

Evaluating *Cryptosporidium* and *Giardia* concentrations in combined sewer overflow

Russell D. Arnone and Joyce Perdek Walling

ABSTRACT

Since the first identified *Cryptosporidium* outbreaks occurred in the 1980s and the massive 1993 Milwaukee, WI outbreak affected more than 400,000 people, the concern over the public health risks linked to protozoan pathogens *Cryptosporidium* and *Giardia* has grown. *Cryptosporidium* and *Giardia*, found in streams, rivers, groundwater, and soil, form hardy, disinfection-resistant oocysts and cysts. Both organisms are recognized causative agents of gastrointestinal illnesses linked to the consumption of contaminated surface or groundwater. This study, the first in a planned series to estimate the urban contribution to the total *Cryptosporidium* and *Giardia* receiving-water loads, focused on combined sewer overflow (CSO). CSOs are discharges of mixed untreated sewage and stormwater released directly into receiving waters during rainfall. This engineered relief is necessary to accommodate hydraulic strain when the combined rain and sanitary flows exceed the system capacity. Limited comprehensive data are available assessing the CSO discharge contribution as a source of these two pathogens. Works by States *et al.* and Gibson *et al.* each found *Cryptosporidium* and much greater *Giardia* concentrations in CSOs draining parts of Pittsburgh, PA. This project estimated the relative detection frequency and concentration of *Cryptosporidium* and *Giardia* in CSO. Analytical results were obtained using a modification of Method 1623, originally developed for much cleaner environmental samples. These data are useful for drinking water treatment plants located downstream of CSOs. It is also significant in determining the potential concentrations of parasites at treatment plant intakes and for assessing health risks for water contact and fishing activities. Commonly monitored indicator organisms (total coliform, fecal coliform, *E. coli*, *Enterococcus*, and fecal streptococcus), endospores, and selected physical and chemical parameters were analyzed to further describe the samples. CSO from urban areas was not found to be a significant contributor of *Cryptosporidium*, however, it was found to be a *Giardia* source.

Key words | combined sewer overflow, *Cryptosporidium*, *Giardia*, U.S. EPA Method 1623

Russell D. Arnone (corresponding author)

Joyce Perdek Walling

United States Environmental Protection Agency,
Urban Watershed Management Branch (MS-104),
Edison, NJ 08837,
USA

Tel: +1-732-321-6791

Fax: +1-732-321-6640

E-mail: arnone.russell@epa.gov;

walling.joyce@epa.gov

BACKGROUND

Cryptosporidium

Cryptosporidium is a protozoan parasite that produces an unpleasant gastric and diarrheal illness known as cryptosporidiosis. Able to survive extended periods under harsh environmental conditions, *Cryptosporidium* oocysts are roughly spherical and 4 to 6 μm in diameter. Although the

U.S. Environmental Protection Agency (EPA) lists six species of *Cryptosporidium*, only *Cryptosporidium parvum* is known to infect humans. *Cryptosporidium* is frequently waterborne. Infections occur through contact with contaminated drinking water supplies, contaminated food, contaminated swimming pools and other recreational waters, as well as zoonosis. At present, there is no effective pharmaceutical therapy for the treatment of

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cryptosporidiosis. *Cryptosporidium*, therefore, can cause some alarming public health problems, particularly for people with weakened immune systems, especially Acquired Immunodeficiency Syndrome (AIDS) patients, in whom severe and protracted diarrhea can persist for months with considerable weight loss and mortality (Gerba *et al.* 1996; Rose 1997). Individuals may become infected with as few as 30 oocysts (AWWA 1999).

Giardia

Giardia lamblia, also known as *Giardia duodenalis* or *Giardia intestinalis*, is the most common cause of human protozoan infection. Sometimes referred to as “beaver fever,” “hiker’s disease,” or “camper’s disease,” *Giardia* infection, giardiasis, causes abdominal cramps, diarrhea, and bloating. The infection is transmitted by tiny spores or egg-like cells called cysts measuring 9 to 12 μm in length. There are several species of *Giardia*, but humans are usually infected by *Giardia lamblia*. Due to its thick wall, the *Giardia* cyst can survive weeks or months in fresh water, although it is less hardy than the *Cryptosporidium* oocyst (Rosen 2000). The infective dose for *Giardia* cysts varies from ten to one million viable cysts, depending on the immune system of the host. Giardiasis can be treated with drugs, including metronidazole, furazolidone, trinitazole, and paromomycin, therefore, it is not regarded as a fatal disease. Many individuals are asymptomatic carriers of *Giardia* (Rockwell 2002).

HISTORICAL DATA

The largest recorded outbreak of cryptosporidiosis occurred in Milwaukee, WI in 1993, where an estimated 403,000 people were infected, and approximately 50 to 100 area residents with compromised immune systems died after becoming infected (Fox & Lytle, 1996; Blair 1994; Hoxie *et al.* 1996). There have been more than 20 outbreaks of waterborne *Giardia* in the U.S. from recreational and surface drinking water contact between 1986 and 2000 (Levine *et al.* 1990; Herwaldt *et al.* 1992; CDC and U.S. EPA 1995; Kramer *et al.* 1996; Levy *et al.* 1998; Barwick *et al.* 2000; Lee *et al.* 2002).

The occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in receiving waters has been documented in Table 1.

Limited data exists for these protozoa in CSO. In the analysis of five CSO samples, States *et al.* (1997) found *Giardia* cysts in 100% (geometric mean 28,681 cysts/100l) and *Cryptosporidium* oocysts in 80% (geometric mean 2,013 oocysts/100l). In analyzing eleven end-of-pipe CSO samples, Gibson *et al.* (1998) reported *Cryptosporidium* oocysts in 100% (geometric mean = 6,036 oocysts/100l) and *Giardia* cysts in 100% (geometric mean = 35,402 cysts/100l) of the samples. However, each CSO is confined to its unique sewershed, therefore comparisons must be carefully considered. Differences in CSO *Cryptosporidium* and *Giardia* concentrations are due to each watershed’s unique characteristics. These differences also reflect the infected individuals within the watershed.

Encystment can protect protozoa from disinfection efforts (Frey *et al.* 1998), making prevention and physical removal the preferred protection mechanism. Well operated drinking water plants can physically remove 99% of oocysts from infected raw waters. Therefore, efforts to protect source waters from this organism are critical. Identifying and characterizing *Cryptosporidium* and *Giardia* in discharges to drinking water supplies provide a basis for designing source water protection programs.

The U.S. EPA Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) (U.S. EPA 2006), improves the control of *Cryptosporidium* in drinking water systems with the greatest risk levels. The LT2ESWTR promulgated January 2006, will supplement existing regulations by targeting additional *Cryptosporidium* treatment requirements to higher risk systems. U.S. EPA estimates that full implementation of this rule will reduce the incidence of cryptosporidiosis cases by 256,000 to 1,000,000 annually, with an associated reduction of 37 to 141 premature deaths. The cost of this rule will be between \$73.5 million and \$111 million annually.

OBJECTIVES

This study was conducted to expand the database on the expected occurrence and concentration of *Cryptosporidium* and *Giardia* in CSO, primarily the waters serving as a drinking water source. These hardy and viable parasites, transmitted in the encysted form, can withstand adverse conditions for extended periods. The longevity of the

Table 1 | Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in surface waters

Water type	No. of samples	% Samples positive <i>Giardia</i>	% Samples positive <i>Crypto.</i>	Conc. range per 100 l (GM)# <i>Giardia</i>	Conc. range per 100 l (GM)# <i>Crypto.</i>	Reference
Surface	51	39	39	–	–	(A)
Rivers/lakes	181	15	51	<1–140 (3)*	<1–4400 (43)*	(B)
Allegheny River	24	63	63	0–420 (34)	0–2230 (31)	(C)
Youghiogheny River	24	54	63	0–530 (120)	0–1470 (58)	(C)
Stream, dairy farm water	24	54	82	0–1570 (82)	0–1105 (42)	(C)
River diversion	19	21	50	0–625 (22)	0–24000 (109)	(D)
Lake outlet	20	40	50	0–222 (8)	0–2200 (58)	(D)
Stream/river	11	–	78	–	200–11200 (2510)*	(E)
Surface water	107	–	77	–	4–1800 (91)	(D)
Reservoir Inlet	60	13	5	0.7–24 (19)	0.7–2.4 (1.2)	(F)
Reservoir outlet	60	15	12	1.2–107 (6.1)	1.7–31 (8.1)	(G)
Surface water	85	81	87	4–6600 (277)	7–48400 (27000)	(G)
Raw source waters	262	45	52	2–4380 (200)	6.5–6510 (240)	(H)

(GM)# = geometric mean.

* = arithmetic mean.

(A) Barthe & Brassard 1994.

(B) Rose *et al.* 1991.(C) States *et al.* 1997.

(D) Rose 1988.

(E) Ongerth & Stibbs 1987.

(F) LeChevallier *et al.* 1997.(G) LeChevallier *et al.* 1991.

(H) LeChevallier and Norton, 1995.

encysted form allows viable parasites to travel large distances, particularly in river systems, from the CSO to water plant intakes.

SAMPLING, ANALYSIS AND QUALITY CONTROL

Sampling

CSOs from three outfall sites were sampled and analyzed for *Cryptosporidium* and *Giardia*. All three CSO outfalls discharge to receiving waters that is a source for potable water. Samples were collected from two overflow events at each site for a total of six samples. Due to the probability of

high particulate matter concentration found in CSO clogging the filter, the analysis required modifying Method 1623 (U.S. EPA 2001). The modification eliminated the filtration step, replacing it with centrifugation. Additional analysis measured the concentrations of several physical and chemical properties, five bacterial indicators (total coliform, fecal coliform, *E. coli*, *Enterococcus* and fecal streptococcus) and endospore concentrations in the CSO samples collected.

The first CSO sampling site was at the McDaniel Street Pump Station in Atlanta, GA. The sewershed drained a highly-impervious, commercial area. Sample collection occurred upstream of the station's sodium hypochlorite disinfection facility. Samples were collected using a con-

tainer suspended within the overflow. Precipitation at this outfall was monitored using the United States Geological Survey (USGS) gauging station 02336300 located on Peachtree Creek in Atlanta. Near real-time precipitation data can be accessed on the internet at <http://waterdata.usgs.gov/ga/nwis/rt>.

The other two CSO catchments sampled were in Louisville, KY. Both CSO sites were equipped with automatic sampling equipment. The outfalls selected discharge drainage from areas with different land use characteristics. The primary difference between the two catchments is the large portion of commercial and office use facilities draining to Outfall E. Public parks and semi-public land area together with single-family residential land dominant the sewershed draining to Outfall F. Rainfall data for both sites came from the Louisville USGS gauging station 03294500 near the Ohio River. Real time rainfall data is accessible online at <http://waterdata.usgs.gov/ky/nwis/rt>.

Samples were collected from each combined sewer outfall during two wet-weather events at each site. Following the U.S. EPA National Pollution Discharge Elimination System (NPDES) sampling guidance (U.S. EPA 1992), the dry weather duration before each sampling event was recorded. The required dry weather duration prior to each sampling event was at least 72 hours. When this dry period has occurred, weather forecasts were monitored for potential storms likely to cause an overflow.

Upon visual confirmation of an overflow event, an effort was made to start sampling within five minutes of the start of overflow. Sample collection typically required 15 to 30 minutes. This sampling approach was based on the theory that the greatest number of oocysts and cysts would be found in the “first-flush,” the period within the flow containing an elevated concentration. Although researchers have confirmed that the greatest concentrations of certain contaminants in some watersheds occur immediately after an overflow begins (Burton and Pitt 2002), data produced by Gibson *et al.* (1998) to demonstrate this occurrence for *Cryptosporidium* and *Giardia* are inconclusive. Both an increase and decrease of the concentrations of these protozoan parasites occurred during the “first-flush.” Verifying “first-flush” for *Cryptosporidium* and *Giardia* for the outfalls studied was outside the scope of this project.

The sample volume collected during the first two sampling events of eight 4-l samples was found to be excessive. After the first two events, the sample size was decreased to four 4-l samples. Using grab or automatic sampling as specified for the particular outfall, each CSO aliquot was transferred to a common container until the total volume was collected. The combined sample was homogenized using an electric mixer for at least 5 minutes before transferring to pre-disinfected 4-l containers with a disinfected plastic ladle and funnel. The sample was then placed in an insulated cooler, iced to maintain temperature between 0°C and 8°C, sealed with a custody seal, and shipped for overnight delivery to the laboratory for analysis.

Analysis

Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA quantifies *Cryptosporidium* and *Giardia* in water by filtration, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. This method is validated for surface water, but may be used in other waters provided the laboratory demonstrates that the method's performance acceptance criteria are met. Method 1623 does not identify the species of *Cryptosporidium* or *Giardia* or the host species of origin, and cannot determine the viability or infectivity of detected oocysts and cysts.

Method 1623 is summarized as follows:

1. Filter sample volume, normally 10-l for drinking water.
2. Elute cysts and oocysts from the filter.
3. Centrifuge and aspirate the supernatant.
4. Subject the pellet containing cysts and oocysts to IMS separating the cysts and oocysts from other material using magnetic beads attached to antibodies.
5. Stain the cysts and oocysts using fluorescently labeled antibodies by FA.
6. Visually enumerate under fluorescence microscopy.

CSO samples contain high levels of interfering organisms, substances and materials that impede the concentration process and reduce accuracy. The IMS procedure defined in Method 1623 limits the volume of the centrifuged pellet to a maximum of 0.5 ml per aliquot. CSO solids are typically at concentrations 100 times of that found in drinking water supplies. Therefore, a small volume of sample was required

to achieve the appropriate pellet volume. States *et al.* (1997) bypassed filtration, directly centrifuging 2-l samples of CSO to analyze for *Giardia* and *Cryptosporidium*. Similarly, in this study, the centrifugation step was used directly on the CSO sample, eliminating the filtration and elution steps.

The following is a summary of the modified Method 1623 used for CSO analysis in this study:

1. 1-l aliquots from each sample were poured into 1-l centrifuge tubes. Each sample was centrifuged at $1500 \times G$ for 15 minutes and allowed to coast to a stop. Pellet volume mass was measured to see if the 0.5 ml volume required was met. If the pellet was significantly smaller than the required volume, the supernatant was decanted and an additional sample was added for further centrifugation. If the pellet was significantly larger than the required volume, it was resuspended and the sample volume was reduced and then centrifuged. The supernatant was withdrawn from each tube and a small amount of reagent water was added.
2. The pellet and reagent water from each 1-l centrifuge tube were decanted into a separate 50-ml centrifuge tube. Each 1-l tube was rinsed using reagent-grade water. Rinses from each tube were composited into the sample's respective 50-ml tube. The tubes were then subjected to secondary centrifugation for 15 minutes at $1500 \times G$. The supernatant was carefully aspirated to 5 ml using a Pasteur pipette and the pellet volume recorded.

Samples were subjected to IMS and analyzed per Method 1623. The final volume analyzed was recorded and reported.

Each sample was analyzed in quadruplicate for *Cryptosporidium* and *Giardia* to assess method variability, with results expressed in mean log concentration. CSO samples were also analyzed for endospores and five bacterial indicators by membrane filtration. Table 2 provides a summary of bacterial analytical procedures.

The Standard Methods (SM) for the Examination of Water and Wastewater (1998) suggests sample volumes yielding colony counts from 20 to 60 colonies per membrane for fecal coliform, *Enterococcus*, and fecal streptococcus. It also suggests sample volumes yielding counts from 20 to 80 colonies per membrane for total coliform and *E. coli*. Sterile buffer water (SM 9050) dilutions were established using literature-reported concentrations. Camp, Dresser and McKee (1997) reported the expected density (colonies forming units (CFU) /100 ml) in CSO for total coliform (10^5 to 10^7), fecal coliform (10^4 to 10^6), *E. coli* (10^4 to 10^6) and *Enterococcus* (10^4 to 10^6). Olivieri *et al.* (1977) reported the expected density in CSO for fecal streptococcus (10^4 to 10^6). The aerobic spore procedure has been used by U.S. EPA on both sewage and drinking water. Raw wastewater was found to contain spore densities on the order of 10^5 CFU/100ml and treated effluent contained densities on the order of 10^4 CFU/100 ml

Table 2 | Summary table of analytical procedures

Target parameter	Standard Methods (SM) * Method Number	Method Title
Fecal coliform	SM 9222D	Fecal coliform membrane filter procedure
Total coliform	SM 9222B	Standard total coliform membrane filter procedure
Fecal streptococcus	SM 9230C	Membrane filter techniques
<i>Enterococcus</i>	SM 9230C	Membrane filter techniques
<i>E. coli</i>	SM 9222G	Membrane filter partition procedures
Endospores	N/A	U.S. EPA, ORD, NRMRL, WSWRD, Microbial Contaminants Control Branch – Aerobic Spore Procedure, MCCB SOP 63

*Standard Methods for the Examination of Water and Wastewater 1998.

(Meckes 2001). CSO concentrations were anticipated to be in this range.

Samples were also analyzed for particle size distribution (PSD), chemical oxygen demand (COD), total solids (TS), and total suspended solids (TSS). These non-critical measurements further describe the sample. PSD was measured using Coulter Particle Characterization Unit (Model No. Delsa 440 SX). TS and TSS were measured using SM 2540B (Total Solids Dried at 103–105 °C) and SM 2540D (Total Suspended Solids Dried at 103–105 °C). COD was analyzed following SM 5220. Upon arrival at the laboratory, pH, turbidity, and temperature were measured.

Quality control

The use of antibodies positive for *Cryptosporidium* and *Giardia* antigens (protein structures on the surface of the cell) during the IMS and FA microscopy portions of Method 1623 can be affected by the chemical and biological characteristics of the matrix, particularly if interfering organisms or substances are present. For this reason, a matrix spike (MS) sample was analyzed, and the percent recovery (R) was used to quantify the matrix's interference with the analysis. The matrix spike duplicate (MSD) sample consisted of a second spiked sample, and the relative percent difference (RPD) was used to quantify the analytical precision, which can be affected both by differences in sample matrix between samples and the laboratory analytical precision. Easy Seed[™] spiking solution, manufactured by Biotechnology Frontiers (www.biotechfrontiers.com) and containing a 5-ml flow-cytometer-counted specimen containing 99 *Cryptosporidium* oocysts and 99 *Giardia* cysts, was used for spiking purposes. Manufacturer QA/QC specifications required a standard deviation of less than 2.5 cysts/oocysts per package and a stain rate of at least 95%. The minimum recovery was set at 20%, and the RPD had to be less than or equal to 71%. The laboratory must analyze precision and recovery for each 20 samples or conduct an analysis weekly, whichever is more frequent, and demonstrate a recovery greater than 20%. Analysis of one laboratory blank must result in no false positives.

RESULTS

The microbial, physical and chemical properties measured (see Table 3) were consistent with literature-reported values for CSO samples.

Table 3 presents the results of the six CSO samples collected and analyzed. Two were from an Atlanta, GA outfall and four were from two Louisville, KY outfalls. The Atlanta site historically required approximately 1 in. per hour of rainfall for CSO discharge to receiving waters. The Louisville sites required rainfall at a rate of approximately 0.1 in. and 0.25 in. per hour for CSO discharge to receiving waters. Sample volumes centrifuged to obtain 0.5-ml pellets as specified in Method 1623 ranged from 0.51 to 1.01. The comparatively small processed volume caused high detection limits for the samples.

Cryptosporidium was not significantly found in the CSO. The organism was detected in only 12% of the samples, but at two of the three sites. Analysis was performed in quadruplicate with at most 2 oocysts found per volume examined. The reported geometric mean concentration per 100l for samples where no oocysts were detected was calculated using 50% of the detection limit. MS and MSD recovery results were greater than 20%, meeting QC requirements. RPD results met QC requirements for *Cryptosporidium*.

The CSO samples contained significant *Giardia* concentrations. *Giardia* was detected in most samples (96%). Analysis was performed in quadruplicate concurrently with *Cryptosporidium* analysis. The number of *Giardia* cysts found in the sample volume examined ranged from non-detectable to 186. Results are extrapolated to obtain results per 100l, ranging from 200 to 30,000 cysts per 100l. The modified version of Method 1623 produced replicate precision with standard deviations of log transformed data ranging from 1.07 to 2.13 for each sampling event. All but one matrix spike and matrix spike duplicate results met QC requirements of greater than 20% recovery. RPD results met QC requirements for *Giardia*.

Cryptosporidium was not significantly found, therefore a meaningful relationship could not be tested with bacterial indicator or endospore concentrations. Bacterial indicator and endospore concentrations were not well correlated with *Giardia*. The best correlated indicators were *Enterococcus*

Table 3 | Urban CSO characterization parameters summary

	Date	Volume		<i>Cryptosporidium</i>			<i>Giardia</i>				
		Examined	per 100l *	%	MS	MSD	per 100l †	Std. Dev.***	%	MS	MSD
	Liters		Detection	%R	%R			Detection	%R	%R	
Louisville "F" a	9/27/02	1.0	59**	25	31	38	500	1.42	100	20	15****
Louisville "F" b	3/19/03	0.67	75**	0	59	42	200	2.13	75	39	30
Louisville "E" a	10/11/03	0.5	100**	0	49	36	30000	1.14	100	30	57
Louisville "E" b	2/14/03	0.5	100**	0	40	53	15000	1.07	100	38	54
Atlanta "B" a	12/10/02	1.0	84**	50	39	51	14000	1.09	100	36	38
Atlanta "B" b	2/26/03	0.5	100**	0	44	38	4200	1.18	100	52	40
	Total	Fecal	<i>E.coli</i>	Endospore	Fecal	<i>Enterococcus</i>	PSD*****	Turbidity	TSS	TS	COD
	Coliform	Coliform		Streptococcus			mean	NTU	mg/l	mg/l	mg/l
	CFU/100 ml	CFU/100 ml	CFU/100 ml	CFU/100 ml	CFU/100 ml	CFU/100 ml	um				
Louisville "F" a	420,000	88,000	70,000	8,100	32,000	11,000	0.128	20	59	168	104
Louisville "F" b	370,000	160,000	44,000	30,000	43,000	37,000	0.124	84	206	396	40
Louisville "E" a	5,700,000	430,000	900	3,700	210,000	300,000	0.271	38	101	238	83
Louisville "E" b	2,300,000	330,000	53,000	320,000	49,000	38,000	0.12	71	227	1062	99
Atlanta "B" a	310,000	34,000	23,000	2,900	27,000	19,000	0.131	67	77	167	107
Atlanta "B" b	3,100,000	37,000	29,000	38,000	23,000	26,000	0.266	95	14	388	99

where no oocysts or cysts were detected. The detection limit was calculated by dividing 100l by the volume examined.

**Cryptosporidium* and *Giardia* values are the geometric means calculated from four replicates, N = 4.

**A value of 50% of the detection limit was used in calculating the geometric mean for the replicates where no oocysts or cysts were detected. The detection limit was calculated by dividing 100l by the volume examined.

***Standard deviation of log transformed data.

****Outside quality control range.

*****Particle Size Distribution - by number.

($R^2 = 0.5091$) and fecal streptococcus ($R^2 = 0.4528$). Recognizing the limited data set ($N = 6$), strong correlations were not expected.

CONCLUSIONS

Data suggest that CSO from urban areas is not a significant source of *Cryptosporidium*. However, the detection limit was high (calculated by dividing 100 l by volume examined) because of the small sample volume needed to achieve the 0.5 ml pellet volume required by Method 1623. The low MS/MSD recoveries, although in line with most reported values, suggest a possibly greater propensity of *Giardia* than reported. Sanitary sewage, a component of CSO, is believed to be the major contributor of *Giardia* because a portion of the general population is asymptotically affected with this pathogen. Low matrix spike recovery indicates a possible greater propensity of *Giardia* than reported.

The modification to Method 1623 that eliminates the filtration procedure appears satisfactory for CSO *Cryptosporidium*/*Giardia* analysis. MS/MSD recovery results are comparable to results exemplified by the unmodified Method 1623.

Detection and measurement of *Cryptosporidium* and *Giardia* are difficult, expensive and time consuming because of the low concentrations and the labor intensity of Method 1623. Although the method met QC requirements for most samples, the complexity of the CSO matrix compounds the difficulty of *Cryptosporidium* and *Giardia* enumeration. Limited correlations between *Giardia* and both *Enterococcus* and fecal streptococcus were found.

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