Efficacy of Protocols for Cleaning and Disinf ecting Infant Feeding Bottles in Less Developed Communities

Li Ma, Guodong Zhang, Balasubr Swaminathan, Michael Doyle, and Anna Bowen*
Center for Food Safety, University of Georgia, Griffin, Georgia; Division of Foodborne, Bacterial, and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Abstract. Although breastfeeding is the best choice for most infants, infant formula is used widely, commonly introduced during the neonatal period, and usually given to infants in bottles that can be difficult to clean. We artificially contaminated infant feeding bottles with low and high inocula of bacterial enteric pathogens and evaluated the efficacy of several cleaning and chlorine disinfection protocols. Rinsing with soapy water followed by tap water was the most effective cleaning method and reduced pathogen load by 3.7 and 3.1 log10s at the low and high inoculum levels, respectively. Submersion in 50 ppm hypochlorite solution for 30 minutes produced a 3.7-log10 reduction in pathogens, resulting in no identifiable pathogens among bottles. This result was comparable to boiling. When combined with handwashing, use of safe water, and appropriate storage of prepared infant formula, these simple, inexpensive practices could improve the microbiological safety of infant formula feeding in less developed settings.

INTRODUCTION

Although the World Health Organization (WHO) recommends exclusively breastfeeding infants until 6 months of life and continued breastfeeding until age 2 years or older, infant formula is used widely and is commonly introduced during the neonatal period.1-3 Because HIV can be transmitted through breast milk, health agencies in many industrialized and some developing countries promote infant formula use for infants of women infected with HIV. Although WHO suggests breast milk replacement only where acceptable, feasible, affordable, sustainable, and safe,1 national programs include provision of free infant formula as part of the strategy to prevent mother-to-child transmission of HIV in some less developed countries, including Thailand, Botswana, Brazil, Nigeria, Vietnam, and Argentina.4-9 Furthermore, early weaning (~4-6 months) from breast milk is encouraged as part of mother-to-child HIV prevention in many HIV prevention programs in developing countries; weaned infants should receive breast milk replacements until ~1 year of life. As a result, large numbers of infants in developing countries are routinely encouraged to take breast milk substitutes as part of HIV prevention strategies, and an additional, unclear number of parents choose to use infant formula for other reasons. Many of these infants are fed breast milk substitutes with bottles and nipples. Demographic and Health Surveys conducted in the past 5 years showed that, among 43 developing countries, a median of 11% (range, 1-71%) of children <36 months old have consumed fluids from a bottle with a nipple; exposure to feeding bottles is likely to be even higher among infants born to HIV-infected mothers.10

Undoubtedly, safe use of infant formula is challenging in developing world settings. Because water and sanitation infrastructure are often suboptimal in these communities, infants who are not breastfed remain at high risk of morbidity and mortality caused by diarrhea pathogens.11-12 Infant feeding bottles can be very difficult to clean, and prepared formula can become contaminated through a number of mechanisms.13-15 Studies in developing countries have found home-prepared infant formula feedings frequently contaminated with 10^2-10^6 coliforms/mL; often, multiple pathogens are isolated from a single prepared feed.16-20 Salmonella species and Escherichia coli, particularly enteropathogenic E. coli, have been isolated from prepared infant formula and have caused multiple outbreaks of diarrheal disease among formula-fed infants.21-27 Great care must be taken while preparing and feeding infant formula in these settings to prevent microbiological contamination.

Government programs for prevention of mother-to-child prevention of HIV transmission have recommended the following procedures for formula feeding infants of mothers with HIV:28 Infant caretakers are to sterilize bottles and utensils by boiling for 10-20 minutes before use. At the time of feeding, infant formula powder should be mixed with cooled, previously boiled water inside a sterilized bottle or cup. If followed correctly, these guidelines are useful in minimizing microbial contamination of infant formula. However, they are often inconvenient and expensive for mothers in developing countries, particularly in rural communities, because of the lack of sufficient, affordable firewood or other fuel and the burden of transporting it. Consequently, it is unlikely that the current guidelines have been fully adopted. For example, a study conducted in Nigeria showed that infant formula was most often prepared more than half a day in advance of needs,17 whereas in rural South Africa, some caretakers rinse bottles with water but do not wash them with soap, whereas others wash bottles and nipples with soap and water but do not disinfect them.29 To increase the likelihood that infants who receive formula products are fed in a microbiologically safe manner, and thereby are more likely to survive infancy, more practical methods of preparing infant formula and bottles for formula feeding are needed.

Because of its low cost and ease of handling, chlorination could be a suitable alternative to boiling as a disinfection method for infant feeding equipment in rural communities. Although exclusive breastfeeding is clearly the optimal feeding choice for most infants worldwide and controversy continues to surround feeding modalities in developing countries for infants of mothers infected with HIV,20-21 infant formula is used widely nonetheless, is generally administered in bottles rather than cups, and is frequently prepared under suboptimal conditions. Therefore, we aimed to develop inexpensive, practical protocols to assist caregivers choosing to use infant formula to do so more safely. In this study, we evaluated the efficacy of...
CLEANING AND DISINFECTION OF INFANT FEEDING BOTTLES

CLEANING AND CHLORINE DISINFECTION PROTOCOLS FOR INFANT FEEDING BOTTLES DESIGNED FOR USE IN DEVELOPING WORLD COMMUNITIES WHERE INFANT FORMULA IS USED.

MATERIALS AND METHODS

We conducted two related sets of laboratory experiments for cleaning and disinfecting infant feeding implements. During the first experiment (Figure 1), we explored efficacy of several cleaning and disinfection procedures for artificially contaminated feeding bottles. Based on the results of the first experiment, we conducted a second experiment on contaminated feeding bottles and nipples to develop more practical and efficacious methods of cleaning and disinfection (Figure 2).

**Bacterial strains, medium, and preparation of inocula.** A mixture of three strains of enteropathogenic *Escherichia coli* (EPEC), 607-54 (O55:H6), 833-60 (O119:H6), and B170, and three strains of *Salmonella*, K0501 (S. Newport), K1082 (S. Braenderup), and K0061 (S. Berta), was used for the inoculation experiments. All strains were obtained from the culture collection of the Enteric Diseases Laboratory Branch at the Centers for Disease Control and Prevention (CDC, Atlanta, GA). Working cultures were made from frozen stock (−80°C) and maintained on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) plates at 4°C and transferred monthly for up to 3 months. Before inoculation, the cells of each strain were grown in tryptic soy broth (TSB; Difco) at 37°C for three consecutive 24-hour intervals. The inocula were titered by mixing equal volumes of each strain and enumerating serial dilutions in Butterfield phosphate buffer (BPB, pH 7.2; Difco) on TSA and MacConkey agar plates.

**Infant formula and feeding equipment.** Nestle's NAN starter infant formula with iron, (Nestle, Ibia, Brazil) was purchased in Botswana. Infant feeding equipment consisting of polycarbonate bottles, nipples, rings, and caps (Sun kids, China) and infant bottle brushes (Sun kids) were also purchased in Botswana.

**Dishwashing liquid and soapy water preparation.** Dishwashing liquid (soap) (Sunlight; Unilever, Boksburg - Gauteng, South Africa), containing 20–40% anionic detergents, <15% solubilizers, colorants, and perfume, was purchased in Botswana. For use, 2 mL of the dishwashing liquid was added to 1 L of tap water to make a soapy water solution.

**Preparation of infant formula and feeding equipment.** Before initial use, feeding equipment was boiled in water for 10 minutes as instructed on the labels. Between experiments,
the feeding equipment was boiled in water for 20–30 minutes, soaked in soapy water for ~25 minutes, brushed with a bottle brush for ~15 seconds, rinsed three times, and air dried.

Infant formula was reconstituted according to manufacturer’s instructions and artificially contaminated at low (~10^4 CFU/mL of formula) and high (~10^6 CFU/mL of formula) levels or left unadulterated (background control). The low and high contamination groups were inoculated with the six-strain mixture of test bacteria. All bottles were filled with 120 mL of formula with or without inocula and incubated at 25°C for 3 or 6 hours. After incubation, the formula was poured off, and the bottles were drained for 15 minutes on sterile paper towels.

In the second experiment, infant feeding equipment was prepared with the following modification. The bottles containing the inoculated formula were inverted three times before being incubated at 25°C. This procedure was designed to ensure contact between formula and nipple as occurs in typical use situations.

**Preparation of disinfection solution.** Reagent-grade 5% sodium hypochlorite was purchased from Aldrich (Milwaukee, WI). Working chlorine solutions were prepared by diluting 5% sodium hypochlorite in tap water, and the concentration of free chlorine was measured using the N, N-diethyl-p-phenylenediamine (DPD) free- and total-chlorine assay kit (Hach, Loveland, CO) following the manufacturer’s instructions. Tap water was supplied municipally (Griffin, GA) and contained no detectable free chlorine.

**Cleaning.** The following cleaning procedures were evaluated with the bottles prepared according to the protocols described above: 1) no cleaning treatment; 2) rinse twice with tap water (RR); 3) rinse three times with tap water (RRR); 4) rinse while brushing with tap water followed by rinse with tap water (RbR); and 5) rinse while brushing with soapy water followed by rinse with tap water (RbsR). A rinse was defined as filling the bottle with 125 mL of tap water, swirling (until water reached the top of bottle) three times, and inverting three times with nipple, ring, and cap on, and pouring off the water completely. RbR was similar to RR except a brush (2.25-in-diameter nylon-bristle brush) was inserted into the bottle (2.125 in diameter) during the first rinse, and the bottle was brushed in an up-and-down motion lasting ~6 seconds. RbsR was similar to RbR except soapy water (prepared as above) was used during the first rinse. After the cleaning procedure, all bottles except those used as negative controls (contaminated inoculated infant formula and not subjected to any cleaning procedure) and background controls (infant formula with no inoculum and no cleaning procedure) were drained for 15 minutes on sterile paper towels. Microbiological analysis of each bottle was carried out to evaluate the efficacy of cleaning procedures. Microbiological analyses of negative control and background control bottles were performed immediately after 15 minutes of draining the formula. Three replicate trials were performed for the cleaning efficacy study.

In the second experiment, in addition to RR and RbsR, cleaning procedures consisting of a single rinse with tap water (R) and a rinse with soapy water followed by rinse with tap water (RsR) were included in lieu of RbR.

**Disinfection.** Only inoculation of formula with ~10^6 CFU of the pathogen mixture/mL of formula and incubation at 25°C for 6 hours were used to prepare the infant feeding equipment for the first experiment. The bottles were drained for 15 minutes after the formula was poured out. The following procedures were evaluated for disinfection of the drained bottles: 1) rinsing with tap (control; no detectable free chlorine) or chlorinated water (50, 125, or 200 ppm free chlorine) followed by rinsing with tap water and 2) submerging in tap (control) or chlorinated water (50, 125, and 200 ppm free chlorine) for 5 or 10 minutes with or without pre-cleaning treatments. The pre-cleaning treatments included none and rinse once, twice, and three times with tap water. Bottles (3 bottles/container) were completely horizontally submerged into 2.5 L of either tap water or chlorine solution, whereas the nipples, ring, and cap were separated and submerged individually. All bottles were drained for 5 minutes on sterile paper towels before being rinsed with tap water (120 mL) and subjected to microbiological analysis (see below).

In the second experiment, inoculation of formula with both low and high contamination levels and incubation at 25°C for 6 hours was used to prepare the infant feeding equipment (with contaminated nipples) for the disinfection study. Submerging in tap (control) or chlorinated water (50 ppm) for 5 or 30 minutes with or without pre-cleaning treatments was evaluated. The pre-cleaning treatments included none, R, RR, RsR, and RbsR. The submersion treatment was conducted in beakers instead of rectangular-shaped containers. Because air bubbles were observed trapped within the submerged bottles during the first experiment when the bottles were horizontally submerged, bottles (3 bottles/container) were vertically submerged right-side up into beakers (Nalgene polypropylene Griffin 4-L beaker; Nalge Nunc International, Rochester, NY) holding 5 L of either tap water or chlorine solution during the disinfection experiment. The beakers, although labeled as having a 4-L capacity, could hold >5.5 L of liquid. Nipples and rings were left attached to each other to simulate possible practice in the community, but caps were separated and submerged individually.

In the cleaning and disinfection experiments, rinse and bath water solutions were kept at room temperature, or ~21°C.

Three contaminated infant feeding implements prepared as above were boiled for 10 minutes in water and subjected to microbiological analysis according to the procedure described below to serve as positive controls in all the disinfection experiments.

**Microbiological analysis.** One hundred twenty milliliters of universal pre-enrichment broth (UPB; Difco, Becton Dickinson, Sparks, MD) was added to each bottle. The bottles were topped with nipples, rings, and caps, inverted 10 times, and agitated in a digital incubator shaker (Innova 4000; New Brunswick Scientific, Edison, NJ) at 150 rpm at room temperature for 10 minutes. Serial dilutions (1:10) in BPB were prepared from this suspension, and 0.1 mL of appropriate dilutions was surface plated in duplicate onto MacConkey agar (Mac) plates for the enumeration of *E. coli* and *Salmonella*. In addition, *E. coli* and *Salmonella* in the original UPB suspension were enumerated by plating a total of 1 mL (undiluted of the UPB rinse) onto four Mac plates (0.25 mL each) when low bacterial counts were anticipated. All plates were incubated at 37°C for 18–24 hours before enumeration. To qualitatively determine the presence of EPEC and *Salmonella* in the UPB rinse suspension, all bottles with remaining UPB were incubated at 37°C overnight, and a loopful of growth culture from each of these bottles was streaked on Mac plates.

In a preliminary study to determine the appropriate recovery medium for enumerating inocula after treatments, Violet Red
Bile Agar (VRB), Mac, and TSA (control) were compared. VRB and Mac performed equally well in suppressing background microorganisms even after overnight enrichment in UPB; however, Mac was less inhibitory to Salmonella strains than VRB. Therefore, Mac was used as the selective recovery medium.

### Statistical analysis
All experiments were done at least in duplicate, and triplicate samples from each treatment were analyzed. Mean cell numbers of Salmonella and EPEC in UPB were subjected to an analysis of variance and the Duncan multiple range test (SAS Institute, Cary, NC) to determine significant differences (\( P < 0.05 \)) between treatments.

### RESULTS

Growth of the inocula was observed in the bottles during incubation. At high inocula, bacterial concentrations increased from an initial population of 6.95 log\(_{10}\) CFU/mL of formula to 7.45 and 7.92 log\(_{10}\) CFU/mL of formula when held at 25°C for incubation. At high inocula, bacterial concentrations increased cantile differences (\( t < 0 \)) was used in statistical analysis. With each inoculum level, means followed by the same uppercase or lowercase letter are not significantly different (\( P > 0.05 \)).

#### Table 1
Efficacy of different cleaning procedures of bottles with clean nipples in reducing EPEC and Salmonella contamination

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Cleaning procedure</th>
<th>EPEC and Salmonella (log(_{10}) CFU/mL in UPB†)†</th>
<th>3-h incubation‡</th>
<th>6-h incubation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>None</td>
<td>1.07 ± 0.66 a</td>
<td>2.27 ± 1.2 a</td>
<td>0.60 ± 0.43 a</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>2.27 ± 0.03 B</td>
<td>0.36 ± 0.37 B</td>
<td>0.12 ± 0.37 B</td>
</tr>
<tr>
<td></td>
<td>RbR</td>
<td>&lt; 0 b</td>
<td>&lt; 0 b</td>
<td>&lt; 0 b</td>
</tr>
<tr>
<td></td>
<td>RbsR</td>
<td>ND b</td>
<td>ND c</td>
<td>ND c</td>
</tr>
<tr>
<td>High</td>
<td>None</td>
<td>6.53 ± 0.22 A</td>
<td>6.73 ± 0.24 A</td>
<td>0.36 ± 0.56 C</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>3.46 ± 0.41 B</td>
<td>4.04 ± 0.19 B</td>
<td>0.36 ± 0.56 C</td>
</tr>
<tr>
<td></td>
<td>RbR</td>
<td>3.52 ± 0.28 B</td>
<td>3.80 ± 0.57 B</td>
<td>0.36 ± 0.56 C</td>
</tr>
<tr>
<td></td>
<td>RbsR</td>
<td>0.60 ± 0.66 C (d3, 4/9)</td>
<td>0.36 ± 0.56 C</td>
<td>&lt; 0, 6/9</td>
</tr>
</tbody>
</table>

* Low inocula ranged from 1.0 × 10\(^3\) to 3.1 × 10\(^3\) CFU/mL; high inocula ranged from 6.9 × 10\(^6\) to 7.5 × 10\(^6\) CFU/mL.
† UPB = universal pre-enrichment broth.
‡ Values are means ± SD of nine replicates in three trials.
§ UPB, universal pre-enrichment broth.
\‡ Low inoculum ranged from 2.9 × 10\(^3\) to 3.5 × 10\(^6\) CFU/mL; high inoculum ranged from 3.4 × 10\(^6\) to 4.8 × 10\(^6\) CFU/mL.

Efficacy of different cleaning procedures of bottles with contaminated nipples in reducing EPEC and Salmonella contamination

\( P < 0.05 \) was no significant difference between the RR and RbR cleaning procedures for bottles held at 25°C for 3 or 6 hours, suggesting that brushing was not a contributing factor in reducing of the inoculated bacterial cell numbers in bottles during cleaning. However, RbsR, compared with all other cleaning treatments, substantially reduced inoculated bacterial cell numbers; hence, this cleaning procedure was used in the second experiment.

Results of the efficacy of cleaning procedures of feeding equipment from the second experiment are summarized in Table 2. At a low level of initial contamination, a single rinse (R) significantly reduced EPEC and Salmonella cell numbers in bottles, with greater than a 1-log\(_{10}\) reduction compared with the controls. A further > 1-log\(_{10}\) reduction was achieved with an additional rinse (RR). Rinsing in soapy water (RsR) or rinsing and brushing in soapy water (RbsR) provided a reduction of > 1-log\(_{10}\) compared with RR, or a > 3-log\(_{10}\) reduction compared with the controls. The same pattern of reduction of EPEC and Salmonella was obtained when the initial contamination level was high, except for rinsing once (R) and twice (RR). There was no additional reduction of E. coli and Salmonella with an additional rinse, suggesting a stronger association of the bacterial inoculum with the bottles when the initial contamination level was high. Again, brushing was not a contributing factor in the reduction of inoculated bacteria in bottles because there was no significant difference between the RsR and RbsR cleaning procedures.

#### Table 2

<table>
<thead>
<tr>
<th>Cleaning procedure</th>
<th>Low inoculum†</th>
<th>High inoculum‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.85 ± 0.19 A</td>
<td>5.96 ± 0.12 A</td>
</tr>
<tr>
<td>RR</td>
<td>2.53 ± 0.30 B</td>
<td>4.70 ± 0.32 B</td>
</tr>
<tr>
<td>R</td>
<td>1.33 ± 0.06 C</td>
<td>4.66 ± 0.05 B</td>
</tr>
<tr>
<td>RsR</td>
<td>0.12 ± 0.29 C (0.5/6)</td>
<td>2.87 ± 0.44 C</td>
</tr>
<tr>
<td>RbsR</td>
<td>0.20 ± 0.31 C (0.4/6)</td>
<td>3.33 ± 0.19 C</td>
</tr>
</tbody>
</table>

* UPB = universal pre-enrichment broth.
† Values are means ± SD of six replicates in two trials ≤ 0, not detectable by enumeration (detection limit \( 1 \) CFU/mL). UPB but positive after enrichment; < 0 was used in statistical analysis. With each inoculum level, means followed by the same uppercase or lowercase letter are not significantly different (\( P > 0.05 \)).
‡ Low inoculum ranged from 2.9 × 10\(^3\) to 3.5 × 10\(^6\) CFU/mL; high inoculum ranged from 3.4 × 10\(^6\) to 4.8 × 10\(^6\) CFU/mL.

#### Table 3

<table>
<thead>
<tr>
<th>Disinfection procedure</th>
<th>Free chlorine concentration (ppm)</th>
<th>EPEC and Salmonella (log(_{10}) CFU/mL UPB†)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse in tap water (RR)</td>
<td>3.99 ± 0.32 A</td>
<td></td>
</tr>
<tr>
<td>Rinse in chlorine solution (RcR)</td>
<td>50</td>
<td>2.98 ± 0.17 B</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>3.01 ± 0.56 B</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>3.14 ± 0.45 B</td>
</tr>
</tbody>
</table>

* Initial inoculum ranged from 6.2 × 10\(^3\) to 7.5 × 10\(^6\) CFU/mL.
† UPB = universal pre-enrichment broth.
‡ Values are means ± SD of six replicates in two trials. Means followed by the same uppercase or lowercase letter are not significantly different (\( P < 0.05 \)).
Table 4
Disinfection of bottles with clean nipples by horizontal submersion in tap or chlorinated water

<table>
<thead>
<tr>
<th>Disinfection procedure</th>
<th>Free chlorine concentration (ppm)</th>
<th>Cleaning procedure</th>
<th>Submerge for 5 minutes</th>
<th>Submerge for 10 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND &lt; 0 Others</td>
<td>ND &lt; 0 Others</td>
</tr>
<tr>
<td>Submerge in tap water</td>
<td>None</td>
<td>R</td>
<td>4.60 ± 0.15</td>
<td>4.34 ± 0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RR</td>
<td>4.44 ± 0.14</td>
<td>1.64 ± 1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RRR</td>
<td>2/6 0/6 1.93 ± 0.27</td>
<td>2/6 1/6 1.93 ± 0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>1/6 5/6</td>
<td>1/6 5/6</td>
</tr>
<tr>
<td>Submerge in chlorine solution</td>
<td>50</td>
<td>None</td>
<td>1/9 6/9 1.65 ± 1.34</td>
<td>1/9 6/9 2.48 ± 1.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>1/9 6/9 0.54 ± 0.34</td>
<td>2/9 6/9 1.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RR</td>
<td>2/6 1/6 1.57 ± 0.76</td>
<td>2/6 4/6 1.57 ± 0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RRR</td>
<td>2/6               0.75 ± 0.05</td>
<td>2/6               0.75 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>1/9 7/9 3.32</td>
<td>1/9 5/9 0.75 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>1/9 7/9 0.9</td>
<td>2/9 7/9 1.28 ± 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RR</td>
<td>2/6 3/6 1.42 ± 1.08</td>
<td>2/6 3/6 1.42 ± 1.08</td>
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<tr>
<td></td>
<td></td>
<td>RRR</td>
<td>3/6 3/6</td>
<td>3/6 3/6</td>
</tr>
</tbody>
</table>

* Initial inoculum ranged from $6.9 \times 10^3$ to $9.1 \times 10^6$ CFU/mL.
† UPB = universal pre-enrichment broth.
‡ Numbers represent six or nine replicates in two or three trials. ND = not detectable after enrichment; < 0 = not detectable by enumeration (detection limit 1 CFU/mL UPB) but positive after enrichment.

The results of disinfection of contaminated bottles with clean nipples by horizontal submersion in tap or chlorinated water are presented in Table 4. The following inferences may be drawn from the data presented in Table 4: 1) for bottles submerged in tap water (controls), there was a significant reduction in numbers of EPEC and Salmonella in bottles pre-rinsed once and submerged for 10 minutes compared with submersion for 5 minutes. Additional rinses further reduced EPEC and Salmonella counts in the bottles, with none detectable in one of the six bottles in two trials. 2) Without a prior rinse (none) or rinse once (R), compared with the control, submerging the bottles in chlorine solutions (50, 125, and 200 ppm) for 5 or 10 minutes generally reduced contamination in bottles significantly, because pathogens could not be detected by enumeration in most of the bottles. 3) Chlorine concentration (50–200 ppm) in the submersion water had little effect on reducing EPEC and Salmonella contamination in the bottles. 4) Increasing submerison time, 5 versus 10 minutes, in chlorine solution (50–200 ppm) for 5 or 10 minutes generally reduced contamination in bottles significantly, because pathogens could not be detected by enumeration and only 1 or 2 CFU/mL detected in the other two bottles. Cleaning by R, RR, or RsR before disinfection further reduced the residual pathogen loads in four of six bottles (two replicating trials) below the detection limit by enumeration.

In the second experiment, infant feeding implements were prepared by inverting and shaking the bottles with artificially contaminated formula to inoculate the nipples before the formula was subjected to time-temperature abuse. Both low and high levels of pathogen inocula were used, and all bottles were incubated at 25°C for 6 hours. Results of the efficacy of bottle disinfection with or without previous cleaning treatments for the second experiment are summarized in Table 5. At a low initial contamination level and no previous cleaning step, submerging the bottles in chlorinated water for 5 minutes significantly reduced residual pathogen loads, with residual pathogen loads in four of six bottles (two replicating trials) below the detection limit by enumeration and only 1 or 2 CFU/mL detected in the other two bottles. Cleaning by R, RR, or RsR before disinfection further reduced the residual pathogen loads.

Table 5
Disinfection of bottles with EPEC and Salmonella-contaminated nipples by vertical submersion in tap water or chlorine solution

<table>
<thead>
<tr>
<th>Disinfection procedure</th>
<th>Cleaning procedure</th>
<th>Low inoculum† 5 minutes§</th>
<th>High inoculum† 5 minutes§</th>
<th>High inoculum† 30 minutes§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ND &lt; 0 Others</td>
<td>ND &lt; 0 Others</td>
<td>ND &lt; 0 Others</td>
</tr>
<tr>
<td>Submerge in tap water</td>
<td>None</td>
<td>1.76 ± 0.41</td>
<td>4.57 ± 0.06</td>
<td>3.7 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>4/6 0/3 0.3</td>
<td>4/6 1.51 1.68</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>6/6</td>
<td>4/6 1.15 1.26</td>
<td>1/6 5/6</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>6/6</td>
<td>4/6 0.78 1.20</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td>RsR</td>
<td>6/6</td>
<td>6/6</td>
<td>1/6 5/6</td>
</tr>
<tr>
<td></td>
<td>RbsR</td>
<td>4/6 0.3 0.48</td>
<td>6/6</td>
<td>Not done</td>
</tr>
</tbody>
</table>

* UPB = universal pre-enrichment broth.
† Numbers represent six replicates in two trials. ND = not detectable after enrichment; < 0 = not detectable by enumeration (detection limit 1 CFU/mL UPB) but positive after enrichment.
‡ Low inoculum ranged from $5.9 \times 10^6$ to $7.6 \times 10^6$ CFU/mL.
§ Bottles with or without prior cleaning, were submerged in tap or chlorinated water (50 ppm) for 5 or 30 minutes.
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loads to undetectable by enumeration in all the bottles tested in the replicating trials. When the contamination levels were high and bottles were submerged for 5 minutes, the residual pathogen contamination was reduced to undetectable by enumeration in all bottles tested only when either R or RsR were included in the cleaning procedures. Although a significant reduction in residual pathogen contamination was obtained in bottles submerged in chlorinated water either without a cleaning step or with R or RR, rinsing once or twice did not further reduce pathogen contamination compared with no cleaning. Increasing the submersion time from 5 to 30 minutes in chlorine solution greatly increased pathogen reduction, with all bottles having no detectable pathogens by enumeration when no cleaning step was included. When a single rinse in tap or soapy water was included, greater pathogen reduction occurred, with only one of six bottles having residual pathogen contamination detectable by enrichment culture.

In general, prior rinse of bottles with tap water increased the number of bottles with undetectable residual pathogen cells either by direct enumeration or enrichment culture after submersion in chlorine solution, especially when the submersion times were short (5 or 10 minutes). Such a prior cleaning step became insignificant when the submersion time was increased to 30 minutes, because the residual pathogens were not detected by direct enumeration from any of the bottles (six of six) that had no prior cleaning treatment, compared with five of six for both bottles that were rinsed once with tap or soapy water. However, a prior cleaning step, either rinsing once or rinsing with soapy water, produced one of six bottles that was free of contaminating pathogens (not detectable by enrichment culture) after submerging in chlorine solution for 30 minutes.

**DISCUSSION**

Although exclusive breastfeeding is recommended for most infants until 6 months of age and continued breastfeeding recommended until age 2 years or older, breast milk replacements are used commonly worldwide and are frequently fed to infants in bottles. Several alternatives to boiling are available for disinfecting the water used to prepare infant formula 33–37 ; however, few low-cost alternatives are available for disinfecting infant bottles in settings without reliable access to electricity, potable water, and cooking fuel. In this study, we incubated artificially contaminated infant formula in bottles at 25°C for 3 and 6 hours before cleaning to simulate use of infant formula in developing world settings and evaluated the microbiological effectiveness of several low-cost methods for cleaning and disinfecting feeding bottles.

Infant bottle cleaning practices that were evaluated included no rinse, rinsing with tap water one to three times, and rinsing with soapy water with or without brushing. Rinsing bottles with soapy water provided the greatest reduction of contaminating bacterial pathogens (Tables 1 and 2). A 2-log reduction in the number of inoculated bacterial pathogens was achieved by rinsing bottles with soapy water, whereas only a 1-log reduction was provided by the disinfection procedure of rinsing with chlorinated water.

Interestingly, brushing did not contribute significantly to the reduction of bacterial pathogens in the bottles, although such mechanical action should facilitate the removal of attached pathogens or residual formula. The brushes we used were designed for cleaning infant feeding bottles and were purchased in Africa. The brushes were slightly larger in diameter (2.25 versus 2.125 in) than the interior of the bottles; therefore, they were in close contact with the bottles’ walls when inserted. The vertical brushing movement used in this study may not have been as effective as twisting movements that might be used by infant care-givers. Furthermore, although contaminated bottles, nipples, and rings were held at warm temperatures for up to 6 hours, the infant formula might not have had sufficient time to dry and tightly adhere to the feeding implements—a scenario in which brushing might be more effective than rinsing as a cleaning step.

Cleaning steps such as rinsing remove much of the organic matter remaining on the feeding equipment after use and provide some reduction in bacterial counts. However, a disinfection procedure is needed after cleaning to ensure the microbiological safety of infant feeding bottles, especially when the initial pathogen contamination levels are high (Tables 1 and 2). Commercial hypochlorite solution has been used to decontaminate infant bottles and breast pump collection kits that include bottles38,39, at 0.14 USD for a single-day supply, this product may be outside the financial means of many parents in developing countries. In these studies, only disinfection, not sterilization, was achieved by immersing the kits in hypochlorite solution, a similar finding as in this study.38,39 Nevertheless, submerging contaminated infant feeding bottles in 50 ppm chlorine solution for at least 30 minutes can reduce the contaminating bacterial pathogens to a very low level (Table 5).

To achieve optimal decontamination, complete contact between contaminated infant feeding bottles and chlorine solution is necessary. Because inoculated pathogens were not detected by enumeration or enrichment culture in most of the bottles treated with chlorine solution, such sporadic occurrences of pathogen contamination suggest that some sites in a few bottles were not in contact with chlorine solution. Bottles that are horizontally submerged tend to trap air bubbles; therefore, they should be placed vertically in the disinfection solution with the right-side up. Infant care-givers should use containers higher than infant feeding bottles but narrower in diameter than the bottles’ height to prevent horizontal submersion of bottles. Such containers should also be properly labeled and tightly covered to minimize the risk of ingestion or drowning where young children are present.

Although these cleaning and disinfection steps showed promise in the laboratory, they require field testing for microbiological effectiveness and acceptability to users. The regrowth of bacteria we observed during incubation of the bottles suggests that pathogens were early log-phase cells instead of stationary phase cells before undergoing cleaning and disinfection treatments. It is well known that stationary phase cells are more resistant to chlorine than log-phase cells; therefore, chlorine disinfection steps may not be as effective under field conditions. Nonetheless, the conditions used in this study were intended to simulate home-use conditions in which infant formula is prepared minutes or hours before consumption, resulting in growth of contaminating pathogens in the formula. An additional consideration is that under the conditions used in this study, chlorine solutions cannot completely attenuate certain pathogens, such as *Cryptosporidium*, that frequently infect infants.40 However, dilute chlorine solutions are used to improve drinking water throughout much of the
developing world, and a recent meta-analysis suggested they reduce diarrhea incidence among children in these settings by 29%.34 Third, it is unclear whether field practices such as allowing formula to desiccate within bottles or adding viscous substances other than formula to the bottles might increase chlorine demand beyond the levels observed under our laboratory conditions. Furthermore, within 24 hours of using disinfection solution to bathe bottles that had not been subjected to a prior cleaning step in our study, free chlorine residuals in the disinfection solution decreased substantially (data not shown). Consumers might attempt to use disinfection solution for multiple batches of used infant feeding equipment, thereby reducing the effectiveness of the disinfection steps. Acceptability and feasibility of these cleaning and disinfection procedures should also be studied, because some cultures or individuals may also object to the scent or taste of chlorine solution or find it difficult to complete the sequence of steps required for adequate bottle disinfection.34,43

In summary, rinsing infant feeding equipment with soapy water effectively removed contaminating pathogens. Disinfection of such equipment by submerging in chlorine solution was not able to consistently sterilize bottles but is still a practical, low-cost option for improving the microbiological safety of infant formula in rural Africa or other communities in the developing world where infant feeding bottles are used. Ideally, infant caregivers would first rinse bottle equipment with soapy tap water and then submerge it in chlorine solution; such steps would reduce the residual pathogen load as well as organic material that may interfere with chlorine’s antimicrobial activity. However, even without prior rinsing, submerging highly contaminated bottles in 50 ppm chlorine solution for 30 minutes can reduce the bacterial pathogen contamination substantially, by 3.7 log10. If combined with appropriate hand-washing with soap, use of safe drinking water when preparing formula, and appropriate refrigeration and storage of formula, these infant bottle cleaning and disinfection steps could help protect the health of vulnerable children who receive infant formula.

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Authors’ addresses: Li Ma, Center for Food Safety University of Georgia, 1109 Experiment Street, Griffin, GA 30223; Tel: 770-228-7283 ext. 144, Fax: 770-229-3216, E-mail: lima@uga.edu. Guodong Zhang, Center for Food Safety University of Georgia, 1109 Experiment Street, Griffin, GA 30223; Tel: 770-228-7283 ext. 144, Fax: 770-229-3216, E-mail: guodongzhang63@hotmail.com. Balasubr Swaminathan, CDC, 1600 Clifton Road NE, MS A-38, Atlanta, GA 30333; Tel: 404-639-2206, Fax: 404-639-2205, E-mail: abowen@cdc.gov.

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