Evaluating appropriate maximum holding times for private well water samples

Allison Maier,* Julia Krolik,* Stephanie Fan,[†] Patricia Quintin,[‡] Danielle McGolrick,[†] Alan Joyce,* and Anna Majury*,^{†,§,¶}

*Public Health Ontario, 181 Barrie St., Kingston, ON

[†]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON

[‡]Department of Applied Science, St. Lawrence College, Kingston, ON

[§]Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON

[¶]Department of Public Health Sciences, Queen's University, Kingston, ON

Abstract: Privately maintained groundwater wells are often located at a great distance from laboratories, creating a barrier to bacteriological testing (a necessity for determining drinking water potability). Extending the acceptable holding time between testing and collection could potentially diminish this barrier. Using seven *Escherichia coli* strains isolated from private well waters, the acceptability of Ontario's current allowable sample maximum holding time (48 h) was compared with time of collection. Additionally, the acceptability of extending the holding time from 48 h to 72 h was investigated. All analyses were performed using noninferiority statistical approaches to determine if later holding times had no meaningful difference in bacterial growth (determined by colony forming unit enumeration). All strains did not statistically decrease below the acceptable 10% difference during the two time periods. However, variations in the survival rates of isolates were observed, suggesting that a risk management approach should be employed when determining maximum holding times.

Key words: holding times, well water, *Escherichia coli*.

Routine bacteriological testing of drinking water is essential as it provides an indication of water potability. In most North American jurisdictions, privately maintained water supplies, predominantly groundwater wells, are the responsibility of the owner and testing is not regulated. Most of these wells are located rurally; thus, travel time from the site of the well to the laboratory may be significant and a potential barrier to testing for some private well owners. As such, the maximum acceptable holding time between the collection and testing of the water sample is a vital consideration for well owner populations.

Currently, there are inconsistent maximum acceptable holding times globally: the United States Environmental Protection Agency (EPA) allows a maximum of 30 h for groundwater samples (US Environmental Protection Agency 2008), whereas Health Canada states 24 h is acceptable (8 h is preferred), though up to 48 h may be allowable for remote areas, but these recommendations are not specific for wells (Health Canada 2012). The World Health Organization's drinking water recommendations are also not specific to groundwater and state that 6 h is ideal and 24 h is the maximum allowable (World Health Organization 2003). Furthermore, there is limited and inconsistent scientific

evidence supporting these policies, none of which is specific to groundwater samples. Previous studies have investigated surface water (lakes and rivers, often those receiving municipal or agricultural waste) (Standridge and Lesar 1977; Dutka and El-Shaarawi 1980; Pope et al. 2003; Aulenbach 2010), municipal and industrial effluent (Standridge and Lesar 1977; Dutka and El-Shaarawi 1980; Selvakumar et al. 2004), storm-water (Selvakumar et al. 2004), and water from within municipal distribution systems (McDaniels et al. 1985). Survival rates were variable for different fecal indicator bacteria (FIB), with total coliforms often decreasing shortly after collection (Mendez Novelo et al. 2010), whereas fecal coliforms generally survived longer, possibly up to 62 h (Aulenbach 2010). Some studies observed significant decreases in *Escherichia coli* (*E. coli*) density by 18–24 h (Selvakumar et al. 2004, Aulenbach 2010), while other studies suggested that up to 48 h was acceptable (Pope et al. 2003). Notably, across all of these studies, there was very little power to analyze holding times greater than 48 h.

In Ontario, testing of samples from private water supplies is available through the Public Health Ontario Laboratories (PHOLs). Well owners collect water samples and are instructed to keep them cold (refrigerated at approximately 4°C, but not frozen) until they can drop off the sample directly at one of 11

Corresponding author: Anna Majury (email: anna.majury@oahpp.ca)

laboratories located across the province or at a drop off location operated by local health units (typically at their offices or municipal offices) (Public Health Ontario 2014). The PHOLs are licensed to provide testing of well water samples by the Ontario Ministry of the Environment and Climate Change (MOECC), whose testing protocols are used by all licensed laboratories. According to these MOECC protocols, samples must be tested within 48 h of collection (MOECC 2012), and are often rejected for being “too old” because of various delays between the time of collection and laboratory testing, as seen Figure 1. Prior to 2012, the PHOLs allowed for samples to be accepted for up to two days (i.e., a maximum of 56 h) instead of the stricter two day definition of 48 h—this protocol change resulting in, on average, an additional 1500 rejected submissions. Once samples arrive at the laboratory, bacteriological analysis is performed using membrane filtration and differential coliform enumeration for both *E. coli* and total coliform colony forming units (CFUs).

The limitations posed by the 48 h window are particularly problematic given that earlier work reveals very low levels of testing among well owners; a recent five-year review of well water submissions in southeastern Ontario suggested that over 99% of wells are not tested according to local guidelines (three samples without evidence of bacterial contamination each year) (Maier et al. 2014). This is further corroborated by a survey conducted in southern Ontario, where 65% of well owners stated that they tested their water less than once a year (Kreutzweiser et al. 2008). Previous research suggests that many owners do not test because of inconvenience, including the strict 48 h requirement (Jones et al. 2006; Imgrund et al. 2011). As such, increasing the acceptable maximum holding time might increase the rate of testing. This, in turn, would increase the number of well owners who are making informed decisions regarding well water consumption based on bacteriological potability.

To address the lack of groundwater-specific evidence available to inform policy development, a study was designed to evaluate existing protocols. Current and potential maximum

acceptable holding times for *E. coli* enumeration were evaluated: specifically (i) the current practice in Ontario of 48 h as compared with initial collection and (ii) extending the 48 h to 72 h.

Methods and rationale

Study design

The study was designed to simulate the effects of holding times on enumeration of *E. coli* in private well water samples, utilizing wild type strains (in this context, bacteria which were cultured directly from environmental samples as compared to standard laboratory strains). Five wild type *E. coli* were isolated from well waters submitted to the Kingston PHOL (WT-K1 through WT-K5). Efforts were undertaken to ensure wild types were isolated across multiple seasons. To test the geographic generalizability of the results, two wild types were isolated at different PHOLs (one from Hamilton (WT-H) and Orillia (WT-OR) respectively).

Three holding times were considered within this analysis: 0 h (to represent the well water sample as first collected), 48 h (the current maximum holding time allowed by the MOECC), and 72 h (an additional 24 h period after the current maximum holding time). To simulate the conditions between collection and testing (advised to be between 2° C and 8° C), samples were stored in a refrigerator at 4° C.

All testing was performed utilizing a well water matrix to simulate the sample environment. The matrix was obtained by autoclaving well water, which was pooled from incoming private well water submissions, and for which prior testing produced a “no growth” result. The matrix consisted of 10–15 samples representing local yet geographically diverse wells.

Plate counting methods have an inherently high variability (International Organization for Standardization 2000). To minimize the effects of this, plating was performed in triplicate. During design, data normality of all plate counts was

Fig. 1. Percentage and number of samples rejected for being “too old” by the testing laboratory (PHOL) from 2003–2013, inclusive (unpublished data, Majury 2013).

confirmed. Subsequently, means of each triplicate set were used for analysis. Additionally, each wild type was tested by a single analyst (whose reproducibility had been previously confirmed) as between-person variability is known to be an issue (International Organization for Standardization 2000). In total, only two separate analysts took part in this study to minimize, but not discount entirely, variability. Finally, to account for the high variability (and therefore the high variance in the data), a sufficient sample size ($n = 25$) was chosen for statistical analysis.

Laboratory methods

The wild type presumptive *E. coli* isolates were confirmed as *E. coli* using API E20 strips (Biomérieux, Durham, NC). For each trial conducted, *E. coli* was cultured on Columbia blood agar with 5% sheep blood (Oxoid, Nepean, ON) and incubated at 37° C for 18–24 h. A bacterial suspension, using the well water matrix, was created to an optical density of 1 at 600 nm (with an acceptable range between 0.950 and 1.049) using the NanoDrop 2000c (Thermo Scientific, DE) (henceforth known as an OD1 suspension). It was previously determined (through testing 10-fold serial dilution from 10 to 10⁻⁷) that 10⁻⁶ dilutions resulted in growth between the suggested range for counting (25–250 colonies per plate) (Sutton 2011). Therefore, for each wild type, 25 OD1 suspensions were created and subsequently serially diluted to 10⁻⁶, plated in triplicate, incubated at 37° C for 18–24 h, and enumerated. A schematic diagram depicting the process is shown in Figure 2.

Statistical analysis

Mean CFU counts (CFUCs) (of the 25 triplicate means) were determined for each strain and holding time, and normality for each was checked using both skewness and kurtosis methods. Additionally, any outliers were identified and removed where they affected normality. These analyses were performed using IBM SPSS Statistics 22 (IBM Corp., Armonk, NY). The means were subsequently graphed for observational purposes.

As the goal of this study was to evaluate the validity of the current maximum holding time, including whether it could be increased from 48 h to 72 h, it was necessary to determine that the CFUCs did not decrease over time. Traditional statistical methods (employed by the majority of holding time literature

(Dutka and El-Shaarawi 1980; McDaniels et al. 1985; Pope et al. 2003; Selvakumar et al. 2004; Aulenbach 2010)) prove significant differences between means of groups by rejecting the null hypothesis (equivalent means) in favour of an alternate hypothesis (the means are different) (Streiner 2003). However, in these methods, failing to disprove the null (an insignificant result) is not equivalent to proving the null hypothesis is true (Mascha and Sessler 2011). A new methodology, which aims to test for equivalence has been developed for clinical purposes (i.e., proving two therapies have equivalent outcomes) and can be applied here. More specifically noninferiority testing, a one-sided test similar to equivalence that is used to determine that one treatment is no worse on the outcome measure than the original (Mascha and Sessler 2011), can be used prove that mean CFUCs over multiple holding times have not decreased. These statistical methods reverse the traditional null and alternate hypotheses and again prove the alternate hypothesis by rejecting the null. However, as variability is inherent in the data, it is not possible to prove two means are exactly the same and instead the tests focus on trivial versus nontrivial differences (i.e., differences that have a practical/clinical impact). Specifically, for noninferiority testing, the new mean (μ_1) should be no lower than the original (μ_0) and the null hypothesis becomes (Equation 1):

$$\mu_1 - \mu_0 \leq -\delta \quad (1)$$

where δ is the value where the difference becomes meaningful as determined by practice.

In this case the alternate hypothesis is (Equation 2):

$$\mu_1 - \mu_0 > -\delta \quad (2)$$

which is interpreted as the new mean is better or at least no more than δ worse than the original (Mascha and Sessler 2011).

For this study, the meaningful difference was determined to be 10% of the original mean CFUC. This was based on the probability of misclassifying water samples as either positive or negative for *E. coli* (i.e., a 10% difference represents one in 10 *E. coli* cells dying and a 10% probability of misclassifying a water sample that originally contained only one *E. coli* CFU and a 1% probability of misclassifying a water sample that originally contained two *E. coli* CFUs).

Fig. 2. Diagram of testing process.

Fig. 3. Change in mean CFUC for the seven strains at 48 h and 72 h, as a percentage of 0 h mean CFUC.

To meet the two goals of the study (to compare 0 h and 48 h and then 48 h and 72 h), noninferiority tests compared mean CFUCs at 0 h with 48 h, and separately mean CFUCs at 48 h and 72 h. For each analysis, the δ was determined to be 10% of the mean CFUC at the lower time period. Given that the means were found from the same OD1 suspension held for different times, a paired t -test design was utilized. The paired t -test formula (t), adjusted for the noninferiority test, is (Equation 3):

$$t = \frac{\bar{d} - \delta}{\left(\frac{Sd}{\sqrt{N}} \right)} \quad (3)$$

where degrees of freedom = $n - 1$

The test is one-sided and statistical noninferiority was defined as a $p \leq 0.05$.

Results

The skewness and kurtosis values for mean CFUCs for all strains were acceptable, and therefore the data were normal. One strain (WT-H) had two outliers removed, resulting in a final sample size of 23 (due to the repeated measures nature, an outlier at one time period resulted in the OD1 suspension being removed across all time periods).

Figure 3 shows the mean CFUCs over time for the strains as a percentage of the original mean for comparison across strains. Strains are grouped by colour to represent their origin (Kingston PHOL (black) vs. other PHOL (grey)). The 10% decrease determined to be the meaningful difference for this study is overlaid on the graph. The actual mean CFU counts (and standard deviation (σ)) for each strain at each time period can be seen in Table 1.

Table 1. Mean CFUC and standard deviation (σ) for each strain at each time period.

Wild type	No.	0 hs		48 h		72 h	
		Mean	σ	Mean	σ	Mean	σ
WT-K1	25	94.4	11.7	95.0	10.5	96.3	7.0
WT-K2	25	90.9	8.1	93.8	9.6	91.6	10.2
WT-K3	25	90.5	10.3	91.1	8.3	92.1	10.3
WT-K4	25	119.9	7.8	113.6	9.0	108.8	9.3
WT-K5	25	72.8	5.8	87.5	8.2	84.1	6.2
WT-H	23	97.7	5.0	93.1	5.18	87.1	7.4
WT-OR	25	129.3	9.9	136.5	9.8	129.1	7.7

All wild types were tested for noninferiority (with 10% of the original mean CFUC being designated as the meaningful difference). The results of these tests are presented in Table 2. The results show that both objectives regarding no meaningful difference across holding times were met. Specifically, no statistical inferiority was found for all wild types between (i) 0 h and 48 h and (ii) 48 h and 72 h. This would suggest the current policy of 48 h is acceptable, but also that it would be equally acceptable to extend the maximum allowable holding time to 72 h, as no meaningful decrease in CFUCs was observed for any of the wild types tested.

However, when combined with observational results this conclusion is weaker. Five wild types do appear to remain close to 100% of the original after 72 h, with some wild types having increasing mean CFUCs by 48 h and (or) 72 h. However, two wild types do show a general downward trend from 0 h to 48 h to 72 h. This suggests that behavioural variability exists within

Table 2. Statistical results from noninferiority paired t-tests.

Wild type	0 h to 48 h comparison				48 h to 72 h comparison			
	10% difference (CFUC)	df	t	P	10% difference (CFUC)	df	t	P
WT-K1	9.4	24	5.0	<0.01	9.5	24	5.9	<0.01
WT-K2	9.1	24	5.1	<0.01	9.4	24	2.5	0.01
WT-K3	9.1	24	8.3	<0.01	9.1	24	5.7	<0.01
WT-K4	12.0	24	3.1	<0.01	11.4	24	3.4	<0.01
WT-K5	7.3	24	11.2	<0.01	8.7	24	3.0	<0.01
WT-H	9.8	22	3.2	<0.01	9.3	22	1.8	0.04
WT-OR	12.9	24	8.8	<0.01	13.6	24	2.66	0.01

Note: Df is the degree of freedom of the test. Statistically significant results are in bold.

survival rates amongst wild types and that the appropriateness of either 48 h or 72 h holding times may be questionable.

Discussion

This study was a simulation of *E. coli* survival rates in well waters; that is, not an exact replica of laboratory procedures for testing well water samples. Given variability of the underlying phenomenon and the complexity in measuring, the study design has some limitations. For example, most of the well water samples received for testing at the laboratory have less than 10 *E. coli* CFUs, but this range is not quantitative (International Organization for Standardization 2000) and so a higher concentration was necessary for the study. These higher concentrations might have an impact (positively or negatively) on survival rates, making it challenging to generalize the findings to lower concentrations, though a study by Dutka and El-Shaarawi (1980) provided evidence that bacterial density does not appear to be a factor in survival rates of water samples.

Overall, the generalizability of these results may be limited. Although a sufficient within wild type sample size was utilized for statistical purposes, the high time and resource requirements of the study only allowed for seven wild types to be included, all of which were from southern Ontario. Furthermore, the confirmed *E. coli* wild types were chosen at random (with some control for season); therefore, very little is known about them. As such, they may not adequately represent all wild types in the region and may not be generalizable to other regions. Additionally, the high resource demand limited the number of holding time periods that could be reasonably analyzed by the study. There may be key points in time where survival rates change (either between 0 h and 48 h, between 48 h and 72 h, or after 72 h); if so, these would be the most appropriate maximum holding times. Finally, only *E. coli* was modelled in this study, though the PHOL, like many other laboratories, also reports total coliform CFUs, which may behave differently than *E. coli*. However, an *E. coli* result is considered to be evidence of fecal contamination of water, whereas total coliforms are only considered to be evidence of

bacterial contamination, so *E. coli* was the preferable indicator to model.

Contradictory results regarding the holding of *E. coli* for 48 h have been found by earlier studies (Pope et al. 2003; Selvakumar et al. 2004). No previous investigations have had the power to analyze a 72 h holding time. Furthermore, comparison between the results of this study and previous studies is challenging for two major reasons: (i) different source waters and (ii) different statistical approaches. As evidenced by this study and previous literature, considerable variability exists within survival rates of *E. coli* and other FIB (notably total coliforms) from similar water sources, across multiple sites (i.e., different rivers or lakes, effluents from different facilities, or in the case of this study different isolated wild types). Given the variability within similar water sources, it is inadvisable to extrapolate these results across multiple water sources (for example surface water compared with groundwater). As such, the evidence presented by this study provides new insight into possible acceptable holding times that are specific for groundwater. Additionally, most studies employ traditional statistical methods (determining statistically significant differences) to develop survival models for FIB to evaluate holding times. However, as noted previously, these approaches do not have the capability of determining if CFUCs are meaningfully the same across possible holding times. Therefore, this paper provides a novel statistical framework for evaluating acceptable holding times for all water sources.

Both of the holding times evaluated by this study are longer than the maximum holding time utilized by many jurisdictions, including the EPA. The 72 h holding time is longer than the current maximum allowable holding time according to the MOECC. Given the persuasive, albeit limited, results of this study (and the limited current body of literature), a risk management approach should be employed when determining interim protocols as well as investment in further research to support development of evidence-based policies. Specifically, maximum acceptable holding times must balance the risk between: (i) the likelihood of reporting a false negative due to decreased survival after an extended holding time with (ii) the

rates of specimens not tested due to rejection or not submitted due to owners' inability to submit a sample on time. Additionally, although this study was focused on private well waters, increasing the acceptable maximum holding times for well water samples from public systems may also have an impact. Rural and (or) remote community systems, which are at a great distance from the closest certified laboratory, may currently find it difficult and (or) cost-prohibitive to test well water samples regularly. Increasing the acceptable holding time might allow these systems greater access to testing.

This was a time- and resource-consuming study; to test the seven wild types across three time periods required 175 OD1 suspensions, resulting in over 1500 plates used. Further investigation is required to fully evaluate acceptable holding times, notably examining a larger geographic region, various FIB, and additional holding times, to fully substantiate the most appropriate window.

Acknowledgements

The authors would like to acknowledge all the employees at the Public Health Ontario Laboratories, especially Kayla Poirier and Sandra Edelsward.

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