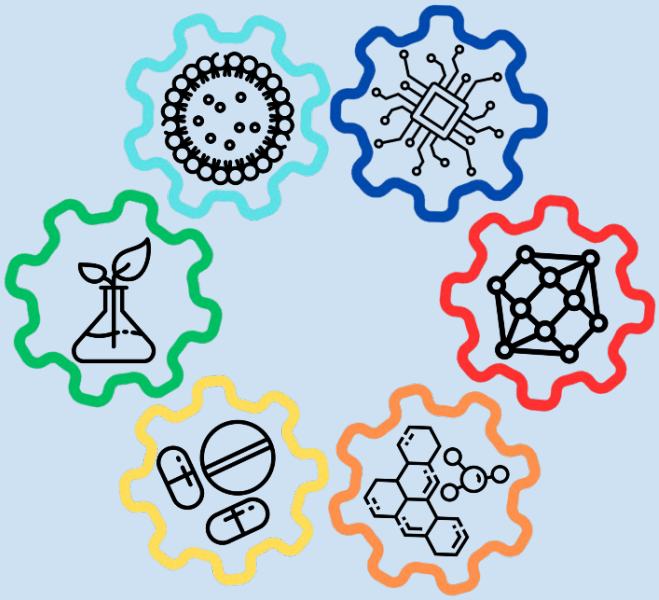
GENERALITAT VALENCIANA
Conselleria de Educación, Universidades y Empleo

idm



XVI·IWOSMOR

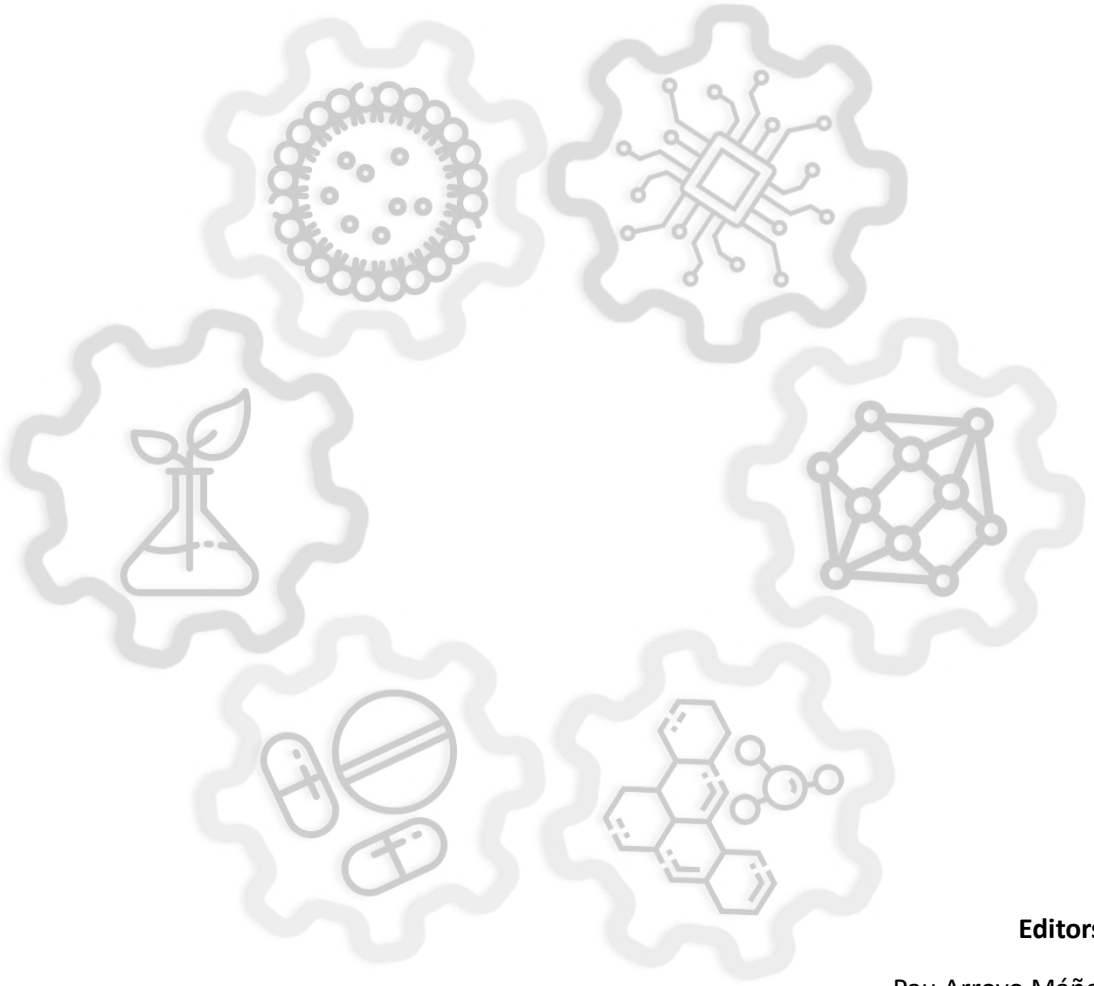
INTERNATIONAL WORKSHOP ON SENSORS AND MOLECULAR RECOGNITION

July, 6 and 7th 2023

BOOK OF ARTICLES

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

VNIVERSITAT DE VALÈNCIA



Editors:

Pau Arroyo Máñez

Margarita Parra Álvarez

Eva María Garrido García

Mariana Barros Nogueira

Paula Rodrigo Martínez

Jordi Hernández Contreras

ISBN 978-84-09-55991-6

- INDEX -

DETECTION OF A CLIMATE CHANGE MARKER (*VIBRIO VULNIFICUS*) BY A LATERAL FLOW TEST STRIP ASSAY

Pedro Quintero-Campos[a], Belen Fouz[b], Carmen Amaro[b], Luis Tortajada-Genaro[a,c], Ángel Maquieira[a,c]

[a]*Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València-Universitat de València, Camino de Vera s/n, E46022 Valencia, Spain. e-mail: pedquica@upvnet.upv.es*
[b]*Instituto Universitario BIOTECMED, Universitat de València. Burjassot, Valencia, Spain*
[c]*Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, E46022 Valencia, Spain*

Keywords: *Vibrio vulnificus* ● Biosensor ● DNA ● Lateral flow ● Climate change

ABSTRACT

Climate change is causing rapid changes at all ecological scales, including coastal ecosystems. This situation alters the distribution patterns of some pathogens susceptible to these changes, which can be used as markers of climate change. One of the most critical aquatic pathogens in human health that is experiencing a growth parallel to the increase in coastal temperature is *Vibrio vulnificus*. This pathogen is associated with aquaculture and can be transmitted from diseased fish to humans (zoonosis). Currently, *V. vulnificus* is detected in water and animals by combining cultural and qPCR methods, which requires specialized personnel and equipment. It would therefore be interesting to have a rapid and accurate detection procedure that requires neither operators nor equipment in field conditions, and that could be implemented on a global scale to monitor both the pathogen and the progress of climate change.

This communication presents the development and fine-tuning of a simple and rapid detection method for *V. vulnificus* using PCR combined with a lateral flow device. The method was tested on fish samples. Preliminary results show that the limit of detection is 0.01% of pathogenic DNA *vs.* fish DNA with a total assay time of less than one hour. These results are promising and suggest that the new procedure could be used for aquaculture, climate change monitoring, and environmental remediation.

INTRODUCTION

Climate change is affecting the planet in an unprecedented manner, causing rapid changes at all ecological scales. Changes in weather patterns due to global warming alter the infection patterns of some pathogens that are particularly sensitive to environmental changes. [1] The vibrios are an important group of aquatic pathogens experiencing rapid growth due to this situation. The genus *Vibrio* comprises more than 100 species that are natural inhabitants of warm water ecosystems of low to moderate salinity. [2]

The ecology of *Vibrio* is inextricably linked to two main environmental factors: seawater temperature and salinity. These two elements have been identified as the main components governing the distribution and abundance of *Vibrio* in coastal waters. [3] Basically, salinity defines the areas suitable for these organisms, while temperature modulates their abundance in areas suitable for their presence. [1]

Ocean warming, changes in precipitation, river runoff, and ice melt associated with climate change may lead to temporary reductions in salinity in many coastal areas, which could contribute to ideal environmental conditions for vibrio growth (Figure 1). As a result of these changing conditions, the number of days suitable for vibrio growth in water has increased considerably since a baseline in the early 1980s, and the overall

suitability for vibrio presence on the coast has increased by 9.9% during this period. [1] These associations have led to *Vibrio* species being collectively recognized as a "microbial barometer of climate change".

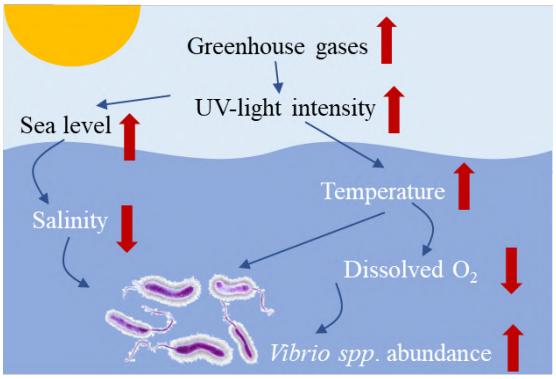


Figure 1. Correlation of *Vibrio spp.* abundance and effects of global warming on aquatic systems. Adapted from [3].

Among the species of the genus *Vibrio* most susceptible to climate change, *V. vulnificus* stands out as a zoonotic agent associated with cases of rapid death by septicemia in humans. This pathogen can infect both by contact with water or contaminated/diseased animals and by ingestion of raw or undercooked seafood [4]. So far, this pathogen is detected by combining cultural microbiological methods with qPCR detection. Consequently, it would be very interesting to develop rapid and accurate identification methods that would require neither trained operators nor equipment in field conditions and could therefore be implemented in developing countries not only to monitor the presence of the pathogen but also the progress of climate change.[5]

Considering the above, the aim of this work has been to develop one of these methods. The new approach combines PCR and lateral flow and detects and quantifies the presence of *V. vulnificus* DNA in less than 1 h.

METHODOLOGY

DNA extraction

Pure cultures of *V. vulnificus*, *V. harveyi*, *V. alginolyticus*, and *V. anguillarum* were obtained in LB-1 after 18 h of incubation at 28ºC. Volumes of 1 mL of each culture were centrifuged at 12,000 rpm for 4 min at 4ºC. Bacterial pellets were resuspended in 100 µL of PBS (Phosphate Buffered Saline 1 %, pH 7), boiled at 100ºC for 5 min, kept on ice for 5–10 min and, then, centrifuged again at 12,000 rpm for 4 min at 4 ◦C (Gyrozen 1730R, Controltecnica, Spain). DNA present in the supernatant was stored at -20 ◦C and used as a PCR template. In parallel, DNA from blood cells of gilthead seabream was extracted using the same protocol. The DNA concentration and quality per sample was determined using NanoDrop 2000 (Thermo Fisher Scientific, Spain).

Amplification by PCR

A forward and reverse primers set was designed targeting *V. vulnificus* and labelled at the 5' ends with fluorescein isothiocyanate (FITC) and biotin, respectively. The final conditions were set in a 12.5 µL volume containing 1.25 µL of PCR Buffer (Biotools, Spain), 0.55 µL of a 50-µM solution of $MgCl_2$ (ThermoScientific, EEUU), 0.25 µL of 10 mM-dNTPs (ThermoScientific, EEUU), 0.3 µL of each 10 µM-primer, 0.25 µL of Taq DNA polymerase (Biotools, Spain), 8.25 µL DNAse-free water, and 1 µL of template. DNA from *V. vulnificus* was mixed in different ratios with DNA from gilthead seabream (1:1, 0.75:1, 0.5:1; 0.25:1, 0.1:1, 0.01:1 and 0.001:1) and the mixture was used as a template to stablish the sensitivity of the assay. The selectivity of the method was determined using DNA extracted from other phylogenetically close pathogenic vibrios (*V. harveyi, V. alginolyticus and V. anguillar*um). The reactions were carried out in UNO96 thermocycler (VWR, EEUU).

Lateral flow assay

A positive PCR generates amplicons labelled with FITC and biotin. The amplification product binds to the gold-labelled anti-FITC antibody in the strip and is immobilized to the streptavidin band of the test line, producing a positive band. The control band captures the unbound gold particles, resulting in a colored band at the end of the strip. The procedure was as follows: a volume of 2 µL of the PCR products was mixed with 120 µL Lateral Flow Assay Buffer (PBS-T) in a well of an ELISA plate. The Lateral Flow Strip was then immersed in the well, and the solution was allowed to flow through the strip for 5 minutes. The strip was then scanned, and the image obtained was analyzed using an image processor. The test was positive if a red band appeared at the bottom of the test strip and no band appeared on the negative control specimen. The detection limit of the lateral flow assay was determined by image analysis of the strip testing multiple dilutions of *V. vulnificus* DNA as a product.

RESULTS

DNA extraction

The concentration of bacterial DNA was measured by absorbance, and the purity of the DNA in each of the samples was determined using the A260/A280 and A260/230 ratios (Table 1):

Table 1. DNA concentration and quality analysis results.

Sample	[DNA] ng/µL	$A_{260/280}$	$A_{260/230}$
V. vulnificus 1	177,9	1,86	2,22
V. vulnificus 2	88,1	1,91	2,45
V. vulnificus 3	106,3	1,91	2,30
V. vulnificus 4	215,9	1,91	2,10
V. vulnificus 5	120,1	1,85	1,86
V. harveyi	263,3	1,88	1,81
V alginolyticus	178,9	1,89	1,81
V. anguillarum	84,0	1,95	2,95

Amplification by PCR

As shown in Figure 2, PCR amplification of *V. vulnificus* using the labelled primers resulted in a fragment of approximately 220 bp. In the absence of DNA, no amplification was produced.

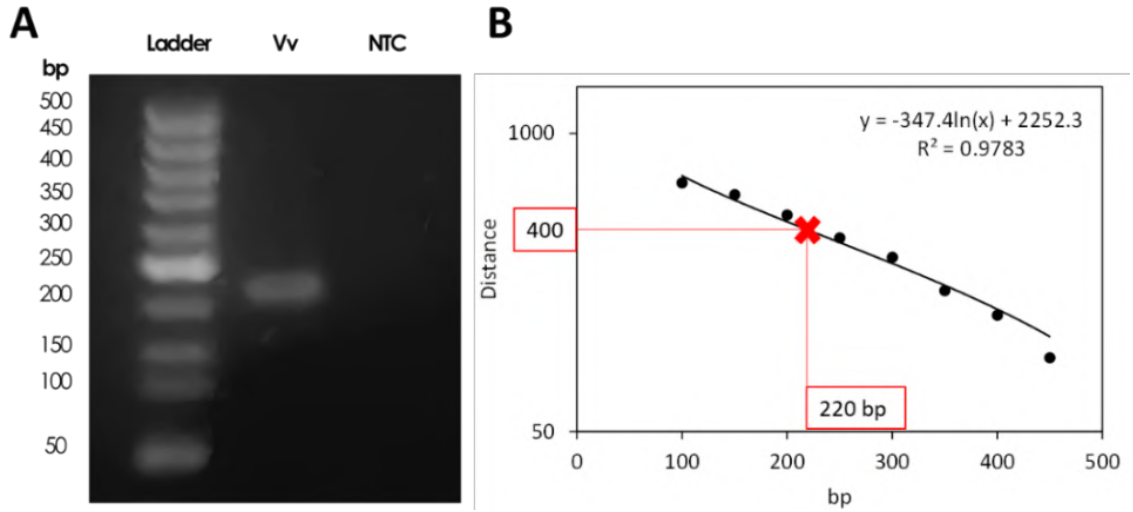


Figure 2: Characterization of the positive amplicons. A single band corresponding to the PCR amplification product of *V. vulnificus* was observed in 2% agarose gels (A). The band size was 220 pb (B). NTC: non-template control.

Lateral flow assay

A schematic of the lateral flow DNA detection method is shown in Figure 3A. Application of this methodology to PCR products showed clear positive results for all samples containing *V. vulnificus* DNA but not for samples containing DNA from other species (Figure 3C). Next, we determined the detection limit of the procedure using samples containing bacterial and fish DNA in different proportions. As shown in Figure 3B, the detection limit is 0.01% which is equivalent to 0.05 ng of bacterial DNA per 49.95 ng of animal DNA.

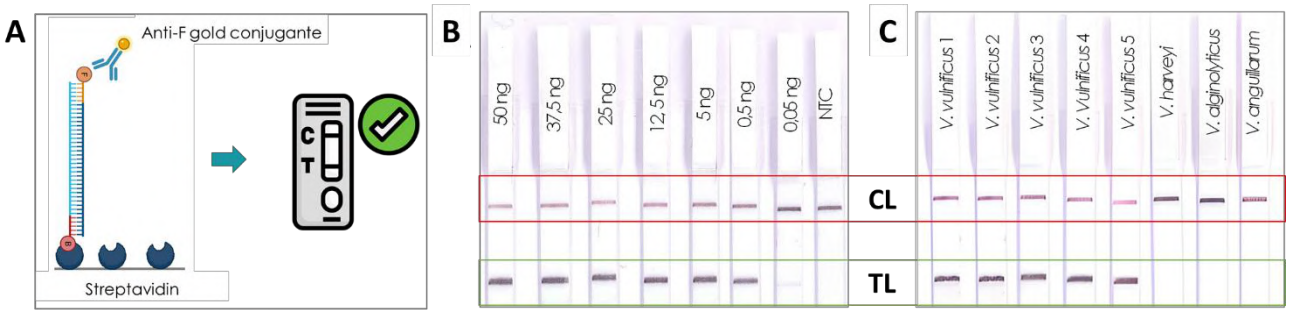


Figure 3: (A) Schematic of the positive PCR detection method using a lateral flow device. **(B) Sensitivity study:** test strip after PCR amplification of target DNA diluted in non-target DNA (values in ng correspond to 100%, 75%, 50%, 25%, 10%, 1%, 1%, 0.1% of bacterial DNA *vs*. animal DNA. **(C) Selectivity study:** A positive band was observed only for *V. vulnificus* DNA. ***CL= Control Line, TL= Test Line.**

CONCLUSIONS

A simple and rapid method for the detection of *V. vulnificus* by PCR combined with a lateral flow test has been developed and fine-tuned. The designed primers allow the generation of double-labeled amplicons that can be detected with a lateral flow strip in less than 5 minutes, developing the complete protocol (including PCR) in less than 1 h. The test was specific for *V. vulnificus* and did not react with DNA from 3 other closely related *vibrios* (*V. harveyi*, *V. alginolyticus*, *V. anguillarum*). Preliminary studies conducted show that the detection limit of the pathogen DNA in relation to fish DNA is around 0.01%, a value that is within the detection limits of the pathogen in fish. Consequently, these results are promising and constitute the first step in developing and implementing a new rapid detection tool for this pathogen applicable out of the laboratory and in the field. This new tool would be useful not only for pathogen detection, but also for disease diagnosis and could eventually be used to monitor climate change, as *V. vulnificus* is a biomarker of global warming.

ACKNOWLEDGMENTS

This study was supported by Spanish grants THINKINAZUL/2021/010 and THINKINAZUL/2021/027 from MCIN (Ministerio de Ciencia e Innovación de España) with funds from European Union NextGeneration EU (PRTR-C17.I1) and GV (Generalitat Valenciana), PID2020-120619RB-I00 funded by MCIN/AEI/10.13039/501100011033 and CIAICO/2021/293 funded by "Conselleria de Innovacion, Universidades, Ciencia y Sociedad Digital" (GV, Spain).

BIBLIOGRAPHY

[1] Trinanes, J. et al. *Lancet Planet Health*. **2021**, *vol. 5*, e426–35.
[2] Baker-Austin C, et al. *Nature Reviews Disease Primers*. **2018**, *vol.* 4, 1-19.
[3] Wang X, et al. Environmental Microbiology. **2020**, *vol.* 22 *(10)*, 4438-4455.
[4] Ceccarelli, D. *et al. Food Microbiology.* **2019**, *vol.* 13*(3)*, 347-388.
[5] Montánchez, I. *et al. Marine environmental research.* **2020**, *vol. 154*, 104850.