The Survival of Mycobacteria in Pure Human Urine

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ABSTRACT

Most mycobacteria pathogens are excreted via human urine through infected humans. This may lead to high numbers of pathogenic mycobacteria in the urine and therefore could establish a new transmission routes for disease infection during the recycling of urine for crop fertilization. In this study, *M. aurum* DSM 4399 and *M. fortuitum* ATCC 6841T were used as examples of fast-growing mycobacteria and *M. avium* ATCC 15769 and a clinical *M. bovis* BCG strain were used as examples of slow-growing mycobacteria to study their survival. The tests were done in fresh human urine (< one day old) and stored human urine (> six months old) at different temperatures 15°C and 30°C. The results of present study revealed that all the mycobacterial strains studied survived within one week in stored urine at 30°C with pH of around 9.0. Mycobacteria has the best survival time up to five weeks in fresh urine stored at 15°C. There were negative correlations between the increasing pH and the number of mycobacteria in the spiked urine. In conclusion, when recycling human urine for plant fertilization, it is advisable to store urine for more than five weeks at storage temperature for at least 30°C in order to prevent the exposure route for pathogenic mycobacteria.
Key words human urine*, M. aurum, M. avium, M. bovis,* and *M. fortuitum*

INTRODUCTION

Human urine contains important mineral nutrients necessary for plant growth and development. Urine consists of micronutrients and macronutrients which are readily in plant available form. The use of human urine as an alternative source for crop fertilization has been attracted by many researchers around the world. Previous studies have been shown that urine fertilization yield slightly higher or similar yield of harvested food crops when compared with chemical fertilizers (Heinonen-Tanski *et al*. 2007, Pradhan *et al*. 2009, Germer *et al.* 2011).The recycling of urine in agricultural application can help in alleviating some of the global problems such as food insecurity, global phosphorus depletion, poverty and hunger. The reuse of human urine for plant fertilization can be associated with hygienic risk and occupation hazards due to the presence of pathogenic micro-organisms from cross faecal contamination or via excretion from disease infected person.

Many pathogenic mycobacteria species causes a wide range of humans and animals diseases worldwide. In 2010, there were 8.8 million incident cases of tuberculosis globally and 1.4 million deaths occurred (WHO 2011). Infection in the kidney and other tuberculosis-like infection in different human tissues may result in the excretion of the pathogens via urine. Previous clinical studies revealed that pathogenic mycobacteria are excreted through human urine (Hillemann *et al.* 2006, Chan *et al.* 2008, Cannas *et al.* 2008, Siatelis *et al.* 2011 and Caleffi *et al.* 2012). The high numbers of pathogenic mycobacteria in the urine may indicate contamination of the environment during urine application for crop fertilization. This could introduce a new transmission route for disease infection. The presence of pathogenic mycobacteria in human urine is a possible source of infection for individuals involved in the application work.

Previous survival studies have been reported on different environmental reservoirs which harbors mycobacteria. The survival time varies depending on the environmental conditions. *Mycobacterium (M.) paratuberculosis* has been reported to have a longer survival time of up to 8 months in culturable pig and cattle slurry stored at 5°C than at 15°C (5 months)(Jøgensen 1977). *M. paratuberculosis* survived up to 6 to 18 months in water (Whittington *et al*. 2005, Cook *et al*. 2010) and for up to 48 weeks in sediments (Whittington *et al*. 2005). The survival time of *M. bovis* in liquid manure stored at 5°C was up to 176 days (Dokoupil 1964) and up to 21 months in soil (Young *et al.* 2005). *M. tuberculosis* survived in sterilized manure kept at room temperature for up to 172 days (Scanlon and Quinn 2000). The treatments of animal

manure and sewage sludge with chemicals have been reported to shorten the survival time of pathogenic mycobacteria. *M. bovis* survived less than 2 weeks in cattle slurry treated with 1% of ammonium hydroxide (Scanlon and Quinn 2000). *M. paratuberculosis* survived slurry up to 4 weeks after treated with 2% calcium cyanamide (Ley and Böhm 1993). It can be concluded that the treatments of manure can reduce the survival time of mycobacteria.

The survival studies of pathogenic micro-organisms in human urine have been carried out on viruses, enteric and gastrointestinal pathogens at different storage temperatures. Enteric bacteria and coliphage MS2 survived in less than one week in stored urine at 30°C (Chandran *et al*. 2009). However, there has been little information on the survival of mycobacteria in human urine. The only survival studies were carried out on *M. bovis* and *M. tuberculosis* by Vinnerås *et al*. (2011). The results of the study suggested that storage time of five weeks at temperature below 20°C or storage time of two weeks at temperature above 20°C is sufficient to cause the inactivation of mycobacteria during urine treatment for crop fertilization. In this present study, the survival of different mycobacterial strains fast growing (*M. aurum* and *M. fortuitum*) and slow growing (*M. avium* and *M. bovis*) were investigated in different human urine samples stored urine (> 6 months old) and fresh urine (< 1 day old) at different storage temperatures at 15°C and 30°C to represent the two typical world's climates, temperate and tropical climates. In addition, the anti-microbial properties of the urine samples at different temperatures in relation to pH were examined.

MATERIALS AND METHODS

Test organisms

The survival studies were conducted with four mycobacterial strains, two rapid growers, *M. aurum* DSM 43999 and *M. fortuitum* ATCC 6841T and two slow growers, *M. avium* ATCC 15769 and *M. bovis* BCG strain obtained from the Kuopio University Hospital The survival of the mycobacterial strains were studied in the following pure human urine samples; (i) stored urine samples > 6 months old collected from many urine separating toilets in an eco-village near Tampere, Finland. (ii) Fresh urine < 1 day old obtained from healthy students in Kuopio. All experiments were performed in four parallel runs.

Preparation of inocula and urine samples

M. aurum was inoculated onto Petri dish containing Tryptic Soy Agar (TSA) medium (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated for 1 week at 30°C. *M. bovis* and *M. avium* were inoculated separately onto Petri dish containing Mycobacteria 7H11 agar) medium (M7H11) (Becton Dickson and Company, Sparks, MD 21152, USA and incubated for 4 weeks at 36°C and *M. fortuitum* was inoculated onto Petri dish containing Mycobacteria 7H11 agar (M7H11) medium and incubated for 1 week at 30°C. About 10^{8} cfu g^{-1} of the test organism from the pure culture was suspended into a small amount of sterile water in a test tube against glass wall using sterile culture loop. The mixture was vortexed to form homogeneous suspension without clumps. The duration for vortexing varies from 1 minute to 10 minutes depending on the smoothness and coarseness of the test organism. Sterile water was added to the homogeneous suspension to a volume of 9 ml at room temperature. About 3.5 ml of the fresh inoculum from the homogeneous suspension was pipette into two sterile bottles (1 litre) each containing 700 ml of fresh and stored urine. The spike urine samples were shook carefully. The inoculated urine samples were subdivided into sterile 100 ml bottles with three replicates per experimental treatments. The pH of the samples was measured weekly using pH meter (Ino Lab pH 720 WTN 82362 Weiiheim Made in Germany).

Dilution series of 10^{-4} through 10^{-7} made from the spike urine samples were plated onto the agar media TSA or M7H11) and the inoculated plates were incubated at temperature 30 ± 0.5^{o}C or 36 ± 0.5^{o}C depending on the *Mycobacterium* strain. Follow-up of the samples were conducted weekly up to 8 weeks. The enumeration of the bacterial colonies was done by hand and counted once a week for at least one month. The detection limit was 10 cfu ml^{-1} for all the test organisms. The end of the experiment depends on the results. If two

consecutive follow up analyses gave negative results no further experimental analyses will be performed. All experiments were conducted in an aseptic environment (microbiological safety cabinet). The decay rates over time (K-value) of the different mycobacterial strain were calculated from the survival curves of viable cells in the human urine of initial cfu ml^{-1} count until no viable cells are detected.

Decontamination procedures for slow growing mycobacteria

1ml of the spike urine samples were pipette into 10 ml sterile centrifuge tubes. 1ml of 0.1% of CPC solution was added to the tubes containing the urine samples. The tubes were centrifuged at 8000rpm (Jouan MR22i) for 15 minutes at 4°C. The supernatant was discarded carefully. 20 ml of sterile water was added to the remained sediments in the centrifuge tubes and shake thoroughly. Centrifuge was repeated as told above. All the supernatant was poured off carefully. 1.5 ml of sterile water was to the sediment and shake well. Serial dilutions and plating dilution are made from the decontaminated samples.

Statistical analysis

The results were analyzed by Spearman rank correlation analysis (non-parametric test) using pH of the urine samples as independent variables and the cfu ml^{-1} as dependent variables. All statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, IL. version 17.0). If a result was less than detection limit (usually 10 cfu ml^{-1}) half of this has been used for logarithm transformations. Graphpad Prism 5 software (Graphpad software Inc version 5.03) was used for graphical analysis and the exponential decay k-value calculations.

RESULTS

Survival of *M. fortuitum*

The survival curves of *M. fortuitum* in both fresh and stored human urine at temperatures 15°C and 30°C are depicted (Figure 1). The initial concentration of *M. fortuitum* in fresh urine samples was 6.6 x 10^{6} cfu ml^{-1} and in stored urine was 6.1 x 10^{6} cfu ml^{-1}. In stored urine at 30°C, *M. fortuitum* was inactivated rapidly within 1 week and up to 4 weeks in stored urine at 15°C (Figure 1) *M. fortuitum* survived for up to 4 weeks fresh urine stored at 30°C and survived up to five weeks in fresh urine stored at 15°C (Figure 1). The pH values of all the urine samples increase gradually at every week interval of the experiment (Table 1). The complete inactivation of *M. fortuitum* after 6 weeks of the experiment was observed and no colony was detected in any of the inoculated or spiked selective media. The declined in cfu ml^{-1} of *M. fortuitum* in both fresh and stored human urine at 15°C and 30°C in relation to pH of the urine samples were observed (Table 1). All the urine samples exhibited negative correlation between pH and cfu ml^{-1} of *M. fortuitum* and they were all statistical significant ($p \leq 0.05$) except for *Mycobacterium fortuitum* in stored urine at 30°C (Table 3). The gradual increase in pH can be explained better with fresh urine than in stored urine due to the pH of the stored urine has already reached a constant pH values around 9.0. The exponential decay values (K-values) were negative for the survival of *M. fortuitum* in all the urine treatments (Table 2).

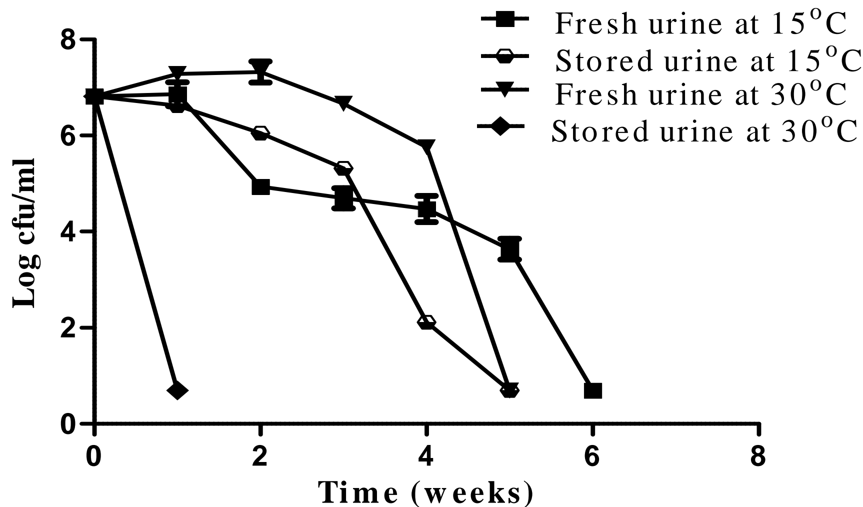

Figure 1 Survival curves of *M. fortuitum* in pure human urine (mean±SD)

Table 1 The reduction in cfu ml^{-1} of *M. fortuitum* in pure human urine at different storage temperatures in relation to pH of the urine samples

Week	Fresh urine at 15°C		Stored urine at 15°C		Fresh urine at 30°C		Stored urine at 30°C	
	pH	cfu/ml	pH	cfu/ml	pH	cfu/ml	pH	cfu/ml
0	6.95	6.6×10^6	8.97	6.1×10^6	6.95	6.6×10^6	8.97	6.1×10^6
1	7,12	7.2×10^6	8.97	4.2×10^6	7.12	1.9×10^7	9.03	l.d.l
2	7.66	8.5×10^4	8.97	1.2×10^6	7.65	2.1×10^7	9.19	l.d.l
3	8.18	4.9×10^4	8.97	2.1×10^5	8.35	4.5×10^6	9.27	
4	8.35	2.9×10^4	8.97	1.3×10^2	8.60	5.6×10^5	9.32	
5	8.87	4.3×10^3	9.10	l.d.l	8.97	l.d.l	9.32	
6	9.03	l.d.l	9.10	l.d.l	9.27	l.d.l	9.32	

Cfu/ml = colony forming units per milliliters l.d.l = less than the detection limit

Survival of *M. bovis*

The survival curves of *M. bovis* in both fresh and stored human urine stored at 15°C and 30°C are shown (Figure 2). The initial concentration of *M. bovis* in fresh urine was 3.6×10^5cfu ml^{-1} and in stored urine was 1.8×10^5 cfu ml^{-1}. *M. bovis* survived less than one week in stored urine samples incubated at 15°C and 30°C and survived up to 2 weeks in fresh human urine samples stored at 15°C and 30°C (Figure 2). Table 3 showed the negative correlations between the weekly increase in pH value of urine and the cfu ml^{-1} of the *M. bovis* in all urine treatments. The K-values for the survival of *M. bovis* in all the urine samples were all negative (Table 2). *M. bovis* was highly sensitive to the decontamination procedures which produced negative results for all the urine samples. Therefore the decontamination was not used throughout the experiments.

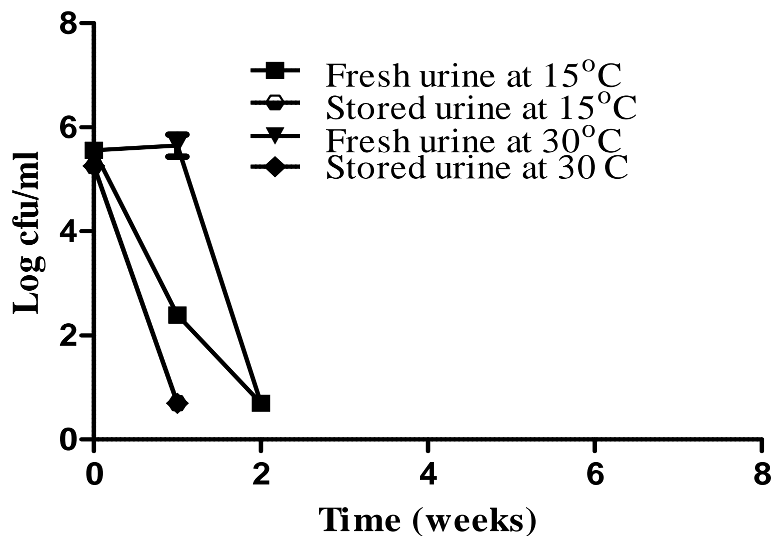

Figure 2 Survival curves of *M. bovis* in pure human urine (mean±SD)

Table 2 The K-value (log cfu/ml week^{-1}) of the exponential decay of mycobacteria in fresh and stored human urine at storage temperatures 15°C and 30°C

Test organism	**K- value**			
	FU 15	**FU 30**	**SU 15**	**SU30**
M. fortuitum	-0.1800	-0.1681	-0.2355	-2.278
M. bovis	-0.9143	-0.4870	-2.017	-2.017
M. avium	-0.1801	-0.2341	-0,3001	-0.5630
M. aurum	-0.3662	-1.178	-0.3952	-1.044

FU 15 = fresh urine at 15°C, FU 30 = fresh urine at 30°C, SU 15 = stored urine at 15°C, SU 30= stored urine at 30°C.

Table 3 Spearman rank correlation coefficients between the pH of human urine and the cfu ml^{-1} of different mycobacterial strains survival in urine at different temperatures.

Variable	**cfu ml^{-1}**	
	r	***p***
M. fortuitum in fresh urine at 15°C	-0.964	<0.01
M. fortuitum in stored urine at 15°C	-0.798	<0.05
M. fortuitum in fresh urine at 30°C	-0.847	<0.05
M. fortuitum in stored urine at 30°C	-0.635	
M. bovis in fresh urine at 15°C	-0.360	
M. bovis in stored urine at 15°C	-0.333	
M. bovis in fresh urine at 30°C	-0.722	
M. bovis in stored urine at 30°C	-0.707	
M. avium in fresh urine at 15°C	-1.000	<0.01

M. avium in stored urine at 15°C	-0.608	
M. avium in fresh urine at 30°C	-1.000	<0.01
M. avium in stored urine at 30°C	-0.949	<0.05
M. aurum in fresh urine at 15°C	-0.852	<0.05
M. aurum in stored urine at 15°C	-0.795	<0.05
M. aurum in fresh urine at 30°C	-0.490	
M. aurum in stored urine at 30°C	-0.802	<0.05

r = Spearman rank correlation coefficient ***P*** $\leq$ 0.05 = Statistical significant.

Survival of *M. avium*

The initial concentration of *M, avium* in fresh urine was 8.9 x 10^6 cfu ml^{-1} and in stored urine was 8.1 x 10^6 cfu ml^{-1}. The test organisms reduced rapidly within 1 weeks in the stored urine samples at 30°C and it survived up to 2 weeks in the stored urine at 15°C (Figure 3). In fresh urine samples stored at 15°C and 30°C the organisms had a better survival up to 4 weeks before rapid decline of their colony (Figure 3). *M. avium* was also highly sensitive to the decontamination procedures which produced negative results for all the urine samples. The correlation between pH and cfu ml^{-1} of the organism was statistically significant ($p \leq 0.01$) for fresh urine samples incubated at 15°C and 30°C and stored urine sample at 30°C. Negative correlations between the increase in pH of the urine and cfu ml^{-1} of the *M. avium* were observed (Table 3). The exponential decay values (k-values) for the survival of *M. avium* for all the urine treatments were negative (Table 2)

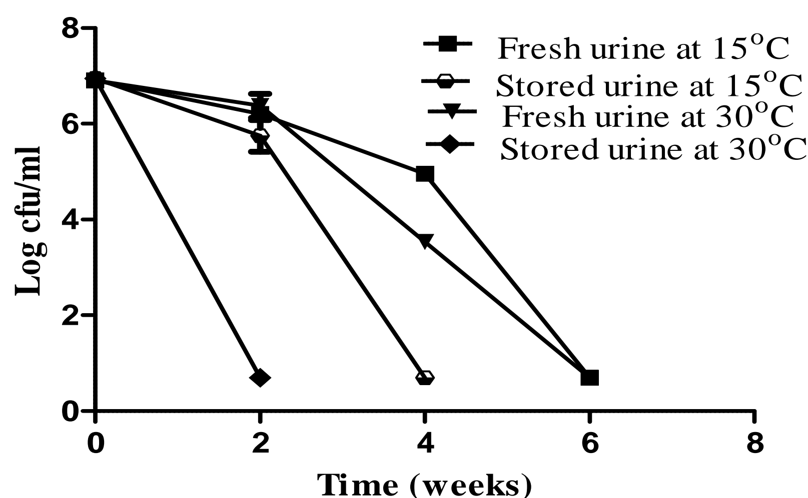

Figure 3 Survival curves of *M. avium* in pure human urine (mean±SD)

Survival of *M. aurum*

The survival curves of *M. aurum* in stored and fresh urine at temperatures 15°C and 30°C are shown in Figure 4. The initial concentrations of cfu ml^{-1} of *M. aurum* in fresh urine was 9.2 x 10^6 cfu ml^{-1} and in stored urine was 2.4 x 10^6 cfu ml^{-1}. The micro-organism declined rapidly within 1 week in stored urine at 30°C and survived up to 2 weeks in fresh urine stored at 30°C (Figure 4). The organism survived up to 3 weeks in the fresh urine samples incubated at 15°C and 2 weeks in the stored urine samples at 15°C (Figure 4). After 6 weeks of the experiment negative results were observed in all the spiked selective media. The correlation between the pH of pure human urine at different temperatures and cfu ml^{-1} of *M. aurum* were statistically significant ($p \leq 0.05$) except for the fresh urine samples at 30°C. Negative correlation between the pH and cfu ml^{-1} of *M. aurum* were observed for all the urine samples (Table 3). Negative exponential decay values (K- values) were observed for all the urine treatments spike with *M. aurum* (Table 2).

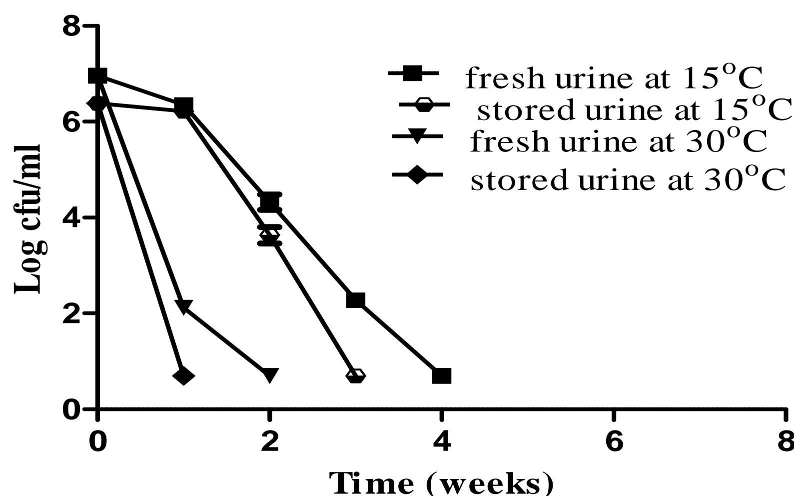

Figure 4 Survival curves of *M, arurum* in pure human urine (mean±SD)

DISCUSSION

There has been little information on the investigation of survival of mycobacteria in pure human urine in view of urine as a fertilizer for crop production. In this study, we carried out the investigation on how mycobacteria survived in pure human urine using four test organisms; the two fast-growing *M. aurum* and *M. fortuitum* and the two slow-growing; *M. avium* and *M. bovis* considering hygienic risk and occupational hazard involved in it reuse in agricultural application. Our findings indicate that all the test organisms studied had a low survival rate in stored human urine. They all survived in < 1 week in stored human urine at 30°C with pH around 9.0 being the most inactivation rate for mycobacteria when compared to other treatments, thus supporting previous studies conducted on the survival of *M. tuberculosis* and *M. bovis* in human urine (Vinnerås *et al*. 2011) and findings conducted on the survival of enteric bacteria and coliphage MS2 in pure human urine (Chandran *et al*. 2009). The temperature conditions were similar to that of Chandran *et al.* (2009) study and slightly different from the investigation of Vinnerås *et al*. (2011). The results of the present study revealed that mycobacteria survived better in stored urine at 15°C at high pH level around 9.0 than that of stored urine at 30°C and the survival time varied from one to four weeks depending on the strains. The present findings indicate that the survival time of mycobacteria in fresh urine at 15°C varied from two to five weeks and in fresh urine at 30°C, the survival time was two to five weeks when the pH had raised to around 9.0.

In this present study, the results on survival time of mycobacteria in human urine was promising when compared to previous survival studies conducted on mycobacteria species in different environmental reservoirs. Previous studies have showed that mycobacteria survival longer period of time in different

environment. The results of the current study revealed that the survival time of mycobacteria in human urine was vary from one week up to six weeks depending on the pH and the storage temperature of the urine samples. Recently, the only other study of mycobacteria survival in human urine was conducted by Vinnerås *et al.* (2011). They reported that mycobacteria survived from 2.3 days up to 28 days in human urine around pH 9.0 at temperatures 22°C and 4^{0}C which is in agreement with the results of the present study.

In the current study, there was weekly increase in pH level of the fresh urine. The increase in the pH level of the fresh urine stored at 30°C was more rapid when compared to that of the fresh urine stored at 15°C. Probably this may be due to the enzyme urease was more favored at temperature 30°C. Similar to our findings, other studies have shown that higher temperature prefers urea hydrolysis and it was higher at temperature 30°C than at temperature 15°C (Zhigang *et al.* 2008). The breakdown of urea in human urine was caused by an enzymes urease to produce increase the concentration of ammonia in the urine solution therefore increase the pH of the urine. The results of the present study indicate that the pH of the fresh urine increased to a value around 9.0 within the period of 5−8weeks depending on the storage temperature. Similar results were also reported by (Zhigang *et al.* 2008 and Chandran *et al.* 2009).

The survival of the mycobacteria strains in both fresh and stored urine was dependent on the pH of the urine and the storage temperatures. In this study, the result revealed that the increase of pH of the urine lead to the progressive reduction of the colony forming units of the test organisms. This means that higher pH values caused more negative effects on the survival of the test organisms than the neutral (pH 7.0). The results of the present study suggested that, for all the urine samples studied at pH around 9.0 there was rapid inactivation of the test organisms. This evidence was supported by the results of previous studies, that high pH level around 9.0 was responsible for the antimicrobial properties of human urine. Thus our results are similar to those reported by Schönning and Stentröm (2004), Chandran *et al*. (2009) and Vinnerås *et al.* (2011). Iivanainen *et al.* (1993) reported that the number of mycobacteria correlated negatively with the pH of the environment. According to results of the present study it was revealed that the numbers of mycobacteria in the spiked urine correlated negatively with the increase in pH of the urine, these results are in agreement with the findings of environmental factors affecting the occurrence of mycobacteria in brooks waters (Iivanainen *et al.* 1993).

In previous survival study using human urine, it was reported that micro-organisms showed poorer survival at higher temperatures and better survival at lower temperatures (Chandran *et al*., 2009, Vinnerås *et al*., 2011) and this was similar observations found in the present study. The findings of the current study revealed that the K-values of the survival of mycobacteria were negative for all the urine treatments and the values are higher in stored urine at 30°C when compared to other treatments. This implies the decay rate increases more rapidly with higher pH and higher storage temperatures of the urine, thus this was similar to the findings of Vinnerås *et al*. (2011). The experimental work focused on both tropical and temperate climates based on the storage temperatures. Despite that urine stored at 30°C proved most efficacy in the survival study. Generally, the maximum survival time of mycobacteria observed in the present study was five weeks. In conclusion, the results of the current study recommend a storage period of urine more than five weeks will be sufficient for the inactivation of the micro-organisms in both climates. This could help to prevent the hygienic risk associated with human urine reuse for plant fertilization. Hence, these findings were in accordance with the recommendation on risk reduction by storage treatment of urine recycling for crop fertilization (Vinnerås *et al.* 2011).

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