Viral contamination and depuration of oysters under field conditions

Contamination virale et décontamination des huîtres dans le milieu naturel

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Abstract

Shellfish consumption is clearly associated with the transmission of enteric viruses. The channel of transmission of enteric viruses to human beings could be decreased particularly if the systems of depuration of shellfish were optimized, standardized and if their viral efficiency was controlled. The purpose of this work was to study the contamination of oysters and to assess the efficiency of several depuration processes.

Our study have demonstrated that in some cases, 50% of oysters samples contain enteric viruses or H.A.V. antigen and the quantities of viruses range from 17 to 246 FF for the rotaviruses and from 124 to 200 for the enteroviruses per 100 g of tissue.

According to our results, the exclusive use of a bacteriological criterium to determine the sanitary quality of oysters is insufficient.

Regarding the methodologies of the depuration of oysters, it appears that maintaining naturally contaminated oysters in disgorging tanks for 3 days gives satisfying bacteriological results but does not yield in every cases to the disappearance of the hepatitis A antigen.

Keywords: Oysters, contamination, depuration, enterovirus, rotavirus, Hepatitis A virus.

Résumé

La consommation de coquillages est clairement associée à la transmission de virus entériques. La voie de transmission des virus entériques à l'homme pourrait être réduite, notamment en optimisant et en normalisant les systèmes de purification des coquillages et en contrôlant leur efficacité contre les virus. Ces travaux ont pour objectif d'étudier la contamination chez l'huître et d'évaluer la performance de plusieurs procédés de purification.

Notre étude a mis en évidence que, dans certains cas, 50 % des prélèvements d'huîtres contiennent des virus entériques ou l'antigène HAV, avec des quantités de virus entre 17 et 246 FF pour les rotavirus, et de 124 à 200 pour les entérovirus, par 100 g de tissu.

Au vu de nos résultats, l'usage exclusif d'un critère bactériologique pour définir la qualité sanitaire des huîtres est insuffisant.

En ce qui concerne les méthodes de purification des huîtres, il apparaît que le maintien d'huîtres naturellement contaminées dans des bassins de dégorgement pendant 3 jours produise des résultats bactériologiques satisfaisants, mais n'aboutisse dans tous les cas à la disparition de l'antigène HAV.

Mots-clés : Huîtres, contamination, purification, entérovirus, rotavirus, virus de l'hépatite A.

INTRODUCTION

Shellfish consumption is clearly associated with the transmission of enteric viruses. Epidemics were reported in many countries (table I). The recent epidemics with viral aetiology transmitted by molluscs are gastro-enteritis (*Norwalk* virus and small round viruses) and hepatitis A (table I).

The transmission of enteric viruses by molluscs is perfectly understandable when one considers their alimentation system by filtration of great quantities of water. They are thus capable of accumulating the pathogenic micro-organisms living in sea water.

The channel of transmission of enteric viruses to human being could be decreased if a virological control of shellfish was established, if an accrued protection of the coastal zones was put in place and finally if the systems of depuration of shellfish were optimized, standardized and if their viral efficiency was controlled.

The objective of this work was to study the contamination of oysters in natural and artificial medium and to assess the efficiency of several depuration processes.

Table I: Viral epidemics related to shellfish consumption (from Schwartzbrod, 1991)

Years	Shellfish	Country	Number of cases of illness	Number of epidemic	Virus	References
1980	Oysters	Florida, USA	6	1	Norwalk	Gunn *et al.*, 1982
1980	Oysters	Philippines	7	1	Hepatitis A	Rao *et al.*, 1986
1980-1981	Cockles	Great-Britain	424		Hepatitis A	O'Mahony *et al.*, 1983
1982	Clams	New York, USA	150	14	Norwalk, Hepatitis A	Rao *et al.*, 1986
1982	Clams	New York, USA	813	103	Norwalk	Morse *et al.*, 1986
	Oysters	New York, USA	204		Norwalk	Morse *et al.*, 1986
1983	Oysters	London, U.K.	181	1	Norwalk	Gill *et al.*, 1983
1983-1984	Clams	New Jersey, USA	136	6	Gastroenteritis (Norwalk)	Porter and Parkin, 1987
1984	Cockles	Singapore	322	1	Hepatitis A	Goh, 1984
1985	Oysters	Great-Britain	13		Gastroenteritis (Norwalk)	Heller *et al.*, 1986
1985	Clams	New York, USA	5	1	Gastroenteritis	Vogt et Satkin, 1986
1988	Clams	Shangaï, China	292.301	1	Hepatitis A	Yao, 1990

Material and methods

Shellfish: oysters (*Crassostrea gigas*), harvested in Brittany coastal areas, were used in this study.

Virus: the simian rotavirus SA11 (ATCC VR 899) was used as a model for the artificial contamination of oysters. The rotavirus strain was grown and titrated in MA104 cells.

Cell culture: MA104 and BGM cell lines were grown at 37°C in 5% CO_2 in MEM (Eurobio) supplemented with 10% or 2% heat inactivated fœtal calf serum (Labsystem) and containing 100 IU/ml of penicillin and 100 µg/ml of streptomycin. When the MA104 cells were inoculated, MEM is not supplemented with fœtal calf serum and contains 8 µg trypsin per ml (Difco 1 : 250).

Virus extraction-concentration procedures: first, oysters tissues (100 g) are finely ground for 5 minutes in a Warring-Blendor at 10,000 rpm. Then viruses are eluted by blending tissues in 200 ml of a borate buffer 0,1 M–3% beef extract pH 9. After an ultrasonication and a centrifugation at 10,000 x g for 45 minutes, the supernatant is collected and viruses are concentrated by addition of PEG 6000 to a final concentration of 10% (wt/vol) and 1.5% NaCl. The sample is kept overnight at 4°C, the precipitate is collected by centrifugation (10,000 x g for 90 mn at 4°C) and the pellet suspended in 40 ml of borate-beef extract buffer. After a magnetic stirring, this suspension is centrifuged (10,000 x g for 30 mn) and the supernatant is neutralized and submitted to a second concentration by adding PEG 6000 to a final concentration of 10% (wt/vol) and 1.5% NaCl. After one night at 4°C, the precipitate is collected by centrifuging (10,000 x g for 30 mn) and the pellet suspended in 10 ml of PBS buffer pH 9 (Biziagos, 1989). The concentrate is then detoxified by filtration through a Sephadex LH20 gel as previously described (Béril *et al.*, 1991).

Rotavirus assay: samples (100 µl) of diluted and undiluted detoxified concentrates are inoculated into 96-wells microtiter plate with confluent monolayer of MA104 cells. After a 48 hours incubation, the cell cultures are fixed with alcohol-acetone and the rotaviruses are revealed by an indirect immunofluorescence method. Results are expressed as fluorescent foci (F.F.) per g of tissue.

Enterovirus assay: samples (0.5 ml) of diluted or undiluted detoxified concentrates are inoculated into 6-wells cell culture plates with confluent cultures of BGM cells. After 2 hours of adsorption at 37°C, the inoculum is carefully removed and 5 ml of maintenance medium (MEM supplemented with 2% fœtal calf serum and antibiotics) is added in each well. Incubation is continued at 37°C. Even if no cytopathogenic effect is observed within 12 days, the cells are frozen 3 times and subjected to a second passage. The cytopathogenic agents are quantified according to Fisher's table method.

Hepatitis A antigen detection (HAV Ag): a solid-phase radio immunoassay (RIA) is performed, based on a method described by Purcell *et al.* (1976). Antibodies to HAV obtained from a convalescent human serum (Ig G titer 200 IU/ml) are diluted 200 fold before use. One hundred µl of these human antibodies are coated onto wells of a polyvinyl microtiter plate for 4 days at 4°C. After washing, 50 ml of Ig G anti-HAV labelled with ^{125}I are added in each well

and the plate is incubated at room temperature overnight. After repeated washes, the bound radioactivity of each well is counted with a gamma counter. A ratio of sample value (P) to negative control value (N) $\geq 2,1$ is considered as positive.

A confirmation test, based on a competition between a negative and a positive serum, is performed for each positive sample.

Oysters contamination

Contamination in coastal area: The oysters were placed in Morlaix bay (Brittany-France) for a few months. The water in Morlaix bay is contaminated by urban and agricultural pollution.

Contamination in a pilot plant: 6 baskets containing 12 to 15 kg of oysters were dipped into 3 different locations of a 5,000 litres-tank for one hour. This tank was full of natural sea water artificially contaminated by 5.10^9 rotaviruses previously fastened on unicellular algae (*Dunaliella primolecta*).

Oysters depuration: The oysters harvested from the coastal area were placed in a disgorging tank filled with natural aerated sea water for 72 hours.

The oysters previously contaminated in the pilot plant were depurated in two 1,000 litres experimental tanks during 72 hours. Two different processes were used. Three oysters baskets collected at 3 different locations in the contamination tank, were placed in a closed circuit in which natural sea water was continuously treated by U.V. radiations (94 $mW.s^{-1}.cm^{-2}$). The remaining 3 baskets of oysters were placed in a semi open circuit in which non-treated sea water was changed once a day.

Oysters sampling: before any depuration treatment, oysters samples are collected to evaluate their initial contamination. To determine the quantity of viruses present in the oysters contaminated in the pilot plant, a sample from each basket is submitted to an individual analysis. Then, oysters samples were drawn after 72 hours of disgorging, from the coastal oysters and from the 3 baskets placed in a semi open circuit. Oysters samples were drawn after 24, 48 and 72 hours from 3 baskets placed in a closed circuit tank with U.V. radiations.

Results

Oysters contamination

• ***Oysters harvested in Morlaix bay***

The results are shown in table II: it appears that 8 of the 12 samples collected from the bay of Morlaix are positive to the virological analysis :

- 2 samples contained rotaviruses,
- 3 samples revealed the presence of HAV antigen,
- 2 samples contained enteroviruses,
- 1 sample contained both rotaviruses and HAV antigen.

• ***Oysters artificially contamined***

The results are shown in table III: the shellfish contamination varied from 390 to 53,150 FF of rotaviruses per 100 g of tissue. The degrees of contamination appear very heterogeneous. This is probably due to a poor distribution of the rotaviruses in the sea water during the contamination period, leading to an insufficient exposure of some oyster baskets to the viral contamination.

Table II: Microbial contamination of oysters harvested in Morlaix bay

		VIRAL CONTAMINATION			BACTERIAL CONTAMINATION		
Month	Sample	Rotavirus (F.F./100 g of tissue)	HAV Ag	Enteroviruses (/100 g of tissue)	Fæcal coliforms (/ 100 g of tissue)	Fæcal Streptococci (/ 100 g of tissue)	Salmonella (/ 25 g of tissue)
March	1	0	–	0	258	2 580	–
	2	0	–	0	258	1 440	–
April	3	**17** (4–90)*	–	0	7 200	840	–
	4	0	–	0	2 790	2 790	–
May	5	0	–	0	4 500	2 790	+
	6	**246** (6–1 370)	–	0	>72 000	13 800	+
June	7	**135** (3–750)	+	0	1 290	270	–
	8	0	+	0	330	<90	–
July	9	0	+	0	>72 000	N.A.	+
	10	0	+	0	2 250	N.A.	–
September	11	0	–	**200** (71 – 310)	108	690	+
	12	0	–	**124** (68 – 295)	2 790	220	–
October	13	0	–	0	<90	450	–
	14	0	–	0	7 200	690	–
November	15	0	–	0	13 800	7 200	+
	16	0	–	0	4 500	33 000	–

HAV Ag: Hepatitis A antigen
N.A.: No Assay

* 95% confidence limits

Table III: Virus recovery from artificially contaminated oysters

Localization of the baskets of oysters	Recovery of rotaviruses (F.F./100 g of tissue)
Place 1	**390** (20 – 2216)
Place 2	**36 620** (30 910 – 42 962)
Place 3	**53 150** 44 611 – 62 645)

Oysters depuration

• ***Oysters, from Morlaix bay, placed in a disgorging tank:***

The microbial contamination of the oysters after 72 hours of depuration is shown on table IV.

Table IV: Microbial contamination of oysters before and after 72 hours in the disgorging tank

(before : samples 7, 9, 11, 13, 15, 17, 18, 19, 20) ;
(after : samples 7', 9', 11', 13' 15', 17', 18', 19', 20')

		VIRAL CONTAMINATION			BACTERIAL CONTAMINATION		
Month	Sample	Rotavirus (F.F./100 g of tissue)	HAV Ag	Enteroviruses (/100 g of tissue)	Fæcal coliforms (/ 100 g of tissue)	Fæcal Streptococci (/ 100 g of tissue)	Salmonella (/ 25 g of tissue)
March	1	0	–	0	258	2 580	–
	1'	0	–	0	<90	<90	–
April	3	**17** (4–90)*	–	0	7 200	840	–
	3'	0	–	0	<90	<90	–
May	5	0	–	0	4 500	2 790	+
	5'	0	–	0	<90	<90	–
June	7	**135** (3–750)*	+	0	1 290	270	–
	7'	0	+	0	<90	<90	–
July	9	0	+	0	>72 000	N.A.	+
	9'	0	–	0	<90	<90	–
September	11	0	–	**200** (10–310)	108	690	+
	11'	0	–	0	<90	<90	–
October	13	0	–	0	<90	450	–
	13'	0	–	0	<90	690	–
November	15	0	–	0	13 800	7 200	+
	15'	0	–	0	<90	450	–
December	17	0	–	0	270	258	–
	17'	0	–	0	N.A.	N.A.	–
January	18	0	–	0	1 290	<90	–
	18'	0	–	0	<90	108	–
February	19	0	–	**10** (1–80)	2 790	N.A.	–
	19'	0	–	0	<90	<90	–
March	20	0	–	0	1 290	7 200	–
	20'	0	–	0	<90	<90	–

HAV Ag: Hepatitis A antigen
N.A.: No Assay

* 95% confidence limits

After 72 hours of decontamination, 4 of the 5 contaminated samples were negative to the virological analysis. One sample, initially contaminated with rotaviruses and HAV Ag was still HAV Ag positive. All of the samples satisfied the french bacteriological public health standards (<300 fecal coliforms per 100 ml tissue). It appears that this disgorging procedure is not efficient enough to completely eliminate viral antigens, but all the bacteriological standards were reached.

• ***Oysters artificially contaminated and depurated in the experimental plant***

The viral contamination of oysters after 72 hours of depuration in the semi open circuit is shown on the table V. None of the samples were positive for rotaviruses.

The viral contamination of the oysters after 24, 48 and 72 hours of depuration in the closed circuit, is shown on the table VI. In the closed circuit setting, none the samples were still contaminated with rotaviruses after 24, 48 and 72 hours of depuration. Likewise, a 72 hours decontamination in the semi open circuit was a completely efficient treatment to eliminate the rotaviruses. Both depuration processes (water disinfected with UV or completely changed once a day) seem to give good results with the rotaviral model.

DISCUSSION

Regarding the level of natural contamination of the oysters in the bay of Morlaix, 50% of the samples contain enteric viruses or HAV Antigen.

Furthermore, the quantities of viruses found are important and range from 17 to 246 FF for the rotaviruses and from 124 to 200 for the enteroviruses per 100 g of tissue. Only one sample was doubly contaminated (rotaviruses + HAV Ag). Several conflicts may be shown when comparing the virological

Table V: Viral contamination of oysters after depuration in the semi-opened circuit

	ROTAVIRUS RECOVERY
Oysters from the place 1	N.D.
Oysters from the place 2	N.D.
Oysters from the place 3	N.D.

Table VI: Viral contamination of oysters after depuration in the closed circuit

	ROTAVIRUS RECOVERY		
	After 24 h	After 48 h	After 72 h
Oysters from the place 1	N.D.	N.D.	N.D.
Oysters from the place 2	N.D.	N.D.	N.D.
Oysters from the place 3	N.D.	N.D.	N.D.

N.D.: Non detected

results with those originating from the bacteriological analysis. Indeed, when 6 virus-positive samples have a fecal coliform count largely above the salubrity norms (300 per 100 ml of tissue), in one sample containing HAV antigens, only 330 fecal coliform and less than 90 fecal streptococci per 100 g of tissue were found. Finally, one sample shows a great contamination (200 per 100 g) with enteroviruses when it fully satisfies to the bacteriological norms (108 fecal coliforms and 690 fecal streptococci per 100 g of tissue). An obvious discrepancy is observed between bacteriological and viral contaminations. Indeed, in some cases enteric viruses are present with a small bacteriological contamination, whereas in other cases the bacteriological contamination is very large when no viruses could be isolated from the sample. This proves that bacteria indicators of fecal contamination are bad indicators of viral contamination.

The depuration by treatment in a disgorging tank for 3 days of naturally contaminated oysters is satisfying on the bacteriological point of view. It eliminates the rotaviruses and the enteroviruses. However, in one case out of two, the HAV antigen remains present in the oysters after depuration. If the HAV Ag is regarded as an indicator of viral contamination, not only the depuration method used is defective but also the determination of the sanitary quality of the shellfish using bacteriological criteria seems inadequate in the case of a viral contamination.

The contamination in the 5,000 litres tank is widely variable according to the location of the samples in the tank. The extreme heterogeneity of the oyster contamination which had the same origin and which did not show any difference in their physiological state may be explained with several hypotheses. It may be the consequence of a poor distribution of the viral particles in the contamination tank due to a poor water circulation. It may also be due to a poor circulation of the water through the baskets, thus preventing an optimum contact between the oysters and the contaminating water. Finally, it may be due to possible differences in the filtration activities of the oysters.

CONCLUSION

According to our results and as numerous authors have written (Gerba *et al.*, 1980; Grabow *et al.*, 1989; Havelaar, 1987), the exclusive use of a bacteriological criterium to determine the sanitary quality of oysters is insufficient. It is imperative to pursue research programs aimed at determining a viral contamination indicator.

Regarding the methodologies of the depuration of oysters, it appears that maintaining naturally contaminated oysters in disgorging tanks for 3 days gives satisfying bacteriological results but does not yield in every cases to the disappearance of the Hepatitis A antigen.

Finally, regarding oysters artificially contaminated with rotaviruses, the depuration in closed circuit as well as in semi open circuit gives satisfying virological results. However, in order to conclude that the depuration methodology is effective and trustworthy, the results must be confirmed by more experimentation after contamination with rotaviruses as well as with other enteric viruses.

REFERENCES

Béril-Stien C., S. Boher, L. Schwartzbrod, 1991. Detoxification by Sephadex LH20 of seafood concentrates for rotavirus from mussels and oysters. *Wat. Sci. Tech.*, **24,** 417-421.

Biziagos E., 1989. Virus de l'hépatite A : méthodes d'études dans les milieux hydriques. Effet de substances antivirales sur la multiplication *in vitro*. Thèse de doctorat, Université Claude-Bernard, Lyon. 170 p.

Gerba C.P., S.M. Goyal, I. Lech, G.F. Bogdan, 1980. Bacterial indicators and environmental factors as related to contamination of oysters by enteroviruses. *J. Food Prot.*, **43**, 99-101.

Gill O.N., W.D. Cubitt, D.A. Mc Swiggan, B.M. Watney, C.L.R. Barlett, 1983. Epidemic of gastroenteritis caused by oysters contaminated with small round structured viruses. *Br. Med. J.*, **27**, 1532-1534.

Goh K.T., 1984. An epidemic of cockles - associated hepatitis A in Singapore. *Bull. WHO*, **62**, 893-897.

Grabow W.O.K., G.K. Idema, P. Coubrough, B.W. Batteman, 1989. Selection of indicator systems for human viruses in polluted seawater shellfish. *Wat. Sci. Tech.*, **21**, 111-117.

Gunn R.A., H.T. Janowski, S. Lieb, E.C. Prather, H.B. Greenberg, 1982. Norwalk virus gastroenteritis following raw oyster consumption. *Am. J. Epidemiol*, **115**, 348-351.

Havelaar A.H., 1987. Bacteriophages as model organisms in water treatment. *Microbiol. Sci.*, **4**, 362-364.

Heller D., O.N. Gill, E. Raynham, T. Kirkland, P.M. Zadick, R. Stanwell-Smith, 1986. An outbreak of gastrointestinal illness associated with consumption of raw depurated oysters. *Brit. Med. J.*, **292**, 1726-1727.

Morse D.L., J.J. Guzewich, J.P. Hanrahan, R. Stricof, M. Shayegani, R. Deibel, J.C. Grabau, N.A. Norwak, J.E. Herrmann, G. Cukor, N.R. Blacklow, 1986. Widespread outbreaks of clam and oysters associated gastroenteritis. Role of Norwalk virus. *N. Engl. J. Med.*, **314**, 678-681.

O'Mahony M.C., C.D. Googh, D.A. Smyth, A.J. Thrussel, C.L.R. Bartlett, N.D. Noha, 1983. Epidemic hepatitis A from cockles. *Lancet,* **1**, 518-520.

Porter J., W. Parkin, 1987. Outbreak of clam associated gastroenteritis in New Jersey : 1983-1984. *N. Eng. J. Med.*, **84**, 649-651.

Purcell R.H., D.C. Wong, Y. Moritsugu, J.L. Dienstag, J.A. Routenberg, J.D. Boggs, 1976. A microtiter solid-phase radio immuno assay for hepatitis A antigen and antibody. *J. Immun.*, **116**, 349-356.

Rao V.C., T.G. Metcalf, J.L. Melnick, 1986. Human viruses in sediments, sludges and soils. *Bull. WHO,* **64**, 1-13.

Schwartzbrod L., 1991. Virologie des milieux hydriques. Tec. et Doc. Lavoisier éd. Paris, France, 301 p.

Vogt R.L., F. Satkin, 1986. Clam associated *gastroenteritis N. Engl. J. Med.*, **315**, 582-583.

Yao G.B., 1990. Clinical and natural history of hepatitis A in an epidemic in Shangaï 1988. International Symposium on viral hepatitis and liver disease. Houston.