STUDY ON THE RADIATION SENSITIVITY OF PATHOGENIC *VIBRIONACEAE* AND *ENTEROBACTERIACEAE IN VITRO* AND AFTER INOCULATION INTO OYSTERS (*CASSOSTREA BRASILIANA*)

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Abstract

In vitro studies were conducted to evaluate the effects of ionizing radiation on various biotypes and serotypes of *Vibrio cholerae* (different biotypes and serotypes from group O1 and one strain from group O139); *V. parahaemolyticus*, *V.vulnificus*, *V. fluvialis*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*; *Salmonella* typhi, *S. enteritidis*, *S. typhymurium*, *Shigella flexneri* and *Escherichia coli* O157:H7. *In vivo* tests were also conducted in oysters allowed to self-contaminate with *V. cholerae* and *S. enteritidis* cultures in sea water tanks through the natural feeding process of the mollusks. Bacterial populations irradiated (0.5–3.0 kGy) in pure culture in liquid broth or in oysters varied from 10^6 to 10^{10} colony forming units per mL or gram (CFU/g or mL), respectively. The decrease in viable cells through the radiation dose range applied varied from 4 to 10 $\log_{10}$. The lowest radiation resistance was found in *Vibrio* spp. *Aeromonas hydrophila*, and *E. coli* O157:H7, whereas *S. enteritidis* and *S. typhimurium* proved to be the most resistant species tested. A dose of 1.5 kGy was determined to be appropriate for elimination of up to 10^{10} of bacteria tested except *Salmonella* spp. particularly, *S. enteritidis*, which required 3.0 kGy for complete elimination. Radiation doses of up to 3.0 kGy were not lethal to oysters.

1. INTRODUCTION

Bivalve mollusks are marine organisms which are often consumed in raw form while still alive (De Paola *et al.*,1983; Varnam and Evans, 1991). They inhabit coastal areas and are considered to be "sentinels" of the environment in which they live because of their food intake pattern, which involves an hourly filtration of up to 6 L of water that causes mollusks to concentrate microorganisms, including potential human pathogens, in their digestive system (Madden *et al.* 1982; Costa, 1983; Rodriguez *et al.* 1990; Donini *et al.* 1993). As a result, bivalve mollusks are potential vehicles for foodborne diseases (Saliba and Helmer, 1990), and are thus frequently implicated in outbreaks of cholera, salmonellosis, shigellosis, and other serious diseases (Klontz *et al.* 1987; IOM, 1991; Varnam and Evans, 1991; Madden *et al.* 1982; PAHO, 1991a). Therefore, decontamination processes that may contribute to reduce the microbiological hazard posed by bivalve mollusks are or should be of interest to public health (Eyles and Davey, 1984; Saliba and Helmer, 1990).

Ionizing radiation is a technology that can be used to eliminate microbiological hazards in foods (Ley, 1966; ICMSF, 1980; Sang *et al.*, 1987; FAO/OIEA/OPS, 1992; Jay, 1992; Loaharanu, 1994). Moreover, ionizing radiation is one of the most effective available technologies for control of microbial hazards in bivalve mollusks for human consumption (Ley, 1966; Wood, 1970; ICMSF, 1980; Kilgen *et al.*, 1987; WHO, 1994; Jay, 1992; FAO/OIEA/OPS (OMS), 1997). In 1984, the Codex Alimentarius issued a standard to regulate the treatment of food with ionizing radiation (Anonymous, 1984). Several countries have since enacted regulations allowing this technology (IAEA, 1995). Varnam and Evans (1991) reported that a radiation dose of 3 kGy was sufficient to completely eliminate

pathogenic bacteria belonging to the *Vibrionaceae*, as well as *Salmonella* spp., in mollusks. Sang *et al.* (1987) used ionizing radiation to control *Salmonella* spp. in frog legs.

Laboratory experiments designed to study the efficiency of ionizing radiation to decontaminate bivalve mollusks, in general, have been conducted after sterilizing the mollusks prior to controlled inoculation, or using the naturally occurring bacterial flora for determining treatment effects. In contrast, experimental models using live oysters and natural feeding patterns to contaminate the mollusks, allow an evaluation of irradiation effects in the presence of the natural background flora (Gelli *et al.*, 1998). Moreover, these models allow contamination of oysters with high levels of pathogenic enteric bacteria which are less frequent, but possible, in natural environments.

The seventh cholera pandemia reached South America in 1991, establishing its devastating presence first in Peru (Tauxe and Blake, 1990; PAHO, 1991b; Ries *et al.*, 1992; Quevedo, 1993). This epidemic highlighted the need for urgent sanitary measures to control microorganisms having ample environmental dissemination (Loaharanu, 1994; Levine, 1991; PAHO, 1991b; Ries *et al.*, 1992; Swerdlow *et al.*, 1992). Such measures must be scientifically sound, technologically feasible, and economically viable. Irradiation has been proven to fulfill all of these conditions (WHO, 1994). For that reason, the International Atomic Energy Agency (IAEA) and the Pan American Health Organization (PAHO) organized a Co-ordinated Research Project to evaluate the applicability of irradiation as a public health intervention measure which, among other objectives, would evaluate the effect of irradiation as a decontamination method of bivalve mollusks (FAO/OIEA/OPS, 1992). The present study was undertaken as part of that project.

The objectives of the present study were: a) To evaluate the *in vitro* effects of ionizing radiation on selected pathogenic strains of *Vibrionaceae* and *Enterobacteriaceae*; b) to evaluate the effects of irradiation on some of the same bacterial strains (i.e. *V. cholerae* and *S. enteritidis*) *in vivo* after incorporation into live oysters (*Crassostrea brasiliana*) via natural oyster feeding patterns; and c) to determine oyster survival post-irradiation.

2. MATERIALS AND METHODS

1. *Bacterial strains and culture preparation*:

The strains used in the study were *Vibrio cholerae* O1, El Tor, Ogawa, non toxigenic; *V. cholerae* O1, El Tor, Ogawa, toxigenic; *V. cholerae* O1, El Tor, Inaba, toxigenic; *V.cholerae* O1, Classic, Ogawa, toxigenic; *V. cholerae* O139; *V. parahaemolyticus*; *V. fluvialis; V. vulnificus*; *Aeromonas hydrophila*; *Plesiomonas shigelloides; Salmonella typhi*; *S. enteritidis*; *S. typhimurium*; *Shigella flexneri;* and *Escherichia coli* O157:H7, verotoxigenic. All bacterial strains were obtained from the Bacterial Culture Collection Section of the Instituto Adolfo Lutz, Sao Paulo. The non-toxigenic strain of *V.cholerae* was isolated from sea water from the coast of the state of Sao Paulo. The El-Tor Inaba strain was isolated from a cholera patient in Peru, in 1991, whereas the El-Tor Ogawa strain was similarly isolated in Bolivia, in 1992. The *Salmonella enteritidis* and *S. typhimurium* strains used were isolated at the Instituto Adolfo Lutz from foods implicated in foodborne disease outbreaks.

Bacterial cultures were kept lyophilized until used. Prior to use, all cultures except the *Enterobacteriaceae* were grown in alkaline peptone water; the *Enterobacteriaceae* were grown in buffered peptone. Upon incubation of cultures at 37°C for 18–24 h, cultures were

reinoculated into the same media and incubated as before to ensure that actively growing cultures were used.

2. *Inoculation of oysters*:

Oysters from the genus *Cassostrea brasiliana* were obtained from a depuration plant in Cananeia, on the southern coast of the state of Sao Paulo. Oysters were inoculated by placing them in 40-L tanks filled with sand-filtered sea water in which the desired bacterial cultures were diluted according to the procedure described by Gelli *et al.* (1998). Oysters were left in the tank and allowed to feed in a natural way for 24 h.

3. *Irradiation*:

A ^{60}Co Gammacell II 220 irradiator (Atomic Energy of Canada, Ltd., AECL) belonging to the Coordenadoria de Aplicaciones en Inginieria e Industria, Instituto de Pesquisas Energeticas (IPEN/CNEN), Sao Paulo, was used to irradiate the samples. Fricke dosimeters were used to determine the absorbed dose in oysters.

4. *In vitro studies*:

Three tubes, each containing 3 mL of the suspension of one of the pure cultures described earlier, were prepared. One of the tubes was used to determine the initial numbers of viable cells. The second tube was irradiated, and the third served as non-irradiated control to determine possible adverse effects of transportation and time of irradiation on the cultures. Irradiation doses tested were 0.0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 kGy.

5. *In vivo studies*:

Twenty oysters inoculated as described earlier were used for every experiment. Five oysters were used to determine the initial bacterial load, which provided a measure of inoculum uptake by the oysters through natural feeding; the remaining oysters were irradiated as described earlier for pure cultures.

6. *Microbiological analyses*:

Standard methods were used for the microbiological analyses. Oysters were aseptically shucked from the shell, homogenized in a blender, and a 25-g sample was taken for dilution with 225 mL alkaline peptone water (*Vibrio* spp., *Aeromonas* and *Plesiomonas*) or buffered peptone water (*Enterobacteriaceae*). A sample of non-inoculated, non-irradiated oysters from each batch received in the lab was examined to determine the possible presence of naturally occurring microorganisms of the same species as those being inoculated.

One-mL aliquots were taken from the initial oyster meat homogenate and from the 5-mL tube containing the pure culture of each bacterium, for the *in vivo* and *in vitro* studies, respectively, to prepare serial dilutions according to standard methods. Serial dilutions were incubated at 35°C for 18–24 h. For *Vibrio* spp., the methods used were those described in APHA (1992) and AOAC (1994). For the *Enterobacteriaceae*, dilutions were incubated at 35°C for 24h, except for *Shigella flexneri* and *E. coli* O157, which were kept at that temperature for 6–8h and 18–24h, respectively.

Selenite-cysteine and modified Rappaport-Vassiliades media, and 42°C, were used for *Salmonella* spp. enrichment. No selective enrichment was used for *S. typhi*, and serial dilutions were plated directly on Salmonella-Shigella (SS) agar. This medium was also used for *Shigella*, whereas *E. coli* was recovered in Mac-Conkey-Sorbitol agar. Characteristic colonies of each bacterium were isolated from the corresponding media and identified using biochemical and serological profiles (APHA, 1992; AOAC, 1994).

V. cholerae cultures isolated from irradiated oysters (*in vivo* studies) or pure cultures (*in vitro* studies) were examined for possible radiation-induced changes in specific characteristics, such as biotype and toxin producing capacity, following AOAC (1994) methods.

7. *Evaluation of radiation effects on oysters*:

Non-inoculated oysters were irradiated at the same doses used for inoculated samples to test the lethality of various radiation doses. Oysters thus irradiated were kept under refrigeration (5°C) for up to 7 days, and their survival was determined daily.

All experiments were replicated three times.

3. RESULTS AND DISCUSSION

The oyster samples used in this study were found to be free of pathogenic microorganisms which were used for the artificial inoculation of the test samples. The data also indicated that transportation of cultures and oyster samples to the irradiation facility, as well as the time necessary for irradiation, did not have any effect on bacterial numbers. There were no alterations in *V. cholerae* biotype and toxin producing capacity, or on the biochemical characteristics of the cultures, due to irradiation.

A comparison of the data on bacterial counts obtained from *in vitro* studies (Tables 1 and 2) indicated wide variation in radiation resistance among the cultures selected for the study. As a group, the *Vibrionaceae* were more radiation sensitive than the *Enterobacteriaceae*. Whereas a dose of 1.5 kGy was enough to eliminate an initial contamination of 10^{10} colony forming units (CFU)/mL in pure culture suspensions of *Vibrio* spp., 2.5 kGy were necessary to achieve similar reductions in *Enterobacteriaceae* cultures. These results agreed with those of Kilgen *et al.* (1987). A comparison of Tables 1 and 2 show that the radiation resistance of *Salmonella typhi*, *Shigella dysenteriae*, and *E. coli* 0157:H7 was no different from that of the *Vibrio* spp., *Aeromonas* and *Plesiomonas* cultures tested. However, *Salmonella enteritidis* surviving cells were found in culture suspensions irradiated at doses as high as 2.0 kGy.

Similar results were obtained from *in* vivo studies using inoculated oysters (Table 3). No surviving cells of non-toxigenic *Vibrio* spp. culture tested were detected in oysters treated at doses above 1.5 kGy. In contrast, 3.0 kGy were needed to ensure complete elimination of 10^6 CFU/g *S. enteritidis* similarly inoculated into oysters.

A comparison of the data in Tables 1 and 2 with those in Table 3 highlights the protective effect of the oyster matrix on inoculated *Salmonella enteritidis* but not on *Vibrio cholerae* O1 Ogawa against radiation injury. A radiation dose of 3.0 kGy was necessary to achieve elimination of 10^6 CFU/g *S. enteritidis* in oysters than was needed to bring 10^{10} CFU/mL cells of *Vibrio cholerae* to non-detectable levels in pure culture suspensions.

Table 1: *In vitro* Effects of Ionizing Radiation on Potentially Pathogenic *Enterobacteriaceae*

Culture	Initial Count (CFU/mL)	Radiation Dose (kGy) and Bacterial Counts (CFU/mL)					
		0.5	1.0	1.5	2.0	2.5	3.0
S. *typhi*	10^8	10^5	10^3	10^1	ND	ND	ND
S.tm.	10^{10}	10^6	10^4	10^3	ND	ND	ND
S.e.	10^{10}	10^7	10^{-4}	10^3	10^1	ND	ND
Sh.f.	10^8	10^3	10^1	ND	ND	ND	ND
O157	10^9	10^4	10^2	ND	ND	ND	ND

S.tm — *Salmonella typhimurium.*
Sh.f. — *Shigella flexneri.*
S.e. — *Salmonella enteritidis.*
O157 — *Escherichia coli* O157:H7.
ND – None detected.

To determine the optimal radiation dose necessary to ensure absence of these pathogens in naturally contaminated oysters, it is essential to ascertain their naturally occurring levels, which may be seasonal and variable depending on the potential for fecal pollution of sea water in each area. This information may be specially important when oyster beds are located in coastal waters close to urban areas. According to Varnam and Evans (1991), 10 CFU/g *S. enteritidis* could cause salmonellosis in the most susceptible groups of consumers; thus the importance of tight control of water quality in bivalve mollusk production and extraction areas (Wood, 1970; IOM, 1991; APHA, 1992). Assuming that the mollusks are grown and collected using Good Primary Production Practices, doses lower than 3.0 kGy should provide a reasonable level of safety against *S. enteritidis*, and hence, against potentially pathogenic *Vibrionaceae.*

In relation to *Vibrio cholerae* O1, human ingestion of bacterial cells in numbers lower than 10^3/g are reported not to result in infection (Levine *et al.*, 1981). Irradiation at a dose of 1.5 kGy would suffice to eliminate high numbers of this pathogen, something desirable because, being a halophilic, aquatic microorganism, it can proliferate in sea waters rich in nutrients, and is able to also grow in numbers during commercialization of mollusks (Cook & Ruple, 1986).

Table 2: *In vitro* Effects of Irradiation on Pathogenic *Vibrionaceae, Aeromonas hydrophila* and *Plesiomonas shigelloides*

Culture	Initial Count (CFU/mL)	Radiation Dose (kGy) and Bacterial Counts (CFU/mL) 0.5	1.0	1.5	2.0	2.5	3.0
V.c. NT	10^{-10}	10^{-6}	10	ND	ND	ND	ND
V.c. El Tor Inaba	10^{-10}	10^{-6}	10^{-2}	ND	ND	ND	ND
V.c. El Tor Ogawa	10^{-10}	10^{-5}	10	ND	ND	ND	ND
V.c. Classic	10^{-10}	10^{-5}	10^{-1}	ND	ND	ND	ND
V.c. O139	10^{-10}	Not done	Not done	ND	ND	ND	ND
V.v.	10^{-10}	10^{-5}	ND	ND	ND	ND	ND
V.p.	10^{-10}	10^{-5}	10^{-2}	ND	ND	ND	ND
V.f.	10^{-9}	Not done	10	ND	ND	ND	ND
P.s.	10^{-8}	Not done	10	ND	ND	ND	ND
A.h.	10^{-9}	Not done	10	ND	ND	ND	ND

V.c – *Vibrio cholerae.* NT – Non toxigenic.
V.v.- *Vibrio vulnificus.* V.p. – *Vibrio parahaemolyticus.*
V.f.- *Vibrio fluvialis.* P.s. – *Plesiomonas shigelloides.*
A.h.- *Aeromonas hydrophila.*
ND – None detected.

Table 3: Effect of Ionizing Radiation on Numbers of *Vibrio cholerae* O1 Ogawa and *Salmonella enteritidis* Inoculated into Oysters via Natural Feeding

Bacterial Culture	Initial Count (CFU/g)	Radiation Dose (kGy) 0.5	1.0	1.5	2.0	2.5	3.0
3. V.c.[a]	10^6	10^3	10^2	ND	Not done	Not done	Not done
S.E.[b]	10^6	10^4	10^3	10^2	10	+[c]	ND

V.c. – *Vibrio cholerae* O1 Ogawa, non toxigenic.
S.E. – *Salmonella enteritidis.*
[a] – Mean of 3 replications.
[b] – Mean of 2 replications.
+ = <10 but positive.
ND – None detected.

Irradiation tests conducted using non-inoculated oysters revealed that their viability, as well as their common organoleptic characteristics, are not affected by even the highest radiation dose used in this study, 3.0 kGy, which would allow live, refrigerated post-irradiation commercialization. The survival of oysters belonging to the species used in this study, *Crassostrea brasiliana*, was determined on the basis of difficulty to open the valves; this species of oyster only opens its valves during feeding or after death.

4. CONCLUSIONS

Used in conjunction with Good Primary Production and Processing Practices, as required by international standards, irradiation allows hygienization of oysters without affecting the survival of the mollusks for subsequent live, in-the-shell commercialization. A radiation dose of 1.5 kGy is sufficient to ensure the safety of raw *Crassostrea brasiliana* against pathogenic *Vibrionaceae*, including *V. cholerae*, as well as against *Aeromonas hydrophila, Plesiomonas shigelloides, Shigella flexneri*, and *Escherichia coli* O157:H7, but may not ensure elimination of *Salmonella typhi*, *S. enteritidis* or *S. typhimurium* if initial contamination is high (10^8–10^{10} CFU/g). To achieve safety levels against *Salmonella* spp., particularly *S. enteritidis*, in raw oysters having high initial contamination (10^8–10^{10} CFU/g), a dose of 3.0 kGy is advisable.

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