ABSTRACT

Tuberculosis is a zoonotic disease that mainly causes respiratory infection. However, it can also infect other organs such as the kidneys and bladder, which can lead to high counts of the organisms in the urine. Introducing urine diversion systems and reuse of the urine in agriculture may introduce new transmission routes for infection, increasing the risk of spread. This study evaluated the inactivation rate of mycobacteria in human urine for ensuring safe reuse in agriculture and examined whether current World Health Organization recommendations on storage time are sufficient for inactivating *Mycobacterium tuberculosis* and *Mycobacterium bovis*. In this study, a decimal reduction in *M. tuberculosis* and *M. bovis* in human urine containing 7 and 3 g NH₃-N L⁻¹, respectively, was obtained in just over 10 days at 4 °C and below three days at 22 °C. This is considerably faster than previously reported reduction rates of mycobacteria in animal slurry at similar temperatures. Based on the present results, a storage time of five weeks at temperatures below 20 °C or of two weeks at temperatures above 20 °C is sufficient to prevent transmission of mycobacteria when recycling human urine. These values lie within the WHO recommended storage period.

**Key words** | ammonia sanitization, human urine, hygiene, *M. bovis*, *M. tuberculosis*

INTRODUCTION

In 2008, it was reported 9.6 million incident cases and 11.1 prevalent cases and approximately 1.8 million deaths worldwide in that year (WHO 2009). Tuberculosis in humans is almost exclusively caused by *Mycobacterium (M.) tuberculosis*, but there are some other *Mycobacterium* species that can also cause tuberculosis in humans (Schulze-Röbbecke 1993). In areas where *M. bovis* is found among cattle it is often also found in humans, whereas in areas with low rates of animal infection the primary infection of cows with *M. bovis* is via humans (O’Reilly & Daborn 1995). Human tuberculosis is mainly an airborne infection. Pulmonary disease is the most common manifestation, and the main transmission pathway is via aerosols from coughing and spitting. However, some organisms also end up in the faeces through swallowing of bacteria expectorated from the lungs. Tuberculosis lesions can also develop in organs other than the lungs, e.g. peripheral lymph nodes, kidneys, brain and bone. Infection in the kidneys may result in excretion of bacteria in the urine, and there have been several reported cases of transmission of *M. bovis* from man to cattle via animal bedding contaminated by renally excreted mycobacteria (O’Reilly & Daborn 1995). Several investigations show between 2 and 10% of patients with *M. tuberculosis* manifestation are excreting *M. tuberculosis* in the urine (Bentz et al. 1975; Nzerue et al. 2000). The actual numbers of *M. tuberculosis* or *M. bovis* excreted in human urine are unclear. An extrapolation from the amount of *M. bovis* bacilli excreted in the urine of tubercular badgers indicates that it may be in the order of 300 000 cfu mL⁻¹ urine (O’Reilly & Daborn 1995).

Studies on the survival of *M. tuberculosis* and *M. bovis* bacteria in the environment report a high variability in the survival times, but most cases show long survival. In sputum, the survival time in some cases has been found to be more
than 300 days (Walther & Ewald 2004). Survival times of over 142 days have been reported in sand (Walther & Ewald 2004), of 21 months in soil (Young et al. 2005), of >194 days in sewage sludge and up to 450 days on a sewage sludge drying bed (Mitscherlich & Marth 1984). The survival of the closely related species Mycobacterium paratuberculosis in manure has been observed to be long-lasting. Berg Jørgensen (1977) showed longer survival time at 5°C (252 days) than at 15°C (182 days) for pig, cattle and mixed pig and cattle slurry. In that study there was a great reduction in organisms between day 0 and day 7, and then little or no change was detected in the count until no organisms were detected (252 days at 5°C and 182 days at 15°C for pig, cattle and mixed pig and cattle slurry). The reason for the small changes in numbers of organisms cultivated during that study after day 7 can be that M. paratuberculosis forms aggregates, making quantitative analysis difficult as one colony can be formed by several organisms. These aggregates also affect the actual survival rate, as organisms inside an aggregate are protected by organisms on the outside. Later studies on the survival of M. tuberculosis inoculated into sterilised manure at room temperature reported a survival time of 172 days, with a decimal reduction time of 27 days (Scanlon & Quinn 2000). It can be concluded that the survival of mycobacterium in manure is long-term and that low temperatures increase the survival time.

In cattle, infection mainly occurs through aerosols or from organisms adsorbed to inhaled particles, e.g. dust or soil particles (Walther & Ewald 2004). Dhand et al. (2009) showed that at pH 3–7 of the soil particles, M. avium subsp. paratuberculosis could adsorb to a wide range of soil types. Therefore it can be expected that if tuberculosis bacteria are spread onto arable soil, there is a risk of infection if soil dust is inhaled or if soil is ingested. However, as mycobacteria are susceptible to gastric enzymes in the stomach, a high infective dose (10^7 bacilli) is required for oral infections in order to cause disease (Corner 2006).

Human urine contains large amounts of plant-available nutrients (Vinnerås et al. 2006) and therefore its agronomic value is high (Guzha et al. 2005; Winker et al. 2009). Separate collection of urine at source makes it possible to collect and recycle large quantities of high quality plant nutrients with very low concentrations of heavy metals (Vinnerås & Jönsson 2002; Vinnerås et al. 2006).

Earlier studies of pathogen survival in human urine have concluded that appropriate treatment of urine before use as a fertiliser for food and feed crops is storage for about 2 months at ≥20°C and ammonia concentrations of about 50 mM NH\textsubscript{3} (Vinnerås et al. 2008; Nordin et al. 2009b). This has been shown to achieve sufficient inactivation of the indicator organisms Enterococcus faecalis, bacteriophage Salmonella Typhimurium 28B, enterobacteria phage MS2 and coliphage Φx 174 and the pathogens Salmonella spp., Escherichia coli O157 and Ascaris spp. (Vinnerås et al. 2008; Nordin et al. 2009a). At higher temperatures the inactivation is even faster. At temperatures below 20°C the inactivation slows down, especially for viruses (Vinnerås et al. 2008; Chandran et al. 2009; Nordin et al. 2009a) and Ascaris spp. (Nordin et al. 2009b). The current recommendation is that storage at temperatures below 20°C should be combined with restricted usage, with the urine not being used for crops intended to be eaten raw by humans (WHO 2006). This recommendation is based on rapid inactivation of zoonotic organisms such as Salmonella spp. The survival of M. tuberculosis and M. bovis, which can also be found in human urine, is mentioned in the WHO guidelines (WHO 2006), but to our knowledge no investigation has been performed on the survival of these organisms in urine during storage. There is a risk of long survival times of M. tuberculosis and M. bovis compared with Salmonella spp., as the former have shown very long survival times in animal manure.

**OBJECTIVES**

The main objectives of this study were to evaluate the inactivation rate of mycobacteria in stored human urine in order to determine the risk of disease transmission associated with the reuse of potentially contaminated urine in agriculture; and to evaluate whether current WHO recommendations on storage time are sufficient to inactivate M. tuberculosis and M. bovis.

**MATERIALS AND METHODS**

Urine was collected from three healthy humans with no tuberculosis infection and under no medication. Urease (0.05 g) was added to the urine and the mixture was shaken at 37°C overnight to obtain pH 9. The pH was analysed at room temperature with an Inolab 720 pH meter (WTW, Weilheim, Germany). For quantification of total ammonia (NH\textsubscript{tot} = NH\textsubscript{3} + NH\textsubscript{4}\textsuperscript{+}), a 1 g subsample of urine was analysed spectrophotometrically on a Thermo Aquamate (Thermo Electron Ltd, Cambridge, UK) using the indophenol blue method (Merck, Whitehouse Station, NJ, USA). The urine was then diluted using physiological saline solution (NaCl 0.9%), National Veterinary Institute, Sweden) to obtain
a concentration of 7 g NH$_3$-N L$^{-1}$, which corresponds to undiluted urine, and 5 g NH$_3$-N L$^{-1}$, corresponding to urine diluted with 50–60% flush water as generally occurs in Swedish urine diverting systems (Vinnerås & Jonsson 2002). The urine was divided into four 50 mL centrifuge tubes per concentration, which were stored at 4°C or at room temperature (22°C) in the laboratory. One tube per temperature and urine concentration was inoculated with mycobacteria. One tube per concentration and temperature was not inoculated and was used for pH and ammonium measurements. These measurements were performed outside the laboratory where the studies on the mycobacteria were conducted, but the two sets of tubes for pH and ammonia measurements were handled similarly to the samples inoculated with mycobacteria.

The samples with 7 g NH$_3$-N L$^{-1}$ were inoculated with a strain of *M. tuberculosis* originally isolated from human urine (Swedish Institute for Infectious Disease Control, Sweden), and the urine samples with 3 g NH$_3$-N L$^{-1}$ were inoculated with a strain of *M. bovis* originally isolated from a cattle lung (SVA, Sweden). The urine was then sampled on days 0, 3, 7, 14, 28 and the final samples were taken on day 45 for 7 g NH$_3$-N L$^{-1}$ and on day 56 for 3 g NH$_3$-N L$^{-1}$. Upon sampling, the inoculated urine was treated with sodium dodecyl sulphate and NaOH for 30 minutes. The samples were then centrifuged at 3000 g for 15 minutes. The pellet produced was suspended and neutralised with 0.09% H$_2$SO$_4$ and again centrifuged at 3000 g for 15 minutes. The supernatant was discarded except for a portion to suspend the pellet for culture and for a ten-fold serial dilution in 0.9% NaCl. Triplicate samples (0.1 mL) from the 3 and 7 g NH$_3$-N L$^{-1}$ urine samples and each dilution were spread on Löwenstein Jensen medium slants and incubated for four weeks at 37°C. The reduction rate was calculated based on the decay curve of viable cells in the urine from the initial count until no viable cells were detected.

**RESULTS**

There was a reduction in the bacterial count at both temperatures and both ammonia concentrations tested (Table 1). At 22°C no viable organisms of either bacterial species were detected at day 14, while at 4°C no viable organisms of *M. tuberculosis* were detected at day 28 or of *M. bovis* at day 56.

Microbe inactivation at the higher temperature (22°C) was considerably faster, with a time for decimal reduction (D-value) of 2.3 days for *M. tuberculosis* with 157 mmol L$^{-1}$ uncharged ammonia (NH$_3$) and 2.8 days for *M. bovis* with 67 mM NH$_3$. This can be compared with the inactivation at 4°C, with D-value of 9.6 days for *M. tuberculosis* with 51 mM uncharged ammonia (NH$_3$) and 10.4 days for *M. bovis* with 22 mM NH$_3$ (Table 2).

**DISCUSSION**

The observed reduction rates in *M. tuberculosis* and *M. bovis* in the samples seemed to be mainly associated with the treatment temperature, with inactivation at 22°C being considerably faster than inactivation at 4°C, irrespective of the ammonia concentration (Figure 1).

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**Table 1** | Cell counts of *Mycobacterium tuberculosis* and *M. bovis* (log$_{10}$ cfu per mL urine) during treatment at 4°C or 22°C and with 3 or 7 mg NH$_3$-N L$^{-1}$

<table>
<thead>
<tr>
<th>Day/Treatment</th>
<th>4°C, 7 g NH$_3$-N L$^{-1}$</th>
<th>22°C, 7 g NH$_3$-N L$^{-1}$</th>
<th>4°C, 3 g NH$_3$-N L$^{-1}$</th>
<th>22°C, 3 g NH$_3$-N L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.0</td>
<td>3.5</td>
<td>5.3</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>2.8</td>
<td>2.2</td>
<td>4.8</td>
<td>3.8</td>
</tr>
<tr>
<td>7</td>
<td>2.4</td>
<td>0.5</td>
<td>4.6</td>
<td>2.5</td>
</tr>
<tr>
<td>14</td>
<td>2.3</td>
<td>BDL$^1$</td>
<td>4.0</td>
<td>BDL$^1$</td>
</tr>
<tr>
<td>28</td>
<td>BDL$^1$</td>
<td>BDL$^1$</td>
<td>2.1</td>
<td>BDL$^1$</td>
</tr>
<tr>
<td>47</td>
<td>BDL$^1$</td>
<td>BDL$^1$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>-</td>
<td>-</td>
<td>BDL$^1$</td>
<td>BDL$^1$</td>
</tr>
</tbody>
</table>

$^1$BDL below detection limit, 1 cfu mL$^{-1}$
The uncharged ammonia concentration at 22°C for treatment of *M. tuberculosis* was 157 mmol L⁻¹, while that for *M. bovis* was 67 mmol L⁻¹ (Table 2). However, the inactivation rate of the two differed only slightly, with D-values of 2.3 and 2.8 for *M. tuberculosis* and *M. bovis*, respectively. At the lower temperature investigated (4°C), the difference in the reduction rate was larger in absolute values (days) but smaller calculated as a percentage. The reduction rate at 4°C for *M. tuberculosis* and *M. bovis* gave D-values of 9.6 and 10.4 days, respectively. The ammonia concentration at the lower temperature (D-value of approximately 10 days and 9.6 for *M. tuberculosis*, 10.4 for *M. bovis*) was considerably, with the D-value for *M. bovis* being 3.5-fold that for *M. tuberculosis*. This indicates a combined effect on the inactivation that can be attributed to both the temperature and the free ammonia content, as reported for the survival of several other organisms in urine and during ammonia treatment (Höglund et al. 2000; Vinnerås et al. 2003, 2008; Vinnerås 2007; Nordin et al. 2009a, b). The change in sensitivity in connection to temperature and ammonia content vary between organisms and a study by Ottoson et al. (2008) showed that *Enterococcus* spp. were more sensitive to the change in treatment temperature, from 4 to 14°C compared to the sensitivity for change in free ammonia concentration. For Gram-positive bacteria such as *Salmonella*, it has not been possible to detect such an effect. The main reason for the difference in sanitisation efficiency at different temperatures has been suggested to be related to temperature-dependent cell permeability (Wharton 1980).

The survival time of *M. tuberculosis* bacteria in urine at 4°C was considerably shorter than the decimal reduction in manure reported by Scanlon & Quinn (2000) of almost 27 days at room temperature (assumed 20–25°C), while the decimal reduction in urine at 22°C was less than 3 days, even for urine containing 3 g NH₃-N L⁻¹ (*M. bovis*). A similar effect was observed for both NH₃-N treatments at the lower temperature (D-value of approximately 10 days and approximate survival time of up to 50 days), whereas Berg Jørgensen (1977) reported a survival time of over 250 days at 5°C in swine and cattle slurry. This indicates that the inactivation effect of the urine, which is most probably due to its ammonia content, gives a fast reduction in *M. tuberculosis*

Table 2 | Inactivation data, as reduction over time (k) and as decimal reduction (D-value), and supporting data for the factors affecting the inactivation, i.e. pH, temperature and ammonia content (total and uncharged)

<table>
<thead>
<tr>
<th>Factor/Treatment</th>
<th>4°C, 7 g NH₃-N L⁻¹</th>
<th>22°C, 7 g NH₃-N L⁻¹</th>
<th>4°C, 3 g NH₃-N L⁻¹</th>
<th>22°C, 3 g NH₃-N L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td><strong>M. tuberculosis</strong></td>
<td><strong>M. bovis</strong></td>
<td><strong>M. bovis</strong></td>
<td></td>
</tr>
<tr>
<td>K value (&lt;i&gt;Log&lt;sub&gt;10&lt;/sub&gt;&lt;/i&gt; day⁻¹)</td>
<td>-0.1046</td>
<td>-0.4264</td>
<td>-0.0957</td>
<td>-0.3603</td>
</tr>
<tr>
<td>D-value (days)</td>
<td>9.6</td>
<td>2.3</td>
<td>10.4</td>
<td>2.8</td>
</tr>
<tr>
<td>pH</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>4</td>
<td>22</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>N-NH₃ tot (g L⁻¹)</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Available NH₃ (mmol L⁻¹)</td>
<td>51</td>
<td>157</td>
<td>22</td>
<td>67</td>
</tr>
</tbody>
</table>

Figure 1 | Reduction over time (days) in counts (<i>log<sub>10</sub></i> cfu mL⁻¹) of *M. tuberculosis* at 4°C (●) and 22°C (●●) with 7 g NH₃-N and in *M. bovis* at 4°C (▲) and 22°C (⋆) with 3 g NH₃-N.
and M. bovis during storage of urine at 4°C and above, although with a much faster reduction rate at 22°C. The effect of the temperature was much higher than that of the ammonia even if it seems like ammonia needs to be present for proper inactivation as studies of inactivation in other material, e.g. manure, showed considerably longer survival times at similar conditions (Berg Jørgensen 1977; Scanlon & Quinn 2000). Additionally, the studies did show that even though aggregate formation by M. tuberculosis probably facilitates somewhat longer survival time than that of M. bovis both organisms had a fast inactivation at both temperatures. In studies of urine storage, lower concentrations of ammonia in urine (1.5 g NH₃-N L⁻¹) have resulted in efficient bacterial reduction (Vinnerás et al. 2008) and it is therefore most probable that the inactivation of M. tuberculosis bacteria would occur and that the rate would be faster than the reduction in manure, even when the urine is more dilute than that investigated in the present study. However, for efficient reduction of pathogenic organisms it is important to keep the urine as concentrated as possible. To determine the NH₃ concentrations needed for proper inactivation of M. tuberculosis, further studies with a larger span of temperatures and urine dilutions are needed.

Introducing a new transmission route, as it will be done with urine reuse in agriculture, will always increase the risk for transmitting diseases as long as present transmission routes remain. As M. tuberculosis is a zoonotic disease it is important to assure very low increase of risk for disease transmission to the farm animals is associated with this new praxis of nutrient reuse. In a urine-diverting system the actual numbers of M. tuberculosis bacteria can be expected to be low. In a worst case scenario concentrations up to 10⁵ organisms mL⁻¹ can occur. This is based on the assumption that infected urine will be diluted by 99% non-infected urine. This dilution is based on the assumption that 10% of the population are infected with tuberculosis (to be compared with the global infection rate of 0.3% of the population), of the infected will 10% excrete M. tuberculosis in the urine (Bentz et al. 1975). The concentration of M. tuberculosis is assumed to be 10⁵ cfu mL⁻¹ of urine as reported for badger urine (O'Reilly & Daborn 1995). Based on this worst case assumption, the recommended time of treatment with a total nitrogen concentration of at least 3 g N L⁻¹ is five weeks at 4°C and two weeks at temperatures above 20°C. This treatment will achieve at least a 3 log₁₀ reduction and thereby give a low risk of finding viable M. tuberculosis or M. bovis in the stored urine.

The results from this study indicate that the reduction rate of M. tuberculosis and M. bovis in human urine with at least 3 g NH₃-N L⁻¹ is sufficiently rapid to ensure that even high concentrations of mycobacteria in the urine are inactivated within the WHO (2006) recommended time of storage for safe reuse of human urine (unrestricted use after 6 months at >20°C and restricted use (processed food and raw fodder) after 6 months storage at <20°C).

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