Chapter 27

Hygiene Indicator bacteria in sea-foods and aquaculture

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INTRODUCTION

Indicator bacteria are types of bacteria that are used to provide an indication of poor hygiene, inadequate processing and or post- process contamination in seafood's, water, feed, ice, equipments, workers, etc. Absence of indicator bacteria in seafood and aquaculture products provides a degree of quality assurance that the fishery products are hygienically good and employed proper processing methods whereas their presence clearly indicates either severe problem or failure occurred during the processing p, under-processing or post-process contamination. The indicator bacteria in fish and fishery products are mainly from enterobacteriaceae and coliforms group of bacteria. Traditionally, indicator micro-organisms have been used to suggest the presence of pathogens (Berg 1978) and it includes the general microbial Process indicators, Faecal indicators, andIndex and Model indicator organisms. The Process indicator is a group of organisms that indicates the efficacy of a processing and tested by Total Plate count (TPC)/ Total heterophilic count or toal coliforms (MPN method). Fecal indicator is a group of organisms that indicates the presence of fecal contamination and could be tested by fecal coliforms, thermotolerant coliforms (MPN method) and *E.coli* (EMB and IMViC test). An Index and model organisms is a group of organisms that indicates the presence of pathogens such as *E.coli* as an index for Salmonella and coliphages as model of human enteric viruses. The pathogenic indicator bacteria viz., Coliforms, Fecal coliforms, *E.coli*, *Klebsiella, Enterobacter, Citrobacter,* Fecal Streptococci,Sulphite reducing clostridia, *Clostridium perfringens*, Bifidobacteria, Bacteriophages, coliphages and *Bacteroides fragilis* baeriophages etc**.,** are associated with food poisoning. In addition, pathogenic viruses, protozoa and parasites also present in fecal matter. Fecal contamination is mainly from sewage of human source, livestock, poultry manure, pets and wildlife. Infection caused by these pathogens is mainly depending on the level of microbial load.

TYPES OF INDICATOR ORGANISMS

The indicator bacteria include **total coliforms** (Gram- negative, non-spore sproe-forming, oxidase- negative, rod shaped, facultative anaerobic, ferment lactose), **thermotolerant coliforms** (produce gas and acid from lactose at 44.5^{o}C ± 0.2^{o}C within 24 ± 2 hrs) and **fecal coliforms**(produce gas and acid from lactose at 37^{o}C ± 0.2^{o}C within 24 ± 2 hrs), which are found in the intestinal tracts of warm blooded animals. Total coliforms were used as fecal indicators by public agencies in the US as early as the 1920s. These organisms can be identified based on the fact that they all metabolize the sugar lactose, producing both acid and gas as byproducts. Fecal coliforms indicate that recent fecal contamination is being occurred. ***Escherichia coli*** (*E. coli*) and **enterococci** (all fecal streptococci grow at pH 9.6, between 10^{o}C and 45^{o}C, 6.5% NaCl and hydrolyzing 4-methlumbelliferyl-B-D-glucoside of thallium acetate, nalidixic acid and 2,3,5-triphenyltetrazolium chloride) are commonly used as indicators. The **Sulphite reducing clostridia** (SRC)-Gram-positive, spore forming, non- motile, strictly anaerobic rods that reduce sulphite to H_2S), ***Clostridium perfringens*** *(*As for SRC but also ferment lactose, sucrose, and inositol with the production of gas, produce stormy clot fermentation with milk, reduce nitrate, hydrolyse gelatin and produce lecithinase and acid phosphatase), **Bifidobacteria** (obligately anaerobic, non-acid fast, non spore forming, non motile, Gram positive bacilli which are highly pleomorphic and may exhibit branching bulbs-bifids, clubs, coccoid, coryneform, V and Y forms. All are catalase- negative and ferment lactose), **Bacteriophages** (bacterial viruses and are ubiquitous in the environment and are used as model to human eneteric viruses, eg., Somatic coliphages, male specific RNA coliphages and pahges phages infecting *Bacteroides fragilis*), **Coliphages** (Somatic coliphages attack *E.coli* strains via the cell wall and sex pili) and ***Bacteroides fragilis*** **baeriophages** (infect most abundant bacteria in the gut, eg. *B. fragilis* HSP40) etc., are also considered as fecal indicator microorganisms.

Total coliform bacteria are defined as the organisms that ferment lactose to produce acidic condition and change the colour of the medium to yellow within 24 ± 2 hours when incubated at 35.0 ± 0.5°C on MacConkey Broth and utilize BGLB (2%) by the observable growth and gas production within ± 2 hours of incubation at 35.0 ± 0.5°C.

Fecal coliform bacteria are defined as the organisms that grow and produce gas in *E coli* broth (EC broth) in 24 ± 2 hours when incubated at 35.0 ± 0.5°C.

E. coli are defined as the organisms that produce growth and gas production in tryptone broth (indole medium) in 24 ± 2 hours when incubated at 44.5 ± 0.2°C and is confirmed on Eosine Methylene Blue

(EMB) agar (a sterile platinum loopful of culture from EC broth is streak-dilution method and incubated at 37°C for 18-24 hrs produces well isolated colonies, 2-3mm dia with a greenish metallic sheen by reflected light & dark purple centre by transmitted lightand **IMViC** test (Indole-Degrade the amino acid tryptophan and produce indole, **Methyl Red**-*E.coli* use the mixed acid pathway, which produces acidic end products such as lactic, acetic, and formic acid. These acidic end products are stable and will remain acidic. When methyl red is added, if acidic end products are present, the methyl red will stay red, **Voges–Proskauer** - utilization of glucose to acetyl methyl carbinol (acetoin) and it will react with alpha-naphthol (VP reagent #1) and potassium hydroxide (VP reagent #2) to form a red color and**Citrate utilization**- Organisms which can utilize citrate as their sole carbon source by the presence of enzyme citrase or citrate-permease and convert the ammonium dihydrogen phosphate to ammonia and ammonium hydroxide-alkaline environment. At pH 7.5 or above, bromthymol blue turns royal blue as positive. So the IMViC tests for *E. coli* as +, +, -and -).

Fecal streptococci are defined as the organisms that produce red or pink colonies within 48 ± 2 hours when incubated at 35.0 ± 0.5°C on Kennel Fecal Streptococcal medium.

Enterococci are defined as the organisms that produce pink to red colonies with a black or reddish-brown precipitate after primary culture for 48 to 50 hours at 41.0 ± 0.5°C on m-Eenterococcus medium followed by incubation for 20 minutes at 41.0°C on Eusculin Iron Agar medium (EIA medium).

DEVELOPMENT OF INDICATORS

Coliforms: Use of bacteria as indicators of the sanitary quality of water probably dates back to 1880 in human faeces (Geldreich 1978). In 1891, Franklands came up with the concept that organisms characteristic of sewage must be identified to provide evidence of potentially dangerous pollution. In 1893, 'Wurtz method' of enumerating *B. coli by direct plating of water samples on litmus* lactose agar was being used by sanitary bacteriologists, using the concept of acid from lactose as a diagnostic feature. This was followed by gas production, with the introduction of the Durham tube (Durham 1893). Sanitary significance of finding various coliforms along with streptococci and *C. perfringens were recognised by bacteriologists* by the start of the twentieth century (Hutchinson and Ridgway 1977). MacConkey (1905) described his now famous MacConkey's broth, which was diagnostic for lactose-fermenting bacteria tolerant of bile salts.

Coliform identification schemes: Various classification schemes for coliforms have emerged. MacConkey (1909) which has recognized 128 different coliform types. Bergey and Deehan (1908) identified 256. In early 1920s, differentiation of coliforms had come to a series of correlations that suggested indole production, gelatin liquefaction, sucrose fermentation and the Voges–Proskauer reaction were among the more important tests for determining faecal contamination (Hendricks 1978). These developments culminated in the IMViC (Indole, Methyl red, Voges–Proskauer and Citrate) tests for the differentiation of so-called faecal coliforms, soil coliforms and intermediates (Parr 1938); these tests are still in use today. Simpler to identify coliform group, despite being less faecal-specific and broader (*Escherichia, Klebsiella, Enterobacter* and *Citrobacter* were considered the most commongenera) was targeted. One of the first generally accepted methods for coliforms was called the Most Probable Number method (MPN) by Multiple-Tube Fermentation Test.

Faecal streptococci and enterococci: A group of Gram-positive coccoid bacteria known as faecal streptococci (FS) and investigated as an important pollution indicator bacterium.

Faecal streptococci: Until 1957, with the availability of the selective medium that enumeration of FS became popular. Since then, several media have been proposed for FS and/or enterococci to improve on the specificity. Taxonomically FS are represented by various *Enterococcus spp., Streptococcus bovis and S. equinus* (WHO 1997).Of the faecal streptococci, the preferred indicators of faecal pollution are the enterococci. The predominant intestinal enterococci are *E. faecalis, E. faecium* and *E. durans*. In addition, other *Enterococcus species* and some species of *Streptococcus* (*S. bovis and S. equinus)* may occasionally be detected. These streptococci however, do not survive for long in water and are probably not enumerated quantitatively. Thus, for water examination purposes enterococci can be regarded as indicators of faecal pollution, although some could occasionally originate from other habitats.

Sulphite-reducing clostridia and other anaerobes: Until bifidobacteria were suggested as faecal indicators by Mossel, 1958. *C. perfringens* was the only obligately anaerobic, enteric micro-organism seriouslyconsidered as a possible indicator of the sanitary quality of water. Anaerobic sulphite-reducing clostridia are much less prevalent than bifidobacteria in human faeces. But their spore-forming habit gives them high environmental resistance (Cabelli, 1978). *C. perfringens* is the species ofclostridia most often associated with the faeces of warm-blooded animals (Rosebury, 1962), but is only present in 13–35% of human faeces. Although *C. perfringens* has been considered a useful indicator species formore than a hundred years (Klein and Houston 1899). Perfringens as a faecal indicator

and could present in the environment for long duration,which is considered to be significantly longer than enteric pathogens (Cabelli, 1978). Bonde (1963) suggested that all SRC in receiving waters are not indicators of faecal pollution, hence *C. perfringens* is the appropriate indicator.

Bacteriophages: Viruses which infect bacteria, first described from the intestinal tract of man in the early 1900s. Use of phages as models for indicating the likely presence of pathogenic enteric bacteria first appeared in the 1930s. Direct correlations are exists between the presence of certain bacteriophages and the intensity of faecal contamination. Evolving role for phages to coliforms, known as coliphages-model human enteric viruses.

Major groups of indicator coliphages (Leelere et al., 2000)	
Family	**Phage examples**
Myoviridae	T2, T4, T6
Siphoviridae	λ, T5
Podoviridae	T7, T3
Microviridae	ϕX174, S13
Inoviridae	SJ2, fd, AF-2, M13

EMERGING MICROBIOLOGICAL METHODS

Fast detections using chromogenic substances: Chromogenic substances are modified either by enzymes (bacteria) or by specific bacterial metabolites. Chromogenic substance changes its colour or fluorescence, easy detection of those colonies, avoids the need for isolation of pure cultures and confirmatory tests. Time required for the determination of different indicator bacteria can be reduced between 14 to 18 hours. Extended Spectrum of Beta Lactamase (ESBL) producing *E.coli* (ESBL) and *E.coli* O 157 are examples.

Application of monoclonal and polyclonal antibodies: Mab have been successfully used for the detection of indicator bacteria in water samples (Hübner *et al.* 1992;Obst *et al.* 1994).Detection of 'viable' indicators is the combination of immunofluorescence with a respiratory activity compound. Detection of *E. coli O157:H7, S. typhimurium* and *K.pneumoniae* in water(Pyle *et al.* 1995). Antibody technology is often used in medicine with enzyme amplification (ELISA).

IMS/culture and rapid culture-based methods: Immunomagnetic separation offers an alternative approach to rapid identification of culturable and non-culturable micro-organisms (Safarik *et al.* 1995).

Principles and application of the method are based on suitable antibody specificity. Purified antigens are typically biotinylated and bound to streptoavidin-coated paramagnetic particles. Raw sample is gently mixed with the immunomagnetic beads, then a specific magnet is used to hold the target organisms against the wall of the recovery vial, and non-bound material is poured off. Target organisms can then be cultured or identified by direct means. IMS approach may be applied to recovery of indicator bacteria from water, but is possibly more suited to replace labour-intensive methods for specific pathogens such as recovery of *E. coli* O157 from water(Anon, 1996).

Gene sequence-based methods: Based on the recognition of specific gene sequences. Usually rapid and can be tailored to detect specific strains of organisms. PCR (polymerase chain reaction), FISH (fluorescence *in situ*hybridization),uses gene probes with a fluorescent marker, typically targeting the 16S ribosomal RNA (16S rRNA).

LIMITATIONS IN DETECTIONG INDICATOR BACTERIA

- Some of the indicator bacteria are environmental origins i.e. environmental reservoirs
- Some of the indicator bacteria are both fecal and non-fecal origin
- Some of the tests for fecal indicator may also detect non-fecal microbes.

FUTURE DEVELOPMENTS:

- Microarrays and biosensors
- Biosensors based on antibody technology, with the antigen triggering a transducer or linking to an enzyme amplification system.
- Microarrays using DNA/RNA probe-based rRNA targets may be coupled to adjacent detectors.

CURRENT APPLICABILITY OF FAECAL INDICATORS

- Members of the total coliform group and faecal coliforms
- *E. coli* is considered as main source of recent faecal contamination and is now considered to be *E. coli* and enterococci.

Clostridium perfringens is considered as alternative indicators to *E. coli* and enterococci.

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