Occurrence of *Aeromonas* spp. in a random sample of drinking water distribution systems in the USA

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ABSTRACT

Aeromonads are aquatic bacteria found in drinking water supplies worldwide. Some species, such as *Aeromonas hydrophila*, can cause disease in humans. For this survey, 293 United States public water systems were selected using random sampling, stratified by water source and system type. Water samples were collected during one year from three sites (six samples per site) in each system. Temperature, pH, turbidity, total and free chlorine were measured using standard methods. Aeromonads were detected in 130 of 5,042 valid samples (2.6%) from 42 (14.3%) systems using the ampicillin-dextrin agar with vancomycin culture method with oxidase, trehalose and indole confirmation tests. Concentrations of aeromonads in positive samples were 0.2 to 880 (median 1.6) colony-forming units (CFU) per 100 mL. Adjusted odds ratios of *Aeromonas* detection were 1.6 (95% confidence limits 1.0, 2.5) during the summer season, 3.3 (1.8, 6.2) for turbidity above 0.5 nephelometric units and 9.1 (3.5, 24) at 0 mg/L compared with 0.25 mg/L total chlorine. Geographic region, system size and type of water source were not significant predictors of *Aeromonas* detection in multivariate regression analysis. The results of this survey demonstrate the importance of maintaining adequate residual chlorine and low turbidity for preventing drinking water contamination with aeromonads.

Key words | *Aeromonas*, chlorine, distribution systems, drinking water

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INTRODUCTION

The genus *Aeromonas*, belonging to the family Aeromonadaceae, includes Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria. Several *Aeromonas* species, collectively known as aeromonads, occur ubiquitously and autochthonously in aquatic environments worldwide. They have long been recognized as fish and reptilian pathogens. A variety of human diseases including gastroenteritis, wound infections and septicemia have been attributed to species of this genus (Janda & Abbott 1998; Huys *et al.* 2002).

Aeromonads have been isolated from chlorinated drinking water supplies in the USA and other countries (LeChevallier *et al.* 1982; Kuhn *et al.* 1997; Gavriel *et al.* 1998; Razzolini *et al.* 2008; Yu *et al.* 2008; Pablos *et al.* 2009). Although these bacteria are susceptible to chlorine disinfection (Knochel 1991; Gerba *et al.* 2003), it has been shown that they are capable of growth and persistence in distribution system biofilms (Chauret *et al.* 2001; Bomo *et al.* 2004). Aeromonads have been detected in drinking water more frequently during the summer months (Moyer 1987; King *et al.* 1992) and from distribution system sites with low chlorine residual or long water residence time (Burke *et al.* 1984; Van Der Kooij 1988; Stelzer *et al.* 1992; Gavriel *et al.* 1998).

Some studies have demonstrated an association between *Aeromonas* detection in feces of gastroenteritis patients and their consumption of tap water (Holmberg *et al.* 1986; Moyer 1987) or the presence of these bacteria in samples of their drinking water (Burke *et al.* 1984). Other studies found little similarity between *Aeromonas*

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isolates from diarrheal patients and their drinking water samples (Havelaar *et al.* 1992; Borchardt *et al.* 2003).

The 1996 Safe Drinking Water Act Amendments direct the United States Environmental Protection Agency (EPA) to publish a list of unregulated contaminants (known as the Contaminant Candidate List or CCL) that may require a drinking water regulation after further evaluation. *Aeromonas* was included in the first and second editions of this list because of its association with human disease, occurrence in drinking water supplies and ability to grow in distribution system biofilms (USEPA 1998). Therefore, EPA has conducted a nation-wide survey to characterize the occurrence of this pathogen in a representative sample of public water systems (PWSs) through the authority granted by the Unregulated Contaminant Monitoring Regulation (UCMR) (USEPA 2008a). This manuscript presents the results of statistical analysis of *Aeromonas* data and other concurrently collected water quality parameters from the nation-wide survey of *Aeromonas* occurrence.

METHODS

Sampling design

PWSs for this survey were selected using stratified random nation-wide sampling as previously reported (USEPA 2008a). Briefly, sampling strata were defined using a combination of system size and water source categories. A total of 293 PWSs representing 49 States and Puerto Rico have participated in this survey (Figure 1).

Three sampling sites were identified in each PWS: the midpoint of the distribution system (MD), the maximum water residence time site (MR), and the lowest disinfectant residual site (LD). Water samples were collected during a 12-month period, monthly during the third quarter and quarterly during the rest of the year, for a total of six samples from each site. Sample volume for analysis of *Aeromonas* was 500 mL. Concurrent water samples were examined for temperature, pH, turbidity, free residual chlorine and total residual chlorine using standard monitoring methods.

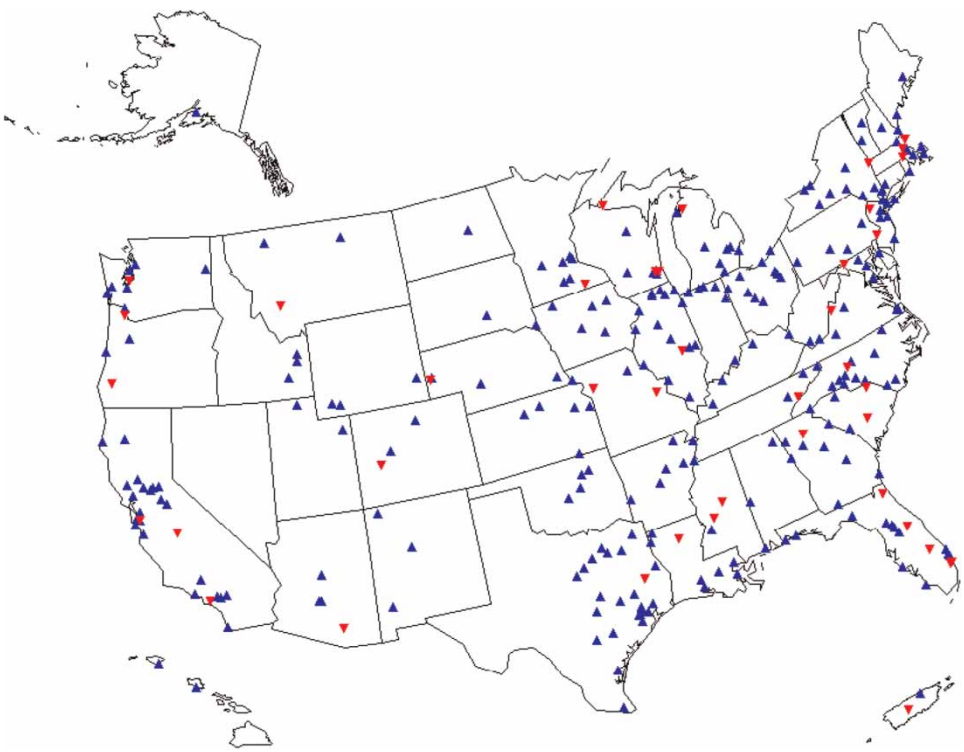

Figure 1 | Locations of public water systems that participated in this study. Blue triangles indicate public water systems (PWSs) without *Aeromonas* detection; inverted red triangles indicate PWSs with *Aeromonas* detection.

Aeromonas detection and speciation

Water samples were analyzed for aeromonads by EPA-approved laboratories using the EPA Method 1605 (USEPA 2001a). Briefly, water samples were filtered using 0.45 μL pore membrane filters and incubated at 35 °C on Ampicillin-Dextrin Agar with Vancomycin (ADA-V) medium for 24 ± 2 h. Presumptive *Aeromonas* colonies were identified based on the yellow color and characteristic morphology, and counted. Up to ten presumptive *Aeromonas* colonies per sample were subjected to confirmation testing using sequential oxidase, trehalose fermentation, and indole production tests (USEPA 2001a). Only presumptive isolates that were positive for all three confirmation tests were identified as *Aeromonas*.

Plates were reported as too numerous to count (TNTC) when too many aeromonads were present or when the plate was overgrown with other organisms. Samples resulting in TNTC plates were excluded from further analyses. Replacement water samples were collected as soon as possible (typically, within a month). The exceptions occurred during the summer months when replacement samples were not collected because regularly scheduled samples were collected monthly. Samples collected from the same site following a TNTC sample were split into aliquots of 1, 10 and 100 mL volume. Each aliquot was filtered and analyzed separately. The concentration of *Aeromonas* was determined using the filter with the optimum number of presumptively positive colonies.

Aeromonas species were identified employing the phenotypic classification scheme developed by Abbott *et al.* (2003). The API 20E strips (bioMerieux, Inc., Hazelwood, MO, USA) were used for phenotypic characterization of isolates. Overnight cultures of *Aeromonas* were inoculated into API 20E strips according to the manufacturer's instructions and incubated for 24 ± 2 h at 35 °C. The following tests were scored as positive or negative: lysine decarboxylase (LDC), ornithine decarboxylase (ODC), arginine dihydrolase (ADH), Voges-Proskauer (VP), and carbohydrate fermentation tests (arabinose, mannitol, sucrose, sorbitol and rhamnose). In addition, an esculin hydrolysis test was performed using Bile Esculin Agar (Difco, Sparks, MD, USA) with plates incubated overnight at 35 °C. Gas from glucose results were obtained using 1% dextrose in phenol red broths (BD, Franklin Lakes, NJ, USA) with incubation at 35 °C for 24 ± 2 h. When atypical test results were obtained, the identification of the isolate was based on the closest match using the scheme developed by Abbott *et al.* (2003).

Statistical analysis

This study used the *Aeromonas* occurrence data that is publicly available at the EPA website (http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/data.cfm#ucmr2005 [accessed 25 May 2011]) as well as other concurrently collected water quality parameters. Data analysis was conducted using SAS 9.2 software (SAS Institute, Cary, North Carolina, USA). *Aeromonas* concentration data were dichotomized either at the sample level (sample level analysis) or the PWS level (PWS level analysis). For the sample level analysis, the effects of predictor variables were evaluated in univariate and multivariate models. Semi-parametric models were employed to characterize deviations from linearity and select appropriate transformations of covariates. A final multivariate predictive logistic regression model included selected significant predictor variables and necessary data transformations.

At the first step of descriptive analysis at the sample level, continuous predictor variables, such as pH, water temperature, turbidity and residual chlorine concentration, were categorized at quartiles of their distributions or other cut-off points when appropriate. The effect of each predictor variable was analyzed in a separate mixed-effect regression model for binary outcome (SAS procedure NLMIXED) with a random intercept and data clustering at the PWS level.

The objective of this step was to develop preliminary multivariate predictive models. It involved semi-parametric generalized additive models (SAS procedure GAM) with spline smoothing functions and linear components. Logarithmic and power transformations of continuous predictor variables as well as piece-wise (broken stick) regression models were explored in order to identify those that produced statistically significant linear associations and non-significant smoothing functions (linearity conditions satisfied).

The last step involved the development and verification of a multivariate mixed-effect predictive logistic regression model using the NLMIXED procedure. Variants of models developed at the previous step were further evaluated and refined using the Corrected Akaike's Information Criterion (AICC). Appropriate knot (break point) locations for piece-wise components and cut-off values for dichotomized predictors were identified at this step. The resulting multivariate models were verified using two random subsets of sample data. The subsets were selected using a random Bernoulli variable associated with the system ID. The model development process described above was conducted iteratively until a parsimonious predictive model that produced consistent results in both random subsets of data was developed.

The separate PWS level analysis employed logistic regression models. For this analysis, water quality data were dichotomized using cut-off values selected at the sample level regression analysis. For each PWS, numbers of samples exceeding the dichotomization cut-off value of each parameter were estimated and then used as predictors in regression models. The outcome variable was the detection of aeromonads in at least one sample from a specific PWS.

RESULTS

Descriptive analysis of *Aeromonas* data at the sample level

Aeromonads were isolated from 130 (2.6%) out of 5,042 water samples (Table 1). The mean and median *Aeromonas* concentrations in positive samples were 34.4 and 1.6 CFU/100 mL respectively, while the mean *Aeromonas* concentration in all 5,042 samples was 0.9 CFU/100 mL. Figure 2 shows the upper portion (positive samples only) of the cumulative probability distribution of *Aeromonas* concentrations. The range of measured *Aeromonas* concentrations was from 0.2 to 880 CFU/100 mL. Only 13 samples (10% of positive samples) had concentrations greater than 40 CFU/100 mL.

A total of 411 colonies that were previously confirmed as aeromonads using the EPA method 1605 from 100 (76.9%) positive samples were further tested to determine the *Aeromonas* species using API 20E bioMerieux strips (Table 2). All 100 positive samples tested contained at least one colony where species was successfully determined. *A. salmonicida* and *A. hydrophila* were the most common species. Almost half of all isolates belonged to potentially

Table 1 | Characteristics of public water systems participating in the study

Type of water source	System size by population served	Number of PWSs	Number (percentage) of PWSs with *Aeromonas* detected	Number of samples analyzed	Number (percentage) of samples with *Aeromonas*	Mean (SD) *Aeromonas* concentration, CFU/100 mL
Ground	25–500	36	6 (16.7)	590	28 (4.7)	1.4 (17.3)
	501–3,300	51	13 (25.5)	869	35 (4.0)	0.2 (2.0)
	3,301–10,000	34	4 (11.8)	586	23 (3.9)	0.3 (3.0)
Surface	25–500	15	3 (20.0)	260	14 (5.4)	6.6 (66.0)
	501–3,300	15	0 (0)	247	0 (0)	0 (0)
	3,301–10,000	24	3 (12.5)	416	5 (1.2)	0.10 (1.5)
All types	All small PWSs (25–10,000)	175	29 (16.6)	2,968	105 (3.5)	1.0 (21.1)
Ground	10,001–50,000	27	2 (7.4)	484	2 (0.4)	0.01 (0.1)
	>50,000	26	6 (23.1)	453	7 (1.5)	0.01 (0.1)
Surface	10,001–50,000	33	2 (6.1)	573	8 (1.4)	2.6 (41.3)
	>50,000	32	3 (9.4)	564	8 (1.4)	0.1 (2.3)
All types	All large PWSs (>10,000)	118	13 (11.0)	2,074	25 (1.2)	0.8 (21.8)
Ground	All sizes	174	31 (17.8)	2,982	95 (3.2)	0.4 (7.9)
Surface	All sizes	119	11 (9.2)	2,060	35 (1.7)	1.6 (32.1)
All types	All sizes	293	42 (14.3)	5,042	130 (2.6)	0.9 (21.4)

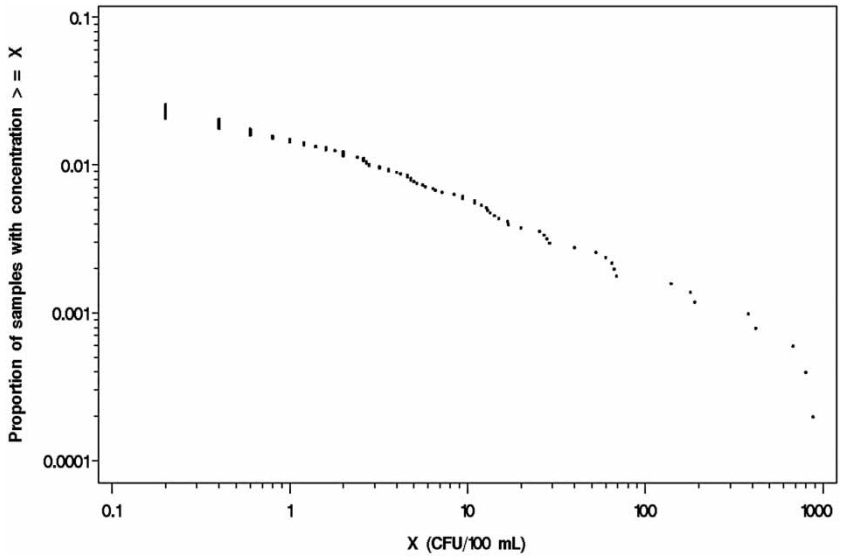

Figure 2 | Cumulative probability distribution of *Aeromonas* concentrations in water samples collected from public drinking water systems.

Table 2 | Summary of *Aeromonas* species data in 100 positive water samples

Aeromonas species	No. (percentage) of isolates	Percentage of samples where this species was detected[a]
A. salmonicida	116 (28.2)	35
A. hydrophila	101 (24.6)	29
A. jandaei	54 (13.1)	14
A. bestiarum	34 (8.3)	18
A. popoffii	30 (7.3)	11
A. eucrenophila	22 (5.4)	13
A. veronii bv *sobria*	19 (4.6)	10
A. schubertii	5 (1.2)	4
Aeromonas spp. (species not determined)	30 (7.3)	
Total	411 (100)	

[a]A total is greater than 100% because some samples contained more than one species.

pathogenic species: *A. hydrophila*, *A. jandaei*, *A. veronii* and *A. schubertii*, as defined by Janda & Abbott (1998).

Chlorine and turbidity data were available for 4,905 (97.3%) samples. Median concentrations of free and total chlorine were substantially lower in *Aeromonas*-positive than *Aeromonas*-negative samples: 0.02 vs. 0.40 mg/L and 0.07 vs. 0.62 mg/L, respectively. Water samples were categorized based on the presence of detectable residual chlorine and, if total chlorine was present, the proportion of combined chlorine or chloramine (Table 3). Most (85.4%) samples with no detectable total chlorine were collected from groundwater systems. Aeromonads were detected in 12.2% of these samples, a markedly higher rate of detection than in samples containing free chlorine (1.8%) or containing mostly chloramine (3.6%).

The rates of *Aeromonas* isolation were just slightly higher during the third quarter (July–September 'summer season') than during the remaining three calendar quarters (Table 4). The average *Aeromonas* concentrations, however, were much higher during the summer season. Further analysis (not shown) demonstrated that similar summer seasonal peaks of *Aeromonas* concentration were present in subsets of PWSs located in the northern and southern halves of the country as well as those using surface water and groundwater sources.

Descriptive analysis of *Aeromonas* data at the PWS level

Aeromonads were detected in 42 (14%) PWSs (Table 1 and Figure 1). The number of positive samples in these 42 systems ranged from 1 to 14 (mean 3.1 samples). Thirty-two (10.9%) PWSs produced from one to three positive samples and the remaining ten (3.4%) PWSs produced from 6 to 14 positive samples each. The latter ten systems produced 85

out of 130 (65%) *Aeromonas*-positive samples in this study. These ten systems represented different geographic regions and system size categories (Table 5). Seven out of ten used groundwater sources. However, the highest average concentrations of aeromonads were detected in surface water systems. Residual chorine was either completely absent or very low in all ten systems. Mean and median level in all samples from these ten PWSs were 0.11 and 0.02 mg/L, respectively. In contrast, mean and median free chlorine levels in 251 PWSs with no *Aeromonas* detected were 0.59 and 0.45 mg/L.

Information on the type of water treatment was available only for a subset of PWSs that participated in this survey. Using the data on the presence and the predominant type of disinfectant in water samples (see the sample level classification described above and in Table 3), all PWSs were categorized as follows: 31 systems with no detectable chlorine in all samples were categorized as 'non-disinfecting'; 33 systems that had at least 50% of chloramine containing samples were categorized as 'maintaining chloramine residual'; and the remaining 228 systems were categorized as 'maintaining free chlorine residual'. The available data on water treatment were used to develop and verify this categorization.

Regression analysis of predictors of *Aeromonas* detection at the sample level

At the univariate analysis step, the *Aeromonas* data were dichotomized at the limit of detection (0.2 CFU/100 mL) or at 10 CFU/100 mL (Table 6). Continuous predictor variables were categorized at quartiles of their distributions except turbidity which was categorized at 33rd, 67th and

Table 3 | Categories of water samples by residual chlorine composition

Chlorine residual category	N (percentage of total)	*Aeromonas* detected %	*Aeromonas* > 10 CFU/100 mL %	Total chlorine, mean/ median	Free chlorine, mean/ median	Percentage from groundwater systems	Percentage from small systems
No residual chlorine present	254 (5.2)	12.2	1.6	0/0	0/0	85.4	42.1
Free chlorine containing (free chlorine $\geq$ 25%, chloramines <75% of total chlorine)	4,096 (83.5)	1.8	0.4	0.76/0.60	0.66/0.50	60.7	66.3
Chloramine containing (chloramines $\geq$75%, free chlorine <25% of total chlorine)	555 (11.3)	3.6	1.3	1.63/1.60	0.12/0.08	36.2	18.7

Table 4 | Seasonality of *Aeromonas* occurrence in water samples from public water systems

Time period	Number of samples analyzed	Number (percentage) of *Aeromonas* positive samples	Number (percentage) of samples with *Aeromonas* >10 CFU/100 mL	Number (percentage) of samples with *Aeromonas* >40 CFU/100 mL	Mean *Aeromonas* concentration, CFU/100 mL
January–March	864	21 (2.4)	5 (0.6)	0	0.13
April–June	856	15 (1.8)	2 (0.2)	0	0.08
July	814	29 (3.6)	7 (0.9)	5 (0.6)	1.98
August	823	19 (2.3)	8 (1.0)	4 (0.5)	2.68
September	838	25 (3.0)	6 (0.7)	4 (0.5)	0.51
October–December	847	21 (2.5)	1 (0.1)	0	0.06

80th percentiles (the latter corresponded to the regulatory threshold value of 0.5 NTU). The system size (dichotomized at 10,000 people served using the standard EPA classification) was not a significant predictor of *Aeromonas* isolation. The effects of finer system size categories were also not significant: the odds ratio of *Aeromonas* detection in samples from the smallest systems (25 to 500 people) vs. largest systems (more than 50,000 people) was 2.6 (0.6, 10.0), $p = 0.2$ (not shown in Table 6). The type of water source (ground vs. surface) and geographic region (southern region defined, for the purpose of this analysis, as HI, CA, NV, UT, AZ, NM, CO, TX, OK, KS, LA, AR, MO, MS, FL, AL, GA, TN, SC, NC, KY, VA, WV, MD, DE and PR, and contrasted with the rest of the country) were not significant predictors of *Aeromonas* occurrence in this analysis.

Aeromonas was significantly more likely to be detected in samples collected from sampling sites with the lowest chlorine residual than from system mid-points. The odds of *Aeromonas* detection in samples with turbidity greater than 0.5 NTU were not significantly greater than in samples with turbidity below 0.1 NTU. However, when turbidity data were dichotomized at the 0.5 NTU, the effects of turbidity above vs. below this cut-off value became significant: odds ratios 3.17 (1.72, 5.83) for *Aeromonas* detection and 5.29 (2.04, 13.7) for *Aeromonas* concentration above 10 CFU/100 mL (not shown in Table 6). Low concentrations of free chlorine or total chlorine were strongly associated with *Aeromonas* isolation. The summer season (third calendar quarter) was a significant predictor of *Aeromonas* detection and, especially, concentration above 10 CFU/100 mL while water temperature was not a significant predictor.

Further multivariate analysis using generalized additive models with spline smoothing functions for continuous predictor variables confirmed that system size (large or small), type of water source and other system-level characteristics were not significant predictors of *Aeromonas* after adjusting for chlorine and turbidity. The summer season remained a significant or nearly significant predictor in all models while high water temperature had no effect after control for the season. Interaction effects of chlorine and temperature, the effects of pH and interaction of free chlorine and pH were not significant in all models. There was a non-linear association between turbidity and the odds of *Aeromonas* detection with a step increase at approximately 0.5 NTU, which was parsimoniously modeled using a binary variable.

Figure 3 shows plots of predicted odds ratios of *Aeromonas* detection vs. total chlorine residual in the sample (zero chlorine serves as the reference point). This figure was produced using a GAM model with spline smoothing function for square root of total chlorine (the predicted values were plotted using non-transformed chlorine

Table 5 | Public water systems with six or more *Aeromonas*-positive samples sorted by average *Aeromonas* concentration

	***Aeromonas* data**									
Rank order	**Average conc., CFU/100 mL**	**Max. conc., CFU/100 mL**	**Number of positive samples**	**Number of samples >10 CFU/100 mL**	**Source**	**Median free chlorine, mg/L**	**Median total chlorine, mg/L**	**Median turbidity, NTU**	**System size category (population served)**	**State or territory**
1	93.8	800	12	3	Surface	0.14	0.17	0.20	25–500	Puerto Rico
2	82.5	880	6	3	Surface	0.05	0.10	0.53	10,001–50,000	Massachusetts
3	40.5	380	6	6	Ground	0.21	0.24	0.50	25–500	Georgia
4	5.2	69	6	2	Ground	0.10	0.22	0.10	3,301–10,000	Illinois
5	3.8	25.4	12	2	Ground	0.01	0.04	0.38	501–3,300	Washington
6	3.4	40	7	2	Ground	0.00	0.00	0.10	501–3,300	Oregon
7	1.8	11	14	1	Ground	0.00	0.00	0.25	25–500	Washington
8	1.8	11	10	1	Ground	0.08	0.09	0.05	3,301–10,000	Nebraska
9	1.1	12.8	6	1	Surface	0.00	0.10	0.15	>50,000	California
10	0.9	6.4	6	0	Ground	0.02	0.03	0.05	3,301–10,000	Wisconsin

Table 6 | Results of single predictor mixed effect regression analysis of *Aeromonas* detection in water samples

Parameter	Category	Positive/ negative samples with valid data	% of positive samples	Odds ratio of *Aeromonas* detection	Number (%) of samples with *Aeromonas* > 10 CFU/100 mL	Odds ratio of *Aeromonas* > 10 CFU/100 mL
System size	Large (>10,000)	25/2,049	1.2	1	5 (0.2)	1
	Small (25–10,000)	105/2,863	3.5	3.1 (0.8, 12)	24 (0.8)	4.6 (0.8, 27)
Source type	Surface	35/2,025	1.7	1	11 (0.5)	1
	Ground	95/2,887	3.2	3.6 (0.9, 15)	18 (0.6)	1.1 (0.2, 4.9)
Geographic area	South	49/2,758	1.7	1	11 (0.4)	1
	North	81/2,136	3.7	1.5 (0.4, 5.0)	18 (0.8)	5.0 (0.98, 25)
Sampling site	Mid-point	34/1,647	2.0	1	6 (0.4)	1
	Max residence	42/1,638	2.5	1.4 (0.8, 2.5)	5 (0.3)	0.8 (0.2, 2.8)
	Lowest chlorine	54/1,627	3.2	2.1 (1.2, 3.5)*	18 (1.1)	3.6 (1.3, 9.7)*
Turbidity (turb), NTU	turb $\leq$ 0.1	43/1,773	2.4	1	7 (0.4)	1
	0.1 < turb $\leq$ 0.3	39/1,711	2.2	0.8 (0.5, 1.6)	5 (0.3)	0.5 (0.1, 1.7)
	0.3 < turb $\leq$ 0.5	8/482	1.6	0.5 (0.2, 1.5)	1 (0.2)	0.4 (0.04, 3.4)
	turb > 0.5	39/889	4.2	1.8 (0.9, 3.6)	16 (1.7)	2.4 (0.7, 7.9)
Free chlorine (Cl free), mg/L	Cl free > 0.8	4/1,227	0.3	1	0 (0)	1
	0.4 < Cl free $\leq$ 0.8	7/1,129	0.6	2.2 (0.6, 8.7)	3 (0.3)	
	0.1 < Cl free $\leq$ 0.4	16/1,160	1.4	4.6 (1.3, 17)*	4 (0.3)	1.7 (0.3, 8.7)
	Cl free $\leq$ 0.1	99/1,286	7.1	21 (6.1, 75)*	21 (1.5)	8.3 (1.9, 36)*
Total chlorine (Cl tot), mg/L	Cl tot > 1.13	8/1,220	0.7	1	1 (0.1)	1
	0.6 <Cl tot $\leq$ 1.13	6/1,210	0.5	1.1 (0.3, 3.7)	4 (0.3)	3.2 (0.3, 35)
	0.2 <Cl tot $\leq$ 0.6	13/1,235	1.0	1.8 (0.6, 5.7)	1 (0.1)	0.4 (0.02, 8.1)
	Cl tot $\leq$ 0.2	98/1,151	7.8	11 (3.8, 31)*	22 (1.8)	11 (1.1, 116)*
pH	pH $\leq$ 7.3	29/1,403	2.0	1	12 (0.8)	1
	7.3 < pH $\leq$ 7.6	30/1,404	2.1	0.7 (0.3, 1.5)	4 (0.3)	0.6 (0.1, 2.5)
	7.6 < pH $\leq$ 7.9	32/885	3.5	1.0 (0.4, 2.2)	5 (0.5)	1.1 (0.3, 4.7)
	pH > 7.9	36/1,139	3.1	1.2 (0.5, 2.9)	8 (0.7)	1.5 (0.4, 6.6)
Season	Quarters 1, 2, 4	57/2,510	2.2	1	8 (0.3)	1
	3rd quarter	73/2,402	3.0	1.6 (1.1, 2.5)*	21 (0.9)	3.2 (1.4, 7.7)*
Water temperature (t), degrees Celsius	t $\leq$ 14.7	36/1,222	2.9	1	7 (0.6)	1
	14.7 < t $\leq$ 19.7	41/1,195	3.3	1.4 (0.7, 2.6)	8 (0.6)	0.98 (0.3, 3.0)
	19.7 < t $\leq$ 24.2	27/1,223	2.2	1.5 (0.7, 3.1)	8 (0.6)	1.5 (0.5, 5.0)
	t > 24.2	24/1,219	1.9	1.7 (0.7, 4.0)	6 (0.5)	1.6 (0.4, 6.9)

*$p < 0.05$.

values of the X axis). As the concentration of total chlorine increases, the predicted odds of *Aeromonas* detection initially decline steeply in both types of systems. At higher chlorine concentrations, the association becomes less steep in chlorinating systems and levels off in chloraminating systems.

The final linear predictive mixed effect model involved a piece-wise component for total chlorine with one knot at 0.25 mg/L concentration (Table 7). The effect of total chlorine in systems maintaining free chlorine or chloramine was adequately modeled using a linear association and a common slope in the interval from 0 to 0.25 mg/L total chlorine. Above this value, the effect of total chlorine was present only in PWSs maintaining free chlorine (it was modeled using an interaction term between square root of chlorine and an indicator variable for the system type). The final model also included binary variables for summer season and turbidity above 0.5 NTU. Figure 4 shows graphs of predicted odds ratios of *Aeromonas* detection with zero total chlorine serving as a reference point (the

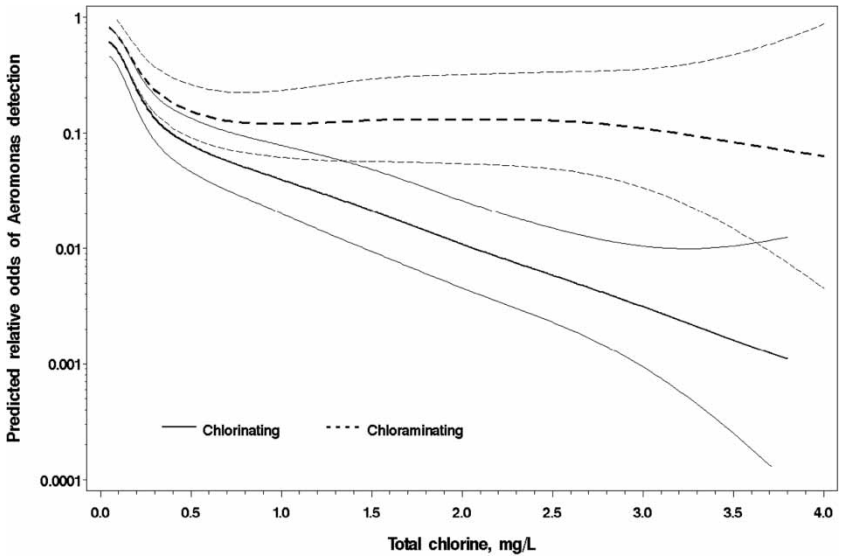

Figure 3 | Predicted relative odds of *Aeromonas* detection (with 95% confidence limits for the mean) in chlorinating and chloraminating public water systems. This graph was produced using a generalized additive model with spline smoothing functions and five degrees of freedom.

Table 7 | Results of multivariate (multiple predictor) mixed effect regression analysis of *Aeromonas* detection at the sample level

Parameter	*Aeromonas* detection All 292 systems with chlorine and turbidity data	Random subset 1 (163 systems)	Random subset 2 (129 systems)	*Aeromonas* >10 CFU/100 mL All 292 systems with chlorine and turbidity data
Fixed effect intercept (at 0.25 mg/L of total chlorine)	−7.1 (−8.2, −5.9)	−7.2 (−8.8, −5.5)	−7.1 (−8.8, −5.5)	−9.7 (−12, −7.4)
Season (3rd quarter vs. 1st, 2nd and 4th quarters)	0.5 (−0.02, 0.9)	0.4 (−0.3, 1.0)	0.6 (−0.1, 1.3)	1.5 (0.5, 2.5)
Turbidity (>0.5 NTU vs. less than 0.5 NTU)	1.2 (0.6, 1.8)	1.5 (0.6, 2.4)	0.9 (0.0, 1.7)	2.0 (0.9, 3.1)
Total chlorine up to 0.25 mg/L (all systems)	−8.8 (−13, −5.0)	−6.6 (−12, −1.3)	−12 (−18, −6.1)	−8.3 (−15, −1.6)
Square root of total chlorine above 0.25 mg/L (chlorinating systems only)	−2.3 (−4.2, −0.3)	−2.1 (−4.7, 0.4)	−2.3 (−5.6, 0.9)	−2.4 (−6.3, 1.4)

effects of turbidity and summer season are not shown on this graph).

This final regression model (Table 7, column 1) was successfully verified in two random subsets of PWSs (columns 2 and 3). Parameter estimates are comparable in these subsets. A separate analysis using the entire data set and *Aeromonas* data dichotomized at 10 CFU/100 mL (Table 7, column 4) demonstrated that total chlorine, high turbidity and summer season were strong predictors of *Aeromonas* presence at this relatively high level.

The odds ratio estimates for binary predictors and selected contrasts in total chlorine values are presented in Table 8. The relative odds of *Aeromonas* detection at zero total chlorine compared with 0.25 mg/L total chlorine was 9.1 (3.5, 24) in all systems. Further increase in total chlorine from 0.25 mg/L to 1 mg/L (0.5 units of square root-transformed chlorine data) was associated with 3.1 (1.2, 8.3)-fold reduction in the odds of *Aeromonas* detection in chlorinating systems only. The summer season had a strong effect on the odds of *Aeromonas* concentration above 10 CFU/100 mL.

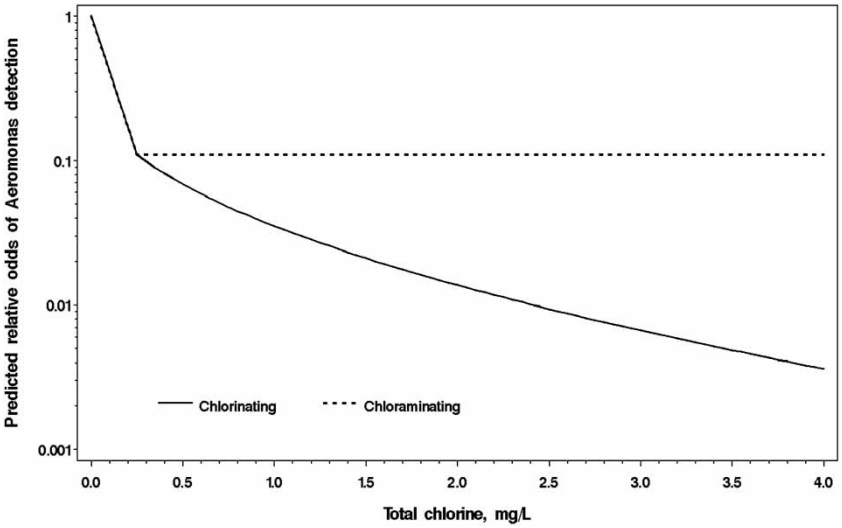

Figure 4 | Predicted relative odds of *Aeromonas* detection in samples collected from chlorinating and chloraminating public water systems (piece-wise polynomial regression model with interactions).

Table 8 | Estimated odds ratios with 95% confidence intervals for *Aeromonas* detection or *Aeromonas* concentration greater than 10 CFU/100 mL. Estimates of the effects of chlorine are presented for selected contrasts in chlorine concentrations

Predictor variable	Contrast	Odds ratio of *Aeromonas* detection	*p* value	Odds ratio of *Aeromonas* > 10 CFU/100 mL	*p* value
Season	3rd quarter vs. 1st, 2nd and 4th quarters	1.6 (1.0, 2.5)	0.06	4.3 (1.6, 12)	0.005
Turbidity	0.5 NTU or greater vs. less than 0.5 NTU	3.3 (1.8, 6.2)	0.0003	7.3 (2.5, 21)	0.0003
Total chlorine (continuous predictor)	No chlorine vs. 0.25 mg/L of total chlorine in all systems	9.1 (3.5, 24)	<0.0001	8.0 (1.5, 42)	0.01
	0.25 mg/L vs. 1 mg/L in systems maintaining free chlorine residual	3.1 (1.2, 8.3)	0.02	3.4 (0.5, 23)	0.2

Regression analysis of predictors of *Aeromonas* occurrence at the PWS level

Univariate logistic regression analysis demonstrated that groundwater systems had significantly greater odds of having at least one *Aeromonas*-positive sample than surface water systems (Table 9). The effect of system size was short of being statistically significant. For this system-level analysis, continuous data on total and free chlorine were dichotomized at the 25th percentile (0.2 mg/L and 0.1 mg/L, respectively), and turbidity data were dichotomized at the 0.5 NTU cut-off value. In separate models, counts of samples with low free chlorine, low total chlorine or high turbidity were predictive of *Aeromonas* detection in a PWS. *Aeromonas* was detected in a significantly greater proportion of PWSs that did not maintain residual disinfectant than systems that maintained free chlorine residual. The effect of maintaining predominantly chloramine was short of being significant.

In multivariate logistic regression analysis of the system level data, all models were reduced to a single predictor variable, the count of samples with low total chlorine (or low free chlorine in separate models). Turbidity, system size and type of water source were not significant predictors of

Table 9 | Results of univariate logistic regression analysis at the PWS level (*$p < 0.05$)

Parameter	Category	Number of systems with *Aeromonas* detection/number of systems without *Aeromonas* detection	Percentage of systems with *Aeromonas* detection	Odds ratio of a PWS having at least one positive sample
Source water type	Surface	11/108	9.2	1
	Ground	31/143	17.8	2.1 (1.02, 4.4)*
System size	Large	13/105	11.0	1
	Small	29/146	16.6	1.6 (0.8, 3.2)
Number of samples with free chlorine $\leq$ 0.1 mg/L	0	5/114	4.2	1
	1 to 6	13/77	14.4	3.8 (1.3, 11)*
	7 to 12	8/31	20.5	5.8 (1.9, 19)*
	13 to 18	16/29	35.6	13 (4.3, 37)*
Number of samples with total chlorine $\leq$ 0.2 mg/L	0	6/116	4.9	1
	1 to 6	14/82	14.6	3.3 (1.2, 8.9)*
	7 to 12	10/26	27.8	7.4 (2.5, 22)*
	13 to 18	12/27	30.8	8.6 (3.0, 25)*
Number of samples with turbidity > 0.5 NTU	0	7/93	7.0	1
	1 to 6	26/126	17.1	2.7 (1.1, 6.6)*
	7 to 18	9/32	22.0	3.7 (1.3, 11)*
Main type of disinfectant present in the distribution system	Chlorine	24/204	10.6	1
	Chloramine	7/26	21.2	2.3 (0.9, 5.8)
	No disinfectant	11/20	35.5	4.7 (2.0, 11)*

Aeromonas detection in a public water supply system after adjusting for the number of low chlorine samples.

DISCUSSION

In this study, drinking water samples from 293 distribution systems throughout the USA were tested for culturable *Aeromonas* bacteria using EPA method 1605. The method is based on the previously developed ADA method for *Aeromonas* in drinking water (Havelaar *et al.* 1987) with important modifications that include the addition of vancomycin to the growth medium (ADA-V medium) and a new battery of confirmation tests (USEPA 2001a). Vancomycin reduces the growth of *Bacillus* spp. and other non-aeromonads bacteria without inhibiting most aeromonads (USEPA 2001b). It has been shown that the presence of cytochrome oxidase and the ability to ferment trehalose are among biochemical characteristics of practically all isolates of *Aeromonas* (Abbott *et al.* 2003). The ability to produce indole is another characteristic feature of most aeromonads that was utilized in this method in order to improve its specificity (USEPA 2001b).

This survey used a culture-based monitoring method and simple biochemical tests for the identification of species that could be employed, at a reasonable cost, by microbiological laboratories of water utilities if monitoring of aeromonads in water supplies were to become a standard requirement. The lack of molecular testing is, therefore, an inherent limitation of this large-scale survey.

Two factors in the design of this study contributed to the overestimation of the isolation rate and average concentration of *Aeromonas* in public water supplies in the USA. First, in order to better characterize the occurrence of *Aeromonas* in water supplies during its known seasonal peak period, water samples were collected at a higher frequency (monthly) during the third quarter than during the rest of the year when sampling was conducted quarterly. Second, water samples were only collected from sites in distribution systems with low chlorine residual or medium to high water residence time where aeromonads were more likely to be present.

Three other factors might lead to an underestimation of *Aeromonas* concentration. First, samples with overgrowth of presumptive aeromonads as well as overgrowth of other organisms (TNTC samples) were excluded from further

analyses. Replacement samples after TNTC samples were collected during the colder season only (first, second and fourth quarters). The second factor was the use of ampicillin in the growth medium. Although most aeromonads are resistant to ampicillin, *A. trota* is an exception that rarely possesses this characteristic (Abbott *et al.* 2003). However, it is not among the most common species of aeromonads and it is not commonly associated with human disease (Janda & Abbott 1998). Finally, most isolates of *A. schubertii* are not capable of indole production (Abbott *et al.* 2003). Such isolates likely failed the indole production test and, therefore, were not counted as aeromonads.

The summer peak of *Aeromonas* found in this study is consistent with a majority of previous studies (Burke *et al.* 1984; Gavriel *et al.* 1998; Chauret *et al.* 2001) which showed greater *Aeromonas* isolation rates in the summer or at a higher water temperature. In contrast, a recent study in Spain demonstrated a greater rate of aeromonads isolation from drinking water in the winter and fall at water temperature below 14 °C (Pablos *et al.* 2009). In the present study of public water systems from different climatic regions of the USA, the effect of water temperature was not significant after adjusting for the summer season. *Aeromonas* was detected at similar rates in the northern and southern parts of the country, and the summer peak of *Aeromonas* occurrence was present in both geographic areas. This suggests that factors other than water temperature might contribute to the summer peak of this pathogen in drinking water supplies. A similar conclusion was previously reached by Burke *et al.* (1984).

The strong inhibiting effect of chlorine residual on *Aeromonas* demonstrated in this study is consistent with previously published monitoring results (Burke *et al.* 1984) and susceptibility of the suspended form of this pathogen to chlorine disinfection (Knochel 1991; Gerba *et al.* 2003). The weaker effect of combined chlorine (chloramine) is consistent with the weaker inactivation potential of this chemical on suspended bacteria. Monochloramine (the predominant form of chloramine in water samples) is approximately 1,000 times less effective for inactivation of suspended HPC bacteria than hypochlorous acid, which is the predominant form of free chlorine at pH below 7, and approximately 30 times less effective than hypochlorite ion, which is the predominant form of free chlorine at pH above 8 (LeChevallier *et al.* 1988).

Information on the water treatment process was available only for a subset of PWSs participating in this study. The data analysis demonstrated, however, that the type of chlorine residual present in the distribution system samples (free or combined) had a strong effect on *Aeromonas* occurrence. Therefore, PWSs were categorized for further analysis as 'maintaining free chlorine residual' or 'maintaining chloramine residual' using the available data of total and free chlorine concentrations in distribution system samples.

The results of this nation-wide survey demonstrated that exposures to aeromonads in drinking water supplies are widespread. However, aeromonads were not detected in most PWSs or were detected at very low concentrations. The evaluation of public health impact of waterborne exposure to aeromonads was beyond the scope of this survey. A subsequent analysis (USEPA 2008b) taking into account these monitoring results as well as the lack of reported waterborne outbreaks of *Aeromonas* and moderate severity of its health effects concluded that this pathogen does not present a substantial public health hazard and, therefore, should not be included in the third version of the Contaminant Candidate List (USEPA 2009).

CONCLUSIONS

The data from this investigation show that aeromonads are much more likely to be present in drinking water when a PWS does not maintain an adequate concentration of residual chlorine throughout the distribution system or delivers high turbidity water to its customers. Low residual chlorine and high turbidity may also indicate a potential growth of other harmful microorganisms in distribution system biofilms. Maintaining adequate water quality throughout the distribution system is, therefore, essential for reducing exposures to multiple potentially harmful microorganisms.

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