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THE INCIDENCE OF NON-CHOLERIC VIBRIO ISOLATION IN CRUSTACEAN CAPTURED FROM SOTHERN'S ROMANIAN WATERS

INCIDENŢA IZOLĂRII VIBRIONILOR NON-HOLERICI DIN CRUSTACEE CAPTURATE DIN APELE SUDICE ROMÂNEŞTI

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ABSTRACT | REZUMAT

The investigations have searched the harvesting frequency of bacterial species of non-choleric Vibrio from aquatic shell-fish. These researches are continuing the last years began studies, for realizing completed and detailed statistics for the prevalence of aquatic shell-fish from Romanian waters and different types of aquatic ecosystems. Between 1997 and 2018 had been collected and analysed by complex bacteriological exams a total number of 11,916 crustaceans substantiations from the aquatic medium; the crustaceans samples had been directly captured from aquatic medium or trapped by fisherman. The samples have been brought to labs where has been made complex bacteriological exams for isolating and identifying the possible non-choleric Vibrio's species. From 11,916 prelevated and prepared substantiations it was isolated 191 stems of non-choleric Vibrio's species, representing a total harvesting frequency of 1.603 and has been identifying the following species: *V. alginolyticus* (71 stems), *V. parahaemolyticus* (57 stems), *V. vulnificus* (46 stems), *V.mimicus* (9 stems) şi *V.furnissii* (8 stems). The annual harvesting frequency has been: in 1997 of 2.07 %, in 1998 of 1.95 %, in 1999 of 2.48 %, in 2000 of 1.81 %, in 2001 of 1.15%, in 2002 of 1.03%, in 2003 of 1.33%, in 2004 of 1.57%, in 2005 of 2.11%, in 2006 of 1.28%, in 2007 of 2.44 %, in 2008 of 0.71 %, in 2009 of 1.36 %, in 2010 of 1.54 %, in 2011 of 1.37 %, in 2012 of 1.93 %, in 2013 of 1.53 %, in 2014 of 2.30 %, in 2015 of 1.57%, in 2016 of 0.81%, in 2017 of 1.37%, and in 2018 of 1.56 %.

Keywords: vibrio's, non-choleric, shell-fish, isolation, the incidence

Investigaţiile au urmărit evaluarea frecvenţei de izolare a vibrionilor non-holerici din diferite probe de crustacee capturate din mediul acvatic. Aceste cercetări continuă seria investigaţiilor iniţiate în urmă cu 10 ani în scopul întocmirii unor statistici detaliate referitoare la prevalenţa vibrionilor non-holerici în apele din România, precum şi în diferitele tipuri de ecosisteme acvatice. Între anii 1997–2018 au fost prelevate şi investigate prin examene bacteriologice complexe, un număr total de 11.916 probe de crustacee acvatice, capturate fie direct din bazinul hidrografic al zonelor de sud şi sud-est ale ţării (inclusiv zona litorală a Mării Negre) fie din capturile accidentale ale pescarilor. Probele au fost transportate în laboratorul de analiză unde au fost executate examene bacteriologice complexe în scopul izolării şi identificării eventualelor specii Vibrio non-holerice. Din numărul total de 11.916 probe analizate au fost izolate un număr de 191 tulpini aparţinând speciilor: *V. alginolyticus* (71 tulpini), *V. parahaemolyticus* (57 tulpini), *V. vulnificus* (46 tulpini), *V. mimicus* (9 tulpini) şi *V. furnissii* (8 tulpini), reprezentând o prevalenţă totală de 1,603. Frecvenţa anuală de izolare a fost: în 1997 de 2,07 %; în 1998 de 1,95 %; în 1999 de 2,48 %; în 2000 de 1,81 %; în 2001 de 1,15 %; în 2002 de 1,03 %; în 2003 de 1,33 %; în 2004 de 1,57 %; în 2005 de 2,11%; în 2006 de 1,28%; în 2007 de 2,44%; în 2008 - 0,71%; în 2009 de 1,36%; în 2010 de 1,54%; în 2011 de 1,37%; în 2012 de 1,93%; în 2013 de 1,53%; în 2014 de 2,30%; în 2015 de 1,57%; în 2016 de 0,81%; în 2017 de 1,37%, iar în 2018 de 1,56%.

Cuvinte cheie: vibrioni, non-holerici, crustacee, izolare, incidenţă

The bacterial species which are included in *Vibrio* genus which had a clinical significance (they are pathogens for humans and different species of animals) are usually present in the environment and form a native micro-flora from swam, pools, lakes with fresh-water and salted water, estuary, delta, seas, and oceans, especially for Temperate and Tropical Area (3, 14). Some specialists consider that are ubiquitary species because they survive and multiply in the most various medium, in the food the majority of isolation being quoted from fish, mollusks, or crustacean samples (3, 10). In recent years, there have been demonstrated some evidence of vibrion's existence in different environment types (settlings, plankton) the bacteria being taken and concentrated in the body by the aqua-

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tic animals. The study made in Seacoast waters of the USA and Australian Continent demonstrated the existence of real reservoirs of non-choleric vibrio in the crustacean, shellfish, and mollusks. That is why in some countries (for example the USA), the main infection source for the human is represented by the oyster's consumption and some of the food toxic-infections appear of the sea origin food mainly meat untreated thermal from the sea species (especially seafruit salads, sauces and lobster meat salad, and the uncooked shrimps).

The prevalence of food-borne produced with different *non-choleric Vibrio* species can touch in some countries (for instance Japan) values of over 70 % from the total of the infection cases with bacterial etiology. The number of cases from each episode varies from a person to several hundred persons. These studies made in other countries started our researches to state the existence of the non-choleric *Vibrio* species in Romanian waters, the isolation frequency from different types of environments, and biological samples.

MATERIALS AND METHODS

It was sampled and harvested aquatic crustacean samples from Danube Delta to Black Sea Confluence, Black Sea seacoast Area, salted lakes (Razelm area), and from different pools and rivers of South and Southeast Area.

The samplings were focused on representative aquatic crustacean species that are living in the mentioned areas. For example: from Danube Delta to Black Sea Confluence it was harvested samples of *Gammarus* sp., samples of *Corophium* sp., samples of *Daphnia* sp., samples of *Mesomysis kowalewski*, samples of *Artemia salina*; from Black Sea seacoast Area it was harvested samples of crab (*Pachygrapsus marmoratus*, *Macropipus holsatus* and *Carcinus mediterraneus*), samples of shrimps (*Crangon crangon*, *Palaemon adspersus*, *Palaemon elegans*), samples of marine *isopods* - "sea-fleas" (*Idothea baltica*, *Sphaeronema serratum*); from salted lakes of Razelm Complex it was harvested samples of *Artemia salina*, the most representative crustacean for this environment which is the nutritional base for seacoast fishes; from different pools and rivers of South and Southeast Area it was harvested samples of crawfish (*Astacus leptodactylus*, *Austropotamobius torrentium*, *Astacus astacus*), samples of *Daphnia sp.*, samples of *Cyclops sp.* and samples of *Gammarus pulex*. The study has been done in 22 years, from 1997 to 2018.

The samples were processed by special methods for isolating and identifying the bacterial species included in the Vibrio genus. The methods generally followed the stages stipulated by standardized methods to identify the *Vibrio parahemolyticus* and other *Vibrio* species in food and biological samples. The method was adjusted to reveal all the *non-choleric vibrio's* which might exist in samples. Many samples were processed using the method suggested by James O. Oliver in 1997 or using the method suggested by L. Tudor in 1999 (both methods were often used simultaneously to compare the results) (3, 5, 8, 11). The confirmation was made by using PCR method (14, 15). The samples were prepared and the content was extracted and homogenized, afterward being obtained 1:1 and 1:10 dilutions in alkaline buffered water (ABW), through their introduction in 50 and 180 ml ABW, respectively. The samples (0.2 g from 1:1 dilution and 0.1 ml from the decimal dilution) were distributed in plates with agar containing 1 % tryptone and 3 % sodium chloride, in duplicates. These plates were further on incubated at 37 °C for 18-24 h. The *DNA extraction* - the bacterial cultures and the crustacean and shellfish samples were centrifuged at 5000 rpm for 10 min and the sediment was collected using a QIAamp DNA kit.The DNA quantity and its purity were determined using spectrophotometry, obtaining the percentages for 260/280 nm. The concentrated DNA (a result of the ethanol precipitation) was subjected to vacuum evaporation, through centrifugation. The sediment was suspended again in 50 or 100 µl of Tris-EDTA buffer solution (pH=8; 10 mM Tris,1 mM EDTA) and incubate at 65°C for 10 min to solubilize the DNA. The concentrated DNA was stored at -20°C (14, 15).

The number of yearly samples harvested and processed and species of crustacean identified is presented in Table no. 1. From the total number of samples, 191 *Vibrio* strains were isolated, belonging to *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. mimicus*, and *V. furnissii* species.

RESULTS AND DISCUSSIONS

From a total number of 11916 aquatic crustaceans samples harvested for 22 years long,191 *Vibrio* strains were isolated, the isolation prevalence being established at 1603. *V. alginolyticus* species was the most frequent isolated one (71 strains), followed by *V. parahaemolyticus* (57 strains) and *V.vulnificus* (46 strains) and the less isolated species were *V. mimicus* (9 strains) and *V. furnissii* (8 strains).

The results of the isolation by years and sampling areas are presented in Tables 2 and 3.

Following the processing of the samples, one important observation has been revealed concerning the non-choleric Vibrio species eco-biology: in the case of the samples harvested from the saltwater crustaceans, the majority vibrions strains were isolated from the visceral parts, while in the case of the samples harvested from the freshwater crustaceans the most of the isolations have been done from the cephalophorous seg-

ment. Moreover, the prevalence of non-choleric vibrions isolation is higher from the saltwater crustaceans samples harvested from the salt lakes or the seaside water, comparatively to the samples harvested from the fresh waters. As the scientific data reveals (2,3), the frequency of non-choleric vibrions isolation is relatively high in species on superior levels of trophic aquatic ecosystem pyramid. This correlation was noticed by

Table 1

Non-choleric Vibrio strains isolated from aquatic crustacean samples

Sampling Area	Year	Total number of samples	Total number of isolated stems	Year	Total number of samples	Total number of isolated stems
Danube Delta to Black Sea Confluence	1997	92	3 stems	2008	87	2 stems
	1998	88	2 stems	2009	89	3 stems
	1999	99	3 stems	2010	92	1 stem
	2000	104	4 stems	2011	79	2 stems
	2001	82	2 stems	2012	85	2 stems
	2002	85	1 stem	2013	87	1 stem
	2003	85	2 stems	2014	81	5 stems
	2004	93	2 stems	2015	84	2 stems
	2005	97	3 stems	2016	92	1 stem
	2006	81	1 stem	2017	85	3 stems
	2007	78	4 stems	2018	88	2 stems
	Total period		**1,933 samples - 51 stems**			
Black Sea seacoast Area	1997	185	2 stems	2008	185	1 stem
	1998	203	3 stems	2009	164	2 stems
	1999	171	4 stems	2010	192	3 stems
	2000	193	2 stems	2011	188	2 stems
	2001	197	2 stems	2012	181	2 stems
	2002	183	3 stems	2013	197	5 stems
	2003	192	2 stems	2014	184	2 stems
	2004	206	2 stems	2015	179	1 stem
	2005	154	3 stems	2016	167	2 stems
	2006	162	4 stems	2017	183	1 stem
	2007	178	2 stems	2018	174	3 stems
	Total period		**4,018 samples - 53 stems**			
Razelm Area	1997	61	3 stems	2008	72	1 stem
	1998	54	2 stems	2009	68	2 stems
	1999	59	2 stems	2010	52	3 stems
	2000	67	3 stems	2011	74	2 stems
	2001	72	2 stems	2012	65	3 stems
	2002	65	2 stems	2013	66	1 stem
	2003	62	4 stems	2014	58	6 stems
	2004	71	4 stems	2015	59	3 stems
	2005	58	3 stems	2016	61	1 stem
	2006	66	2 stems	2017	67	2 stems
	2007	57	5 stems	2018	64	2 stems
	Total period		**1,398 samples - 58 stems**			
South and Southeast Area Rivers and Pools	1997	193	3 stems	2008	221	0 stems
	1998	220	4 stems	2009	195	0 stems
	1999	154	3 stems	2010	184	1 stem
	2000	243	2 stems	2011	169	1 stem
	2001	257	1 stem	2012	187	3 stems
	2002	247	0 stems	2013	172	1 stem
	2003	261	0 stems	2014	198	0 stems
	2004	268	2 stems	2015	188	2 stems
	2005	259	3 stems	2016	173	0 stems
	2006	238	0 stems	2017	175	1 stem
	2007	178	1 stem	2018	187	1 stem
	Total period		**4,567 samples - 29 stems**			
South and Southeast Area of Country	**Total period**		**11,916**		**191 stems**	

the authors within the marine ecosystem (the shore area, the Razelm – Sinoe Complex area, the Delta flow area), where the vibrions isolation frequency is higher comparatively to the statistic of other aquatic species isolation (on inferior levels of trophic pyramid) or comparatively to water and sediment isolation (8, 11, 13).

Table 2

Yearly prevalence of non-choleric Vibrio species isolated from crustaceans

Sampling Area	Annual Prevalence (%)										
	1997	1998	1999	2000	2001	2002	2003	2004	2005	**2006**	**2007**
Danube Delta to Black Sea Confluence	3.26	2.27	3.03	3.85	2.44	1.18	2.35	2.15	3.09	**1.23**	**5.13**
Black Sea Seacoast Area	1.08	1.48	2.34	1.04	1.02	1.64	1.04	0.97	1.95	**2.50**	**1.12**
Razelm Area	4.92	3.70	3.39	4.48	2.78	3.08	6.45	5.63	5.17	**3.03**	**8.77**
South and Southeast Area Rivers and Pools	1.55	1.82	1.95	0.82	0.39	0	0	0.75	1.16	**0**	**0.01**
South Area (total number of samples)	**2.07**	**1.95**	**2.48**	**1.81**	**1.15**	**1.03**	**1.33**	**1.57**	**2.11**	**1.28**	**2.44**

Table 3

Yearly prevalence of non-choleric Vibrio species isolated from crustaceans

Sampling Area	Annual Prevalence (%)										
	2008	2009	2010	2011	2012	2013	2014	2015	2016	**2017**	**2018**
Danube Delta To Black Sea Confluence	2.30	3.37	1.09	2.53	2.35	1.15	6.17	2.38	1.09	**3.53**	**2.27**
Black Sea Seacoast Area	0.54	1.22	1.56	1.06	1.10	2.54	1.09	0.56	1.20	**0.55**	**1.72**
Razelm Area	1.39	2.94	5.77	2.70	4.61	1.51	8.62	5.08	1.64	**2.98**	**3.12**
South and Southeast Area Rivers and Pools	0	0	0.54	0.59	1.60	0.58	0	1.06	0	**0.57**	**0.53**
South Area (total number of samples)	**0.71**	**1.36**	**1.54**	**1.37**	**1.93**	**1.53**	**2.30**	**1.57**	**0.81**	**1.37**	**1.56**

Table 4

The total prevalence of non-choleric Vibrio species isolated from crustaceans

Sampling Area	Total number of samples	Total Prevalence (%)
Danube Delta to Black Sea Confluence	1,933	2.64
Black Sea seacoast Area	4,018	1.32
Razelm Area	1,398	4.15
South and Southeast Area Rivers and Pools	4,567	0.64
South Area (total number of samples)	11,916	1.603

CONCLUSIONS

The total prevalence of bacteria isolation from *Vibrio* genus (non-choleric vibrio's) for the period of the study was 1.603%. The yearly variations were low, between 2.48 % maximum and 0.71 % minimum. By statistical analysis of obtained data, it was established that the *non-choleric vibrio's* isolation frequency for the samples harvested in Razelm area (8.77% maximum in 2007) is higher than in the river areas (maximum 1.95% in 1999).

The most frequent isolated species were *V. alginolyticus* (71 strains), followed by *V. parahaemolyticus* (57 strains) and *V. vulnificus* (46 strains) and the less isolated ones were *V. mimicus* (9 strains) and *V. furnissii* (8 strains) species. There is a direct link within aquatic ecosystems concerning the *non-choleric vibrio's* contamination of the species from the different levels of trophic pyramid. The main contamination source is represented by invertebrates which generate the zooplankton contaminating the secondary and tertiary consumers (crustaceans and fishes).

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