Inactivation of *Cryptosporidium parvum* under chlorinated recreational water conditions

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ABSTRACT

Cryptosporidium is a chlorine-resistant protozoan parasite and the etiological agent in many disinfected recreational water outbreaks. While previous studies have reported disinfection C*t* values for *Cryptosporidium parvum* using sodium hypochlorite, these studies have employed conditions and procedures which are not ideal for establishing public health remediation recommendations for chlorinated recreational water venues. In the present study, free chlorine C*t* values were measured at pH 7.5 using young oocysts (<1 month old) and tissue culture to determine oocyst viability. Two different oocyst isolates were used: one originating from Iowa and one from Maine (USA). This study determined that the C*t* values for a 3-log reduction in oocyst viability were 10,400 (Iowa) and 15,300 (Maine) at pH 7.5. These C*t* values are higher than the Centers for Disease Control and Prevention (USA) currently recommends ($Ct = 9{,}600$) for achieving a 3.0-log inactivation of *Cryptosporidium* oocysts during remediation of recreational water venues following fecal diarrhea accidents.

Key words | *Cryptosporidium parvum*, C*t* value, hyperchlorination, parasitic protozoa, recreational waters, swimming pool

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NOMENCLATURE

C*t*	concentration of disinfectant (mg/L) × exposure time (minutes)
AGI	acute gastrointestinitis
ID_{50}	median infective dose
ppm	parts per million also mg/L
HOCl	hypochlorous acid
CDF	chlorine demand-free
DPD	*N*, *N*-diethyl-*p*-phenylenediamine
MDCK	Madin-Darby canine kidney
PBS	phosphate buffered saline
BSA	bovine serum albumin
DABCO	1,4-diazabicyclo-[2.2.2]-octane

INTRODUCTION

Cryptosporidium spp. are obligate, intracellular parasites of humans that infect the epithelial cells of the small intestine which ultimately results in the release of environmentally-resistant, and immediately infectious oocysts (Fayer *et al.* 1997). Infection causes a self-limited, acute gastrointestinal illness (AGI; watery diarrhea, vomiting, weight loss and abdominal cramps) with an incubation period of approximately 7 days (Chen *et al.* 2002). The two primary *Cryptosporidium* species of concern for human health, *Cryptosporidium parvum* and *Cryptosporidium hominis* have both been implicated in outbreaks associated with drinking and recreational water, food, animal-to-person contact, and person-to-person contact. *Cryptosporidium*'s low infectious dose and exceptional chlorine resistance have contributed to the pathogen becoming the leading cause of AGI associated with chlorinated swimming venues. From 2003 to 2004, *Cryptosporidium* was implicated in 11/18 (61.1%) AGI outbreaks associated with chlorinated swimming venues (Dziuban *et al.* 2006).

The median infectious dose (ID_{50}) for cryptosporidiosis has been reported to be approximately 132 oocysts

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(DuPont *et al.* 1995) measured using the excretion of oocysts or as few as 35.7 oocysts (Messner *et al.* 2001) using diarrheal illness as an indication of cryptosporidiosis. A single 100 g fecal accident from an infected person could contain over 1×10^6 oocysts per gram of stool (Goodgame *et al.* 1993; Castor & Beach 2004). Diarrhetic feces can become dispersed very quickly throughout a pool and since the average swimmer ingests 49 ml and 21 ml (children and adults, respectively) of pool water per hour on average (recalculated since the original data was collected after 45 minute exposures) (Dufour *et al.* 2006), the swimming pool environment can be an effective means of transmitting *Cryptosporidium*.

The calculated C*t* value [concentration of free chlorine in milligrams per litre (or parts per million) multiplied by time in minutes] typically recommended for swimming pool remediation after a diarrhea accident is 9600 at pH 7.0, which is based on data collected for use with drinking water (Korich *et al.* 1990) rather than for recreational water settings (which are typically maintained at pH values between 7.2 and 7.8). Considering the strong effect that pH has on the degree of dissociation of hypochlorous acid (White 1999), the relative percentage of HOCl *versus* hypochlorite (OCl^-) is substantially reduced in the higher pH environments typically present in swimming pools. In addition, the presence of organic and inorganic compounds can substantially reduce the effective free chlorine concentration available for disinfection. Thus, continuous or frequent monitoring of free chlorine levels is important for accurately estimating the *Ct* value for disinfection experiments. Unfortunately, such frequent monitoring has not been reported in most studies.

Excystation has been used in most *C. parvum* disinfection studies, with mouse infectivity occasionally employed to verify the calculated disinfection rates (Korich *et al.* 1990; Driedger *et al.* 2000; Corona-Vasquez *et al.* 2002). Excystation assays tend to underestimate the rate of inactivation because such measurements do not account for oocysts that are able to excyst and release sporozoites, which while capable of infiltrating cells are unable to produce an actual infection (Black *et al.* 1996; Bukhari *et al.* 2000; Joachim *et al.* 2003). While mouse infectivity is considered a gold standard (Black *et al.* 1996; Bukhari *et al.* 2000), it is an expensive and cumbersome method. Tissue culture has been compared to mouse infectivity with favorable results (Arrowood 2002; Rochelle *et al.* 2002; Joachim *et al.* 2003). Tissue culture, while demanding special skills, is less expensive than mouse infectivity and far less labor-intensive. Since large numbers of oocysts can be applied to each monolayer (up to 1×10^6), a three to four-log reduction in oocyst viability can easily be measured.

This study reports a revised *Ct* value suited for use in recreational water settings employing chlorine as a disinfectant. Considering the strong effect which pH has on the degree of dissociation of hypochlorous acid (HOCl) (White 1999), this study measured the new *Ct* using 1) a more swimming pool-specific pH, 2) fresh oocysts (<1 month old) to mimic those likely to be found in a pool following a fecal accident, 3) a frequently monitored and maintained hyperchlorination level of 20 mg/L free chlorine (Centers for Disease and Control and Prevention recommended response to a diarrhea contamination event) (MMWR 2001), 4) tissue culture infectivity which better reflects oocyst inactivation, and 5) evaluated two distinct *Cryptosporidium* isolates to investigate potential isolate inactivation variability.

METHODS

Cryptosporidium parvum oocysts

The *C. parvum* oocysts used in this study were produced at the Centers for Disease Control and Prevention. Two different oocyst lines were used; one was originally isolated from cattle in Iowa (Harley Moon isolate) and the other was first collected from a human outbreak in Maine, USA (Millard *et al.* 1994). Both have been identified as *C. parvum* according to the method of Xiao *et al.* (2003) (data not shown). The oocysts were amplified in infected calves and purified as per Arrowood & Donaldson (1996). In short, the collected feces were strained through 20- and 60-mesh stainless steel sieves to remove large debris. Oocysts were separated from fecal material via two series of discontinuous Sheather's sucrose gradients (specific gravity 1.064 g/ml and 1.103 g/ml) with final purification being made through a microscale cesium chloride (specific gravity 1.15 g/ml)

gradient. Solution densities were measured using conventional laboratory hydrometers. The oocysts were washed three times in buffered chlorine demand-free water (centrifugation at 3290 × g for 10 minutes), were stored in sterile chlorine demand-free (CDF) water at 4°C, and counted using a hemocytometer before use. The Iowa oocysts used were from two lots: lot A (26 and 27 day old oocysts at the time of the experiments) and lot B (17, 20, 24, 26 and 27 day-old oocysts). Three lots of Maine oocysts were used: lot A (3 and 5 day-old oocysts), lot B (10, 14, 21, and 29 day-old oocysts), and lot C (4 day-old oocysts).

Chlorine demand-free (CDF) water and glassware

CDF water and glassware were prepared as per *Standard Methods for the Examination of Water and Wastewater* (19th edition) (APHA 1995). In short, de-ionized water was buffered to pH 7.5 with 10 mM sodium phosphate and then sodium hypochlorite (Clorox®, 5.25% sodium hypochlorite, Clorox Company, Oakland, CA) was added to a concentration of at least 5-ppm free chlorine. After 48 hours, the free chlorine was removed through ultraviolet light irradiation. The water was stored and all experiments were performed in chlorine-demand free glassware.

Free chlorine, pH, and temperature monitoring

Free chlorine from sodium hypochlorite (Clorox®, 5.25% sodium hypochlorite) was measured hourly with a Thermo-Orion (Beverly, MA) AQUAfast II photometer using DPD (*N*, *N*-diethyl-*p*-phenylenediamine) chemistry. If the free chlorine concentration dropped more than 5% between measurement time points during an experiment, additional sodium hypochlorite was added until the initial level was reached. The temperature and pH were monitored using a Thermo Orion Model 290Aplus Portable Meter.

Disinfection experiments

A total of seventeen disinfection experiments were performed according to the following protocol. Free chlorine was added to 1 L of CDF water to 20 mg/L (20 ppm), stirred for one hour, and re-checked to ensure that the chlorine level was stable. If the chlorine value was stable, 1 x 10^8 washed *C. parvum* oocysts were added to the water and, within five minutes, the level of free chlorine was measured. At each collection time point, a 40-ml sample was drawn from the beaker and placed in a sterile 50-ml screw-cap tube containing 1 ml of 10% sodium thiosulfate (Sigma, St. Louis, MO) to stop chlorine oxidation, and then gently swirled and inverted to mix. The sample was centrifuged at 3290 × *g* for ten minutes to pellet the oocysts, re-suspended in 1 ml of BioWhittaker™ Ultra-CULTURE media (Cambrex Bio Science Walkersville, Inc, Walkersville, MD), and stored for 5 to 18 hours at 4°C until inoculation onto tissue culture. An aliquot was aseptically removed for counting in a hemocytometer. Time zero oocysts were removed prior to the disinfection experiment and thus represent the initial infectivity of the oocysts.

Determining the Viability of *C. parvum* Oocysts

Cell culture

Tissue culture was used to determine the decrease in oocyst viability (Arrowood 2002). Madin-Darby canine kidney (MDCK) cells were inoculated into culture chambers (Nunc Lab-Tek, Rochester, NY) and allowed to grow for 96 hours at 37°C under 5% CO_2 to form a monolayer. In order to both prevent the over-inoculation of shorter time point samples and to be certain of capturing any viable oocysts from later time points, the number of oocysts inoculated onto the monolayers was based on the time of sampling. For untreated samples 10,000 oocysts were inoculated, 7 hour samples 100,000 oocysts, and 10 to 14 hour samples 500,000 oocysts were inoculated, in triplicate, onto the cell monolayers. The monolayers were then incubated at 37°C for 48 hours under 5% CO_2.

Fixing and staining

After incubation, the monolayers were washed, fixed and stained as described in Arrowood (2002). Briefly, the monolayers were gently washed with sterile 0.01 M phosphate buffered saline (PBS) pH 7.2 and fixed with Bouin's solution for one hour. The Bouin's solution was removed and the cells decolorized with five washes of 70% ethanol. After again washing with PBS, the monolayers were blocked with PBS and 0.1% bovine serum albumin (PBS + BSA) at room temperature. After at least an hour,

the PBS + BSA was removed and the *Cryptosporidium*-specific monoclonal antibody C3C3 conjugated with indocarbocyanine (Research Organics, Cleveland, OH) was added. The labeled antibody was incubated on the monolayers for at least an hour (though typically overnight) in the dark at 4°C. After labeling, the unbound C3C3 conjugate was removed and the monolayers were washed with PBS + BSA. Two drops of a long-term mounting media, 2.4 g polyvinyl alcohol (Sigma, St. Louis, MO), 6.0 g glycerol, 6.0 ml H_2O, 12.0 ml 0.2 M Tris buffer (pH 8.5) and 2.5% (wt/vol) of the antiquenching agent DABCO (1,4-diazabicyclo-[2.2.2]-octane), was placed on the monolayer followed by an 18 − mm^2 glass cover slip. Excess PBS + BSA and mounting medium were carefully aspirated from the slide. These mounted chambers were allowed to sit overnight in the dark at 4°C before microscopic examination.

Counting

The life cycle stages to be counted, meronts and gamonts, were determined by their size (~3–5μm) and donut shape. All slides were examined visually at 250x magnification and counted; for all of the samples, at least two slides were counted and the results averaged. The parasite asexual and sexual stages were enumerated by dividing the slide into 30 rows; the stages in five evenly distributed rows were then counted and multiplied by six. Replicate slides were then averaged.

Calculations and statistical analysis

C*t* values were calculated for 2.0 and 3.0-logs of oocyst inactivation using the equation: C*t* = concentration of free chlorine x oocyst contact time in disinfectant (in minutes). Averages and standard deviations were calculated using Excel (Microsoft). Given 1) the small sample size, 2) the inability to assume a normal distribution and 3) appropriate restrictions on variance necessary for regression analysis, the Wilcoxon signed-rank (two-sided) test was deemed most appropriate for detecting differences between the two oocyst sources and the individual oocyst lots. Data for the analysis are listed in Tables 1 and 2 and the resulting averages are illustrated in Figure 1. Linear interpolation was used between data points in Figure 1 to graphically communicate the experimental results and do not represent a suggested disinfection kinetics model.

RESULTS AND DISCUSSION

Temperature, pH, and free chlorine concentration monitoring

The temperature, pH, and free chlorine were monitored in all inactivation experiments. Tables 1 and 2 show the average values of the physical and chemical parameters monitored for the Iowa and Maine oocyst disinfection experiments. The pH and temperature were consistent across all experiments: the pH ranged from 7.5 to 7.6 and

Table 1 | Oocysts, physical chemical conditions for each experiment and the resultant minutes and C*t* values of *C. parvum* Maine oocysts

Oocysts		Experimental conditions			Minutes for reduction in viability		C*t* values	
Lot	Age (days)	Free chlorine (mg/L) [*]	pH	°C	2-log	3-log	2-log	3-log
A	3	21.6 [1.4]	7.5	22	444	654	9590	13500
A	5	20.6 [1.0]	7.6	22	480	654	9890	13500
Lot A Average					462	654	9740	13500
B	10	20.3 [0.37]	7.5	23	555	ND	11300	ND
B	14	20.0 [0.39]	7.5	23	546	ND	11900	ND
B	21	20.1 [0.30]	7.5	23	546	ND	11000	ND
B	21	19.9 [0.30]	7.5	23	555	ND	11100	ND
B	29	20.0 [0.40]	7.5	23	556	ND	11200	ND
B	29	19.8 [0.40]	7.5	23	563	ND	11200	ND
Lot B Average					554		11300	
Lot B Standard deviation					6		319	
C	4	19.8 [0.63]	7.5	21	594	822	11800	16300
C	4	20.0 [0.40]	7.5	21	564	810	11300	16200
C	4	20.4 [0.73]	7.5	21	573	834	11700	17100
Lot C Average					577	822	11600	16500
Lot C Standard deviation					13	10	216	403
Lots A, B and C Average					543	755	11100	15300[†]
Lots A, B and C Standard deviation					43	92	731	1700[†]

Values are rounded to three significant digits.
ND = not done.
[*]standard deviation of chlorine readings for individual experiment.
[†]Only for lots A and C.

Table 2 | Oocysts, physical chemical conditions for each experiment and the resultant minutes and C*t* values of *C. parvum* Iowa oocysts

Oocysts		Experimental conditions			Minutes for reduction in viability		C*t* values	
Lot	Age (days)	Free chlorine (mg/L) [*]	pH	°C	2-log	3-log	2-log	3-log
A	26	20.8 [1.04]	7.6	23	353	529	7350	11000
A	27	19.7 [0.61]	7.6	23	340	511	6700	10100
Lot A Average					347	520	7020	10600
B	17	19.9 [0.52]	7.5	21	311	467	6200	9300
B	20	21.0 [0.51]	7.5	24	347	520	7300	11000
B	24	20.3 [0.29]	7.5	24	336	504	6830	10300
B	26	22.1 [0.59]	7.5	24	318	477	7040	10600
Lot B Average					328	492	6840	10300
Lot B Standard deviation					14	21	407	628
Lots A and B Average					334	501	6900	10400
Lots A and B Standard deviation					15	22	391	587

Values are rounded to three significant digits.
[*]standard deviation for individual experiment.

the temperature ranged from 21 to 24°C. The average free chlorine values ranged from 19.7 to 22.1 mg/L and varied little within each experiment (Tables 1 and 2).

C*t* values and inactivation curves for Maine and Iowa oocysts

Tables 1 and 2 also show the per-experiment and the average 2- and 3-log C*t* values calculated for Maine and Iowa oocysts and the minutes needed for oocyst inactivation at 20 mg/L free chlorine. The three lots of Maine oocysts [ranging in age from 3 to 29 days old (Table 1)], exhibited some variability in their susceptibility to free chlorine inactivation. Lots B and C had similar 2-log reduction C*t* values of 11,300 and 11,600, respectively, while those oocysts from lot A were more easily inactivated with an average 2-log reduction C*t* value of 9,740. This trend continued with 3-log reductions in oocyst activity observed with lots A and C exhibiting 3-log reduction C*t* values of 13,500 and 16,500, respectively, for an average C*t* of 15,300.

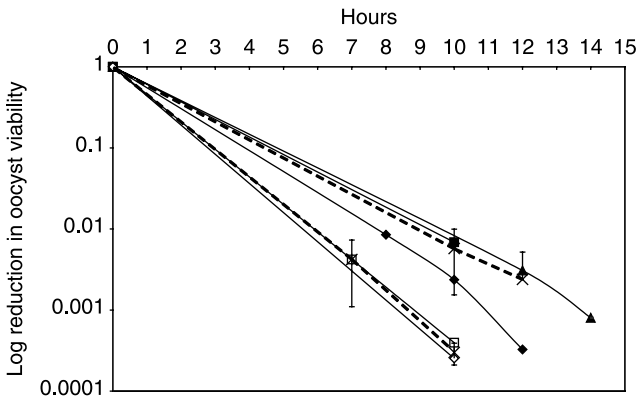

Figure 1 | Graph of log reductions in oocyst viability for the Iowa oocyst lots (A □, B ◇, and All ∗) and the Maine oocyst lots (A ◆, B ■, C ▲, and All ×) for the time points tested. The dashed lines represent the average log reductions for the Iowa and Maine oocysts. Vertical bars are 2-standard deviations at 7, 10 and 12 hour time points.

Two Iowa oocyst lots ranging from 17 to 27 days old exhibited similar susceptibility to chlorine inactivation with an average 3-log reduction C*t* value of 10,400. The Maine oocysts appear to be less susceptible to chlorination taking on average four hours longer to reach a 3-log reduction than the Iowa oocysts tested.

Figure 1 shows the disinfection curves for all experiments as well as the averages and two standard deviations. While previous work suggests that *Cryptosporidium* oocyst inactivation follows a second-order kinetic model (Korich *et al.* 1990; Rennecker *et al.* 1999; Driedger *et al.* 2000), the focus of this study was to determine end-point values for public health remediation purposes. Thus, the linear interpolation approach used to communicate experimental results in Figure 1 should not be viewed as suggesting a kinetic model for the actual oocyst disinfection process. No significant differences in disinfection rates were measured between Iowa oocyst lots A and B or Maine oocyst lots A, B and C; although the small sample size limited the power of the statistical analysis of Maine lot A data *versus* Maine lots B and C. However, a statistically significant difference was measured between the inactivation rates of the Maine and Iowa oocysts after 10 hours of exposure ($p = 0.005$) by the Wilcoxon signed-rank (two-sided) test.

The results from this study indicate that a free chlorine C*t* value of 15,300 (at pH 7.5) is needed to achieve a 3-log inactivation of *C. parvum* oocysts originating from a Maine outbreak and 10,400 for oocysts originating from calves in Iowa. While previous studies of *C. parvum* disinfection rates focused on inactivation using free chlorine, most were focused on drinking water conditions and have used excystation and/or mouse infectivity to determine oocyst

viability. In 1990, Korich *et al.* (1990) published a study which examined oocyst disinfection at pH 7.0 and employed both excystation and mouse infectivity assays to determine oocyst viability. A C*t* value of 9600 for a 3.0-log reduction of *C. parvum* oocyst infectivity was extrapolated from this study for use in developing recreational water remediation recommendations following fecal accidents involving diarrhea (MMWR 2001).

Driedger *et al.* (2000) examined the effect of pH on chlorine disinfection of *C. parvum* oocysts and included pH 7.5. Their C*t* value at pH 7.5 was extrapolated to be approximately 16,000 for a 3.0-log reduction for oocysts originating from Iowa, which is substantially greater than the value determined in the present study (C*t* = 10,400). This difference in C*t* values is likely due to the use of excystation to determine viability. It has been strongly suggested that excystation assays underestimate the inactivation rate because sub-populations of treated oocysts can excyst but be unable to cause infection (Black *et al.* 1996; Bukhari *et al.* 2000; Joachim *et al.* 2003).

Another compounding factor may be oocyst age; studies noting the age of the oocysts used in disinfection experiments are rare. Rennecker *et al.* (2000) and Corona-Vasquez *et al.* (2002) used oocysts 11 to 52 and 19 to 75 days old, respectively, and their experiments were performed at pH 6.0. While Driedger *et al.* (2000) examined disinfection rates at pH 7.5; they used oocysts ranging from 67 to 98 days old in their experiments. When examining disinfection rates for application to drinking water, it is necessary to use a broad range of oocysts ages, especially older oocysts, to reflect transport times between the source of oocysts and exposed individuals. However, because it is likely, if not certain, that the *Cryptosporidium* oocysts in a swimming pool-associated outbreak are very young, a goal of this study was to use the youngest oocysts possible. All oocysts used were less than one month old and two of the five lots were less than a week old. The Maine oocyst data in Table 1 suggest that oocyst age may be important (lot A vs. lot B), but any relationship between oocyst age and susceptibility to free chlorine was not consistently indicated by the data in the present study. There was no significant difference in inactivation rates between lots A and C, both of which represented oocyst ages of 3 to 5 days. However, as this study did not investigate oocyst ages older than 29 days (an age above which many studies are performed), further research is needed to determine whether oocyst age is an important variable for *Cryptosporidium* disinfection studies.

The measured differences in the C*t* values of oocysts originating from Maine *versus* Iowa suggest that *C. parvum* oocysts from the Iowa (calf) and Maine (human) isolates have different susceptibilities to chlorine inactivation. Differences in infectivity and viability in *C. parvum* oocysts from different geographical sources have been noted in oocysts isolated in Spain and Columbia (Vergara-Castiblanco *et al.* 2000). One factor responsible for the differences noted in the present study may be oocyst surface characteristics. For instance, Iowa oocysts are often easier to purify than the Maine oocysts, which may be due to differences in the oocyst surface characteristics. While these differences in the Iowa oocysts might discourage clumping and binding to debris, they may facilitate a more rapid absorption of the chlorine ions into the oocysts. In future disinfection studies it may be prudent to evaluate *Cryptosporidium* oocysts originating from multiple geographical locations to further investigate this variability in the susceptibility of *Cryptosporidium* to free chlorine. Thus, in order to better compare disinfection studies it would be valuable to specify source (or lineage) of the oocysts used in disinfection studies.

C. parvum was used in these experiments because of the availability of the calf model system to produce oocysts. However, many pool-associated outbreaks are due to *C. hominis* (Xiao *et al.* 2003). These experiments should be repeated when a system to produce sufficient quantities of *C. hominis* oocysts becomes available. Unfortunately, most *C. hominis* oocysts are derived from diarrhea outbreaks; after genotyping and other tests, oocysts numbers are often insufficient for disinfection experiments or they are too old.

CONCLUSION

The results of this study indicate that the C*t* values for inactivation of *C. parvum* using free chlorine at pH 7.5 are higher for oocysts isolated from a human outbreak in Maine; C*t* values for oocysts isolated from a bovine source in Iowa are only slightly higher as compared to experiments by other researchers conducted at pH 7.0. The increased C*t* value for the Maine isolate reported in the present study may be due to

the age of the oocysts (<1 month) or oocyst characteristics related to its source (geographic location or human *versus* bovine origin). Given the relative similarity between the present study and previous studies using Iowa oocyst isolates, the results from this study suggest that oocysts originating from different geographical locations or those oocyst lines isolated from human outbreaks may have a greater resistance to inactivation by chlorine. Given its greater resistance to free chlorine, the Maine oocyst C*t* value will be used to update CDC guidelines on the length of time used by pool operators for hyperchlorination in response to observed or suspected diarrhea fecal accidents.

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DISCLAIMER

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

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