specific for *V. cholera*. *V. parahemolyticus* and *V. vulnificus* respectively. The oligonucleotide probes were designed based on the nucleotide sequences internal to the amplified segments of the respective targeted genes.

In order to test the method. three reference strains. *V. parahaemolyticus* ATCC 17802 (*toxR* +. *tdh* -. *trh* +). *V. vulnificus* ATCC 27562 and *V. cholera* N16961. were used as positive control and *Listeria spp.* from internal collection of University of Sassari was used as negative control respectively.

In order to prevent and control contamination. each PCR test included reagent control (without DNA).

Each multiplex PCR amplification was performed with a 25 µl reaction volume consisting of 10 ng of purified genomic DNA. 10 mM deoxynucleoside triphosphates (Sigma). 5 U of AmpliTaq DNA polymerase

(Promega Corporation. Madison. Wis.). PCR buffer 10X and 50 mM MgCl2. Amplification of the targeted genes for *V*. *cholerae* and *V*. *vulnificus* was carried out with 25 µM each oligonucleotide primer. However. for the detection of *V*. *parahaemolyticus*. 10 µM each of oligonucleotide primers F-vvh were used in order to achieve optimum multiplex PCR amplification All PCR amplifications were performed by using a GeneAmp PCR System 9700 (Applied Biosystems. USA). Reaction parameters was as follows:



After bringing the final temperature at 4°C. amplifieds were run on 1.2% agarose gel (Sigma) in 1x Tris-Borate-EDTA (TBE).and ethidium bromide (0.5 µg/ml). Images of the stained gels were visualized and acquired by

using a Gel Doc digital photo-documentation system (Bio-Rad Lab., Hercules. CA. USA).

Primers sequences (Roche diagnostics. Milan. Italy) used in this protocol are set in the table below:

Vibrio spp.	Gene	Primers	Amplicone size
V. cholerae	VC-toxR	**F:** 5'- CCT TCG ATC CCC TAA GCA ATA C -3' **R:** 5'- AGG GTT AGC AAC GAT GCG TAA G -3'	779 bp
V. parahemolyticus	VP-toxR	**F:** 5'- GTCTTCTGACGCAATCGTTG - 3' **R:** 5'- ATACGAGTGGTTGCTGTCATG - 3'	368 bp
V. vulnificus	VV-vvhA	**F:** 5'- TTC CAA CTT CAA ACC GAA CTA TGA C -3' **R:** 5'- ATT CCA GTC GAT GCG AAT ACG TTG -3'	205 bp

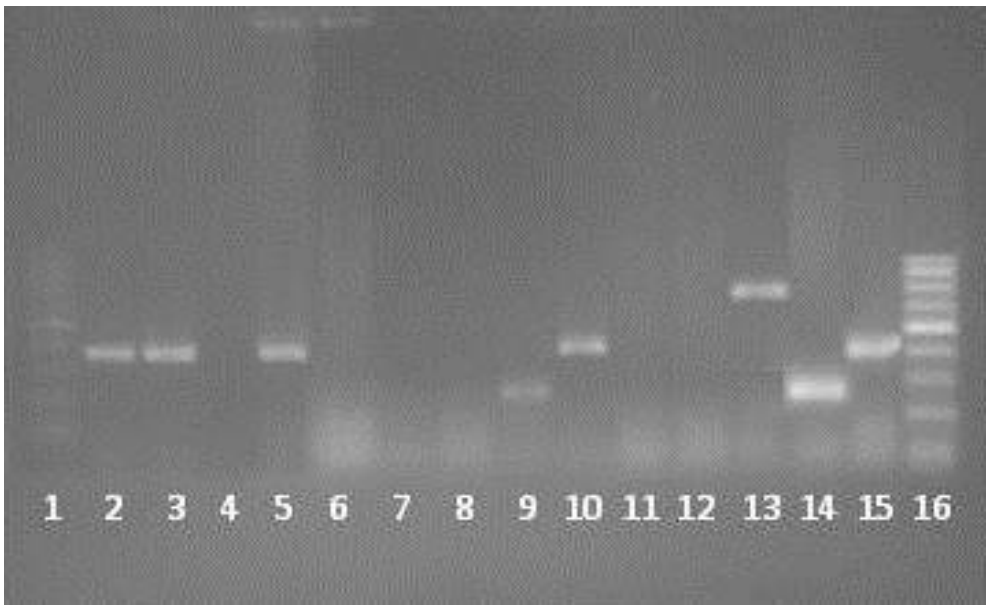


Figure n.1 - Gel electrophoresis on an agarose gel of mPCR products; 1, 16= molecular weight marker DNA Ladder; 2-3-5-10=*V. parahaemolyticus* strains; 9=*V. vulnificus strain*; 11=negative control; 12=reagent control;13-14-15=*V. cholera, V. vulnificus* and *V.parahaemolyticus* positive control.

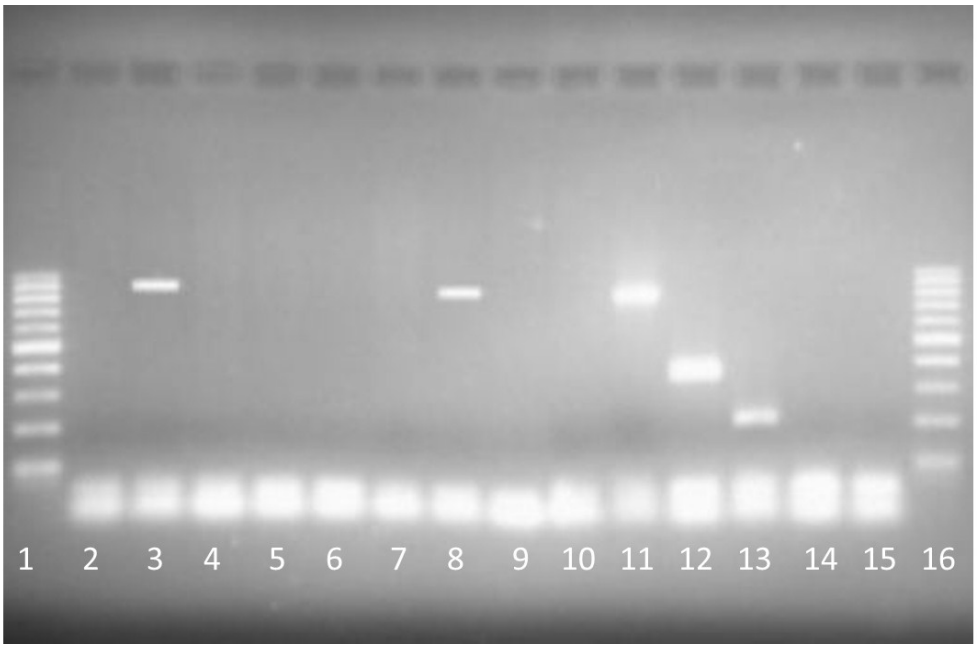


Figure n.2 - Gel electrophoresis on an agarose gel of mPCR products; 1, 16= molecular weight marker DNA Ladder; 3-8=*V. cholerae* strains*;* 11-12-13= *V. cholera, V.parahaemolyticus V. vulnificus* positive control*; 14*=negative control; 15=reagent control.

PCR analysis of virulence genes

In this study the virulence genes of *V.spp.* core was detected for *V. parahaemolyticus* only. Two different PCRs were standardized in order to detect the following 2 virulence-associated genes of *V. parahaemolyticus*: PCR 1:trh and PCR 2: tdh by modifying the protocols of Bej et al.(1999).

Primers sequences used in this protocol are set in the table below:

Gene	Primers	Amplicone size
tdh	**F**: 5' - GTAAAGGTCTCTGAC TTTTGGAC - 3' **R**: 5' - TGGAATAGAACCTTC ATCTTCACC - 3'	269 bp
trh	**F**: 5' - TTGGCTTCGATATTTTCAGTATCT - 3' **R**: 5' - CATACAAAC ATATGCCCATTTCCG - 3'	500 bp

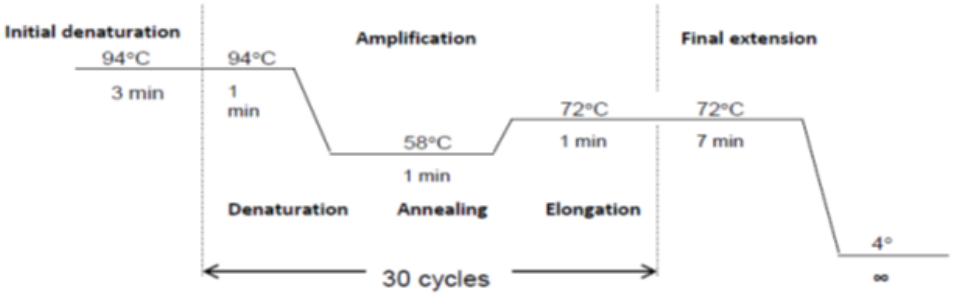
- PCR 1: aimed to amplify the trh gene to identify thermostable direct hemolysin-related. The oligonucleotide were designed based on the nucleotide sequences internal to the amplified segments of the respective targeted genes.

In order to test the method. a reference strains. *V. parahaemolyticus* ATCC 43996 (*toxR +*. *tdh +*. *trh +*) was used as positive control and *Listeria spp.* from internal collection of University of Sassari was used as negative control.

To prevent and control contamination. each PCR test included reagent control (without DNA).

Each PCR amplification was performed with a 25 µl reaction volume consisting of 10 ng of purified genomic DNA. 10 mM deoxynucleoside triphosphates (Sigma). 5 U/µl of AmpliTaq DNA polymerase (Promega

Corporation. Madison. Wis.). PCR buffer 10X and 50 mM MgCl2. Amplification of the targeted genes was carried out with 10 µM each oligonucleotide primer. All PCR amplifications were performed by using a GeneAmp PCR System 9700 (Applied Biosystems. USA). Reaction parameters were as follows:



PCR products were separated by electrophoresis on 1.2% (w ⁄ v) agarose gel and visualized under ultraviolet light after ethidium bromide (0.1 mg/ml) staining. A Gene Ruler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas) was used as a DNA size marker.

The gel images were visualized by Quantity-One software (Bio-Rad. USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad. Hercules. CA. USA).

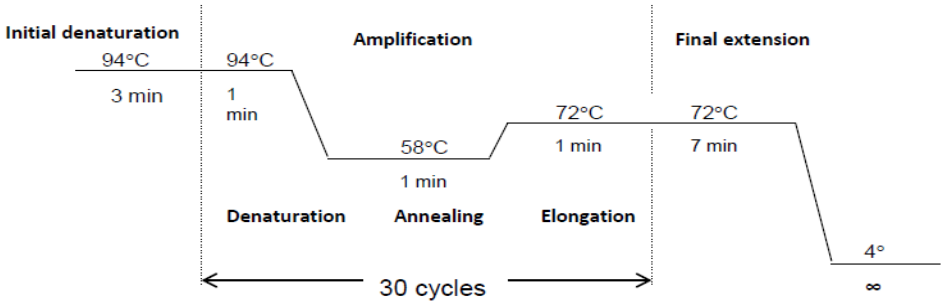
- PCR 2: aimed to amplify the *tdh* gene to identify thermostable direct hemolysin. The oligonucleotide were designed based on the nucleotide sequences internal to the amplified segments of the respective targeted genes.

In order to test the method. a reference strains. *V. parahaemolyticus* ATCC 43996 (*toxR* +. *tdh* +. *trh* -) was used as positive control and *Listeria spp.* from internal collection of University of Sassari was used as negative control.

To prevent and control contamination. each PCR test included reagent control (without DNA).

Each conventional PCR amplification was performed with a 25 µl reaction volume consisting of 10 ng of purified genomic DNA. 10 mM

deoxynucleoside triphosphates (Sigma). 5 U/µl of AmpliTaq DNA polymerase (Promega Corporation. Madison. Wis.). PCR buffer 10X. 25 mM MgCl2 and BSA (Bovine serum albumin) 2%. Amplification of the targeted genes was carried out with 25 µM each oligonucleotide primer. All PCR amplifications were performed by using a GeneAmp PCR System 9700 (Applied Biosystems. USA). Reaction parameters was:



PCR products were separated by electrophoresis on 1.5% (w ⁄ v) agarose gel and visualized under ultraviolet light after ethidium bromide (0.1 mg/ml) staining. A Gene Ruler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas) was used as a DNA size marker.

The gel images were visualized by Quantity-One software (Bio-Rad. USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad. Hercules. CA. USA).

Results

Multiplex PCR for detection of *Vibrio spp.*core confirmed the presence of the three different sub-species found with biochemical tests.

A total of 472 samples were analyzed with mPCR: n 185 samples of *M. galloprovincialis* before depuration and n 59 samples after; n 193 samples of *R. decussatus* directly from harvest and n 35 samples from depuration process.

Only two samples were *vvhA* positive. indicating that *V. vulnificus* was present in the samples. Both samples were from *R. decussatus* harvested in Rearing O before depuration process.

Instead 8 samples were *V. cholera* positive; n 6 of these samples were from *R. decussatus* and n 2 samples from *M. galloprovincialis*; all the samples were form Rearing O and from the same season.

A total of 114 samples were identified as. *V. parahaemolyticus.* All the samples were tested with conventional PCR for *trh* and *tdh* factors. Only 2 samples were *tdh+/trh-* and 2 were *tdh-/trh+.* Not a single sample was *tdh+/trh+*.

Strain code	Mollusc	Date	*Vibrio spp.*
C20-Plant A	*R. decussatus*	June 2011	*V. vulnificus*
C28-Plant A	*R. decussatus*	June 2011	*V. vulnificus*
I13-Plant O	*R. decussatus*	January 2012	*V. cholerae*
I14-Plant O	*R. decussatus*	January 201	*V. cholerae*
I15-Plant O	*R. decussatus*	January 201	*V. cholerae*
I16-Plant O	*R. decussatus*	January 201	*V. cholerae*
I17-Plant O	*R. decussatus*	January 201	*V. cholerae*
I18-Plant O	*R. decussatus*	January 201	*V. cholerae*
L1-Plant O	*M. galloprovincialis*	March 2012	*V. cholerae*
L2-Plant O	*M. galloprovincialis*	March 2012	*V. cholerae*

Table n.9 – Results of multiplex PCR for detection of *Vibrio spp.*core in relation with the date of harvest.

Discussion

Conventional phenotypic methods for the accurate identification of different *Vibrio species* are problematic. especially when requiring the

discrimination of closely related species (Kwok et al., 2002). because of the greater variability in biochemical characteristics (Thompson et al., 2004). The existence of atypical phenotype strains isolated from clinical samples as well from the environment has been reported (Vieira et al., 2001; Tarr et al., 2007). This fact further complicates the difficulties regarding the *Vibrio species* identification site by phenotypic characteristics. Hence the need for test samples with molecular methods.

Previous studies showed that in order to identify a potentially pathogenic strain. it is necessary to target multiple genes for PCR amplification. In this study. species-specific VP-*toxR*. pathogenic strain-specific *tdh* and *trh*. were selected for the detection of *V*. *parahaemolyticus* in shellfish by multiplex PCR (Bej et al. 1999) Similarly. species-specific *vvhA* was selected for the detection of *V*. *vulnificus* (Panicker et al., 2004) and species-specific *VC-toxR* for *V. cholerae* (Rivera et al. 2001).

A total of 472 samples were tested using a mPCR that was got ready selecting an appropriate target genes. oligonucleotide primers. PCR-reaction and cycling-parameters resulted in the amplification of three target genes (VP-*toxR*. *vvhA.* VC-*toxR*) simultaneously in a single PCR reaction. In addition. PCR amplification of multiple genes instead of a single gene from each of the *Vibrio spp*. would help to reduce false-negative identification during testing of a complex matrix such as shellfish tissue. However. multigene PCR amplification is limited to individual *Vibrio spp* how was demonstrated by other study (Panicker et al. 2004).

Samples tested in this study were selected from suspect colonies isolated after enrichment from ChromAgar and mCPC Agar and after biochemical positive tests.

The mPCR confirmed the biochemical tests only for two samples of *V. vulnificus*. that were isolated from *R. decussatus* harvested in rearing A in the same sampling on June 2011. *Vibrio spp*. showed differing sensitivity to

environmental conditions but it is difficult to assess the effect of a single parameter. as some factors are interactive as reported in previous study (Cavallo et al., 2002). Water temperature was considered one of the most important factor. with salinity. governing the distribution and abundance of pathogenic vibrios. This was confirmed also by this study. how demonstrated in the table n.9.

Among the *V. cholerae* isolates. interestingly was to notice that all the samples were isolated from rearing O and from the winter season. The mPCR confirmed the presence of *V. cholera* in both the molluscs (6 samples from *R. decussatus* and n 2 samples from *M. galloprovincialis*). In the temperate region. vibrios can be cultured and detected from seawater and seafood in the spring/summer period. while in general. they are found in scant amounts in winter when the temperature declines. but reappear in high concentration in the warm season (Igbinosa et al., 2008). This is not in accordance with the results of this study because *V. cholera* was present in

winter season. but didn't reappear in spring/summer. The results are showed in the table below. Seawater features were not relayed for rearing *O*. Research carried out by various authors on edible molluscs has shown the presence of *V. parahaemolyticus* in Italy. particularly in summer months (Cioglia et al.,1982; Gelosa L. 1981)

Also in our study *V. parahaemolyticus* was identified during this period of the year but not on January like reported in the study of Cavallo (2002). This is in accordance with different studies that demonstrate high presence of *V. parahaemolyticus* during all the seasons but low presence in winter. Moreover the fair detection of *Vibrio parahaemolyticus* and the scarcity of confirmation for *V. cholerae* and *V. vulnificus*. is in line with that reported in other studies. in relation to the lower spread of these species in proportion to the first ((Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al.

2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)(Sulla et al. 2009)Serratore et al., 2009; Serratore et al., 2006). In fact a total of 114 samples were identified as *V. parahaemolyticus* (24%). against 2% of *V. cholera* and 0.40% of *V. vulnificus*. Only the 7.7% of samples that were resulted *V*. *parahaemolyticus* using biochemical tests were confirmed by mPCR. This fact showed how is not enough to investigate Vibrios on the base of the phenotypical characteristic. but is necessary to use biomolecular techniques.

The data set of *V. parahaemolyticus* distribution were not in agreement with those of other Italian researchers (Croci *et al*., 2001; Ottaviani *et al*., 2005) who reported a high frequency of isolation during warmer months. while in this study most of the bacteria were isolated from April 2011 to March 2012 (76/114). Besides we had a high presence of *V*. *parahaemolyticus* in *R. decussatus* (61%) than in *M. galloprovincialis* (23%) in contrast with the last study about investigation of *V. parahaemolyticus*

in Italian marine coastal water (Ligurian Sea. central Adriatic Sea. North Adriatic Sea. South Adriatic Sea and Tyrrhenian Sea). (D. Ottaviani et al. 2012).

All 114 *V. parahaemolyticus* strains were tested for the gene encoding the thermo-stable direct haemolysin (*tdh*) and a *tdh*-related haemolysin gene encoding for *trh*. two haemolysins associated with *V. parahaemolyticus*-related illnesses (Nishibuchi and Kaper 1985; Shirai *et al*. 1990). The selected oligonucleotide primers were highly specific for their respective target gene segments and did not show any amplification when purified genomic DNA from bacterial species other than *V*. *parahaemolyticus* were subjected to PCR.

Only 2 samples were *tdh+/trh-* and 2 were *tdh-/trh+.* Not a single sample was *tdh+/trh+*. The 2 samples *tdh+/trh* were from clams of the two different coastal area (rearing A and rearing O). but from different season (May and December 2011). On May in rearing A the salinity. temperature

and pH were respectively 40 ppt. 19.6°C and 8.7. In December in rearing O were 38.9 ppt. 13.1°C and ph was 9.5.

The two samples *tdh-*/*trh+* were both from clams and from the same rearing and period (December 2011 and plant O).

This is in contrast with Ottaviani (2012). where positive *trh* samples were 13% and there was none for *tdh.* while in our study was 0.9% for each factor. As regard the molluscs. the dates .in accordance with the above-mentioned work. were more prevalent in the clams than in the mussels.

This result suggests that strains with these characteristics transited in our country only during a limited period of time or that they are found extremely rarely or are difficult to detect. In this regard. recent studies have reported an apparent inverse relationship between water temperature and prevalence of *tdh*-positive strains. suggesting that these toxigenic strains might have a selective advantage in surviving colder conditions or that they would be the first strains to have appeared in the

environment and are gradually being replaced by non-pathogenic strains as the water warms (DePaola *et al*., 2003b; Rodriguez-Castro *et al*., 2010).

It is also known that a series of environmental factors affect the occurrence and abundance of vibrios and that their impact varies in the different geographic areas (Martinez-Urtaza *et al*., 2008; Caburlotto *et al*., 2012). The salinity is the key factor influencing the occurrence of *Vibrio* and pathogenic *Vibrio* species while the water temperature is a variable influencing only secondarily.

For these reasons. the incidence and concentration of *Vibrio* spp. in similar or close coastal areas (such as those within the Mediterranean Sea) might be very different.

Stage 3 – Molecular characterization. typing and phylogenetic analysis

The last stage of this study was carried on in the "Departamento de Microbiologia y Parasitologia. Centro de Investigationes Biologicas (CIBUS)" of the Faculty of Biology (USC) in Santiago de Compostela (Spain) from March 2012 to July 2012. N 99 *V. parahaemolyticus* strains collected in the University of Sassari were analyzed in USC. The selection were from sample collected from May 2011 to December 2011 from *Mytilus* and *Ruditapes* of all harvest areas before and after filtration process.

All the 99 strains were re-processed to confirm the species. First of all they were sowed on TCBS. *V.parahaemolyticus* where colonies are green or blue green on the agar due to sucrose fermentation. Eight strains were resulted yellow (C1; C39. C43. D25. F55; F80. G25. H60) and 5 were not growed (C29. C30; D29; E59; F73). All the strains were sowed on TSA-s agar (1% NaCl). Remaining 86 samples were subject to multiplex-PCR to confirm the species.

A few samples that not were identified like *V. spp*. were sequenced using 16s ribosomal RNA (16s rRNA). The genes coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies as it is highly conserved between different species of bacteria.

Besides samples were subject to typing and phylogenetic analysis using PCR-based methods (ERIC-PCR. REP-PCR and BOX-PCR) to produce fingerprints of bacteria genomes.

DNA extraction

An isolated bacterial colony by TSA-s was suspended in 1 ml of PBS (Phosphate-buffered saline) in a microfuge tube. The cell suspension was centrifuged for 1 min at 12.000 rpm. The supernatant was removed. was added 200µl of InstaGene matrix (BioRad) to the pellet and was incubated at 56°C for 30 minute. The complex was vortexed and placed in a boiled waterbath for 8 minutes at 100°C. The cell suspension was vortexed and

centrifuged at 12.000 for 3 minute. The supernatant contained genomic DNA was ready to be used as a template for detection of *V.parahaemolyticus.*

Confirm of *V. parahaemolyticus* by conventional PCR

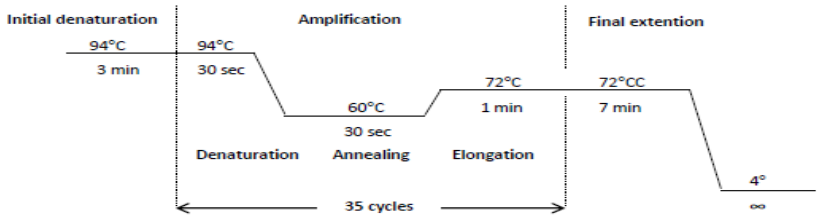
Conventional PCR that was carried out on 86 samples and was based on the Nhung et al.(2007) protocol that set primers including 1 universal forward primer (VM-F) and 5 reverse primers (MmR) specific for 5 species (*V. cholerae. V. parahaemolyticus. V. mimicus* and *V. alginolyticus*). Was used the modified protocol to look for *V. parahaemolyticus* only as reported in the following table:

V. spp.	Primer	Sequence (5' → 3')	Tm (°C)	Amplicone size
	VM-F[a]	CAGGTTTGYTGCACGGCGAAGA	73.2	
V. parahaemolyticus	Vp-MmR[b]	TGCGAAGAAAGGCTCATCAGAG	67.7	96 bp

[a]: Universal forward primer for 5 *Vibrio* species
[b] :Reverse primers specific to *Vibrio* species

Amplification reactions contained 12.5 µl of PCR MasterMix (2X) (Thermo Scientific). 1 µl of each primer 10 µM and 0.5 µl of the template in a final

reaction volume of 25 µl. PCR amplification was carried out in a thermal cycler (Eppendorf Mastercycler Engine DNA Thermal Cycler PCR) as follows:



PCR products were separated by electrophoresis on 1.5% (w ⁄ v) agarose gel and visualized under ultraviolet light after ethidium bromide (0.1 mg/ml) staining. A Gene Ruler^{TM} 100 bp DNA Ladder Plus (MBI Fermentas) as a DNA size marker. reference strain was *V. parahaemolyticus* ATCC 43996 and a negative control with H_2O were used.

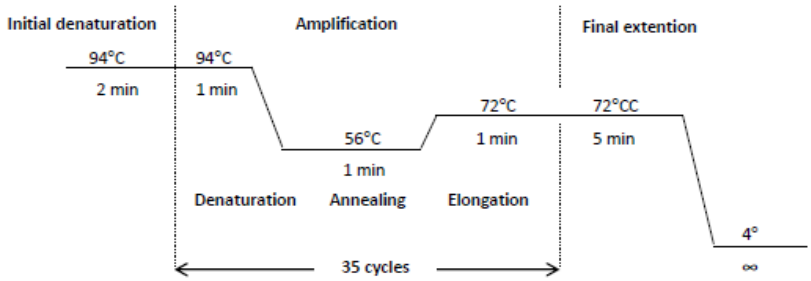
The gel images were visualized by Quantity-One software (Bio-Rad. USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad. Hercules. CA. USA).

PCR subunit 16s rDNA

A total of 14 samples were not identified as *V. spp.* and were compared to select the most similar on the base of the profile bands in the mPCR. N 4 samples were selected (D2. F1. F73. H13) and sequenced using 16s ribosomal RNA. It is approximately 1.5kb (or 1500 nucleotides) in length. Universal PCR primers were used to amplify the 16S rRNA gene providing the phylogenetic information. The following primer sets specific to the 16S rRNA gene of bacteria were used for PCR: 27FY: 5'-AGAGTTTGATCMTGGCTCAG -3' and 1510R: 5'-TAC GGY TAC CTT GTT ACG ACT T- 3' (Lane. D.J. 1991).

Ready-To-Go Pcr Beads (GE Healthcare) containing Taq Dna Polymerase. dntp's. Magnesium Chloride. reaction Buffer and stabilizers were used to set a conventional PCR to amplify 16s rRNA gene. The final reaction volume is 25 µl. In Each Ready-To-Go Pcr Beads tubes was added 1 µl of the

suspension of extracted template DNA. 22 µl of sterile distilled water and 1 µl of each primer. PCR was performed for as following in a thermal cycler (Eppendorf Mastercycler Engine DNA Thermal Cycler PCR):



The result of PCR amplification was examined by electrophoresis on 1.5% agarose gels and was dispatched to STAB VIDA laboratories (Universidade Nova de Lisboa-Monte da Caparica Campus-Life Sciences Department). The sequences was analyzed using the software DNASTAR Lasergene. The application SeqMan was used for contig. assembly and analysis while EditSeq for importing and editing file types. The programs to compare

nucleotide sequences with sequences databases and calculates the statistical significance of matches were BLAST (http://blast.ncbi.nlm.nih.gov/) and EzTaxon.(http://147.47.212.35:8080/index.jsp) (Tab 2).

ERIC-PCR

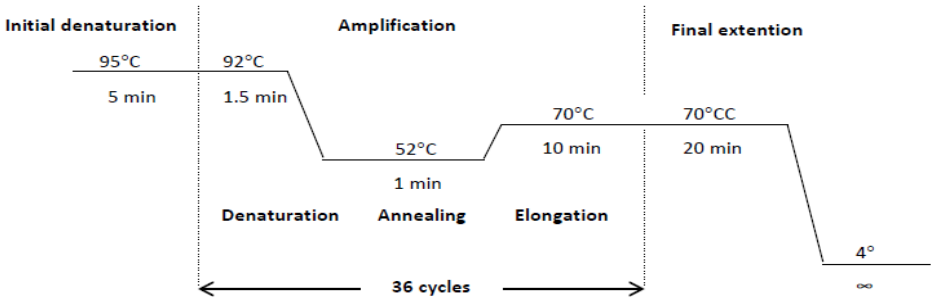
Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR for typing n 72 *V. parahaemolyticus* samples was carried out as indicated in the protocol of Versalovic (Versalovic et al.,1991) and the oligonucleotide sequences used were as follows:

Primer	Oligonucleotide Sequence
ERIC1R	5'-ATG TAA GCT CCT GGG GAT TCA -3'
ERIC2	5'- AAG TAA GTG ACT GGG GTG AGCG – 3'

The PCR mixture (pr. sample) was made using PCR MasterMix (2X) (Thermo Scientific) adding $MgCl_2$ (25 mM). 4 µl of each primer 100 µM. BSA (Bovine

Serum Albumin) (2%) and 1000 ng of the template in a final reaction volume of 25 µl.

PCR amplification was performed in an automated thermal cycler (Eppendorf Mastercycler Engine DNA Thermal Cycler PCR) using the following thermo cycling programme:



Of each PCR reaction 12 µl were then electrophoresed directly on 1 % agarose gels containing 1 xTAE (Tris acetate-EDTA). 0.5 µg/ml ethidium bromide. Were used the FastRuler™ Ladder 1 kb (Thermo Fisher Scientific) molecular weight marker to confirm amplification of the DNA fragments. reference strain *V. parahaemolyticus* ATCC 43996 and a negative control with H_2O.

The gels were visualized by Quantity-One software (Bio-Rad. USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad. Hercules. CA. USA).

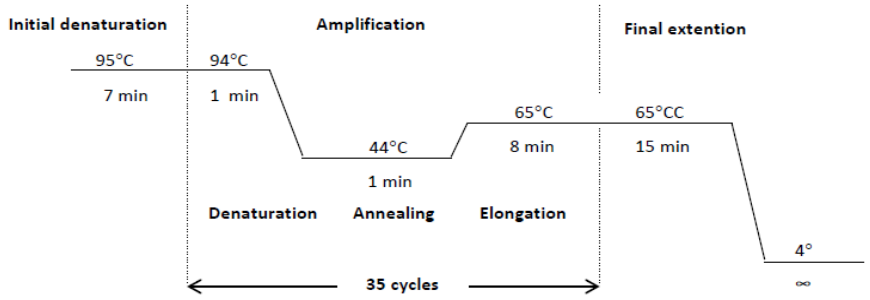
A dendrogram was constructed using the data matrix of all 72 strains of bacteria based on unweighted pair group method with arithmetic means (UPGMA) (Sneath and Sokal. 1973) using the Bionumerics software. version 6.6.4 (Applied Maths NV).

Rep-PCR

Repetitive Extragenic Palindromic (Rep) PCR analysis was performed in accordance with Versalovic et al.,(1991b) modified protocol to group all 72 *V. parahaemolyticus* strains. Primers that anneals so-called repetitive DNA elements distributed more or less randomly over the genome. were used as following:

Primer	Oligonucleotide Sequence
REP1D	5'- NNN RCG YCG NCA TCM GGC - 3'
REP2D	5'- AGC GCT CTT ATC ACG GCC TAC - 3'

Each 25 µl PCR reaction contained PCR MasterMix (2X) (Thermo Scientific). 100 µmol each of 2 opposing primers. 1000 ng of template (genomic) DNA. $MgCl_2$ (25 mM) and BSA (Bovine Serum Albumin) (2%). PCR amplifications were performed in an automated thermal cycler (Eppendorf Mastercycler Engine DNA Thermal Cycler PCR) with the following conditions:



PCR-products were separated by agarose gel electrophoresis (120 V. 1 h).preparing a 1.5 % agarose gel with ethidium bromide (0.5 µg mL^{-1}) in 1×TBE. running chamber was filled with 1×TBE (10×TBE = 108 g Trisbase/l. 55 g boric acid/l and 40 ml of 0.5 M EDTA. pH 8.0). 12 µl of PCR-products

were mixed with 5 µl of Loading Dye (Sigma) and was loaded up gel. Was used the FastRuler™ Ladder 1 kb (Thermo Fisher Scientific) molecular weight marker to confirm amplification of the DNA fragments. reference strain *V. parahaemolyticus* ATCC 43996 and a negative control with H_2O.

The gels were visualized by Quantity-One software (Bio-Rad. USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad. Hercules. CA. USA).

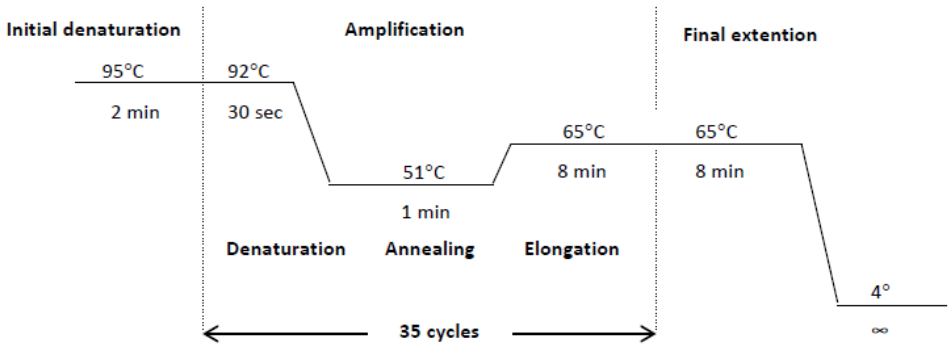
Cluster analysis were carried out using BioNumerics software. version 6.6.4 (Applied Maths NV) and UPGMA as the clustering algorithm. A dendrogram was constructed using the data matrix of all 72 strains.

BOX-PCR

BOX-PCR fingerprint technique was performed on the 72 *V. parahaemolyticus* samples using highly conserved repeated DNA element and was used a single primer how indicated by Smith et al.(2001).

Primer	Oligonucleotide Sequence
BOXA1R	5'- CTA CGG CAA GGC GAC GCT GAC G- 3'

25 µl PCR reaction mix for each sample was compounded by PCR MasterMix (2X) (Thermo Scientific). 100 Mmol primer. 1500 ng of template (genomic) DNA. $MgCl_2$ (25 mM) and BSA (Bovine Serum Albumin) (2%). PCR amplifications were performed in an automated thermal cycler (Eppendorf Mastercycler Engine DNA Thermal Cycler PCR) with the following conditions:



10 µl of each PCR reaction were electrophoresed directly on 1 % agarose gels containing 1 xTAE (Tris acetate-EDTA). 0.5 µg/ml ethidium bromide. We used the FastRuler™ Ladder 1 kb (Thermo Fisher Scientific) molecular

weight marker to confirm amplification of the DNA fragments. reference strain *V. parahaemolyticus* ATCC 43996 and a negative control with H_2O .

Gels were visualized by Quantity-One software (Bio-Rad. USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad. Hercules. CA. USA).

A dendrogram was constructed using the data matrix of all 72 strains of bacteria based on unweighted pair group method with arithmetic means (UPGMA) using the Bionumerics software. version 6.6.4 (Applied Maths NV).

Results

A total of 99 strains of *V. parahaemolyticus* isolated in our laboratories of University of Sassari were analyzed in USC laboratories. N 8 strains were resulted yellow on TCBS agar (C1; C39. C43. D25. F55; F80. G25. H60) and 5 were not growthed (C29. C30; D29; E59; F73). N 72 samples were

confirmed *V. parahemolyticus* and n 14 were non confirmed by Nhung protocol.

A PCR protocol-subunit 16s rDNA was applied on 4 most representatives samples (D2. F1. F73. H13) of the remaining 14 samples that were not identified by Nhung protocol; the sequenceses of the 16s rDNA were compared with nucleotide sequences present in two different database: EzTaxon.(http://147.47.212.35:8080/index.jsp) and Genebank (http://www.ncbi.nlm.nih.gov/genbank/). Search. align and calculation of the statistical significance of matches databases sequences to a query sequence was performed using BLAST (Basic Local Alignment Search Tool). In the Table __ was reported the results.

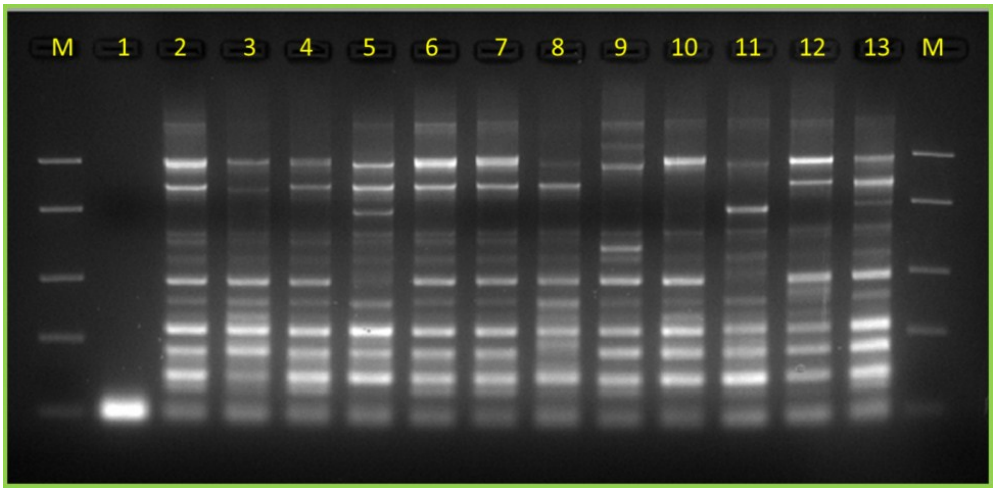
Table_:. Results of 16s rDNA sequences

Sample	Genebank %	EzTaxon
F1	V. alginolyticus 99% V. azureus 99% V. parahaemolitycus 99%	V. owensii 99.44% V. azureus 99.31%
D2	V. owensii 99% V. azureus 99% V. natriegens 99%	V. azureus 99.46% V. owensii 99.44%
F73	V. azureus 99% V. parahaemolitycus 99% V. owensii 99%	V. owensii 99.44% V. azureus 99.31%

H13	V. alginolyticus 99% V. azureus 99% V. parahaemolitycus 99%	V. alginolyticus 99% V. azureus 99.31%

Seventy-two samples were confirmed *V. parahaemolyticus* by conventional PCR and were further evaluated ERIC-PCR. REP-PCR and BOX-PCR methods for fingerprinting with a typical profile as reported in the images below:

Figure n.1 - Gel electrophoresis on an agarose gel of ERIC-PCR products; M= molecular weight marker DNA Ladder; 1= negative control; 2-12= bacterial isolates; 13= *V.parahaemolyticus*



Figuren.2 - Gel electrophoresis on an agarose gel of REP-PCR products; M= molecular weight marker DNA Ladder; 1= negative control; 2-12= bacterial isolates; 13=*V.parahaemolyticus*

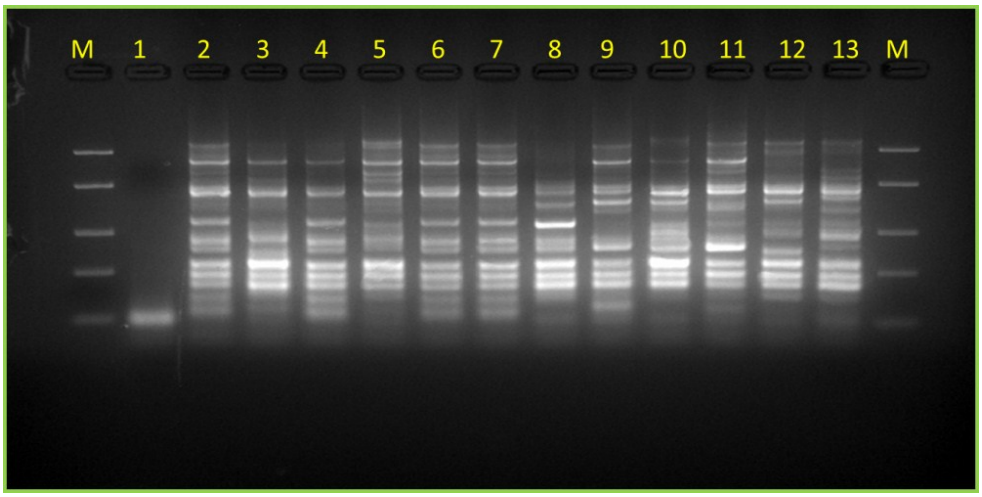
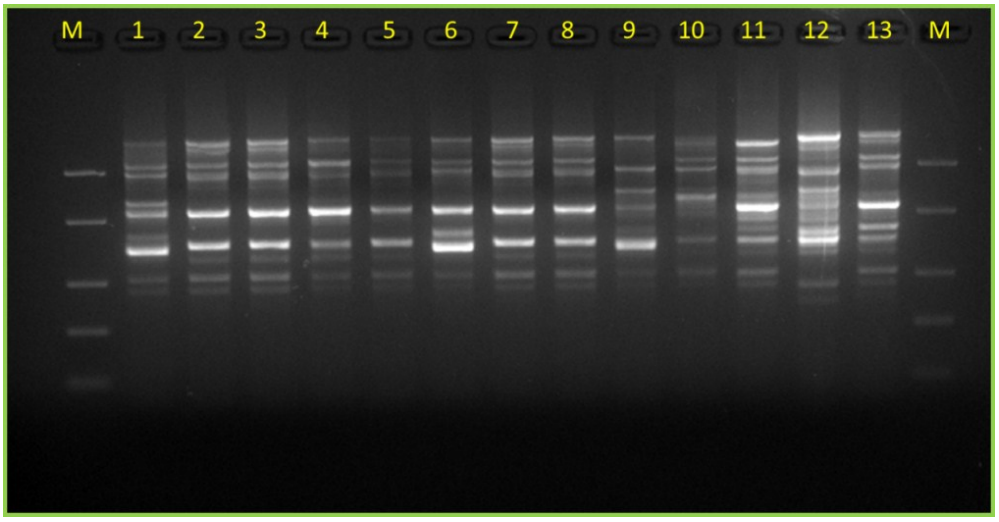


Figure n.3 - Gel electrophoresis on an agarose gel of BOX-PCR products; M= molecular weight marker DNA Ladder; 1= negative control; 2-12= bacterial isolates; 13= *V.parahaemolyticus*



For the analysis with ERIC- REP- and BOX-PCR. a reference strain (VP ATCC 43996) was used in every single experiment in order to evaluate the

reproducibility of all the PCR amplification process and the gel electrophoresis profiles.

Gel images were saved as TIFF files. normalized with the above mentioned molecular size marker. and further analysed by BioNumerics software. To consctruct the dendrograms. levels of similarity between the profiles were calculated by using the band-matching Dice coefficient (S_D) (Dice. 1945).and the cluster analysis of similarity matrices was calculated with unweighted pair group method with arithmetic averages (UPGMA) (Sneath & Sokal. 1973). Isolates were assigned to a different type when any band differences were observed.

How reported in the dendrogram of ERIC-PCR all strains included in cluster A were isolated in autum. Strains *trh+* (1 isolate) and *tdh+* (1 isolates) were also grouped in this cluster. Moreover cluster indicted with the asterisch grouped strains isolated majority in December. The rest of strain were spread among the other sub-cluster.

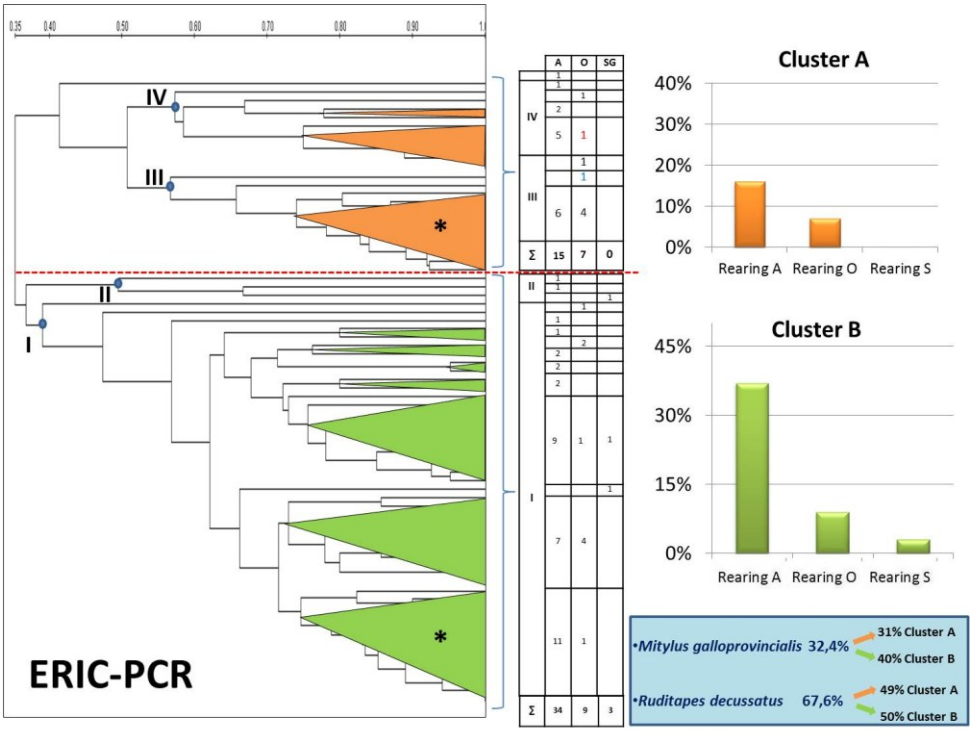


Figure n.4 - Dendrogram of the 72 *V. parahaemolyticus* isolates from different coastal areas of Sardinia based on ERIC-PCR fingerprint analysis (Dice similarity coefficient and UPGMA clustering). Alphabetic letters indicate the geographical origin. *: groups of strains isolated in December. The coefficient of similarity is 70%. The prevalence of distribution of strains in relation with mussels is showed.

The dendrogram about REP-PCR showed how the strains grouped in the cluster A were all from *R. decussatus* and just one from *Mitylus galloprovincialis* also were isolated in autumn as well as ERIC-PCR method. Cluster B I grouped strains isolated majority in December as well as in ERIC-PCR method.

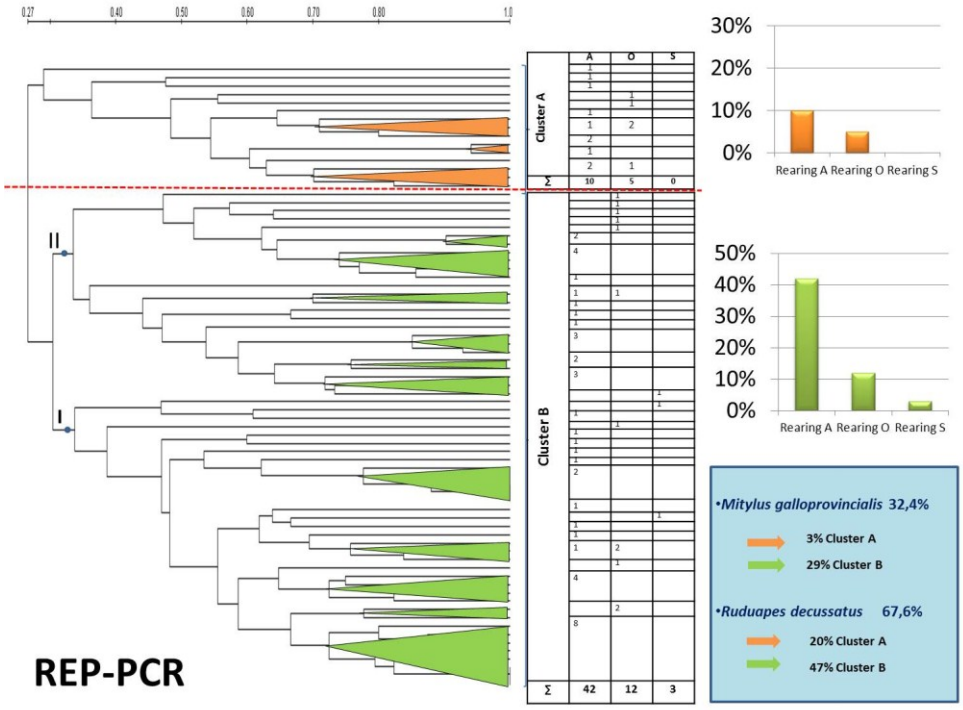


Figure n.5 - Dendrogram of the 72 *V. parahaemolyticus* isolates from different coastal areas of Sardinia based on REP-PCR fingerprint analysis (Dice similarity coefficient and UPGMA clustering). Alphabetic letters indicate the geographical origin. The coefficient of similarity is 70%. The prevalence of distribution of strains in relation with mussels is showed.

The last dendrogram regarding BOX-PCR reported how the strains classified in the cluster A did not a particular temporal or geographic distribution. but a lot of the strains collected during December 2011 were grouped in the cluster B and that were also grouped in the cluster B using ERIC-PCR and REP-PCR methods. Finally in ERIC-PCR and REP-PCR methods the samples G110. G111. G115 were detected as clones.

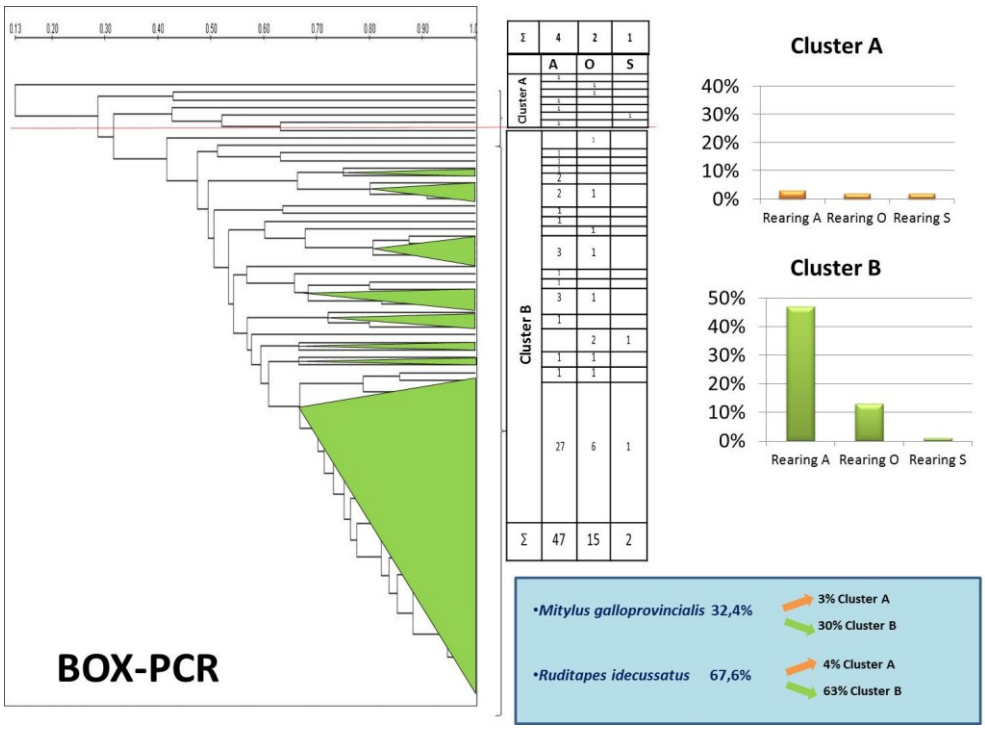


Figure n.6 - Dendrogram of the 72 *V. parahaemolyticus* isolates from different coastal areas of Sardinia based on BOX-PCR fingerprint analysis (Dice similarity coefficient and UPGMA clustering). Alphabetic letters indicate the geographical origin. The coefficient of similarity is 70%. The prevalence of distribution of strains in relation with mussels is showed.

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Genotyping methods allowed the identification of profiles with amplification bands ranging. Genotyping by ERIC-PCR allowed the identification of profiles with 9-13 amplification bands ranging from 90 to2780 kb that originated 2 clusters. Genotyping by REP-PCR allowed the identification of profiles with 9-15 amplification bands ranging from 102 to2128 kb that originated 2 clusters.

Genotyping by BOX-PCR allowed the identification of profiles with 8-10 amplification bands ranging from 98 to2780 kb that originated 2 clusters.

The dendrogram reported in Fig.:__ show similarity of strains profiles comparing the data set of three typing method and demonstrate how the results of the different technique grouped strains in 2 clusters in accordance with each singular dendrogram. The high number of amplification bands was in REP-PCR method demonstrating a major discrimination power. This fact is proved by the highest number of different DNA fragment patterns (33 patterns in REP-PCR. 31 patterns in ERIC-PCR and 30 in BOX-PCR).

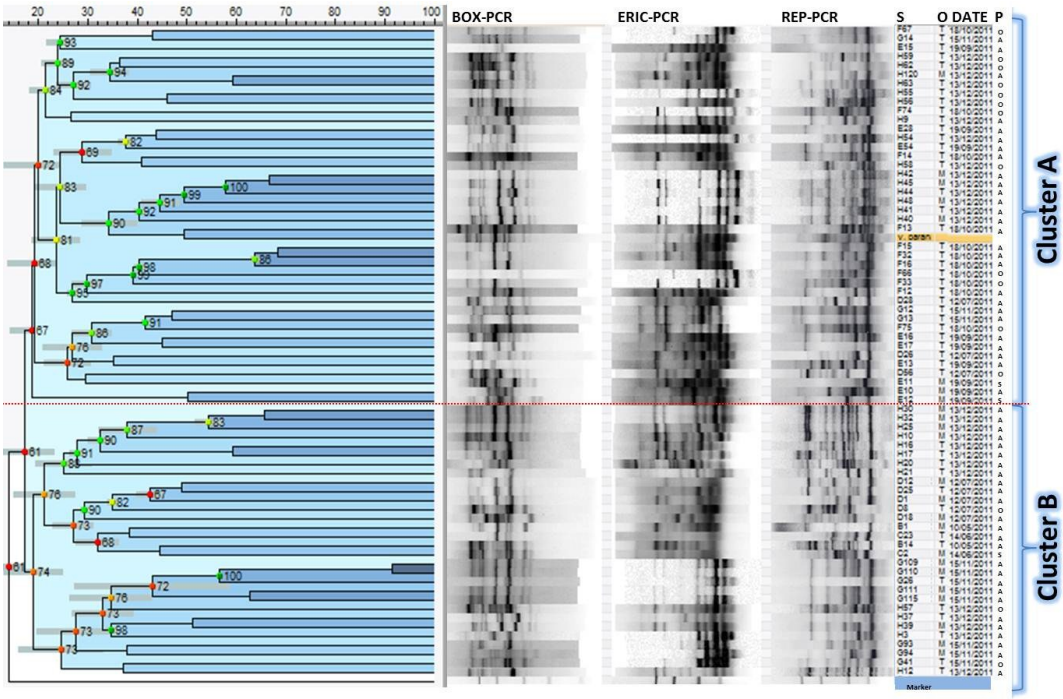

Figure n.7 - Dendrogram obtained using Dice similarity coefficient and UPGMA analysis comparing data set of ERIC-. REP- and BOX-PCR methods . Dendrogram show phylogenetic relationship of *V. parahaemolitycus* strains from three fingerprint PCR-based method. The numbers reported in the dendrogram show average of the individual matrices; S: sampe name; O: origin (M=*M. galloprovincialis*; T=*R. decussatus*); P: .

SAMPLE	MOLLUSC	REARING	DATE	TCBS GROWTH	V.p. tox R	V.p. Mm-R	tdh+	trh+	T°C	ppt	pH
B1	M. galloprovincialis	A	May 2011	g	+	+	0	0	19.64	40	8.72
C1	M. galloprovincialis	O	June 2011	y	+	-	0	0	-	-	-
C2	M. galloprovincialis	S	June 2011	g	+	+	0	0	21.29	33	8.37
D1	M. galloprovincialis	A	July 2011	g	+	+	0	0	22.81	40	7.93

D12	M. galloprovincialis*	A	July 2011	g	+	+	0	0	22.81	40	7.93
D17	M. galloprovincialis*	A	July 2011	g	+	0	0	0	22.81	40	7.93
D18	M. galloprovincialis*	A	July 2011	g	+	+	0	0	22.81	40	7.93
D2	M. galloprovincialis*	A	July 2011	g	+	0	0	0	22.81	40	7.93
E10	M. galloprovincialis	A	September2011	g	+	+	0	0	23.7	40	7.92
E11	M. galloprovincialis	S	September2011	g	+	+	0	0	22.94	36	8.55
E12	M. galloprovincialis	S	September2011	g	+	+	0	0	22.94	36	8.55
F1	M. galloprovincialis	A	October 2011	g	+	0	0	0	20.79	40	7.92
G93	M. galloprovincialis	A	November 2011	g	+	+	0	0	17.86	39	7.58
G94	M. galloprovincialis	A	November 2011	g	+	+	0	0	17.86	39	7.58
G109	M. galloprovincialis*	A	November 2011	g	+	+	0	0	17.86	39	7.58
G110	M. galloprovincialis*	A	November 2011	g	+	+	0	0	17.86	39	7.58
G111	M. galloprovincialis*	A	November 2011	g	+	+	0	0	17.86	39	7.58
G115	M. galloprovincialis*	A	November 2011	g	+	+	0	0	17.86	39	7.58
H39	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H42	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H120	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H25	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H45	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H40	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H32	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H30	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H48	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53
H1	M. galloprovincialis	O	December 2011	g	+	0	0	0	-	-	-
H10	M. galloprovincialis	A	December 2011	g	+	+	0	0	13.12	38	9.53

Table n.9 - *M. galloprovincialis* isolates in relation with parameters of seawater. *: depurated sample; y: yellow; g: green; ng: not grouted; +: positive; -: not investigate; 0: negative.

SAMPLE	MOLLUSC	REARING	DATE	TCBS GROWTH	V.p. toxR	V.p. Mm-R	*tdh+*	*trh+*	T°C	ppt	pH
B14	R. decussatus	A	May 2011	g	+	+	+	0	19.6	40	8.7
B31	R. decussatus	O	May 2011	g	+	0	0	0	-	-	-
B41	R. decussatus	O	May 2011	g	+	0	0	0	-	-	-
C23	R. decussatus	A	June 2011	g	+	+	0	0	21.5	40.12	8.2

C29	R. decussatus	O	June 2011	ng	+	-	0	0	-	-	-
C30	R. decussatus	O	June 2011	ng	+	-	0	0	-	-	-
C39	R. decussatus	O	June 2011	y	+	-	0	+	-	-	-
C4	R. decussatus	O	June 2011	g	+	+	0	0	-	-	-
C43	R. decussatus	S	June 2011	y	+	-	0	0	21.3	33.03	8.4
D25	R. decussatus	A	July 2011	y	+	+	0	0	22.8	40.1	7.9
D26	R. decussatus	A	July 2011	g	+	+	0	0	22.8	40.1	7.9
D28	R. decussatus	A	July 2011	g	+	+	0	0	22.8	40.1	7.9
D29	R. decussatus*	A	July 2011	ng	+	-	0	0	22.8	40.1	7.9
D5	R. decussatus	O	July 2011	y	+	0	0	0	-	-	-
D56	R. decussatus	O	July 2011	g	+	+	0	0	-	-	-
D8	R. decussatus	O	July 2011	g	+	+	0	0	-	-	-
E13	R. decussatus	A	September 2011	g	+	+	0	0	23.7	40.5	7.9
E15	R. decussatus	A	September 2011	g	+	+	0	0	23.7	40.5	7.9
E16	R. decussatus	A	September 2011	g	+	+	0	0	23.7	40.5	7.9
E17	R. decussatus	A	September 2011	g	+	+	0	0	23.7	40.5	7.9
E28	R. decussatus	A	September 2011	g	+	+	0	0	23.7	40.5	7.9
E54	R. decussatus	A	September 2011	g	+	+	0	0	23.7	40.5	7.9
E59	R. decussatus	O	September 2011	ng	+	-	0	0	-	-	-
F12	R. decussatus	A	October 2011	g	+	+	0	0	20.8	40.1	7.9
F13	R. decussatus	A	October 2011	g	+	+	0	0	20.8	40.1	7.9
F14	R. decussatus	A	October 2011	g	+	+	0	0	20.8	40.1	7.9
F15	R. decussatus	A	October 2011	g	+	+	0	0	20.8	40.1	7.9
F16	R. decussatus	A	October 2011	g	+	+	0	0	20.8	40.1	7.9
F32	R. decussatus	A	October 2011	g	+	+	0	0	20.8	40.1	7.9
F33	R. decussatus	O	October 2011	g	+	+	0	0	-	-	-
F55	R. decussatus	O	October 2011	y	+	-	0	0	-	-	-
F64	R. decussatus	O	October 2011	g	+	0	0	0	-	-	-
F66	R. decussatus	O	October 2011	g	+	+	0	0	-	-	-
F67	R. decussatus	O	October 2011	g	+	+	0	0	-	-	-
F72	R. decussatus	O	October 2011	g	+	0	0	0	-	-	-
F73	R. decussatus	O	October 2011	ng	+	-	0	0	-	-	-
F74	R. decussatus	O	October 2011	g	+	+	+	0	-	-	-
F75	R. decussatus	O	October 2011	g	+	+	0	0	-	-	-
F78	R. decussatus	S	October 2011	g	+	0	0	0	23.4	36.24	8.5
F80	R. decussatus	S	October 2011	y	+	0			23.4	36.24	8.5

SAMPLE	MOLLUSC	REARING	DATE	TCBS GROWTH	V.p. toxR	V.p. Mm-R	*tdh+*	*trh+*	T°C	ppt	pH
G12	R. decussatus	A	November 2011	g	+	+	0	0	17.9	39.5	7.6
G13	R. decussatus	A	November 2011	g	+	+	0	0	17.9	39.5	7.6
G14	R. decussatus	A	November 2011	g	+	+	0	0	17.9	39.5	7.6
G24	R. decussatus*	A	November 2011	g	+	0	0	0	17.9	39.5	7.6

G25	R. decussatus*	A	November 2011	y	+	0	0	0	17.9	39.5	7.6
G26	R. decussatus*	A	November 2011	g	+	+	0	0	17.9	39.5	7.6
G41	R. decussatus	O	November 2011	g	+	+	0	0	-	-	-
G64	R. decussatus*	O	November 2011	g	+	0	0	0	-	-	-
H17	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H21	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H41	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H37	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H44	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H16	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H12	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H13	R. decussatus	A	December 2011	g	+	0	0	0	13.1	38.9	9.5
H20	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H3	R. decussatus	A	December 2011	g	+	+	0	+	13.1	38.9	9.5
H5	R. decussatus	A	December 2011	g	+	0	0	+	13.1	38.9	9.5
H9	R. decussatus	A	December 2011	g	+	+	0	0	13.1	38.9	9.5
H54	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H55	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H56	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H57	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H58	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H59	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H60	R. decussatus	O	December 2011	y	+	-	0	0	-	-	-
H61	R. decussatus	O	December 2011	g	+	0	0	0	-	-	-
H62	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-
H63	R. decussatus	O	December 2011	g	+	+	0	0	-	-	-

Table n.10 - *R. decussatus* isolates in relation with parameters of seawater. *: depurated sample; y: yellow; g: green; ng: not grouted; +:positive; -: not investigate; 0: negative.

Discussion

Thirty-six strains were isolated from *M. galloprovincialis* samples. while 88 were from *R. decussatus*. A subset of 86 strains was characterized by conventional PCR-based and 72 were confirmed as *V. parahaemolyticus*. Among these. 66 strains were isolated from molluscs before the depuration process. and only 6 strains from molluscs after depuration process.

Molecular methods have given significant advances over conventional phenotypic methods to the identification and differentiation of closely related species. In the literature many genetic approaches for the identification of Vibrio species have been reported(Kim et al., 1999; Nandi et al., 2000; Blackstone et al., 2003; Panicker et al., 2004; Takahashi et al., 2005; Gubala. 2006). The multiplex PCR modified protocol used in the USC laboratories represented a valid alternative molecular approach for specific and rapid detection of vibrios (Nhung et al. 2007). Furthermore is known that vibrios seem to have fewer mobile genetic elements. e.g., transposons

and phages. and DNA regions with a G+C content that differ from the whole-genome average. which are indicative of recent horizontal transfer (Heidelberg et al., 2000; Makino et al., 2003). Nevertheless, it has been demonstrated by studies in the last few years that horizontal gene transfer has contributed to plastic of vibrio genomes. For this reason in this work the *V. parahaemolyticus* samples collected in Sardinia were re-tested to confirm the real belonging to the sub-species.

Four representatives samples not confirmed as *V.parahaemolyticus* were subjected to the 16S rRNA gene sequences to elucidate the phylogenetic structure of the vibrios. Several *Vibrionaceae* species have nearly identical 16S rRNA gene sequences like demonstrated by result reported in the table__ where two different databases showed the high similarity between *V. parahaemolyticus* (99%) and other different sub-species (*V. alginolyticus* 99%. *V. azureus* 99%. *V. owensii* 99%. *V. natriegens* 99%). This is in accordance with a study that reported the overall level of similarity among

some Vibrio 16S rRNA gene sequences (85.6%). Seven species appeared to be closely related. sharing an overall level of sequence similarity of 91.3% (*V. alginolyticus. V. campbellii. V. harveyi. V. natriegens. V. parahaemolyticus. V. vulnifcus* and *V. proteolyticus*)(Dorsch et al., 1992).

In recent years. how mentioned above. phenotypic methods used to characterize strains belonging to a bacterial species are not sufficient and have been largely replaced by genotypic methods. followed by various PCR-based fingerprinting techniques (Versalovic et al., 1991; Olive et al., 1999; van Belkum et al., 2001).

In this study the 72 strains confirmed *V. parahaemolyticus* were subjected to PCR-based fingerprinting with short random primers or primers directed against repetitive sequences in the bacterial genome (Versalovic et al., 1991;

Colombo et al., 1997; Olive et al., 1999; Maluping et al., 2005; Rodrìguez et al., 2006).

ERIC- and REP-PCR have been applied recently to investigate the genetic variability among *V. parahaemolyticus* and *Vibrio tapetis* strains (Maluping et al., 2005; Rodrìguez et al., 2006; Silva-Rubio et al., 2008; Kingston et al., 2009; Rao & Surendran. 2010): both methods revealed good discriminative ability and the authors concluded that they can be used as a rapid means of comparing Vibrio strains for epidemiological investigation.

For this reason. in the present study. these two methods. in addition to BOX-PCR technique. have been used to genotype a collection of *V. parahaemolyticus* isolated from shellfish in the marine environment of Sardinia Sea (Italy).

Regarding the evaluation of the three genotyping methods used. we have focused on the discriminatory power that defines the ability of a typing method to distinguish different strains. in that their reproducibility has been demonstrated to be comparable in our hands.

Several strains rendered a unique pattern regardless of the typing method used which indicated the high discriminatory power of the methods. Also the exist high heterogeneity among the *V. parahaemolyticus* strains.

The majority of strains isolated in autumn were grouped in a unique cluster by all the typing method used. These results are in agreement with previous data reported in the literature by other research groups (Maluping et al., 2005) on this bacterial species.

Strain E15 was also revealed to be very different genetically from reference strain using ERIC-and BOX-PCR methods (similarity of 11.8% with ERIC-PCR and 13.3% with BOX-PCR). while using REP-PCR the same strain had a similarity of 50%.

A number of strains were grouped into clones by the three molecular typing techniques: with ERIC-PCR G109. G110 and G115 were included in cluster A. with REP-PCR G110. G111 and G115 were included in cluster B while with BOX-PCR G110. G111. G115 and H30 were included in cluster B. The

sampling date. the site and other characteristics of the strains supported the inclusion of the strains in the specific clone.

A number of examples suggest a relationship between some strains and a geographic site. such as the case of BOX-PCR where the majority of strains of the group I in the cluster B have been isolated from rearing A. The majority of strains that were grouped in the same cluster B using ERIC- and REP-PCR were from the same site mentioned above.

As concerns the isolation period. we had identified some strains isolated from September 2011 to December 2011 that were grouped in the same cluster (cluster A with ERIC-and REP-PCR and cluster B group I with REP-PCR) while BOX-PCR did not show clusters or groups in relation to season.

In relation with molluscs the most important result was the cluster A of REP-PCR that grouped strains isolated only from *R. decussatus.*

Using the three techniques we obtained two cluster (A and B) for each methods. In the clusters B were grouped always the greater number of

strains from clams and mussels. but the highest percentages were from clams with all methods.

Another important observation was made regarding the persistence of some genetic clusters over time: in some cases. strains showing very similar genetic profiles were isolated for two or three subsequent months such as reported by Caburlotto et al.(2011).

Strains B14 and F74 (*tdh+/trh-*) were grouped in the same cluster with ERIC-PCR method. Using BOX-PCR method strains B14. F74 (*tdh+/trh-*) and H3 (*tdh-/trh+*) were grouped in the same cluster B. REP-PCR method grouped B14 (*tdh+/trh-*) and H3 (*tdh-/trh+*) strains in the cluster B. These results supporting the possibility for virulence genes to be acquired. via horizontal genetic transfer in accordance with other studies (Caburlotto et al. 2011).

Low incidences of toxigenic (carrying *tdh* and/or *trh* genes) *V. parahaemolyticus* in the marine environment have been reported many times (Nishibuchi and Kaper. 1995; US FDA. 2001; DePaola *et al*., 2003a).

However. Ottaviani et al. (2012). showed results about *V. parahaemolyticus* *trh*-positive strains in the Mediterranean Sea confirmed their presence in all the geographical areas. Prevalence (13%). in agreement with previous italian studies (Ottaviani *et al*., 2010a; Serracca *et al*., 2011). was higher with respect to that reported in extra-European and European countries (Robert-Pillot *et al*., 2004; Bauer *et al*., 2006; Wagley *et al*., 2008; Rodriguez-Castro *et al*., 2010). In our study the prevalence for each virulent factor was 0.9% and they were isolated in spring and autumn and only from *R. decussatus.* This was in accordance with extra-European and European countries studies where the seawater conditions were different from the Mediterranean Sea.

In summary. the intraspecific typing of environmental *V. parahaemolyticus* can be of great interest for the recognition of clonal relationships between environmental. foodborne and for studying the geographical and temporal distribution of this human and fish pathogen.

Moreover. the use of multiple typing methods would allow a more accurate characterization of the genetic profiles of isolates and the identification of clones hardly revealed through the common techniques.

Genetic profile and dendrograms are showed in figure_ _ _ and in table _ _ _ . The typing method used in this study displayed a more accurate characterization of the strain genetic profiles and the identification of clones hardly revealed through common techniques. In this work. how is possible to observing the genetic profile. ERIC-and REP-PCR method showed a higher discriminatory power than BOX-PCR method.

Finally. the comparative analysis focusing on the obtained genetic profiles and the presence of virulence genes further supports the possibility for typing methods to discriminate strains with similar phenotypic establishing the level of genetic relatedness among strains and the presence of possible genetic clones. In some cases. these typing methods have also been shown

to be capable of distinguishing among very similar strains isolated on the same date. but at different and close sampling sites.

Chapter 6: CONCLUSION

The major objective of this study was to determine the incidence of *Vibrio spp.* core and the potential pathogenicity in shellfish samples collected from

Sardinia coastal (Italy) that vary in water temperature and salinity during a year. Furthermore analyses were performed using traditional phenotypic systems. bimolecular techniques and multiple typing methods allows a more accurate molecular characterization of *Vibrio parahaemolyticus* strains.

The three rearing (A. O and S) selected in this research are located in different coastal areas of Sardinia and are subjected to the influences of land features (rivers that transport products of agricultural. rearing. urban and industrial activity) and influences of marine features (transitional Mediterranean deep water represents a source of heat and salt for the colder and less saline resident waters at depth. thus progressively raising their temperature and salt content).

These features have influence on the growth of *Vibrio spp.* in relation with the molluscs considered. *M. galloprovincialis* lives on ropes suspended from

floating rafts in the rearing zones. *R. decussatus* lives in muddy-sand sediments of shallow coastal areas.

Prevalence. count and biochemical tests confirmed a high presence of *V. spp.* in clams. This result was confirmed by biomolecular assay. Probably this is connected with different habitat of two molluscs in relation with salinity and temperature as was reported in several studies.

An important result of this work was the presence of *V. spp.* after the depuration process for shellfish of the breeding of “class B areas” (89% in *Mytilus* and 88% in *Ruditapes*) demonstrating the inefficacy of filtration technique as reported by previous authors.

Aquaculture has become the world’s largest growing food industry (Costa-Pierce. 2002). The shellfish farming represented an annual growth of 10% compared to 2 to 3% of other major food sectors (Karthik et al., 2005; Lovatelli. 2006; Subasinghe. 2006). For this reason is most important to value the presence of potentially pathogens. Bedsides no relationship was

observed between presence of *Vibrio. spp.* and fecal pollution in this study where the presence of *Salmonella* and *E. coli* was within the limits provided by law (data not showed). Several authors have confirmed the lack of correlation between traditional indicators and the presence of *Vibrio spp.* (Hood & Ness. 1982; Marino et al., 2005; Normanno et al., 2006; Ripabelli et al., 1999). Seasonal variations in the indigenous bacteria populations make it extremely difficult to select safe waters for mollusc harvest (Croci et al., 2002). Fecal indicators provide an inadequate index of microbiological safety for naturally occurring vibrios and underestimate the efficiency of the depuration process. *Vibrio spp*. has a different response to the depuration process from that of *E. coli.* It is possible to obtain edible shellfish from anthropogenically-contaminated shellfish. but the same measure cannot be used with shellfish contaminated by naturally occurring bacteria.

Likely. regulation N° 854/204/EC that establishes specific attributes on the organization of official controls on products of animal origin intended for human consumption. is inadequate in the light of the data gathered in aforesaid studies to minimize the probability of shellfish contamination by vibrios. from harvesting to consumption. and in the protection of public health.

Moreover our results suggest that clams were the seafood most at risk for transmission of *trh/tdh*-positive *V. parahaemolyticus* to humans. Recently. a decrease has been reported worldwide in the proportion of infections by pandemic *V. parahaemolyticus.* as well as the more frequent involvement of non-pandemic strains in large outbreaks (McLaughlin *et al*., 2005; Wootipoom *et al*., 2007; Harth *et al*., 2009). Similarly. in 2009 was reported a case in Italy of acute gastroenteritis related to a *V. parahaemolyticus trh*-positive strain isolated from indigenous mussels (Ottaviani *et al*., 2010b).

Clinical cases of infection due to ingestion of seafood contaminated with *V. parahaemolyticus* increased epidemiological interest in genotyping of the different vibrios strains in order to identify the source of virulent strains and to analyze the possible persistence. in time and space. of particular clones.

The data presented here further confirms that *V. parahaemolyticus* is potentially an emerging public health problem in Italy. More research is needed to identify the environmental factors that contribute to the persistence of toxigenic *V. parahaemolyticus* in the environment. as well as to assess differences in virulence among various toxigenic strains. regardless of their pandemic potential. In our study the prevalence for each virulent factor was 0.9% (all collected in spring and autumn). In this regard. recent studies have reported an apparent inverse relationship between water temperature and prevalence of *tdh* and *trh*-positive strains. suggesting that these toxigenic strains might have a selective advantage in surviving colder conditions or that they would be the first strains to have

appeared in the environment and are gradually being replaced by non-pathogenic strains as the water warms (DePaola *et al*., 2003b; Rodriguez-Castro *et al*., 2010).

One of the aim of the present study was to develop of a methodology to identify the origin *V. parahaemolitycus*. It is important both for assessing the degree of risk posed to public health and for developing strategies to mitigate the environmental loading of pathogens associated with shellfish borne disease transmission. Three genomic fingerprinting based-PCR methods. enterobacterial repetitive intergenic consensus (ERIC)-PCR. repetitive element palindromic REP-PCR and other fingerprinting for molecular typing BOX-PCR. were assessed for their potential in differentiation of 72 *V. parahaemolitycus*. An important consideration emerged from this study was that the majority of strains collected from clams and in autumn were grouped by the three typing method in the same cluster. This in accordance with a recent investigation (Ottaviani et al. 2012)

suggest that strains with these characteristics transited in our country only during a limited period of time or that they are found extremely rarely or are difficult to detect.

The final step of this research was made using BioNumerics software to compare the three fingerprint PCR-based method. It showed the same characteristics of each dendrogram dividing all strains in two cluster using a similarity of 70%. The high intensity of the color reported in the dendrogram (Fig.__) indicated strains more similar between them and the node reported the similarity percentage.

Our investigation emphasizes the need of enhancing shellfish-borne disease control strategies. focusing the attention also on potentially pathogenic *V. parahaemolyticus* strains. which are often harbored by molluscs.

Preventive measures to enhance the quality of living bivalve shellfish when commercialized have included the monitoring and improvement of the water quality found at the harvest areas. Nevertheless, bacterial indicators

used for shellfish health evaluation were announced, in different reports, as inadequate predictors of the presence of autochthonous bacteria and human enteric viruses. Considering the results of these findings, in order to ensure public health, more accurate indexes of water quality and bivalve microbiological safety are required since they are still not available.

However, the development of a local diagnostic scheme for direct detection and identification of the existing pathogens for monitoring bivalve health is probably a future tendency. Future investigations should address the relationships between indicator microorganisms survival with regard to the *V. spp*., Further work is required to establish a scientific agreement among those considered potential indicators, or others to be discovered, and also to understand the implications of their introduction into legislation.

Conventional methodology, applied to predict the level of contamination by *V. spp*., needs to be improved in specificity and reduced in time. Detection

by new molecular methods may be more sensitive and specific, which will allow for a faster response to health safety problems.

Methods of detecting several sub-species of vibrios should be implemented so that the assessment of microbial contamination can be more closely associated with the results produced by epidemiological studies.

Depuration and relaying helps to improve shellfish quality but if prevention of human or animal-induced pre-harvest contamination can be achieved, natural causes will always be present. A better knowledge of the parameters affecting the kinetics of the processes of depuration is still needed. More sensitive, reliable, and universally accepted depuration procedures must be developed, so that standardized methodologies can enable the comparison between the experimental results. Technological advances should also be employed.

Consumer protection involves both the knowledge of the risk associated to the ingestion of raw shellfish and the preventive actions that take into

account shellfish specificity, shellfish contamination and adequate regulations. The combination of new depuration approaches and a more accurate quality assessment will help to relieve public concern regarding foodborne diseases associated with shellfish products.

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ACKNOWLEDGMENTS

This study was supported by a grant from Sardiania Region- Council

Department of Planning (Regional law 7 of 7 August 2007).

SUMMARY