Validation of the membrane filtration method for the detection of *Vibrio cholerae* in marine waters

Validación del método filtración por membrana para la detección de Vibrio cholerae en aguas marinas

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Abstract

This work describes the validation of the membrane filtration method for the detection of Vibrio cholerae in marine waters. The reference method of the Standard Methods for the Examination of Water and Wastewater (23rd edition) section 9260H was taken into account. The selective culture medium was Thiosulfate Citrate Bile Sucrose (TCBS) agar, the differential medium was CHROMagar Vibrio, and 1 % Alkaline Peptone Water (APW) was used as the enrichment medium. To confirm the results, string, oxidase and API20E tests were performed. The parameters determined were detection limit, sensitivity, selectivity/specificity, negative and positive rate, precision, accuracy and uncertainty, which are recommended for qualitative methods. The parameters evaluated had an acceptance criterion of 95 %. For the performance parameters, the results obtained met the optimal acceptance criterion. The method is sensitive and specified for the mycobacterium of interest.

Keywords: Validation, Vibrio, qualitative method, quality

Resumen

En el presente trabajo se describe la validación del método de filtración por membrana para la detección de Vibrio cholerae en aguas marinas. Para tal fin, se tuvo en cuenta el método de referencia descrito en el Standard Methods for the Examination of Water and Wastewater. 23th edition. 9260H. Se utilizó como medio de cultivo selectivo el agar Tiosulfato Citrato Bilis Sacarosa (TCBS), el medio diferencial ChromAgar Vibrio y como medio de enriquecimiento el Agua Peptona Alcalina al 1 % (APA). Como pruebas confirmatorias (cuerda, oxidasa y API20E). Los parámetros de validación determinados fueron límite de detección, sensibilidad, selectividad/especificidad, tasa de negativos y positivos, precisión, exactitud e incertidumbre, los cuales son recomendados para los métodos de tipo cualitativo. Los resultados demostraron un criterio de aceptación del 95 % y óptimo, este último de acuerdo a los parámetros de rendimiento. Adicionalmente, el método resulta ser sensible, reproducible y específico para el microorganismo de interés y apto para su aplicación en laboratorio en la matriz agua de mar evaluada.

Palabras clave: *validación, Vibrio, método cualitativo, calidad.*

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Introduction

The identification of the types of microorganisms existing in an environment is of great importance in different areas. For example, in environmental samples, it makes it possible to establish the quality of surface water used for recreational or domestic activities, as well as to know the dynamics of an ecosystem by identifying the existing microbiota. In the area of public health, the detection of pathogenic bacteria is essential to prevent local or regional outbreaks.

In order to have reliable and accurate procedures that allow the adequate isolation and identification of the different bacteria in the laboratory, the verification and/or validation of microbiological analytical methods has been defined according to ISO 8402:1995 as "confirmation by examination and provision of objective evidence that specified requirements have been fulfilled for a specific use" (International Organization for Standardization [ISO], 1994). Also, ISO/IEC 17025:2017 specifies validation as "provision of objective evidence that a given item fulfils specified requirements, where the specified requirements are adequate for an intended use" (ISO, 2017).

In terms of analysis methods, they can be standardized, non-standardized and alternative. Standardized methods refer to methods that have been developed by established and recognized bodies or institutions. Non-standard methods are developed by the laboratory itself or other parties, or adapted from standardized and validated methods, but with modifications (Dirección General Marítima [Dimar], 2020; Eurachem, 2005). Alternative methods, for their part, are those that have been validated against a standardized reference method and are recognized as equivalent. An example of these are those developed by equipment manufacturers.

Regardless of the type of microbiological analysis method to be implemented, a verification or validation can be carried out taking into account the required parameters, a positive result being evidence of the correct performance of the method. These parameters include: limits of quantification and detection, accuracy, precision, specificity, negative and positive rate and robustness, among others. It is important to be clear about which ones apply to the particular case. In general, the steps to be taken in a verification and/or validation process are summarized in Figure 1.

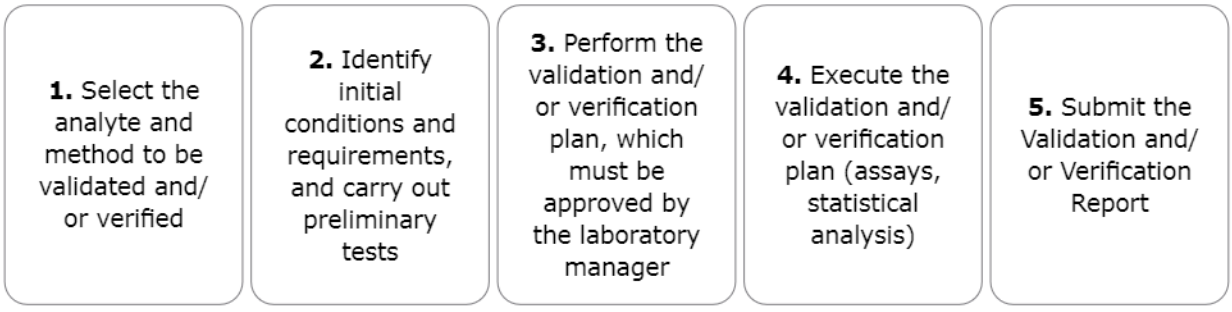


Figure 1. Stages in a validation and/or verification process.

The laboratory of Dimar's Center for Oceanographic and Hydrographic Research (CIOH), has developed processes to validate and/or verify different methods for detecting microorganisms in seawater samples. This work evidences the validation process carried out for the detection of *Vibrio cholerae* using the membrane filtration method, which consists of concentrating the bacteria contained in a sample volume using a sterile cellulose membrane or filter (which can also be made of a similar polymer material), with a pore size of 0.45 µm, in which after a period of incubation in a specific culture medium for the type of microorganism of interest, it is possible to see the formation of colonies. These colonies are counted to calculate the bacterial density present in the sample analyzed (Mulvany, 1969; Forster, 2015). This method is noteworthy due to the time saving, highly reproducible processing and the possibility that it can be carried out *in situ*. The latter is of great impact for research supported by scientific

expeditions or oceanographic campaigns (Mulvany, 1969; Forster, 2015).

Vibrio cholerae is defined as a gram-negative mobile bacillus, belonging to the genus Vibrio of the family Vibrionaceae. It is native to aquatic ecosystems and has been identified in marine, estuarine and riverine waters. This bacterium is distributed worldwide and is pathogenic to humans, causing severe diarrheal diseases known as vibriosis or cholera when serogroups O1 and O139 are present (Boucher, Orata and Alam, 2015; Onohuean, Agwu and Nwodo, 2022; Osunla and Okoh, 2017). The detection of this bacterium is of great importance for the economic, environmental and public health sectors, as its presence can cause sporadic local outbreaks (Escobar *et al*. 2015). Currently, its identification in environmental samples by Colombian laboratories is limited, and the data collected mostly belong to clinical samples (Instituto Nacional de Salud [INS], 2020).

To determine its presence, the standardized method described by APHA, AWWA, WEF (2017) in Section 9260H of the Standard Methods for the Examination of Water and Wastewater (23rd edition) was used as a reference. The validation parameters determined were limit of detection, sensitivity, selectivity/specificity, negative and positive rate, precision, accuracy and uncertainty, all of which were applicable to qualitative methods.

Methodology

The validation for *Vibrio cholerae* was carried out using the methodology set out in the procedures established by the Dimar-CIOH laboratory, which indicate the steps for determining and detecting *Vibrio cholerae* based on the Standard Methods for the Examination of Water and Wastewater section 9260H (SM926OH).

Viability and purity of the working strain

The working strain was acquired by donation from the Colombian Institute of Health (INS) and was subjected to viability and purity tests in the CIOH laboratory to confirm its species-typical characteristics and its viability for use in the validation process. The tests consisted of growth on a Thiosulphate Citrate Bile Sucrose (TCBS) Selective Agar culture medium, accompanied by string and oxidase tests; growth on a CHROMagar culture medium, where the colonies were round, and turquoise or light blue in color; and finally, standardized and miniaturized biochemical identification tests were performed using API 20E.

Separately, a growth curve was performed on the working strain in order to know its behavior under laboratory conditions. The methodology used for this purpose was measurement of the microbial mass by optical method (turbidimetry). For this purpose, a bacterial suspension of the *Vibrio cholerae* strain was prepared and measurements were taken at a wavelength of 600 nm every 30 minutes for 24 hours.

Method validation

The parameters that were evaluated were the following: limit of detection, sensitivity, selectivity/specificity, negative and positive rate, precision, accuracy and uncertainty parameters, all of which are applicable to qualitative methods. For this purpose, a bacterial suspension was prepared using serial dilutions starting from a known concentration of cells, and compared to McFarland turbidity standard # 1. The suspensions were made using natural seawater that was previously sterilized, in the case of the limit of detection and growth curve. The suspensions required for the other parameters were prepared using natural seawater in order to take advantage of the conditions and accompanying microbiota.

Limit of detection

A 10^{-8} dilution of the above-mentioned suspension product was used. For this purpose, 11 replicates of this dilution were made and filtered using sterile nitrocellulose filters with pore size 0.45 µm and 47 mm diameter and subsequently placed on TCBS agar and incubated at 36.0 °C for 18 to 24 hours.

Determination of method performance parameters

The parameters of precision (concordance), sensitivity, specificity/selectivity, relative

accuracy, false positive and false negative rate are considered (SEIMC, 2013). For the analysis, suspensions were prepared at 10^{-7} dilution (for the working strain this corresponds to a concentration of 16 CFU/100mL). Afterwards, 7 samples were membrane filtered using cellulose nitrate filters with a pore size of 0.45 µm and then placed on TCBS selective agar to determine the presumptive positive strains of *Vibrio cholerae*. Likewise, 7 samples were processed and placed on m-FC agar for the isolation of *E. coli* as a negative control as the count was negative. The petri dishes were then incubated for 18 to 24 hours at 36.0 °C for *Vibrio cholerae* and 44.5 °C for *E. coli*. Once the incubation time had elapsed, counts were taken and the 2x2 matrix was filled in (Table 1).

Table 1. 2x2 matrix or contingency table for false positive and false negative rates.

	Presumptive count	
Confirmed count	Positive (+)	Negative (-)
Presumptive (+)	a	b
Presumptive (-)	c	d

In which:

a: True positives

b: False negatives

c: False positives

d: True negatives

Once the data were obtained, the equations described in Table 2 were used to determine the validation parameters.

Table 2. List of validation parameters to be determined and their equations.

precision (concordance) $k = \frac{p_0 - p_e}{1 - p_e}$ Where: *Po*= (a+d)/n *Pe*= [((c*(a+c))+((a+b)*(b+d))]/n²	**Sensitivity** $Sensitivity\ \% = \frac{a}{a+b} * 100$
Specificity/selectivity $Specificity\ \% = \frac{d}{c+d} * 100$	**Relative accuracy** $Relative\ accuracy\% = \frac{a+d}{n} * 100$
False positive rate $False\ positive\ rate\ \% = \frac{c}{a+c} * 100$	**False negative rate** $False\ negative\ rate\ \% = \frac{b}{b+d} * 100$

Robustness

The assay was carried out taking into account the incubation time of the enrichment step (6-8 hours) of *Vibrio cholerae* at a temperature of 36.0 °C. The test consisted of evaluating two enrichment times of 6 and 18 hours, in order to verify if there was any difference in the concentration of the target micro-organism with this variation. To determine if there were significant differences between the times evaluated, an ANOVA test was performed, which compares the variance of the means between each of the groups of data to be evaluated, and is significant when the p-value is greater than 0.050. This analysis was carried out using the statistical program MiniTAB 19.20.

Uncertainty

Expanded uncertainty (Eurachem, 2015), defined as a value that represents the dispersion of the data, is calculated by initially using the value of the standard and combined standard uncertainty, which includes the uncertainties of the equipment, materials, reagents and operator involved in the validation. In the case of the equipment, materials and reagents, the uncertainty in the calibration and analysis certificates were used, as applicable. Operator uncertainty can be taken into account with the coefficient of variation derived from repeatability tests. Once these data are available, the expanded uncertainty is calculated with the following equation:

$$xU = \mu_c(y) * k \qquad \textbf{(Eqn. 1)}$$

Where:

μc (y): combined standard uncertainty

k: Coverage factor = 2 (95 % confidence level)

Membrane filtration method in the detection of Vibrio cholerae

Once the validation parameters had been determined, the method described in section 9260H of the Standard Methods for the Examination of Water and Wastewater (23rd edition) and adopted by the Dimar-CIOH laboratory was applied. For this purpose, 7 seawater samples were taken. From each of these, 100 mL were taken for the filtration process, using a 0.45 µm membrane filter and the process proceeded with enrichment in 1 % Alkaline Peptone Water (APW). Finally, it was incubated for a period of 18 to 24 hours at a temperature of 36.0 ºC. After the APW incubation time, seeding was carried out using TCBS Agar, which was incubated for 18 to 24 hours at 36.0 °C. Subsequently, for complementary tests, BHI agar and CHROMagar were used for string, oxidase and API 20E tests.

Results and discussion

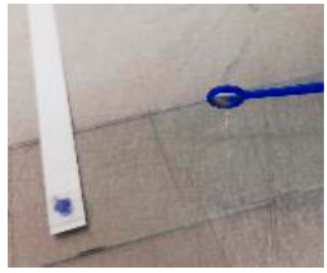
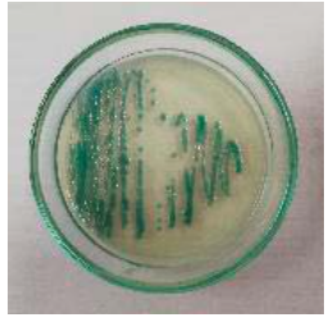
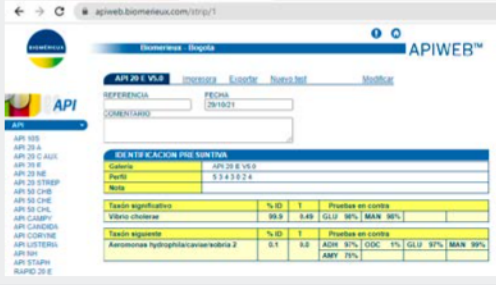
Viability and purity of the working strain

Viability and purity tests performed on the *Vibrio cholerae* MR-VcO1 working strain were satisfactory, as shown in Table 3. Colonies characteristic of the species were obtained on the two agars used, TCBS and CHROMagar. TCBS agar is known to be an efficient selective agar for Vibrio species due to its composition, in which the NaCl percentage allows their growth, since they are considered halophilic bacteria. Taking into account that this agar is used for the isolation and selection of bacteria according to their morphological characteristics, it is advisable to complement with other tests such as those performed in this study: biochemical sucrose (on TCBS agar), string, oxidase and API 20 tests. These yielded positive results for the species under study (Table 3). As for the use of CHROMagar, being a chromogenic medium, it allowed colonies of the species under study to be chosen more selectively.

The growth curve showed that the stationary phase of the *Vibrio cholerae* MR-VcO1 strain began after approximately 15 hours of incubation (Fig. 2). Thus, it was decided to use the inoculum from 18 hours of incubation for all validation assays, a timeframe consistent with the findings of other research (Martinez, Megli and Taylor, 2010).

Table 3. Results of viability and purity tests of the working strain of *Vibrio cholerae.*

TCBS (thiosulfate citrate bile sucrose) Agar Sucrose (+/-)	Colonies are round, with translucent edges, opaque centers and sizes of approximately 2 mm Sucrose (+)	

Oxidase (+/-)	Positive	
String (+/-)	Positive	
CHROMagar	Blue-green and/or turquoise-blue colonies	
API 20E*		

*The figure obtained from the reading of the API 20E tests after the incubation time (24 hours at 36.0 °C), when entered into the bioMérieux application, showed that the strain corresponds 99.9 % with the species *Vibrio cholerae*.

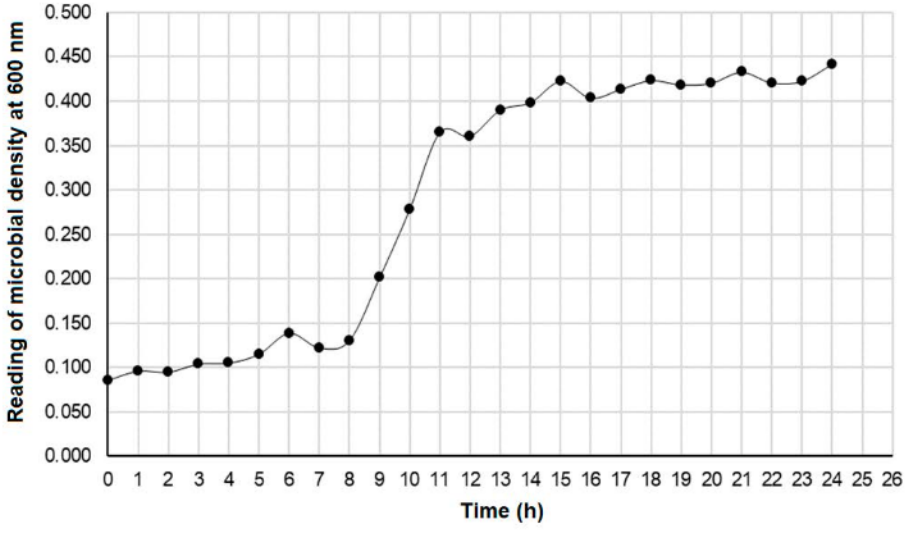


Figure 2. Growth curve of the *Vibrio cholerae* MR-VcO1 donor strain. Behavior for a period of 24 hours with readings at 30 minute intervals.

Limit of detection

The test performed gave a 90 % probability of positive replicates (Table 4), i.e. with the presence of the *Vibrio cholerae* strain. By achieving a low limit of detection, as shown in Figure 3 with the growth of a colony, the probability of finding lower concentrations in the samples tested increases, which is important in the detection of potentially pathogenic micro-organisms.

Table 4. Limit of detection determined by two analysts.

Limit of quantification			
#	Dilution (base 10)	1 CFU*	1 CFU
		Analyst1	Analyst2
1	-8	Present	Absent
2	-8	Present	Present
3	-8	Absent	Present
4	-8	Present	Present
5	-8	Present	Present
6	-8	Present	Present
7	-8	Present	Absent
8	-8	Present	Present
9	-8	Present	Present
10	-8	Present	Present
11	-8	Present	Present
# data present		10	9
# data absent		1	2
Average data with expected result (present)		9.5	
Average absent data		1.5	
%		95 %	
Must be >90%			

* Colony forming units (CFU) .

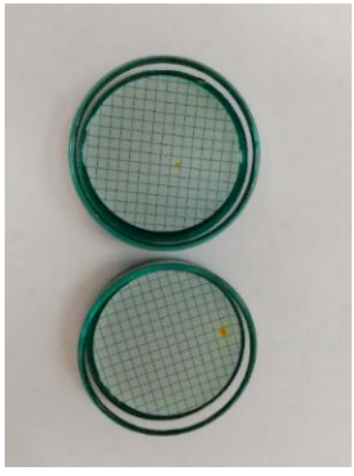


Figure 3. Limit of detection determined by two analysts.

Method performance parameters

The results are shown in Table 5. Precision was determined by means of the Kappa concordance index and the result obtained was 0.82, which indicates that there is a very good concordance between the tests carried out for its determination and the method used. The values for a satisfactory result must be greater than 90 %, and our results had values of 90 % for sensitivity and 94 % for specificity.

Table 5. Count of positive and negative strains in a 2x2 matrix.

	Presumptive count	
Confirmed count	Positive (+)	Negative (-)
Presumptive (+)	18	2
Presumptive (-)	2	31

Parameter	Result
Precision (concordance)	0.82
Sensitivity	90 %
Specificity/selectivity	94 %
Relative accuracy	92 %
False positive rate	10 %
False negative rate	6.0 %

The relative accuracy, like the sensitivity and specificity parameters, is expected to have a value greater than 90 %. For the experiment carried out in this validation, a value of 92 % was obtained, which is an acceptable and satisfactory result. This indicates that the culture media used are suitable for isolating the bacteria under study, as they allow their growth, supplying the basic nutritional needs of the microorganisms, while also being specific and limiting the growth of other bacteria.

The false positive and negative rates, in contrast to the previous parameters, are expected to have low percentages (< 10 %), as it is related to the capacity of both the method and the analyst to identify typical or non-typical colonies. The results obtained correspond to 10 % and 6 % respectively, which indicates that there is a low possibility of obtaining false positives with the method used. Taking into account the specificity and/or selectivity characteristics of the method in terms of the culture medium selected, it limits the growth and development of other organisms, which, even if they develop with the complementary biochemical tests, are discarded before being included in the final calculation of the total bacterial concentration.

Robustness

Microbiological methods are not robust due to their nature, taking into account that most of the impurities or interferents are living organisms. However, in the case of the validated method, it was possible to identify the robustness by performing tests and using variables such as temperature and incubation time, among others.

Table 6 shows the counts obtained for the test. The ANOVA statistical analysis carried out showed that there were no significant differences between the concentrations when the enrichment time was varied significantly, since p=0.086, which indicates that the changes in enrichment time do not modify the expected results. Of course, if this enrichment time is extended beyond 18 hours, it should be evaluated in order to extend the hypothesis, since this stage aims to recover the cells already present in the sample and not to increase the microbial mass, which can happen with long enrichment times.

Table 6. Count data to estimate the robustness of the method.

Enrichment time (hours)	Day	Replicas	CFU Count	CFU Log	Average	SD	FINAL AVERAGE CFU
6	Day 1	1	432	2.635	2.543	0.054	349
		2	387	2.588			
		3	297	2.473			
		4	287	2.458			
		5	311	2.493			
		6	342	2.534			
		7	415	2.618			
	Day 2	1	368	2.566			
		2	355	2.550			
		3	347	2.540			
		4	311	2.493			
		5	367	2.565			
		6	348	2.542			
		7	342	2.534			

Enrichment time (hours)	Day	Replicas	CFU Count	CFU Log	Average	SD	FINAL AVERAGE CFU
18	Day 1	1	335	2.525	2.431	0.105	270
		2	318	2.502			
		3	390	2.591			
		4	355	2.550			
		5	287	2.458			
		6	223	2.348			
		7	245	2.389			
	Day 2	1	298	2.474			
		2	197	2.294			
		3	258	2.412			
		4	227	2.356			
		5	295	2.470			
		6	170	2.230			
		7	295	2.470			

Uncertainty

In the case of qualitative analysis methods, as in the case of the validation, the uncertainty is considered negligible; therefore, it is not taken into account. Even so, in order to obtain the result of this parameter, the uncertainties of different sources involved in the test were identified, which allows the identification of the instruments and/or equipment that contribute the greatest variation to the result. The value obtained for the combined uncertainty was 1.741 and the expanded uncertainty was 3.483.

Conclusions

The validation process is a systematic experimental approach that gives a clear idea of the capabilities and limitations of an analytical method, as well as the sampling technique to be considered in a testing and calibration laboratory.

Knowing that the working strain is in a suitable condition is an essential step before performing the validation assays, because if the strain has lost its genotypic characteristics, it can limit the validation process. For its part, the limit of detection carried out in the validation of the method to detect *Vibrio cholerae* had a probability for the values of 95 %, which was above the acceptance criterion of 90 %. For the performance parameters, the results obtained are within the optimal acceptance criteria. The method was found to be sensitive and specific for the micro-organism of interest.

This study demonstrated the selectivity of the TCBS culture medium used to identify presumptive strains of *Vibrio cholerae*, in which the negative control (*E. coli*) did not show growth in any of the dilutions performed, but did show growth in its FC-selective agar. With the results obtained for each of the parameters, it can be considered that the methodology evaluated is suitable for the detection of *Vibrio cholerae* in marine waters.

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