Viability of *Vibrio cholerae* 01 on Frog Legs under Frozen and Refrigerated Conditions and Low Dose Radiation Treatment

FLORENCE C. SANG*, MARTIN E. HUGH-JONES, and HARRY V. HAGSTAD

Department of Epidemiology and Community Health, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803

(Received for publication October 23, 1986)

**ABSTRACT**

Frog legs were contaminated with *Vibrio cholera* 01, Inaba serotype, ElTor biotype. The organism remained viable for more than 28 and 2 d when stored at -20°C and 4°C, respectively. Exposure to a multicurie ^90^Cobalt source of 50 and 100 kilorads eliminated *V. cholerae* from both the frozen and fresh frog legs.

Cholera has been endemic in the delta of the Ganges and Bahmaputer rivers in Eastern India for centuries, escaping periodically in a series of pandemics to bring death and suffering to other parts of the world. This was caused by *Vibrio cholerae* 01, serotype Ogawa, classical biotype (8,10). The seventh pandemic started in Asia in 1961 and is still continuing. It is caused by *V. cholerae* 01, Inaba serotype of ElTor biotype (10).

From 1911 until 1978, there was only one naturally acquired case of cholera reported in the United States. This occurred in Port Lavaca, Texas, in 1973. No source of infection was ever identified (1). In August 1978, a case was identified in Vermilion parish, Louisiana. *V. cholerae* 01, Inaba serotype was isolated. Later 10 other people were involved in the outbreak, 6 in Vermilion parish and 4 in Lafayette parish (1a,3,9). The vehicle of transmission was shown to be inadequately cooked crab (1,1a,3). Intensive surveillance of sewage and diarrheal illness in southern Louisiana was initiated; *V. cholerae* 01, Inaba serotype was isolated from sewage systems in six municipalities. Environmental samples of water and live seafood were taken from implicated Louisiana coastal waters. Among hundreds of samples of crabs, oysters and shrimp, only one sample of shrimp was positive.

Despite intensive surveillance, only two isolates were recovered between 1979-1980; one from sewage and the other from a patient. Both isolates were *V. cholerae* serotype Inaba (1b,5). All strains of *V. cholerae* isolated from Texas in 1973 and the Louisiana isolates showed striking similarities in hemolytic reactions and phage types (6). It is possible that *V. cholerae* has been present along the Gulf Coast waters for a long time and recognized recently because it was fortuitously tested for (6,7).

How *V. cholerae* 01, Inaba reached Louisiana and Texas is unknown. Frog legs are a locally popular food for which the demand cannot be met from wild Louisiana frogs. The hypothetical potential exists for the importation of *V. cholerae* with foreign frog legs. The total annual volume of frog legs imported to the U.S.A. in 1985 was 8.8 million pounds with about 8.0 million pounds originating from cholera endemic areas, (2). This study was designed with the following objectives: (a) to determine the viability of *V. cholerae* under normal food storage conditions (4°C and -20°C), (b) to determine whether *V. cholerae* multiplies in frog legs during thawing, and (c) to determine the efficiency of irradiation in the elimination or reduction of *V. cholerae* in frog legs.

**MATERIALS AND METHODS**

Frog legs

Six dozen commercially processed frog legs were purchased from a local supermarket and sterilized with ultra violet light. Four legs were sampled randomly and assayed to ensure freedom from *V. cholerae*. All frog legs were stored at -20°C till needed.

*Vibrio cholerae*

The strain used in these experiments was *V. cholerae* 01, Inaba serotype of ElTor biotype. It was made available by Dr. Henry Bradford of the Public Health Laboratory in New Orleans, Louisiana. Identification and characterization of *V. cholerae* used in the experiments and re-isolated from frog legs was according to the standard criteria (12) by Dr. Siebeling, Department of Microbiology, Louisiana State University, Baton Rouge, Louisiana.

Inoculating and treating frog legs

Frog legs were contaminated by putting all the legs for each experiment in a biohazard bag, size 12 cm × 24 cm (American Scientific Products) and adding 10 ml of overnight growth of *V. cholerae* diluted in 190 ml of 1% alkaline peptone water (APW). The bag was sealed, shaken and then rolled for 1-2
min to thoroughly contaminate the test material. The test material was then removed from the bag and transferred to a metal rack to drain for 30 min. Four legs, matched in size, were put in each biohazard bag as a set for subsequent experimental treatment. One set was sampled randomly to determine the initial level of contamination, which was expressed as the number of colony forming units per ml (CFU/ml) of wash solution. For enumeration, each frog leg was placed aseptically in a biohazard bag, into which 20 ml of 1% APW was added as a wash solution. The bag was sealed and rotated gently for 1-2 min. One ml of the wash solution was withdrawn and added to a tube containing 9 ml of sterile 1% APW for sequential ten-fold dilutions which was diluted to $10^{-6}$. Two, 0.01-ml portions of each dilution were transferred separately onto blood agar plates and incubated at 37°C for 24 h. Colonies were counted and the total number of CFUs determined by applying the appropriate dilution factor.

The experiments were done in three parts: (a) Viability of *V. cholerae* at -25°C and during thawing period. Eight sets of frog legs were contaminated and stored at -20°C. Two sets were removed on days 1, 14, 21, and 28. The legs were allowed to thaw at room temperature for 30 min and 8 h, respectively. Each frog leg was assayed for *V. cholerae*. (b) Viability at 4°C. Two sets of frog legs were contaminated and stored at 4°C. One set was removed on day 1 and the second on day 2 for *V. cholerae* assay. (c) Irradiation. A multicurie $^{60}$Co radiation source was used for irradiation treatment of frog legs. Fricke dosimetry (4, 11, 13) was employed to calculate the dose of irradiation.

The average dose rate was 4,000 rads per minute. Five sets of frog legs were contaminated and irradiated. Two sets were thawed and each was irradiated for 12.5 and 25 min, respectively. The other two sets were frozen over night at -25°C and during thawing period. Eight sets of *V. cholerae* in frog legs were contaminated and stored at -20°C. One set was removed on day 1 and the second on day 2 for *V. cholerae* assay.

**RESULTS**

**Viability after storage at -20°C and 4°C**

The initial mean level of contamination of frog legs stored at -20°C was $6.9 \times 10^7$ CFU/ml. The mean counts after 7, 14, 21, 28 days are shown on Table 1. Freezing did not kill the organism but reduced the number by the 28th day to a mean of $2.6 \times 10^4$ CFU/ml. There was no difference in number between those thawed for 0.5 and 8.0 h. This indicates that *V. cholerae* did not multiply in frog legs during a longer time at room temperature. The mean initial level of contamination for frog legs stored at 4°C was $4.8 \times 10^2$ CFU/ml. The mean count after day 1 and 2 were $2.5 \times 10^7$ and $1.5 \times 10^7$ respectively. *V. cholerae* was stable at 4°C for 2 d without any significant decline.

**Effects of irradiation**

The mean initial level of contamination of frog legs, fresh and frozen, were $3.8 \times 10^6$ and $3.4 \times 10^6$ CFU/ml respectively. Treatments with levels of 50 Krad or above eliminated *V. cholerae* from the legs.

**DISCUSSION**

This series of experiments demonstrated that *V. cholerae* remained viable in frog legs for at least 28 d when frozen (-20°C) and 2 d when chilled (4°C). Thus it may be concluded that frog legs remain contaminated throughout the probable transport period and the accepted shelf life of these products.

The analysis of reports on cholera in Louisiana indicates that cholera outbreaks have been associated with contaminated seafoods. Data from the National Marine Fisheries Service show that 91% of frog legs imported to the USA originate from cholera-endemic areas. In these developing countries with low standards of environmental sanitation and food hygiene, *V. cholerae* could contaminate and be exported with frog legs. Irradiation was shown to be an effective method of eliminating *V. cholerae* in both fresh and frozen frog legs. Even with a relatively low dose of 50 Krad, there was complete elimination of *V. cholerae*. On April 16, 1986, the Food and Drug Administration (FDA) accepted the use of low dose radiation (up to 100 Krad) as a process of sterilizing foods (14). If this procedure is used on all imported frog legs, the health risk will be substantially reduced.

**ACKNOWLEDGMENTS**

This work was supported by the Department of Epidemiology and Community Health, School of Veterinary Medicine, Louisiana State University. We thank Dr. Henry Bradford of the Public Health Laboratory, New Orleans, Dr. Edward Lambremont of Nuclear Science Center, LSU, and Dr. R. Siebeling of the Microbiology Department, LSU for their help.

**REFERENCES**


Shaw, et al., con't. from p. 657