SURVIVAL AND GROWTH OF SHIGELLA FLEXNERI, SALMONELLA ENTERITIDIS, AND VIBRIO CHOLERAE O1 IN RECONSTITUTED INFANT FORMULA

FONE MAO WU, LARRY R. BEUCHAT, MICHAEL P. DOYLE, ERIC D. MINTZ, JOY G. WELLS, AND BALASUBR SWAMINATHAN

Center for Food Safety, University of Georgia, Griffin, Georgia; Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Abstract. Formula feeding is an alternative method to prevent mother-to-child infection with human immunodeficiency virus through breast-feeding in developing countries. Growth of bacterial pathogens in reconstituted infant formula has become a health hazard when contaminated water is used for rehydration. This study was conducted to assess bacterial safety risk of using contaminated water to reconstitute infant formula. Survival and growth characteristics were determined for three bacterial pathogens, Vibrio cholerae O1, Shigella flexneri, and Salmonella enterica serovar Enteritidis, inoculated into sterile tap water (3.2–3.4 log_{10} colony-forming units [CFU]/ml) and infant formula (1.5–1.7 and 3.2–3.4 log_{10} CFU/ml) and incubated at 4°C or 30°C for up to 24 hours. Vibrio cholerae O1 was the most sensitive of the three pathogens when inoculated into water, with no viable cells detected within 2 hours at 4°C or 30°C. The rate of inactivation in water was greater at 30°C than at 4°C. Vibrio cholerae O1, Shigella flexneri, and Salmonella enterica serovar Enteritidis grew rapidly in infant formula at 30°C, reaching populations of 9.2, 8.7, and 9.2 log_{10} CFU/ml, respectively, at 24 hours. Populations of all three pathogens did not change significantly after incubating infant formula for 24 hours at 4°C, but continuously decreased in water throughout incubation for 24 hours, regardless of temperature. Results suggest that unless refrigerated, reconstituted infant formula should be consumed soon after preparation to avoid increased risk of illness associated with increases in populations of pathogenic bacteria that may be introduced by contaminated water.

INTRODUCTION

Breast-feeding can be a mode of transmission for human immunodeficiency virus (HIV) type 1 from infected mothers to infants. In an area such as West Africa in which acquired immunodeficiency virus (AIDS) is prevalent, breast-feeding and the use of expressed breast milk are of concern. One alternative is to feed infants with microbiologically safe and sufficient quantities of infant formula; however, the use of infant formula in developing countries has caused higher rates of diarrheal morbidity and mortality, possibly because contaminated water is often used to prepare infant formula and because the high nutrient contents and low acidity of infant formula provide a good growth medium for bacterial pathogens. In a survey conducted in Abidjan, Côte d’Ivoire for the safety of using drinking water to rehydrate infant formula by a Centers for Disease Control and Prevention (CDC) task force in the joint United Nations Program on HIV/AIDS, it was found that 90% of infants by the age of one month had been given drinking water without further treatment. Only 12% of caretakers fed formula to infants because of common concerns of risk of diarrhea on infants, need of tedious preparation, and reduced nutritional values of infant formula. Most bottles were prepared from tap water (66%) and local bottled water (17%) without further treatment.

The purpose of this study was to determine in infant formula after rehydration and incubation at 4°C and 30°C (ambient temperature in tropical areas) the survival and growth characteristics of bacterial pathogens known to occasionally contaminate water in some regions of sub-Saharan Africa. Data obtained will be used to assess safety risks associated with consumption of rehydrated infant food stored for up to 24 hours after reconstitution.

MATERIALS AND METHODS

Bacterial strains and media. Five strains each of Shigella flexneri (strains F141, serovar 2a; F5204, serovar 2a; F3423, serovar 6; BU137X1, serovar 3a; and F1852, serovar 2a), Salmonella enterica serovar Enteritidis (strains S276, S294, S293, S421, and S492), and toxigenic Vibrio cholerae O1 (strains F8805, F4166, F5071, F5200, and S938T1) were obtained from the CDC (Atlanta, GA). Shigella flexneri and Salmonella enterica serovar Enteritidis were grown individually in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI). Vibrio cholerae O1 was grown in heart infusion broth (HIB) (Difco) at 37°C for 24 hours.

Inoculation. A loopful of overnight (16–18 hours) culture was transferred to TSB (HIB for V. cholerae) at three consecutive 24-hour intervals before being used as inoculum. One milliliter of cell suspension of each strain was sedimented by centrifugation at 3,000 × g for 2 minutes and the cell pellet was resuspended in 1 ml of sterile 0.85% saline solution. Equal volumes (200 μl) of cultures of each strain of a specific pathogen were combined and serially diluted in sterile saline. Portions (0.06–0.32 ml) of appropriately diluted cell suspension were added to 40 ml of sterile tap water to give high (approximately 2,000 colony-forming units [CFU]/ml) or low (approximately 20–50 CFU/ml) inoculum populations of each pathogen when combined with 6.7 g of infant formula (SMA Classic; Sodilac Lait, Inc., Isigny, France), which contains 6.3 mg of iron per 100 grams. This formula has been used in Côte d’Ivoire to replace breast milk for infants 0.5 months to one year of age.

Enumeration of pathogens in infant formula and water. Infant formula containing high or low inocula was incubated at either 30°C or 4°C for up to 24 hours. At incubation times of 0 (within 5 minutes after inoculating formula), 2, 4, 8, and 24 hours, samples were analyzed for populations of pathogens. Sterile tap water inoculated with the high-population inoculum and uninoculated infant formula served as controls. Samples were serially (1:10) diluted in sterile saline solution and duplicate 0.1-ml portions were surface plated onto MacConkey agar (Oxoid, Unipath, Ltd., Hampshire, United Kingdom) for enumeration of Shigella flexneri or Salmonella

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**enterica** serovar Enteritidis, and onto thiosulfate citrate bile salt sucrose (TCBS) agar (Eiken Chemical Co., Ltd., Tokyo, Japan) for enumeration of *V. cholerae*. When low bacterial populations in infant formula or water were anticipated, undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) were surface plated on recovery media. Plates were incubated at 37°C for 24 hours before enumeration of colonies. Presumptive colonies of each pathogen selected from plates inoculated with the highest dilution of samples were confirmed by agglutination assays using species-specific antisera (*Salmonella* O, group D. antiserum and *Shigella flexneri* polyvalent antiserum types 1a to 6; CDC, Atlanta, GA; *V. cholerae* polyvalent antiserum; Lee Laboratories, Grayson, GA). Enrichment of samples with low populations of *Shigella flexneri*, *Salmonella enterica* serovar Enteritidis, or *V. cholerae* was done by adding equal volumes of double-strength gram-negative broth (Difco), selenite broth (Difco), or alkaline peptone water, respectively, to inoculated water or infant formula followed by incubation at 37°C for 18–24 hours. Enrichment broth was streaked onto MacConkey agar (for *Salmonella enterica* serovar Enteritidis and *Shigella flexneri*) or TCBS agar (for *V. cholerae*) for detection of presumptive colonies of each pathogen.

**pH.** Two milliliters of water or infant formula was collected at each sampling time and frozen at −30°C in 15-ml plastic centrifuge tubes. Samples were subsequently thawed in a water bath at 21°C for 15 minutes before the pH was determined with a pH meter (Model 350; Corning Co., Corning, NY).

**RESULTS**

**Survival of pathogens in water.** All three pathogens inoculated into sterile tap water with a chlorine content of less than 0.06 parts per million (ppm) progressively decreased to undetectable levels when incubated at 4°C or 30°C. *Vibrio cholerae* O1, with seawater as its natural habitat, was highly sensitive to the reduced osmotic condition of tap water (Figure 1). Within 10–15 minutes after inoculation of water at a population of 3.2 log_{10} CFU/ml, *V. cholerae* decreased to less than 1.0 log_{10} CFU/ml. Populations of *Shigella flexneri* (Figure 2) and *Salmonella enterica* serovar Enteritidis (Figure 3) also decreased at 4°C and 30°C, but not as rapidly as *V. cholerae*. *Salmonella enterica* serovar Enteritidis was not detected in water after 8 hours at 30°C or 24 hours at 4°C. *Shigella flexneri* was the most resistant of the three pathogens. An initial population of 3.3–3.4 log_{10} CFU/ml survived in water at 30°C for 8 hours but not for 24 hours, and at 4°C for 24 hours. Populations of all three pathogens decreased in water more rapidly at 30°C than at 4°C.

**Survival and growth of pathogens in infant formula.** Changes in populations of *V. cholerae* O1, *Shigella flexneri*, and *Salmonella enterica* serovar Enteritidis in reconstituted infant formula stored at 4°C or 30°C for 24 hours are shown in Figures 1, 2, and 3, respectively. In contrast to the rapid decrease in populations in water, cell numbers of the three pathogens in reconstituted infant formula stored at 4°C did not change substantially during the 24-hour incubation period. Infant formula supported growth of all three pathogens.
at 30°C. *Vibrio cholerae* O1, with a lag phase of at least two hours at 30°C, grew from 1.6 and 3.0 log_{10} CFU/ml to 9.0–9.2 log_{10} CFU/ml within 24 hours. *Shigella flexneri* and *Salmonella enterica* serovar Enteritidis, at initial populations of approximately 1.5 log_{10} CFU/ml, increased to 8.5 and 9.2 log_{10} CFU/ml, respectively, within 24 hours.

A substantial change of pH of infant formula inoculated with pathogens occurred after incubation for 24 hours at 30°C. The pH decreased from 7.2–7.3 to 6.4–6.8 (Table 1). The reduction of pH did not prevent the pathogens from reaching populations of 8.5–9.2 log_{10} CFU/ml.

### DISCUSSION

In this study, we observed that populations of all three pathogens decreased in water more rapidly at 30°C than at 4°C. Similar results were observed in a study of the survival of *Escherichia coli* O157:H7 in water stored at 8°C, 15°C, and 25°C for up to 91 days, with the greatest survival occurring at 8°C and the least at 25°C.6

The observed rapid decrease in populations of *V. cholerae* upon inoculating into water might not reflect the actual number of viable cells. *Escherichia coli* and *V. cholerae* can enter a dormant state, in which they are viable but not culturable (VNC) in media used for their detection in artificial seawater.7 A similar VNC state has been observed in *Salmonella enterica* serovar Enteritidis,8 *Campylobacter jejuni*,9 *V. cholerae*,10 and *V. vulnificus*.11 The public health significance of the presence of VNC cells remains to be established, since it is unclear whether they still retain the ability to infect humans.

In this study, all the three pathogens grew very well in infant formula at 30°C, with a short lag time after the pathogen-inoculated water was used to reconstitute infant formula. Other common pathogenic bacteria to which infants may be particularly susceptible, including enteropathogenic and enteroinvasive *E. coli*, probably behave similarly. The rapid growth of pathogens in milk at an elevated temperature (> 4°C), regardless of the presence of competitive background microflora, has been described. *Escherichia coli* O157:H7 grew in unpasteurized milk at 8°C, 15°C, and 22°C during the first few days of incubation.12 Increases of 1–2 log_{10} CFU/ml and 3–5 log_{10} CFU/ml occurred at 8°C within the first four days and at 15°C within the first three days, respectively. In another study, growth characteristics of *E. coli* O157:H7 in infant rice cereal reconstituted with milk and stored at 15°C, 21°C, and 30°C were determined.13 At an initial inoculum of 1.9 log_{10} CFU/ml, the population in reconstituted cereal stored at 21°C and 30°C increased to approximately 8.7 and 8.4 log_{10} CFU/ml within 48 and 24 hours, respectively. A similar growth trend was reported in a study of enterotoxigenic *Bacillus cereus* in infant rice cereal reconstituted with milk.14

The pathogen, at an initial level of 2.1 log_{10} CFU/gram, reached populations of 7.1, 7.2, and 7.4 log_{10} CFU/gram, within 12, 48, and 72 hours when stored at 30°C, 21°C, and 15°C, respectively. The decreased pH of infant formula in our study did not inhibit the growth of pathogens to a high population after 24 h of incubation; however, a further decrease in pH may inhibit growth of the test pathogens in infant formula. In a growth and survival study of *E. coli* O157:H7 in milk, a rapid decrease in pH to less than 4.0 within four days resulted in a decrease in population of the pathogen to an undetectable number of viable cells within 14 days in milk stored at 22°C.12

Water can serve as a vehicle for transmitting enteric pathogens to humans. Among the pathogens examined in our study, *V. cholerae* is most often waterborne. It is excreted into the environment in enormous quantities by infected persons with profuse watery diarrhea, and by asymptotically infected individuals, and can persist indefinitely in untreated surface water in which conditions of salinity and acidity are favorable.15 Epidemics occur where sanitation and access to safe drinking water are lacking, a situation which unfortunately prevails in many areas where the prevalence of HIV is also high. In recent years, sub-Saharan Africa, which has the highest prevalence of HIV infection among women of childbearing age, also accounted for the highest numbers of cholera cases reported to the World Health Organization.16

When food is prepared using water contaminated with any of the three pathogens, growth may occur that results in population densities high enough to cause severe illness.17 In our study, all populations of test pathogens progressively decreased in water; however, some cells could have entered a VNC state. This may partially explain the behavior of *V. cholerae* in water during the first two hours of storage at 30°C.

To prevent infant diarrhea in sub-Saharan Africa caused by formula rehydrated with contaminated water, a prerequisite is to use pathogen-free water. Because implementation of adequate public water treatment and distribution systems or the practice of boiling water before use is not feasible in many areas, alternative point-of-use water treatment strategies have been suggested to improve drinking water quality.18 A common problem of home water storage in developing countries is that water becomes contaminated with pathogens by hands or utensils dipped into the water at the open orifice of water storage vessels.19 To prevent stored water from becoming contaminated, storage vessels with small mouths and spigots have been used in intervention studies. Interventions involving the use of narrow-mouthed vessels, disinfection of water by treatment with calcium hypochlorite or bleach, and education of consumers, were successful in reducing fecal coliform bacteria and *E. coli* in water in a Bolivian community20 and in street-vended beverages in Guatemala.21 A similar strategy was used to reduce water contamination in Africa. In an intervention study for producing safer oral rehydration solution in a cholera epidemic in Guinea-Bissau, special vessels were used for preparing, storing, and dispensing water disinfected with hypochlorite.22 Addition of two drops of bleach to one liter of water reduced populations of coliforms and *E. coli* from 3.4 × 10^7 and 6.2 × 10^6 CFU/100 ml

### TABLE 1

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Initial population</th>
<th>pH 0 hours</th>
<th>pH 8 hours</th>
<th>pH 24 hours</th>
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</thead>
<tbody>
<tr>
<td>Shigella</td>
<td>3.37 ± 0.08</td>
<td>7.22 ± 0.08</td>
<td>7.22 ± 0.02</td>
<td>6.42 ± 0.16</td>
</tr>
<tr>
<td>flexneri</td>
<td>1.52 ± 0.14</td>
<td>7.26 ± 0.03</td>
<td>7.26 ± 0.01</td>
<td>6.54 ± 0.18</td>
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<tr>
<td>Salmonella</td>
<td>3.19 ± 0.15</td>
<td>7.27 ± 0.05</td>
<td>7.30 ± 0.03</td>
<td>6.77 ± 0.12</td>
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<tr>
<td>enterica</td>
<td>1.65 ± 0.11</td>
<td>7.31 ± 0.01</td>
<td>7.34 ± 0.04</td>
<td>6.69 ± 0.14</td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td>3.23 ± 0.15</td>
<td>7.32 ± 0.05</td>
<td>7.22 ± 0.22</td>
<td>6.38 ± 0.03</td>
</tr>
<tr>
<td>O1</td>
<td>1.63 ± 0.02</td>
<td>7.34 ± 0.02</td>
<td>7.37 ± 0.03</td>
<td>6.42 ± 0.12</td>
</tr>
</tbody>
</table>

* Values are the mean SEM log_{10} colony-forming units/ml.
to $3.6 \times 10^3$ and 0 CFU/100 ml, respectively. In another study, a combination of point-of-use water disinfection by an electrolytically produced disinfectant, safe storage of water in closed plastic vessels with a narrow mouth for pouring, and community education resulted in significantly reduced infant diarrhea.25

A diluted solution of sodium hypochlorite was used for point-of-use water treatment. At this concentration, approximately one-tenth the concentration of sodium hypochlorite in commercial laundry bleach, there is no danger of acute poisoning, even if it is ingested straight from the bottle by a small child or infant.24 Long-term effects of chlorination by-products are poorly characterized. Studies in the United States, where the majority of the population, including young children and infants, routinely drink chlorinated water, have suggested a potential association with slightly elevated risks of bladder cancer and of birth defects.25, 26 In the setting of high infant mortality due to acute infections with waterborne bacterial pathogens such as V. cholerae, Shigella, and Salmonella, the potential long-term risks of providing infants and children with chlorinated water need to be weighed against the proven benefits.27 If point-of-use disinfection were available for treating drinking water in the house of a newborn, the same methods could be used to prepare water for rehydration of infant formula, but precautions should still be taken to prevent pathogenic bacteria that may survive the treatment from proliferating in rehydrated infant formula.

The results of our study demonstrate that V. cholerae, Shigella, and Salmonella grew rapidly within a short period of lag time when contaminated water is used to reconstitute infant formula. Since some pathogens, e.g., Shigella spp. and Salmonella spp., have a low infectious dose, we suggest that reconstituted infant formula should be either refrigerated or consumed immediately after preparation to avoid increased risk of illness associated with increases in population of pathogenic bacteria that may be introduced by contaminated water.

Authors’ addresses: Fone Mao Wu, Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797 and Foodborne and Diarrheal Diseases Branch, Mailstop C03, Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333. Larry R. Beuchat and Michael P. Doyle, Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797, Telephone: 770-228-7284, Fax: 770-229-3216; E-mail: mdoyle@csf.griffin.peachnet.edu.

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