Training manual

Advanced Microbiological and Molecular Techniques for Improving The Competence of Students in Biological Sciences (Under SCSP)

(17th July - 22nd July 2023)

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Chapter 1

An overview of seafood borne bacterial pathogens

Minimol V.A and Toms C. Joseph

Seafood is generally considered microbiologically safe when cooked and offers several health benefits including reduction of cardiovascular diseases, contribution to improving bone strength and congenital developments in infants, reduction of joint pains and inflammations etc. However, when the seafood is consumed in raw form such as fresh, live, partially cooked etc, despite having these advantages, are associated with foodborne illness. Rapid industrialization has resulted in the release of sewage and other industrial effluents into natural water bodies, increasing the chances of seafood borne diseases.

The seafood-borne outbreaks are mainly caused by bacteria, viruses, and parasites. The major risk recognised for the contamination of seafood by pathogenic bacteria is by the exposure of food chain to contaminated water. The water runoff from polluted areas such as waste waters from agricultural, industrial and sewage will significantly change the microbial flora of the harvesting water bodies and culture ponds which will result in the contamination of seafood with pathogens like, pathogenic *E. coli,* S*almonella, Campylobacter* etc. or viruses such as Hepatitis A, Norwalk etc. The consumption of raw or partially cooked seafood especially bivalve molluscs can be one of the major contributing factors for the spread of seafood borne pathogens. Another reason for the spread of contaminating pathogens in seafood is the poor personal hygiene of workers and food handlers. Inadequate storage temperature and use of poor-quality raw material in the preparation of seafood etc will increase the risk of illness due to bacteria. Many of the pathogens grow rapidly at room temperature. Fish or fishery product left at ambient temperature is easily spoiled and contaminated with pathogens. This chapter covers the details of major seafood borne bacterial pathogens including emerging pathogens that are causing serious threat to food safety measures.

Salmonella

Infection caused by *Salmonella* continues to be the major cause of seafood borne outbreaks globally. The main source of contamination are associated with raw oyster, salmon, tuna, value added products of tuna, sole etc. Infection due to *Salmonella* causes gastrointestinal disease and typhoid fever in human. *Salmonella* induced seafood borne outbreaks are reported from several countries worldwide. Non typhoidal serovars are generally associated with seafood borne outbreaks. It was reported that USA alone contributes about 1 million cases of food borne non-typhoidal disease globally. In India, the prevalence of *Salmonella* is ranged between 30.5% in fish to 34.1%. The prevalence rates were low in cold temperate regions such US, Spain and Mexico, ranging from 1.5% to 16.4%. The major serovars of *Salmonella* reported from seafood samples of fishing harbours and fish markets in Cochin (India) were S. Weltevreden, *S.* Rissen, *S.* Typhimurium and *S.* Derby. *Salmonella* infection occurs either through the contact with infected animals, or through the consumption of contaminated seafoods.

Pathogenic *Escherichia coli*

Escherichia coli is a commensal bacterium commonly in the intestinal tracts of warm-blooded animals including humans. Hence, the presence of this bacterium in food products indicates faecal contamination. There are around 700 serotypes of *E. coli* that are generally non-pathogenic in nature, however, there are certain pathotypes that are pathogenic to human being; enterohemorrhagic *E. coli (EHEC),* enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Shiga toxin-producing *E. coli* (STEC). This classification is based on their O:H antigen types, virulence characteristics and clinical syndromes. ETEC causes gastroenteritis in humans and low dose of toxin production is sufficient for the excessive fluid secretion and diarrhoea in humans as well as in infants. EPEC causes infantile diarrhoea and the outbreak is mostly seen in least

developed countries due to the poor sanitation and hygiene habits. The STEC is highly virulent and is grouped under enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7 of EHEC category cause diarrhoea and hemolytic uremic syndrome (HUS) in humans and several infections have been reported in many parts of the world. Virulence in STEC is due to the presence of virulence genes such as either *stx1,* or *stx 2,*and both*, ehxA* and *eae* genes. The minimal dose of less than 100 cells are able to cause food poisoning in humans.

Staphylococcus aureus

Staphylococcal food borne illness is due to the consumption of food contaminated with membrane-damaging, invasive, one or more staphylococcal toxins. The presence of Enterotoxigenic *S. aureus* in fishery products and fish processing environments have been reported from India (Murugadas, 2017). Infection due to Methicillin resistant *Staphylococcus aureus* (MRSA) is mostly hospital acquired and the high prevalence of this bacterium in health care sector is reported from all over the world. There are only two incidences of food borne outbreaks due to MRSA. MRSA outbreak that resulted in mortalities were reported from Netherlands where banana was implicated as the source of infection. The ingestion of contaminated shredded pork barbeque and coleslaw resulted in food poisoning outbreak due to MRSA in United States. The prevalence of *S. aureus* in Indian seafood ranged from 9 % to 23 % during the period from 1985 to 2016.

Vibrio parahaemolyticus

The food borne outbreaks caused by *Vibrio parahaemolyticus* are associated with consumption of raw, partially cooked seafood especially bivalve mollusc. This bacterium was first reported as an entero-pathogen in a food borne diseases in Japan in 1950 due to the consumption of partially cooked sardine. It has been considered as the one of the leading causes of food poisoning agent globally. Food borne illness due to the presence of these bacteria have been frequently. It has been detected in many seafood samples

including eel, octopus, squid, shrimp, oyster, sardine, tuna, mackerel, perch, pompano, etc. Most of the environmental strains are non-pathogenic and does not cause any infections. Pathogenic strains are characterized by the presence of haemolysin genes such as *tdh* and/ or trh gene (Okuda, 1997). Most of the pathogenic environmental strains carry *trh* gene whereas presence of *tdh* gene is more in clinical strains that cause infection. Main symptoms of infection include gastroenteritis, wound infection and in rare cases, septicemia can occur. No dominant serovars were involved in food poisoning until the appearance of O3:K6 pandemic serotype in India in 1996.

Vibrio cholerae

The transmission route of *V. cholerae* to human occurs mainly through aquatic environments particularly water. There are reports of this pathogen in fish and fishery products from several parts of the world. Several cases of rejections of consignments of seafood in international trade due to the presence of *V. cholerae* has been reported. Generally environmental strains are non-pathogenic and do not possess any virulence related genes such as *ctx, zot, ace*, and *tcp*A. The survival and evolutionary dynamics of *V. cholerae* in water causes the emergence of diverse sero and biovariants of *V. cholerae* due to the gene transfer mechanisms. The horizontal and lateral gene transfer mechanism causes the acquisition of virulence genes, antigenic types such as O1 and O139 etc. Toxigenic *V. cholerae* of classical biotype, had been responsible for infections previously and many epidemic outbreaks were reported in the 19th century which was gradually replaced with an emerging strain of the El Tor biotype in 20th century. Re-emergence of classical biotype together with El Tor strains were reported in Bangladesh during 1982 and these strains were frequently reported in gastroenteritis and diarrhoea from this area until 1993. Another epidemic strain of *V. cholerae* carrying O139 antigen was first reported in 1992 in Southern Asia. The incidence of cholera due to O139 and O1 Biotype El Tor strains gradually increased thereafter in India and Bangladesh. Subsequently, the variant of O1 El Tor (hybrid) which carry *tcp*A classical genes or classical *ctx*

A or *ctx* B genes have been reported from clinical cases of cholera from Bangladesh. The non-toxigenic strains of O1 are different in terms of its biochemical and serological properties. Clinical and environmental origin of non-toxigenic strains of O1 have been reported from several countries. However, the non-toxigenic strains lacking toxigenic genes also has the potential of causing diarrhoea in human. The mechanism of virulence and pathogenicity of this strain remains unknown.

Listeria monocytogenes

Listeria monocytogenes is major concern in lightly preserved food products and the prevalence of this bacterium in considerably increased in ready to eat fishery products. Seafood has the highest risk among the minimally processed products. *L. monocytogenes* enters into seafood by cross-contamination and the presence of this pathogen in seafood have been reported from different seafood products. Prevalence rate of this pathogen in seafood products varies from 0 to 17 %. However, the prevalence in seafood is relatively low compared to other food products such as dairy and other animal products. The mortality rate due to *L. monocytogenes* infection is very high ranging from 20% to 30% in immunocompromised patients and hence an important public health concern. The symptoms of infection include septicemia, meningitis, gastroenteritis, pneumonia, and spontaneous abortion. Regulatory agencies such as FDA, ISO, WHO, etc. have included this pathogen in zero tolerant category in processed food products due to its survivability in wide environmental conditions. This pathogen is able to withstand high NaCl concentration of upto 20%, pH range of 4.3 to 9.8, temperature range of 0.5 to 45^{o}C, and low water activity of 0.91. This pathogen is very well adapted to grow in refrigerated condition, and pose serious risk to the chilled and frozen products once it is contaminated.

***Yersinia* spp.**

The genus *Yersinia* belongs to *Enterobacteriaceae* family. Presently, it comprises of 16 species and two species (Y. *enterocolitica* and *Y.*

pseudotuberculosis) are pathogenic to human. *Y. enterocolitica* is widely distributed in aquatic and animal reservoirs with swine serving as a major reservoir. Yersiniosis is caused by *Y. enterocolitica* of which virulence biotypes associated with infections are biotypes 1B, 2, 3, 4, and 5. The spectrum of disease ranges from mild diarrhoea to acute gastroenteritis, enterocolitis and pseudo appendicitis in humans. *Y. enterocolitica* is able to withstand freezing for long period of time and remain viable after extended frozen storage which raises public health concerns in the low temperature preservation and processing of seafood.

Clostridium botulinum

C. botulinum are grouped under Gram positive bacteria, and are anaerobic spore producing bacilli of important public health concern in seafood industry. This bacterium is autochthonous to the aquatic environment and aquatic sediments and forms major reservoir of this pathogen. The toxigenic types of *C. botulinum* belong to type A, B, E and F. The major risk factors in seafood are due to the presence of these toxigenic types. Botulinum food poisoning is due to the consumption food contaminated with preformed toxins of *C. botulinum* and low oral dose of 70 µg is sufficient to causes illness in human. Its prevalence in seafood depends upon several factors such as topographical location, culture practices, detection methods etc. The fish poses serious risk due to its direct contact with sediment and the ingestion of spores through contaminated feed/sediment. This bacterium is a major concern in packaged seafood products where cold chain is not maintained during storage, transport and distribution chain. The favourable condition for the growth of *C. botulinum* in preserved products such MAP or vacuum-packed products include, pH of about 4.6, water activity of 0.93%, low salt upto 3%, temperature range of 3^{o}C to 50^{o}C.

Emerging pathogens in seafoods

Apart from the well reported seafood borne pathogens, several other pathogens are also emerging throughout the world irrespective of the geographical conditions, and able to cause infectious diseases in the current

century. It is not always true that emerging pathogens are a new category of microorganisms, instead it can be already established pathogens in which the virulence or resistance to disease characteristics is high as a result of stressful conditions such as changes in the habitat, climate, overdose of antibiotics etc. It is important to study the time of emergence of particular bacteria of infectious category to the food chain via source tracking and establishment of national network of surveillance system, so that the epidemic spread can be controlled by effective implementation of the mitigation measures and re-emergence can be prevented.

Vibrio vulnificus

Vibrio vulnificus a halophilic bacterium belonging to *Vibrionaceae* and widely distributed in brackish water and marine environments. High concentration of these bacteria can be seen in filter feeding bivalves that inhabits coastal polluted waters. So, the major risk factor for the food borne outbreak is the consumption of contaminated raw or partially cooked shellfishes. Infection can also occur through open wounds and may lead to septicaemia in fatal cases. The fatality rate of *V. vulnificus* infection ranges from 20 to 60%. Recently, this bacterium has emerged as public health significant bacteria due to its high fatality rate all over the world.

***Campylobacter* spp.**

Campylobacter spp. causes gastrointestinal disease termed camplyobacteriosis and one of the leading causes of food borne outbreaks in developed countries. Since 2005 to 2019, this bacterium has been implicated in gastrointestinal disease of more than 2,20,000 people in EU and ranks first in foodborne outbreak followed by *Salmonella* and *Yersinia*. The USA reports 8.45 lakh cases of *Campylobacter* infection per year. The outbreak is mainly due to ingestion of contaminated food products, where the chicken alone contributes to about 25% of the infections. The incidence of *Campylobacter* spp. have been reported in other types of food animals such as cattle, pig, cows, sheep etc. *Camplyobacter pleridis* and *C. lari* subsp.*concheus* were isolated from shell fish. The *Campylobacter* spp. is a

commensal bacterium to poultry and the intestinal tract carry huge amount of this bacterium. The rupture of intestinal tract while processing can disseminate the content to skin. Cross contamination with shellfish harvesting area and handlers can result in seafood borne outbreak. Shellfish associated campylobacterosis was first reported during 1980s where 28 persons were infected after eating raw clams.

***Cronobacter* spp.**

Cronobacter species belongs to the family *Enterobacteriaceae* and is considered as an opportunistic pathogen in neonates. Among 7 species of *Cronobacter*, three species are pathogenic to human, namely C. *sakazakii*, *C. malonaticus and C. turicensis*. Out of these, *C. sakazakii* causes high mortality rate of about 40-80% in neonates. This bacterium has been isolated from wide range of food sources such as dairy products, plant-based products, dried fish, shrimp, seaweeds and minimally proceeds products. This bacterium is considered as an emerging pathogen of seafood recently due to its survivability in low moisture foods such dried fish product. However, the seafood borne outbreak due to this bacterium was not reported so far.

Arcobacter spp.

Arcobacter is an emerging zoonotic pathogen, belongs to *Campylobacteraceae* and is closely related to the Genus *Campylobacter.* They are able to survive in low oxygen condition, and well adapted to temperature of less than 30°C. *Arcobacter* causes bacteraemia, gastroenteritis and diarrhoea. Out of 27 species, three species are major pathogenic strains causing disease, namely *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. Food borne infection associated with chicken and vegetables have been reported. Seafood borne outbreak due to *Arcobacter* was not reported so far, however reports of isolation of *Arcobacter* from fish, shellfish, and seawater are available.

Vibrio mimicus

Vibrio mimicus is an important emerging zoonotic pathogen in seafood that causes disease in aquaculture fishes as well as gastroenteritis in human. Major reservoir of this pathogen are raw oysters, fish, turtle eggs, shrimps, cray fish. Davis *et al.* (1981) studied the biochemical characteristics of atypical *V. cholerae* by biochemical tests revealed new species of sucrose negative strain for which the name *Vibrio mimicus* sp. nov. strain was proposed. *V. mimicus* carrying ctx gene is reported as pathogenic strain that can cause severe watery diarrhoea and gastrointestinal disorders. In India, there were only few reports of this organism from seafoods.

Food safety with respect to seafood pathogens is an important in terms of public health perspectives as over 200 types of diseases are due to the consumption of contaminated foods. (To ensure food safety, routine microbiological screening tests should be validated in real time so that the contaminated food products get detected. National regulations shall be enforced for ensuring food safety that includes the strict implementation of food hygiene and sanitation programme through Hazard analysis and critical control point (HACCP), together with Good management practices (GMP), standard operating Procedures (SOPs), Sanitation standard operating procedures (SSOPs) practices from production to consumption stages, there by the product becomes safe at all stages of production, processing and distribution levels. The harmonization of these practices in international trade ensures the safety of seafood products, globally.

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Chapter 2

Laboratory techniques in microbiology

G. K. Sivaraman

When eating contaminated food, people are more likely to contract illnesses, the bacteria are thought to be the primary cause of these food-borne illnesses. Food deterioration and potential contamination are indicated by changes in colour, texture, odour or consistency. Food-borne pathogens are the subject of much research due to the high frequency of outbreaks of food-borne infections in many nations, including developed countries. Advances in food safety practices have emerged in the food business as a result of rising customer awareness of the safety of food products and the demand that goes along with it for products to be acceptable and fresh. Many foods that are not subjected to a microbiological examination turn into health risks because they can result in a variety of illnesses. So that establishes microbiological standards to assure food safety and safeguard consumer health. The establishment of standards for the microbiological inspection of foods entails, setting the analytical procedure to be followed to ensure adherence to these standards such as ISO/ FDA/ FSSAI can be considered as reference methods. The ISO procedures are now being improved through simplification and the introduction of new technology in order to make them more useful in food control laboratories. In order to get similar results without sacrificing accuracy and assuring the quality and safety of the foods under control, laboratories can employ internationally recognized testing techniques by using standardized procedures for conducting microbiological studies. Because of this, it is becoming more frequent for accreditation bodies to use these standardized methods as a reference when establishing microbiological criteria are considered (EU Regulations 2073/2005 and 1441/2007), where the global standards created by the ISO Committee are regarded as reference methods.

Laboratory techniques in microbiology

A number of techniques are routine in microbiology laboratories that enable microorganisms to be cultured, examined and identified.

Common test methods

A wide range of technologies are employed for the identification and verification of microorganisms, therefore following different types of methods are most frequently used the examination of seafood.

1. Direct Examination
2. Culture Media
3. Enumeration methods
4. Alternative methods
5. Immunoassay
6. Rapid Methods
7. Advanced molecular methods

Direct Examination: Prepare a slide for direct microscopic analysis, the original product or a low serial dilution of it should be used if suspected that a food has contaminated and could cause foodborne illness or has suffered microbial deterioration. From the shape and Gram strain of bacteria may indicate the necessity for additional testing. Even if the food may have undergone cooked/heat treatment and the relevant microorganisms may no longer be viable, a microscopic investigation must be performed. The presence of Gram-positive cocci (clusters) may be indicated that the presence of potential enterotoxigenic *Staphylococcus aureus* strains. Similarly, the presence of spore-forming, Gram-positive rods in a frozen food indicate the presence *Clostridium perfringens* and Bacillus cereus may also be present in the food. The presence of several Gram-negative rods under microscopic examination raising suspicion for its presence of following bacteria, such as S*almonella, Shigella, Escherichia, Yersinia, Vibrio,* or *Campylobacter*. Also, consider the symptoms, and its duration of

appearance and incubation may also take in to account while microscopic examination.

Culture Media: The formulation of a culture medium will depend on the overall goal of the study, which may be to promote the growth of the broadest range of organisms possible, to be selective or elective for a single species or small group, to resuscitate damaged but viable bacteria, or to provide diagnostic information. Media is used in microbiological labs to cultivate various types of microorganisms in order to identify and detect them. A culture medium often contains a variety of nutrients to promote microbial growth. The cultural methods are the test of choice for both fresh produce and ready-to-eat food products including seafood.

For the development of non-picky, heterotrophic microorganisms, general purpose media such as nutrient agar and plate count agar for bacteria are used. Although they don't contain any inhibitors, but be selective due to a lack of certain nutrients that more picky species need. The majority of organisms are inhibited by one or more substances found in selective media, but the species or group of species, that need to be isolated are substantially less so. It should be emphasized that all selective media will typically be somewhat inhibitory to the organisms to be selected because they are predicated on the presence of inhibitory chemicals. Without a resuscitation phase to allow them to heal, target organism cells that have sustained sub-lethal damage might not be able to proliferate on the medium. A selective medium should continue to be inhibitory to undesirable species even as the necessary organisms are expanding. Conversely, elective media are made to promote the faster growth of a particular species or group of microorganisms so that they can compete with others even in the absence of inhibiting chemicals. Thus, *Clostridium perfringens* can develop quickly in cooked-meat broth kept at 43 to 45 °C, where it can take over as the dominant organism after only 6 to 8 hours. The use of elective media presents the issue that the development of the desired species may alter the medium in a way that now promotes the development of other species.

A bacteria that has been sub-lethally damaged by a previous condition, such as heat treatment, chilling, drying, or exposure to radiation, can be recovered using resuscitation media. In addition to being more vulnerable to the inhibitory substances inherent in selective medium, such injured microorganisms may also be destroyed if exposed to circumstances that promote the rapid growth of healthy cells. Resuscitation fluids often have poor nutritional value and may contain substances that scavenge free radicals like those that may be produced by the metabolism of oxygen. Due to the presence of a particular metabolic pathway or even just a single enzyme, diagnostic media contain a reagent or reagents that offer a visual response to a particular reaction, making it feasible to identify distinct species or groups.

Now the ready-made media available are in practice combine selective reagents, elective components and diagnostic features, for example Baird-Parker agar used for the presumptive isolation of *Staphylococcus aureus*. The addition of egg yolk provides the diagnostic properties while sodium tellurite and lithium chloride serve as the selective and elective agents are sodium pyruvate and glycine, respectively. The production of black colonies due to the reduction of tellurite is characteristic of S. aureus and also other organisms such as other species of Staphylococcus, Micrococcus and some species of Bacillus able to grow on this medium. The appearance of an opaque zone caused by lecithinase activity surrounded by a halo of clearing caused by proteolytic activity is another diagnostic trait that is shared by the majority of S. aureus strains.

Enumeration methods:

Plate Count: The most accurate way to determine whether a bacterium is alive is to grow until it forms an obvious colony. This serves as the foundation for the conventional pour plate and spread plate, both of which are still commonly utilized in microbiology laboratories.

Pour plate method: Using a sterile pipette, a predetermined volume of inoculum (often 1 ml) is poured into the centre of a sterile petri plate. The

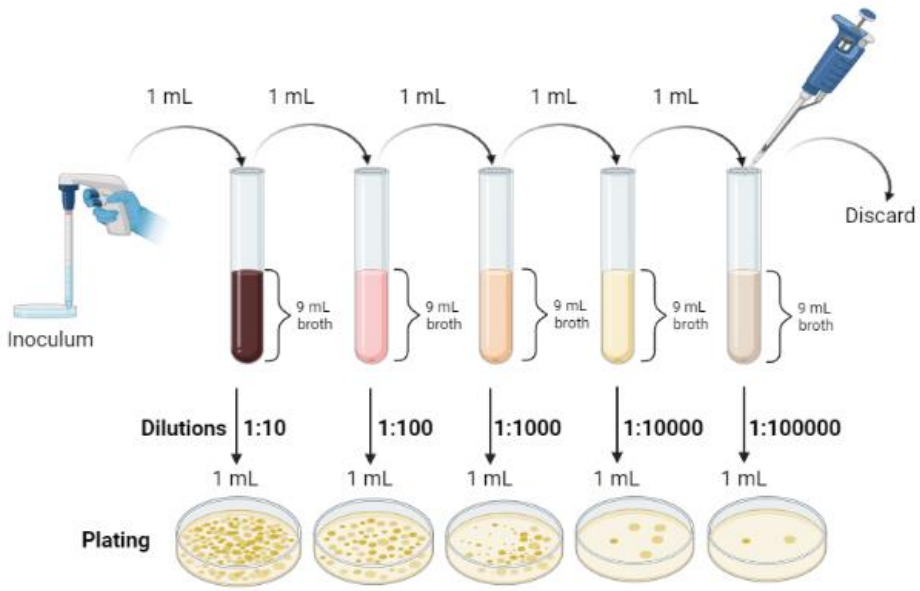
petri plate holding the inoculum is then filled with molten, cooled agar (about 15-18mL), which is stirred thoroughly. The plate is inverted and incubated at 37°C for 24-48 hours after the agar has solidified. Both inside the medium and on its surface, microorganisms will continue to multiply. All types of colonies are counted carefully and each colony represents a "colony-forming unit" (CFU).

So the number of microorganisms present in the sample is determined by:

CFU/mL= CFU x dilution factor

CFU/mL using the formula: = (number of colonies x dilution factor) / volume of culture plated

The ideal number of colonies per plate for accurate counting should be between 25 and 250 (thump rule).



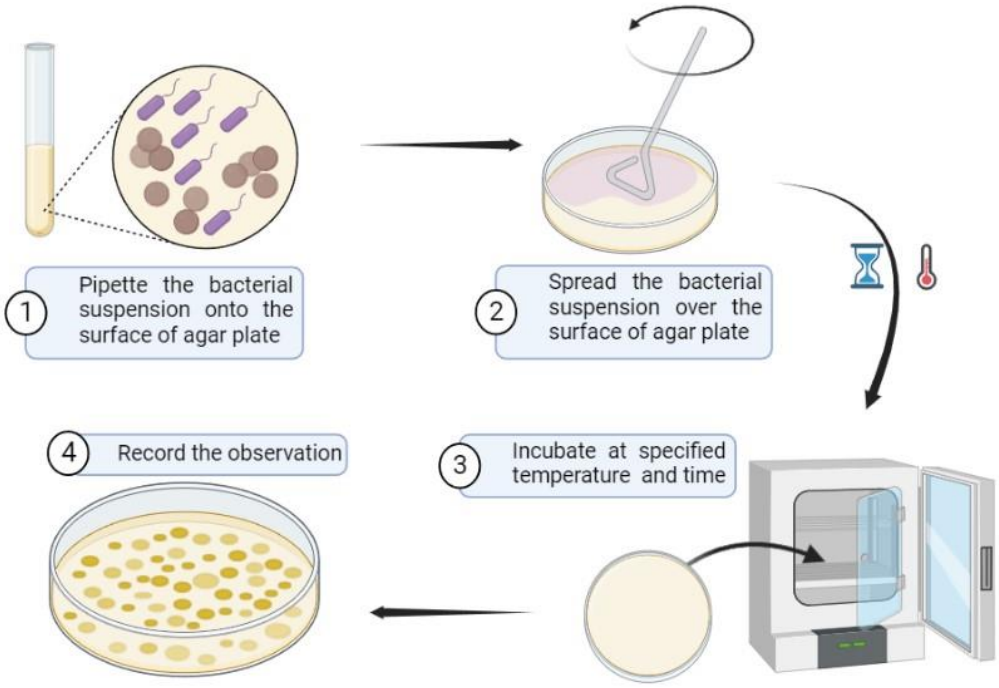
Drawbacks of pour plate method

- The pour plate approach requires more preparation time than the spread plate or streak plate methods.
- Heat-sensitive organisms that come into touch with heated agar lose their viability.

- Compared to colonies that are on the surface, embedded colonies are substantially smaller.
- The reduced growth rate of obligate aerobes in the depth of the agar.

Spread plate method: The homogenized fish sample is diluted in a succession of tubes filled with physiological saline. The material is then serially diluted 10 times and plated in an appropriate. Each tube has 0.1 mL taken from it, which is then applied to the agar surface. A sterile, bent glass rod is then used to disperse the sample uniformly across the agar surface. The scattered cells grow into independent colonies during incubation. The total number of viable organisms initially present in the sample is determined by counting the colonies on the plate and multiplying that number by the dilution factor. It is possible to quantify the spread plate approach to count the quantity of bacteria in a sample. The spread plate technique is most frequently used to isolate and identify a variety of microbial flora present in environmental samples as well as for microbial testing of foods and other samples.

In order to identify or count the bacteria in a food sample, the spread plate technique is a reliable counting approach. By strewing it across the agar plate's surface, the inoculum is diluted. The inoculum is diluted while streaking in consecutive parts of the plate until the point where just one bacterial cell is deposited on the surface of the agar plate. Picking well-isolated colonies and re-streaking them on brand-new agar plates will provide pure cultures. An isolated colony of bacteria is typically thought to be the offspring of a single bacterial cell.



To calculate the colony-forming units (CFU) present per ml in the original sample, count the number of colonies and multiply that number by the appropriate dilution factor.

CFU/ml = (number of colonies x dilution factor) / volume of culture plated

Most Probable Number counts: The most probable number (MPN) is a statistical technique that uses the extinction dilution principle to estimate the viable populations of bacteria in a food samples by inoculating broth at 10-fold dilutions. It is frequently applied to the estimation of microbial populations in agricultural and meat products, water, and soils. When samples contain particulate matter that obstructs plate count enumeration techniques, MPN tests are particularly helpful. In order to determine whether the water is safe to drink in terms of the number of bacteria present, MPN is most frequently used to assess the quality of water. Fecal contamination of water is indicated by a kind of bacteria known as faecal coliforms. In contrast, the presence of large numbers of faecal coliform bacteria would indicate a very high probability that the food could contain disease-producing organisms making it unsafe for consumption. The presence of very few faecal coliform bacteria would indicate that water probably contains no disease-causing organisms.

When testing food is serially diluted and inoculated in lactose broth, any coliforms that may be present use the lactose in the medium to create acid and gas. The media changes colour when there is acid present, and gas is found when gas bubbles gather in the inverted Durham tube that is also present in the medium. The number of tubes producing positive results (i.e., both colour change and gas production) is counted, and the pattern of positive results (the number of tubes exhibiting growth at each dilution) is compared with conventional statistical tables to determine the total number of coliforms.

MPN test is performed in 3 steps

- Presumptive test
- Confirmatory test
- Completed test

Presumptive test: A screening test called the presumptive test is used to check a food sample for the presence of coliform germs. In the event that the presumptive test returns negative results, no additional testing is carried out and the food source is deemed microbiologically safe.

Confirmatory Test: In addition to coliforms, some additional microbes use lactose fermentation to produce acid and gas. A confirmatory test is performed to confirm the presence of coliform. Incubate the inoculated lactose-broth fermentation tubes at 37°C for 24±2 hours, and then check for gas production. If there is no gas generation, continue incubating for a total of 48± 3 hours to check for gas production. The presence of a coliform group member in the food under investigation is indicated by the generation of gas in lactose broth and the appearance of Gram-negative, non-spore-forming bacilli. A negative test results from either the absence of gas generation in lactose broth or the inability to identify Gram-negative, non-spore-forming bacteria (no growth).

Alternative methods: Routine cultural methods are relatively labour intensive and require time for adequate growth to occur. Food producers

and testing labs are urgently need knowledge on the microbiological quality of foods and raw materials.

Dye-Reduction Tests: Redox dyes have the capacity to absorb electrons from an active biological system, which causes a change in colour. The triphenyltetrazolium salts are a significant exception to the rule that the reduced form is colourless and the oxidised form is coloured. Most commonly used yes are redox dyes, methylene blue, resazurin and triphenyltetrazolium chloride.

Electrical Methods: Growing microorganisms alter the chemical composition of the growth medium, which may also affect the electrical properties of the medium. It is possible to measure the electrical properties of a growth medium by growing organisms supplied with two metal electrodes.

ATP Determination: All living cells contain adenosine triphosphate, which serves as the universal carrier for the conversion of free energy from catabolic to anabolic processes. A system for creating light by the activity of enzymes known as luciferases on substrates known as luciferins has evolved in a fire fly. These processes necessitate the presence of magnesium ions and ATP and result in the hydrolysis of one ATP molecule through a succession of intermediates in order to produce one photon of light. The creation of an enzyme-substrate complex, which is then oxidised by molecular oxygen to an electrically excited state, is facilitated by an ATP molecule. With the discharge of a photon of light, the molecule's excited state dissociates to release the luciferase enzyme once more before returning to its lower energy ground state.

Now equipment's are developed for the accurate measuring of light emission as well as pure luciferin and luciferase from even at very low levels. i.e very sensitive assay for ATP by the photomultiplier tubes by the microorganisms.

Immunoassays: There has been a lot of work put into developing immunoassays employing polyclonal or monoclonal antibodies for food

microbiology because of their potential for specificity. Now commercial immunoassay kits for a number of different foodborne microorganisms and their toxins, including mycotoxins. It is quite simple and possible to directly raise antibodies to specific surface antigens of microorganisms or to macromolecules like staphylococcal or botulinum toxins. Immunoassays employ a variety of different forms, but the binding of the antibody to the antigen is their fundamental component. Sandwich ELISA (enzyme-linked immunosorbent assay) is a regularly used methodology in which a capture antibody is mounted on a solid surface (microtitre plate). Following the addition of the antigen-containing sample to the well, mixing, and removal, any antigens still present are left linked to the antibodies and then detected by adding a second antibody that is connected to an enzyme like alkaline phosphatase or horseradish peroxidase. This antibody will also bind to the antigen producing an antibody sandwich. By adding a chromogenic substrate for the enzyme connected to the second antibody and evaluating the colour generated, binding is determined. Alternative methods of detection are employed, including the binding of antibodies to latex and the search for agglutination in the presence of the antigen, as well as the use of fluorescently labelled antibodies that can be used to detect target organisms using a fluorescence microscope or flow cytometry. Commercial ELISAs are available for pathogens like Salmonella and Listeria monocytogenes.

Molecular methods for the detection of pathogens: Real time PCR assays: Real time PCR (also called quantitative PCR or qPCR) is now getting popular for detection of foodborne pathogens due to the possibility of quantitating pathogens and eliminating the need for gel-based detection of PCR products. There are two methods of quantifying the PCR products (a) use of fluorescent dyes that intercalate with double stranded DNA and (b) use of modified oligonucleotide probes that fluoresce when hybridised with complementary DNA. Dyes such as SYBR Green that bind dsDNA would bind to all dsDNA including non-specific products or primer dimmers, but are less expensive and can be used for any target to be amplified. The fluorescent reporter probes, on the other hand need to be specifically

synthesised for each reaction. The TaqMan assay is an example of this, where a single stranded oligonucleotide probe complementary to a segment of 20-60 nucleotides within the DNA template and located between the two primers is used. In this assay, a fluorescent reporter (e.g. 6-carboxyfluorescin) and quencher (e.g. tetramethylrhodamine) are covalently attached to the 5'and 3" ends of the probe respectively. The single stranded probe does not show fluorescence due to close proximity of fluorochrome and quencher. During PCR, the 5' to 3' exonuclease activity of Taq polymerase degrades the proportion of probe that has annealed to the template, releasing the fluorochrome from proximity to the quencher. Thus, fluorescence is directly proportional to the fluorophore released and amount of DNA template present in PCR product. With both types of assays, the exponential increase in fluorescence is used to determine the cycle threshold (Ct) which is the number of PCR cycles at which significant exponential increase in fluorescence is detected. Using a standard curve for Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made.

DNA microarray assays: The use of DNA microarray technology for study of foodborne pathogens has been attempted by some investigators. Some of the approaches used include PCR amplification and hybridisation with microarray. The targets for PCR include one more universal gene like 16S rRNA or 23S rRNA, or pathogen-specific genes such as virulence associated genes or random or arbitrary fragments or genomic probes selected by comparative genomics. Studies related to seafood are not common and the complexity and cost of the assay limits its application in routine seafood testing.

Loop-mediated isothermal amplification (LAMP): LAMP is a novel nucleic acid amplification method based on the principle of autocycling strand displacement DNA synthesis performed by the Bst DNA polymerase, which offers a number of advantages compared to PCR. All reactions can be carried out under isothermal conditions ranging from 600 to 650C. Six primers recognizing eight distinct regions on the target nucleotides are used to

acquire an extremely high specificity and the main advantage lies in that detection is simplified by visual judgment with the unaided eye without post-amplification electrophoresis. Thus, a LAMP assay is independent of expensive equipment, and it is specific, fast, and easy to perform. These features represent a simple, rapid, and cost- effective analysis that is desired in modern detection methods for foodborne pathogens. Furthermore, the increase in the turbidity of the reaction mixture in accordance with the production of precipitate correlates with the amount of DNA synthesized and is suitable for quantitative and real-time assays.

Recently, derivative LAMP assays, such as reverse-transcription LAMP assay, multiplex LAMP assay, in situ LAMP assay, and real-time reverse-transcription LAMP assay have been developed and employed for the detection of various foodborne pathogens, including porcine parvovirus, *Campylobacter jejuni* and *Campylobacter coli, Vibrio parahaemolyticus, Aeromonas caviae, Escherichia coli, Staphylococcus aureus,* etc.

LAB-ON-A-CHIP (LOC): Rapid advances in miniaturization relating to nanotechnology and microfluidics have enabled the development of "Lab-on-a-Chip" (LOC) devices which are small enough to be portable. These devices are fully-integrated, miniaturized systems which are capable of performing sample preparation together with detection in a simple and automated manner. LOCs consists of a chip containing wells, channels, electrodes and filters designed for buffer and sample storage, sample preparation, PCR and target DNA detection. These compartments are interconnected through microchannels to create microfluidic networks. At the microscopic scale, physical processes such as osmotic movement and surface interactions are enhanced and reaction volumes, costs and assay time are significantly reduced, making these devices useful for field application. Moreover, LOC technology allows exposure to hazardous materials to be minimized and a reduction in waste generation. Recently a biochip devised for pathogen detection consisting of microfluidic mixers, chambers, pumps, valves, channels, heaters and DNA microarray sensors. This chip was used to detect pathogenic bacteria in whole blood samples. Currently, several chip-

based systems are in development or already on the market. Companies such as Agilent Technologies (Palo Alto, CA), Affymetrix (Santa Clara, CA), ACLARA Biosciences (Mountain View, CA) have products on the market based on the microfluidic LOC technology which should have significant impact on environmental microbial monitoring by permitting detection and identification of targets within minutes at the sampling site with a sensitivity level of a single cell.

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Chapter 3

Methods for the microbiological examination of seafood

S. Visnuvinayagam

Aerobic plate count

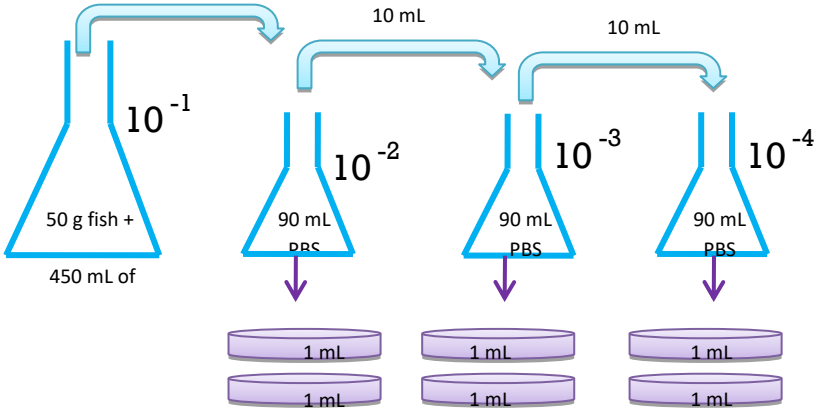
The Aerobic plate count method is used for examining frozen, chilled, pre-cooked, or prepared foods. It is also known as the total mesophilic count, total bacterial count, and viable aerobic bacteria.

- Take 50 g analytical unit fish to determine aerobic plate count
- Add 450 ml Butterfield's phosphate-buffered dilution water to a blender jar containing 50 g analytical unit and blend for 2 min (This results in a dilution of 10^{-1}). Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and further as appropriate.
- Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 s. and reshake dilution bottle 25 times in 30 cm arc within 7 s if it stands more than 3 min before it is pipetted into a petri dish.
- Pipette 1 ml of each dilution into separate, duplicate, appropriately marked Petri dishes.
- Add 12-15 ml plate count agar (cooled to 44 ± 1°C) to each plate within 15 min of original dilution.
- After solidification of agar, Invert the solidified Petri dishes and incubate promptly for 48 ± 2 h at 35°C. Do not stack plates when pouring agar or when agar is solidifying.
- Select plates with 25-250 colonies including those of pinpoint size

$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$

where: N = Number of colonies per ml or g of product;$\sum$ C = Sum of all colonies on all plates counted;n_1 = Number of plates in the first dilution counted; n_2 = Number of plates in the second dilution counted;d = Dilution from which the first counts were obtained

Aerobic Plate count (APC)



50 g of fish sample + 450 ml of sterile Butterfield's phosphate buffer (BPBS)

⇩

Macerate in a blender

⇩

Prepare tenfold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},)

⇩

Add 1 ml from each dilution to sterile empty and add plate count agar (PCA)

⇩

Incubate at 37° C for 48 hrs

Enumeration of Faecal Coliform and *Escherichia* (Most Probable Number technique - 3 tubes)

I. Presumptive test for coliforms, faecal coliforms and *E. coli*

- Weigh 50 g of food into a sterile high-speed blender jar (for frozen samples can be softened by storing for <18 h at 2-5°C, but do not thaw)
- Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min.

- Prepare decimal dilutions (1:10) with sterile Butterfield's phosphate diluents or equivalent in a test tube.
- Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 s.
- Using at least 3 consecutive dilutions, inoculate 1 mL aliquots from each dilution into 3 LST/Lactose broth tubes for a 3 tube MPN analysis
- Incubate LST tubes at 35°C± 0.5°C.
- Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of the medium in fermentation vial or effervescence when tubes are gently agitated.
- Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. Perform a confirmed test on all presumptive positive (gas) tubes.

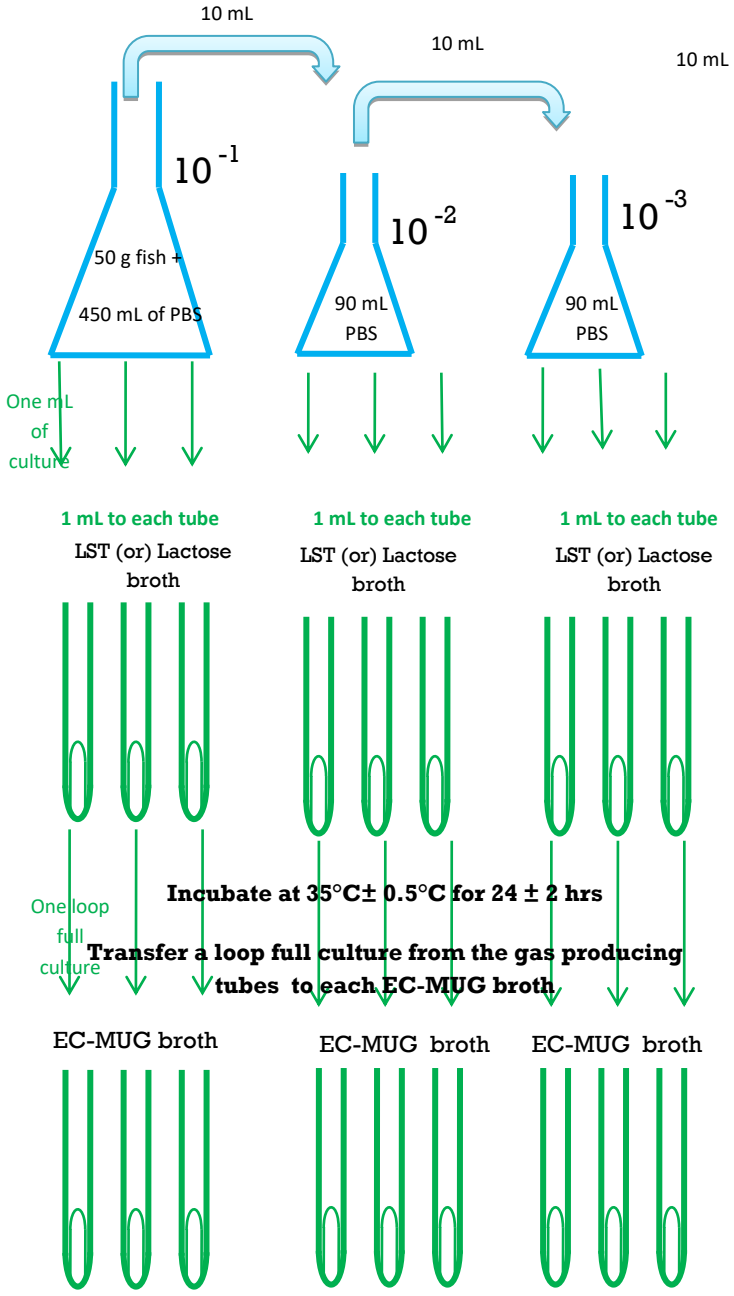
II. MPN - Confirmed test for Faecal Coliforms

- From each gassing LST or Lactose broth tube from the presumptive test, transfer a loopful of each suspension to a tube of EC-MUG broth
- Incubate EC-MUG tubes 24 ± 2 h at 45.5 °C and examine for gas production.
- If negative, re-incubate and examine again at 48 ± 2 h.
- Use the results of this test to calculate faecal coliform MPN.

II. MPN - Confirmed test for *E. coli*

- Only positive tubes (gas production) checked for the MUG positive in UV-light 356nm
- Bluish fluorescence in the test tube indicates the presence of *E. coli*
- Use the results of this test to calculate faecal coliform MPN

Enumeration of *E. coli* by MPN method



Incubate at 24 ± 2 h at 45.5 °C (in water bath) and examine for gas production in normal eye vision

⇩

Only posive tubes (gas) check for the MUG utilization in UV-light (356nm)

MPN Table (3 tube method)

Table 21-3. Most probable number (MPN) index for 9-sample method.

Number of + tubes				Number of + tubes			
0.1	0.01	0.001	MPN/ 100 g	0.1	0.01	0.001	MPN/ 100 g
0	0	0	<3.0	2	2	0	21
0	0	1	3.0	2	2	1	28
0	1	0	3.0	2	2	2	35
0	1	1	6.1	2	3	0	29
0	2	0	6.2	2	3	1	36
0	3	0	9.4	3	0	0	23
1	0	0	3.6	3	0	1	38
1	0	1	7.2	3	0	2	64
1	0	2	11	3	1	0	43
1	1	0	7.4	3	1	1	75
1	1	1	11	3	1	2	120
1	2	0	11	3	1	3	160
1	2	1	15	3	2	0	93
1	3	0	16	3	2	1	150
2	0	0	9.2	3	2	2	210
2	0	1	14	3	2	3	290
2	0	2	20	3	3	0	240
2	1	0	15	3	3	1	460
2	1	1	20	3	3	2	1100
2	1	2	27	3	3	3	>1100

Source: Bacteriologic *Analytic Manual*, 8th ed. Maryland: Food and Drug Administration, 2010.

Enumeration of *S. aureus*

- Take 50 g analytical unit fish to determine aerobic plate count
- Add 450 ml Butterfield's phosphate-buffered dilution water to blender jar containing 50 g analytical unit and blend 2 min (This results in a dilution of 10^{-1}). Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and further as appropriate.
- For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml).
- Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates).
- Invert plates and incubate for 45-48 h at 35°C.

- Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*.
- Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle.

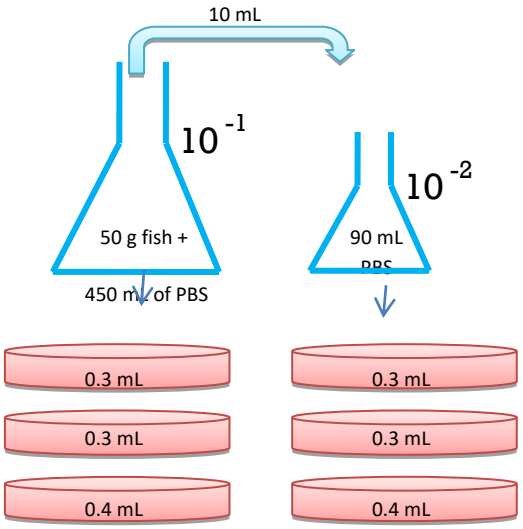
Note: Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used.

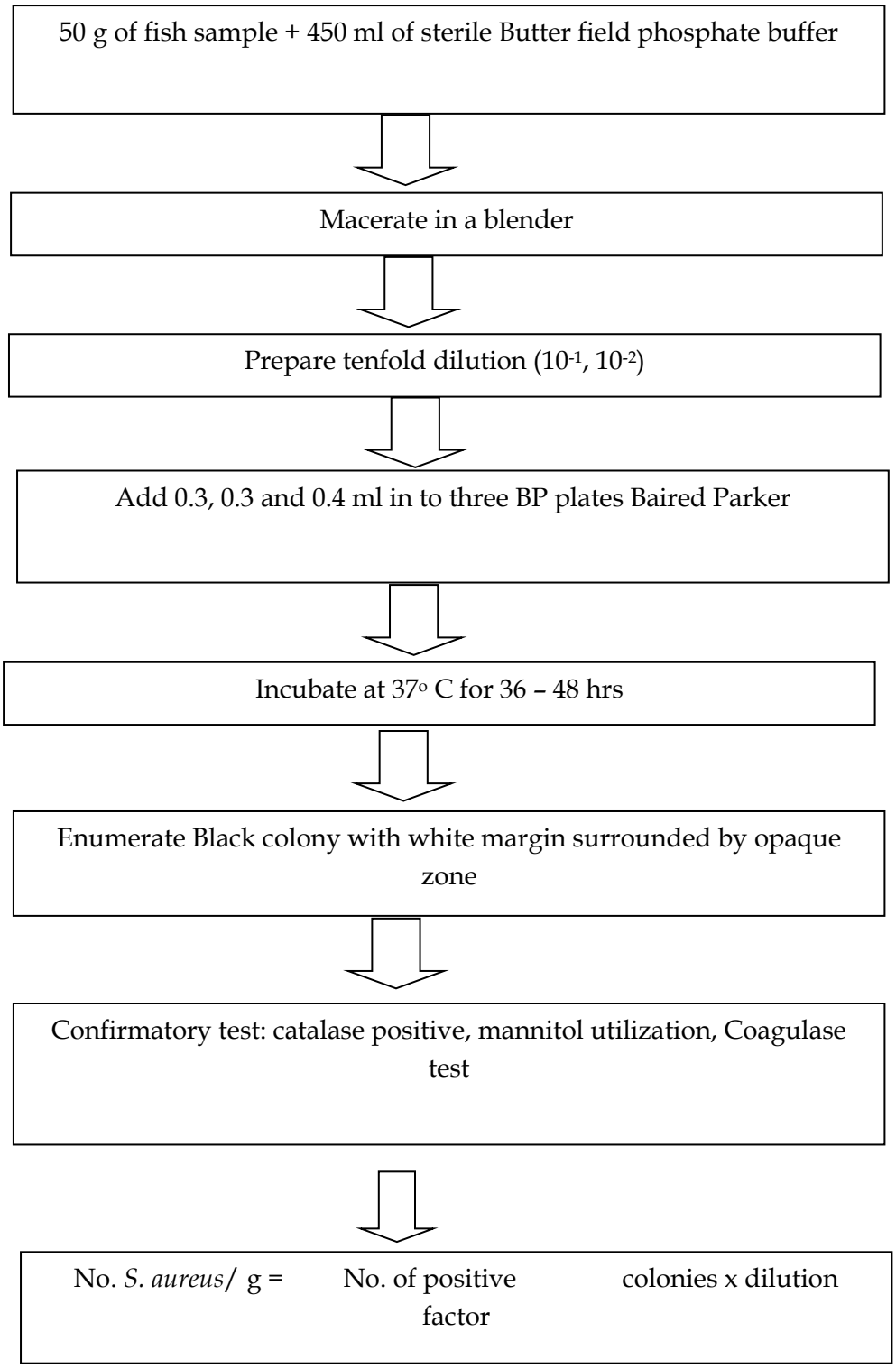
Test for confirmation of *S. aureus*

- Catalase test: Positive
- Grams stain: Gram-positive cocci
- Utilization of mannitol
- Coagulase test: Coagulase production for confirmation

TPC/g = No. of colonies x dilution factor CFU/Gram

Enumeration of *Staphylococcus aureus*




50 g of fish sample + 450 ml of sterile Butter field phosphate buffer
Macerate in a blender
Prepare tenfold dilution (10^{-1}, 10^{-2})
Add 0.3, 0.3 and 0.4 ml in to three BP plates Baired Parker
Incubate at 37° C for 36 – 48 hrs
Enumerate Black colony with white margin surrounded by opaque zone
Confirmatory test: catalase positive, mannitol utilization, Coagulase test
No. *S. aureus*/ g = No. of positive colonies x dilution factor

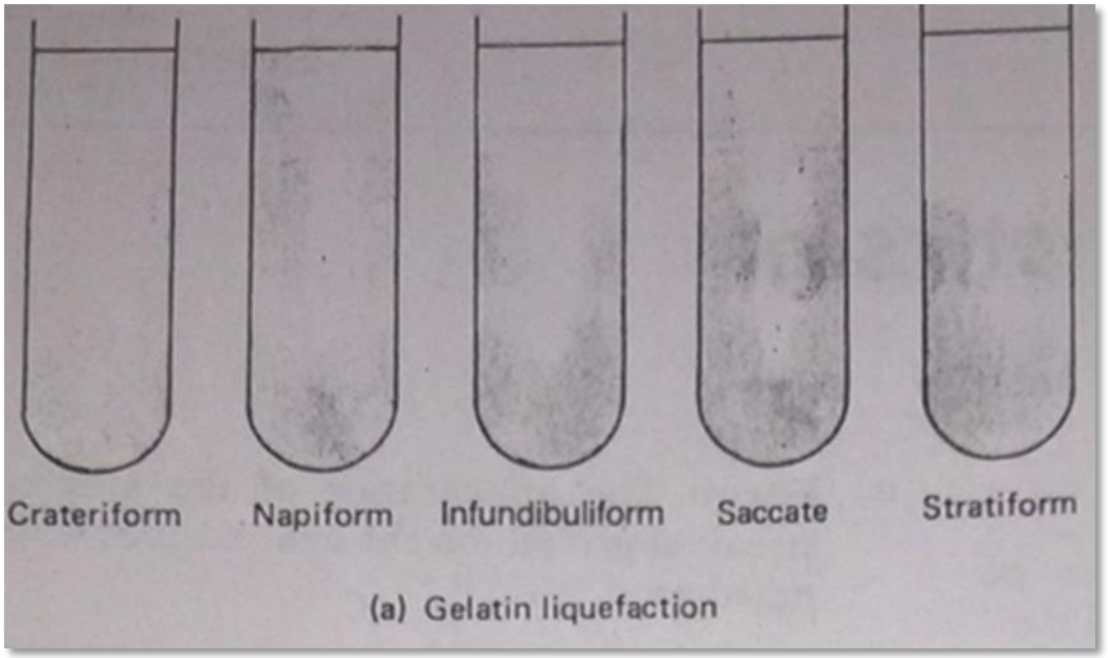
Chapter 4

Overview of biochemical methods for the identification of bacteria

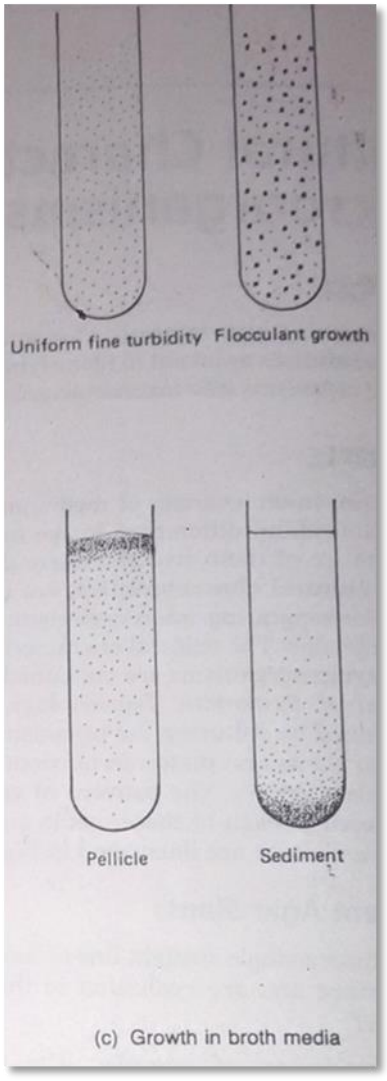
Murugadas Vaiyapuri, Rekha. M, Reshmi. K and Prinetha. U. P

Bacteria are identified up to genus or species level using set of streamlined protocols. They are categorized as polyphasic taxonomy or systematic taxonomy which involves different methods starting from grams reaction, other macromolecular structure characterization, biochemical characterization, characterization for the fatty acid profile and molecularly based on 16s rDNA sequencing analysis, and DNA-DNA hybridization.

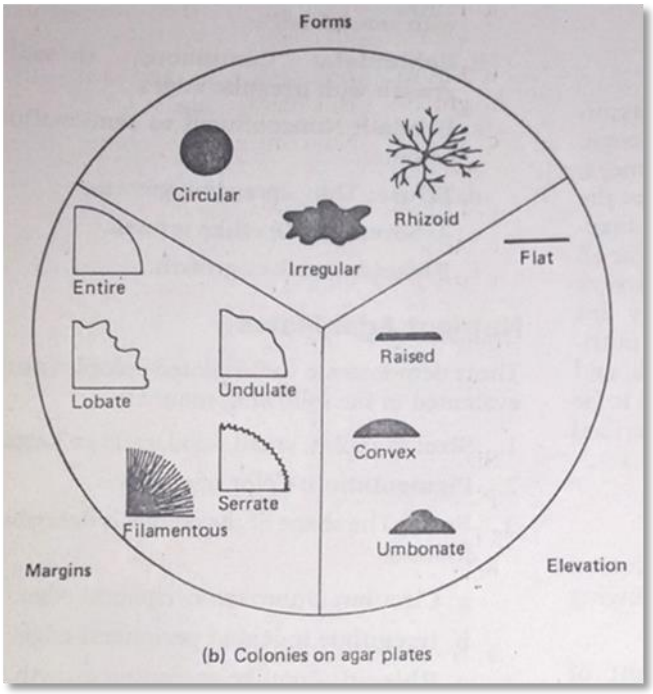
Among these methods' identification based on colony morphology and culture characteristics; (cell arrangement, Gram's reaction, characteristics of other structures) based on staining and microscopy; and biochemical tests are considered as conventional methods of identification of bacteria. These difference in culture characteristics, macromolecule structures, etc plays vital role in the detection or differentiation of the bacteria to the genus or species level. Observation for the abundance of growth, pigmentation, optical characteristic, form of the culture etc in the line of streaking on the nutrient agar or any other non-selective enrichment slants. Colony characteristics on the nutrient agar plates or any other non-selective enrichment plates. Observe for the size, pigmentation, form, margin, elevation in the colony. Observe for the distribution and appearance of the growth in the nutrient broth such as uniform turbidity, flocculants, pellicle, sediment characteristics. Observe in the nutrient gelatine stab culture for the liquefaction pattern. All these characteristics are explained in the diagrammatic representation below and the individual conventional method of identification are addressed in each chapter.



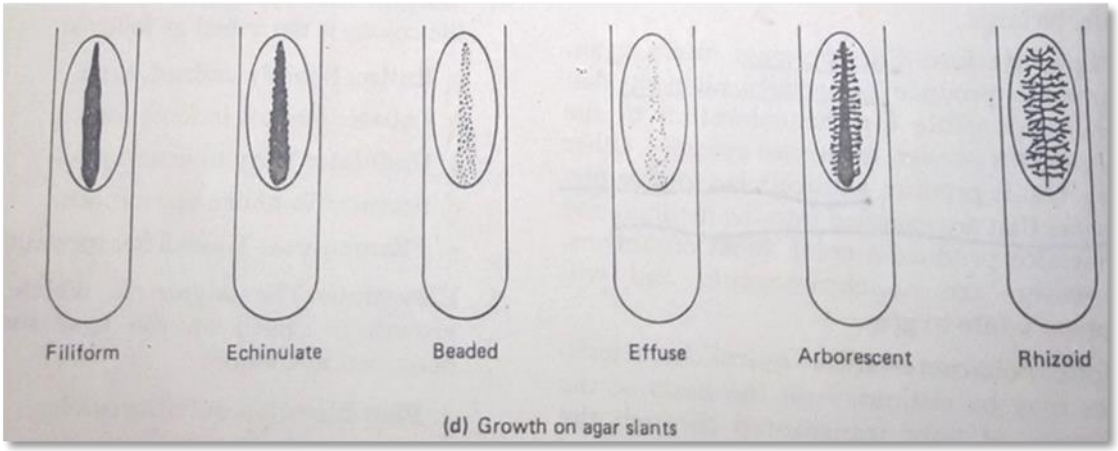
(a) Gelatin liquefaction



(c) Growth in broth media



(b) Colonies on agar plates



(d) Growth on agar slants

Motility test

Organ of locomotion in bacteria is flagella. Flagella mostly identified in Rods with exception of few cocci. Hence bacteria possessing flagella are motile. Bacteria may be flagellated either single or two or in clusters and they located either at ends or throughout the body. In rare case non-motile variants of motile bacteria do exist. Non-motile bacteria generally lack flagella.

Principle

To determine the bacteria is motile or non-motile. Motility of the bacteria can be determined in liquid medium by their movement. Motility can be observed at high power objective (40X)

Materials required

1. Cavity slide
2. Cover slip
3. Motility test medium

Method

Hang's motility test (Direct Method)

- Grow cultures to be tested for motility in broth or on agar slant
- Add a small drop of overgrown cultures or young culture from slants and emulsify with a drop of distilled water on middle of the cover slip.
- Place paraffin jelly on the sides of the cavity slides.
- Invert the slide on the cover slip and make it attached to it.
- Invert it back and observe the drop of culture in 40X objective of microscope at the periphery for the motility of bacteria.

Motility test medium (Semisolid medium)

- Grown the culture to be tested for motility in the plate or slant.
- Stab the centre of the medium with an inoculating needle to a depth of 1/2 inch and incubate it at their desired temperature.

Observation

Positive test means motile ie organism migrate from the stab line and diffuse into the medium as turbidity or as fuzzy streaks of growth. Negative test means no growth or turbidity and clear surrounding the inoculated line. In general, incubate the motility medium at 22-25^{o} C rather than 37^{o} C even if the optimum growth temperature is 35^{o} C. Incubate the tube at 35 for first two days and at 22-25^{o} C for next 5 days.

Biochemical tests

Bacteria do have the biochemical fingerprints that are properties controlled by the cellular enzymatic activity. Biochemical test always has to be related to the bacterial metabolism. Biochemical characterization of bacteria is based on the extracellular enzyme activity and intracellular enzyme activity. Extracellular enzymes are elaborated out of the bacterium

and usually performs the action of hydrolysis to break down complex molecules to simpler building block units which can be further utilized by the bacteria after transporting into the cell. Whereas on the other hand the intracellular enzyme functions inside the cell for the metabolism and the metabolic products are excreted out of the bacterium. This metabolic product accumulated outside of the bacterium is detected in the biochemical test. Biochemical methods involve the identification of activity of both the types of enzymes.

Tests used to identify the extracellular enzymes activity are starch hydrolysis, lipid hydrolysis, casein hydrolysis, chitin hydrolysis etc.,

Tests used to identify the intracellular enzyme activity basically identifying the end product of the reaction are carbohydrate fermentation, litmus reaction, H_2S production, nitrate reduction, catalase, oxidase, IMVC, TSI etc.,

For the starch, lipid and protein hydrolysis test starch, tributyrin, skim milk powder is added in the nutrient agar or composition mentioned in appendix section and checked for their respective activity.

Starch hydrolysis

The degradation of starch molecule by amylase to shorter polysaccharides maltose and dextrin. Overnight grown cultures were streaked onto the starch agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 or 48h. Pour potassium iodide solution or gram's iodine solution over the colony and observe it under the light.

Observing a zone of clearance against the dark blue background is the positive and no clearance zone around the colony is negative for the starch hydrolysis test.

Lipid hydrolysis

The degradation or hydrolysis of lipid molecule by lipase to shorter fatty acid molecule and glycerol or alcohol. Overnight grown cultures were streaked onto the tributyrin agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 to 48h. observe it under the light.

Observing a zone of clearance around the colony is considered as positive and no clearance zone around the colony is negative for the lipid hydrolysis test.

Protein hydrolysis

The degradation or hydrolysis of high molecular weight protein molecule by protease to shorter peptides. Overnight grown cultures were streaked onto the skim milk or casein agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 to 48h. observe it under the light.

Observing a zone of clearance around the colony is considered as positive and no clearance zone around the colony is negative for the lipid hydrolysis test.

Carbohydrate fermentation test

Bacteria obtain their energy through series of enzymatic reactions by majority of cases oxidation of carbohydrate substrates. Some bacteria utilize sugars either in aerobic respiration or through fermentation pathway. Whereas the facultative anaerobes use both pathways. Some of the bacteria do not use sugar also.

Bacteria can be differentiated based on the carbohydrate fermentation for many types of sugars. The media required for the carbohydrate fermentation are described in appendix.

Inoculate overnight grown fresh cultures into the carbohydrate fermentation broth incorporated individually with various sugar. Incubate at various temperature according to the requirement of bacteria and incubate for 24h to 48h. Observe it for the characteristic colour change.

Oxidase test

During aerobic respiration, oxidase enzymes (intracellular cytochrome) catalyzes the oxidation of reduced cytochrome by molecular oxygen which results in the formation of H20 or H2O2 depending on the type of enzyme system they possess.

Oxidase activity was found in the aerobic, facultative anaerobes and microaerophiles. Obligate anaerobes were negative for the oxidase activity. In general, Gram positive organism was oxidase negative with exception of

Bacillaceae and Gram negative in exception to the Enterobacteriaceae were found in majority of the cases.

Principle

Determination of ability of bacteria to produce cytochrome oxidases. This is confirmed by the oxidization of light pink substrate (p-aminodimethyl alaniline oxalate) as electron donors and the substrate is oxidized to the blackish compound in the presence of free oxygen and oxidase enzyme.

Method

- Prepare for the young culture in TSA slant or plate
- Add directly the substrate containing solution as 1% or 0.5% on the colony or pour the solution on to the Whatman filter paper No.1 and pick a colony of the young culture and streak onto the filter paper loaded with substrate.

Observation

Dark pink, maroon, finally black or purple colour development denotes positive for oxidase test. No colour change or light pink indicates negative for oxidase test. The result should be read within 10 to 30 seconds.

Catalase test

In aerobic respiration the bacteria produce hydrogen peroxide and toxic superoxide. Accumulation of these toxic compound result in death of cell. In order to avoid this the bacteria, produce catalase to rapidly degrade hydrogen peroxide. Superoxide dismutase is the enzyme used for the degradation of the toxic superoxide. So, catalase production can be determined by the addition of 3% H_2O_2 and observe for the bubbles of free oxygen as gas in the slide.

Keep three drops of 3% H_2O_2 and add a minute quantum of culture picked out from individual isolated colony or drop H2O2 on to the colony and observe for bubbling or foaming.

Other biochemical test is Indole production, H_2S production, Methyl red test, Voges Proskauer test, Citrate utilization, Urease test, Gelatin liquefaction test.

The list of test performed such as grams reaction or any other staining methods to detect or identify the macromolecular structure such as capsule,

spore, or acid fastness or shape and arrangements, cultural characteristics, and other biochemical tests such as oxidase, catalase, H2S production, IMVC, urease, gelatin liquefaction, nitrate reduction, carbohydrate fermentation, O-F test, starch hydrolysis, lipid hydrolysis, and protein hydrolysis should be tabulated and should be checked with the standard reference books such as Bergey's manual of Systematic Bacteriology for arriving at genus or sometimes species level identification of bacteria.

Miniaturized biochemical identification systems

In order to reduce the consumption of media, reagents in the biochemical test where larger volume are consumed for identifying different group of bacteria several modifications were done in the biochemical identification scheme. In economic way multitest kits were developed with identification schemes. The bacteria were first grouped based on Gram's reaction, oxidase, catalase, motility test and then identified with the help of multitest kits where in bacteria can be identified even up to species level.

These are comprehensive biochemical identification kit proposed to identify gram positive bacteria, gram negative bacteria, *Enterobacteriaceae*, non-*Enterobacteriaceae* with twenty biochemical tests in majority of the cases.

Numerous commercial kits are available in the market and our laboratory concentrate on the API kit sold by Biomerieux. In case the definitive identification could not be done with this method few more supplementary tests should be performed. The results of these kits should be correlated with the type and source of sample, colony morphology etc.

It is absolutely essential to use the manufacturers instruction and guidelines to perform the test and read the result and interpretation of results. Some of the kits used by our laboratory for the identification of bacteria based on the conventional identification scheme with the help of kit are API 20E for *Enterobacteriaceae*, API 20NE for non-*Enterobacteriaceae*, API 50CHB for *Bacillus*, and API STAPH for species differentiation of *Staphylococcus*.

Recently completely automated systems have been developed such as vitek, bactec, remel, biolog systems for the rapid identification of bacteria based on biochemical profiles are now in the market.

Chapter 5

Isolation of *Salmonella* from seafood

T. Muthulakshmi and Greeshma .S.S

Salmonella is a Gram-negative, catalase positive, oxidase negative, facultative anaerobe food borne pathogen which belongs to *Enterobacteriaceae* family. *Salmonella* was first isolated from animal intestine samples by American bacteriologist named Daniel salmon in 1800s. They normally inhabit the intestine of living organisms and compete for food supply. When the *Salmonella* count increases it cause disease collectively called as Salmonellosis. Nausea, fever, vomiting, diarrhea and abdominal cramps are the common symptoms in Salmonellosis. Salmonellosis can be caused by eating uncooked food contaminated with feces of affected warm-blooded animals.

There are more than 2200 serotypes found to exist. Salmonella serotyping is based on somatic(O) antigen, Capsular (Vi) and flagella(H) antigen. In epidemiological point of view, *Salmonella* can be divided into typhoid group, Animal group and food poisoning group. Animal group consists of the particular species adopted for an animal or bird. Food poisoning group don't have particular host preference. If affects all animals and human-being.

Salmonella are resistant to various environmental factors. They can tolerate and grow in temperature from 8-45^{0}C, water activity above 0.94, pH range of 4-8. *Salmonella* will be completely eliminated in the temperatures above 70^{0}C, but they are resistant to some extend for most of the processing methods like chilling, drying, salting, chlorine and other surface contamination cleaning method.

Isolation from seafood

Salmonella is a fastidious organism, generally the occurrence is less in seafood, so that it requires a pre-enrichment and enrichment prior to plating on selective media. Salmonella should be absent in 25 g of seafood tested

Materials required for isolation

1. Lactose broth
2. Tetrathionate broth
3. Rappaport Vassiliadis enrichment broth
4. Bismuth sulphate agar
5. Hectoen enteric agar
6. Triple sugar iron agar
7. Lysine iron agar
8. Urea agar
9. Simmons citrate agar
10. Brain heart infusion broth
11. Malonate broth
12. Trypticase soy broth
13. Sugars lactose, sucrose, Dulcitol

Pre-enrichment:

Generally, 25g of sample will be pre-enriched in 225ml Lactose broth. But for RTE(Ready to eat) products 225g sample should be pre-enriched in 2.025L of Lactose broth.

Media required: Lactose broth:

Beef extract – 3g

Peptone – 5g

Lactose – 5g

Distilled water – 1L

pH – 6.9 ± 0.1

Blend 25g of sample in 225 ml Lactose broth in stomacher bag with stomacher blender for 30 seconds. Transfer to conical flask and incubate at 37°C for 24 hours. *Salmonella* is lactose negative organism, it cannot ferment lactose to acidic products. Then also we are using lactose broth as Pre-enrichment media because all bacteria other than *Salmonella* can ferment lactose and change media pH to acidic which limits their survival. Only Salmonella can survive in acidic pH.

Selective enrichment

Tetra thionate Broth (TTB)

Tetra thionate Broth (TTB)base: 4.5 g (BD and DifCo)

Distilled water: 100ml

Dissolve properly TTB base in distilled water in boiling water bath. Do not autoclave. Check pH and add 2ml iodine solution (6.5g Iodine crystals and 5g Potassium iodide in 20ml distilled water). Mix properly and transfer 10 ml to sterile tube.

Rappaport Vassiliadis broth (RV Broth)

RV broth base: 26.6g (BD and DifCo)

Distilled water: 1L

pH: 5.1 ± 0.2

Dissolve RV broth base distilled water and transfer 10 ml to tubes. Autoclave at115° C, 10lbs for 20 minutes

USFDA advices the usage of both Tetra thionate Broth (TTB) and Rappaport-Vassiliadis media (RV) for selective enrichment of *Salmonella*. Transfer 1ml of pre-enriched lactose broth to 10 ml TTB and incubate at 37°C for 24 hours. Simultaneously transfer 0.1ml of pre-enriched lactose broth to 10 ml RV media and incubate at 42°C for 24 hours.

Selective plating

Streak one loopful culture from both enrichment media on to pre dried selective plating mediums like Hectoen Enteric Agar (HEA), Xylose Lysine Desoxycholate Agar (XLD) and Bismuth sulphite Agar (BSA). Incubate all plates at 37°C for 24 hours and select typical colonies of *Salmonella* for further confirmation.

Media preparation

Hektoen Enteric Agar (HEA)

Hektoen Enteric Agar (HEA) – 76g (BD and DifCo)

Distilled water – 1L

pH – 7.5 ± 0.2

Do not autoclave the medium

This used to differentiate between *Salmonella* and *Shigella.* HEA contains sugars like lactose, sucrose and salicin and bromo thymol blue and acid fuschin as acid/base indicators. The ferric salt in this media will act as H_2S indicator. *Salmonella* cannot ferment all the above three sugars and will appear as blue to green colour colony with or without black centre in HEA

while all other can ferment sugars and appear as yellow to orange colour colonies.

Xylose Lysine Desoxycholate Agar (XLD)

XLD : 55g(BD and DifCo)

Distilled water: 1L

pH – 7.4 ± 0.2

Do not autoclave the medium

XLD contains sugars like xylose, sucrose, lactose and lysine. Phenol red will act as the indicator and ferric ammonium citrate in the medium will act as H_2S indicator. *Salmonella* cannot ferment lactose and sucrose, but it can ferment the xylose and change the media pH to acidic. But the decarboxylation of lysine by *Salmonella* will produce a basic product called cadaverine which neutralizes the acidic pH and changes the reaction to alkaline. Hence *Salmonella* colonies on XLD will appear a s red to pink colonies with or without black center (due to H_2S production by some *Salmonella spp).* Others will ferment sugars like lactose and sucrose will appear yellow to orange coloured colonies.

Bismuth sulphite Agar (BSA)

BSA : 52g (BD and DifCo)

Distilled water: 1L

pH – 7.0± 0.2

Do not autoclave the medium

BSA contains brilliant green and Bismuth sulphite which suppress the growth of Gram positive organisms and other coliforms. This medium contains glucose as the fermenting sugar. The metallic ions like Bi++ and Fe++, present in the medium will stains salmonella colonies and the surrounding media to black/ brown colour. Typical *Salmonella* forms black to brown colonies with metallic sheen followed by a black to brown background.

Typical colonies from all plates can be picked and confirmed as *Salmonella* by various biochemical reactions, PCR and serotyping.

Table: Biochemical reactions for *Salmonella*

Sl no	Biochemical test	Result	Remarks
1	Gram staining	negative	
2	motility	motile	
3	TSI	Alkaline slant Acid butt	H_2S positive, Gas positive
4	LIA	Alkaline slant, alkaline butt,	
5	Indole	Negative	
6	Urease	Negative	
7	Glucose	Positive	
8	Lactose	Negative	
9	Sucrose	Negative	
10	Dulcitol	Positive	Acid and gas production
11	Salicin	Negative	
12	MR test	Positive	
13	VP test	Negative	
14	Lysine decarboxylase	Positive	
15	Malonate	Negative	
16	Citrate	Positive	

Protocol for the isolation of *Salmonella* from fish

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Pre enrichment- Mix 25 g of pooled sample with 225 ml of Lactose broth(pH-6.8), macerate in a stomacher blender

⇩

Incubate overnight at 37^0 C

⇩

Tranfer 1 ml of pre-enrichment to Tetrathionate broth, 0.1 ml of pre-enrichment to Rappaport vassiliadis broth

⇩

Incubate TTB in 37 ^{0} C and RV broth to 42 ^{0} C

⇩

Streak to predried HEA, BSA and XLD plates

⇩

Incubate overnight at 37^0 C

⇩

Pick typical colonies (HEA green with or without black centre, XLD pink with or without black centre, BSA black colonies)on to TSA slants Proceed for biochemical tests

Serological confirmation: *Salmonella* suspected cultures giving typical biochemical reactions are confirmed by agglutination test with *Salmonella* polyvalent antiserum in the slide with one drop of culture and anti-sera

Positive result- agglutination in text mixture, no agglutination in the saline control

Negative result- No agglutination in text mixture, no agglutination in the saline control

Nonspecific- agglutination both in test and control

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Chapter 6

Detection of pathogenic of Vibrios: *Vibrio mimicus*, *Vibrio harveyi* and *Vibrio parahaemolyticus*

Ezhil Nilavan S., Muthulakshmi, T. and Minimol V A.

Aquaculture is one of the growing vibrant food production sectors worldwide. However, the major drawbacks in this system are the sudden onset of diseases, especially by *Vibrio* spp. This is becoming a great concern in larval and juvenile penaeids. Hence, the monitoring of aquaculture environments for pathogenic Vibrios is essential to control the spread of Vibrio infections. The members of the genus *Vibrio* are the most important food-borne and aquatic pathogens which are responsible for illness in humans and cause large-scale mortality in the aquaculture sector. Nowadays in the international trade of marine fishes, testing of *Vibrio* species has become a criterion of microbiological testing. Even though *Vibrio* species are a common inhabitant of the aquatic environment, some species are emerging as pathogens which can cause up to more than 50% of deaths of all clinical cases. Major *Vibrio* sp. viz. *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. mimicus, and V. splendidus* are usually associated with shrimp diseases. *V. harveyi* is associated with luminescent vibriosis in shrimps e.g., *Litopenaeus vannamei* and *Penaeus monodon* and it is the most important etiological agent for mass mortality in *P. monodon*. The mode of infection in fish mainly consists of penetration of bacterium to the host tissue mainly by the chemotactic activity, followed by deployment of the iron sequestering system and eventually damages the fish through extracellular products i.e., hemolysin and protease.

Traditional method of detection of pathogenic *Vibrio* species

There are well-established isolation and biochemical confirmation procedures for pathogenic *Vibrio* spp. Which were described in ISO and BAM protocol for Vibrios. First stage in traditional detection methods exploits the ability of *Vibrio* species to grow rapidly at relatively high pH values. Media

containing sodium chloride and with a pH of about 8.6, such as alkaline saline peptone water (ASPW), are used for enrichment. Typically, a 6-hour preliminary enrichment (at 41.5°C for fresh products, or 37°C for frozen or salted products) is followed by a second enrichment in ASPW at 41.5°C (for *V. cholerae* and *V. parahaemolyticus*) or 37°C (for other species) for 18 hours. Preliminary identification based on colony appearance on TCBS agar is traditionally confirmed using classical biochemical tests. The second enrichment culture is inoculated onto thiosulphate citrate bile salts sucrose (TCBS) agar and one other optional selective medium and incubated at 37°C for 24 hours. On TCBS agar, *V. mimicus* colonies are green, *V. parahaemolyticus* colonies appear blue-green and *V. harveyi* colonies are green in color. Selective chromogenic agar media specifically designed for the differentiation of pathogenic *Vibrio* species are also available.

Protocol for the isolation of *V. mimicus* from fish

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW, macerate in a stomacher blender

⇩

Incubate APW at 35 ± 2 ^{0}C for 16 to 18 hours and transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate TCBS Plates overnight at 35 ± 2 ^{0}C

⇩

V. mimicus appears as small 2-3 mm, smooth green colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 2% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- Gram negative short rods
- String test positive
- Arginine decarboxylase-Negative
- Lysine, Ornithine decarboxylase- positive
- Sucrose Negative
- Growth in 0% salt, no growth in 6% salt

Protocol for the isolation of *V. harveyi* from aquaculture samples

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW, macerate in a stomacher blender

⇩

Again, transfer a transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate overnight at 35 ± 2 ^{0} C

⇩

V. harveyi appears as large 2-3 mm, green colony

⇩

streak to marine agar and check for fluorescence

⇩

Pick typical colonies on to TSA slants with 2% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation: These bacteria are

1. Gram negative, Motile rods
2. Oxidase- and catalase-positive
3. Green colonies on TCBS agar
4. These isolates do not utilize inositol, sorbitol, and melibiose
5. lysine decarboxylase, ornithine decarboxylase, and gelatinase-positive,
6. Sensitive to the vibrio static reagent, 0/129.
7. Glucosamine positive.

Protocol for the isolation of *V. Parahaemolyticus* from seafood

All the media used for the biochemical identification of *Vibrio parahaemolyticus* should contain 2 or 3% NaCl

25 g of Sample
(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 3% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Streak a loopful from APW onto a TCBS plate with 3% NaCl. Incubate APW overnight at 35 ± 2 ^{0}C

⇩

V. Parahaemolyticus appears as round, opaque, green or bluish colonies 2-3 mm in diameter on TCBS

⇩

Pick typical colonies on to TSA slants with 3% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation

- Oxidase positive
- Gram negative, straight/ curved rods
- Non H_2S producer
- Growth in 3 %, 6%, 8% NaCl, No growth in 0 % NaCl
- *V. parahaemolyticus* can be differentiated from other Vibrios by ONPG, Salt tolerance and lactose reactions
- Resistance to 10 μg of O/129, sensitive to 150 μg of O/129

Protocol for the isolation of *V. alginolyticus* from aquatic samples

All the media used for the biochemical identification of *Vibrio algionlyticus* should contain 2 or 3% NaCl

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 3% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Streak a loopful from APW onto a TCBS plate with 3% NaCl. Incubate APW overnight at 35 ± 2 ^{0}C

⇩

V. alginolyticus appears as large yellow colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 3% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive, Gram negative
- Growth in 3 %, 6%, 8%, 10% NaCl, No growth in 0 % NaCl
- Decarboxylase test results–Arginine negative, Ornithine positive, Lysine positive.
- Sugar test results- Sucrose, D-Mannose, D-Mannitol -Positive, D-cellobiose, Lactose, Arabinose –negative,
- Resistance to 10 μg of O/129, sensitive to 150 μg of O/129.

Protocol for the isolation of *V. vulnificus* from aquatic samples

All the media used for the biochemical identification of *Vibrio vulnificus* should contain 1-2% NaCl. *Vibrio vulnificus* is a waterborne bacteria which affect the vulnerable population with iron storage disorders. This bacteria can cause ulcerative disease in cultured fishes and can cause of mass mortality

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 2% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Streak a loopful from APW onto a TCBS plate with 2% NaCl. Incubate APW overnight at 35 ± 2 ^{0}C

⇩

V. vulnificus appears as 2-3 mm green colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 2% NaCl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- Catalase positive
- Gram negative
- Growth in 1 %, 3%, 6%, NaCl, No growth in 0 % NaCl
- Decarboxylase test results–Arginine negative, Ornithine positive, Lysine positive for biotype 1. Lysine positive arginine, ornithine negative for biotype 2
- Salicin, mannitol, cellobiose, lactose, indole positive.
- Citrate, acetoin production and urease negative
- Resistance to 10 μg of O/129, sensitive to 150 μg of O/129.
- Resistant to colistin

Molecular methods

A simple and rapid identification method of *Vibrio* causing the disease to aquaculture settings is essential for taking preventive and curative measures in aquaculture. PCR-based identification is a suitable alternative because it is comparatively easy, less expensive, and can be completed within several hours. However, the success of this method depends on the selection of target genes, which should be species-specific, widely distributed, and also stable in the genome. PCR assays for the identification of pathogenic vibrio species are listed in table 1.

Table 1: PCR detection of pathogenic Vibrios

Pathogen	Primer name	Primer	PCR conditions & product size.	Product size
V. parahaemolyticus	*Species specific toxR-F* *toxR-R*	F:5'-GTCTTCTGACGCAATCGTTG-3' R: 5'-ATACGAGTGGTTGCTG	94°C, 03 min 30; 94°C for 01min 63°C for 1.5 min	368bp

		TCATG-3’	72^{o}C for 1.5 min 72^{o}C for 03 min	
	Virulence specific *tdh*	F**:**5’-TGACTGTGAACATTAATGA-3’ R**:**5’-CGATTCTTTGTTGGATATAC-3’	94^{o}C, 03 min 30; 94^{o}C for 30 sec 45^{o}C for 30 sec 72^{o}C for 01 min 72^{o}C for 05 min	263 bp
	Virulence specific *trh*	F**:**5’-TTGGCTTCGATATTTTCAGTA TCT-3’ R**:**5’-CATAACAAACATATGCCCATT TCCG-3’	94^{o}C, 03 min 30; 94^{o}C for 01min 58^{o}C for 01 min 72^{o}C for 01 min 72^{o}C for 03 min	500bp
Vibrio harveyi	*Tox R*	F:5’-GAAGCAGCACTCACCGAT-3’ R:5’-GGTGAAGACTCATCAGCA-3’	94^{o}C, 05 min, 30; 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 1 min, 72^{0}C for 10 min	382 Bp
V. mimicus	*Vmh*	F:5’-GGTAGCCATCAGTCTTATCACG-3’ R:5’-ATCGTGTCCCAATACTTCACCG-3’	95^{o}C for 30 cycles; 95^{o}C for 45 sec 55^{o}C for 45 sec 72^{o}C for 35 sec	390 bp

			72°C for 05 min	
V.alginolyticus	*VAL*	F:5'-CGA GTA CAG TCA CTT GAA AGC C-3', R: 5'-CAC AAC AGA ACT CGC GTT ACC-3'	95° C for 15 min,30; 94°C for 30 s, 57°C for 30 s, 72°C for 60 s, 72°C for 5 min	737 bp
Vibrio vulnificus	*vvhA*	F-CGCCGCTCACTGGGG CAGTGGCTG R- CCAGCCGT TAACCGAACCACCCGC	95° C for 10 min,25 cycles 94°C for 60 s, 62°C for 60 s, 72°C for 60 s, 72°C for 5 min	518 bp

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Chapter 7

Introduction to automated microbial system

G. K. Sivaraman

BD Pheonix™ M50 system is an Automated Microbioal System – An *In vitro* Rapid identification (ID) and quantitative determination of Antimicrobial Susceptibility Test (AST) by Minimum Inhibitory Concentration (MIC) of bacteria. It adheres strictly to Clinical Laboratory Standard Institute (CLSI) categorical interpretive criteria for reporting susceptibility patterns. The combination panels (ID/AST) using 51 wells includes an ID side with dried substrates for bacterial identification and an AST side with varying concentrations of antimicrobial agents, growth and fluorescent controls at appropriate well locations. The system can provide rapid identification of most clinically significant Gram-negative and Gram-positive bacteria, as well as yeast. The BD Phoenix™ M50 system offers 85 wells for antimicrobial dilutions on standard AST-only panels and combination ID/AST panels. The BD Pheonix NMIC/ID – 55 panel has been designed for identification and MIC of Gram negative Enterobacteriaceae and Non Enterobacteriaceae along with ESBL detection. The BD Pheonix NMIC-500 panel (has been specially designed for carbapenem minimum inhibitory concentrations (MICs), and carbapenamase-producing organisms (CPO) detection. The CPO utilizes meropenem, doripenem, temocillin and cloxacillin alone and in combination with various chelators and beta-lactamase inhibitors in amount required for the detection and classification of CPOs.

The BD Phoenix™ M50 system panels test for several resistance markers such as:

For Gram-positive bacteria

- HLAR—High Level Aminoglycoside Resistant *Enterococcus*
- iMLSb—Inducible Clindamycin Resistance
- MRSA—based on Oxacillin Interpretation with *Staphylococcus* species
- *mecA* – detection of *mecA*-medicated resistance in *Staphylococcus aureus*
- BL- *Staphylococcus* ß-Lactamase (Nitrocefin based test)
- VRSA—Vancomycin-Resistant *Staphylococcus Aureus*
- VRE—based on Vancomycin interpretation

For Gram-negative bacteria

- CPO—Carbapenemase Producing Organism
- ESBL—offered on Gram-negative panels and requires no additional disc diffusion or E-Test confirmation.

For detecting Carbapenemase-Producing Organisms (CPOs)

- The BD Phoenix™ CPO detect test, available on certain BD Phoenix™ Gram-negative panels, provides information, including Ambler classification, to help guide clinicians in their treatment decisions.
- This test is available in two-panel configurations that allow microbiology labs to test for CPOs as part of routine susceptibility testing on BD Phoenix™ panels:
  - 2-well configuration—provides detection of Carbapenemase-producing organisms in *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*
  - 9-well configuration-provides detection of Carbapenemase-producing organisms and Ambler classification (A, B or D) in *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

- The BD Phoenix™ CPO detect test is currently the only phenotypic test on an automated AST system that provides CPO detection and Ambler classification.
- On average, the BD Phoenix™ CPO test detects and classifies CPOs from isolated colonies within 6 to 11 hours.

Procedure for the identification and AST of *E. coli* & *S. aureus*:

Briefly, bacterial colonies were transferred to the ID broth (BD Difco diagnostic systems) and the inoculum density is adjusted to 0.5 McFarland using BD PhoenixSpec nephelometer. Twenty five microlitres of the adjusted ID broth is transferred to the AST broth (BD Difco diagnostic systems) with the AST indicator which is a resazurin-based dye. The suspensions (ID broth inoculum and AST broth inoculum) are then poured through the respective fill port on both sides of the panel. Panels were loaded to the instrument within 30 minutes of inoculation. Quality controls were also performed using the quality control strains *E. coli* ATCC 25922.

Two distinct ID-AST combo panels (NMIC/ID-55 for *E. coli* and PMIC/ID-80 for *Staphylococcus*) are used for identification and antibiotic susceptibility testing. The combination panel includes an ID side with dried substrates for bacterial identification and an AST side with varying concentrations of antimicrobial agents, growth and fluorescent controls at appropriate well locations. Procedures are performed according to manufacturer's instructions.

BD Phoenix™ M50 instrument and ID/AST combo panels

List of antimicrobial agents/ resistance markers featured on the panels

Gram Negative	Gram Positive
Antibiotics	
Amikacin	Ampicillin
Amoxicillin/Clavulanate	Cefazolin
Ampicillin	Cefoxitin
Aztreonam	Clindamycin
Cefazolin	Erythromycin
Cefoperazone/Sulbactam	Gentamicin
Cefotaxime	Gentamicin-Synergy
Cefoxitin	Inducible Macrolide resistance test (iMLSb)
Ceftazidime	Levofloxacin
Chloramphenicol	Linezolid
Ciprofloxacin	Beta-Lactamase (Nitrocefin-based)
Colistin	Moxifloxacin
Gentamicin	Nitrofurantoin
Imipenem	Norfloxacin
Levofloxacin	Oxacillin
Meropenem	Penicillin
Piperacillin	Quinupristin/Dalfopristin
Piperacillin/Tazobactam	Rifampin
Tetracycline	Teicoplanin
Trimethoprim/Sulfamethoxazole	Tetracycline
ESBL	Trimethoprim/Sulfamethoxazole (SXT)
Cefotaxime/Clavulanate (ESBL)	Vancomycin
Ceftazidime/Clavulanate (ESBL)	
Cefpodoxime-proxetil (ESBL)	
Ceftazidime (ESBL)	
Ceftriaxone/Clavulanate (ESBL)	

Chapter 8

Introduction to Antimicrobial Resistance

G. K. Sivaraman

Introduction

Antimicrobial resistance (AMR) is the ability of the microorganisms (bacteria, fungi, viruses and parasites) resist the action of antimicrobials, it's very difficult to treat the common infections and could cause severe illnesses, chance of spreading of infections and finally lead to death. If we are failure to tackle AMR as a pandemic issue, it could lead to 10 million deaths annually by 2050 and costing $100 trillion lost from global GDP (O'Neill 2014). AMR is a major cause of death globally, with a burden likely to be higher than that of HIV or malaria. A comprehensive systematic study estimated that globally AMR was associated with 4.95 million deaths, including the direct contribution to 1.27 million deaths, in 2019 and India had one of highest burdens of AMR and maximal resistance trends in Asia (Murray et al., 2022). Moreover, AMR threat has long been signaled from the Recommendation-Commission on antibiotic resistance in 2013 and the O'Neil report, Global Antimicrobial Resistance and Use Surveillance System (GLASS) by WHO in 2015, Fleming Fund, 2015 and G7 Finance Ministers issued statements to support antibiotic development in 2021. But action has been episodic and uneven, resulting in global inequities in AMR. However, surveillance on AMR, diagnostics, treatment, control, vaccines, discovery of new antibiotics are extremely in slow progress. Moreover, the recent SARS-COVID-19 pandemic could have been worsened the emergence of AMR due to unexpected and unpredicted prescriptions of antibiotics (Hsu, 2020).

Aquaculture farming and use of antibiotics in aquaculture

Due to consumer's food habit and awareness on health, fish and fisheries products get more attention across the globe due to nutrient

contents namely essential protein, poly unsaturated fatty acids (PUFA), micro, and macro-nutrients. Now the per capita consumption of fish in the world was 9.0 kg in the year 1961, which grew to 20.5 kg in 2017 (FAO, 2018). Because of its higher demand and exponential population growth, the intensified aquaculture culture farming system is becoming blooming on every years as becomes an intensive and super intensive aquaculture farming system. So the intensive aquaculture often demands the use of formulated feeds, antibiotics, disinfectant's, water, soil treatment compounds, algaecides, pesticides, fertilizers, probiotics and prebiotics etc. (Subasinghe et al., 2000; Bondad- Reantaso et al., 2005 and Rico et al., 2013) which could cause severe stress on fishes that lead to disease outbreak (Rottmann et al., 1992) and with high mortalities. So the fish farmers are often bound use antibiotic to control the diseases. Generally the antibiotics are administered through feed or applied directly into the aquaculture ponds (Heuer et al., 2009, Pham et al., 2015, Okocha et al., 2018). Moreover, the administered antibiotics are not metabolize completely by the fishes and almost 75% of the consumed antibiotics are excreted in to the pond through feces and directly applied antibiotics in the ponds will remains for a certain period (varied days of withdrawal period for different antibiotic). As on now, there is no defined antibiotics are produced for the control of fish diseases, often veterinary antibiotics are being used in fish farming (Chi et al., 2017).

Trends of antibiotic consumption

Global antimicrobial consumption in aquaculture in 2017 was estimated at 10,259 tons and antimicrobial consumption in aquaculture is expected to increase 33% between 2017 and 2030 and mainly due to its expansion of aquaculture farming. The four countries with the largest share of antimicrobial consumption in 2017 were all in the Asia-Pacific region: China (57.9%), India (11.3%), Indonesia (8.6%), and Vietnam (5%) and they represented the largest share of aquatic animal production output in 2017: China (51.2%); India (9.9%); Indonesia (9.8%); and Vietnam (5.7%) (Schar et al., 2020). India accounts for about 3% of the global consumption of

antimicrobials in food animals (Van Boeckel et al., 2015). By 2030, global antimicrobial use from human, terrestrial and aquatic food producing animal sectors is projected to reach 236,757 tons annually. On an equivalent biomass basis, estimated antimicrobial consumption in 2017 from aquaculture (164.8 mg kg−1) is 79% higher than human consumption (92.2 mg kg−1) and 18% higher than terrestrial food producing animal consumption (140 mg kg−1), shifting to 80% higher than human (91.7 mg kg−1) consumption and remaining 18% higher than terrestrial food producing animal consumption projected in 2030 (Schar et al., 2020).

Antibiotics used in Aquaculture

Globally, the most commonly used classes of antimicrobials were quinolones (27%), tetracyclines (20%), amphenicols (18%), and sulfonamides (14%) (Lulijwa Ronald et al., 2020). Most frequently reported antibiotic compounds in Asian aquaculture production were sulphonamides: sulphadiazine, sulfamethoxine; beta-lactams: amoxicillin and florfenicol [Rico et al., 2012]. Food and Drug Administration (USFDA) has approved oxytetracycline, florfenicol, and Sulfadimethoxine/ormetoprim antibiotics for use in aquaculture (Romero et al., 2012).

Factors influence of antimicrobial resistance (AMR)

Aquaculture system may act as the source of AMR pathogen by collecting from all possible settings and potential source for dissemination of AMR across the settings since its well interconnected system in India. The aquaculture system either use the natural water bodies (rivers, lakes, streams, marine backwater and sea cage) and human made aquaculture farming (fin fishes and shell fish farming) are frequently getting a chance of contracting with the AMR pathogen, antibiotic residues and AMR contributing factors such as biocides, chemical residues (cu, selenium, lead etc), heavy metal contaminations, pesticides,, global warming and water quality parameters (pH, salinity, DO, ammonia, nitrate, nitrites, etc) through domestic, industrial and hospital sewage and agricultural runoff. Whereby the existing potential normal microflora of the aquatic system would acquire

these ARGs through HGT or vertical and development resistance against these pollutants and influence the transfer of ARGs between them which lead to the accumulation of AMR pathogens and risk to the clinical settings (Michael et al., 2013). Antimicrobial resistant bacteria can be transferred from food animals to humans either through direct contact with animals, contaminated foods, or indirectly through contaminated environments (Sharma et al., 2018, Argudín et al., 2017, Muloi et al., 2018).

The important listed AMR pathogens by FAO/ WHO/ OIE tripartite are the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) whereas, numerous publications are pouring in the recent years with non- pathogenic bacteria are also harboring from a few to more than 10 numbers of antimicrobial resistance genes (ARGs) and also harbor virulence and toxigenic genes. So these non-pathogenic antibiotic resistant bacteria in this aquatic system are either ignored or not monitored properly since these species could act as potential reservoir for the dissemination of AMR. However, a clear cut understanding of the origin and environmental factors that account for the clinical appearance of ARGs is still lacking. Moreover consistent study is warranted to prevent the extent of AMR amplification and its dissemination under the influence of the environmental selection pressure/ factors and to evaluate of its risks (pathogenicity) to human, animal and aquatic animal health. So thereby prevent the spread of AMR infection through proper sanitation, hygiene, use of protective gears, proper disposal of waste and infection prevention measures, proper treatment of effluent from hospitals, manufacturing waste and impact of antibiotic discharges, reducing unnecessary use in aquaculture, promote development of new rapid AMR diagnostics, promote the development of vaccines, immune-modulators, antimicrobial peptides, digestible enzymes in feed, endolysins, hydrolases, and new drugs, enhance the potential of existing antibiotics and finding alternatives to the antibiotics (bacteriophage therapy, pre and probiotics) and CRISPR- cas9 genome editing etc.

Regulation of antibiotics used in aquaculture

The use of antibiotics in aquaculture in India is regulated by government agencies such as Coastal Aquaculture Authority of India (CAA), Marine Products Export Development Authority (MPEDA), Export Inspection Agency (EIA), Food Safety Standard Authority of India (FSSAI) and State Government have aligned their antibiotics regulations and Maximum Residual Limits (MRLs) with the European Council (EC) and the FDA requirements, to meet export requirements. India, government authorities have listed antibiotic compounds authorized and banned for use in aquaculture (CAA) have adopted European Countries (Maximum Residue Levels) MRLs to meet export requirements of the importing consuming countries.

Conclusion

It is imperative to identify and mitigate the source and spread of AMR as they contributing serious antimicrobial resistance, alterations of microbial community, causes of health hazards to the stakeholders, food safety and quality issues, and economic loss worldwide. It is well known that AMR is a one health approach that includes connections between humans, animals, and the environment as a cause and a solution. Thus for eliminating the contamination of antibiotics and resistance genes in the aquaculture field, it is necessary to implement better management practices, effective biosecurity measures, and employ other disease prevention measures instead of chemotherapy.

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Chapter 9

Determination of AMR by phenotypic method – Disk Diffusion assay

G. K. Sivaraman and U. Prinetha

Over 70 years have passed since the disc diffusion testing principle was first applied in microbiology labs. Drs. Bauer, Kirby, Sherris, and Turck meticulously developed by considering all parameters including the media, temperature, and depth of agar and were published in the year 1966. The essential operational stages from the Bauer paper's disc diffusion reference approach were adopted by CLSI. The CLSI is approved by FDA-USA and recommended by WHO.

Disk diffusion assay: One phenotypic technique that can be used to assess the antibiotic resistance is disc diffusion testing i.e. in vitro susceptibility testing of antimicrobial resistance (antibiogram). A standard inoculum of the bacteria (McFarland Standard 0.5 = ~1.5×10^8 CFU/mL) is used to inoculate agar plates, and then an antimicrobial disc is placed on the inoculated agar plate. Following the recommendations of the Clinical and Laboratory Standards Institute (CLSI), the plate is incubated under controlled circumstances. When in contact with the surface of the agar, the antimicrobial agent (set concentration, as per CLSI) contained in the discs used for a disc diffusion experiment diffuses into the agar. A "zone of inhibition" forms around the disc as a result of the antimicrobial drug diffusing into the agar during incubation and preventing bacterial growth. The diameter of this zone is measured and the findings are classified as resistant, moderate, or susceptible (CLSI M7, M31 and M100) and the inhibition zone's size reveals the level of resistance. This disk diffusion assay is extremely sensitive to changes in the following factors: bacterial concentration, media composition, pH, agar depth, diffusion rate of the antibiotics, growth rate of the bacteria, and incubation time. Internal quality control testing must be carried out on a regular basis as advised by CLSI

(CLSI M2) to ensure the accuracy and repeatability of antimicrobial susceptibility test results

Practical

Sample Preparation: The purified, single, and young culture (18-24 hrs) grown on non-selective agar must be used.

Media required

- Sterile saline solution (0.85%) 3-4 mL each tube
- Mueller-Hinton agar plates (4 mm)
- Antimicrobial Disks (stored in -10°C to -20°C)
- Nutrient agar plates/ non-selective agar
- Quality control Strain

Equipment

- McFarland standard 0.5/ nephelometer
- Vortex
- Disk dispenser/ forceps
- Micropipette & tips (100 µl)
- Bunsen burner
- Small sterile cotton swabs/ spreader
- Ruler or caliper

Composition and preparation of culture media and reagents

- **Mueller Hinton Agar:** Mueller-Hinton Agar may be prepared from a commercially available base. Ensure that the Mueller-Hinton agar formulations have met the quality standards prescribed by CLSI document M6 *Protocols for Evaluating Dehydrated Mueller-Hinton Agar*.
- **Nutrient agar** (ISO 6579:2002)

Meat extract 3.0g

Peptone 5.0g

Agar 12g to 18g

Water 1000 mL

Adjust pH to ~7.0 after sterilisation,

Autoclave at 121^{O}C for 20 min.

- **Saline solution**

  Sodium chloride 8.5g

  Water 1000 mL

  Adjust pH to 7.0.

  Autoclave at 121^{o}C for 20 min

Procedure

1st Step: Select colonies

Check the bacteria and the quality control strains are pure and well isolated colonies on the grown agar plates and free of any visible contamination. Colonies cannot be more than 18 to 24 hours old when using the direct colony suspension method. Except for staphylococci, most quickly growing organisms are studied using the log phase approach.

2nd Step: Prepare inoculum suspension:

Pick up at least 4 to 5 well isolated colonies with a sterile loop or swab and transfer to the tube of saline and emulsify the inoculum on the inside of the tube to avoid clumping of the cells. Make sure that the microorganism suspension is thoroughly mixed, and vortex it.

3rd Step: Standardize inoculum suspension:

Prepare the inoculum standard to a 0.5 McFarland by compare turbidity to that in the 0.5 McFarland standards using a paper with black lines or nephelometer and adjust it accordingly.

4th Step: Inoculate plate:

Dip a sterile cotton swab into the inoculum, rotate the swab several times and press firmly on the inside wall of the tube above the fluid level to remove

excess inoculum. The adjusted suspensions should be used within 15 minutes. Streak the swab over the entire surface of the Mueller Hinton agar plate. Keep the plates 3-5 minutes to allow the excess moisture to be absorbed.

5th Step: Add antimicrobial disks

Apply the disks containing the antimicrobial agents within 15 minutes of inoculating the MHA plate. Dispense the antibiotic disks on the agar surface with a dispenser or sterile forceps (5 disks on a 100 mm plate). To ensure complete, level contact with the agar, firmly press each disc into the surface. Once a disc was placed on the agar, it should not be repositioned. Select the FDA approved products and antibiotic disks with the specified contents as listed in the CLSI standards (Table 1 and 2).

6th Step: Incubate plate

Incubate the plate at 35±2°C for 18-24hrs within 15 minutes of standardizing the inoculum suspension. For nonfastidious bacteria, incubate in ambient air at 35°C for 16–18 hours (refer as per CLSI recommendations conditions).

7th Step Measure inhibition zones

Check for the growth is even and confluent and the zone of inhibition is of very clear. Measure the diameter of the clear inhibition zones margin. Measure zone of inhibition with respect to each antibiotic using reflected light from the back of the plate for the *Enterobacteriaceae*, *Staphylococci*, and *Enterococci* (except for oxacillin and vancomycin). Use transmitted light for *Staphylococci* with oxacillin and *Enterococci* with vancomycin. Measure the zone of inhibition where there is a clear distinction of growth and no growth. Even if the swarm type (*Proteus mirabilis)* are tested, only measure the obvious zone. It may be challenging to interpret zones with trimethoprim-sulfamethoxazole and sulfonamides and trimethoprim alone since these antibiotics may not prevent bacterial growth until the bacteria have undergone several generations of growth. Therefore, measure the zone at the point when there is an 80% reduction in growth within the zone. If

there is no zone of inhibition, the disk's diameter needs to be recorded as 6mm.

8th Step: Interpret and report of the results:

Refer the CLSI Guideline M100-S22, S-20: Performance Standards for Antimicrobial Susceptibility Testing, Table 2A- 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) and report as sensitive (S), intermediate (I) or resistant.

Recently World Health Organization (WHO) has developed the software viz. WHONET for the analysis of antibiotic sensitive test (AST) to derive multiple interpretations with world unified protocol to support clear and error-free concept.

NOTE: The situation with respect to susceptibility testing of bacteria isolated from aquatic animal is essentially different. In general there are not a wide number of testing protocols that have been developed by different national agencies. Only one international agency, CLSI, has started to develop standardized testing protocols suitable for bacteria isolated from aquatic animals that require incubation at temperatures <35°C or longer than 16-20hrs. Although the CLSI protocols do not yet cover all the diverse species encountered in aquatic animals, the progress they have made is substantial.

Table 1: List of antibiotics for susceptibility testing of *Staphylococci*

Antibiotic	**Disc content**	**Zone diameter interpretive criteria nearest whole mm**		
		S	**I**	**R**
Penicillin	10 units	≥ 29	-	≤ 28
Cefoxitin	30 µg	≥ 22 ≥ 25 (for CONS)	- -	≤ 21 ≤ 24 (for CONS)

Gentamicin	10 µg	≥ 15	13-14	≤ 12
Tetracycline	30 µg	≥ 19	15-18	≤ 14
Ciprofloxacin	5 µg	≥ 21	16-20	≤ 15
Trimethoprim-Sulfamethoxazole	1.25/23.75 µg	≥ 16	11-15	≤ 10
Chloramphenicol	30 µg	≥ 18	13-17	≤ 12
Erythromycin	15 µg	≥ 23	-	≤ 13
Linezolid	30 µg	≥ 21	-	-

Table 2: List of antibiotics for susceptibility testing of *E. coli*

Antibiotic	**Disc content**	**Zone diameter interpretive criteria nearest whole mm**		
		S	**I**	**R**
Ampicillin	10 µg	≥ 17	14-16	≤ 13
Amoxicillin-clavulanic acid	20/10 µg	≥ 18	14-17	≤ 13
Ceftriaxone	30 µg	≥ 26	23-25	≤ 22
Cefpodoxime	10 µg	≥ 21	18-20	≤ 17
Ceftazidime	30 µg	≥ 21	18-20	≤ 17
Aztreonam	30 µg	≥ 21	18-20	≤ 17
Cefotaxime	30 µg	≥ 26	23-25	≤ 22
Cefoxitin	30 µg	≥ 18	15-17	≤ 14
Imipenem	10 µg	≥ 23	20-22	≤ 19
Amikacin	30 µg	≥ 17	15-16	≤ 14
Tetracycine	30 µg	≥ 15	12-14	≤ 11
Ciprofloxacin	5 µg	≥ 21	16-20	≤ 15
Nalidixic acid	30 µg	≥ 19	14-18	≤ 13
Chloramphenicol	30 µg	≥ 18	13-17	≤ 12
Colistin	10 µg	≥ 11	-	≤ 10

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Chapter 10

Introduction to nanotechnology and its application in fisheries and aquaculture sector

S. Visnuvinayagam

Nanotechnology has emerged as one of the most significant and fascinating fields in recent years. Utilizing materials at the nanoscale to create novel products or processes is referred to as nanotechnology. It is a leading-edge discipline in biology, engineering, physics, and chemistry. Being a new and inventive approach, nanotechnology has paved the way to open up new perspectives for the analysis of bio-molecules, targeted drug delivery, protein or cell production, clinical diagnosis, the creation of non-viral vectors for gene therapy, DNA transporters as disease therapeutics, etc. It will alter the course of technical development in a variety of applications. Although the term "nanotechnology" is still relatively new, the existence of nanometer-sized, functional structures and devices is very old. The measurement unit for nanometers is a billionth of a meter. Nanomaterials' distinctive physical and chemical features can be used for applications that benefit society.

Nanoparticles:

Nanomaterials (NMs) are unique materials with at least one dimension of 100 nm or with a primary size in the 1-100 nm range that have been created by humans. In general, it is believed that nanoparticles are made up of several atoms or molecules that are joined together at a radius of 100 nm. Nanomaterials may be formed into a variety of forms, including nanoparticles (NPs), nanorods, nanotubes, nanospheres, and nanowires. Metal nanoparticles, carbon-based nanomaterials i.e., Carbon nanotubes

(CNTs), and fullerene spheres are having to wide applications in almost all sectors.

Method for nanoparticle preparation:

Two ways are employed to generate nanoparticles i.e. Bottom-up approach and the Top-down approach. In general, the bottom-up technique refers to the creation of nanoparticles by chemical interactions between atoms/ions/molecules. Top-down incorporates mechanical ways of crushing/breaking bulk into multiple sections in order to generate nanoparticles.

Application in fishery and aquaculture sector:

Fisheries and aquaculture may utilize nanotechnologies and generate new uses. Nanotechnology's advantages for fisheries and aquaculture must be weighed against environmental and worker health issues.

Food industry: In the food industry, nanotechnology is already being used. Nano-polymers and coatings could be used in fisheries to strengthen food packaging and protect delicate fish fillets. The use of antibacterial nano-coatings and transparent polymer films that can help exclude oxygen from around the food product can help extend the shelf life of fish and shellfish. Nano-sensors on food packaging can also detect the deterioration of fish or shellfish.

Aquaculture: Nanotechnological applications for fish health in aquaculture include antibacterial surfaces in the aquaculture system, nano-delivery of veterinary products in fish food utilizing porous nanostructures, and nano-sensors for detecting infections in the water.

Aquaculture Engineering: From an engineering standpoint, there are several innovative construction materials, textiles, and fabrics that might be employed in aquaculture engineering and fishing boats. Carbon nano-fibers, which are more than 100 times stronger than steel, might be employed in cage construction, nets, and mooring lines. The antibacterial capabilities of

NMs have the potential to be employed to reduce biofouling on maritime constructions.

Aquaculture feed: Fish will consume NM-containing foods, and nanotechnology may be employed to optimize the delivery of micronutrients or unstable substances in aquafeeds. The employment of nanoencapsulation technology for fat-soluble vitamins, minerals, and fatty acids, for example, may be useful. Some NMs may alter the physical qualities of the meal (for example, buoyancy and hardness).

Purification of water: Measurement techniques for NM environmental monitoring and NM surveillance in goods, particularly food such as fish fillets, are required. Nanotechnology has several uses in water purification, including the removal of bacteria, organic compounds, and metals.

Toxicity of nanoparticles:

There is a substantial corpus of information about NM occupational health and safety. Employers may get guidance notes from many government bodies, including the Health and Safety Executive in the United Kingdom. Few persons will be exposed to raw NMs or free particles, the estimated occupational health effects from NMs are likely to be modest for fishery workers. There is guidance provided for scientific employees in research labs to ensure safe handling. The potential advantages of nanotechnology to the fishing and aquaculture sectors should be evaluated against environmental hazards. The colloidal behaviour of NPs is discussed briefly. Laboratory investigations demonstrate that free NMs at mg l1 levels are hazardous to various fish and invertebrates. Many of the effects seen with classical chemicals, including respiratory distress, gill damage, metabolic abnormalities, and impacts on embryonic development, So far, ecotoxicity statistics imply that produced NMs may be less harmful than certain existing compounds. However, there are several data gaps, including a paucity of data on critical aquaculture and fishery species.

Conclusions

Based on current evidence, the benefits of using nanotechnology in fisheries and aquaculture are considerable, and the Potential risks should not be allowed to hinder innovation. So, the right use of nanotechnology in fisheries and aquaculture should be encouraged with minimal risks.

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Chapter 11

Extraction of DNA and RNA

S. Ezhil Nilavan, Vineetha Das, Sreejith V.N,

Procedure

1. Inoculate the bacterial culture in non-selective enrichment broth viz., TSB or BHI or NB or LB and incubate overnight at optimum temperature (may vary with your bacterial culture).
2. Take 1 mL of overnight grown bacterial culture in 1.5mL centrifuge tube. Label the tubes with the sample id.
3. Centrifuge it at 8000rpm for 5 min.
4. Discard the supernatant.
5. Resuspend the pellet in 0.5mL of 1X Tris-EDTA buffer (pH 8.0) using a vortex or micropipette.
6. Repeat the centrifugation step at 8000rpm for 5 min.
7. Discard the supernatant. To the pellet, add 0.5 ml of Tris-EDTA buffer
8. Resuspend the cells using a vortex or micropipette.
9. Heat the tubes either by placing them in a heating plate or in a water bath at 95°C for 5 minutes.
10. Immediately transfer the tubes to ice or -20^{0}C (if not used immediately).
11. Incubate on ice for 10 min.
12. Centrifuge at 8000 rpm for 5 minutes.
13. The supernatant can be used as template for PCR reactions.

1. Cell lysate preparation from bacterial colonies

Procedure

1. Use micropipette to add 1ml of sterile DNase-free water to the tube.

2. Use a sterile pipette tip to 'touch' a series of bacterial colonies on plate or overnight grown culture on slants and mix it with the DNase free water in the tubes. Avoid picking up a large amount of bacterial cells. Too much cellular material might inhibit the PCR reaction.
3. Centrifuge at 8000 rpm for 5 minutes and discard the supernatant.
4. Add 0.5 ml of Tris-EDTA buffer to the pellet.
5. Resuspend the cells using a vortex or micropipette.
6. Heat the tubes either by placing them in a heating plate or in a water bath at 95°C for 5 minutes.
7. Immediately transfer the tubes to ice or -20^{0}C (if not used immediately).
8. Incubate on ice for 10 min.
9. Centrifuge at 8000rpm for 5 minutes.
10. The supernatant can be used as template for PCR reactions.

2. DNA extraction from fish tissue

CTAB METHOD

1. Collect 100–200 mg tissue in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-lauryl sarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg ml–1 proteinase K added just before use)
2. Using a disposable stick, homogenize the tissue in the tube thoroughly
3. After homogenization, incubate at 65°C for 1 hour.
4. Add 5 M NaCl to a final concentration of 0.7 M. Next, slowly add 1/10 volume of N-cetyl N,N,N trimethyl ammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly.
5. Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24/1) and mix gently. Centrifuge at 13,000 g for 5 minutes and then transfer the aqueous

solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol.

6. Mix gently and centrifuge at 13,000 g for 5 minutes. Collect the upper layer solution and repeat the phenol extraction process once or twice.
7. Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24:1) and centrifuge at 13,000 g for 5 minutes.
8. Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20°C for 30 minutes or –80° C for 15 minutes.
9. Centrifuge at 13,000 g for 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µl sterilized double-distilled water at 65°C for 15 minutes. Use 1 µl of this DNA solution for one PCR.

3. Dneasy blood & tissue kit (qiagen)

1. Cut tissue into small pieces, and place in a 1.5 ml micro centrifuge tube.
2. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing and incubate at 56°C until completely lysed. Vortex occasionally during incubation.
3. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
4. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing
5. Pipette the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
7. Place the spin column in a new 2 ml collection tube; add 500 µl Buffer AW2 and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.

8. Transfer the spin column to a new 1.5 ml or 2 ml micro centrifuge tube.
9. Elute the DNA by adding 100 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.

4. RNA extraction from fish tissue

The sample size depends on the type of sample (the following steps shall be performed at a temperature under 4° C):

(1.1) for large (juvenile or adult) shrimp, take 25-75 mg shrimp tissue and add 150 µL trizol. Crush, and then add 850 µL trizol for a final volume of 1,000 µL.

(1.2) For PL shrimp, take 300 shrimp and add 1,000 µL trizol. Crush, then take just 150 µL of the mixture and add 850 µL trizol for a final volume of 1,000 µL.

(1.3) if the sample is haemocytes, add 750 µL trizol and mix for 20 seconds.

(2) Incubate the sample from (1) at 25°C for 5 minutes.

(3) Centrifuge at 12,000g at 4°C for 10 minutes; pipette up the supernatant and transfer it to a new micro centrifuge tube.

(4) Add 200 µL chloroform and mix for 20 seconds.

(5) Incubate at 25° C for 10 minutes.

(6) Centrifuge at 12,000g at 25°C for 10 minutes; pipette up the supernatant and transfer it to a new micro centrifuge tube.

(7) Add 670 µL iso-propanol and mix.

(8) Incubate at 25°C for at least 10 minutes or incubate at -20°C over night or at -70°C for 1 hour.

(9) Centrifuge at 12,000g at 25°C for 10 minutes: pipette off the supernatant and discard.

(10) Rinse the pellet with 0.5 ml of 70% ethanol for atleast 30 minutes at 25°C.

(11) Centrifuge at 12,000g at 25°C for 10 minutes. Pipette off the supernatant and discard.

(12) Leave at room temperature for 20 minutes or until the pellet is dry
(13) Add 150 µL EDTA for every 50 mg of original sample tissue, or if the original sample was 250 µL haemocyte, add 75 µL EDTA. Incubate at 55°C for 15 minutes. Mix gently and use for the next step to create complementary DNA.

5. cDNA synthesis from extracted RNA

This protocol is specifically used for Revert Aid H minus First Strand synthesis kit from Thermo Fisher Scientific
Add total RNA (RNA extracted using the Trizol method) (1 µg to 5 µg- up to 4µL) in a sterile nuclease free, 0.2 mL PCR tube
Add 1 µL of Primer; oligo (dT) 18 primer or random hexamer primer. If gene-specific primer is used add to a final concentration of 15-20 pmol.
Add RNase-free Water to a final volume of 12 µL. Gently flick the tube a few times to mix, and then spin briefly (~5 seconds).
Incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.
Add the following components in the indicated order:

5X Reaction Buffer	4 µL
Ribo Lock RNase Inhibitor (20 U/µL)	1 µL
10 mM dNTP Mix	2 µL
Revert Aid H Minus M-MuLV Reverse Transcriptase (200 U/µL)	1 µL
Total volume	20 µL

5. For oligo (dT) 18 or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C. For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C.

Note. For GC-rich RNA templates the reaction temperature can be increased up to 45°C.

6. Terminate the reaction by heating at 70°C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

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Chapter 12

Polymerase chain reaction and its types

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Polymerase chain reaction (PCR) is one of the fundamental techniques in various molecular microbiology experiments and refers to a set of procedures for the *in vitro* enzymatic amplification of a desired DNA fragment or gene from the whole genome of an organism. PCR enables the synthesis of more than 10 million copies of a target DNA sequence from a few initial copies of the sequence. PCR technique is used widely in various diagnostics and forensic investigations, and becomes essential for many common procedures such as cloning, sequencing, microaarays etc.

History

PCR was discovered by Dr. Kerry Mullis in 1983 at the Cetus Corporation in Emeryville, CA and he was awarded the Nobel Prize in Chemistry for his work on PCR in 1993. Moreover, the *in vitro* DNA synthesis using two primers was initiated by Gobind Khorana in 1971, but the main constraints were primer synthesis and polymerase purification issues. Though the previous techniques were used for isolating a specific gene by gene cloning method, it is laborious and time-consuming processes. Prior to 1988, a PCR reaction was carried out by a series of water baths by adding a fresh aliquot of Klenow fragment of *E. Coli* DNA polymerase I after each denaturation step. The Klenow fragment was not highly specific, can amplify only about 400bp and could produce an incompletely pure target product and the target sequence should be confirmed by specific hybridization probe. Obviously, it is a tedious process and was eliminated by the introduction of a thermostable DNA polymerase, the Taq DNA polymerase and is highly specific to the target sequence as well as can withstand in the repeated heating PCR cycling conditions. This increased specificity also could increase the yield of the target sequence and can amplify the PCR products up to 10 kb. The introduction of DNA polymerase enzymatically assembles a new DNA strand

from target DNA and nucleotides, for initiation of DNA synthesis. Recently, *Pyrococcus furiosus* (Pfu) DNA polymerases and *Thermococcus Litoralis* (VENT) are becoming more widely used because of the proof reading 3' to 5' exonuclease activity which is lacking in *Taq* polymerase.

Components and Reagents used in PCR Mixture

Typical PCR mixtures consists of a 10X reaction buffer for the Taq polymerase, forward and reverse primers, deoxyribonucleotide triphosphates (dNTPs), Taq polymerase and the template DNA. Each of these components is described below.

Template DNA

DNA template is nothing but the target DNA to be amplified. The quantity, quality and integrity of the template DNA is an important factor to determine the success of PCR. About 1 ng of plasmid or phage DNA and 10-100 ng of pure genomic DNA generally yield expected amplifications. Higher amounts of template DNA usually result in nonspecific PCR amplifications. Still higher quantities might result in the inhibition of amplification. The contaminants such as heparin, heme, formalin, Mg^{2+}-chelating agents, as well as detergents etc. if any will also result in the inhibition of amplification process and need to be eliminated.

10X Buffer for *Taq* polymerase

10X buffer provide suitable condition for optimum activity and stability of the DNA polymerase. The reaction buffer consists of Tris-HCl, EDTA, KCl, Nonidet P40, tween 20 and glycerol. However, this can vary depending on the type of polymerase and the source. Buffers are supplied at 10× concentration along with the polymerase. It is not advisable to interchange buffers and polymerases from different suppliers, as this may not yield expected results.

$MgCl_2$ concentration

The range of $MgCl_2$ concentration in a reaction mixture varies between 1 to 4 mM, with a standard concentration of 1.5 mM. The 10X buffers are available either with or without $MgCl_2$. If the buffer does not contain

concentration of $MgCl_2$, it has to be added separately in to the reaction mixture. The Mg^{2+} ions form a soluble complex with dNTPs which is essential for dNTP incorporation. It stimulates polymerase activity, and increase the T_m of primer/template interaction (and therefore they stabilise the duplex interaction). The optimal concentration of $MgCl_2$ prevent Mg^{2+} ions to form complexes with dNTPs, primers and DNA templates. Too low Mg^{2+} ions result in low yield of PCR product, and too high increase the yield of non-specific products.

Primers

Primers are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target. PCR primers are usually 18-25 nucleotides in length with an average GC content of 40-60%. To avoid non-specific priming, primers should not have more than three G or C nucleotides at the 3'-end. The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin loop formation. The standard concentration of primer/oligonucleotide is usually 1μM which will be sufficient for at least 30 cycles of amplification. The presence of higher concentration of pimer can can cause amplification of undesirable non-target sequences. Conversely, the PCR is inefficient with limiting primer concentration.

Deoxynucleoside triphosphates (dNTPs)

Deoxynucleoside triphosphates (dNTPs) are the building blocks from which the DNA polymerases synthesize a new DNA strand. The equimolar concentration of each dNTP (dATP, dCTP, dGTP & dTTP) is required in a PCR mixture and usually at 200 μM each. Unequal concentrations lead to misincorporations of nucleotides resulting in altered sequences which might affect post-PCR experiments such as sequencing, cloning and expression of protein.

***Taq* DNA polymerase**

Taq polymerase attaches to the target sequence and can withstand repeated heating PCR cycling conditions. Typically, DNA polymerases can only incorporate nucleotides from the 3' end of a polynucleotide. The first thermostable DNA polymerase used was the *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus*. Even though this enzyme is probably the most widely used in PCR applications, several other DNA polymerases are commercially available. The concentration of *Taq* polymerase is very crucial in a PCR. The recommended concentration is 1-1.5 units in a 50 µl reaction mixture involving amplifications of up to 1 kb. When larger than 1 kb is to be synthesized, the concentration of *Taq* may be increased. It is important to follow manufacturer's recommendations regarding the concentrations to be used since this can vary considerably depending on the source of the enzyme.

PCR Cycle and Principles

The PCR process consists of repetitive series of three fundamental steps called PCR cycle. The cycling is often preceded by a single temperature step called Initialization at a high temperature (>90°C) and one final elongation for any remaining single-stranded DNA is fully extended. The Final hold step is at 4°C for short term storage of the amplified PCR products.

In PCR, the double stranded DNA is denatured by heat and then the temperature is lowered to allow annealing of two specific primers by complementary base pairing on the opposite strands of the DNA. *Taq* polymerase directs the synthesis of the new strand from the primed sites in both directions that results in double stranded DNA and the procedure is repeated for 25-40 times in a thermocycler. In each cycle, the target DNA is replicated by a factor of 2 so that, after the completion of PCR, millions of copies of DNA are available for subsequent manipulations.

Initial Denaturation

Initial Denaturation ensures complete denaturation of the double stranded DNA at the start of the PCR cycling. A 5 min denaturation at 95°C should be sufficient for templates with a GC content of 50% or less. The denaturation time should be extended up to 10 min for GC-rich templates.

After initial denaturation, the following 3 steps are repeated 30-40 times.

1. **Denaturation**/ The double-stranded DNA template denaturation: The double-stranded DNA template denatured into two complementary single strands of DNA by disrupting the hydrogen bonds between complementary bases. Usually DNA undergoes rapid denaturation at 94–98 °C with 30 seconds to 2 minutes.

2. **Annealing**/ Annealing of two oligonucleotide primers to the single-stranded template: After denaturation, temperature is lowered to 50–65°C for 20–60 seconds allowing annealing of the primers to the single-stranded DNA template and preventing immediate reannealing of long DNA strands. The primers rapidly anneal to the single strands of DNA because of their small size and Taq polymerase will binds to them. Generally the annealing temperature is 136 about 3-5 degrees Celsius below the Tm of the primers used. Once the stable DNA-DNA hydrogen bonds are formed, the polymerase binds to the primer-template hybrid and begins DNA synthesis.

Annealing temperature of the primers is calculated using the following formula

Tm = 4 (G + C) + 2 (A + T)

Annealing temperature (°C) = Tm – 5°C

Where, Tm = Melting temperature; G, C, A, T =number of respective nucleotides in the primer.

3. Extension

Enzymatic extension of the primers to produce copies that can serve as templates in subsequent cycles: The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. The DNA polymerase will polymerize a thousand bases per minute. At each

extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final extension

After the completion of cycling, a final extension for 5-15 min at 72°C is done to complete incompletely synthesized strands.

Preparation of PCR Reaction Mixture

A master mix can be prepared by adding the required concentration reagents of PCR reaction in a given volume and then be aliquoted appropriately to individual tubes depending upon the testing requirement. This procedure can minimize the possibility of pipetting errors in case of handling low volume and also saves time. Transfer the required water, buffer, $MgCl_2$ dNTPs, primers and *Taq* DNA polymerase in a single tube be aliquoted into individual tubes. Various template DNA to be amplified are then added to the individual tubes and to be labelled for further recording of the results. A positive control (known DNA sample) & a negative PCR control (water) should also be included to ensure the reliability of the procedure. PCR reaction mix preparation is follows

Thaw all the reagents such as water, buffer, $MgCl_2$ dNTPs, primers and template DNA except *Taq* DNA polymerase (it should keep always under ice) vortex gently and spin all solutions. Prepare master mix by adding the following reagents except template and transfer aliquot to individual tubes (PCR tubes 0.2ml) placed on ice. Then add DNA templates to the respective tubes. The final concentration for 30 ml reaction mixture is given in the table

Component	**Final concentration**	**Volume required (µl)**
Sterile deionized water	-	15.4
10X *Taq* buffer	1X	3
10 mM dNTP mix	0.2 mM of each	0.5
Primer Forward	1 µM	3

Primer Reverse	1 µM	3
Taq DNA Polymerase	1.5 u	0.3
25 mM $MgCl_2$	1.5 mM	1.8
Template DNA	100 ng	3
Total volume		**30**

Vortex each PCR tubes and spin to collect all drops from the walls of tube. Place the PCR tubes in thermocycler and start PCR. Once the PCR cycle is over, the PCR product is visualized by electrophoresis in agarose gel stained with 0.5 µg/ml of ethidium bromide.

Agarose gel electrophoresis

Agarose gels are cast by melting the appropriate percentage of agarose (1-2%) in convenient buffer (1X TAE) until a clear, transparent solution is achieved. The melted gel is then cooled (50°C), poured on a tray containing combs and allowed to solidify. Upon solidification, the comb is removed and gel is placed in the electrophoresis chamber and then pour the buffer to cover the gel. DNA samples are loaded into the sample wells by mixing with loading dye (2 µl for 10 µl of the PCR product) and gel is run at a voltage for a time period that will perform optimal separation. When electric field is applied across the gel, DNA migrates toward the anode. When running of the gel is completed, the separated bands on agarose gel are labeled or stained for interpreting. One method of staining DNA is to expose it to the dye ethidium bromide (EtBr) (0.5 µg/ml). EtBr intercalates between the stacked bases of nucleic acids and fluoresces red–orange when illuminated with ultraviolet (UV) light. EtBr is a carcinogen and should be handled with care.

Specialised PCR

In addition to the amplification of a target DNA sequence by the typical PCR procedures already described, several specialized types of PCR have been developed for specific applications.

RT-PCR (or Reverse Transcription PCR)

RT-PCR (or Reverse Transcription PCR) is used when the target nucleic acid is RNA. The central dogma in molecular biology explains about the direction or flow of information in which the DNA of the organism encodes the genetic information, intern transfer to RNA by the process of transcription and then to protein via translation process. As RNA is highly unstable and enzymatic amplification is difficult and need to reverse transcribed to cDNA for amplification. The reverse transcriptase, an enzyme that converts RNA into cDNA. This cDNA can be used for PCR and reverse transcription process may be combined in a tube, as the initial heating step of PCR being used will inactivate the transcriptase enzyme. The Tth polymerase is used for the enzymatic amplification due to its inherent RT activity, and can carry out the entire reaction. As the phenotype of an organism is explained by the RNA or protein fractions. So, RT-PCR is used in expression profiling of specific gene or gene products. It can also used in RNA transcript analysis where in transcription start and termination sites are determined. Also it enables the mapping of exons and introns of the gene sequence.

RT-PCR can also be very useful in the insertion of eukaryotic genes into prokaryotes. Because most eukaryotic genes contain introns, which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the exact (without regard to the error-prone nature of reverse transcriptase) DNA sequence that would be directly translated into protein after transcription. When these genes are expressed in prokaryotic cells for the sake of protein production or purification, the RNA produced directly from transcription need not undergo splicing as the transcript contains only exons. (Prokaryotes, such as *E. coli*, lack the mRNA splicing mechanism of eukaryotes). RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as Influenza virus A and retroviruses like HIV.

Nested PCR

Nested sets of primers can be used to improve PCR yield of the target DNA sequence. In nested PCR, two primer sets are used in which the first round of PCR is performed with one primer set for 15-30 cycles, then second set of primer is used for second round PCR, for an internal region of the first amplified DNA for an additional 15 to 30 cycles. The PCR product of the first round of PCR is used as DNA template for the second PCR. Thus, the nested PCR method increases the sensitivity and specificity of DNA amplification. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products. This is because after the first round of PCR any non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity, and great care must be taken when performing such PCRs, particularly in a diagnostic laboratory.

Multiplex PCR

Multiplex PCR enables simultaneous amplification of many sequences or gene using two or more set of primers in one PCR. The presence of many PCR primers in a single tube could cause many problems, such as the increased formation of misprimed PCR products, "primer dimers", and the amplification discrimination of longer DNA fragments. For this type of PCR amplification, primers are chosen with similar annealing temperatures. The lengths of amplified products should be similar; large differences in the lengths of the target DNAs will favour the amplification of the shorter target over the longer one, resulting in differential yields of amplified products. In addition, Multiplex PCR buffers contain *Taq* polymerase additive, which decreases the competition among amplicon and the discrimination of longer DNA fragments during Multiplex PCR. Multiplex PCR products can be further hybridised with a gene-specific probe for verification.

Quantitative PCR

Quantitative PCR is used to measure the amount or quantity of target nucleic acid (DNA or RNA) in a sample. The amount of fluorescence generated during the the phase of true exponential stage is directly measures the amount of nucleic acid or target. Special thermal cyclers fitted with light source and suitable filters for wavelength selection are used for the real time detection or monitoring of PCR product. Fluorescent dyes used are Sybr Green, or flurorophore-anchored DNA probes, such as TaqMan, to measure the amount of amplified product as the amplification progresses. Quantitative PCR is also used by microbiologists working in the fields of food safety, food spoilage and fermentation and for the microbial risk assessment of water quality (drinking and recreational waters) and in public health protection.

The antibacterial assay Virtual Colony Count utilizes a data quantification technique called Quantitative Growth Kinetics (QGK) that is mathematically identical to QPCR, except bacterial cells, rather than copies of a PCR product, increase exponentially. The QGK equivalent of the threshold cycle is referred to as the "threshold time".

Colony PCR

In Colony PCR, bacterial colonies are screened directly by PCR, for example, the screen for correct DNA vector constructs. Colonies are sampled with a sterile pipette tip and a small quantity of cells transferred into a PCR mix. To release the DNA from the cells, the PCR is either started with an extended time at 95 °C (when standard polymerase is used), or with a shortened denaturation step at 100 °C and special chimeric DNA polymerase.

Applications of PCR

1. Selective DNA amplification

- PCR allows selective amplification of a specific region of DNA/ gene from genomic DNA which can be utilized for direct sequencing, genomic cloning, DNA typing, detection of infectious microorganisms, sitedirected mutagenesis, prenatal genetic disease diagnosis and analysis of sequence variations.

• PCR 'fingerprints' methods can be used to identify genetic relationships between individuals (parentchild, between siblings and paternity testing) and microbial identification, evolutionary relationships among individuals/ organisms and forensic analysis. • PCR may also be used in the analysis of ancient DNA.

• Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample and quantitative determine the levels of gene expression. 2. PCR in diagnosis of diseases

• A diagnostic application in microbiology for the detection of infectious agents and the differentiation of non-pathogenic from pathogenic strains. • Identification of non-cultivatable or slow-growing microorganism 's like viruses mycobacteria, anaerobic bacteria.

• Viruses can be detected before the onset of disease and/ or immediately after the infection.

• Utilize for early diagnosis of cancer research

. • Amount of virus in an infected patient can be quantified.

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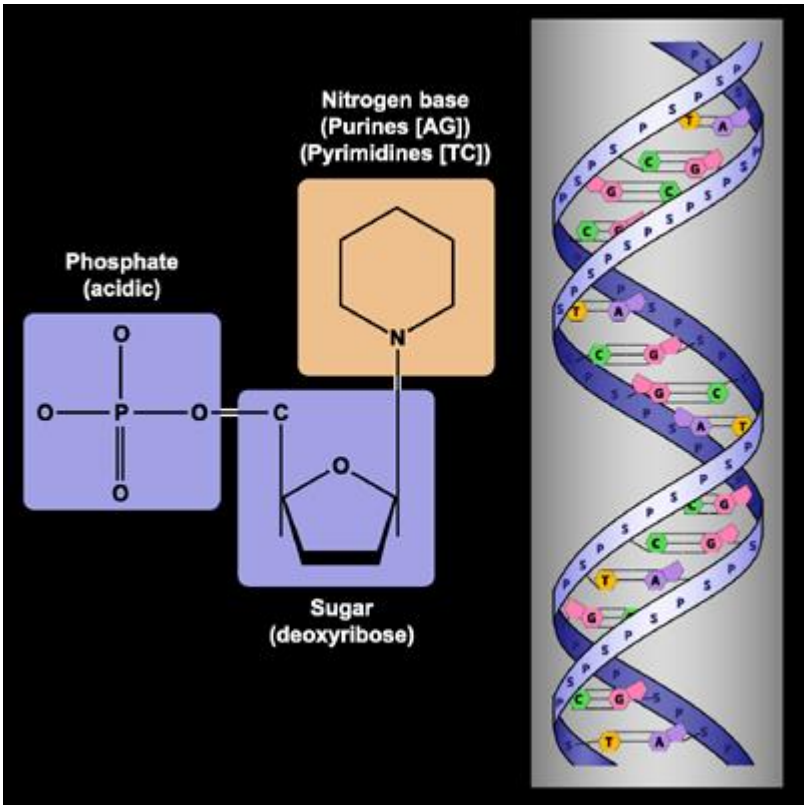
Chapter 13

Introduction to Sanger sequencing

T. Muthulakshmi and Vishnuvinayagam S.

DNA is the cell's genetic material, storing cell data as a nucleotide which contains 1. Phosphate group, 2. Deoxyribose sugar 3. A carbon-nitrogen ring (Base). Based on the base present, the nucleotide can be of 4 types: Adenine, Guanine, Cytosine, and thymine. In this adenine and guanine, double rings of carbon and nitrogen are called purines. The cytosine and thymine have a single ring of carbon and nitrogen called pyrimidine bases. DNA is double-stranded. The chains are attached by hydrogen bonds. A purine always bonds with a pyrimidine. Adenine bonds with thymine, and Cytosine bonds with guanine. One chain of double strands is complementary to the other.



Sequencing determines the order of the four chemical building blocks - called "bases" - that make up the DNA molecule. The sequence tells about the kind of genetic information of the particular region. It can code for a gene or protein or sometimes a non-coded region. Sequence data can also tell about the changes of the original sequence, which caused mutation related to diseases.

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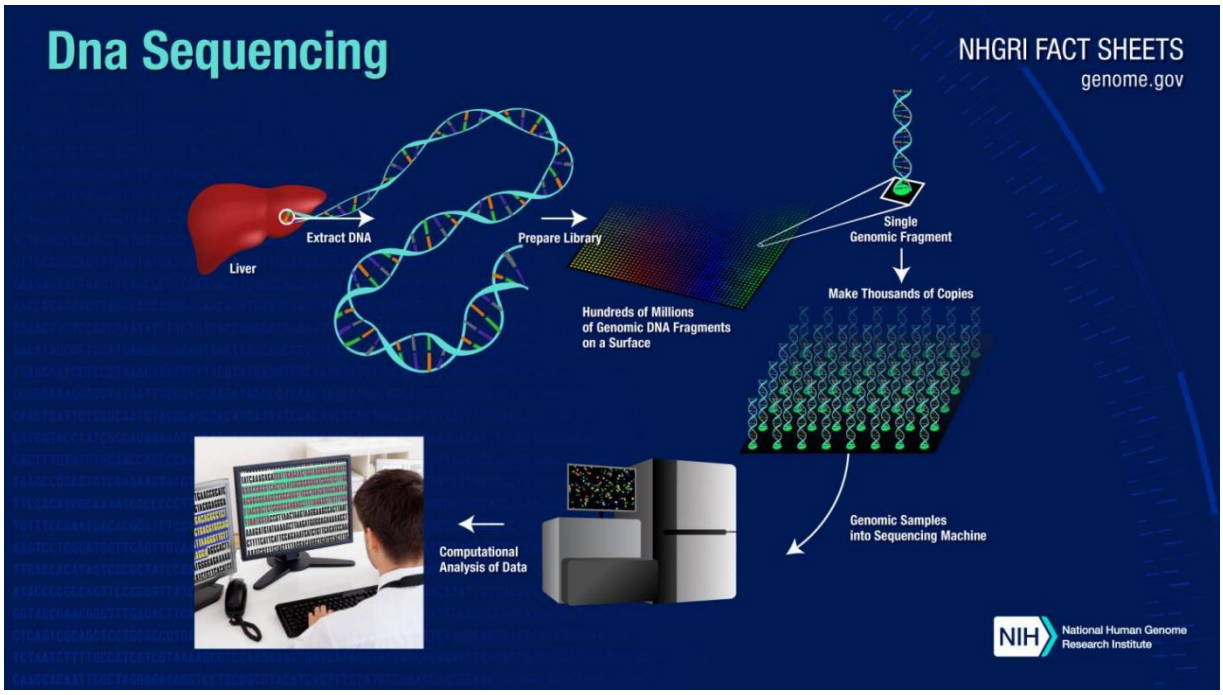
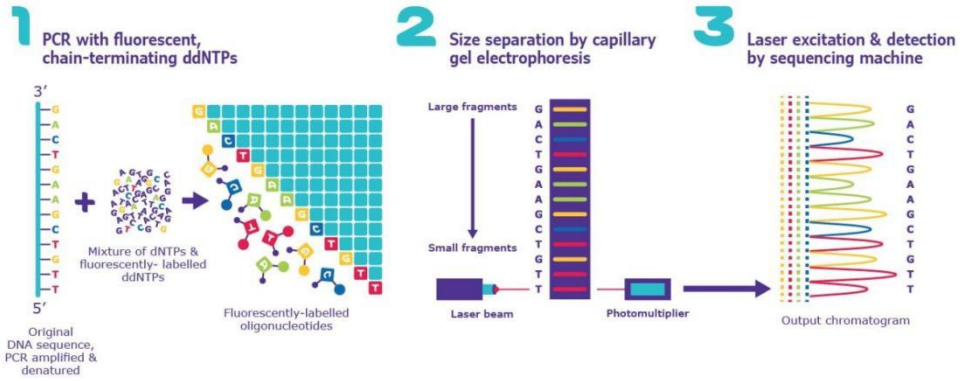


Sanger sequencing, also known as the "chain termination method, "is a method for determining DNA nucleotide sequences. The technique was developed by Nobel Laureate Frederick Sanger and his colleagues in 1977, hence the name the Sanger Sequence. The Sanger sequencing is considered the gold standard for validating DNA sequences. Even with advanced sequencing methods, the Sanger sequencing was performed to determine the purity of the sequence. The regions are divided into less than 1000 bp regions and sequenced using Sanger sequencing. The human genome project made the total human genome into more minor sequences and walked through multiple rounds. The fragments were aligned to assemble the sequences.

Steps in Sanger sequencing



Sanger sequencing has 3 main steps

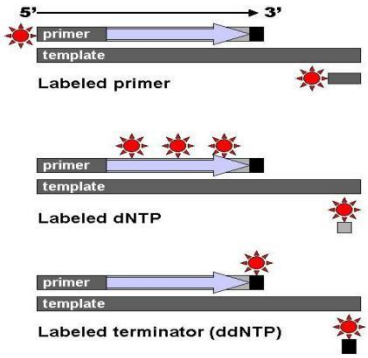
There are three main steps in Sanger sequencing.

1. PCR with Florescent chain terminating dNTPs

2. Size separation by capillary gel electrophoresis

3. Laser excitation and detection, gel analysis

1. PCR with Florescent chain terminating dNTPs



In Sanger sequencing DNA to be sequenced is used as a template for the PCR. It is similar to the standard PCR; instead of normal DNTP, it is

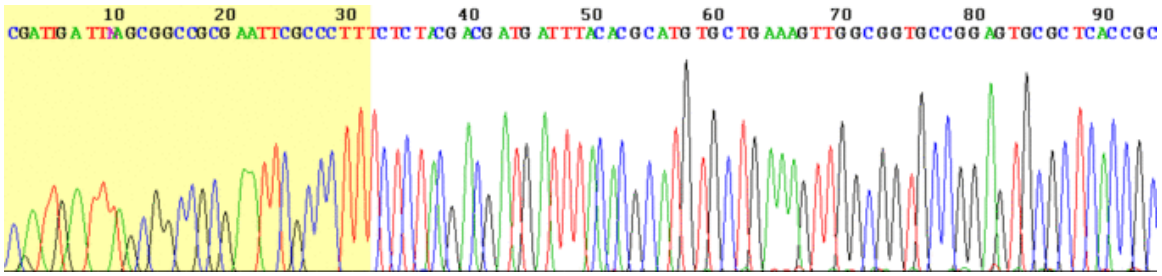
supplemented with modified DNTP. The modified dNTP s have deoxyribonucleotides. The DNA polymerase adds dNTP to the growing DNA strand in the extension steps. There the phosphodiester bond is formed between two nucleotides. A free OH group is necessary for the nucleotides to continue this chain. Since the modified nucleotide lacks the OH group, the chain will be terminated in the step. So it is called the chain termination PCR. Whenever the modified dNTP joined, it terminated the chain formation. So millions of oligonucleotide copies form by chain termination. In manual Sanger sequencing, four PCR reactions are set up, whereas, in automated Sanger sequencing, all four DNTP have unique fluorescent probes.

Gel electrophoresis for size separation

The DNA sample to be sequenced is loaded in gel electrophoresis after PCR. DNA will move to the positive electrode. Because the oligonucleotides are small, they will move according to their size. The smaller the fragment faster the movement. The resultant gel has oligonucleotides arranged from more minor to larger. All nucleotides will be run separately from the 4 PCR reactions in manual Sanger sequencing. In automatic Sanger sequencing, the gel is run in single gel electrophoresis.

Determination of Sequence through gel analysis

The gel analysis is done through software in automated Sanger sequencing. It is done manually in the manual Sanger sequencing. In manual sanger sequencing, if the bottom of the band is found in the column corresponding to DGTP, it is G in the first place. Same way next one will be read. In automatic sequencing, the computer reads the gel and identifies the corresponding DNTP.



Chapter 14

Introduction to molecular fingerprinting tools and bioinformatic analysis

Murugadas Vaiyapuri and Radhakrishnan V N

Antimicrobial resistance (AMR) is becoming a major concern for human health. The World Health Organization (WHO) has prioritised the diseases for which research and development programmes are urgently needed. Pathogens are prioritised depending on the determination to create new antibiotics or preserve current medications for treatment or control techniques. Pathogens are classified as critical, high, or medium priority. Priority 1 bacteria include carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and ESBL-producing *Enterobacteriaceae*; priority 2 bacteria include vancomycin-resistant Methicillin-resistant *Enterococcus faecium* Vancomycin-intermediate-resistant *Staphylococcus aureus* Cephalosporin-resistant *Staphylococcus aureus*, fluoroquinolone-resistant Fluoroquinolone-resistant *Neisseria gonorrhoeae* Fluoroquinolone-resistant *Salmonellae* Clarithromycin-resistant *Campylobacter* spp. Priority 3 includes fluoroquinolone-resistant *Helicobacter pylori*. Ampicillin-resistant *Shigella* sp. Penicillin-resistant *Streptococcus pneumoniae*, *Haemophilus influenzae* (WHO, 2017). Gram-negative bacteria, such as *A. baumannii*, *P. aeruginosa*, ESBL producing Enterobacteriaceae (*Klebsiella, E. coli, Serratia*, and *Proteus*), *Campylobacter sp, Helicobacter pylori, Salmonella sp, Shigella sp*, N. *gonorrhoeae*, and *H. influenzae*, are the key targets for management. *S. aureus, Enterococcus faecium,* and *S. pneumoniae* are three more major Gram-positive bacteria that contribute to the urgency of AMR.

The challenge of regulating AMR begins with giving proof of its prevalence as well as recording the genotype of the prevalent bacteria. Molecular methods remain crucial for understanding regional and global epidemiology, as well as the point of genesis and transmission of infections based on clone relatedness and genetic diversity down to the strain level (Barrett et al., 2006; Vaiyapuri et al., 2019). The valuable evidence gathered

will be an important factor in developing methods for their control (Ranjbar et al., 2014). AMR resistance molecular approaches determine the existence of AMR genes or particular mutations linked with antibiotic resistance (WHO, 2019). Molecular approaches may supplement phenotypic methods by giving additional information, such as the particular gene or mutation behind a resistance phenotype, boosting our knowledge of the amount of resistance in a given situation as well as the underlying processes responsible for resistance (Bissonnette et al., 2017; Burnham et al., 2017; WHO, 2019).

Molecular fingerprinting tools are categorized into 4 based on the approach

1. Sequencing based method
2. Amplification based method
3. Hybridization based method
4. Restriction digestion-based method

1. Sequence based molecular fingerprinting tools

Multi-locus sequence typing

The molecular typing procedure which involves the sequencing analysis of more than a single locus is called multi-locus sequence typing (MLST). The first MLST procedure was demonstrated during 1998 for *Neisseria meningitides* and later on the MLST analysis was extended to more than 100 species or genera of bacteria (Enright & Spratt, 1999; Maiden *et al.*, 1998). The MLST analysis can be used for epidemiological investigations in public health, animal health and food borne disease investigations. This is one of the best molecular tools for fingerprinting of bacterial pathogens. Mostly implemented in global and local epidemiology. The MLST analysis is targeted on housekeeping genes of clinically important bacterial pathogens. The MLST analysis is divided into three steps, amplification of housekeeping genes, sequencing of the amplicons and analysis of sequences for assigning the sequence types. In general, the amplicons range between 450 to 500bp for analysis purposes. In exceptional cases, the amplicon size may vary. This tool has high reproducibility, more discriminating power, portability of data

as it is sequence based, speed of the completion, easy interpretation and inter-laboratory comparison. Example of *S. aureus* / MRSA- MLST- analysis. The MLST scheme for the *S. aureus* (MRSA and MSSA) was developed by Enright et al. (2000). The MLST can be performed by amplification of fragments, elution and sequencing, bioinformatics analysis.

Staphylococcal Protein A typing

Staphylococcal Protein A typing (SPA) is the single locus amplification and sequencing method. The SPA gene's variable region XR domain is the target area for SPA typing in *S. aureus* or MRSA. It is often used for Staphylococcal protein A (spa) typing (Frenay et al., 1999). This approach involves amplification, sequencing, and SPA type designation, and it is often employed in local epidemiology since it accumulates genetic alterations very slowly (Koreen et al., 2004). Open source or software from StaphType (Ridom GmbH, Wurzburg, Germany) and Based upon Repeat Pattern (BURP) are used to perform minimum spanning tree-based clustering of spa kinds (Harmsen et al., 2003; Sammeth et al., 2006; Aires de Sousa et al., 2006).

Whole genome sequencing

Whole-genome sequencing (WGS) is a thorough approach for studying bacterial isolates' whole genomes. There are many ways for sequencing whole genomes. In the late 1970s, Maxam and Gilbert's chemical cleavage approach and Sanger sequencing by chain-termination method were used to sequence viral and bacterial genomes (Maxam and Gilbert., 1977; Sanger et al., 1977). In 2008, a transition to a faster, automated sequencing approach was made, allowing for the sequencing of bigger bacterial and eukaryotic genomes. Following-Sanger sequencing technologies are referred to as 'next-generation sequencing.' It can generate massive volumes of sequencing data at a very cheap cost and time. 454-sequencing, pyrosequencing, Illumina sequencing, and Sequencing by Oligonucleotide Ligation and Detection are all examples of second-generation sequencing (SOLiD). While Sanger's sequencing operates on the basis of chain termination by the inclusion of di-deoxynucleotides (ddNTPs), A, T, G, and C, the output data is slightly less

than one kilobase (kb). NGS is the massively parallel sequencing of millions of DNA fragments at the same time, resulting in the generation of millions of nucleotide short reads. The most prevalent NGS technology is undoubtedly Illumina dye sequencing, which employs a "sequence by synthesis" strategy. The genomic DNA is split at random into small pieces and affixed to the inner surface of a flow cell, where sequencing will occur. A solid-phase PCR is then used to generate clusters of clonal populations from each of the individual DNA strands bound to the flow cell. At the end of each cycle, the incorporating nucleotide's identity is recorded using a photodetector by activating the fluorophores with suitable lasers, followed by enzymatic removal of the blocking fluorescent moieties and progression to the next location (Fedurco et al, 2006; Turcatti et al, 2008; Heather and Chain, 2016)

2. Amplification based methods

Repetitive extragenic palindromic-PCR (REP-PCR)

Repeating extragenic palindromic -PCR (Versalovic et al., 1991, 1994) is a fingerprinting technology established on the PCR foundation in a bacterial genome that is based on the amplification of these repetitive elements that are particularly distinct to strains within the species of bacteria. These repeating REP elements were found in a variety of *Enterobacteriaceae* and non-*Enterobacteriaceae* bacteria, and the REP sequences are palindromic, forming a stem-loop structure (Higgins et al., 1982; Stern et al., 1984). Two sets of primers targeting these REP elements, based on 38-bp sequences having degenerate sequences in six places with a 5-bp variable loop among both sides of a conserved palindromic stem, were used for typing in different bacteria. REP element-based typing of AMR bacteria such as *E. coli, Salmonella* sp., and others has been described (Qian and Adhya, 2017).

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

The ERIC sequences, which include a 126-bp sequence that is highly conserved core inverted repeat and are situated in extragenic areas of the bacterial genome, are another type of repetitive DNA sequences utilised for bacterial typing. Initially detected in *E. coli* and *Salmonella Typhimurium*, the

typing approach based on the ERIC pattern is currently being extended to additional bacteria in the *Enterobacteriaceae* (Sharples et al., 1990).

Amplified fragment length polymorphisms (AFLP)

The amplified fragment length polymorphism (AFLP) approach is excellent for fingerprinting DNAs of any origin and complexity. AFLP offers various benefits over other DNA fingerprinting techniques. The ability to check a full genome for polymorphism and its repeatability are the most crucial. AFLP has the potential to become a universal DNA fingerprinting technique since it can be used to any DNA sample, including microbial DNA, human, animal, and plant DNA. The polymerase chain reaction is used to selectively amplify restriction fragments from a digest of total genomic DNA in the AFLP approach. Zabeau and Vos were the first to create this approach (1993). The AFLP process consists of four basic steps: DNA digestion, ligation, amplification, and gel analysis. Two restriction enzymes initially degrade genomic DNA. The DNA fragments are ligated using double-stranded oligonucleotide adapters that are identical to one of the 5' or 3' ends formed during restriction digestion. The ligated DNA fragments are amplified by PCR using primers that are complementary to the adaptor and restriction site sequences, as well as extra selected nucleotides at the 3' end. The employment of selective primers minimises the mixture's complexity. Under precise annealing conditions, only fragments with complimentary nucleotides extending beyond the restriction site will be amplified by the selective primers. AFLP is a RAPD variant that may discover restriction site polymorphisms without previous sequence information by employing PCR amplification for restriction fragment detection. Janssen et al., (1996) found considerable support for the use of AFLP in bacterial taxonomy by comparing newly acquired data to findings produced by well-established genotypic and chemotaxonomic approaches like as DNA hybridization and cellular fatty acid analyses. Screening of DNA markers connected to genetic characteristics and microbiological typing, diagnostics of genetically inherited disorders, pedigree analysis, forensic typing, and parentage analysis are some of its possible uses.

Randomly amplified polymorphic DNA (RAPD)

The RAPD approach is a PCR-based discriminating method in which short arbitrary primers anneal to several random target sequences, resulting in the formation of the test organism's fingerprint profile. The target sequence to be amplified is unknown in RAPD analysis, and a 10base random sequence primer is utilised in the experiment to construct the RAPD profile. The low-stringency annealing conditions required for the RAPD-PCR reaction result in the amplification of randomly sized DNA fragments. The RAPD-PCR multiple band patterns is followed by dendrogram analysis to provide fingerprint profiles for the test organism. This technique may be used to identify clonal variants in bacterial strains. Because RAPD patterns are not always reproducible, hence this approach must be used under carefully controlled settings. The RAPD approach was utilised to identify enteropathogenic *E. coli, Salmonella, Shigella, Vibrio, Aeromonas*, and *Listeria* in food and water samples. RAPD has been used by multiple organisations to identify and characterise LAB strains from diverse sources, including human, food, and milk samples.

3. Hybridization based method

Microarray

A microarray is made up of regularly organised target DNA sequences that are connected to a solid substrate such as glass, silicon wafers, nylon membranes, or other functionalized substrates. The sample's DNA is fluorescently labelled and put to the array (hybridization). A fluorescent microarray detector and a computer application can then identify and evaluate several distinct AMR genes (Holzman, 2003). Fink et al. (2019) created a microarray-based AMR chip that identifies massive ARGs for -lactams and vancomycin. Using 14 probes, an array chip was built for six key classes of antibiotics, including -lactams, macrolides, aminoglycosides, tetracyclines, sulphonamides, and trimethoprim (Card et al., 2014) and found a total of 14 distinct resistance genes conferring resistance to six antibiotic classes. A microarray chip was created for 166 ARGs found in major Gram positive and negative bacteria (Garneau et al., 2010).

Fluorescent in situ hybridization (FISH)

FISH is a method that uses fluorescently labelled oligonucleotide probes to hybridise to the complementary DNA sequences of resistance genes. After the hybridization procedure is completed, any leftover probes are rinsed away. The signal from the bounded probes for ARGs is captured using epifluorescence or confocal laser scanning microscopy (Levsky and Singer, 2003). FISH probe designed to detect ampicillin, macrolide, and chloramphenicol resistance in *Escherichia coli, Helicobacter pylori*, and *Bacillus cereus* (Demiray and Yilmas, 2005; Juttner et al, 2004; Laflamme et al, 2009; Lee et al., 2019).

Restriction digestion based fingerprinting methods

Pulsed-field Gel electrophoresis (PFGE)

PFGE is a "Gold standard" approach for bacterial pathogen molecular subtyping. The approach involves in-situ macro-restriction of isolated genomic DNA in an agarose plug and digestion with restriction endonucleases (Barrett et al., 1994). Later, as part of the PulseNet approach, the gold standard this technique is widely used for disease outbreak analysis from food (Swaminathan et al., 2001). Initially, the Tenover (1995) guideline was employed, and subsequently, software for character-based analysis was created to evaluate genomic DNA based on the band number and positions of the band that emerged in the gel electrophoresis (Barrett et al., 2006). These approaches were also employed for source tracing, as well as local and worldwide epidemiology (Vernile et al., 2009).

Restriction Fragment Length Polymorphism (RFLP)

The restriction enzyme restricts the microbe's chromosomal DNA in RFLP. The approach was originally created and utilised to build linkages in the human genome (Botstein et al., 1980). The same approach may be used to produce bands from any DNA, including PCR products and tagged probes with restriction sites. The fingerprint or banding pattern created by agarose gel electrophoresis shows the availability and distribution of restriction sites throughout the chromosome. The RFLP technique may be used to compare strains within species, and using rare cutting restriction enzymes minimises the number of bands generated when compared to using frequent cutting restriction enzymes. The banded pattern was then utilised in probe

hybridization. As a result of restrictions such as time and labour demanding work for pure DNA extraction, restriction digestion and probe-based hybridization, and recording and analysis of bands, this approach has lost its relevance (Ben-Ari and Lavi, 2012; Ranjbar et al., 2014).

Multi Locus Enzyme Electrophoresis

MLEE is a system established using restriction enzymes for multiple loci of housekeeping genes prior to the introduction of MLST. The banding pattern, including the number and location of the bands, is also examined in this manner (Zahner et al., 1994). The use of MLEE approaches has decreased significantly since the development of the MLST scheme (Kotetishvili et al., 2003).

Bioinformatic analysis of fingerprint data

Bioinformatic analysis of fingerprint data is performed as character-based analysis or sequence-based analysis. In the character-based analysis, the number of bands and position of bands are taken into consideration for the normalization and analysis purpose. In this type of character-based analysis, the image quality is very crucial. There is much software used for character-based analysis, bio numeric is the software paid version and GelJ is the open-source software used in the character-based analysis. In the sequence-based analysis, the sequence data is either compared to the public domain or assigned value for the fingerprint data.

Example for character-based analysis ERIC PCR fingerprint analysis. Using GelJ software normalise the banding pattern visually. Construct the phylogenetic tree of the selected isolates with GelJ software. Keep the DNA ladder (100bp) for the normalization of banding position. Construct the phylogenetic tree based on the similarity calculated by Pearson correlation between the fingerprints with the tolerance of 1%, and grouping of the fingerprints with the help of the unweighted pair group method using arithmetic averages algorithm (UPGMA) (Rasschaert et al. 2005). Example for sequence-based data is multi-locus sequencing typing data. The assignment of alleles number and allelic profile has to be carried out after analysing the quality of the sequences obtained and after assembling. After assigning the allelic profiles, the sequence type's identification will be

carried out in the PubMLST public domain. Assigning the clonal complexes and relationship to the already existing bacterial strains in the public domain can be carried using the Minimum spanning tree development based on algorithm called BURST (based upon related sequence types) developed by Mellman *et al.* 2008. This will bring you the information on how many evolutionary events happened for your strain and what is the diversity of clones prevalent in your local region. As per the information on https://pubmlst.org/organisms/staphylococcus-aureus, there are now 902, 1091, 954, 607, 937, 855, 1024 alleles were identified and available in public domain for *arc, aroE, glpF, gmk, pta, tpi and yqiL* loci respectively. Detailed information on MLST and analysis for bacterial strains can be sought from https://pubmlst.org/organisms/staphylococcus-aureus., MRSA from seafood (Murugadas et al., 2017; Vaiyapuri et al., 2019).

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Chapter 15

Setting up of ERIC PCR and gel documentation and analysis

Murugadas Vaiyapuri, Sreejith V. N., Vineetha Das and Toms C Joseph

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

Highly conserved repetitive DNA sequences used for the typing of bacteria are the ERIC sequences that occurs in the intergenic (between genes coding for a protein) regions of polycistronic operons or present upstream or downstream of the untranslated regions of the bacterial genome. The ERIC sequences are a 126-bp imperfect palindromic sequences that shows similarity to REP sequences in many features. They are detected mainly in *E. coli* and *Salmonella typhimurium*. However, the typing method based on ERIC pattern is now expanded to the other bacteria within *Enterobacteriaceae* (Sharples et al., 1990).

ERIC PCR and analysis has three steps

Setting up of PCR with *E. coli* culture

Perform ERIC-PCR reactions in duplicate for each isolate in 25 µl volume containing 3 µl of *E. coli* genomic DNA, 2.5mM MgCl2, 1U Taq polymerase, 0.2mM dNTPs, 1X PCR buffer, 1µM of each primer (ERIC 1 and ERIC 2), and final volume adjusted with nuclease-free water.

Carry out reaction in 0.2 mL PCR tubes, always in same thermal cycler (Nemoy et al. 2005; Mohapatra et al. 2007).

Gel documentation

Perform the gel electrophoresis of PCR product in 3% agarose gel with 120V for 3h, and gel images are captured in the gel documentation system. Document twice for each gel with low and high exposure of trans UV light.

Bioinformatic analysis

Using GelJ software normalise the banding pattern visually. Construct the phylogenetic tree of the selected isolates with GelJ software. Keep the DNA ladder (100bp) for the normalization of banding position. Construct the phylogenetic tree based on the similarity calculated by Pearson correlation between the fingerprints with the tolerance of 1%, and grouping of the fingerprints with the help of the unweighted pair group method using arithmetic averages algorithm (UPGMA) (Rasschaert et al. 2005).

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Chapter 16

Setting up of quantitative real time PCR

Minimol V A

Real Time PCR is one of the PCR based assays to monitor the amplification of a particular gene /gene product in real time basis without any need for the post amplification process for visualizing DNA such as agarose gel electrophoresis, capillary electrophoresis etc. The fluorescent dye added to the reaction mixture allows the monitoring of the amplification staring from the first cycle of the PCR run and concomitantly the fluorescence is increased to 2 to 1000-fold as amplification progresses. Thus, based on the fluorescence, the DNA can be quantified over wide range of concentrations with the help of standard curves. Further, the data generated from the amplification process can easily be analyzed. The sensitivity and reliability of the result is significantly higher compared to conventional PCR. Real time PCR can be used for viral/bacterial quantification, gene/allele copy number, allelic discrimination assays (SNPs) gene expression, Methylation studies etc.

The real time detection of the nucleic acid amplification is achieved by nonspecific or sequence specific strategies. The nonspecific method uses intercalating dyes which can able to produce fluorescence while binding with ds DNA.The commonly used nonspecific dye in real-time PCR is asymmetric cyanine dye called SYBR Green I. This dye has higher affinity to ds DNA compared to that of ethidium bromide and the intensity of the bound dye is higher (magnitude of 1000 folds) than the free form of syber green. This enables an increase in fluorescence during amplification. However, once the melting of the double stranded DNA after polymerisation causes the denaturation of DNA and signal strength falls off due to the detachment of fluorescent dye. Other dyes of this category include, O-PRO-1, BEBO, YOYO -1. The major advantage of nonspecific dyes are less

expensive, and can be used with any pair of primers/target. The disadvantage is that it binds non-specifically to any ds DNA yielding signal from nonspecific products. However, this can be verified at the end with the help of melt curve analysis by subjecting the amplicon to a temperature range beyond its melting temperature.

The sequence specific strategies employ the use of either hydrolysis probes or hybridization probes. These probes are synthesised based on the sequences of the internal fragments of the two primers. The quantification of the PCR product is done by measuring the fluorescence signal strength based on either quenching or FRET mechanism.

Hydrolysis probes are the probes which are hydrolysed due to 5'-3' exonuclease activity of DNA polymerase during the elongation stage of the PCR cycle. TaqMan Probe is widely known hydrolysis probe for RT PCR application. It is nothing but a oligo sequence labelled with reporter dye in one end (5'end) and quencher dye at the other end. In intact the fluorescence emitted from the reporter dye is banned due to the presence of quencher dye in its close proximity. During PCR run, DNA is denatured and both primer and the probe annealed to the target DNA. However, the Taq polymerase has exonuclease activity will cleave the probe and the reporter and quencher dye get separated, thus allowing the fluorescence emission from the reporter dye when it excited with a suitable light source. As amplification progress, the signal strength gets increased enabling the quantification of DNA. The melting point of the probe should be 10 degrees higher than primer Tm as cleavage of the probe take place only during the elongation step of the PCR. In addition to TaqMan Probe, TaqMan MGB probes are also used. The Minor groove binder increase the melting temperature of the probe and it increase the duplex stability particularly for shorter probes. In case of hybridization probes the fluorescent signal is obtained due to the structure changes in the secondary structure of the probe during hybridization phases. The changes in the structure causes increase the distance between reporter and quencher dye preventing the fluorescence resonance energy transfer (FRET) from a reporter dye to

quencher dye. The probe in its intact form is a hair pin like structure and behaving non-fluorescence chromophore due to close proximity of both quencher and reporter dye. However, the conformation changes during hybridization demands separation of both dyes and the far distance among the dyes prevent the energy transfer through FRET mechanism. Thus, the increased fluorescent signal from the reporter dye enables the quantitative estimation of the DNA. . With both types of assays, the exponential increase in fluorescence is used to determine the cycle threshold (Ct) which is the number of PCR cycles at which significant exponential increase in fluorescence is detected. Using a standard curve for Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made.

Protocol for setting up of Absolute Quantitative real time PCR

Material required

Genomic DNA of Known concentration (50ng/µl)

Primers and probes

2X Taqman universal master mix

DNAse free water

DNAse free TE

Plasticware

Real time instrument

Strategy:

Make standards of known concentration by serially diluting a stock concentration of genomic DNA of known concentration

Set up a real time PCR assay for the standards, unknown and negative control samples.

Plot a standard curve for the Ct values vs the log concentration of the standards from the standard curve determine the concentration of the unknown samples using Ct values.

Procedure:

1 Make 10-fold serial dilution for the genomic DNA to minimum of 4 standard concentrations.

2 Make master mix taking in to consideration about the replicates, NTC, standards, unknown samples

Components	Volume per reaction µl
2X TaqMan master mix	5.0
20X TaqMan Primer probe mix	0.5
Water	2.5
Total	8.0

3 Mix well and centrifuge briefly

4 Prepare the mix for each replicate by four time of 8 µl master mix (step2) with respective DNA/TE/NTC)

5 Disperse the master mix into four individual tubes for each DNA/TE/NTC. Centrifuge the tube briefly

6: Set up the experiment on the instrument using system software.