Evaluation of alternative DNA extraction processes and real-time PCR for detecting Cryptosporidium parvum in drinking water

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ABSTRACT
USEPA Method 1623 is the standard method in the United States for the detection of Cryptosporidium in water samples, but quantitative real-time polymerase chain reaction (qPCR) is an alternative technique that has been successfully used to detect Cryptosporidium in aqueous matrices. This study examined various modifications to a commercial nucleic acid extraction procedure in order to enhance PCR detection sensitivity for Cryptosporidium. An alternative DNA extraction buffer allowed for qPCR detection at lower seed levels than a commercial extraction kit buffer. In addition, the use of a second spin column cycle produced significantly better detection (P = 0.031), and the volume of Tris–EDTA buffer significantly affected crossing threshold values (P = 0.001). The improved extraction procedure was evaluated using 10 L of tap water samples processed by ultrafiltration, centrifugation and immunomagnetic separation. Mean recovery for the sample processing method was determined to be 41% using microscopy and 49% by real-time PCR (P = 0.013). The results of this study demonstrate that real-time PCR can be an effective alternative for detecting and quantifying Cryptosporidium parvum in drinking water samples.

Key words | Cryptosporidium, real-time PCR, tap water, ultrafiltration

INTRODUCTION

Cryptosporidium is a protozoan parasite that is shed in the feces of infected hosts. The organism can contaminate surface water when fecal material is transported into a water body. A study of source water from 66 surface water treatment plants conducted by LeChevallier et al. (1991) found that Cryptosporidium was present in 87% of the locations sampled. If contaminated surface water is consumed without proper treatment, illness can occur. Cryptosporidium can survive conventional water treatment and chlorine disinfection. Outbreaks of Cryptosporidium, associated with drinking water, have been documented in the United Kingdom and the United States since the early 1980s. Since that time, over 150 outbreaks of Cryptosporidium have occurred worldwide (Karanis et al. 2007; Baldursson & Karanis 2011). Thus, effective detection technologies are imperative in order to accurately detect these organisms and prevent outbreaks.

At present, there are three methods that are approved by the United States Environmental Protection Agency (EPA) for the detection of Cryptosporidium in water. These are: Method 1622 (Cryptosporidium in Water by Filtration/Immunomagnetic Separation (IMS)/Immunofluorescence Assay (IFA)) (2005), EPA Method 1623 (Cryptosporidium and Giardia in Water by Filtration/IMS/IFA) (2005), and EPA Method 1623.1 (Cryptosporidium and Giardia in Water by Filtration/IMS/FA) (2005). However, fluorescence microscopy is time-consuming and relatively expensive.

Another option for the analysis of Cryptosporidium in water is real-time polymerase chain reaction (qPCR). Numerous qualitative and quantitative PCR methods have
been used for aqueous matrices, but there are no standardized methods for the analysis of Cryptosporidium with qPCR (Di Giovanni et al. 1999; Kostrzynska et al. 1999; LeChevallier et al. 2000; Sturbaum 2003; Hill et al. 2007; Polaczyk et al. 2008; Yang et al. 2013).

The intent of the current study was to improve the DNA extraction procedure for Cryptosporidium and determine the effectiveness of the procedure with full-size samples. To accomplish this, filtration, concentration, and DNA extraction/purification steps had to be developed. Modifications were performed for various DNA extraction steps, while a qPCR amplification program was used to detect DNA. Since EPA Method 1623 is an approved method for the detection and enumeration of Cryptosporidium by microscopy, samples were split prior to the detection method in order to compare the quantitative PCR results to the oocyst counts recorded during microscopic examination of slides.

METHODS

Water samples

Treated tap water samples were obtained from a laboratory faucet at a water treatment plant in Charlotte, NC. Ten-litre water samples were collected in cubitainers, seeded with oocysts and filtered on the same day as collection. No dechlorination of the samples was performed.

Micro-organisms

Flow-cytometry sorted spiking suspensions containing Cryptosporidium parvum were obtained from the Wisconsin State Laboratory of Hygiene. Each suspension contained between 150 and 180 oocysts and was less than 6 weeks old when seeded into a water sample.

Filtration set-up

The filtration set-up used was the same as reported by Hill et al. (2005). New size #24 (6.4 mm ID) and #73 (8 mm ID) silicone tubing (Nalgene, Rochester, NY, USA) was used for the filtration experiments. All tubing connections and fittings were cleaned after each use. The hollow-fiber ultrafilters (high-flux Fresenius Optiflux 200NR polysulfone dialysis filters) had a surface area of 2.0 m², a fiber inner diameter of 200 μm, and a molecular weight cutoff of approximately 30 kDa (Fresenius Medical Care, Lexington, MA, USA). A new ultrafilter was used for each water sample. A peristaltic pump (Watson-Marlow Model 505S Wilmington, MA, USA) was used for all experiments.

Ultrafiltration procedure

Ultrafilters were wetted immediately before use with 0.1% sodium polyphosphate (NaPP) as described by Hill et al. (2005). The cross flow rate varied from 2.0 to 2.2 L/min, and the pressure was operated between 6 and 9 psi in order to keep the permeate rate between 0.6 and 0.8 L/min for each experiment. Samples were filtered until approximately 150 mL was left in the retentate (sample) reservoir. The volume that was held in the ultrafilter and tubing was also pumped back into the retentate reservoir for a total sample volume of approximately 250 mL. The ultrafilter was then backflushed. The backflushing solution contained 0.2% Tween-80 (Sigma–Aldrich catalog #P-1754), 0.01% NaPP (Sigma–Aldrich catalog #305553), and 0.01% Antifoam A (Sigma–Aldrich catalog #A-5758). As described by Polaczyk et al. (2008), a 150 mL backwash sample was collected in a beaker at a rate of 600 mL/min. The retentate and backwash were added to a 500 mL centrifuge tube for secondary concentration at 1,200 × g. Figure 1 shows all of the steps used in the analysis of the water samples.

Secondary concentration

Centrifugation was used as a secondary concentration method. The contents of the 500 mL centrifuge tube were vortexed for 30 seconds and poured evenly into two 250 mL centrifuge tubes that were centrifuged for 30 minutes at 1,200 × g. Centrifuge tubes were allowed to coast to a stop. Following the initial centrifugation, the supernatant in each centrifuge tube was aspirated to 35 mL. Centrifuge tubes were vortexed for 30 seconds and the contents were further mixed by 30 seconds of trituration with a 10 mL pipette. Finally, the contents of one centrifuge tube were added
to the second centrifuge tube. The centrifuge tube was rinsed twice with deionized water and the rinsate was added to the second centrifuge tube. The sample was then centrifuged a second time for 15 minutes at $1,200 \times g$. After the second centrifugation, the supernatant was vacuum-aspirated to 5 mL at 2 inHg. The contents of the centrifuge tube were vortexed for 30 seconds and then triturated for 30 seconds. The sample concentrate was then further purified with IMS.

IMS

IMS was performed with a Dynabeads® GC-Combo kit (Invitrogen Dynal, Oslo, Norway). The IMS procedure was performed in accordance with USEPA Method 1623 (USEPA 2005) with the exception of one modification. During the IMS procedure, 100 μL of 0.1 N HCl was added to the microcentrifuge tube, which contained the magnetic beads and attached oocysts. Method 1623 specifies 50 μL acid volumes, but this study used 100 μL volumes so that one-half of each sample could be applied to a slide and stained, while the second half of the sample could be analyzed by qPCR. After the first acid dissociation, 50 μL of the sample was quantitatively placed onto a well slide and 50 μL was placed into a microcentrifuge tube for qPCR analysis. A second acid dissociation was also performed using 100 μL of 0.1 N HCl, and the acid was divided as in the previous dissociation. The sample designated for qPCR had 100 μL of 1 N NaOH added prior to nucleic acid extraction.

Monoclonal antibody staining

After sample slides were dried, samples were stained as specified in USEPA Method 1623 (USEPA 2005). The monoclonal antibody stain used was EasyStain (BTF, Sydney, Australia). Dynal Spot-on slides (Invitrogen Dynal, Oslo, Norway) were examined within 7 days of staining by epifluorescence/differential interference contrast microscopy (Olympus BX61, Center Valley, PA, USA).
Basic nucleic acid extraction procedure

The (Biofire Diagnostics 1-2-3 SWIPE Sample Purification) kit served as the basic extraction/purification mechanism for the DNA preparation, although several modifications to the prescribed protocol (discussed in the following sections) were tested and implemented to increase method sensitivity.

The basic nucleic acid extraction procedure that was used to extract DNA began with the addition of an extraction buffer to a bead tube, which contained 0.10–0.25 mm glass beads. After the extraction buffer was added, either a known number of oocysts or a concentrated sample was added to the bead tube. After sample addition, the bead-beating procedure was performed. The bead-beating consisted of the bead tube being shaken for 5 minutes at maximum speed in a Vortex Genie 2 with the Turbo Mix attachment (Scientific Industries, Bohemia, NY, USA). After bead-beating, the sample was transferred to a spin column (Biofire Diagnostics, Salt Lake City, UT, USA). Following completion of a washing procedure, the spin column was eluted using Tris–EDTA (TE) buffer to produce an eluted DNA volume of approximately 55 μL.

The various modifications that were investigated included: extraction buffer type, extraction buffer addition point, number of spin column/extraction buffer cycles, extraction buffer volume, and TE buffer volume. Extraction comparison experiments were performed using small volume, serially diluted oocyst stock suspensions.

Extraction buffer type

To develop a more effective extraction method, two types of lysis/extraction buffer were tested. The lysis/extraction buffer (Buffer 1) provided in the Biofire Diagnostics kit was compared to the UNEX lysis/extraction buffer (MicroBioLogics, Catalog #MR0501) used in previous studies (Water Research Foundation 2010; Shields et al. 2013; Hill et al. 2013). To assess the efficacy of each buffer, oocysts were added to bead tubes at a concentration that would yield approximately 125 oocysts per reaction. Six qPCR reactions were performed for each extraction buffer.

Extraction buffer addition point

Once an extraction buffer was selected, the addition point was investigated. Extraction buffer was either added to the bead tube (seeded with oocysts) before bead beating or after bead beating. Six reactions were performed to investigate each buffer addition point.

Extraction buffer volume

The volume of extraction buffer used was also tested to determine the optimal volume for method performance. Bead tubes were seeded with small volumes of oocyst stocks, calculated to contain approximately 16–24 oocysts per reaction, and processed through DNA extraction and purification. Twelve reactions were analyzed for samples processed through both spin column cycles with the same 450 μL aliquot of extraction buffer. Alternatively, 12 reactions were performed for samples that received 200 μL of extraction buffer during the first spin column cycle, followed by a second aliquot (250 μL) of fresh extraction buffer that was added during the second spin column cycle.

Spin column cycles

After the volume of extraction buffer was optimized, the number of spin column cycles was examined to determine if an increase in DNA recovery could be achieved (as indicated by an associated decrease in qPCR CT values). Bead tubes were seeded at four different levels ranging from eight to 33 oocysts per reaction, and the DNA extraction/purification procedure was performed. Thirty-one total reactions were performed with one spin column cycle, and 31 total reactions were performed after recovering DNA using two spin column cycles.

Buffer volume

The final DNA extraction modification that was tested involved the volume of TE buffer that was used to extract the DNA template from the spin column. Three volumes (100, 55, and 30 μL) were tested. Eighteen reactions were performed for the 100 μL volume, while 16 reactions were
performed for the 55 μL volume, and 12 reactions were performed for the 30 μL volume.

**Real-time PCR analysis**

Amplification of *Cryptosporidium* DNA was performed using glass capillaries on a RAPID Real-time PCR System (Idaho Technology, Salt Lake City, UT, USA). A TaqMan® assay, reported by Hill *et al.* (2007) and Jothikumar *et al.* (2008), was used to detect *Cryptosporidium parvum*. The optimal amount of DNA template to be added to each capillary was also assessed. These tests were completed after the extraction buffer was selected and the time of addition of the extraction buffer to the bead tube had been determined (but prior to other method development testing). Three different volumes of DNA template (1, 5, and 8 μL) were tested in order to determine which volume enhanced detection and recovery. The amplification protocol used was as follows: denaturation at 95 °C for 15 minutes, followed by 45 cycles of denaturation at 95 °C for 10 seconds, annealing at 55 °C for 20 seconds, and extension at 72 °C for 15 seconds. Each 20 μL reaction contained 10 μL of 2x PCR master mix (QuantiTect Probe PCR Kit, Qiagen, Valencia, CA, USA), 8 μL of DNA, and primers and probe as described by Hill *et al.* (2007).

**Statistical analysis**

Since samples were split into two final detection methods, the total number of organisms seeded was divided by two to calculate recovery efficiency. For slides, the recovery efficiency was calculated by dividing the number of counted organisms by half the number of organisms seeded into the 10 L sample. For qPCR, six reactions per sample were processed, and the mean crossing threshold (CT) was calculated for each sample. The intercept of the standard curve was then subtracted from the mean CT, and the resulting number was divided by the slope of the standard curve. This resulted in a calculated log value that was converted to a number. The recovery efficiency was calculated by dividing the calculated number of oocysts, based on the standard curve, by half the number of organisms seeded into the 10 L sample. Paired t-tests were performed, using statistical analysis software (Minitab 15), to determine if qPCR recovery/calculation was significantly different from that achieved with slide examination. In addition, CT values and standard deviations were calculated for the extraction modifications. Any samples that were non-detects (i.e., CT value > 44) were discarded from the dataset to avoid skewing results in the calculations of these values. Extraction modifications were tested for statistical differences using ANOVA (Minitab 15). For each statistical analysis, the significance level (α) was set at 0.05.

**RESULTS AND DISCUSSION**

**Comparison of extraction buffer types**

The UNEX extraction buffer, used in conjunction with the Biofire Diagnostics 1-2-3 SWIPE Sample Purification kit (but replacing the Biofire Diagnostics kit’s lysis buffer) yielded better qPCR results than when the Biofire Diagnostics kit was used without modification. While none of the capillaries containing oocyst DNA that had been extracted with Buffer 1 tested positive, 83% of the capillaries from the UNEX buffer were positive. Capillaries that produced non-detects as a result were not included in calculations of mean CT values and standard deviation values. Since all capillaries where Buffer 1 was used produced non-detects, no mean CT could be calculated. For the UNEX buffer, the mean CT was calculated to be 39.3 (SD = 1.78).

**Extraction buffer addition point**

When the UNEX buffer was added after bead beating, only two of six (33%) capillaries had detections, while six of six (100%) capillaries had detections when the buffer was added prior to bead beating. Although there was no statistically significant reduction in the mean CT value for samples processed with the buffer added before bead beating (P = 0.894), the percentage of positive samples increased. Therefore, for the remainder of testing, UNEX extraction buffer was added prior to bead beating.

**Extraction buffer volume**

When 450 μL of UNEX buffer was added in a single application, the mean CT value for all capillaries seeded with
16–24 oocyst equivalents per capillary was 37.3 (SD = 2.72), while the mean CT increased slightly to 37.6 (SD = 1.15) when the extraction buffer was added as two aliquots of 200 μL and 250 μL, respectively. The slight increase in CT produced by using two smaller volume aliquots was not statistically significant (P = 0.686). However, more positive capillaries were achieved through all seed levels, as summarized in Table 1, when the single addition of 450 μL of extraction buffer was used, so this was maintained throughout the remainder of processing.

### Spin column cycles

When a seed value of eight oocysts per reaction was used, 40% of samples that passed through the spin column once were positive (mean CT = 40.6; SD = 2.85) and 83% were positive (mean CT = 39.5; SD = 1.40) when passed through twice (P = 0.507). At seed doses of 13 (mean CT = 39.6; SD = 1.30), 16 (mean CT = 39.2; SD = 1.52), and 33 (mean CT = 37.5; SD = 1.66) oocysts per reaction, all reactions were positive when the spin column procedure was performed twice. Alternatively, when one spin column cycle was used with 13 oocysts per reaction, only 50% of the reactions were positive (mean CT = 37.2; SD = 1.11) (P = 0.051). However, with higher seed doses of 16 (mean CT = 37.7; SD = 1.69) (P = 0.051) and 33 (mean CT = 35.2; SD = 3.29) (P = 0.124) oocysts per reaction, all reactions were positive when one spin column cycle was performed. Overall, 71% of the samples passed through the spin column once were positive while 97% of samples passed through the spin column twice tested positive for Cryptosporidium. Thus, this modification was continued throughout the remainder of the study.

### Buffer volume

Once an extraction procedure had been determined, different volumes of TE buffer were tested in order to determine the optimum volume for removal of DNA template from the spin column filter. Three volumes ranging from 100 to 30 μL were tested. Samples contained in 100 μL of TE buffer had a mean CT value of 36.8 (SD = 1.87) but were positive for only 72% of the samples. When a 50 μL TE buffer volume was used, the mean CT was higher (40.3, SD = 2.77), and the number of positive samples also decreased. In between these two volumes, a 55 μL volume was tested. For samples contained in 55 μL of TE buffer, the mean CT value was 36.7 (SD = 1.51). The use of 55 μL of TE buffer allowed for more positive results, as shown in Table 1. The change in mean CT value between all TE buffer volumes was statistically significant (P = 0.001). More specifically, the CT values for 30 μL were significantly higher than both the 100 μL volume (P = 0.006) and 55 μL volume (P = 0.001). The 55 μL TE buffer volume was retained for use in order to optimize recovery and sensitivity of Cryptosporidium since the mean CT value was lower, the standard deviation was lower, and the number of samples with detections was higher.

### DNA template volume

Once the addition point of the extraction buffer was determined, tests were conducted to determine the amount of extracted DNA template to be used. Three volumes (i.e., 1, 5 and 8 μL) were tested. The mean CT for the 1 μL template volume was 37.6 (SD = 0.324), while use of the 5 μL template yielded a mean CT of 38.0 (SD = 2.14). Finally, when 8 μL of template was used, the mean CT was 37.8 (SD = 4.33). There was no significant difference in the mean CT at 120 oocysts per capillary, based upon the volume of template used (P = 0.751), but the use of an 8 μL template was selected for use in future tests since it produced the greatest sensitivity as shown in Table 2.

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**Table 1** Summary of percent positive data and CT data for extraction buffer volume and TE buffer volume modifications

<table>
<thead>
<tr>
<th>Buffer Volume</th>
<th>Percent Positive</th>
<th>Mean CT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extraction buffer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 μL + 250 μL</td>
<td>83% (10/12)*</td>
<td>37.6 (SD = 1.15)</td>
</tr>
<tr>
<td>450 μL</td>
<td>100% (12/12)</td>
<td>37.3 (SD = 2.72)</td>
</tr>
<tr>
<td><strong>TE buffer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μL</td>
<td>72% (13/18)</td>
<td>36.8 (SD = 1.87)</td>
</tr>
<tr>
<td>55 μL</td>
<td>94% (15/16)</td>
<td>36.7 (SD = 1.51)</td>
</tr>
<tr>
<td>30 μL</td>
<td>50% (6/12)</td>
<td>40.3 (SD = 2.77)</td>
</tr>
</tbody>
</table>

*Number of positive reactions out of total number of reactions performed.
The detection limit for this assay was determined to be two oocysts per reaction. A standard curve, shown in Figure 2, was developed and had an $R^2$ value of 0.996, a slope of $-3.39$, and an intercept of 41.3.

Comparison of oocyst recoveries for microscopy versus qPCR

To test the effectiveness of the modifications made to the DNA extraction procedure with full-size samples, a small-scale study was performed. Tap water samples were spiked with known concentrations of oocysts and processed with the ultrafiltration procedure. As shown in Table 3, the number of oocysts calculated by qPCR was higher than the number counted with microscopy for each separate experiment. When compared, the percent recovery of oocysts calculated by qPCR was significantly higher than the percent recovery when microscopy was used ($P = 0.013$).

CONCLUSIONS

Based upon the results of the various modifications that were tested, a final nucleic acid extraction procedure was developed and used for all ultrafiltration samples. Prior to addition of the concentrated sample, 450 μL of the lysis buffer was added to a bead tube. After sample addition, the bead-beating procedure was performed. After bead-beating, the sample was transferred to a spin column and centrifuged at 7,800 × g for 2 minutes. The spin column was then reloaded with the same sample and centrifuged a second time at 7,800 × g for 2 minutes. The spin column was washed once with Buffer 2 and centrifuged for 2 minutes at 7,800 × g. To ensure the complete removal of Buffer 2, the spin column was transferred to a new microcentrifuge tube and centrifuged at 7,800 × g for 3 minutes. Lastly, the nucleic acid was eluted from the spin column through the addition of 55 μL of TE buffer, a 2-minute room-temperature incubation, and subsequent centrifugation for 2 minutes at 7,800 × g. Two incubation and centrifugation cycles were performed for nucleic acid elution.

As shown by the qPCR and microscopy results (Table 3), both methods yielded oocyst recovery estimates that were within the EPA acceptable range of 13–111% for matrix spike samples. In previous research performed with tap water, samples seeded with a similar number of organisms (i.e., 150 oocysts) produced recoveries of 51% (SD = 18) (Hill et al. 2009). In addition, in a study of reagent and surface water samples, ultrafiltration recoveries of 42% for Cryptosporidium were reported (Simmons et al. 2003). Ultrafiltration performed on nineteen surface water samples

Table 2 | Percentage of positive reactions for multiple seed doses at each template volume tested

<table>
<thead>
<tr>
<th>Extracted template volume (μL)</th>
<th>Seed dose (oocysts per capillary)</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0% (0/4)$^a$</td>
<td>0% (0/4)</td>
<td>0% (0/4)</td>
<td>50% (1/2)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>50% (2/4)</td>
<td>25% (1/4)</td>
<td>50% (2/4)</td>
<td>50% (1/2)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>50% (2/4)</td>
<td>33% (2/6)</td>
<td>75% (6/8)</td>
<td>100% (4/4)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Number of positive reactions out of total number of reactions performed.

Table 3 | Percentage of C. parvum oocysts recovered by microscopy versus qPCR

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Percent recovery by microscopy (%)</th>
<th>Percent recovery by qPCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
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<td>3</td>
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<td>5</td>
<td>20</td>
<td>27</td>
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<tr>
<td>Mean</td>
<td>41</td>
<td>49</td>
</tr>
<tr>
<td>Std deviation</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure 2 | Real-time PCR standard curve for Cryptosporidium parvum.
resulted in a mean recovery of 47.9% (Kuhn & Oshima 2002).

Since fluorescence microscopy is time-consuming and relatively expensive, qPCR represents a rapid and cost-effective option for the detection of oocysts from water samples in non-regulatory situations. However, as reported in this study, there is some variability in the ultrafiltration and concentration methods. While recovery, overall, was encouraging, there were two samples that were affected by much lower recovery for both microscopy and qPCR. Therefore, this was not a byproduct of the detection method (i.e., qPCR or microscopy) but was instead consistent for those samples regardless of the detection method.

While this study showed that qPCR could allow for the recovery of Cryptosporidium from water samples, there are still some limitations that were not addressed. The qPCR assay used in this study is not species-specific, and the method performed in this study does not allow for viability to be assessed. In addition, the full-scale water sample testing was limited to only one species of oocyst and the number of oocysts seeded did not vary substantially. To more fully assess ultrafiltration of water samples with qPCR detection of Cryptosporidium, additional water sources and oocyst seed doses, particularly lower seed doses, would need to be examined.

ACKNOWLEDGEMENTS

The use of trade names and names of commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the US Department of Health and Human Services. The findings and conclusions in this presentation are those of the authors and do not necessarily represent those of the Centers for Disease Control and Prevention.

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