Detection of *Vibrio cholerae*, *V. Parahaemolyticus* and *V. Vulnificus* in seafood using real time PCR

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Introduction

The genus Vibrio consists of a Gram-negative straight or curved rods, motile by means of a single polar flagellum. Vibrios are capable of both respiratory and fermentative metabolism. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are serious human pathogens.

Vibrio cholerae colonizes the human small intestine where it produces virulence factors that cause diseases. Two of the principal genetic elements were involved in pathogenesis: (i) the phage CTXΦ harbouring, among others, the *ctxA* and *ctxB* genes encoding the Cholera toxin and (ii) a pathogenicity island harbouring, among others, the *tcp* operon encoding the Toxin Coregulated Pili (TCP).

Vibrio parahaemolyticus causes gastro-enteritis in which the hemolysins, thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH), have been considered to play a crucial role. TDH should act like a porin in the enterocyte plasma membrane and allows the influx of multiple ionic species.

Vibrio vulnificus is a leading cause of death related to seafood consumption in the United States, unlike *Vibrio cholerae* and *Vibrio parahaemolyticus*, diarrhoea and/or dysentery are not the predominant clinical manifestations. The majority of disease caused by *V. vulnificus* is associated with wound infections as a result of the contamination of wounds with water harbouring the organism and causing septicaemia. It seems there isn't *V. vulnificus* non-pathogenic.

Objectives, Primers and Probes

The aim of this work was to develop real time PCR methods in order to detect these three vibrios in seafood. A two steps assay was designed:

- First, PCR targets a gene of interest specific of the targeted pathogen species to wit *ompW* for *V. cholerae*, *tlh* for *V. parahaemolyticus* and *vvp* for *V. vulnificus*. If the test is negative, that means the pathogens are absent and the detection stops here. But, if a positive answer is obtained, it's necessary to pass to the second step. It's called the "species PCR".
-Second, checking for the presence of the virulence factor is required. For that, *ctx* and *tcp* genes for *V. cholerae*, *tdh* and *trh* genes for *V. paraehemolyticus*, both in multiplex PCR, have to be searched. The *V. vulnificus* is considered as always pathogenic. It's callled the "pathogen PCR".

The real-time PCR experiments were realized on the ABI 7300 (Applied Biosystems). The cycles, primers and the probes designed by courtesy of us.

Results of the "species PCR"

Species	*ompW*		*tlh*		*vvp*	
	Ct	Ct	Ct	Ct	Ct	Ct
V. cholerae	15	15	ND	ND	ND	ND
V. cholerae	14	15	ND	ND	ND	ND
V. Cholerae	13	15	ND	ND	ND	ND
V. parahaemolyticus	34	35	10	11	ND	ND
V. parahaemolyticus	37	37	14	16	ND	ND
V. Parahaemolyticus	35	36	13	12	ND	ND
V. vulnificus	ND	ND	37	35	24	23
V. vulnificus	ND	ND	ND	ND	24	24
V. Vulnificus	ND	ND	37	ND	24	25
V. Campbelii	ND	ND	26	26	ND	ND
V. cyclotrophicus	ND	ND	ND	ND	ND	ND
V. fluvialis	ND	ND	ND	ND	ND	ND
V. harveyi	ND	ND	ND	ND	ND	ND
V. metschnikovii	ND	ND	ND	ND	ND	ND
V. mimicus	ND	ND	27	27	ND	ND
V. neonatus	ND	ND	ND	ND	ND	ND
V. alginolyticus	ND	ND	ND	ND	ND	ND
Photobacterium angustum	ND	ND	ND	ND	ND	ND
Salmonella spp.	ND	ND	ND	ND	ND	ND
Salmonella spp.	ND	ND	ND	ND	ND	ND
Clostridium perfringens	ND	ND	ND	ND	ND	ND
E.coli	ND	ND	ND	ND	ND	ND
E.coli O157	ND	ND	ND	ND	ND	ND
Bifidobacterium crudilactis	ND	ND	ND	ND	ND	ND
Yersinia enterocolitica	ND	ND	ND	ND	ND	ND

ND : not detected

All the PCR have been realized two times at least

As you can notice, some vibrios give some false positive results with a late amplification for the *tlh* and the *ompW* PCR. But, in this case, is note a problem. Indeed, for the PCR *tlh*, the dilution limit for the detection of *V. parahaemolyticus* has been established, in the aim to compare the Ct with the other Ct achieved with the others vibrios and be sure of the specificity of the PCR (see results below). Even in the biggest dilution (10^{-8}), the Ct are enough lower than the smallest Ct obtained with an other *vibrio*.

For the *ompW* PCR, only *V. Parahemolyticus* cross-reacts. So, the strains can be easily discriminate by a culture of them on TCBS media.

So, the three PCR can be considered enough specific for the targeted vibrios. But, this is only the first step of the development of the technical. In the next time, the limit of all PCR has to be determined yet. For the moment, only the number of minimal bacteria detected in inoculated shrimps for *V. Vulnificus* has been calcul

Limit of the detection PCR*tl (Ct)*	
V. Parahaemolyticus dilution 10^{-8}	17
Others vibrios : Ct Minimum	26

Detection of Vibrio vulnificus in artificially contaminated seafood

25 g of shrimps were diluted in 225 ml APW 2%, homogenized, inoculated with 1 ml of a suspension of *Vibrio vulnificus* S4 and S6, diluted 10^{0} to 10^{-7} and incubated 18h at 42° C. The DNA was extracted from 1 ml of pre-enrichment broth and the PCR *vvp* was performed.

V. Vulnificus S4			V. Vulnificus S6		
Inoculum (Log cfu)	Ct *vvp*	Internal Contol	Inoculum (Log cfu)	Ct *vvp*	Internal Contol
7	24	+	7	26	+
6	26	+	6	27	+
5	27	+	5	26	+
4	26	+	4	28	+
3	28	+	3	30	+
2	30	+	2	34	+
1	ND	+	1	ND	+
Not inoculated	ND	+	Not inoculated	ND	+
NTC	ND	+	NTC	ND	+
Positive Control	24	+	Positive Control	25	+

ND : Not Detected, NTC : Non-Template Control

In both cases, the detection limit is the inoculum 2 or 24 cfu/ml for S4 and 40 cfu/ml for S6 and no PCR inhibition was detected.

Limit of the detection PCR*vvp*	
V. Vulnificus S4	24 cfu/ml
V. Vulnificus S6	40 cfu/ml

Results of the "pathogen PCR"

Virulence factors					
typed strains	*V. Cholerae* *ctxA*	*tcpI*	typed strains	*V. Parahaemolyticus* *tdh*	*trh*
CTX- / TCP-	-	-	TDH- / TRH-	-	-
CTX- / TCP-	-	-	TDH- / TRH-	-	-
CTX- / TCP-	-	-	TDH- / TRH+	-	+
CTX- / TCP-	-	-	TDH- / TRH+	-	+
CTX+ / TCP+	+	+	TDH+ / TRH-	+	-
CTX+ / TCP+	+	+	TDH+ / TRH-	+	-
CTX+ / TCP+	+	+	TDH+ / TRH+	+	+
CTX+ / TCP+	+	+	TDH+ / TRH+	+	+

The determination of the patothype of the strains is realised with two multiplex PCR : *ctxA*/*tcpI* for *V. cholerae* and *tdh*/*trh* for *V. parahaemolyticus*. The strains have been typed previously, and the PCR results obtained are compared with the results waited.

Conclusion

At this step of the work, we have already three specific PCR for the targeted vibrios and two robust PCR for the detection of the virulence factors of *V. cholerae* and *V. parahaemolyticus*. The interest of this method compared to traditional methods (microbiology), is the swiftness of the detection. It is possible to obtain the results of the presence and the proof of pathogenicity of a bacterium in seafood in two days. Comparatively, the microbiological methods give an answer after 5 days of wok (in the better case). Moreover, the first results give a better sensibility of the molecular methods.

The next steps are to determine the limit of the detection of all the reactions and validate the PCR in accordance with the ISO 16140.

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