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# Evaluation of the contamination of sea products by *Vibrio* and other bacteria in the eastern coast of Algeria

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### ABSTRACT

Seafood and their environment (seawater) constitute a major risk of foodborne diseases that are related to Vibrio species whose repercussions may become sometimes serious and fatal for the consumer. Two hundred samples of seafood (170 shrimp, 20 mussels, 10 crabs) from the regions of Jijel, Colo., Skikda, Annaba and El Kala have been subjected to a double enrichment in NaCl 1% and 5% followed by an isolation on TCBS agar and a biochemical identification on API20E biochemical galleries. Vibrio alginolyticus was isolated in a sample of shrimp taken from the region of Skikda in May (0.6% of the samples). No Vibrio has been detected in other matrices. Other species of bacteria, also dangerous to public health, were isolated on the TCBS agar from 37.2% of the shrimp samples, 75% of the mussel samples and seven seawater samples. These bacteria are, in decreasing order: Citrobacter freundii, Boulkharderia pseudomallei, Aeromonas hydrophila, Proteus vulgaris and Staphylococcus spp. Our study shows results similar to those published in Croatia, Netherlands, Italy, Germany and Morocco, but higher than of Belgium where no Vibrio has been isolated from seafood. This leads us to recommend the development of research methods and more appropriate preventive measures to minimize the risk of biological contamination in seafood.

Keywords: Vibrio spp., Vibrio alginolyticus, seafood, seawater, bacterial contamination.

#### INTRODUCTION

Food safety has become for many countries an issue of high priority. A collective food borne disease may have serious sanitary, political and economical consequences [5].

Infectious diseases of food origin represent throughout the world a considerable number of deaths and, these diseases have particularly severe consequences in developing countries.

Indeed, 1.8 million persons died of diarrheic diseases in 2000, a high proportion of these cases being attributed to the contamination of food and water [21].

Fish and sea food constitute the second source of animal proteins after meat. In 2000, a 130 million tons of fish and sea food have been produced worldwide **[8]**. Raw and undercooked bivalve molluscs (clams, oysters, shrimp and mussels) represent an important marine environment vector of infectious agents and marine biotoxines this is due to the ability of shellfish to concentrate pathogens and toxins during the filter-feeding process **[17]**. Illness can be

associated both to human wastes and to microorganisms indigenous to coastal marine environment. Shellfish contamination from sewage-polluted waters continues to be a problem:

Salmonella have been shown to survive for over a month in the aqueous-sediment microcosm [10], and also thermophilic *Campylobacter* have been isolated from sea water [1]. *Vibrio* are widely distributed in marine and estuarine environments and in seafood throughout the world, which constitutes in many countries a real public health issue.

Apart from cholera, a notifiable disease according to the sanitary legislation of the world health organization (WHO), there is practically no surveillance system for infections with non choleric *Vibrio*.

Actually, because of the recent evolution of the European regulation (CEE n° 2073/2005), the search of *Vibrio* in fishing products intended for export is recommended by the Ministry of Agriculture and Fishing in many European countries. This regulation on microbiological criteria recommends focusing on effective methods for the evaluation of risks related to *Vibrio* in sea products [6].

Our study aimed first at searching for the presence of *Vibrio* in crustaceans (shrimp, crabs), mollusks (mussels) and in sea water of Jijel, Collo, Skikda, Annaba and Al Kala Coast (Algiers), in order to identify the isolated strains and, second, characterize less halophilic bacteria isolated on TCBS agar.

#### MATERIALS AND METHODS

The sea food and sea water samples were taken from five different regions of the Algerian eastern coast: Jijel, Collo, Skikda, Annaba and Al Kala.

Sea water samples were taken together with sea food samples at the same period, in the same coast except for Al Kala coast that is far away from Constantine and consequently the samples could not be analyzed in the required time ( $\leq 6$  hours).

Two hundred samples of sea food were collected and analyzed for *Vibrio*, and one sampling of sea water per month and per coast led to 28 samples.

The material used for the analysis, was the material commonly used in food microbiology according to the standard **[13].** 

The analytical method consisted on 3 steps to search for Vibrio according to the standard

[19]. Briefly, enrichment at 42°C during 6-8h and 18-24h in alkaline peptone water (APW) (Algeria Pasteur Institute, Algiers, Algeria) with different NaCl concentrations (1% and 5%); after initial enrichment, isolation was made on TCBS agar (Algeria Pasteur Institute) upon seeding in surface and in depth incubated during 18-24h at 37°C; after incubation and isolation on TCBS agar, a purification of suspect colonies fermenting saccharose (green colonies) was made for characterization of *Vibrio* as well as identification of non characteristic colonies; biochemical identification of characteristic strains; and finally, results were confirmed by the oxydase test, gram stain, KIA, (Kligler Hajna) (Algeria Pasteur Institute) and API 20 E Galleries (BIOMERIEUX).

According to [2], the samples of sea water were analyzed for *Vibrio* and submitted to the following protocol: five hundred (500) ml of sea water for analysis were passed through a 0.5  $\mu$ m membrane for filtration. The membrane was poured on TCBS agar and left for incubation at 24°C during 24 h then at 35° C during 48h.

The characteristic *Vibrio* colonies were purified and isolated on TCBS agar for identification then confirmed by the biochemical tests cited below.

#### RESULTS

Only one sea food sample out of 200 was contaminated by *Vibrio*: the frequency of contamination is thus 0.5%. This bacteria was isolated in a shrimp sample from the Skikdi coast. 0,6% (170 samples) was found in this region during the month of May. Non-vibrio contamination was detected in mussel samples, crabs and sea water (table 1).

Nature of samples	Number of samples	Number of positive cases	Prevalence %
Shrimps	170	1	0,6
Mussels	20	0	0
Crabs	10	0	0
Total	200	1	0,5

#### Table 1: Distribution of positive cases in samples

After biochemical confirmation of *Vibrio* strain, the isolated colonies with yellow color on TCBS agar (gramnegative, oxydase positive), presented an effective growth in alkaline peptone water at 5% NaCl but not at 1%, confirming that these colonies belong to the species *Vibrio alginolyticus*.

Among the 200 samples of sea food and the 28 samples of sea water analyzed to search for Vibrio *spp* on TCBS agar, 75 sea food samples and 7 sea water samples showed a bacterial contamination. The contaminated samples were for mussels with 75% (20 samples) and shrimps 35,2% (170 samples). No bacterial contamination on TCBS agar was found for the crab samples analyzed.

The shrimp samples mostly contaminated were taken in the region of Collo with 76.4% (17 samples) followed by the region of Skikda 34% (37 samples) and in decreasing order, Jijel 27% (57 samples) and Annaba 26% (69 samples). The contaminated mussels at 75% (20 samples) come from Al Kala region (table 2, figure 1).

Table 2 : Distribution of	positive samples isolated in seafood
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Seafood	Number of samples	Number of postives samples	Prevalence %
Shrimps	170	60	35,2
Mussels	20	15	75
Crabs	10	0	0
Total	200	75	37,5



Figure 1 : Distribution of positives samples in seafood

The results of the analyzed samples in a period of 4 months (May, June, July, December) of the year 2007 and in January and march of the year 2008 have shown clearly a high contamination (93%) in December 2008. and a lower contamination in may, June and July.

Table 3 : Distribution of samples of contamined seafood by region

Regions	Seafood	Number of samples	Number of postives samples	Prevalence %
Region 1 (Jijel)	Shrimps	37	10	27
Region 2 (Collo)	Shrimps	17	13	76,4
Region 3(Skikda)	Shrimps	57	19	24
	Crabs	10	0	54
Region 4(Annaba)	Shrimps	69	18	26
Region 5(El Kala)	Mussels	20	15	75
Total		200	75	37,5

The results for 28 analyzed sea water samples have shown a bacterial contamination in the month of May, June and January. For Skikda and Jijel regions, a contamination was seen in the month of March for the region of Skikda. No

bacterial contamination was observed on TCBS agar in sea water analyzed brought from Collo's and Annaba's region (table 3,4)

	Year 2007			Year 2008			
Samples	May (n=20)	June (n=20)	July (n=10)	December (n=15)	January (n=45)	Februar (n=45)	March (n=45)
Shrimps (n=37)	3	4	0	0	3	0	0
Shrimps (n=17)	8	5	0	0	0	0	0
Shrimps (n=67)	0	0	0	0	3	5	11
Crabs (n=10)	0	0	0	0	0	0	0
Shrimps (n=69)	0	0	0	14	4	0	0
Mussels (n=20)	0	0	0	0	0	7	8
Total	11	9	0	14	10	12	19

Table 4: Summary of samples presenting a contamination according to months

Among 75 contaminated samples, characterization of colonies was done (gram staining, and oxydase, API 20E Galleries). Gram-negative bacteria isolated on TCBS agar has allowed the identification of 4 species. It is by increasing order of importance *Citrobacter freundii*, *Pseudomonas pseudomallei*, *Aeromonas hydrophila* et *Proteus vulgaris*. The seven samples of contaminated sea water allowed to isolate and to show the presence of *Pseudomonas pseudomallei* in 5 samples and that of *Aeromonas hydrophila in* the other two.

In the other side *Staphylococcus* could be isolated among Gram-positive bacteria, in shrimps and mussels (table 5).

Isolated bacterial species	Shrimps (n=170)	Prevalences	Mussels (n=20)	Prevalences	Sea water (n=28)	Prevalences
Citrobacte Freundii	19	11,1%	0	0	0	0
Pseudomonas Pseudomallei,	16	9,4%	2	10%	5	17,8%
Proteus Vulgaris	13	7,6%	0	0	0	0
Aeromonas Hydrophila	5	2,9%	7	35%	2	7,14%
Staphylococcus spp	7	4,1%	6	30%	0	0

Table 5: Prevalences of bacterial species in seafood and sea water

#### DISCUSSION

In our study, among the 200 samples of sea products analyzed, one sample with a prevalence of 0.5% was contaminated by *Vibrio*, it concerns shrimp samples (170) with a result of 0.6% taken from the region of Skikda in the month of May. No positive samples when searching *Vibrio* was found in mussels (20) and crabs (10) analyzed. No *Vibrio* spp. was found in sea water (28) in our studies.

The interest that we can give by searching contaminant agents of sea products is justified by the fact that in Algeria very few studies were devoted to the search of pathogenic agents.

In Algeria, the fishing sector shows a real boom because many fishing harbors are modernized or on going to be a part of the economic dynamic in these regions.

Our study is limited to the Eastern coasts of Algeria whose halieutic production is equal to 13 621.12 tones on a total production of 65 626.81 tones **[9]**.

Regarding the choice of the kind and the number of the analyzed samples, the marine crustaceans mainly shrimps; crabs were responsible of many epidemiologic advances of food origin worldwide [4]. The epidemiology has shown that the kind of the product was the cause of food poisoning due to the proliferation of enteropathogen organisms among them Enterobacteriaceae and Vibrionaceae, and gram-positive bacteria, mainly *Staphylococcus aureus* [4]. With no specific standards, there is a wide diversity of analysis methods to find *Vibrio*, throughout them the standardized operator modes can serve as a reference for the Food and Drug Administration (1998), the Nordic Commitee on Food Analysis (NMKL, 1997) and the Canadian method (MFLP-37, 2006) [18].

In the present study, the technique used is a horizontal method for the search of *Vibrio* spp., method validated by the food microbiology laboratory of the University of Liege (laboratory accredited for the validation of bacteriological methods in food).

This technique revealed less selectivity and sensitivity because it has allowed the isolation of other bacterial species whose morphology is comparable to that of *Vibrio* spp.

The salted alkaline peptone water was used during the present study as an enrichment media and consisted on the preparation of two broth with different percentage of NaCl (1%) and 5%). Because some species of *Vibrio* are halophilic (*V.cholerae* and *V. mimicus*) and not other. The need of NaCl for *Vibrio cholerae* is 3% whereas for the growth of *Vibrio parahaemolyticus* 

The minimal concentration is 0.55% of NaCl [4]. However, this organism is rapidly inactivated by distilled water. The low rate of isolated *Vibrio* in Algeria (0.6%) could be the result of the variation in growth factors (NaCl concentration).

The enrichment media used during our study has allowed to figure out a growing multiplication of bacterial species less halophilic belonging to the family of the Vibrionacae like *Aeromonas* and to other families, these bacteria have multiplied in an enrichment broth at 1% Nacl.

The isolating media used is TCBS media (Thiosulfate Citrate Bile Saccharose, Diagnostics Pasteur, réf. 69456; Oxoid, réf. CM 333) which is the referenced media recommended for the detection and isolation of pathogen *Vibrio*. (Standard NF ISO 8914, and the World Health Organisation recommandations).

The high concentration in bile and citrate associated to a high pH (pH=8.8) permitted the elimination of numerous bacteria. The main source of carbon is saccharose. The use of saccharose results on a decrease of pH and a change of pH indicator from green to yellow.

The positive saccharose colonies, as many species of *Vibrio* especially *V. cholerae* and *V. alginolitycus* appear hence yellow.

The negative saccharose colonies as *Vibrio parahaemolyticus* appear green. However, this is neither sensitive nor specific and many false positive colonies were found (**Oxoid**, **2007**) and the latter justify fully our results. During our study, the colonies similar to *Vibrio* were identified like *Proteus*, colonies that give yellow colonies similar to positive saccharose *Vibrio* as well as *Pseudomonas* which give also yellow colonies on TCBS agar. One of the other disadvantages of TCBS media is that it doesn't generally differentiate the various species of *Vibrio*, it should in fact identify the species of *Vibrio* in the analyzed samples.

Identification of diverse species of *Vibrio* on TCBS media is presumptive and has to be confirmed by supplementary tests. It will be advantageous to identify the presence and to discriminate *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* by other selective media like TTC Agar which is also used, but relatively fastidious to prepare, and needs the regular control and rigorous dosage of potassium tellurite.

Recent studies have allowed to suggest a culture media for the detection and the discrimination of *Vibrio* genus characterized with at least one chromogen agent, in a *Vibrio* culture media (**Oxoid, 2007**). The chromogenic media (*Vibrio* selective Chromagar) permit the differentiation of V. *parahaemolyticus, V.vulnificus and V.cholerae* comparing to other species of *Vibrio*.

*V. parahaemolyticus* appears under a form of purple colonies. *V.vulnificus* and *V.choleare* produce bleu colonies while other species like *V. alginolyticus* form colorless colonies. The principle of the media is based on the simultaneous detection of the  $\beta$ -glucosidase, specific of *V. parahaemolyticus*, and of the  $\beta$ -galactosidase, specific of *V. cholerae* and *V. vulnificus*, in the presence of high concentration of saccharose (Oxoid, 2007).

No method of reference is available for the enumeration of *Vibrio* in fishing products and there is no correlation between the dynamic of *Vibrio* population and those of fecal pollution indicators used usually to judge the sanitary quality of water and food **[18]**.

A new standard ISO/TS 21872-1, validated in 2007, advocates 4 successive phases because *Vibrio* spp. can be present at low number and are much often accompanied to a number much more high of other organisms belonging to Vibrionacae family and other families. Consequently, 2 successive selective enrichments are required to search targeted organisms. The first enrichment consist on incubating the mother suspension at a temperature of  $37^{\circ}C$  during  $6h\pm1h$  for frozen produced or at  $41.5^{\circ}C$  during  $6h\pm1h$  for fresh products, dry or salted. The second enrichment would be in a selective liquid media which is transferred in 1ml of the culture, obtained during the first selective enrichment and taken in surface in a tube containing 10ml of salted alkaline peptone water.

Regarding the isolation and identification, enrichment is made in 2 solid selective media: TCBS media with thiosulfate, citrate, bile and saccharose; and another appropriate solid selective media in addition to TCBS media is salted nutritive agar (SNA).

On the other hand, the use of biochemical tests doesn't usually permit identification of the species and there is a continual need to return to the molecular techniques [5].

Classical methods of identification, determination by PCR techniques of specific genomic sequences of the species, and genus associated to the virulence are more specific.

Thus, the belonging of a strain to *V. parahaemolyticus* species, phenotypically very close, is distinct biochemically only by fermentation of one sugar of *V. alginolyticus* species. And can be confirmed by the amplification of R72H sequences specific to *V. parahaemolyticus* species. Therefore, the R72 H is a good marker for the identification of V. *parahaemolyticus* [18],[23].

It was observed, these last years, that *Vibrio* can react with disadvantageous environmental conditions by entering in a viable phase but they are not cultivable when they are exposed to unfavorable conditions. Salinity, temperature or nutritive elements deprivation can cause lesions that make them undetectable by bacteriological methods. Nevertheless, if the conditions become favorable they may return to their "normal" state and be cultivated again. This phenomenon has as obvious consequences that systematic examinations of the samples taken in the environment for searching of pathogen may be negative [6],[18].

The conventional methods of show cultivable bacterial form of *Vibrio* in water and sea water have to consider the contamination rate that can be reliable of the damaged physiological state of the bacteria searched and the abundance of the accompaniment flora **[18]**.

Our results are difficultly interpretable due not only to the few studies of *Vibrio*, problematic in sea food, but also to the fact probably of the non availability of the required means and the complexity of searching method on TCBS agar **[18]**.

In conclusion, the identification is hard and recognition of *Vibrio* colonies is largely under experience and their aspects can sometimes vary not only from a species to another but also from a culture media lot to another (ISO/TS 21872-1:2007).

Studies of prevalence have shown different rates of contamination by Vibrio species in sea food and sea water. In Italy (Adriatic sea), from September 1997 to January 1998, shell fish water was examined, and Vibrio spp. were isolated from 48.4% of samples; the species most frequently found were V. alginolyticus (32.2%) and V. vulnificus (17.7%), followed by V. cincinnatiensis (3.2%), V. parahaemolyticus (1.6%), V. fluvialis (1.6%) and V. cholerae non-O1 (1.6%) [16]. In another study undergone for three years in raw bivalve mollusks, sold in the Puglia region of Italy. V. parahaemolyticus and V. vulnificus were found in 47 (7.83%) and 17 (2.83%) of the samples, respectively [15]. In Morocco 2007, 220 samples of fishing products were analyzed to search Vibrio. This study shows a prevalence of 8.2% in all the fishing products analyzed, 5.7% in shrimps and 35% in bivalve mollusks for V. alginolyticus [5]. In Netherlands a total of 91 samples of shell were examined from August to October 1999, Vibrio alginolyticus was the predominant species with 44% followed by V. parahaemolyticus (27,4%), other species of Vibrio (6,5%), Vibrio metschnikovii (1%) [20]. In Croatia, during the summer of 2000, 117 samples of marine fish, shrimps, bivalve mollusks were collected in Adriatic sea, Vibrio parahaemolyticus was predominant (47%) followed by Vibrio vulnificus (37,6%), V. alginolyticus (3%) and other Vibrio (12,8%) [7]. Furthermore, in Germany 2007, Mussel samples were taken regularly between June 2004 and May 2005 in seven shellfish-growing areas of the German Wadden Sea. Among Vibrio isolates, Vibrio alginolyticus was the species most frequently detected (51.2%), followed by

*Vibrio parahaemolyticus* (39.5%) **[14].** In Belgium 1998, a total of 1299 samples (live bivalve mollusks, crustaceans, and fish of different kind) were tested for the presence of *V cholerae*, 311 samples for *V. parahaemolyticus* and 82 for other species of Vibrio. None *Vibrio* has been isolated in the samples **[7]**.

These studies allowed to see that the predominant strains isolated from sea food and sea water are different according to the regions. In some regions Vibrio *parahemolyticus* is the predominant strain followed by *Vibrio alginolyticus* and *Vibrio cholerae* (Croatia,2000; Italy,2006). Whereas in other regions *Vibrio alginolyticus* is the predominant strain followed by *Vibrio parahemolyticus* and other *Vibrio* (Italy,1999; Netherlands,2000; Morocco,2007;Germany,2007). The results of prevalence obtained in the present study are lower than those

observed in Croatia, Netherlands, Italy and Morocco and greater than those of Belgium where no *Vibrio* was isolated from sea products. The difference between the reported prevalence may be explained by the large diversity of analysis methods for the finding of pathogen and non pathogen *Vibrio* for human.

In relation to *Vibrio alginolyticus*, it is an important opportunistic bacterial pathogen for both human and aquatic animals **[11]**, **[12]**,**[22]**. They appear after exposition to sea water or after contact with marine animals **[3]**. The infection by *Vibrio alginolyticus* reported mainly are otitis, conjunctivitis, superficial pyodermatitis and gastro enteritis. For immunosupressed subjects they can become severe and potentially fatal. This bacille can infect tissue from cutaneous lesions. Hence, *Vibrio alginolyticus*, has to be part of the list of pathogenic agents in case of cutaneous infections, in particular towards sick persons who were in contact with sea water in hot region or marine animals **[3]**.

Regarding to the other bacteria isolated on TCBS agar, among the 200 samples of sea food and 28 samples of sea water analyzed to search *Vibrio* spp., 75 samples showed a bacterial contamination (other than *Vibrio*) with a prevalence of 37.5% for sea food and 7% samples of sea water. The presence of this bacteria in sea water and in sea food can be explained either by waste water rejects, water rain off and water banks but doesn't exclude the contamination from the fishers and fish traders manipulations. The statistical analysis of obtained results shows that there are significant differences in the sampling, region and period and this can be explained by the fact that there is a predominant contamination in shrimps especially during the month of December and May in the regions of Collo and Al Kala.

#### CONCLUSION

This study has shown the presence of *Vibrio* in shrimps. The result registered is low, 0.6%. These results are difficult to interpret.

Our results justify fully the requirement to use a chromogenic media recommended in actual standards.

The presence of other pathogenic bacteria isolated on TCBS media in sea food and sea water should be considered as a signal of danger for consumer and manipulators; it suggests the need to establish measures and requirements in order to prevent all risks related to these sea products by biological contaminants eating those belonging to *Vibrio* species.

The prevention methods to fight against food infection by *Vibrio* could be the conservation of sea food by cooling, washing sanitation, hands hygiene as well as controlling the market. Furthermore, the consumption of shell, crustaceans, raw or not well cooked fish as well as the cross contamination with food and swimming when injuries preexist has to be avoided.

Care must be taken of, the hygiene, the consumed water during treatment, the ranking order of safe regions and the unsafe regions in harbors. The improvement of the detection methods is required as well.

Finally, the use of complementary studies based on the development of search techniques (PCR), ensures not only a better specifity than classical bacteriological techniques for the determination of species, but also allows to focus on the genus of pathogen bacteria.

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