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REVIEW

Molecular Technologies and Applications in Seafood Safety

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Abstract

Global seafood consumption has significantly increased due to the health concerns of populations. One of the basic requirements to meet the increasing demand for seafood is security and safety. To meet these requirements, detection and identification of seafood pathogens are of importance. For this purpose, besides conventional methods a variety of rapid, sensitive and reliable techniques have been developed. In general, rapid detection methods are generally time-efficient, sensitive, specific and labor-saving. This review emphasizes on the principles and applications of recent rapid methods for the detection of seafood pathogens; including polymerase chain reaction (PCR), multiplex PCR, Random Amplified Polymorphic DNA (RAPD), Real Time Polymerase Chain Reaction or Quantitative Polymerase Chain Reaction (qPCR), Denaturation Gradient Gel Electrophoresis (DGGE), loop-mediated isothermal amplification (LAMP).

Keywords: DNA, rapid method, pathogen, PCR, seafood.

Introduction

FAO declared that total fish production in 2016 reached an all-time high of 171 million tons of which 88% was utilized for direct human consumption. This production resulted in a record-high per capita consumption of 20.3 kg in 2016. Seafood are some of the most traded food in the world today. In 2016, about 35% of global fish production entered international trade (FAO, 2018).

Seafood includes finfish (e.g., salmon and tuna) mollusks (e.g., mussels, oysters, and clams) marine mammals (e.g., seal and whale), fish eggs (roe) and crustaceans (e.g., shrimp, crab, and lobster). Fish, mollusks, and crustaceans can be contaminated by pathogens from various sources. All seafood can be susceptible to surface or tissue contamination originating from the water or sediment. Additionally, in the case of failing required sanitary and hygienic conditions, seafood may become contaminated during handling, preparation, processing. (Lee & Rangdale, 2008). Supporting factors may include the storage and transportation at inappropriate temperatures and packaging in inadequate materials. Furthermore, seafood is often consumed raw or prepared in ways that do not kill organisms (Iwamoto, Ayers, Mahon, & Swerdlow, 2010).

Seafood is responsible for an important proportion of food-borne illness and outbreaks worldwide. The most common pathogens that have been identified in fresh/raw fishery and seafood products are *Escherichia coli, Salmonella* spp., *Staphylococcus aureus, Campylobacter jejuni, Listeria monocytogenes, Vibrio parahaemolyticus, some Bacillus strains.* The infectious doses of many of these pathogens are very low (10-1000 bacterial cells). (Zarei, Maktabi, & Ghorbanpour, 2012; Hazen *et al.*, 2009). Detection and elimination of pathogens contamination are crucial in seafood safety goals (Venugopal & Gopakumar, 2017). Seafood processors have to ensure their products are free from all these abovementioned pathogens.

There are well-established conventional/culture methods from ISO (International Organization for Standardization) have been used for the analysis of the pathogens present in the seafood. These methods generally involve pre-enrichment, selective enrichment, isolation of colonies on selective media, biochemical confirmation and serological tests for the purpose of identification. Completion this procedure generally needs several days. These methods are laborious, relatively slow and less efficient (Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010). There are also some other disadvantages, if the concentration of the microorganisms is low, it is difficult to detect

these by conventional methods. Because of phenotypic characteristics of certain microorganisms, their identification in the culture media may not be expressed (Farber, 1996; Fusco & Quero, 2014).

As the global seafood trade taken into consideration, to meet the modern life necessities it is necessary to use faster, sensitive, efficient and reliable techniques to analyze the pathogens in the seafood safety controls (Corry, Atabay, Forsythe, & Mansfield, 2003; Adzitey & Corry, 2011). In recent years, various molecular techniques have been developed and used frequently (Greiner, & Konietzny, 2007; Pinto, Forte, Guastadisegni, Martino, Schena, Tantillo, 2007).

Compared to conventional methods, molecular techniques enable more rapid and sensitive results in a shorter time (Bavisetty, Benjakul, & Wongkamjan, 2018).

It is particularly important when a risk assessment is required for public health if a first sign of an illness occur less than 24 hours after ingestion of contaminated seafood (Gugliandolo, Lentini, Spano, & Maugeri 2010).

Molecular techniques are culture-independent and based on the analysis of the genetic material of the evaluated microorganisms. These techniques provide the identification and quantification of the entire microbiota present in the contaminated organism and the determination of the expressed genes, translated proteins and produced metabolites. Several researches have shown that seafood-borne pathogens have been identified by using these techniques with satisfactory results (Taminiau, Korsak, Lemaire, Delcenserie, & Daube, G. 2014; Ghanbari, Kneifel, & Domig, 2015; Zhang *et al.*, 2015; Alikunhi, Batang, Aljahdali, Aziz, & Al-Suwailem, 2017; Kim & Lee, 2017; Xu *et al*., 2017).

The aim of this paper is to collect some information about commonly available molecular techniques and applications on detection and characterization of the pathogens isolated from seafood.

Polymerase Chain Reaction (PCR)-Based Assays

One of the most commonly used molecular techniques for the detection of the bacterial pathogens is Polymerase Chain Reaction (PCR). PCR is an in-vitro rapid, sensitive, reliable technique to ensure the safety and the quality of seafood for detection of pathogens. By this method, a specific DNA fragment is amplified during a cyclic 3-step process (Mandal *et al*., 2011). The first step is denaturation (strand separation) the second step is annealing (primer binding) and the third step is extension (new DNA synthesis).

These steps require different working temperatures. These three steps are repeated about 30-35 times in a standard PCR reaction so that the polymerization product is doubled from the previous cycle after every three step cycle. The most important feature of the PCR is that it allows a very small amount of DNA to work with. The development and improvement of the technique for amplifying specific segment of DNA improve the specificity and sensitivity enough for detection even one target DNA molecule (Pinto, Chenoll, & Aznar, 2005).

PCR have been applied in the detection of various foodborne pathogens like *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella* spp. and *Shigella* spp. (Lee & Rangdale, 2008; Alves, Marques, Pereira, Hirooka, & Moreira de Oliveira, 2012; Chiang *et al*., 2012). PCR technique is recommended over the conventional methods as a final step for the identification of *Vibrio* spp. that sometimes questionable situations occur in conventional methods and it requires to check the results in a reference laboratory for confirmation (Kaysner & De Paola Jr, 2004). Koch, *et al*. (1993) recommended the use of PCR in the detection of enterotoxigenic *Vibrio cholera* in foods.

Besides PCR another PCR derivatives have been developed and used in the detection and identification of seafood pathogens. These are; multiplex PCR, Random Amplified Polymorphic DNA (RAPD), and Real Time Polymerase Chain Reaction or Quantitative Polymerase Chain Reaction (qPCR).

Multiplex polymerase chain reaction

Comparing to simple PCR, Multiplex-PCR (m-PCR) offers a more rapid detection of pathogens that allows the simultaneous detection of multiple targets in a single assay. Basically conventional PCR and m-PCR have similar working procedures; but several specific primers sets are used in m-PCR assay; while only one specific primer set is used in conventional PCR assay. Zhao *et al*. (2014) stated that design and concentration of primer sets are significantly important for the development of m-PCR. To obtain successful assay the primer sets should have similar annealing temperature. M-PCR has been successfully applied in detection of different bacterial pathogens in aquatic environments and shellfish. Some of the m-PCR assays developed for the detection of bacteria are *E. coli, Salmonella typhimurium, V. cholera, V. parahaemolyticus and V. vulnificus* in shellfish (Brasher *et al*., 1998). Kong *et al*. (2002) reported for the first time the development of an m-PCR method that permits the simultaneous detection of six different types of waterborne pathogens *Aeromonas* spp., *Salmonella* spp., *Shigella* spp., *V. cholera, V. parahaemolyticus and Y. enterocolitica* in a single tube. The development of a gene-specific DNA microarray coupled with the simultaneous detection of *V. cholera, V. parahaemolyticus and V. vulnificus* in shellfish (Panicker, Myers, & Bej, 2004). Zehari *et al*. (2012) used multiplex polymerase chain reaction (PCR), to

determine the prevalence of *Listeria monocytogenes, Vibrio parahaemolyticus, Staphylococcus aureus and Salmonella* spp. in 245 samples of raw/fresh, frozen, and ready-to-eat (RTE) seafood products marketed in Iran. They concluded that multiplex PCR can provide a rapid and cost-effective method for the surveillance of these pathogens in seafood products. M- PCR has also been widely used for the detection of *Vibrio* spp. Gugliandolo *et al.* (2010) compared conventional and molecular methods in detection of *Aeromonas spp., Salmonella spp., Vibrio cholera*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in mussels and water samples from a farming area. At the end of the research they found that conventional methods were less sensitive than multiplex PCR method; conventional enrichment and isolation procedures allowed only the isolation of *V. parahaemolyticus* and *V. vulnificus* strains, while *Aeromonas* spp., *Salmonella* spp. and *V. cholerae* were not recovered. Lindstrom *et al.* (2001) evaluated the m-PCR assay as alternative method for the detection of *Clostridium botulinum* types A, B, E, and F in food. They explained the assay is sensitive and specific and provides a marked improvement in the PCR diagnostics of *C. botulinum*. Researchers compared m-PCR to conventional PCR and stated that m-PCR provides an important improvement over conventional PCR in the diognastics of *C. botilinum* as the PCR requires more than one step to complete and identification the several *C. botilinum* species. In their study, the total time required by the m-PCR assay, including a two-step enrichment, was 2 to 6 days, depending on the sample material. The detection limit was 10^{-2} to 10^{3} spores/g of sample material. All *C. botulinum* cultures yielded the expected amplification products that, due differences of 150 to 200 bp in the product size, were easily differentiated in low-resolution agarose gels.

Randomly Amplified Polymorphic DNAs (RAPD)

Randomly Amplified Polymorphic DNAs (RAPD) is a PCR-based technique involving use of single arbitrary primer in a PCR reaction, resulting in amplification of many discrete DNA. The technique determines the genetic variations. It provides a quick and efficient screen for DNA sequence based polymorphism at a very large number of loci. The major advantage of RAPD includes that, it does not require pre-sequencing of DNA. The basic principle of the RAPD method is that a single 9-10 bp oligonucleotide randomly selected on the genomic DNA of the species of interest is replicated using PCR anneling temperatures ranging 30-36°C which is lower than conventional PCR working temperatures as 50-60°C (Atienzar & Jha 2006). The amplification product is run on non-radioactive standard gel electrophoresis and observed in the form of bands. Result are evaluated as absent or present of the bands (Williams, Kubelik, Livak, Rafalski, & Tingey, 1990).

RAPD-PCR is a common method to perform genotyping among *Vibrio* spp. as this procedure is known to be able to reveal a high level of DNA diversity within common bacterial isolates, and thus is useful in discriminating the isolates (Silvester, Alexander, Santha, & Hatha, 2016). Vincent *et al.* (2015) conducted a study to assess the rapid molecular identification and characterization of 45 *Vibrio parahaemolyticus* isolates from 15 samples of 3 different types of fish. Polymerase chain reaction (PCR) based confirmation was done targeting the 450 bp fragment of the thermolabile (tl) gene, while DNA fingerprinting was performed using Randomly Amplified Polymorphic DNA (RAPD) PCR with the primer GEN15008. The researchers concluded that RAPD-PCR is effective in detecting polymorphism and estimating genetic distance among different isolates of *V. parahaemolyticus*. Vongkamjan *et al.* (2017) collected a total of 595 samples from raw material, finished seafood products and environmental samples from different sites of a seafood processing plant and *Listeria monostogenesis* were isolated by PCR, and multiplex PCR techniques. They used the RAPD technique to confirm the existence of genetic diversity among *L. monocytogenes* isolates. It was concluded that the techniques used in the research were suitable for the purpose due to the cost, time and prior knowledge required to perform the analysis.

Quantitative Polymerase Chain Reaction (q-PCR)

The q-PCR method is based on quantitative determination of the amount of increased fluorescence as a result of measurable fluorescence in each amplification cycle. The DNA fragments obtained as result of q-PCR can be analyzed during PCR treatment. The compulsory agarose gel electrophoresis used for the determination of the amount of DNA in the PCR method and the steps of imaging under UV light are not needed in the q-PCR method and the process is performed very quickly and reliably (Dorak, 2006). The highest advantage of q-PCR is it is very fast (Bricker, 2011). The amount of the resulting PCR products is proportional to the increase in the signal (Rijpens & Herman, 2002). Also, there is no need to wait until all cycles have been completed to see what the result is (Bricker, 2011). The increase in signal can be displayed in every cycle. The quantitative information in the PCR is obtained from the cycle in which the amount of DNA increases logarithmically. Generally, only 4-5 cycles fall into this range from 30-40 cycles in the logarithmic linear part of the curve (Rijpens & Herman, 2002). With this method, results can be taken in minutes instead of hours or days. Several fluorescent techniques have been improved and used together with q-PCR systems to make the procedure easy and cost effective (Lee *et al.*, 2015). These are SYBR green, TaqMan probes and molecular beacons. SYBR green is a double-stranded DNA

(dsDNA)-binding fluorescent dye, TaqMan probes and molecular beacons are the common alternatives to SYBR green (Hein, Lehner, Rieck, Klein, Brandl, & Wagner, 2001). Among the three fluorescent systems for qPCR, SYBR green is the simplest and the cheapest system as compared to TaqMan probes or molecular beacons (Fukushima, Tsunomori, & Seki, 2003). On the other hand, some researches have displayed that TaqMan-based qPCR is more sensitive than SYBR green or molecular beacons-based q-PCR. However, the sensitivity of PCR-based method basically related with primer specificity, primer sequence and annealing temperature, rather than the choice of detection probe (Klerks, Zijlstra, & van Bruggen, 2004). Compared with conventional PCR, q-PCR is more sensitive and accurate template quantification is allowed over a wide dynamic range (7 - 8 log) and it minimizes the risk of cross-contamination (Bustin et al., 2005; Omiccioli, *et al*., 2009)

Aquatic and seafood pathogens such as *Vibrio parahaemolyticus, Vibrio vulnificus, V. cholera, Vibrio penaeicida* have been detected by this method (Blackstone *et al*., 2003; Panicker *et al*., 2004; Gubala, 2006). Goarant and Merien (2006) described the steps in the successful development of a real-time PCR quantification assay of *V. penaeicida* in shrimp haemolymph, seawater (from ponds or bays) and sediment pore water, including the choice of an accurate extraction technique. They concluded that the entire detection process; including sampling, DNA extraction and real-time PCR amplification, that can be completed within 4 h. q-PCR can be used for simultaneously detection of Salmonella and Listeria in salmon (Amagliani *et al.*, 2010). Taminiau *et al.* (2014) studied on identification and quantification of six spp. of seafood pathogens (*Campylobacter jejuni*, *Campylobacter coli*, enterohemorrhagic *Escherichia coli*, *Salmonella* spp., *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) by using q-PCR protocol. They highlighted that; "real-time PCR were developed for detection of six foodborne pathogens in seafood, the protocols allow a good level of sensitivity and specificity (almost 100%), PCR was more sensitive than ISO methods (EHEC, *Vibrio parahaemolyticus*), in shrimps, ISO methods were more efficient (*salmonella, Vibrio vulnificus*)". Fernández-Álvarez *et al*. (2016) developed and validated a SYBR Green real-time polymerase chain reaction protocol for specific detection of the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* for rapid diagnosis of typical furunculosis. In 2017, ISO expressed the use of PCR or q-PCR method as an alternative method in addition to biochemical tests for identification and confirmation of Vibrio species.

Denaturation Gradient Gel Electrophoresis (DGGE)

Denaturation Gradient Gel Electrophoresis (DGGE) is a separation method based on the principle of electrical field motion on a denaturant urea and formamide gradient polyacrylamide gel of DNA fragments of the same length but in different sequences. Certain denaturing gels are capable of inducing DNA to melt at various stages. As a result of this melting, the DNA spreads through the gel and can be analyzed for single components, even those as small as 200-700 base pairs. There are many applications of the DGGE method in food microbiology and food-related ecosystems. Broekaert *et al*. (2011) indicated that the many potential spoilage bacteria in ice stored marine fishes, including *Photobacterium phosphoreum, Shewanella baltica* and *Pseudomonas fluorescens*, which were previously overlooked by cultivation method, were identified by PCR-DGGE method. Du *et al.* (2017) evaluated the microbiological and chemical changes in Taihu white prawn during ice storage. PCR coupled with denatured gradient gel electrophoresis was performed to investigate the changes in microflora. Vongkamjan *et al.* (2017) collected a total of 595 samples from raw material, finished seafood products and environmental samples from different sites of a seafood processing plant and *Listeria monostogenesis* were isolated by PCR, and multiplex PCR techniques. They used the RAPD technique to confirm the existence of genetic diversity among *L. monocytogenes* isolates. It was concluded that the techniques used in the research were suitable for the purpose of the study due to the cost, time and prior knowledge required to perform the analysis. Duan *et al*. (2018) investigated the succession of bacterial microbiota in tilapia fillets during cold storage at 4°C applying the PCR-DGGE method.

Loop-mediated isothermal amplification (LAMP)

An isothermal nucleic acid amplification method, LAMP has been regarded as a kind of new detection technology. LAMP based on autocycling strand displacement DNA synthesis performed by the *Bacillus stearothermophilus* (Bst) DNA polymerase large fragment. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. One type of enzyme is used in LAMP based reaction under isothermal conditions (ranging 60 to 65°C). Comparing to conventional PCR; because four primers specifically designed for six different regions on the target DNA are used, the reaction has extremely high sensivity; 10 to 100 fold more than conventional PCR, with a detection limit of 10 copy or even more less template. Nagamine *et al.* (2001) stated that LAMP takes less time than conventional PCR to detect virus. The high amplification efficiency of LAMP no needs to choose complex-variable temperature conditions and set different response procedure. LAMP has great advantage over PCR in terms of time consuming; with DNA being amplified 10^9-10^{10} times in 15-60 min, it can save 1 h to complete the reaction

compared with PCR. Greater yield of amplification products provides easier detection (Mori, Nagamine, Tomita, & Notomi 2001). The resulting amplicons can be visualized by adding SYBR Green to the reaction tube (Iwamoto, Sonobe, & Hayashi, 2003). Most of the LAMP based assays have been carried out on detection of *Vibrio parahaemolyticus, Vibrio vulnificus*, *Salmonella spp.* and *Listeria monostogenesis* (Han & Ge, 2010). Srisuk *et al.* (2010) developed a LAMP reaction targeting the *ompW* gene to detect total *V. cholerae* in contaminated seafood. LAMP assay was developed to detect *V. parahaemolyticus* on naturally contaminated seafood samples, fish, shrimp and mussel during 6 h assay time (Wang, Shi, Su, Ye, & Zhong, 2013). Di *et al.* (2015) developed and optimized the LAMP assay by using short enrichment step to detect *Vibrio parahaemolyticus* strains in seafood samples. Researchers stated that they correctly detected all the target strains, but none of the non-target strains. "Very low numbers of *V. parahaemolyticus* (2 colony forming unit (CFU) per gram of seafood) could be detected within 3 h and the minimum time of the whole assay was only 5 h".

Conclusion

Rapid detection of pathogens in seafood is important to prevent outbreaks of seafood-borne diseases and economic losses. Molecular techniques have emerged as a promising tool for the detection of the pathogens present in seafood. Raw materials, processed products or environmental materials can be analyzed for pathogens. Comparing to conventional methods, these methods are generally more sensitive, specific, time and labor-saving and reliable. Detection of very low number of pathogens is also possible. It takes short time to perform the wet-lab procedures. Among these techniques especially LAMP assay combined with a short enrichment period is superior to the conventional culture as well as PCR method. But all these techniques require trained personnel and specialized instruments.

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