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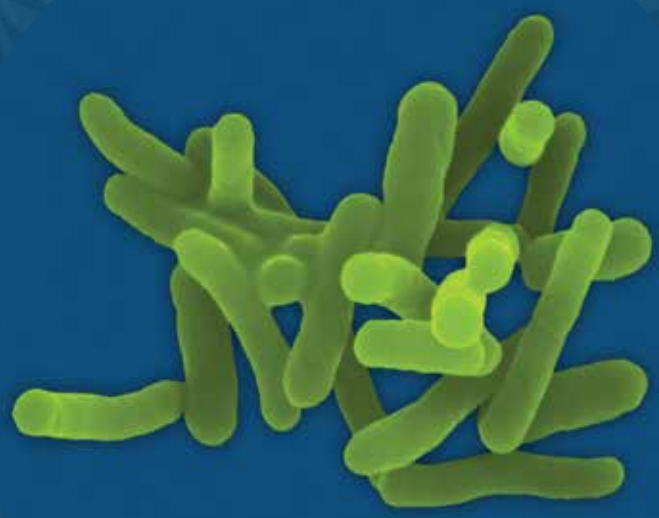
World Health Organization

Selection and application of methods for the detection and enumeration of human-pathogenic halophilic *Vibrio* spp. in seafood

GUIDANCE



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MICROBIOLOGICAL RISK ASSESSMENT SERIES

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MICROBIOLOGICAL RISK ASSESSMENT SERIES

Selection and application of methods for the detection and enumeration of human-pathogenic halophilic *Vibrio* spp. in seafood

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DECLARATIONS OF INTEREST

All participants completed a Declaration of Interests form in advance of the development of the guidance. While several of the experts were working on the area of methodology, none were considered to present any potential conflict of interest, as the purpose of the guidance is not to recommend specific methods.

Preface

This guidance was developed in response to a request to FAO/WHO from the 42nd Session of the Codex Committee on Food Hygiene to provide recommendations on a range of test methods for quantifying *Vibrio parahaemolyticus* (total and pathogenic) and *Vibrio vulnificus i*n seawater and bivalves, and to facilitate performance evaluation of the methods. Their development was initiated at a Joint FAO/WHO Expert Meeting on methodology for detection and enumeration of *Vibrio parahaemolyticus* and *Vibrio vulnificus* associated with seafoods held in Ottawa, Canada, 17–19 October 2011. The draft guidelines were subsequently tested at a regional workshop in Singapore held on 19 – 23 November 2012 with participation of 9 countries. A second pilot testing workshop was convened in Chile on 2- 6 December 2013 with participation of 4 countries. The experience in these workshops was taken into consideration in finalizing the guidance. In addition, the document was also subject to peer review before finalization.

Abbreviations used in this report

AGM	Adjusted Geometric Mean
ANOVA	Analysis Of Variance
AOAC	AOAC International [formerly the Association of Official Analytical Chemists]
APW	Alkaline Peptone Water
ASW	Alkaline Salt Peptone Water
CCFH	Codex Committee on Food Hygiene
CFIA	Canadian Food Inspection Agency
CFU	Colony-Forming Units
EPA	Environmental Protection Agency [United States of America]
EURL	European Union Reference Laboratory
HACCP	Hazard Analysis and Critical Control Points [system]
HPA	Health Protection Agency [United Kingdom]
IEC	International Electrotechnical Commission
IMS	Immunomagnetic Separation
ISO	International Organization for Standardization
ISSC	Interstate Shellfish Sanitation Conference [United States of America]
LAMP	Loop-Mediated Isothermal Amplification [Assay]
LOD	Limit of Detection
LOQ	Limit of Quantification
MLE	Maximum-Likelihood Estimation
MPN	Most Probable Number
NSSP	National Shellfish Sanitation Program
PCR	Polymerase Chain Reaction
PHP	Post-Harvest Processing
RT-PCR	Reverse Transcription-PCR
SD	Standard Deviation
SPB	Salt Polymyxin Broth
SSCA	State Shellfish Control Authority [United States of America]
TCBS	Thiosulphate Citrate Bile Salt Sucrose [agar]
TDH	Thermostable Direct Haemolysin
TRH	TDH-Related Haemolysin
VBNC	Viable But Non-Culturable

Executive summary

One of the challenges in the development of global risk assessments for halophilic *Vibrio* spp. has been the availability of appropriate datasets from around the world which could be considered to be representative of the range of conditions under which halophilic *Vibrio* spp. grow and become a problem for seafood safety. Even when data are available, the different methodology used to generate that data often make it difficult compare and consolidate globally representative datasets. In order to begin addressing this the Codex Committee on Food Hygiene recommended that the range of test methods available for quantifying *V. parahaemolyticus* (total and pathogenic) and *V. vulnificus* in seawater and bivalves be looked at with a view to providing guidance that would facilitate performance evaluation of the methods. This document has been developed in response to that request and is intended to provide sufficient information on methods (but not details of the methods) to inform the selection of the most appropriate methods according to the potential end use of the data generated, for example, harvest area monitoring, post-harvest process verification, end product monitoring, and outbreak investigation.

The methods considered included enumeration by direct plating on selective agars, direct plating on non-selective agars followed by colony hybridization with probes, conventional selective enrichment followed by selective plating and biochemical testing or molecular testing or direct molecular test such as polymerase chain reaction (PCR) on broth, conventional most probable number method involving plating and biochemical testing or plating and molecular testing or direct PCR on broth. The performance characteristics considered whether the methods were qualitative or quantitative, and included sensitivity and specificity, ability to recover stressed cells and detect pathogenic strains, limit of detection and quantification, sample volume requirement, time to result, availability of supplies and skill level required to perform the tests. Guidance on which of these characteristics, and to what extent a method should meet these characteristics, according to how the data generated by the method will be used is also provided. Recommended approaches for in-house verification of *Vibrio* spp. methods for use in national risk assessments are also included.

Aspects of data requirements that could support national and regional risk assessments have also been discussed. These include levels of bivalve production, levels and patterns of consumption, abiotic factors that may affect the levels of halophilic *Vibrio* spp, levels of the organism of concern at harvest, at market, in foods that have caused illness, time temperature profiles of the food chain, growth of the organism in bivalve species, susceptible population and epidemiological data.

1

Introduction

FAO and WHO have published three Microbiological Risk Assessments on specific *Vibrio* species pathogenic for humans:

- Risk assessment of choleragenic *Vibrio cholerae* O1 and O139 in warm-water shrimp in international trade (MRA Series, no. 9; 2005).
- Risk assessment of *Vibrio vulnificus* in raw oysters (MRA Series, no. 8; 2005).
- Risk assessment of *Vibrio parahaemolyticus* in seafood (including Part I – Microbiological risk assessment of *Vibrio parahaemolyticus* in raw oysters; Part II – Microbiological risk assessment of *Vibrio parahaemolyticus* in Anadara granosa (Bloody clam); and Part III – Microbiological risk assessment of *Vibrio parahaemolyticus* in finfish) (MRA Series, no. 16; 2011).

These risk assessments were used by the Codex Committee on Food Hygiene (CCFH) to develop the Codex Guidelines for Application of General Principles of Food Hygiene to the Control of Pathogenic *Vibrio* spp. in Seafood (CAC/GL 73-2010). While working on this, the 41st Session of CCFH recognized the need to provide countries with tools to assist them in the implementation of the guidelines under the various conditions that exist in different regions and countries. Noting that a risk management tool had been developed in the United States of America, based on the conditions and data from that country, CCFH asked FAO/WHO to convene an expert meeting to look into the possibility of applying this tool in other geographical areas and to other species of shellfish. The Joint FAO/WHO Expert Meeting convened in September 2010 concluded that the *Vibrio parahaemolyticus* calculator tool developed in United States of America may be used to estimate relative risk reductions associated with temperature controls (post-harvest refrig-

eration) in areas in which the strain virulence, initial concentration and growth rates of *V. parahaemolyticus* in the bivalve species of concern are similar to that indicated in data from the United States of America. The meeting noted that for development of a tool applicable for other regions or other bivalve species, data from the various regions would be required. Considering the importance of using appropriate methodology for data collection, the 42nd Session of CCFH asked FAO/WHO to continue the work and provide recommendations on a range of test methods for quantifying *V. parahaemolyticus* (total and pathogenic) and *V. vulnificus* in seawater and bivalves, and to facilitate performance evaluation of the methods. In response to this, FAO/WHO implemented a joint Expert Meeting in Ottawa in 2011. Based on the discussions held at this expert meeting, the present document was developed by FAO and WHO to provide guidance on the selection and application of methods for the detection and enumeration of human-pathogenic *Vibrio* spp. in seafood. The collection of data using this guidance document will contribute to the revision of present risk assessment models and assist their application on a wider geographical basis (Figure 1.1). It will also allow national authorities to generate data to support risk assessment on a local basis and determine whether a significant risk may exist in their country and, if so, to evaluate possible risk management strategies.

This guidance document provides sufficient information on methods to inform the selection of methods for several potential end uses. It is not intended to detail all of the methods that have been published or to explicitly compare those methods, especially with regard to the many different bacterial culture media (both enrichment broths and selective plating media) or details of DNA extraction procedures, primers and probes (as relevant) for molecular methods. These factors are obviously important in the practical application of methods. Consideration will be given to the performance criteria that may apply to specific end uses and will describe approaches to the verification of methods for such end uses.

One of the conclusions of the Joint FAO/WHO Expert meeting held in 2010 was: The development of methods, particularly molecular methods for V. *parahaemolyticus* and *V. vulnificus,* is evolving rapidly. This means that the recommendation of any single method for the purposes of monitoring these pathogens is difficult and also of limited value as any method is likely to be superseded within a few years (FAO/WHO, 2016).

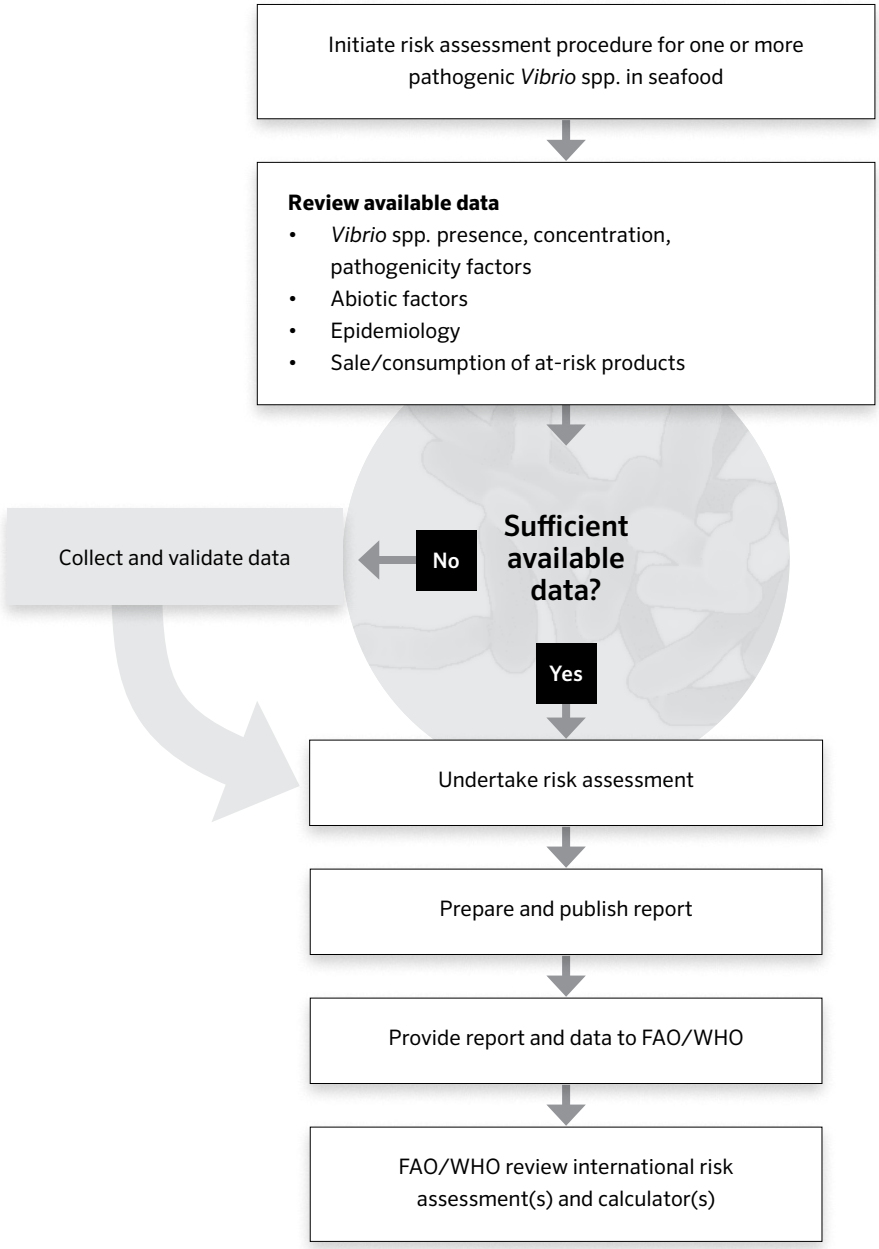

FIGURE 1.1 Flowchart for the conduct of a national risk assessment

Monitoring of *Vibrio* spp. concentrations in the seafood is the only direct way to establish the levels of *Vibrio* in these commodities at the time of harvest and through the production chain. Such data is invaluable when undertaking a risk assessment. However, undertaking monitoring at a sufficient intensity to detect potential health risks on an ongoing basis can pose practical difficulties and will be expensive. Other factors that influence the concentrations of total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in the seafood, and that are practical and less expensive to collect, may be used to indirectly predict concentrations and could be used also to determine risk. Where a potential risk has been identified to exist in a country, cost-effective programmes may incorporate monitoring of such factors to determine areas and seasons with higher risk. Seawater temperature and salinity have been identified as two important abiotic factors for predicting the concentrations of *V. parahaemolyticus* and *V. vulnificus* in some oyster species in some parts of the world, but other ecological factors may be involved elsewhere. In applying risk assessments and risk calculators more widely, such relationships need to be confirmed for the species of interest (if different) and for the specific geographical and environmental situation. The collection of locally relevant data is therefore an important part of the potential application of the international risk assessments.

2

Possible end uses of *Vibrio parahaemolyticus* and *Vibrio vulnificus* testing methodologies

There is a range of possible end uses for testing methodologies, including:

- monitoring the presence, or concentration, of pathogenic *Vibrio* spp. in marine food animals (*sensu lato*) in harvesting areas;
- determining the relationship of the presence or concentration of pathogenic *Vibrio* spp. with climate and environmental factors;
- monitoring at significant stages of the production chain, including final sale;
- testing the effectiveness of post-harvest treatments;
- testing in support of outbreak investigations; and
- use by expert reference laboratories.

For live bivalve molluscs, there is the potential for the testing of seawater in which bivalves will be held post-harvest, whether for depuration, conditioning or simple holding purposes. Additionally, for cooked seafood products there is the potential for testing of environmental surfaces that may be involved in promoting cross-contamination. This section will consider aspects of end uses with respect to seafoods.

2.1 HARVEST-AREA MONITORING

Surveillance of *V. parahaemolyticus* and *V. vulnificus* in the environment has been investigated in many countries by researchers, but monitoring in harvesting areas has not generally been undertaken as part of a control programme for either species. This is at least partly due to the significant growth that can occur between harvest and consumption: control of temperature between harvest and sale is seen as a major element in controlling risk. With non-bivalve species there are additional considerations, as the relationship between the occurrence and concentration of *Vibrio* on the outer surfaces, or in the digestive tract, may not bear a direct relationship to that in the final product, even taking into account any subsequent proliferation, as the parts that are eaten may not include the outer skin or carapace, or the digestive tract.

An example of food safety interventions based on harvesting area monitoring is seen during the summer months in Canada (generally May to October, depending on water temperature); whereas oysters intended for sale in the shell should only be harvested from sites where the levels in the oysters do not exceed 100 Most Probable Number (MPN)/g of *V. parahaemolyticus,* unless a post-harvest processing step validated to reduce *V. parahaemolyticus* levels to equal or less than 100 MPN/g is applied. Elevated levels at the harvesting area should trigger additional industry monitoring. In addition, *V. parahaemolyticus*-specific control measures, such as time and temperature controls, are required during the at-risk season.

A *V. parahaemolyticus* method from Health Canada's Compendium of Analytical Methods (i.e., MFLP-37) based on a 3 or 5-tube multiple dilution MPN format is used. All turbid tubes plus all tubes from the next dilution are streaked onto selective and differential medium thiosulphate citrate bile salts sucrose (TCBS) agar, with one tube streaked onto one plate. Presumptive colonies (3 colonies per plate) are subjected to biochemical identification and/or multiplex polymerase chain reaction (PCR) targeting R72H (unknown function), TDH (thermostable direct haemolysin), and TRH (TDH-related haemolysin) markers (i.e., MFLP-23). Based on these results, an MPN index is determined that translates into *V. parahaemolyticus* MPN/g.

2.2 POST-HARVEST PROCESS VERIFICATION

A post-harvest process (PHP) for *Vibrio* spp is one applied to seafood that is normally eaten raw, and undertaken in order to reduce the concentration of one or more pathogenic *Vibrio* spp. to a value that is deemed to pose an acceptable risk to consumers, while still yielding seafood that has acceptable sensory and

taste qualities for consumers. Such processes may include pasteurization, freezing, ultra-high pressure treatment or gamma irradiation. Conventional depuration of bivalve molluscs does not reduce the concentration of marine *Vibrio* spp. sufficiently to be considered in this context. For seafood that is normally eaten cooked, thorough cooking will provide such a reduction in *Vibrio* concentration.

Demonstration of a significant reduction through a PHP inherently requires a method that demonstrates viability of the target pathogen, and gives a quantitative result, pre- and post-process. The use of PHP is likely to subject *Vibrio* cells to stress and it is therefore essential to ensure that the enumeration method can adequately recover stressed but viable cells. Direct plating on a selective medium may not satisfy this requirement.

The United States National Shellfish Sanitation Program (NSSP) Model Ordinance (FDA, 2013; see also Annex 1) requires that, for a PHP

> "… the dealer must demonstrate that the process reduces the level of *V. vulnificus* and/or *V. parahaemolyticus* in the process to non-detectable (<30 MPN/gram) and the process achieves a minimum 3.52 $\log_{10}$ reduction. Determination of V. *vulnificus* and/or *V. parahaemolyticus* levels must be done using the MPN protocols described in Guidance Documents, Chapter IV, Naturally Occurring Pathogens, Section .02, followed by confirmation using methods approved for use in the NSSP."

Specifically, it identifies that:

- For validation
  - Microbiological testing for initial levels will be by a 3-tube MPN using appropriate dilutions (10^{-1} to 10^{-6}).
  - Microbiological testing for processed samples will be by a single-dilution five-tube MPN, inoculating with either 0.01 g or 0.1 g of shellfish per tube.
- For ongoing verification
  - The monthly sampling shall consist of 30 tubes from a minimum of three samples of 10 tubes, each with an inoculum of 0.01 g. Ideally, this would be done on three separate days of production, spread throughout the month, using a 10-tube MPN each day. If this is not feasible, the 30 tubes can consist of 3 samples from three consecutive days or 3 samples from a given day (from three separate lots if possible).
  - If more than 11 tubes of the 30 most recent 3×10 tube samples within any calendar month are positive, then the process fails for that month.
  - If all 10 tubes are positive for any given sample, this is considered a verification failure.

The guidance therefore includes a number of markedly differing MPN formats and it is difficult to relate the performance characteristics of these formats: the approaches have been selected in terms of target concentrations (or thresholds) and the probability of compliance with the requirements, but not the variability of the test method itself in relation to the concentrations or thresholds. There is a requirement for identification to species level, but not identification of pathogenic principles.

Unless information becomes available to demonstrate that clinical and environmental strains differ in their resistance to one or more PHPs, there is no advantage in using a method that determines the presence of pathogenic principles: i.e. a method for total viable *V. parahaemolyticus* or *V. vulnificus* should be satisfactory.

2.3 END-PRODUCT MONITORING

Some current end-product guidelines and standards for *V. parahaemolyticus* in bivalve molluscs are shown in Tables 2.1 (United Kingdom), 2.2 (Australia and New Zealand), 2.3 (Canada), 2.4 (Japan) and 2.5 (United States of America). In general, where numerical standards or guidelines have not been defined for a pathogen, food microbiology laboratories would usually test for presence/absence of a pathogen in 25 g: this would be assumed to apply for *V. vulnificus* unless otherwise specified. Testing against guidelines and standards has usually been based on conventional isolation and enumeration methods. In the United Kingdom, for example, the method used for determining compliance with the Health Protection Agency (HPA) guideline for *V. parahaemolyticus* (HPA, 2009) used to be direct plating on TCBS, but *V. parahaemolyticus* testing requirement has been withdrawn (R. Lee. *pers.comm.*). Present standards and guidelines are generally specified with regard to total *V. parahaemolyticus,* although the Australian and New Zealand guidelines refer to the potentially unsafe limits relating only to Kanagawa-positive strains (the implication being that the other guideline levels given relate to total *V. parahaemolyticus)*. The United States of America requirement for *V. parahaemolyticus* in ready-to-eat fishery products relates to either Kanagawa-positive or -negative colonies.

NSSP indicates that non-detectable levels for *V. vulnificus* and *V. parahaemolyticus* in PHP bivalves are <30 MPN/g (FDA, 2013).

Where standards or guidelines have been developed for use with methods for all strains of a species (e.g. total *V. parahaemolyticus*), the values will need to be reviewed, and potentially revised, if methods for the enumeration of pathogenic strains (e.g. *tdh*-positive and/or *trh*-positive) are applied instead.

TABLE 2.1. United Kingdom guidelines for pathogenic *Vibrio parahaemolyticus* in ready-to-eat foods

Result CFU/g	Risk category	Interpretation	Cause	Action	Laboratory specialist and reference tests
$>10^3$	High	UNSATISFACTORY Potentially injurious to health and/or unfit for human consumption	Strong evidence for poor processing	Immediate investigation of the food origin, review cooking and subsequent temperature and time controls. Take investigative samples of processed (cooked) food, raw food components (particularly marine products) and the food preparation environment.	Confirmation of identity; typing.
20 – $<10^3$	Moderate	UNSATISFACTORY	Little evidence for poor processing or cross-contamination	Risk will increase proportional to levels detected. Food may not become hazardous provided appropriate levels of control are applied. Consider taking investigative samples of processed (cooked) foods, raw food components (particularly marine products) and the food preparation environment.	Consider referral of isolates, particularly where associated with outbreak investigations.
<20	Low	SATISFACTORY		No further action required.	

Source: Based on HPA, 2009.

TABLE 2.2. Guidelines for *Vibrio parahaemolyticus* in ready-to-eat foods in Australia and New Zealand

Microbiological quality (CFU per gram)			
Satisfactory	**Marginal**	**Unsatisfactory**	**Potentially hazardous**
<3	<3–10^2	10^2–10^4	≥10^4

Note: *V. parahaemolyticus* should not be present in seafoods that have been cooked. For ready-to-eat seafoods that are raw, a higher satisfactory level may be applied (<10^2 CFU/g). The potentially hazardous level of *V. parahaemolyticus* relates to Kanagawa-positive strains. Source: FSANZ, 2001.

TABLE 2.3. Health Canada Interim Guideline for *Vibrio parahaemolyticus* in raw oyster shellstock (end product) intended for raw consumption

Test organism[a]	Product type	Number of Sample Units (n)	Acceptance number (c)	m[b] (MPN/g)	M	Criteria for action
Vibrio parahaemolyticus	Raw oyster shellstock	5	0	100	Not applicable	Reject if any unit is equal to or exceeds m (i.e., ≥100 MPN/g)

[a] Testing should be conducted according to MFLP-37 or any method published in the Health Canada's Compendium of Analytical Methods for *Vibrio* species in which the "application" section is appropriate for the intended purpose (e.g., MFLP-methods)

[b] The enumeration of the concentration of viable *V. parahaemolyticus* via the MPN method should be determined using a 5-tube multiple dilution technique to generate quantitative values that can be assessed for compliance with the interim microbiological guideline for *V. parahaemolyticus*.

Source: Personal Communication Ms. Denise MacGillivray, Director, Bureau of Microbial Hazards, Health Canada (July 2016)

TABLE 2.4. Microbiological standards for *Vibrio parahaemolyticus* in Japanese seafood

Food category	Microbiological standard
Seafood	<100 MPN/g
Seafood for raw consumption	Not detectable in 25 g
Ready-to-eat boiled seafood	Not detectable in 25 g

Notes: Ministry of Health, Labour, and Welfare, Japan, standards, as reported in Hara-Kudo, 2012.

TABLE 2.5. United States of America standards for *Vibrio* spp. in seafood products

Product	Requirement
Ready-to-eat fishery products (minimal cooking by consumer)	*Vibrio cholerae* – absence of toxigenic O1 or O139 or non-O1 and non-O139 in a 25-gram sample
Ready-to-eat fishery products (minimal cooking by consumer)	*Vibrio parahaemolyticus* – levels less than 1 x10^4 MPN/g (Kanagawa-positive or -negative)
Post-harvest processed clams, mussels, oysters, and whole and roe-on scallops, fresh or frozen, that make a label claim of "processed to reduce *Vibrio parahaemolyticus* to non-detectable levels"	*Vibrio parahaemolyticus* – levels less than 30/g (MPN)
Cooked ready-to-eat fishery products (minimal cooking by consumer)	*Vibrio vulnificus* – absence of organism(1)
Post-harvest processed clams, mussels, oysters, and whole and roe-on scallops, fresh or frozen, that make a label claim of "processed to reduce *Vibrio vulnificus* to non-detectable levels"	*Vibrio vulnificus* – levels less than 30/g (MPN)

Note: (1) Stated in document as "presence of organism": it was presumed that the presence would be unsatisfactory. No quantity of product is given. SOURCE: From FDA, 2011.

2.4 OUTBREAK INVESTIGATION

There are two main requirements for methods used for the microbiological testing of foods in outbreak investigations. The first is to determine whether a pathogen is present: this would imply that the method must be capable of detecting pathogenic strains. The second requirement is the ability to characterize any pathogen that is detected to determine that it is indistinguishable from that identified in clinical samples. The latter requirement may be satisfied by methods that yield viable cells that can be subject to further characterization, conventional PCR product that can be sequenced, multiple or multiplex real-time PCR reactions, or genotyping or DNA fingerprint typing that would show the presence of sufficient corresponding characteristics between the strains present in the food and clinical samples such that there is reasonable confidence that the strains may be the same.

It should be noted that only full genome sequencing can actually show strain identity. In principle, all other methods can only reliably show when strains are not identical. Even showing the presence of an identical strain in a foodstuff and a patient does not conclusively prove that the foodstuff was the source of infection for that patient unless there is supporting epidemiological evidence.

Enumeration in food may be of interest in outbreak investigations, but is often impractical given the time for detection of the outbreak, the manner of storing food, and the individual nature of each mollusc. Thus the same pathogen may be present as in the clinical samples, but in concentrations deemed too low to cause an infection. An additional complication is the variability in concentration across a batch of food. If it is possible, enumeration of pathogenic species and strains in foods involved in outbreaks provides valuable information for risk assessments.

Viability may also be significant in the testing of foods potentially related to outbreaks as processed (or even stored) foodstuffs may contain non-infectious pathogens that can be detected by sensitive molecular methods. However, many current molecular approaches for the detection of pathogenic bacteria rely on an enrichment procedure to yield sufficient material for detection and are not sufficiently sensitive in their own right. This enrichment step therefore provides the viability check.

3

Methods

A brief overview of the main available methods is provided. The detail presented on each method is limited, but intended to guide consideration in the selection of appropriate methods, and identification of potential performance characteristics

3.1 ENUMERATION BY DIRECT PLATING ON SELECTIVE AGAR

Surface spread plating of measured volumes of diluted shellfish homogenate on selective agar has been used as a simple means of enumerating *V. parahaemolyticus*. In general, single plates have been used at one or more dilutions of seafood. It provides next day results with relatively low effort and cost. Differential agar media are useful for providing presumptive levels of the target *Vibrio* spp. The theoretical limit of detection (LOD) is determined by the inoculum size, which is generally ~0.1 g (LOD = 10 colony forming units [CFU]/g) with bivalve mollusc tissues, but is generally less with other seafood with lower moisture content, such as crustacea and finfish (~0.01 g; LOD = 100 CFU/g). Increasing the agar content of the plating medium to 2-3% facilitates absorption of the inoculum and reduces colony spreading. It is essential to avoid a moist agar surface during incubation to prevent microbial lawns from forming. Direct plating alone can only provide presumptive results and confirmation is required.

The LOD for direct plating methods is typically less than for MPN methods because agar plating media are capable of absorbing only relatively small volumes

of sample inoculum (0.1 to 1.0 ml). Ignoring the recovery efficiency of a specific medium, the lowest number of colonies counted per spread or pour plate is usually 10, in order to ensure that the count obtained is statistically valid. If 0.5 ml of a 1:10 dilution of seafood has been inoculated, this yields a theoretical lower limit of quantification of 200 CFU per gram (10 colonies in 0.05 g). The actual limit of quantification (LOQ) may be greater than this. The theoretical LOD will be less than 200 CFU/g. However, quoting LODs on the basis of detecting one colony per plate is misleading as there is a high chance of not detecting any colonies at such low concentrations.

If the agar content is increased from the standard 1.5% to 2% or 2.5%, absorption can be enhanced and up to 1.0 ml of liquid samples such as seawater or seafood washes can be spread plated on a standard size Petri dish if placed in an incubator with the lid removed for 15–20 minutes before inverting the plate for incubation (Zimmerman *et al.*, 2007). Bivalve shellfish, especially oysters have high moisture content and 0.1-0.2 ml of inoculum is typically spread plated, resulting in a maximum LOD of 5–10 CFU/g (Kaysner and DePaola, 2004). Finfish and crustacean products are usually diluted 1:10 to facilitate homogenization and this dilution proportionally reduces the LOD (100/g) relative to products that do not require dilution, such as oysters.

It is necessary to use selective media, possibly combined with selective incubation temperatures, for direct *Vibrio* counts in order to suppress the growth of other bacteria that will be present in seafood samples. However, the presence of the selective agents and the use of raised incubation temperatures will also reduce recovery of the target species. This reduction will be more marked if the cells are stressed from refrigeration, PHP or other causes.

Although a large number of selective agars have been developed for the isolation of *V. parahaemolyticus*, the medium that has most often been used for such direct plate methods is TCBS. This medium is relatively inhibitory to some *Vibrio* spp. and is known to vary significantly in its inhibitory characteristic, both by manufacturer and by batch (Nicholls, Lee and Donovan, 1976). The percentage recovery on TCBS from four different manufacturers has been shown to vary from 48% to 62% for *V. parahaemolyticus* and from 54% to 67% for *V. vulnificus* (West *et al.*, 1982). Variability in counts, as determined by the $\log_{10}$-standard deviation, has also been shown to differ between the products from different manufacturers. A range of quality control approaches have been proposed to reduce the recovery and consistency problems with the medium (Taylor and Barrow, 1981; West *et al.*, 1982).

The differential features based on carbohydrate fermentation or production of chromogenic compounds are not reliable for accurate estimates of the target

Vibrio spp. as seafood has a rich and diverse microflora that can often produce false positive reactions. There is therefore the need to undertake confirmation procedures to determine the identification of presumptive colonies. There is also the problem on TCBS, and potentially other selective plating media, of detecting and identifying target *Vibrio* colonies amongst those of non-target bacteria (either other *Vibrio* spp. or non-*Vibrio* spp.). This problem is exacerbated if only a small proportion of the colonies of the species express known pathogenicity markers, as it is very unlikely that subculture of a reasonable number of colonies from present general purpose selective media for *Vibrio* spp. will be successful in detection of a colony exhibiting those markers.

The performance of commercially available chromogenic agars (see Section 4.2) for enumeration of *Vibrio* spp. in a direct plating format, in terms of LOD, LOQ and variability, does not seem to have been assessed.

Direct plating techniques are often regarded as giving continuous results, in comparison with the MPN assays, which clearly give discontinuous values (see Section 3.3). Direct plating actually gives discrete values, although if no dilutions are made, the step between the values will be increments of one. If a dilution is made prior to plating, then increments of single colonies on the plate will actually relate to greater increments in the original matrix (e.g. using a 1:10 dilution of oysters, the step between the concentrations in the original oysters will be 10 CFU. Direct plating is also often regarded as having low variability but this is often not evaluated. Reference has already been made to the change in variation with manufacturer of a single medium. The variability will also be influenced by a range of other factors, including the nature of the medium, the age and storage history of the medium, and the bacterial species (or even strain) being enumerated.

The use of post-harvest storage on ice (refrigeration) induces stress, and cold-stressed *Vibrio* cells enter into a viable-but-non-culturable (VBNC) state. The VBNC bacteria are unable to grow on selective agar plates (e.g. TCBS). A resuscitation step (holding in alkaline peptone water at room temperature for 60–90 minutes) can reverse the stress significantly.

3.2 CONVENTIONAL SELECTIVE ENRICHMENT

A wide variety of selective enrichment broths and selective plating media have been developed and used for the detection of both *V. parahaemolyticus* and *V. vulnificus* from seafoods (Donovan and van Netten, 1995; Sloan *et al.*, 1992). Conventional selective enrichment for *Vibrio* is usually based on the suspension of 25 g of seafood homogenate in 225 ml of either alkaline peptone water or salt colistin (or

polymyxin) broth. After incubation, an aliquot is plated onto one or more selective media. The nominal lower LOD is 1 bacterium in 25 g. However, the actual limit of detection will be greater than this, and will vary according to a number of factors.

The first consideration is the selective enrichment medium. If the complication of competing microflora is initially ignored (both non-target *Vibrio* spp. and other bacteria), the efficiency of the recovery of the target organism will depend on the nutrient content, the pH, the sodium chloride content, the incubation temperature and, where relevant, the type and concentration of inhibitory agent. Where competing microflora are present, the efficiency will depend on the relative effects of the above factors on the target and competing micro-organisms.

Traditionally, the salt content of alkaline peptone water (APW) for the isolation of *Vibrio* spp. in general was 1%, although up to 3% was used for the isolation of *V. parahaemolyticus* from seafood and 0% was used for the isolation of *V. cholerae* from seafood (Furniss, Lee and Donovan, 1978). The salt concentration given in ISO/TS 21872 (International Organization for Standardization/Technical Specification) parts 1 and 2 is 2% (ISO, 2007b). When the medium contains more than 0.5% NaCl, it is often termed alkaline salt peptone water (ASW). The salt concentration traditionally used in salt polymyxin broth and salt colistin broth is 3%.

The pH of the enrichment medium will also affect the growth rate of *Vibrio* spp. and the comparative selectivity against competing bacteria. ASW has been used at pH values ranging from 8.3 to 8.9. However, a pH of 8.6 is more common and that is given in ISO TS 21872 parts 1 and 2. Salt colistin and salt polymyxin broths are used at a pH of 7.4 as the antibiotics are inactivated at high pH.

The traditional peptone concentration in APW is 1% whereas the concentration given in the ISO technical specifications is 2%. It is not known how this affects the performance of the enrichment medium.

Selective enrichment broths were traditionally incubated at a temperature of 35–37°C for the isolation of pathogenic *Vibrio* spp. ISO TS 21872-1 gives an incubation temperature of 41.5°C for the selective enrichment of *V. parahaemolyticus* from most products, but 37°C for deep frozen, dried or salted products (ISO, 2007b). The difference is based on the premise that stressed cells in the latter products may be suppressed at the higher incubation temperature. The temperature given for the selective enrichment of other pathogenic *Vibrio* spp. in ISO TS 21872-2 is 37°C (ISO, 2007c).

In general, 25 g of seafood homogenate has been used in 225 ml of enrichment medium, as is conventional for other pathogens. This yields a 1:10 dilution.

However, it has been shown that a 1:100 dilution is more effective for the isolation of V. *cholerae* from *Crassostrea virginica* (DePaola and Hwang, 1995). This effect has not been explicitly studied for most combinations of *Vibrio* spp. and enrichment medium.

The incubated selective enrichment medium is then plated onto a selective agar medium for isolation of presumptive pathogenic *Vibrio* spp. Although a wide variety of selective agars have been described for this purpose, TCBS agar has been used by most general food microbiologists. In recent years a variety of chromogenic agars have been developed and these are becoming more widely used, primarily due to the drawbacks of TCBS. At least three companies market such media: bioMérieux (chromID™ *Vibrio* agar); CHROMagar (CHROMagar™ Vibrio) and Fluka Analytical [Sigma Aldrich] (HiCrome™ Vibrio Agar). These are generally accepted to be less inhibitory towards some of the pathogenic species and to provide better discrimination between species and from other microflora. However, apart from an initial study on CHROMagar™ Vibrio, there does not appear to have been a systematic evaluation of the performance of these media (Hara-Kudo *et al.*, 2001).

Chromogenic agars are considered to be less inhibitory to *Vibrio* cells than TCBS and to offer easier identification of the presumptive target pathogens on the agar. Hara-Kudo *et al.* (2001) isolated *V. parahaemolyticus* more frequently from naturally contaminated seafood samples using CHROMagar™ *Vibrio* medium than using TCBS following selective enrichment. They also found that a two-step enrichment procedure, using salt trypticase soy broth followed by salt polymyxin broth, was more effective than a one-stage procedure using chromogenic agars.

Traditionally, the conventional selective enrichment followed by selective plating has been followed by biochemical testing, increasingly using commercially produced galleries of tests. This may be followed by further characterization such as serotyping or detection of pathogenicity marker (e.g. TDH for *V. parahaemolyticus*). The maximum number of colonies subject to identification has generally been between five and ten per plate. When either a potential pathogen is present in much lower numbers than non-pathogenic *Vibrio* spp. (of the same or different species, or both), the likelihood of detecting the pathogen from such an approach is low. In addition, many commercial systems do not perform well in identifying *Vibrio* to the species level. PCR assays in the microbiological field were originally developed to confirm the identification of colonies from isolation media rather than for direct detection. Molecular methods may be used for confirmation as an alternative to conventional identification following conventional isolation. However, while the use of such molecular confirmation methods will markedly improve the specificity of identification of selected colonies, they will not overcome the other drawbacks of

these methods, such as the low likelihood of detecting the target bacteria amongst non-target bacteria unless very large numbers of colonies are tested.

3.3 CONVENTIONAL MOST PROBABLE NUMBER (MPN)

A wide variety of MPN formats have been used in food and water microbiology. MPN methods for *Vibrio* spp. are usually based on the conventional selective enrichment and selective plating techniques, with a further one in ten dilution of the initial tenfold (10^{-1}) dilution of the seafood homogenate in the selective enrichment medium being made and then proceeding as follows:

- 10 ml volumes of the 10^{-1} dilution to each of N tubes
- 1 ml volumes of the 10^{-1} dilution to each of N tubes
- 1 ml volumes of the 10^{-2} dilution to each of N tubes

Further dilutions may be made and inoculated into N tubes, depending on the anticipated concentration of the target organism in the sample. After inoculation, the tubes are incubated and subcultured, and identification undertaken as described in Section 4.2. Tables for 3-tube 3-dilution and 5-tube 3-dilution MPN calculation are available from many open sources such as FDA (2010).Alternatively, an MPN calculator available from ISO (http://standards.iso.org/iso/7218/) may be used as long as the likelihood of the MPN combination is taken into account before the resulting value is reported.

A major limitation of the MPN method is that the results are discontinuous, meaning that not all concentration values are possible. At most, there are only as many possible values as there are possible combinations of positive and negative tubes. In practice, there may be fewer values than the number of combinations as some different tube combinations may yield the same MPN value. The MPN approach may therefore be considered semi-quantitative. However, the number of possible outcomes increases rapidly with the number of tubes in the assay. The range over which that number of outcomes is spread depends on the number of dilutions and the dilution factor, with the spread being least with all tubes at the same dilution.

For the 3-tube 3-dilution format, the theoretical standard deviation (SD) of the $\log_{10}$-MPN result is approximately 0.32, provided that results are not at the extremes, i.e. when the tube combinations are from 2,0,0 to 3,3,1; and for the 5-tube 3-dilution format, the standard deviation of the $\log_{10}$-MPN result is approximately 0.24 provided that results are not at the extremes, i.e. tube combinations are from 3,0,0 to 5,5,2 (ISO, 2010). The actual variability will be greater than this. No specific information on the theoretical SD of the $\log_{10}$-MPN result is available for *Vibrio*

spp.; however, as an example, the standard deviation for *E. coli* in the 5 × 3 MPN format has been stated to be approximately 1.7 times the theoretical SD (Prior, Andrews and Russell, 2005).

For the 10-tube, single dilution format used in part of the United States PHP specification (FDA, 2013) (0.01 g per tube), the SD of the $\log_{10}$-MPN result is 0.25 for 3 positive tubes (MPN = 36/g) and is 0.31 for 2 positive tubes (MPN = 22/g). For the latter value, the 95% confidence intervals based on the theoretical variability are (5.4, 92).

Traditionally, n = 3 has been used for the enumeration of pathogens, including *Vibrio* spp. However, a 3-tube MPN series is generally considered to be only semi-quantitative and a 5-tube MPN series is considered to be the minimum that will achieve some form of quantitative outcome. Ignoring for the moment the other factors that complicate the use of these methods, it should be noted that for a 3-tube 4-dilution MPN application, the MPN values around a specified limit of 100/g are 93/g ($\log_{10}$SD = 0.32) and 120/g ($\log_{10}$SD =0.26). The 95% confidence intervals of the two values, based on the theoretical SDs, are (22, 400) and (36, 370). These confidence intervals obviously overlap significantly.

To illustrate this, assume a hypothetical scenario where the test is being applied to an end-product food to be eaten raw and a concentration of <2 CFU/g must be met to demonstrate compliance with a particular standard associated with food eaten raw. The method being applied is MPN. A test using a 3-tube 3-dilution MPN format with dilutions of 1, 0.1, 0.01 ml or gram and results of 2, 1, 1 respectively would lead to a conclusion of 2 MPN/ml, and has an associated 95% confidence interval of 0.7–6.0 MPN/ml. If the requirement to demonstrate <2 CFU/ml is a public health standard, the implication of the variation in the possible concentration should be considered in terms of public health risk. For purposes of illustration, a simplistic application of the dose-response model[1] can highlight the importance of the variability. Assuming an oyster mass of 15 g, application of the dose-response model for *V. parahaemolyticus* used by FAO/WHO (Beta-Poisson; maximum-likelihood estimation (MLE) parameters: alpha = 0.6, beta = 1.31×106) indicates that the risk at a concentration of 2 CFU/g is approximately 1 case per 70 thousand oysters (risk = 1.4×10^{-5}), increasing to approximately 1 case per 9 thousand oysters at 6 CFU/ml (risk = 1.1×10^{-4}) (FAO/WHO, 2011; FDA, 2005). The acceptability of both the level of risk and the likelihood of the risk at the upper end of the confidence limit (and higher) should be considered.

[1] The application is considered simplistic as it is a direct application of the MLE dose-response model for *V. parahaemolyticus* to consumption at the single oyster level. It does not consider the uncertainty in the dose-response model parameters nor the variability in the number of oysters consumed in a serving. It is intended only to illustrate the potential implications of the observed concentrations by MPN (and other methodologies by extension).

A practical problem with using a 5 × 3 MPN format is that, when there is no simple confirmation technique for the target organism (comparable with β-glucuronidase for *Escherichia* coli), several colonies from each subcultured tube will need to be taken from the selective agar culture for identification. If, for example, up to 5 presumptive colonies are taken from each subculture, this will lead to a maximum of 45 identifications for a 3 × 3 MPN format and a maximum of 75 identifications for a 5 × 3 MPN format.

One further consideration is that the ratio of homogenate to selective enrichment medium, discussed in Section 3.2, varies through the dilution series. This means that the selective efficiency of the medium will vary through the dilution series. There is anecdotal evidence that for *V. parahaemolyticus*, as for some other pathogens (e.g. *Campylobacter* spp.), there is a greater than expected incidence of more positive tubes at the higher dilutions than at lower dilutions. When this effect is marked, it will lead to MPN combinations that are considered invalid and which should be reported as void. However, it is possible that less obvious effects could be seen with some samples and this could lead to supposedly valid, but incorrect, tube combinations that would result in an incorrect MPN result being reported. It is sometimes the practice to discount the results of the first row of tubes, but only when these give an obviously anomalous low number of positive results compared with the second row. This is a subjective rather than objective approach, and may not necessarily lead to the correct acceptance/rejection of the first-row results.

3.4 DIRECT PLATING PROBE-HYBRIDIZATION

A robust approach was developed for the identification of *V. vulnificus* by using a spread plate agar medium with relatively low selectivity, followed by DNA probe colony hybridization (Wright *et al*., 1993). Carbohydrate fermentation can guide selection of appropriate dilutions for DNA colony hybridization, but carbohydrate fermentation creates large colonies, which can overcrowd plates and reduce the LOD. The method has been used for the detection and enumeration of *V. vulnificus* in oysters (DePaola *et al*., 1997). A similar approach has been described that uses an immunological method for colony identification following culture on *V. vulnificus* agar (VVA) medium (Senevirathne, Janes and Simonson, 2008).

Direct plating probe-hybridization methods have been used for the detection and enumeration of total and pathogenic *V. parahaemolyticus* (McCarthy *et al*., 1999; DePaola *et al*., 2000). Following growth on a non-selective agar, colonial material is transferred to membranes and subjected to probe-hybridization after bacterial lysis. Probes directed at both the *toxR* and tlh genes have been used for total *V. parahaemolyticus* (Suffredini, Cozzi and Croci, 2009).

Simple media such as T1N3 (1% tryptone, 3% NaCl and 2.5% agar) are often used for halophiles such as *V. parahaemolyticus* because the small colony size and limited selectivity based on elevated NaCl content permit examination of >500 colonies on a standard Petri dish. Samples collected from cold marine environments often contain high levels of *V. alginolyticus*, which can cause spreading colonies that mask other colonies and prevent accurate enumeration by DNA colony hybridization. Lawns can also be formed by the psychrotrophic background microflora of bivalve shellfish at retail markets. It is critical to obtain DNA probes that produce a strong signal with low background in order to reduce the subjectivity in interpreting results.

A draft international standard method is being developed based on the probe-hybridization method for potential application in an official control context. This uses 0.2 g of a 1:1 homogenate, and subsequent ten-fold dilutions, onto saline tryptone soy agar and probes for *toxR*, *tdh* and *trh* genes. The statistics of the counts will, in general, follow those of plate counts. The method is stated to have a nominal LOQ of 10 CFU/g. However, as discussed above, the nominal LOQ should be regarded as higher than this (100 CFU/g if ISO guidelines on enumeration are used) and the actual LOQ may be higher still. Appropriate positive and negative controls should be used in all colony hybridization experiments, as DNA probes tend to degrade with storage.

Some of the methods for direct plating probe-hybridization for the enumeration of *V. parahaemolyticus* use single lifts from separate plates for confirmation of the presence of total *V. parahaemolyticus,* and *tdh*-positive and *trh*-positive strains. Other methods use multiple lifts from single plates. The latter potentially gives a more direct comparison of the different counts, as with the former approach there is plate-to-plate variability to consider. However, as <5% of the *V. parahaemolyticus* colonies are likely to be positive for *tdh* or *trh*, the likelihood of having a valid number of countable colonies from all three lifts from the same plate is small.

The method generally performs well with determining concentrations of total *Vibrio* spp. at harvest and for determining growth rates during storage at ambient temperatures. *Vibrio* levels in samples collected from cold waters and PHP samples are usually below the method LOD, and also contain stressed cells that can be recovered more readily by enrichment. *V. alginolyticus* are often abundant in areas with high salinity and can produce spreading colonies that can mask colonies of the target species. The levels of pathogenic strains in seafood and environmental samples at harvest are usually below the LOD of direct plating and the high rate of non-detectable samples in not informative for determining levels of pathogenic populations.

3.5 CONVENTIONAL PCR

Several conventional PCR assays have been used successfully for the detection of *V. parahaemolyticus* by employing species-specific PCR primers designed on the nucleotide sequences of several targeted genes. The targeted genes have included *tlh, gyrB* and *toxR* (Taniguchi *et al*., 1986; Venkateswaran, Dohmoto and Harayama, 1998; Kim *et al*., 1999). Primers targeted at a fragment of pR72H have been used to detect *V. parahaemolyticus* in shellfish (Lee, Pan and Chen, 1995). The genes encoding major virulence determinants have also been used to characterize enteropathogenic *V. parahaemolyticus* strains (Tada *et al*., 1992). In addition, an assay has been developed to specifically detect *V. parahaemolyticus* strains belonging to the pandemic clone O3:K6 (Nasu *et al*., 2000).

Hill *et al.* (1991) described a conventional PCR method for the identification of *V. vulnificus* in artificially contaminated oysters: the reaction targeted a 519 bp sequence of the cytotoxin-haemolysin gene. For the PCR reaction, 1 ml of a 1:10 oyster homogenate in APW was extracted. Only approximately 10% of the final extract from the 1 ml was used in the PCR reaction. Inoculation of 100 viable cells into the homogenate required incubation at 35°C for 24 h for reliable detection. A nested PCR has been described for use in fish farm environments and this can detect between 12 and 120 cells in artificially seeded samples without the need for enrichment (Arias, Garay and Aznar, 1995). The method produced no bands with a wide range of other bacteria. The first-round PCR used an outer set of universal primers complementary to conserved regions of eubacterial 23S rRNA genes. The second-round PCR used primers complementary to a *V. vulnificus*-specific sequence located within the region amplified by the first set.

Multiplex PCR assays for simultaneous detection of total and enterotoxigenic *V. parahaemolyticus* have also been developed (Bej *et al*., 1999). However, these have been found to lack sensitivity for detection of *V. parahaemolyticus* in food samples. The combination of a multiplex PCR with a colorimetric microwell plate sandwich hybridization assay improved the sensitivity and permitted visualization of the amplified DNA (Lee, Panicker and Bej, 2003).

Panicker, Vickery and Bej (2004a) applied a multiplex PCR for the simultaneous detection of total and clinically significant *V. vulnificus*. This was determined to have a sensitivity of 10^3 cells per ml of culture or per gram of unenriched seeded-oyster homogenate. The sensitivity increased significantly with pre-enrichment.

Reverse transcription-PCR (RT-PCR), which targets short-lived messenger RNA molecules, has been used to assess the viability of bacteria. *V. parahaemolyticus*

RT-PCR, targeting transcripts of the *rpoS* and the *tdh2* genes, has been described to detect cDNA from VBNC cells (Coutard *et al.*, 2005).

3.6 REAL-TIME PCR

Real-time PCR combines the specificity of conventional PCR with the quantitative measurement of fluorescence for determining the presence of specific types of nucleic acids in environmental and food samples. This offers the potential for a more rapid and quantitative analysis for the detection, and potential enumeration, of pathogenic bacteria.

Real-time PCR methods for the enumeration of *Vibrio* spp. have most commonly used SYBR Green or TaqMan probes for the quantification of DNA amplification. Real-time PCR has been applied for quantitative detection of *Vibrio* spp. in seafood and seawater (Panicker, Myers and Bej, 2004b; Blackstone *et al.*, 2007; Takahashi *et al*., 2005; Ward and Bej, 2006). However, detection of these bacteria was possible only after enrichment of tissue homogenates or water. Nordstrom *et al.* (2007) described a multiplex real-time PCR method for the detection of total and pathogenic *V. parahaemolyticus* in oysters. The target genes were *tlh, tdh* and *trh*. The method incorporated an internal amplification control to detect inhibition by oyster matrix. The molecular assays were applied after enrichment of oyster homogenate in APW in a 3 × 3 MPN format. The method was described as giving reliable detection of 4 CFU per reaction of *tdh*-positive or *trh*-positive cells in a background of 7×10^4 per reaction of non-pathogenic *V. parahaemolyticus* cells. The method yielded more positive tubes than did conventional culture and was always positive when a tube was culture positive.

Panicker, Myers and Bej (2004b) reported the development of a SYBR Green-based real-time PCR assay alongside the conventional PCR method described above. As with the conventional PCR method, the real-time PCR method was determined as having a sensitivity of 10^3 cells per ml of culture or per gram of unenriched seeded oyster homogenate, which increased significantly with pre-enrichment.

Cai *et al.* (2006) described detection of *V. parahaemolyticus* directly from naturally contaminated seafood. The number of cells detected varied from 4.3×10^3 to 4.7×10^5 per ml of homogenate. Real-time PCR in such a format therefore lacks sensitivity for direct enumeration and hence is usually performed by using the method as a detection method on enrichment cultures made in APW. Improvements in extraction procedures may overcome, or at least reduce, problems. However, the performance, in terms of LOD, would need to be improved at least one-thousand-

fold to enable assessment of compliance with current guidelines. In general, if the equivalent of 100 µl of bivalve homogenate is used in such an assay, then, assuming no dilution at the homogenate stage and total recovery, the LOD will be at best 10/g and the LOQ will be higher than this.

Campbell and Wright (2003) developed a TaqMan real-time PCR assay for the detection and enumeration of *V. vulnificus* in oyster homogenates. The method was determined to be specific for *V. vulnificus*, to have a detection limit in oyster homogenates of the order of 100 cells per gram and to show a linear response with increasing cell numbers above this limit.

Commercial kits are available for the application of real-time PCR to the detection and identification of pathogenic *Vibrio* spp. These kits are currently relatively expensive, but should have the benefit of reducing variability, especially in laboratories that do not have significant expertise in preparing and running such reactions using in-house reagents.

3.7 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY

The Loop-Mediated Isothermal Amplification Assay (LAMP) has been developed for the detection of *V. parahaemolyticus* and *V. vulnificus* and other *Vibrio* spp. (Nemoto *et al*., 2008; Yamazaki *et al*., 2010). LAMP involves gene amplification at a constant temperature with detection based on turbidity analysis, with the amount of turbidity being proportional to the amount of synthesized DNA. Amplification can also be detected using fluorescent dyes, as in real-time PCR. Fluorescence detection may be visual (yielding a presence/absence result) or by fluorimeter (in which case it is currently regarded as semi-quantitative). LAMP is said to be faster, easier to perform and more specific than conventional PCR assays, while not requiring expensive equipment (when the turbidity analysis approach is applied). The reaction may be performed on growth on agar plates or APW enrichment cultures (e.g. after overnight incubation). The *V. parahaemolyticus* assays are based on the detection of *tdh, trh1* and *trh2* genes and may be undertaken in multiplex format, yielding a positive turbidimetric result if any pathogenicity factor is present. The *V. vulnificus* assay is species-specific. The assays detect in the region of 1 cell per reaction tube, which corresponds to approximately 10 cells per ml of APW: the actual LOD varied between the different targets included in the multiplex system. It should be noted that LAMP assays are usually run in simplex format as the turbidimetric detection system cannot distinguish between the reactions with different targets and there is usually interest in determining which pathogenicity factor is present in a strain.

Enumeration can be performed using LAMP by employing the initial APW selective enrichment in an MPN format. A sensitive and specific approach has been reported that combines a conventional MPN procedure, the LAMP method and a newly developed immunomagnetic separation (IMS) targeting virulent strains (M. Nishibuchi, *pers. comm*.). The range of K antigens targeted in the IMS has been established based on the analysis of available clinical strains: this method may therefore be helpful for screening clinically important *V. parahaemolyticus* strains. The conventional IMS protocol has been replaced by a new IMS system using a "Pick Pen kit". Evaluations undertaken to date indicate that the MPN-IMS-LAMP method is promising. Further evaluation is intended in a tropical environment where the concentration of virulent strains is consistently high. Recent findings on the discrepancy in results for environmental strains obtained by conventional PCR and LAMP methods suggests a distribution of the trh-positive subpopulation in the environment that is distinct from the trh-positive clinical population.

3.8 COMPARISON OF METHODS

Blanco-Abad *et al*. (2009) evaluated a combination of conventional and molecular protocols previously described for *V. parahaemolyticus*. A total of 259 samples of zooplankton (103), mussels (48) and seawater (108) were examined by a presence/absence method and 118 samples of zooplankton (70) and mussels (48) were analysed by a MPN method. All samples were processed by a two-step enrichment procedure, firstly with APW broth and then with salt polymyxin broth (SPB) as a selective secondary broth. Detection of *V. parahaemolyticus* was by PCR and by plate culture on TCBS and CHROMagar Vibrio, after sample enrichment in APW and SPB. The PCR used primers for *toxR, tdh* and *trh*. Three to five colonies were subcultured from each selective agar plate for further identification. With the presence/absence method, *V. parahaemolyticus* was detected in 23.6% of samples by PCR, whereas only 11.2% of samples were positive with the plate culture method. With the MPN method, *V. parahaemolyticus* was detected in 54.2% and 27.1% of the samples by PCR and plate culture respectively. The MPN format therefore produced a markedly higher number of positive results than did presence/absence for both conventional isolation and PCR. This may have been due to the multiple enrichments used for each sample in the MPN format. No significant differences were observed between the use of a single (APW) or two-step enrichment (APW+SPB) by direct-PCR with presence/absence or MPN, although a significantly higher proportion of samples were positive for *V. parahaemolyticus* detected by plate culture in both protocols with the two-step enrichment procedure. Overall, PCR after sample enrichment in APW broth was the most successful method for detection of *V. parahaemolyticus* with both the presence/absence procedure and MPN enumeration. Better detection was obtained with MPN than with the

presence/absence procedure. At the same time, the plate culture procedure showed better results with the two-step enrichment protocol with CHROMagar Vibrio as the selective agar.

Kim *et al*. (2008) compared the performance of conventional, nested and real-time PCR assays for the detection of *V. vulnificus* in clinical blood samples using the *toxR* gene. Conventional and nested PCR gave sensitivities of 45 and 86% in comparison with conventional blood culture. Real-time PCR was determined to have a sensitivity and specificity of 100%. The LODs for the three methods, determined using cells from bacterial cultures, were: conventional PCR 5×10^3/µl; nested PCR 5×10^2/µl; and real-time PCR 5×10^0/µl.

In a ring trial to compare the efficacy of identification methods, the European Union Reference Laboratory circulated swabs inoculated with strains of *V. vulnificus, V. parahaemolyticus, V. cholerae, V. mimicus* and *V. fluvialis* (EURL, 2011). The *V. parahaemolyticus* strain was positive for both *tdh* and *trh*. A protocol was provided for the isolation of *Vibrio* spp. by conventional double enrichment at two different temperatures (37 and 41.5°C) followed by selective plating on TCBS and a medium of the laboratory's own choice. Laboratories were asked to confirm colonies using conventional biochemical methods and PCR. For the swab containing *V. vulnificus*, 19 laboratories (83%) reported the presence of the species at 37°C and 20 (87%) reported its presence at 41.5°C, when using biochemical identification. Sixteen laboratories (94%) using PCR for identification reported the presence of *V. vulnificus* using the *vvh* primer set. For the swab containing *V. parahaemolyticus*, nineteen (83%) laboratories reported the presence of *V. parahaemolyticus* at 37°C or 41.5°C, or both, using biochemical identification. Three laboratories incorrectly reported the presence of *V. mimicus* and/or *V. vulnificus* in this sample. Of the 17 laboratories reporting results for PCR, 16 laboratories reported the presence of a positive toxR reaction and therefore identified the presence of *V. parahaemolyticus*. For the detection of the *tdh* pathogenic marker alone, one laboratory reported the presence of *tdh* using a VP21/VP22 primer set, whilst all 17 laboratories performing PCR using the L-*tdh*/R-*tdh* primer set detected the *tdh* pathogenic marker. For the detection of the *trh* pathogenic marker, 12 laboratories (71%) detected the presence of trh using the S1/S2 primer set, whilst 15 laboratories (88%) detected the presence of trh using the L-*trh*/R-*trh* primer set. PCR therefore performed better than biochemical identification for both species. Identification to species level by PCR was not absolute, even with the pure cultures provided, and there were also some problems with the detection of the *V. parahaemolyticus* pathogenic markers.

Jones *et al*. (2012) compared two different real-time PCR methods and LAMP for the detection of total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in

MPN enrichments of oysters and fish intestines using APW. The overall conclusions of the comparisons were that the various methods were generally comparable when appropriate preparation methods were used prior to assay. Some problems of decreased sensitivity were seen when real-time PCR was undertaken in a multiplex format.

4

Considerations with regard to method performance characteristics for different end uses

4.1 INTRODUCTION

The purpose of this section is to provide general guidance on selection of methods fit for purpose to determine pathogenic *Vibrio* spp. levels for risk assessment. The CCFH guidance adopted in 2009 recognized that the risk-based tool used in the United States of America provided a useful approach that followed the risk assessment framework, but needed local data, including levels of total and pathogenic *Vibrio* spp., to be applicable to other regions. Data needs included the relationship between *Vibrio* levels and harvest water temperature, salinity and possibly other ecological factors; growth rates in local shellfish; and levels at various points along the food chain from harvest to consumption in order to determine the impact of industry and consumer practices on levels, and ultimately human health risk. The method performance requirements vary for each of these purposes, and especially between determining total levels of the species and pathogenic subpopulations. Direct plating and sample enrichment in an MPN format are the two major approaches to determining *Vibrio* levels.

The following are some parameters that need to be taken into account when selecting a method in relation to the end uses presented in Chapter 2.

- Does the organism×end-use combination indicate the use of a presence/absence or quantitative method?
- If presence/absence, what volume of product should the method challenge?
- Should assessment of the method be based on the nominal or actual LOD?
- If quantitative, what is the LOQ necessary to meet the lower limit in the standard or guidelines?
- What level of variability is acceptable?
- If an MPN method, what breadth of interval between levels is considered acceptable?
- Is it necessary to demonstrate viability?
- If the format of a molecular method allows demonstration of viability (e.g. by the use of a conventional enrichment stage), will the method potentially give false-positive results with PHP processed product that originally contained high concentrations?
- Is it necessary to determine the presence or concentration of pathogenic strains or is the presence or concentration of all strains of the species adequate?
- Can the guideline or standard be adjusted to allow the presence or concentration of all strains of the species?
- Is it necessary to be able to type the strain(s) in some way?

To achieve a specific aim (e.g. comparing strains isolated from both food and clinical samples), the method of achieving the aim may vary according to the methodology. Where different methods use the same basic format, e.g. MPN of a specified format with conventional isolation and identification, conventional PCR detection, real-time PCR detection and LAMP detection, an approach needs to be developed to determine how to compare performance. Overall performance will be an outcome of the method format, the conventional enrichment step and the detection step. Certain stages in the method may be limiting in terms of performance.

4.2 PERFORMANCE CHARACTERISTICS OF METHODS

The methods selected to detect and enumerate pathogenic *Vibrio* species in bivalve molluscs should be carefully considered so that they are appropriate to the end use, and are also within the capabilities of laboratory(ies) required to perform tests. For example, availability and costs of reagents, as well as level of technical proficiency, may influence the decision to use culture-based versus molecular-based test methods.

The process to select test methodologies should also consider:

- the desired level of test sensitivity (e.g. sample size) and test frequency;
- whether a presence/absence or a quantitative test is more appropriate;
- whether detection of subpopulations (e.g. virulence markers) is necessary; and
- whether typing (e.g. serotype) of pathogenic strains is required.

The following sections provide background information about various test characteristics.

4.2.1 Presence/absence versus quantitative tests

There are a number of reasons to select a presence/absence or quantitative approach. The intended use of the test is a key factor, and applications are considered below.

Presence/absence

- Monitoring or surveillance of a harvest area.
- Baseline information if looking at an area with no previous information.
- To investigate seasonality in prevalence and identify "risk periods" where further sampling may be undertaken.
- To demonstrate compliance with standards that control concentration (for example "<100 MPN/g"), depending upon the expected concentrations.
- To demonstrate compliance with zero-tolerance plans, such as "absence in 25 g".

Quantitative approaches

- Monitoring of harvesting areas for detection of high levels during risk periods.
- To demonstrate compliance with standards that control concentrations (for example, where a marginal level of concentration is acceptable).
- To help understand the relationship between dose and illness in outbreak investigations.
- To determine ratios of pathogenic to non-pathogenic *V. parahaemolyticus.*
- To determine growth or survival rates.

4.2.2 Test volume

The test-portion size in a presence/absence analysis depends on the purpose of the analysis and the likely influence on regulatory decisions and risk management. In general, larger test portions are more appropriate when the analyte is expected to be at low prevalence or abundance, or presents a severe risk. Typically, presence/absence analysis for pathogens is applied when "zero tolerance" has been established. The normal test portion size for food analysis is 25 g for presence/absence testing. Among *Vibrio* spp., the policy of zero tolerance usually only applies to

toxigenic *V. cholerae*. In products that have an allowable tolerance, such as the guidelines or specifications given for *V. parahaemolyticus* in Chapter 2, an MPN format test may be applied to obtain the necessary precision and confidence that levels do not exceed the established tolerance, based on the required risk management criteria. In cases where resources are limited, a presence/absence approach may be applied to obtain rough estimates of prevalence or abundance if multiple dilutions are analysed.

Vibrio spp. levels vary greatly even within a product lot harvested at the same time and location. A presence/absence approach may be more informative for determining spatial or temporal occurrence, or both, at a certain threshold if a relatively large number of samples are tested on a single tube or dilution basis rather than testing a few samples with more precise MPN analysis employing multiple tubes and dilutions. The appropriate portion size or range of sample portion sizes for multiple dilutions would depend on some expectation or knowledge of the distribution of the target species or subpopulations. At one extreme would be analysis of a product harvested from cold waters (<15°C) for pathogenic *V. parahaemolyticus* using *tdh* or *trh* as markers for pathogenicity. In order to avoid a high proportion of non-detectable results, it may be necessary to analyse relatively large portions (e.g. >100 g). In an attempt to gather data on *V. parahaemolyticus* levels in Chile when no *V. parahaemolyticus* cases were being reported, a 3-tube 3-dilution MPN was used with 10 g amounts in the first dilution (DePaola *et al.*, 2010a). In contrast, bivalve molluscs from temperate and tropical countries may have *Vibrio* spp. levels >100 000 CFU/g and practically all samples would be positive unless a very small sample portion were analysed.

4.2.3 Culture-based versus non-culture-based detection

In general, direct detection methods have not been successfully applied to seafood samples to detect target *Vibrio* spp., for reasons that include:

- the *Vibrio* spp. may be present below test sensitivity limits (for both culture-based and DNA-based tests);
- selective agars are inhibitory to target bacteria, although usually to a lesser extent than non-target bacteria; or
- shellfish matrices may inhibit polymerase enzymes or growth of target species.

Consequently, enrichment is currently recommended to elevate levels of viable *Vibrio* spp., as well as to dilute-out matrix effects.

Direct application of non-culture-based tests is also not recommended for food samples that are treated by PHP because large quantities of DNA may be released from PHP-treated food (including partially or fully cooked products). Consequent-

ly, if direct molecular methods (such as a conventional PCR or LAMP method) are used to validate PHP, viability should be demonstrated to minimize detection of dead target cells.

This does not preclude the future development of direct detection methods that have the required performance characteristics.

4.2.4 Detection of pathogenic strains

Currently, there are well recognized virulence markers for *V. parahaemolyticus* and *V. cholerae,* but not for *V. vulnificus*. Spatial and temporal variations in the proportion of pathogenic strains within a *Vibrio* spp. are to be expected. For risk managers, it may be necessary to determine the presence or concentration of both pathogenic strains and total strains of a species to detect trends in risk for significant harvest locations and seasons.

The ratio of virulent to total cells of a potentially pathogenic *Vibrio* spp. may vary significantly by season, year and region. Therefore, a specific testing frequency is necessary so that the determined ratio is meaningful. In addition, there are inadequate data demonstrating the virulent: total ratio in foods associated with illness.

The concentration of virulent strains in shellfish samples is generally expected to be low and near the test detection limit. A sensitive and simple method for virulent strain detection must therefore be used. Real-time PCR is easy to run if the system is set up and well maintained, but the price of the equipment is beyond the budget of many laboratories, and the cumbersome maintenance requirement of the equipment is problematic for many developing countries. In this case, laboratories could use methodology to detect Kanagawa-positive *V. parahaemolyticus* strains. However, the Kanagawa test is technically challenging, and other methods are encouraged; use of the test should be limited to experienced staff in laboratories familiar with the technique. Pathogenic strains of *V. parahaemolyticus* are supposed to carry the *tdh* gene encoding for TDH or the *trh* gene encoding TRH. There are no phenotypic methods for detection of TRH-positive strains. Immunological tests like ELISA have been used to detect strains producing TDH and TRH in both clinical and seafood samples, but the performance of this method depends on the level of expression of virulence factors.

In order to avoid incriminating food product that does not contain pathogenic strains, detection of the pathogenicity factors of *V. parahaemolyticus* and *V. cholerae* is necessary when outbreak investigations are undertaken. The pathogenicity factor(s) would be expected to be present in both the seafood samples and the clinical specimens if the seafood were the vehicle for the outbreak.

For the evaluation of PHP, it is not necessary to test for pathogenic strains unless data show that pathogenic strains are more resistant to the treatment process than non-pathogenic strains. In the absence of such evidence, a test for all strains of the species should be adequate.

4.2.5 Strain typing

There may be reasons to apply typing at various points in the production chain or in outbreak situations. Methods currently used for typing *V. parahaemolyticus* strains include serotyping and genotyping, with the latter including the profile of reactions obtained with different PCR primer sets (including those for pathogenicity factors) and DNA fingerprinting (see Table 4.1), although whole-genome sequencing is increasingly being applied for strain differentiation.

There is a wide range of DNA fingerprinting methods that might be used, including pulse-field gel electrophoresis (PFGE), random-amplified polymorphic DNA (RAPD) analysis, and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), and these have all been applied to the discrimination of *V. parahaemolyti-*

TABLE 4.1. Typing methods

Typing	Typical purpose of use	Stage when useful			
		Harvest	PHP	End-product	Outbreaks
Serotyping	Epidemiological analysis	Yes (limited value and primarily pathogenic and pandemic strains of *V. parahaemolyticus*)	No	No	Yes (limited value, useful for monitoring pandemic strains of increased virulence)
Genotyping (PCR primer reaction profile)	Differentiation between virulent and avirulent strains. Detection of a particular population (e.g. a pandemic clone)	Yes – in the context of research and risk assessment, but not for routine monitoring	Maybe	Maybe	Yes
Genotyping (DNA fingerprint typing)	Epidemiological analysis; Phylogenetic analysis	Yes	Maybe	Maybe	Yes

cus and *V. vulnificus* strains (Lee and Rangdale, 2011). They differ in their level of discrimination, but this in itself varies between species and even between different groups of isolates. The level of discrimination required may vary depending on the specific application. The user therefore should consider the published data in conjunction with an understanding of the intended use in deciding which method to apply.

Bacterial cell components responsible for serotyping (K antigen) and phage typing (receptor on the cell surface) may be denatured by treatments in the PHP and end-product stages. Genotyping and DNA fingerprinting target DNA thus are applicable to the bacterial cells containing DNA or to the DNA released from the cells. In general, all current typing methods tend to be applied to pure cultures isolated from relevant samples.

4.2.6 Characteristics of method performance

By considering the end use of the test, a set of factors emerge that need to be addressed. These include, but are not limited to:

- Is the method qualitative (i.e. presence/absence), semi-quantitative or quantitative?
- Is the method able to recover stressed cells?
- Are the detection level (LOD) and quantification level (LOQ) sufficient for the intended application?

Each of the available methodologies can be considered in terms of these factors. Depending upon the end use of the method, a methodology-performance matrix can be developed which, when used in conjunction with knowledge of the requirements of the end use of the test, will facilitate the selection of the appropriate methodology. For example, see Table 4.2, which lists some of the factors associated with methodologies currently available.

Methods incorporating an enrichment step are likely to be more effective than direct plating for data collection when target *Vibrio* populations are low or problematic background microflora are prevalent. Enrichment of large sample portion sizes is possible and such an approach can be applied to determine presence/absence or in an MPN format to obtain quantitative estimates. Traditionally, enrichment has been followed by streaking on differential and selective agar media to aid in selection of presumptive colonies for further characterization for species identification, serology and virulence attributes. The effectiveness of this approach depends on the selective and differential properties of the isolation media; levels or ratios of bacteria with similar colony morphology to the target organism; the number of colonies examined; and the performance characteristics of the con-

TABLE 4.2. Method performance criteria

Performance criterion	Direct Plating		Enrichment presence/absence			MPN		
	No hybridization	Hybridization (non-selective)	Selective plating		No selective plating	Plate		Broth only
			Biochemical	Molecular	Molecular	Biochemical	Molecular	Molecular
Quantitative?	No (presumptive)	Yes	No	No	No	Yes (presumptive)	Yes	Yes
Recovery stressed cells?	depends on medium selectivity	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LOQ and LOD	Depends on format and inoculum size							
Sensitivity/ Specificity	Low/ low	Low/ high	High/ moderate	High/ high	High/ high	High/ moderate	High/ high	High/ high
Variability	low	low	low (but often not estimated)			May be higher depending on MPN format		
Distinguish pathogenicity	No	Yes	No	Yes	Yes	No	Yes	Yes
Possible strain characterization, sub-typing?	Yes (colonies obtained)		Yes (colonies obtained)		No	Yes (colonies obtained)		No
Time to results (approx. estimates)	18–24 h[3]	2–3 days	3–4 days (presumptive)	3–4 days	1–2 days	5–10 days	4–5 days	1–2 days
Availability of Supplies[1]	high	high	high	medium-high	high	high	high	high
Skill level[2]	low (except for specialist medium)	medium	low	medium	high[4]	medium	high	high
Cost	low	medium-high	medium	medium-high	medium-high	highest when multiple colonies per tube confirmed	very high	high
Test volume	limited to 0.1–0.2 g per plate		25 g composite sample is frequently used (replicates advised)			25 g composite sample is frequently used (replicates advised)		

Notes: (1) Availability refers to commercial availability; however, other factors may limit facility of implementation. (2) Even in cases where skill level is not high, training materials are readily available. (3) Assumes that confirmation is not performed (i.e. biochemical). (4) The use of appropriate commercially available kits reduces the skill level required.

firmatory assays. While this approach may be fairly effective for estimating total species levels, it usually results in gross underestimation of levels of pathogenic subpopulations as they typically represent a small fraction of the total species population and are not distinguishable by colony morphology.

In recent years, real-time PCR has been increasingly employed for direct examination of enrichment broths for DNA sequences indicative of *Vibrio* spp. and virulence determinants or markers. This approach produces next-day results and greatly improves sensitivity and efficiency as numerous cells can be tested for single or multiple DNA target sequences simultaneously in a single PCR. An even more recent DNA amplification method, loop-mediated amplification (LAMP), can be applied in a similar manner to real-time PCR for direct examination of enrichment broths, with simple template preparation protocols. More extensive sample preparation is required for conventional PCR in order to remove matrix components that are more inhibitory to conventional PCR relative to real-time PCR and LAMP.

There are complexities associated with these factors, which must not be ignored. For quantitative methods, the reliability and variability of the methodology must be considered, in particular when demonstrating compliance with a quantitative standard (such as <100 MPN/g). For example, MPN methods have an associated confidence interval indicating the possible concentrations in the sample. This is a distribution of concentrations (and their associated likelihood) that could lead to the observed result. A 3-tube MPN with dilutions of 1, 0.1, 0.01 ml or gram and results of 0, 1, 0 estimates an MPN of 0.31/ml (or gram) with a 95% confidence limit of 0.04 – 2.3 MPN/ml (or gram). The confidence interval and other statistics, such as the standard deviation, associated with the MPN should be considered in the context of the end use. If the method is being applied to monitor harvest conditions for research purposes, where general trends in concentration are of interest, then the expected concentration (i.e. the MPN itself) may provide sufficient information and any inherent variation may be acceptable. However, if the method is being applied to demonstrate compliance with a standard that has been established to protect public health, the variation in implied concentration may be more critical in determining whether the method is appropriate.

4.3 REQUIREMENTS OF END USES

4.3.1 Harvest area monitoring and surveillance

Data from previous environmental surveys of V. *parahaemolyticus* and *V. vulnificus*, or from outbreak investigations, are useful for identifying regional and seasonal variations, and determining threshold conditions for acceptable risk. A presence/

absence method could be used to determine whether pathogenic *Vibrio* spp. were present at a specific prevalence threshold. However, a quantitative method may be more appropriate to determine harvest levels of *V. parahaemolyticus* (total and pathogenic) and *V. vulnificus* in bivalves intended for sale during the at-risk season.

The choice of method for harvesting-area monitoring will depend on whether it is required to show that contamination levels are above or below a standard. This is not the case for risk assessment purposes, but will be the case for monitoring undertaken for control plans. However, even for risk assessment purposes, an appreciation of the performance of the method is necessary for proper awareness of the significance of the results.

With regard to monitoring under a control plan, if a harvest standard used is 100 MPN/g, the method should have a LOQ of the target organism that is two $\log_{10}$SDs below the standard or guideline, in order to be able to reliably detect a result that is less than that value. If an actual (rather than theoretical) $\log_{10}$SD of 0.3 is assumed, for example, this would yield an LOQ of 25 per gram. This limit would need to be achieved in the presence of higher concentrations of non-target bacteria (e.g. non-pathogenic *Vibrio* spp.) commonly found in the local environment. The direct plate count has been shown to have a nominal lower limit of quantification of <200/g and thus cannot achieve the required performance needed to show compliance with the standard itself, let alone a lower value intended to ensure reliability of compliance. If an MPN approach was to be used, whatever the final detection method (conventional or molecular), the method should have the following characteristics: it should meet the LOQ recommendation given above, and the number of MPN values should be adequate to properly bracket the standard or guideline of interest. However, it is important to appreciate that the sampling plan is as important as the laboratory method in determining the success of a control plan.

Genotyping and serotyping are most useful for outbreak investigations and studies characterizing local *Vibrio* populations, but provide little value for routine monitoring.

4.3.2 Post-harvest process monitoring

Whether a presence/absence or a quantitative method is required depends on the parameters that are being varied, the concentrations expected and performance of the process in relation to the expected concentrations. For example:

- A single dilution MPN or a presence/absence test may be sufficient to verify a lethality process if expected post-processing concentrations are low.
- Quantitative methods are more appropriate if expected concentrations are high or more variable (such as determining initial concentrations).

- Specific examples of this include:
    - A single-dilution MPN is effective for validation of cooking or other PHP.
    - A presence/absence test would be more efficient for process verification.

In the United States of America, where PHP is most often applied, the approach to determine the effectiveness of reducing *Vibrio* spp. levels has been to examine natural *Vibrio* spp. populations in oysters. Densities of 10 000 to 100 000/g of either total *V. parahaemolyticus* or *V. vulnificus* can usually be obtained by storage of oysters at ambient summer temperatures for up to 24 h. Typically a diverse *Vibrio* population develops with a range of resistance attributes to various processes and provides a robust challenge for PHP validation. However, pathogenic *Vibrio* populations may be low or absent in the natural *Vibrio* flora and this approach provides little information about the effectiveness of a PHP for reducing specific pathogenic strains. It is more efficient and informative to use pure cultures of pathogenic strains to determine their sensitivity to the various processing approaches such as freezing, heat-treatment and high hydrostatic pressure.

Testing following PHP may be required to demonstrate a certain level of contamination, such as <30 MPN/g. Given such a standard, a presence/absence test may be sufficient (where the sample size and number of samples have been determined to provide a sufficient level of confidence of detecting concentrations >30 MPN/g). Whether the method is, presence/absence-based or concentration-based, at this point in the supply chain cells of *V. parahaemolyticus* may be stressed, and therefore any method applied should have the potential to recover stressed cells.

4.3.3 End-product monitoring

Concentrations of virulent strains in shellfish are generally low and close to the detection or quantification limit of most quantitative methods. In the absence of outbreaks and outside of the at-risk period (if identified), a presence/absence method for pathogenic strains may be sufficient. Otherwise, a quantitative method would be needed in order to evaluate the concentrations of pathogenic strains versus total *Vibrio* spp. (ratio) and to compare with those estimated in shellfish harvested from the same growing areas. In general, for *V. parahaemolyticus*, ratios of total to pathogenic strains will be determined for risk assessment purposes, but routine end-product monitoring will be based on total *V. parahaemolyticus*.

For cooked products, it is recommended that testing for total strains is adequate. The *Codex Guidelines on the Application of General Principles of Food Hygiene for the Control of Pathogenic Vibrio species in Seafood* (CAC/GL 73-2010) recognizes that two different control measures can be established for molluscan shellfish to be

consumed (a) raw or alive and (b) partially or thoroughly cooked. In addition, if spatial variation in the ratio of virulent strains to total strains of *V. parahaemolyticus* is reflected in a standard, they may not remain the same.

End-product samples may be based upon a test that demonstrates 'absence in' a specified mass of product. For example, where standards have not been defined, absence in 25 g is often assumed to be an appropriate standard. For such a standard a presence/absence test is sufficient to demonstrate compliance with the standard. As previously identified, the actual LOD of a presence/absence test is usually greater than the theoretical LOD: this has not usually been taken into account when defining standards or the methods to be used for judging compliance with those standards.

Other standards may require a concentration-based methodology. For example, the Canadian interim guideline for *V. parahaemolyticus* in raw oyster shellstock (end product) intended for raw consumption is a concentration-based plan with an acceptance level of $< 10^2$ MPN/g.

Direct plating has been reported to be effective for enumeration of total levels of *V. parahaemolyticus* and *V. vulnificus* in freshly harvested oysters (Cook, Bowers and DePaola, 2002; DePaola *et al*., 2003). However, the same methods were found to be ineffective for enumeration of these pathogens in surveys of United States raw oysters at retail level (Cook *et al*., 2002; DePaola *et al*., 2010b). Interfering microflora crowded and often produced lawns on plates inoculated at appropriate dilutions for enumeration of these pathogens. Probable proliferation of *Pseudomonas* spp. and *Aeromonas* spp. occurred during post-harvest storage of up to two weeks at refrigeration temperatures that support the growth of these psychrotrophs but not *Vibrio* spp. Cold stress further reduces the culturability of *Vibrio* cells, especially on agar media.

The examples of quantitative guidelines given for *V. parahaemolyticus* in Chapter 2 have lower limits of 3, 20, 30 and 100 CFU/g. The last-named value is the same as that considered above for harvesting areas and will not be considered further in this section.

For the guideline values of 3 and 20 CFU/g, the approach taken in Chapter 4 would lead to LOQs of <1 and 5 CFU/g, respectively. The direct plating approach and the direct plating probe-hybridization methods would not be able to achieve such an LOQ. The 5 × 3 MPN test has a nominal lower limit of quantification of 0.2 per gram and the 3 × 3 MPN test has a nominal limit of 0.3 per gram. The actual LOQ of the 5 × 3 MPN for *E. coli* using ISO TS 16649-3 (ISO, 2004) has been shown to be of the order of 1 MPN per gram: if the same applies for *V. parahaemolyticus*, the

example MPN format would be adequate for use with the 20 CFU/g value but not the 3 CFU/g one.

The upper, hazardous, levels given in the guidelines are in the region of 10^3–10^4 CFU/g. These can be assessed by direct plating, direct plating probe-hybridization and MPN methods (conventional or combined with PCR) using additional dilutions. However, conventional direct plating and conventional MPN approaches suffer from limitations with regard to identification, and this problem is exacerbated if only enumeration of pathogenic strains is to be undertaken to determine a hazardous product, as in the Australia-New Zealand guidelines.

Direct application of non-culture-based tests is not recommended, and is especially problematic, for PHP-treated food samples (e.g. partially or fully cooked products; high pressure treated). Molecular-based methods should be applied after sample enrichment for either presence/absence or quantification.

4.3.4 Outbreak investigation

If pathogenic *Vibrio* spp. are found in patient stools, they are generally at high concentrations. In this case, it would be sufficient to apply a presence/absence method with determination of the presence of relevant pathogenicity markers. If resources permit, a quantitative method should be applied to evaluate concentrations of total and pathogenic *Vibrio* spp. strains in food incriminated in outbreaks. These data combined with strain typing can provide valuable insight into the attack rate and dose-response of the outbreak strain, and be used to establish criteria for closure and re-opening of shellfish growing areas.

In cases of isolation of pathogenic strains in clinical and shellfish samples (shellfish samples responsible for outbreaks), further characterization is recommended, such as for virulence markers, genotyping and serotyping.

4.4 CONCLUSIONS

The considerations presented above are summarized in Table 4.3. This may be used as the first stage in selecting a method, or combination of methods, for specific uses. However, the basis for the final selection process should also include any local considerations that may be relevant. Such local considerations might include any prior knowledge of expected concentrations of the target species and the ratio of pathogenic to non-pathogenic strains. The steps to be taken in the selection and application of methods are summarized in Figure 4.1.

TABLE 4.3. The comparative value of various detection and enumeration methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus,* for various end uses

End use	Direct Plating		Enrichment presence/absence			MPN		
	No hybridization	Hybridization	Selective plating		No selective plating	Plate		Broth only
			Biochemical	Molecular	Molecular[3]	Biochemical[4]	Molecular	Molecular[5]
Harvest area monitoring	negligible value (in most regions of the world)	ranges from low to high value (see text Section 3.4)	negligible value	limited value	limited value	limited value	moderate value	high value
Post harvest process verification	negligible value	negligible value	negligible value	limited value or high value[1]	limited value or high value	limited value	moderate value	high value
End Product monitoring (raw or processed)	negligible value	limited value	limited value	limited value or high value[2]	limited value or high value	limited value	moderate value	high value
Outbreak investigation	negligible value	limited value	limited value	high value	high value	limited value	moderate value	high value
Growth studies	negligible value	high value for total *Vp*, limited value for pathogenic subpopulation	negligible value	negligible value	negligible value	limited value	moderate value	high value

Notes: (1) Depends on whether applied before or after the process. (2) High value for processed product where zero tolerance is in effect; lower value for raw product. (3) Generally preferable to Enrichment presence/absence with selective plating. (4) Biochemical tests are not generally as specific as molecular ones, lowering potential value. In addition, limited value does not necessarily acknowledge the fact that cost and skill required for this method may be lower and appropriate depending on available resources. (5) High value does not acknowledge the fact that cost and skill required for this method may be higher.

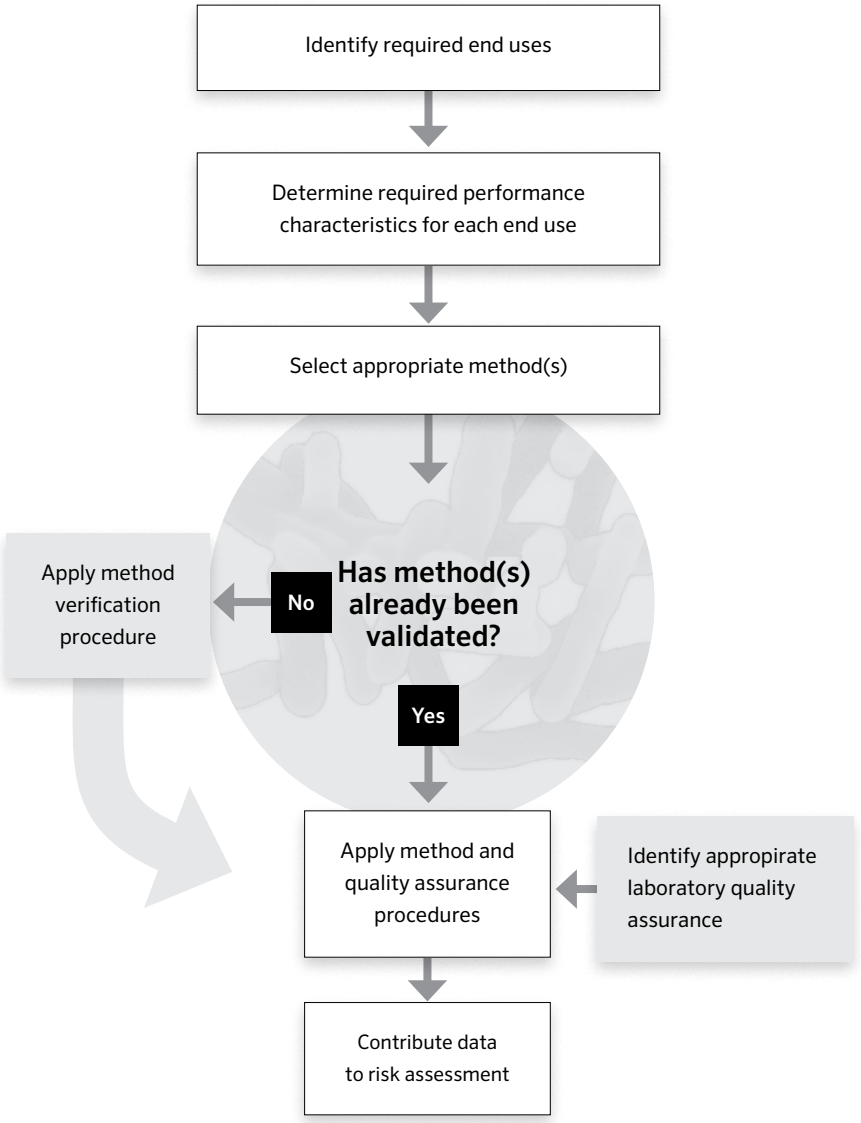

FIGURE 4.1 Steps to be taken in the selection and application of methods

5

Recommended approach for the in-house verification of methods for *Vibrio* spp. for use in national risk assessments

5.1 INTRODUCTION

In the context of microbiological methods:

- Validation means ascertaining whether a method is able to achieve the performance it is intended to deliver.
- Verification means determining whether a single laboratory is capable of achieving the intended performance with a method.

The current procedures for method validation defined by internationally recognized organizations such as the International Organization for Standardization (ISO) and AOAC International (formerly the Association of Official Analytical Chemists) require, amongst other steps, multi-laboratory collaborative studies comparing a reference method with a proposed method (AOAC, 2012; ISO, 2003). The approach is complex, takes a long time (sometimes years), requires significant resources and is extremely expensive. Unlike most other foodborne pathogens, *Vibrio* spp. occur naturally in a single commodity group, seafood, which means that they are applied to a more limited extent than methods with a wider use in food microbiology (such as those for *Salmonella* spp.). There is therefore less

likelihood of the required resource being applied to the validation of a *Vibrio* method, although the European Commission is currently funding the validation of presence/absence methods for *Vibrio* spp. In addition, molecular detection assays are targeting an ever growing list of genes that are often more relevant to risk, and biotech companies are constantly updating their platforms and new companies are regularly entering the market. Application of full validation requirements to rapidly evolving methods does not seem to be appropriate when the method may have been superseded, by the time the validation study is completed. However, it can be envisaged that a full evaluation may be required to underpin a reference method against which other methods can be compared (not necessarily in a full validation). This assumes that a method can be defined that is acknowledged to properly detect or quantify the target species, pathogenic type or gene. Verification procedures are usually less intensive than validation procedures.

It is currently assumed that there are no properly defined reference methods for the detection or quantification of *V. parahaemolyticus* or *V. vulnificus* in seafood. Current standard methods have been shown to grossly underestimate levels of virulent *Vibrio* spp. in naturally contaminated seafood. This complicates the application of accepted validation approaches, which require comparison of an alternative method with a reference method. Even more problematic is that culture-based methods have been shown to perform poorly for detection and enumeration of pathogenic strains (Blackstone *et al.*, 2003; Nordstrom *et al.*, 2007). Inclusion of comparison methods that are not fit for the purpose of detecting pathogenic subpopulations provides little information on method performance, and negative results may be used to misinterpret positive reactions in the molecular test method as false positives. The approach taken in this chapter assumes that a reference method is not available.

If a method appropriate for the intended end use (see Chapter 4) has already been partly or fully validated, then this may be preferred to another, as yet untried, method. Although such appropriate valid methods are not available at the moment, this may not necessarily be the case in the future. A non-validated method may be chosen because it has advantages for the application in question. In that case, the method should be subjected to some assessment to ensure that it performs adequately for the required application: this is the intention of the approach given below. It is not assumed in this chapter that any particular methodology (conventional microbiology, PCR, etc.) will be appropriate: this should be determined by a combination of the end-use requirements, as outlined in Chapter 4, and a determination as to whether the performance characteristics of the method are appropriate to those requirements. The performance characteristics will be obtained either from a previous validation study (or studies), from the method verification approach given in this chapter, or both.

The following is a simplified approach to the verification of a microbiological method for use in acquiring data for the purposes of national risk assessments. It does not constitute validation of a method to determine suitability for regulatory testing. Where previous studies (published or otherwise) have already shown that a method is satisfactory with respect to one or more of the criteria below, assessment of those specific criteria will not need to be repeated. However, if the method has been subject to modification, an assessment will be made as to whether any modification essentially invalidates all or part of the previous validation study (or a verification undertaken under the procedures given in this chapter). If so, the necessary elements will need to be repeated using the modified method (see Table 5.1).

TABLE 5.1 Verification steps recommended for different scenarios

Method type	Parameter	New method	Platform extension	New application	New Laboratory
All	Inclusivity/exclusivity	Yes	No	No	No
	Accuracy	Yes	Yes	Yes	Yes
Qualitative	LOD_{50}	Yes	Yes	Yes	No
Quantitative	Linearity	Yes	Yes	Yes	No
	LOQ	Yes	Yes	Yes	No

It should be noted that this verification approach does not include an inter-laboratory comparison study as an essential step, unlike formal validation procedures. However, some forms of inter-laboratory comparison have been suggested as a means of assessing method accuracy and laboratory competence.

It is assumed that laboratories will follow, in general, the principles given in ISO 7128 (ISO, 2007a). Laboratories that will also apply a method for regulatory purposes may be required to be accredited under ISO 17025 for that specific method (ISO/IEC, 2005). It is not envisaged that laboratories will necessarily be accredited to contribute data to risk assessments, but they should apply good practice and relevant quality assurance procedures.

Some comparative information is given on current ISO validation requirements within the suggested approach in order to emphasize that the verification approach is more limited than a full method validation. The validation requirements are derived from ISO 16140 (ISO, 2003).

Vibrio spp. are naturally occurring bacteria with a wide distribution in estuarine and marine environments, are normally present in most seafood species and par-

ticularly abundant in bivalve molluscs, which are linked to a majority of illnesses associated with ingestion of seafood. AOAC validation criteria for pathogen assays in food matrices indicate a strong preference for using naturally contaminated foods if available; the recommendations for ISO differ depending on the part of the validation study being applied. While seafood with naturally occurring *Vibrio* spp. are readily available during much of the year, variability in natural levels and other problems described below greatly complicate this approach. Additionally, obtaining "control" shellfish that do not contain the target *Vibrio* spp. requires special arrangements to collect shellfish from northern latitudes during cooler months.

Validation of detection methods for *Vibrio* spp. in bivalve molluscs presents many issues that are unusual or unique compared with other pathogen-commodity pairs:

- *Vibrio* spp. naturally inhabit bivalve molluscs and are normally present; and
- *Vibrio* spp. have larger within-species genetic variation compared with other pathogens, including virulent subpopulations, which vary both quantitatively and qualitatively from one oyster to the next within a common collection.

With regard to bivalve molluscs, the following issues also apply:

- they are typically consumed whole, and present a highly complex matrix consisting of many tissue types in comparison with other food commodities; and
- their composition and microbiology are extremely variable and dynamic; these factors affect *Vibrio* distribution, growth and survival as well as method performance.

5.2 GENERAL CONSIDERATIONS RELATING TO VERIFICATION

It is difficult with seafood to achieve uniform levels of *Vibrio* from one animal to the next, and impossible to obtain a "known" inoculum level. The use of samples with natural or enhanced (through incubation) *Vibrio* populations would best simulate "real world" conditions and provide a robust evaluation of method performance, but the variability and uncertainty of *Vibrio* levels, without a known concentration, from one sample to the next would create significant statistical challenges in data analysis.

While there are obvious benefits to running a method in its entirety, the enrichment step in APW is already included in many official or reference methods. The point where culture and molecular detection diverge is the examination of the enrichment for the presence of *V. parahaemolyticus*. Unless there are significant differences in this step (e.g. use of a different peptone or salt concentration, or

different incubation temperature), consideration of the performance of a specific method may be confined to the stages that come after the initial enrichment step.

Another important obstacle is the lack of sufficiently well characterized and diverse strains and defined reference material, including sample matrix. Split samples of naturally contaminated seafood (or other sample matrices such as fish intestinal contents, seawater or sediment) may be distributed under appropriate conditions (e.g. at 4°C) (Jones *et al.*, 2012). For non-cultural methods, frozen or boiled material may be distributed for such purposes or used for longitudinal assessment of method performance. Prior knowledge of the concentrations of the target organism in such material may not be important.

Additional investigations may be needed to confirm results in samples that give substantially different results between methods. Potential causes for such differences could include false positive or negative results by one or more of the methods. Unless an isolate of the target organism can be recovered, it will not be possible to determine the cause of different assay results. The identity of a suspect isolate can usually be determined by use of a reference method or some other reliable means such as rRNA sequence analysis (Jones *et al.*, 2012). If the target organism is not isolated, it should not be assumed to be a false positive reaction. In many cases, it is extremely difficult and resource intensive to isolate strains from pathogenic subpopulations such as *tdh*-positive *V. parahaemolyticus* because they often comprise less than 1% of the total *V. parahaemolyticus* population and are indistinguishable by colony morphology. With real samples, attempts to confirm real-time PCR or LAMP results for pathogenic *V. parahaemolyticus* in APW enrichments fail in most cases (Blackstone *et al.*, 2003; Jones *et al.*, 2009; Nordstrom and DePaola, 2003).

MPN-culture analyses much more often than MPN-PCR analyses yield highly improbable results in which the target organism is detected in smaller sample portions (higher dilution tubes) when it is not detected in larger sample portions. The high frequency of improbable MPN-culture results indicates that this approach is not reliable and the negative results in larger sample portions are actually false-negatives.

The most effective approach for evaluating performance of different methods is to sample from the same homogenate and conduct the analysis using individuals familiar with the assay. *Vibrio* populations are not stable in seafood homogenates for extended periods either refrigerated or frozen, and their levels can decline rapidly in oyster homogenates and probably in homogenates of other bivalve molluscs. Thus it is normally necessary to conduct analyses at the same location if a common homogenate is used. If it is not possible or convenient to process samples at the same location because of travel distance or lack of equipment or supplies,

then another approach would be to split samples from the same collection and ship to all locations where analyses are to be conducted. If naturally contaminated samples are used instead of constructed samples inoculated with pathogens, then shipments are not considered dangerous goods and much of the expense of international shipments can be avoided.

There are two significant issues requiring consideration when employing this approach:

- Care should be taken to assure that samples are handled similarly with regard to time and temperature conditions between collection and analysis to account for potential growth or die off during shipping. This should be documented with a temperature recording device.
- With oysters, limited studies suggest that there is considerable variability in *Vibrio* levels from animal to animal collected simultaneously from the same site. As a result there can be more than one-log differences between replicate samples tested by the same method, due to oyster-to-oyster variability. This variability can be overcome by testing multiple replicates by each test method depending on the degree of variability that has been observed previously or expected based on the literature.

Vibrio strains for use in the verification procedures may be obtained from culture collections, such as the American Type Culture Collection (ATCC), the National Collection of Type Cultures (NCTC, United Kingdom) or the National Collection of Industrial and Marine Bacteria (NCIMB, United Kingdom). A number of other institutions and research groups have also assembled specialized collections and may make strains available to other workers on request. A recent commercial initiative is the provision, at no cost, of a bank of 51 diverse *V. parahaemolyticus* strains by BEI Resources (Biodefense and Emerging Infections Research Resources Repository) in the United States of America. The collection is managed by ATCC.

5.3 VERIFICATION STEPS FOR ALL METHODS

5.3.1 Inclusivity

Select at least 10 pure cultures (in most cases 50 are used for a full validation) of the target *Vibrio* spp. and, if relevant to the test method, cultures showing the respective pathogenic characteristics (e.g. *tdh* and/or *trh*1/*trh*2 for *V. parahaemolyticus*).

For each test strain, a suspension is prepared containing a level 10 to 100 times greater than the theoretical limit of detection of the test (or the LOD_{50}, if this has been determined). The full method is performed on each suspension and the result recorded. Ideally, all target strains should yield a positive result. Where this is not

the case, this will need to be taken into account when interpreting and using the results obtained with the method.

5.3.2 Exclusivity

Select 20 pure cultures (30 are used for a full validation) of non-target micro-organisms. This should include *Vibrio* of non-target species expected to occur in the samples. These might include *V. alginolyticus, V. angullilarum, V. campbellii, V. cholerae, V. harveyi, V. natriegens, V. pelagius, V. splendidus, V. cholerae* non-O1/non-O139, E. *coli, Aeromonas* spp. or *Bacillus subtilis* (preferably a strain isolated from the marine environment). For tests targeting *V. parahaemolyticus*, *V. vulnificus* should be included, and vice versa, in order to ensure that these species can be discriminated.

Where the method is being challenged for a specific characteristic of a species, e.g. a pathogenicity factor, then at least 5 of the strains used should be members of that species that do not exhibit the relevant characteristic.

For each test strain, a suspension is prepared containing a level similar to the greatest level of contamination expected to occur in the food matrices. The full method is performed on each suspension and the result recorded. Ideally, all non-target strains should yield a negative result. Where this is not the case, this will need to be taken into account when interpreting and using the results obtained with the method.

5.3.3 Accuracy

Determination of accuracy is generally relative. There are a number of ways that accuracy can be estimated. In particular, in the absence of a reference method, accuracy can be assessed by:

- Participation in proficiency test distributions with assessment of the results against those of other participants. This assumes that the methods used by the other participants are valid and that the results obtained by the other methods would be expected to be comparable to those of the method to be verified. Where this is not the case, or where too few laboratories are competent with the method, comparison may be made relative to results from one or more expert laboratories: e.g. a reference laboratory or the laboratory that developed the method.
- The use of appropriate microbiological reference materials, preferably those with a certified content of the pathogen relevant to the method to be verified.
- Recovery experiments with spiked samples (normally using a non-selective method to enumerate the inocula). In general, there is a need to challenge

Vibrio methods with mixed cultures containing non-target *Vibrio* spp. as well as other likely members of the flora of samples.

- Split samples of naturally contaminated *Vibrio* populations or those increased by storage at temperatures permitting *Vibrio* growth (where fresh naturally contaminated samples do not contain sufficiently high concentrations).

For molecular methods, boiled suspensions of a strain may be used for LOD_{50}, LOQ, linearity and comparative testing.

5.4 ADDITIONAL VERIFICATION STEPS FOR PRESENCE/ABSENCE METHODS

5.4.1 Limit of Detection

The limit of detection (LOD) is the lowest quantity of the target micro-organism that can be consistently detected in a food sample using the method. It is properly applicable to qualitative (presence/absence) methods, but a limit of detection may sometimes be quoted for a quantitative method; in this sense, such a method is being assessed as a qualitative one. In practice, for microbiological methods, the limit of detection that is used is the concentration where 50% of samples are positive (LOD_{50}).

Grow a representative strain of the target species (and type, if relevant) on a non-selective medium such as saline nutrient agar (see ISO 21872-1 [ISO, 2007b]). Prepare 9 aliquots of a homogenized naturally contaminated seafood matrix. Spike the aliquots with an appropriate volume and dilution of the suspension such that the expected content of triplicate aliquots lies in the range: 3–5 cells; 6–10 cells; 12–15 cells. The amount of homogenized matrix used should be appropriate for the test under consideration. Perform the test and record the results, as in the example in Table 5.2.

The LOD_{50} can be determined by using the Spearman-Kärber test. The spreadsheet given at http://www.nmkl.org/dokumenter/regneark/correctedproc20_2014.xls includes a tool to calculate the LOD_{50} but this does not provide the associated 95% confidence limits.

TABLE 5.2 Example table for the determination of LOD_{50}

Spiking level CFU/25 g	No. of replicates	No. positive	LOD_{50}
5	3	1	
10	3	2	
15	3	3	

5.5 ADDITIONAL VERIFICATION STEPS FOR QUANTITATIVE METHODS

5.5.1 Linearity

Undertake the following exercise at least three times.

Grow a relevant target strain of on a non-selective medium and prepare a suspension that will contain approximately 2000 CFU/ml. Add 100 ml to 900 ml of a 1/10 dilution of a seafood (e.g. oyster) homogenate in APW or another suitable diluent relevant to the method under test. This is regarded as the neat suspension. Prepare doubling dilutions of this using the same diluent up to at least 1/64. Undertake the test in duplicate on each dilution and determine the count per millilitre in the dilution (do not make allowance for the dilution used).

Tabulate the $\log_{10}$ values of the fractions of the dilutions and the $\log_{10}$ counts obtained by the method under test (this assumes that the test yields results that follow a lognormal distribution: this is often the case with conventional microbiological methods). Part of an example table is given as Table 5.3.

TABLE 5.3 Example portion of a table for the determination of linearity

Series	Dilution	$\log_{10}$ dilution	Replicate	Result	$\log_{10}$ result
1	Neat	0	1	220	2.3424
		0	2	310	2.4914
	1/2	-0.301 03	1	50	1.6990
		-0.301 03	2	220	2.3424
	1/4	-0.602 06	1	70	1.8451
		-0.602 06	2	110	2.0414
	1/8	-0.903 09	1	110	2.0414
		-0.903 09	2	40	1.6021
	1/16	-1.204 12	1	40	1.6021
		-1.204 12	2	20	1.3010
	1/32	-1.505 15	1	10	1.0000
		-1.505 15	2	10	1.0000
	1/64	-1.806 18	1	10	1.0000
		-1.806 18	2	10	1.0000
2	Neat	0	1	500	2.6990
		0	2	700	2.8451
	...	...	...	...	...

Initially plot the $\log_{10}$ results against the $\log_{10}$ dilution fraction values to determine that the relationship approximates to a straight line. If not, some other transformation may be appropriate, or the test may not be yielding a linear response with increasing concentration of the micro-organism. There is also the possibility that the relationship may only be linear over part of the range tested. In this case, for the next step use a subset of the data over which the relationship appears at least approximately linear. In this case, the subset of dilutions over which a linear response is obtained will enable the lower and upper limits of concentration for linearity of the test to be determined.

If an approximately linear response is seen, undertake a linear regression of the logged results against the logged dilution fraction values and ensure that the ANOVA table for the regression output includes a lack-of-fit evaluation. The associated probability should not be significant at the 5% level.

The intercept of the line should not be significantly different from zero and the slope should not be significantly different from one.

5.5.2 Limit of Quantification

The method for the calculation of the Limit of Quantification (LOQ) given in ISO 16140: 2003 (ISO, 2003) is:

$$LOQ = 10.s_0$$

where s_0 is the standard deviation of the results obtained from a number of replicates (at least 6, preferably 10) of negative samples.

This method for calculation of the LOQ is not directly applicable to most bacteriological methods or to molecular detection methods that are based on an MPN approach. This is because a sample that does not contain the target micro-organism should give a completely negative result. However, it may be applicable to molecular or other non-culture methods that give a direct measure of quantification and that yield a numerical result for a negative sample (e.g. a direct real-time PCR method where a cycle threshold value will be obtained for a sample that does not contain the target).

For other methods, it is possible to use a definition that the LOQ is that concentration where the probability is 0.99 that the value is not zero. This can be obtained from the linearity regression plot and:

$$LOQ = \text{antilog}\,(a + t_{0.99,n-2} \,.\, S_a)$$

where a is the intercept from the regression equation (EPA, 2009), s subscript a is the standard error of the intercept, t is the critical value of the t statistic and *n* is the number of data pairs.

Where the linearity exercise has shown that the relationship is only linear over a certain range, the LOQ is defined by the lowest concentration at which the linear relationship still applies.

5.6 DETERMINATION OF LABORATORY AND ANALYST PROFICIENCY

Laboratories should periodically assess their proficiency by using one or more of the approaches given in Section 5.3.3 for determining accuracy. Laboratories should also determine the proficiency of analysts, both when first using a method and on an ongoing basis. This can be achieved by different analysts separately undertaking analysis of split samples of the types given in Section 5.3.3 and then comparing their results.

6

Recommendations on the collection of data to support regional and national risk assessments

6.1 INTRODUCTION

Broader application of the international risk assessments for *V. parahaemolyticus* and *V. vulnificus,* including further development of the associated risk calculators, require the availability of data from a wider geographical base. This is necessary both for the input data on potential relevant variables affecting the risk of infection by these organisms, and also for the validation of the outputs. In addition, application of the risk assessments and risk calculators at a regional or national level will require data from that locality, where the patterns may differ significantly from those used in the development of the present tools. The steps to be taken are summarized in Figure 6.1. In addition, there are significant gaps in the dose-response relationship for these pathogens, and the data from a number of the sections below will assist in determining whether the assumptions that have been made about the dose-responses are correct.

All countries are encouraged to provide data for use in the broadening and validation of the risk assessment tools, as data from areas where pathogenic *Vibrio* spp.

are not likely to be a problem will inform the further development of the tools as much as data from areas where illness is perceived, or known, to be a significant risk.

Regions or countries will need to assess whether they require additional data on which to apply a local risk assessment. Such an assessment should consider the following elements:

- Have foodborne pathogenic *Vibrio* infections been identified in the region or area?
- Are any bivalve species eaten raw?
- Do any of the other risk factors, e.g. temperature or salinity, fall within the range that is expected to elevate risk (as identified in the international risk assessments)?

6.2 DATA INPUTS FOR THE PRESENT RISK CALCULATORS

The present risk calculators were originally developed for use in the United States of America. The values of some factors can be entered, on a month by month basis, to give the risk per 100 000 servings (in the case of V. *vulnificus,* in relation to susceptible individuals) through the year. Application elsewhere in their present form will require additional data to validate their application in a specific geographical location. For example, discrepancies have been seen with the application of the *V. parahaemolyticus* risk calculator in Australia, New Zealand and Japan (FAO/WHO, 2016).

6.2.1 *V. parahaemolyticus* risk calculator

The variables that can be entered for this calculator are:
Mean $\log_{10}$ *V. parahaemolyticus*/g at harvest
Air temperature (°C)
Maximum time unrefrigerated (hours)
% pathogenic V. *parahaemolyticus*

6.2.2 *V. vulnificus* risk calculator

The variables that can be entered for this calculator are:
Water temperature (°C)
Air temperature (°C)
Maximum time unrefrigerated (hours)
Maximum time to cooldown (hours)

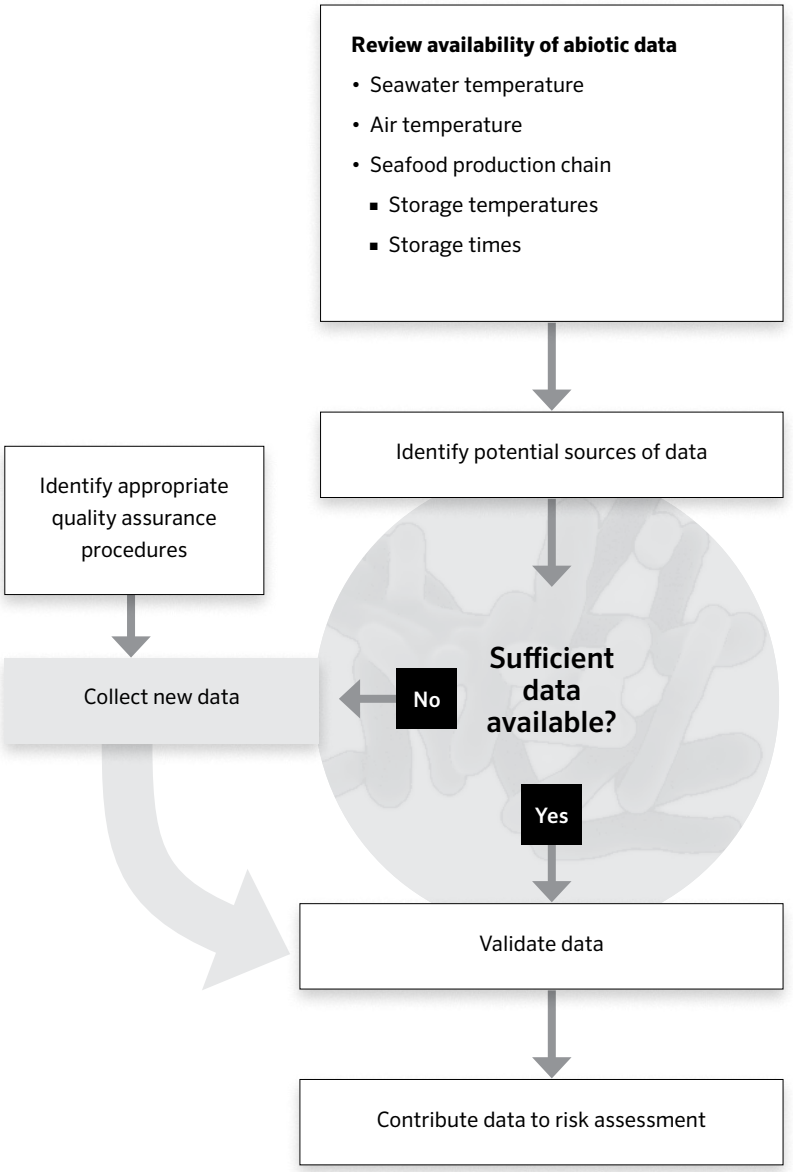

FIGURE 6.1 Steps to be taken for collection of data for risk assessments

6.3 LEVELS OF PRODUCTION AND LEVELS AND PATTERNS OF CONSUMPTION

Knowledge of the levels of production and the levels and patterns of consumption are necessary to place other data into context and to undertake the risk assessment. Levels of production and consumption may differ significantly within a region or country. The situation may also be complicated if there is significant import or export. With bivalve molluscs, the mollusc species of interest will be those eaten raw. Patterns of consumption of raw molluscs are known to vary between regions, between countries and even by area within individual countries. The patterns may also vary between population groups or age ranges, and may change over time. It is therefore important that consumption data not only separately identify those species that are traditionally eaten raw (a general category of 'bivalve molluscs' is not sufficiently informative) but also consider the variability identified above.

Countries should collect data on the production, import, export and consumption of species usually eaten raw within the country. Potential variability in consumption of raw product should be assessed, including consideration of variability between areas and different groups of consumers. The data will need to be reviewed periodically as they may be subject to marked change.

6.4 ABIOTIC FACTORS

Atmospheric and water temperatures and water salinity in harvest areas have been included in the existing international risk assessment models as drivers for the concentration of *V. parahaemolyticus*. Sources will range from satellite imagery to give the big picture, through offshore and near-shore buoys, to local harvesting area spot sample data. Such data may be available from oceanographic and environmental institutes and agencies. For the international risk assessments, the acquisition could be co-ordinated through the Intergovernmental Oceanographic Commission (IOC) or FAO, or both. Data sets available from the National Oceanic and Atmospheric Administration (NOAA) Coastwatch are given in Table 6.1. For the application of the risk assessments at a national level, the competent authority for food safety will need to approach the national oceanographic and environmental agencies for the relevant data. If these data are not available, then they could be collected alongside the sanitary monitoring of harvesting areas. The temporal and spatial resolutions of the data are important.

The atmospheric temperature is also of importance for three reasons:

- it will affect the growth of *Vibrio* spp. in exposed intertidal shellfish;
- it will affect the rate of growth in post-harvest shellfish prior to any cool-down; and
- it will determine the temperature of the post-harvest product and thus affect the time taken to cool the product to a target temperature.

TABLE 6.1. Data available from NOAA Coastwatch

Parameter	Source (sensors)	Resolution	Limitations
Sea surface temperature	AVHRR, MODIS, AMSR-E, TMI, GOES,VIIRS	400 m–800 m (VIIRS) 1.1 km (AVHRR, MODIS) 25 km (AMSR-E, TMI)	Distance to land: ~50 km (microwave sensors)
Ocean colour (Chlor-a, K490, remote sensing reflectances, etc.)	MODIS, MERIS, OCM, VIIRS	250 m (Full Resolution MODIS) 300 m (FR MERIS) 400 m–800 m (VIIRS) 360 m (OCM) 1.1 km (MODIS, MERIS)	
Surface salinity	Aquarius, SMOS	35–50 km (SMOS) 100 km (Aquarius)	Land contamination within ~50 km (value highly variable)
Sea surface height Geostrophic currents	JASON 1 & 2, SIRAL,RA-2, HY-2A, SARAL	6 km (along-track) 25 km (gridded)	Land contamination within 5–18 km

Data on atmospheric temperature will therefore be of importance not only in applying the risk models, but also to the industry in the development of risk reduction strategies. The data will be available through the World Meteorological Organization (WMO) and national meteorological agencies. Local meteorological data may also be available that otherwise is not included in broader data collection systems.

Other factors that may be relevant to the development of new or broader models include thermoclines, haloclines, total suspended solids (TSS), turbidity, total organic carbon (TOC), dissolved organic carbon (DOC), chlorophyll and plankton. Although individual studies have indicated that these may be related to the occurrence and concentration of pathogenic *Vibrio* spp., further research needs to be undertaken to determine whether any relationships are of such significance that they could be considered within risk assessment tools. The information may be available through the same sources as for salinity and seawater temperature.

It has also been identified that the rates of hydraulic turnover of harvesting areas may be of significance with respect to concentrations of pathogenic *Vibrio* spp. (FAO/WHO, 2016). However, the rates will be unique to individual water bodies

(or parts thereof) and will vary according to environmental conditions. This can be considered in the application of national risk assessments.

Countries should therefore collect data on local seawater temperature and salinity values in harvesting areas for use in risk assessment models and management by the authorities and industry. Consideration should be given to the compilation of data on other abiotic factors that may affect concentrations of pathogenic *Vibrio* spp. in harvested product, especially where such data are already collected for other purposes.

6.5 CONCENTRATIONS AT HARVEST (INCLUDING PATHOGENICITY FACTORS)

Robust data exist on the concentration of *V. parahaemolyticus* and *V. vulnificus* in seafood and, for *V. parahaemolyticus*, the ratio of total versus pathogenic strains. The data are only available for a small number of countries and further data are required for the validation of international risk models, and for application of these at a national level. Countries should therefore determine the concentration of pathogenic *Vibrio* spp. in their harvesting areas throughout the harvesting season because:

- if concentrations are such that these are of potential food safety concern, monitoring should continue; and
- if concentrations are not a potential food safety concern, they should be reviewed on a periodic basis.

This monitoring should include determination of species and the presence of relevant pathogenicity factors. The methods used for data collection should be fit for purpose.

6.6 CONCENTRATION IN PRODUCTS ON THE MARKET

While the concentrations of *V. parahaemolyticus* and *V. vulnificus* in product on the market are not necessary as inputs to the current risk models, the data will assist in interpreting the outputs from those models, at both international and national levels. In combination with data on concentrations in the harvest area, market data will assist in assessing the functioning of the cold chain. Countries should therefore determine the concentration of pathogenic *Vibrio* spp. in bivalves in the market in order to provide data to validate the output of the risk assessment models.

6.7 CONCENTRATION IN FOODS THAT HAVE CAUSED ILLNESS (AND RATIOS OF TOTAL VS PATHOGENIC)

In the absence of robust data on the infectious dose from feeding studies, concentrations of pathogenic *Vibrio* spp. in foods incriminated in outbreaks will provide valuable alternative information. Quantification of total and pathogenic strains of *V. parahaemolyticus* in such foods will provide information as to whether the ratio of these in those foods differs from that seen in marketed products not incriminated in outbreaks. Therefore, during outbreak investigations, attempts should be made to quantify and characterize total and pathogenic *V. parahaemolyticus* or total *V. vulnificus* from the incriminated foods. Further characterization of strains (e.g. typing; possession of additional potential pathogenicity markers) may provide information that contributes to the investigation of the outbreak and to the use of the data within risk assessments.

6.8 TIME AND TEMPERATURE PROFILES IN THE FOOD CHAIN

The international risk assessment models include consideration of cool-down time post-harvest and the temperature of product through the food chain. Such information is only available for a limited number of countries and is necessary for validation of the international models and for application in a national context.

Countries should therefore compile data on time and temperature profiles at harvest and during processing, wet storage and associated transport and packaging operations, and during retail. Data on time and temperature of storage in the home environment are also relevant to application of the risk assessments.

6.9 EPIDEMIOLOGY

The extent of epidemiological data on foodborne *Vibrio* infections is extremely limited. It is unlikely that good information will be collected in countries where such infections are not nationally notifiable. In addition, systems need to be put in place, or, where already present, improve them to enhance the detection and investigation of such infections. Where systems are in place, the efficacy of diagnosis and reporting should be assessed. If adequate investigation and notification procedures are not in place in a region or country, it will be necessary to undertake a clinical surveillance study to determine the prevalence of the illness in an appropriate target population. Such surveillance should preferably include follow-up investigation of the possible sources (including foods) of infections. Appropriate information will need to be provided to clinicians, epidemiologists, laboratory workers and others involved in such a surveillance study.

6.10 GROWTH IN BIVALVE SPECIES

Research has shown that *V. parahaemolyticus* does not proliferate at the same rate, or to the same extent, in all species of bivalve: no proliferation was observed in the Sydney Rock Oyster (Fernandez-Piquer, 2011). Variability in growth rate could also occur in non-bivalve seafoods. Where information is not available for a species of local commercial importance (eaten raw), either from international risk assessments or the scientific literature, growth studies should be performed for the *Vibrio* spp. of interest to determine whether the assumptions in current risk models are appropriate for the local bivalve species. The upper range of temperatures should cover the highest temperatures that are known to occur in product between harvest and consumption.

6.11 SUSCEPTIBLE POPULATION

V. vulnificus infections are associated with underlying medical conditions such as liver disease, diabetes, gastrointestinal disorders, haematological conditions and immunodeficiency due to conditions like cancer and treatment of chronic conditions with immunosuppressive agents. The FAO/WHO Risk Assessment for *V. vulnificus* (FAO/WHO, 2005) is based on data on the size of the at-risk population in the United States of America. This could vary considerably in other countries, and could be affected by the prevalence of hepatitis C and HIV/AIDS and rates of alcoholism. For national risk assessment for *V. vulnificus,* data on the relevant at-risk population would be important.

6.12 DATA EXCHANGE NETWORKS

There is a need to identify means by which countries can readily share information relevant to foodborne *Vibrio* infections and associated risk assessments, together with advice on appropriate laboratory methodology and the provision of training. Comparability of the results obtained using the recommended methods will be enhanced by the provision of reference materials and proficiency test material. This will be best achieved by using existing networks.

6.13 DATA RELEVANCE

It is important to ensure that data gathered in support of a regional or country risk assessment are relevant to that risk assessment. This need relates to epidemiological data, *Vibrio* occurrence and concentration in seafood, and abiotic factors.

6.13.1 Epidemiological data

It is important to be able to ensure that epidemiological data relates to the risk being assessed. For example, it is unlikely that information on travel-related *Vibrio* infections will be of relevance to a risk assessment undertaken for seafood consumed in a country or region. However, depending on the scope of the risk assessment, infections related to imported seafood may be relevant.

6.13.2 *Vibrio* monitoring data

Vibrio monitoring data should be relevant to the species under consideration and should include, where appropriate, identification of pathogenic markers. The source (harvesting area; production stage) of samples should be appropriate to the study: it may be necessary to undertake checks to make sure that the samples are representative of the aspect under consideration.

Studies undertaken to generate information on concentrations of pathogenic *Vibrio* spp. in harvesting areas, the production chain, or on the market, should include a sufficiently large number of samples, taken over a pre-determined range of conditions (e.g. with respect to seasons), to yield robust estimates of the occurrence or concentration of the *Vibrio* spp. under study.

6.13.3 Abiotic factors

Data on abiotic factors, such as salinity and temperature, would also be relevant to the purpose of the investigation. Within the marine environment, both salinity and temperature may vary with depth and across an area. Data should be obtained from a location and depth that is relevant to the seafood species of interest. For example, with bivalve species grown on longlines, this might be between 1 and 8 m, depending on local commercial practice. Clams grow in the marine sediment and therefore data from near the seabed is most relevant to those species.

Where data are obtained by remote sensing, the data concerning the effective depth should be determined and a decision should be made as to whether this is relevant to the use. It should also be determined whether the spatial resolution of the data is relevant to the purpose. For example, changes across an estuary may not be seen at spatial resolutions of more than 100 m. Coarse resolutions may also give anomalous data at the coast. For bivalve species that are subject to harvest at low tide, the air temperature may be more relevant than the sea temperature to the concentration of *Vibrio*.

6.14 QUALITY OF DATA

Countries should ensure that data acquired or generated for use in risk assessments are appropriate and have been subject to checks for completeness and quality. The quality will be that relevant to the use to which the data are being put within the risk assessment, and may vary for different types of data and for different uses. Where data are acquired from third parties, information should be obtained from the supplier on the quality assurance criteria that have been applied to the data. In addition, the recipient should undertake separate checks for integrity and quality. These will include determination as to whether the magnitude and range of the data are sensible for the related region or area. Outliers may be valid, but data outside the expected range should be subjected to further checks.

6.14.1 *Vibrio* data

Methods should be used that meet the relevant recommendations given elsewhere in this document. Relevant controls should be used in parallel with the samples under test. These will include target and non-target *Vibrio* spp. and, for molecular methods, relevant positive and negative DNA sequences. Some guidance on the quality of data for use in risk assessments can be found in the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) guidelines for hazard characterization and exposure assessment (FAO/WHO, 2003, 2008).

6.14.2 Abiotic factors

Conventional or digital thermometers should preferably be calibrated against others that are traceable to international standards. Otherwise, they should be calibrated against standard points (melting point of ice; boiling point of distilled water), taking into account the effects of atmospheric pressure.

Salinity meters should be calibrated against a known standard or standards (e.g. certified full strength seawater) and distilled water. The average salinity of seawater is approximately 35 ppt, although this varies with geographical location and depth. A data set containing values greater than that expected for the region should be subject to further validation. Values greater than 40 ppt may be found in hypersaline lagoons and are seen in some tropical saline aquaculture ponds. Salinities in estuaries are usually lower than 35 ppt, although this may not be the case on the flood tide in locations with a large tidal exchange.

Remote sensing data sets should be accompanied by metadata describing their calibration and quality status.

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Note: Specific version references have been given for ISO standards as these contain the recommendations referred to in this document. However, the current version should always be used in applying such standards.

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Annex 1

United States NSSP Post Harvest Processing (PHP) Validation/ Verification Guidance for *Vibrio vulnificus* and *Vibrio parahaemolyticus*[2]

A1.1 PROCESS VALIDATION

Used for the initial validation of a process or when there has been a change to a previous validation process.

TABLE A1.1 Determining adjusted geometric mean (AGM)

AGM Interval	Grams Per Tube	Positive Tubes Allowed
59 995 or greater	0.01	2
37 174 – 59 994	0.01	1
23 449 – 37 173	0.1	4
12 785 – 23 448	0.1	3
10 000 – 12 784	0.1	2

Data on ten processed samples obtained on each of three processing days (total of 30 samples) are required. All samples used on a processing day must come from the same lot of shellfish and be determined to have an adjusted geometric mean (AGM) MPN of 10 000 per gram or greater, as described below for initial load testing. Samples should be distributed throughout the processing day. A sample will consist of a composite of 10 to 12 oysters processed at one time. The zero hour level may be achieved through naturally occurring *Vibrio* levels in shellfish and, where not practical, by time/temperature abuse (inoculated pack samples may be used as appropriate). Analytical methodology to determine *Vibrio* levels should be the official methods previously endorsed by the ISSC as indicated in Model Ordinance Chapter XVI – Post-Harvest Processing. Microbiological testing for

[2] Based on FDA, 2013.

initial levels will be by a 3-tube MPN using appropriate dilutions (10-1 to 10-6). Microbiological testing for processed samples will be by a single-dilution five-tube MPN, inoculating with either 0.01 g or 0.1 g of shellfish per tube, based upon Table A1.1. The numerical value of the endpoint criteria should be less than 30 per gram and achieves a minimum 3.52 log reduction. For the process to be validated, no more than three samples out of 30 may fail. Depending upon the initial load, failure of a single sample is determined according to Table A1.1.

For example, if the AGM equals 50 000, then use the second row because 37 174 ≤50 000 < 59 994. The second row tells to inoculate with 0.01 g of the original oyster homogenate in each tube, and the test fails if more than one of the five tubes is positive.

A1.2 EQUIPMENT VALIDATION

Used to ensure that each new or modified unit of equipment will deliver the validated process. May be accomplished using the following:

- A physical test of the equipment (e.g. thermal distribution study) that is designed to ensure that, when properly operated, it will consistently deliver the validated process.

The process needs to be verified according to Section A1.4 before labelling claims can be made.

A1.3 INITIAL LOAD TESTING

Initial level of vibrios in shellfish for each lot of shellfish used in validation shall be 10 000 MPN per gram or greater, based on the adjusted geometric mean (AGM) of the MPN/g of four samples, where the AGM is given by:

AGM = the geometric mean of the four MPN/g multiplied by an adjustment factor of 1.3

Note: If 4 samples from a lot of shellfish with a true density of 100 000 cells per gram are examined by the MPN procedure, the probability of the geometric mean of the MPNs showing 100 000 or greater is about 50%. In an attempt to improve the probability of samples being accepted when the true density is 100 000/g, an adjustment factor of 1.3 was selected based upon statistical analysis.

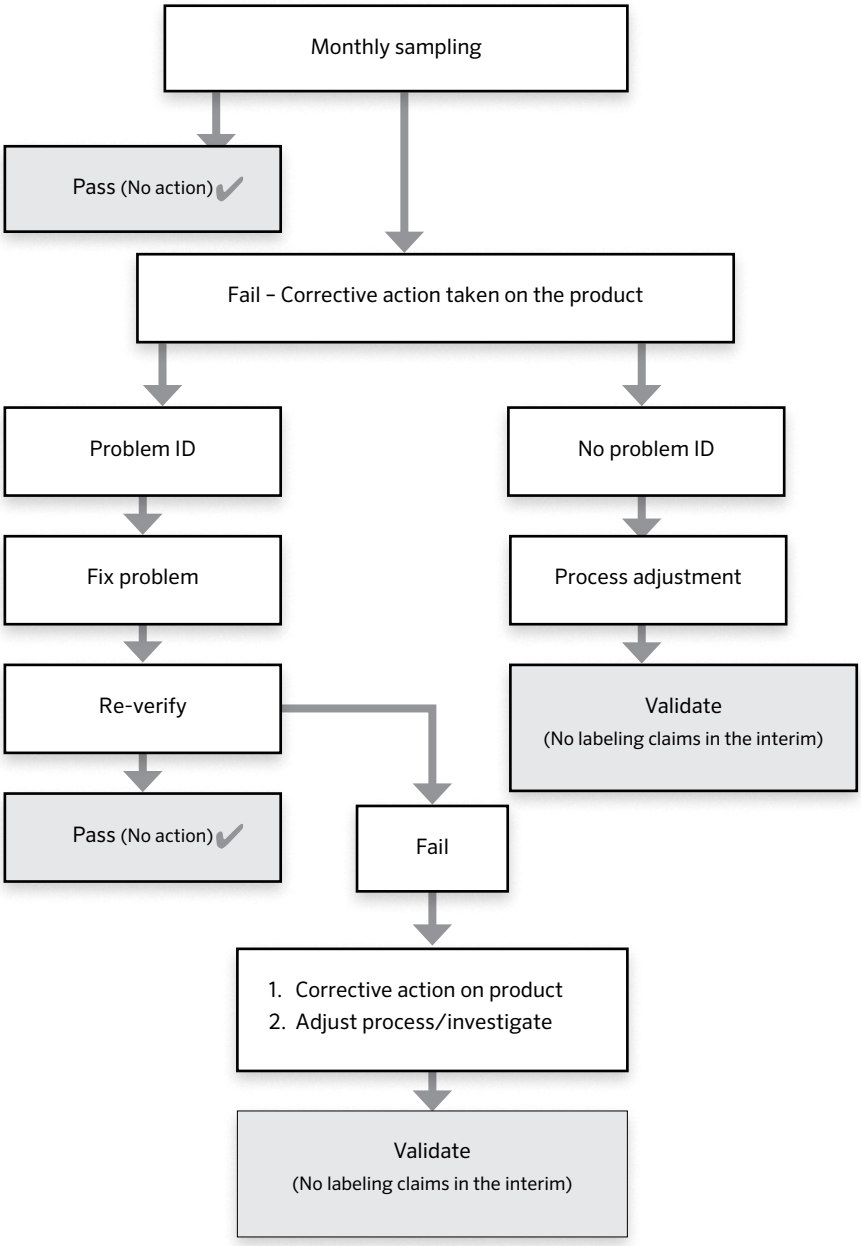
A1.4 VERIFICATION

Used to verify that a previously validated process is working properly.

Process verification by microbiological testing should be done monthly. The monthly sampling shall consist of 30 tubes from a minimum of three samples of 10 tubes each with an inoculum of 0.01 g. Ideally, this would be done on three separate days of production, spread throughout the month, using a 10-tube MPN each day. If this is not feasible, the 30 tubes can consist of 3 samples from three consecutive days, or 3 samples from a given day (from three separate lots if possible).

Each sample will consist of 10–12 oysters. If more than 11 tubes of the 30 most recent 3×10-tube samples within any calendar month are positive, then the process fails for that month. In this case, corrective actions as outlined in the Verification Sampling Plan Decision Tree must be taken and verification must be repeated within one week of the analysis indicating verification failure. Labelling claims may not be used during this time. If all ten tubes are positive for any given sample, this is considered a verification failure and corrective actions must be taken immediately regardless of the result of the other samples for that month. If verification fails twice during a twelve-month period, revalidation is required and product should not be labelled until revalidation occurs.

The dealer, in conjunction with the State Shellfish Control Authority (SSCA), shall annually evaluate the previous 12 months of data and the HACCP plan. The dealer may elect, with SSCA concurrence, to conduct quarterly sampling if the previous 12 verification samples pass.



Note: When a monthly verification fails, the verification must be reported within one week of failure

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Microbiological Risk Assessment Series

One of the challenges in undertaking global risk assessments for halophilic *Vibrio* spp. has been the availability of appropriate datasets from around the world. Such datasets need to be representative of the range of conditions under which halophilic *Vibrio* spp. grow and become a problem for seafood safety. Even when data are available, the different methodology used to generate that data often make it difficult to compare and consolidate globally representative datasets.

This document is intended to provide guidance to inform the selection of the most appropriate methods to generate data on *Vibrio* spp. in seafood. It considers the range of potential methods from culture based to molecular, and proposes the use of performance characteristics to select the most appropriate method according to the potential end use of the data generated, for example, harvest area monitoring, post-harvest process verification, end product monitoring, and outbreak investigation. Aspects of data requirements that could support national and regional risk assessments are also addressed.

This volume and others in this *Microbiological Risk Assessment Series* contain information that is useful to seafood safety risk assessors and risk managers, governments and regulatory agencies, seafood producers and processers, laboratories and other institutions and individuals with an interest in *Vibrio* spp. in seafood.

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