THE SURVIVAL OF MYCOBACTERIA IN PURE HUMAN URINE

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ABSTRACT

Human urine contains high levels of both nitrogen and phosphorus and it may be used as plant fertilizer to enhance sustainable crop production and sustainable sanitation. The risks include the presence of possible pathogens via contamination with faeces or excretion of pathogens from diseased humans. Most mycobacterial pathogens, causing for instance tuberculosis and tuberculosis-like infections in other soft tissues or lymph nodes, are excreted via human urine if the infection is in kidneys. This may lead to high number of the pathogenic mycobacteria in the urine. The application of human urine in crop fertilization could therefore establish new transmission routes for disease infection possible for the persons who are involved in the application work. 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 in urine. The tests were done in fresh human urine (< one day old) and stored human urine (> six months old) at different temperatures, 15^{o}C and 30^{o}C, to mimic Nordic and African temperatures. The results of this study revealed that all the mycobacterial strains studied survived less than one week in stored urine with pH of around 9.0 at 30^{o}C. Survival in stored urine at 15°C was better and varied from one to four weeks depending on the strain. Survival in fresh urine at 15^{o}C was two to five weeks and in fresh urine at 30^{o}C survival time was two to four weeks after which the pH had raised to around 9.0. The number of mycobacteria negatively correlated with the increasing pH of the urine samples. *M. fortuitum* had the best survival but other rapid-grower, *M. aurum*, had rather low survival. *M. bovis* had the least survival time for all the urine samples. In conclusion, when recycling human urine for plant fertilization, it is advisable to store urine for more than five weeks at storage temperatures of at least 15^{o}C and at most 30^{o}C respectively, in order to prevent the exposure route for pathogenic mycobacteria.

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ABBREVIATION AND DEFINITIONS

AFB	Acid-fast bacillus
AIDS	Acquired immune deficiency syndrome
BACTEC	Radiometric growth detection
cfu	Colony forming units
cfu/ml	Colony forming units/millilitres
$CO(NH_2)$	Urea
CPC	Cetylpyridinium chloride
CZ	Carbamazepine
GC/MS	Gas chromatography/mass spectrometry
HCl	Hydrochloric acid
HCO_3^-	Carbonate ion
HEYM	Herrold's Egg Yolk Medium
HIV	Human immunodeficiency virus
H_2O	Water
$H_2PO_4^-$	Dihydrogen phosphate ion
HPO_4	Hydrogen phosphate ion
H_2SO_4	Sulphuric acid
IBU	Ibuprofen
IUTM	International Union of Tuberculosis Medium
LJ	Löwenstein–Jensen medium
M.	*Mycobacterium*
MAC	*Mycobacterium avium* complex
MGIT	Mycobacteria Growth Indicator Tube 960 system
M7H9	Middlebrook 7H9 broth
M7H10	Middlebrook 7H10 agar
M7H11	Middlebrook 7H9 agar
NALC	N-acetayl-L- cysteine
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_3-N	Ammonia nitrogen
NH_4^+	Ammonium ion
NH_4-N	Ammonium nitrogen
$(NH_4)_2SO_4$	Ammonium sulphate
NPK	Nitrogen-phosphorus-potassium fertilizer
PCR	Polymerase chain reaction
pKa	Dissociation constant
PO_4^{3-}	Phosphate ion
PO_4^{3-}-P	Phosphate phosphorus
rRNA	ribosomal Ribonucleic acid
SO_4^{2-}	Sulphate ion
Subsp	Subspecies
TREK-ESP	TREK Diagnostics System
TSA	Tryptic Soy agar
TTC	Triphenyl Tetrazolium Chloride
UV	Ultra-violet light
WHO	World Health Organization

CONTENTS

1 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 alternative source for crop fertilization had been attracted by most researchers around the world. The recycling of urine in agricultural application can help in alleviating some global problems such as food insecurity, global phosphorus depletion, poverty and hunger. The research on human urine as fertilizer in agricultural application and comparison with chemical fertilizer had been investigated by many researchers. Previous studies have shown that urine fertilization yield slightly higher or similar output of harvested food crops when compared to chemical fertilizers (Sundin *et al.* 1999, Heinonen-Tanski *et al.* 2007, Pradhan *et al.* 2009, Germer *et al.* 2011, Akpan-Idiok *et al.* 2012). 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 fecal contamination or via excretion from disease infected person.

Many pathogenic mycobacterial species cause a wide range of human and animal diseases worldwide. In 2010, there were estimated 8.8 million incident cases of tuberculosis globally and 1.3 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 human urine. The results from several studies revealed that pathogenic mycobacteria are excreted through human urine (Hillemann *et al.* 2006, Chan *et al.* 2008, Cannas *et al.* 2008, Alvarado-Esquivel *et al.* 2009). The high number of mycobacteria in the urine may lead to contamination of the environment during urine fertilization. The reuse of human urine in crop fertilization could therefore introduce a new transmission route for disease infection. The presence of pathogenic mycobacteria in human urine is a potential source of infection for individuals involved in the application work.

Previous survival studies have been reported on different environmental reservoirs harboring mycobacteria. The survival time varied depending on the environmental conditions. *Mycobacterium (M.) paratuberculosis* has been reported to have a longer survival time of up to 252 days in pig and cattle slurry stored at 5^{o}C than at 15^{o}C (182 days) (Jørgensen *et al.*

1977). It has been reported that the survival time of *M. bovis* in liquid manure stored at 5°C was up to 176 days (Dokoupil 1964) and survival time in soil was 21 months (Young *et al.* 2005). The survival time of *M. tuberculosis* in sterilized manure kept at room temperature was 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 less than 2 weeks in cattle slurry treated with 1.5% formalin (Genov 1965) and up to 4 weeks after treated with 2% calcium cyanamide (Ley and Böhm 1993). It can be concluded that treatments of manure reduce the survival time of mycobacteria.

The survival studies of pathogenic micro-organisms in human urine were previously carried out on viruses, enteric and gastrointestinal bacteria at different storage temperatures. During urine treatment for reuse in crop fertilization, it has been reported that high pH, high temperature and presence of ammonia may decrease the survival of pathogens in stored human urine (Höglund 2001, Udert *et al.* 2003). In pure human urine, it was revealed that enteric bacteria and coliphage MS2 survived less than one week in stored urine at 30^{o}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^{o}C or storage time of two weeks at temperature above 20^{o}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^{o}C and 30^{o}C to represent the two predominant 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 also examined.

2 REVIEW OF THE LITERATURE

2.1 Characteristics of the genus *Mycobacterium*

Mycobacterium is a genus of actinobacteria and it is the only genus present in the family *Mycobacteriaceae* classified within the order *Actinomycetales*. Mycobacteria are slightly curved or straight non motile rods, which are 0.2–0.6 µm wide by 1.0–10 µm long and sometimes branching. They do not contain endospores or capsules. Most of them are aerobic bacteria but some species may be microaerobics (Falkinham 1996). They are usually considered Gram-positive bacteria but they do not stain well in Gram-staining due to high lipids contents of their cell walls and waxy coat. They are structurally more closely related to Gram-positive bacteria. However, mycobacteria do not fit into the Gram-positive category as the molecules attached to the cell wall are distinctively lipids rather than proteins or polysaccharide (Barrera 2007). Mycobacteria are acid-alcohol fast, which means that they resist decolorization with mineral acids or with acidified alcohol after staining with basic dyes, such as fuchsin, acridine orange or auramine (Barksdale and Kim 1977, McMurray 1996). The cell walls of mycobacteria have high contents of lipids, waxes and characteristics mycolic acids with branched chain of 60 – 90 carbon atoms. The genus *Mycobacterium* has a high guanine + cytosine content (62-70 mol. %) in DNA. The DNA hybridization and 16S rRNA are important tools in determining phylogenic relations on both the generic and subgeneric levels (Hartmans *et al.* 2006). Genes other than 16S rRNA, such genes are *hsp65, rpoB, sodA, smpB, tuf*, tmRNA and recA have been analyzed using polymerase chain reaction (PCR) to estimate the phylogeny of mycobacteria (Mignard and Flandrois 2007, 2008). The closely relatives of mycobacteria belonging to other mycolic acid containing genera are *Corynebacterium, Nocardia, Gordonia, Tsukamurella, Dietzia* and *Rhodococococcus* (Stackebrandt *et al.* 1997, Barrera 2007).

Mycobacteria can be saprophytes, obligate parasites or facultative or opportunistic pathogens. The genus includes about 110 species (Hartmans *et al.* 2006). Mycobacteria are the causal agents of two important diseases, tuberculosis and leprosy in humans and other warm-blooded animals (Holt *et al*. 1994, Hartmans *et al* 2006). The clinical interest of mycobacteria started with the work of Koch (1882), who detected the tubercle bacillus in stained infected tissues.

Within the genus *Mycobacterium* a number of species are grouped into complexes (e.g., *M. avium* and *M. tuberculosis* complexes) that include bacterial species that have a high degree of genetic similarity as well as cause similar disease syndromes (Saviola and Bishai 2006). *Mycobacterium tuberculosis*-complex are formed by the species *M. tuberculosis, M. bovis, M. canetti, M. microti* and *M. africanum,* which are obligate pathogens and cannot grow freely outside the host (Soolingen *et al*. 1997). *Mycobacterium avium* complex (MAC) comprised of two main species *M. avium* and *M. intracellulare*. *M avium* is further subdivided into four subspecies (subsp.) *M. avium* subsp. *avium, M. avium* subsp. *homonissuis, M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* which are potential pathogens (Biet *et al*. 2005, Turenne *et al*. 2008, Lai *et al*. 2010, Shin *et al*. 2010).

Mycobacteria are divided into two major groups on the basis of their growth rate (Table 1). The rapidly growing species form visible colonies on selective media in less than 7 days while the slowly growing species need more than 7 days to form visible colonies. Some species exhibit an intermediate growth rate (Wayne and Kubica 1986). The slow-glowing mycobacteria are often pathogenic and clinically important while the rapid-growers are usually nonpathogenic and ecologically significant. Several studies were published which covered the phylogenetic structure of the rapid and slow-growing mycobacteria (Smida *et al.*, 1988, Stahl and Urbance 1990, Pitulle *et al*. 1992). Generally, most data supported the clustering of mycobacteria according to their growth behavior and indicated that the clinical slow growing strains evolved from their fast growing relatives (Pitulle *et al.*, 1992). Mycobacteria grow slowly with generation times from 2 hours for *M. smegmatis* to 12 days for *M. leprae* (Saviola and Bishai 2006) at optimum growth temperatures between 30^{o}C and 45^{o}C (Allen 1998, Metchock and Wallace 1999). The lipids structure of fast growers and slow growers are different in resistance to decolorization of mycobacteria stained with neutral red and treated with alkali (Wayne 1959). The colony morphology of different species varies from smooth to rough (Fregnan and Smith 1962, Soolingen *et al*. 1997). Many species form whitish or cream-colored colonies, but especially among the rapid growers, there are also many bright yellow or orange species containing carotenoid pigments (David 1984). In some cases, color pigmentation is formed in response to light (photochromogenic species), but most pigmented species also form these pigments in the dark and light (scotochromogenic species) and nonphotochromogenic species do not produce pigments (Timpe and Runyon 1954, Grange 2007). Nevertheless, some strains amongst the nonchromogenic are *M.*

nonchromogenicum (Collins *et al*. 1986, Grange 2007) and *M. avium* (Thorel *et al*. 1990) form pigment.

Table 1. *Mycobacterium species*

Slow grower		Rapid growers	
M. africanum [op]	*M. lentiflavum* [pp]	*M. abscessus* [pp]	*M. houstonense*
M. asiaticum [pp]	*M. lepraemurium* [op]	*M. agri* [np]	
	M. malmoense [pp]	*M. aichiense* [np]	*M. immunogenum*
M. avium [pp]		*M. alvei* [np]	*M. komossense* [np]
M. bohemicum [np]	*M. marinum* [pp]	*M. aurum* [np]	*M. mageritense* [np]
M. botniense	*M. microti* [op]		*M. methylovorum* [np]
M. bovis [op]	*M. montefiorense*	*M. bonickei*	*M. moriokaense* [np]
M. branderi [pp]	*M. nebraskense*	*M. brisbanense*	*M. mucogenicum* [pp]
M. buckleii [pp]	*M. nonchromogenicum* [np]	*M. brumae* [np]	*M. murale* [np]
M. caprae	*M. palustre*	*M. canariasense*	*M. neoaurum* [np]
M. celatum [pp]	*M. parascrofulaceum*	*M. chelonae* [pp]	*M. neworleansense*
M. chimaerae	*M. parmense*	*M. chitae* [np]	*M. novocastrense* [pp]
M. conspicuum [pp]	*M. pinnipedii*	*M. chubuense* [np]	*M. obuense* [np]
M. cookii [np]	*M. saskatchewanense*	*M. confluentis* [np]	*M. parafortuitum* [np]
M. doricum	*M. scrofulaceum* [pp]	*M. cosmeticum*	*M. peregrinum* [pp]
M.genavense [pp]	*M. shimoidei* [pp]	*M. diernhoferi* [np]	*M. pheli* [np]
M. gordonae	*M. shottsii*	*M. duvalii* [np]	*M. porcinum* [pp]
M. gastri [np]	*M. simiae* [pp]	*M. elephantis*	*M. poriferae* [np]
M. haemophilum [pp]	*M. szulgai* [pp]	*M. fallax* [np]	*M. psychrotolerans*
M. heckeshornense	*M. triplex* [pp]	*M. farcinogenes*	*M. pulveris* [np]
M. hiberniae [np]	*M. terrae* [np]	*M. flavescens* [np]	*M. rhodesiae* [np]
M. interjectum [pp]	*M. triviale* [np]	*M. fortuitum* [pp]	*M. senegalense* [pp]
M. intermedium [pp]	*M. tuberculosis* [op]	*M. gadium* [np]	*M. septicum*
M. intracellulare [pp]	*M. tusciae*	*M. gilvum* [np]	*M. shangaiense* [np]
M. kansasii [pp]	*M. ulcerans* [pp]	*M. goodii*	*M. smegmatis* [np]
M. kubicae	*M. xenopi* [pp]	*M. hassiacum* [np]	*M. sphagni* [np]
M. lacus		*M. hodleri* [np]	*M. tokaiense* [np]
		M. holsaticum	*M. vaccae* [np]
			M. vanbaalen

[op] obligatory pathogens [pp] potential pathogens, [np] nonpathogenic

Source: *http://www.dsmz.de/downloads/bacterial-nomenclature-up-to-date-downloads.html*.

Mycobacteria can utilize many carbon compounds for energy, glycerol being very usual carbon source (Ratledge 1982). *M. tuberculosis* is able to metabolize glycerol into pyruvate, whereas *M. bovis* grows much better in the presence of a pyruvate salt as a source of carbon (Barrera 2007). Some environmental mycobacteria are able to metabolize common substrates

such as sugars, alcohols, and organic acids, but also on a large variety of hydrocarbons including branched-chain, unsaturated, aromatic, and cyclic hydrocarbons (Lukins and Foster 1963). Mycobacteria also degrade polycyclic aromatic hydrocarbons, such as pyrene (Heitkamp *et al.* 1988, Dean-Rose and Cerniglia 1996, Willumsen *et al*. 2001, Cheung and Kinkle 2001), phenanthrene (Guerin and Jones 1988), fluoranthene (Rehmann *et al.* 2001) and branched alkane 2,6,10,15,19,23 hexamethyltetracosane (squalane) (Berekaa *et al.* 2000). Some mycobacteria also grow on simple one-carbon compounds methanol and methylamines (Kato *et al.* 1988, Urakami and Yano 1989). Mycobacteria can utilize also a wide variety of nitrogen sources including some amino acids and ammonium. In many common growth media of mycobacteria, asparagine is the nitrogen source (Jenkins *et al.*, 1982). Many species can also reduce nitrate and utilize it as the nitrogen source. Most mycobacterial species do not need special growth factors or vitamins but *M. haemophilum* requires haemin or iron-containing compounds such as ferric ammonium citrate (Wayne and Sramek 1992, Portaels *et al.* 1993). *M. paratuberculosis* requires iron-chelating substance, mycobatin (Thorel *et al*. 1990) and certainly, *M. leprae* which cannot be cultivated *in vitro* (Goodfellow and Wayne 1982).

2.2 Environmental reservoirs of mycobacteria

Mycobacteria can be found in diverse environments as obligate parasites, opportunistic or saprophytes as disease causing organisms. Most species are free living in fresh and salt water, soil and dust. Some of nonpathogenic saprophytes may also occur as opportunistic pathogens (Hartmans *et al*. 2006). The important ecological niche for others such as *M. leprae* and *M. tuberculosis* complex is diseased tissues of humans and warm-blooded animals (American Thoracic Society 1997). *M. bovis*, which causes tuberculosis in humans and cattle, has natural reservoir in ruminants and livestock (Saviola and Bishai 2006). Many species are normal inhabitants of a wide variety of environmental reservoirs, including natural and municipal water, soil, aerosols, protozoan, animals, and humans (Primm *et al.* 2004). Mycobacteria have been isolated from most *Sphagnum* environment throughout the world (Kazda *et al*. 1989, Schröder *et al*. 1992, Thorel *et al.* 2004). Runoff- waters from natural and drained peatlands are important reservoirs for environmental mycobacteria (Iivanainen *et al* 1999). Mycobacteria have been recovered from acidic coniferous forest soils in Finland (Iivanainen

et al. 1997). They have also been isolated form brook water and sediments (Iivanainen *et al.* 1993, 1999). In forest soil, mycobacteria have been found only in the humus layer (Thorel *et al*. 2004). Most species of mycobacteria, including *M. gordonae, M. avium, M. malmoense, M. simiae* and *M. marinum* are found in sphagnum vegetation (Schroder *et al.* 1992, von Reyn *et al*. 1993, Katila *et al.* 1995) as well as acidic, brown waters of the eastern United States (Kirschner *et al.* 1992). *Mycobacterium avium* complex (MAC) which consists of two distinct species *M. avium* and M. *intracellulare* are found everywhere in nature and they have been isolated from fresh water (ponds, lakes, rivers, bogs and swamp), brackish, sea water, wastewater plant, dust and other environmental sources (Falkinham 1996, Torkko *et al*. 2000, Falkinham 2002).

Mycobacteria were isolated from variety of materials such as soil, peat, humus, tufa, sphagnum, and wood, collected in alpine and subalpine habitats (Thorel *et al*. 2004). The most common mycobacteria in soil are *M. terrae, M. fortuitum, M. gordonae* and *M. nonchromogenicum* (Kazda 1983, Jenkins 1991, Katila *et al.* 1995 and Falkinham 1996). *M. fortuitum, M. abscessus* and *M. chelonae* are all water borne and occur in soil as well (Falkinham 1996). *M. flavescens*, *M. austroafricanum* and *M. chlorophenolicum* were found in petroleum-contaminated soil (Chueng and Kinkie 2001). Man–made environment such as water-damaged buildings and their indoor materials also harbor mycobacteria (Anderson *et al*. 1997, Huttunen *et al*. 2000, Jussila *et al.* 2004, Torvinen *et al.* 2006). *M. mucogenicum*, *M. kansasii, M. gordonae*, MAC, *M. fortuitum* and others also occur in public drinking and portable water sources, ice machines and water treatment plant (Covert *et al.* 1999), in chlorinated water such as public swimming pool (Havelaar *et al*. 1985, Leoni *et al* 1999) and whirlpools (Havelaar *et al*. 1985). *M. aurum* was isolated from soil and it is not associated with human diseases (Tsukamura & Tsukamura, 1966). *M. ulcerans* inhabits insects, wild animals and fish (Portael *et al.* 2001). Some environmental mycobacteria have been shown to grow within amoebae (Miltner and Bermudez 2000, Skriwan *et al.* 2002).

2.3 Pathogenicity

Most mycobacteria species have the ability to cause wide range of diseases in humans, livestocks and wildlife. *M. tuberculosis* is a human pathogen and *M. bovis* has a wide host range that includes humans, many wild and domesticated animals (O'Reilly and Darborn

1995). *M. tuberculosis* is transmitted by infectious aerosol from person to person and the mode of transmission of *M. bovis* is not fully understood (Reilly and Darborn 1995). *M. bovis* spreads among cattle by aerosol route and also identified as zoonotic disease transmitted from cattle to human by either a gastrointestinal or an aerosol route (John and Charles 2004, Saviola and Bishai 2006). The most human's tuberculosis cases and extrapulmonary infections are caused by *M. tuberculosis* (Golden and Vikram 2005, WHO 2011). In human, *M. bovis* may cause cervical lymphadenitis, intra-abdominal tuberculosis or pulmonary tuberculosis (Adler and Rose 1996). *M. avium* complex (MAC) is the second most common group causing pulmonary mycobacteriosis (Falkinham 1996, Molina-Gamboa *et al*. 1996, Embil *et al.* 1997, Mangione *et al* 2001, Koh *et al.* 2005, Field and Cowie 2006), cutaneous infection (Sugita *et al* 2000) and hypersensitivity pneumonitis in human (Rickman *et al.* 2002). MAC which includes species of *M. avium*, *M. paratuberculosis* and *M. intracellulare* infect intestinal tissues of humans (Bermudez and Sangari 2000, Sangari *et al*. 2000, 2001, Akgun *et al.* 2002). *M. paratuberculosis* causes Johne's disease in ruminants and it has been proposed to cause Johne's disease in humans (Harris and Lammerding 2001, Naser *et al*. 2004). *M. avium* and *M. intracellulare* cause pulmonary disease, regional lymphadenitis in humans and disseminated disease co-infected with human immunodeficiency virus (HIV) (Saviola and Bishai 2006). *M. africanum* causes disease similar to that of *M. bovis* and *M. tuberculosis* in patient resided in Africa (Saviola and Bishai 2006) and it can be spread by an aerosol route (Alder and Rose 1996).

In humans, *M. fortuitum* causes localized infection of the skin, soft tissues and bones (Saviola and Bishai 2006), pulmonary disease and respiratory tract colonization (Labombardi *et al.* 2002, Gebo *et al.* 2002). The case of nephritis in an immunocompetent woman with a clinical and radiological diagnosis of renal tuberculosis was due to *M. fortuitum* (Serra *et al.* 2007). *M. abscessus* causes sporotrichoid dermatosis (Lee *et al*. 2000). *M. ulcerans* causes ulcerative disease (Buruli ulcer) (Meyers *et al.* 1996, Coloma *et al.* 2005). *M. kansasii* causes diffuse cutaneous disease as well as cervical lymphadenitis in children (Saviola and Bishai 2006) and cellulitis (Hsu *et al* 2002). Water, especially in piped systems, is a source of *M. kansasii*, with aerosols being involved in transmission (Martinkova *et al.* 2001). *M. marinum* causes cutaneous disease and ulcerated nodule in human (Dorronsoro *et al.* 1997, Saviola and Bishai 2006) and water is also an infection route for *M. marinum* through skin abrasions (Adams *et al*. 1970, Engbaek *et al.* 1980). *M. scrofulaceum* is a leading cause of scrofula or

lymphadenitis in children (Wolinsky 1979, Falkinham 1996, Saviola and Bishai 2006). *M. immunogenum* causes hypersensitivity pneumonitis (Shelton *et. al* 1999). *M. leprae* is a major cause of leprosy in human and this disease is probably transmitted from person to person (Saviola and Bishai 2006). *M. genavense* infection in HIV patients often result in abdominal wall thickening, lymphadenopathy and ulceration (Monill *et al*. 2001). The majority of human infected with *M. avium* subsp. *hominissuis* occur in HIV- immunocompromised people and immunocompetent persons with underlying pulmonary disease (Field and Cowie 2006, Alvarez *et al*. 2008). Normal person with strong immunostatus is at low risk of environmental mycobacterial infections and AIDS and other risk groups with weak cell- mediated immunity are at higher risk of infection (Saviola and Bishai 2006). The strain of the mycobacteria, dose of the organism, route of inoculation and the prevailing conditions for growth of the organism may influence the time required to produce disease (Thoen and Barletta 2004).

2.4 Detection, isolation and identification of mycobacteria from different samples

2.4.1 Cultivation

Traditionally, quantification of mycobacteria is done by seeding serial dilutions of bacterial suspensions on suitable media such as Middlebrook 7H10 agar or Lowenstein Jensen followed by counting colony forming units (cfu) (Pathak 2012). However cultivation takes time due to long incubation time for slow growing mycobacteria and this can result in contamination by overgrown colonies of other microorganisms with rapid growth. The growth of rapidly growing microbes can be inhibited by a chemical decontamination to which mycobacteria are more resistant than other microbes (Iivanainen 1999). Owing to this advantage, the resistance of mycobacteria to adverse conditions (acid, base or detergents), decontamination treatments and the use of selective growth media has been developed to increase the efficiency of isolation procedures (Kubica and Good 1981).

2.4.2 Extraction and concentration from source samples

Before decontamination procedures are carried out, the extraction and concentration of mycobacteria must first be prepared. Iivanainen (1999) reported that mechanical shaking in sterile distilled water, saline, nutrient broth, Tween 80 or decontamination agent may be used to extract mycobacteria from solid samples. Concentration of liquid samples is achieved either by centrifugation or filtration before the concentrated sediment can be decontaminated, for urine samples (Hillemann *et al.* 2006, Serra *et al*. 2007, Chan *et al.* 2008, and Vinnerås *et al.* 2011), for blood samples (Naser *et al.* 2004), for sputum (Fukushima *et al.* 2006, Tajedin *et al.* 2011), brook water (Iivanainen *et al.* 1993) and for faeces (Douarre *et al.* 2010). The illustration of conventional (culture) and molecular technique (PCR and DNA microarray) steps involved in the isolation and detection of mycobacteria from urine samples in the laboratory is shown in Figure 1.

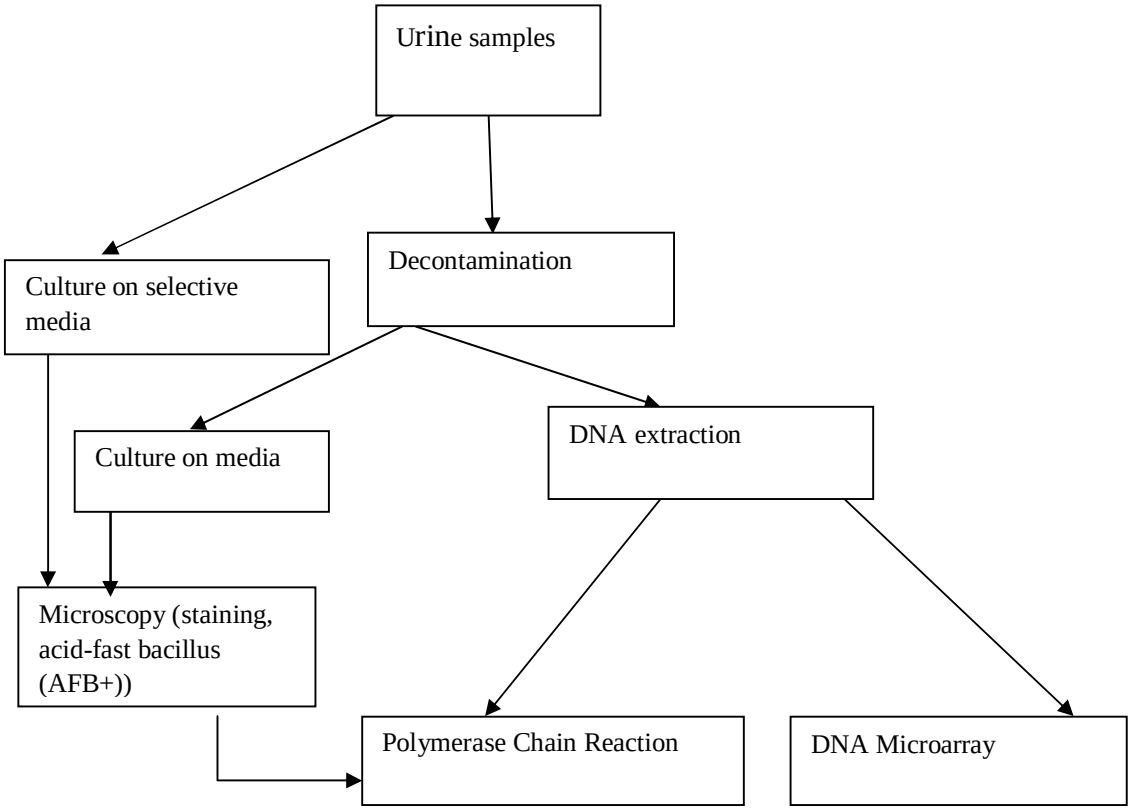

Figure 1. The schematic illustration involving the sequential steps of the isolation and detection of mycobacteria from urine samples

2.4.3 Decontamination

Table 2. Decontamination procedures and growth media used for isolation of different mycobacterial strain from clinical and environmental sources

Sample	Decontamination	Growth medium	Reference
Urine	N-acetyl-L-cysteine + 3%NaOH, neutralized with PO_4^{2-} buffered saline	IUTM, blood agar, chocolate agar, MacConkey agar, brain heart infusion agar, mannitol-salt agar.	Serra *et al*. (2007)
Urine	Sodium dodecyl sulphate & NaOH for 30 min, neutralized with 0.09% H_2SO_4	LJ	Vinnerås *et al.* (2011)
Urine sputum	N-acetyl-L-cysteine + NaOH	LJ	Sener *et al.* (2008), Folugueira *et al.* (1993)
Urine	N-acetyl-L-cysteine + NaOH, 0.1ml Phosphate buffer	LJ, colestos, Middlebrook 7H10 agar.	Moussa *et al.* (2000)
Urine	N-acetyl-L-cysteine + 1-3%NaOH, 1- 1.5ml Phosphate buffer	MGIT 960 tube, BACTEC 12B, LJ	Chan *et al.* (2008), Hillemann *et al.* (2006)
Sputum	NALC-NaOH (2% NaOH, 1.45% sodium citrate, 0.5% NALC) 20 min, phosphate buffer saline pH 6.8 25 min	LJ	Kolk *et al.* (1992), Fukushima *et al.* (2006)
Blood	1 mL of sterile phosphate buffer saline pH 6.8	MGIT, BACTEC 12B	Naser *et al.* (2004)
Bovine feces	0.9% hexadecylpyridinium chloride for 16-24hrs at room temperature	HEYM agar	Douarre *et al.* (2010), Stabel (1997)
Milk	20 ml of 0.75% hexadecylpyridinium chloride for 24-48h at room temp	HEYM	Gao *et al.* (2005)

Sputum	4% NaOH and 3% HCl	LJ	Tajedin *et al.* (2011)
Soil	0.9% hexadecylpyridinium chloride	TREK ESP+ antibiotics	Raizman *et al.* (2011)
Brook water	1 M NaOH 20 mins, 5% of oxalic acid 20 mins+2M H_2SO_4	Egg media	Iivanainen *et al.* (1993)
Food stuff	1.0 N NaOH 10 min, 10 M HCl, 2ml phosphate buffered saline	M7H11, BACTEC 12B	Palmer & Whipple (2006)
Soil, feces, waste and surface water, animal feed, moss	4% NaOH 25- 30 min + 5% oxalic acid 30 min	M7H9	Beewerth & Schürmann (1969)
Soil, surface ground water and tap water	2-3 % Sodium laurylsulphate + approx. 1% NaOH 25min	LJ, LJ+ antibiotics+antifungal, Herrold medium+mycobactin, TTC	Kleeberg & Nel (1973), Kamala *et al.* (1994)
Soil	100ml of 0.9% NaCl 30 min, 3 ml of 1% KOH	Ogawa egg medium	Tsukamura *et al.* (1986)
Soil	5 ml of 0.2% of malachite green, 1 ml of cycloheximide (500µg/ml) neutralize with 1M HCl	Ogawa egg medium	Portaels *et al.* (1988)

HEYM agar = Herrold's Egg Yolk Medium, IUTM = International Union tuberculosis Medium, LJ = Löwenstein-Jensen, M7H9 = Middlebrook 7H9, M7H10 = Middlebrook 7H10, M7H11= Middlebrook 7H11, MGIT 960 = Mycobacteria Growth Indicator Tube 960 system, TTC = Tsukamura minimal medium Tween 80, TREK ESP = (TREK Diagnostics System, Inc Weatlake, OH 44145)

2.4.4 The further analyses

In clinical work each newly isolated human mycobacterial strain is tested for antibiotic resistance in order to find medical treatment for the patient. *M. tuberculosis* resistance to anti-tuberculosis drugs in the Archangels oblast, and the risk factors were revealed for the

development of drug-resistant tuberculosis (Toungoussova *et al.* 2002). The emergence of drug resistance mycobacteria are well known after the first clinical trials (McDermott 1960). The molecular basis for the mechanism of action of antituberculosis agents and the way in which the organisms become resistant have begun to be unraveled (Stephen 2002). The molecular techniques are used to genotype the newly isolated mycobacterial strains in order to detect if the different positive findings among patients join to the same or to different group. This can help to find the spreading routes between humans and the other sources. The molecular mechanisms whereby resistance emerges are useful in controlling the threat of an epidemic of multiple-drug resistance mycobacteria (Stephen 2002).

2.5 Factors affecting the occurrence of mycobacteria in the environment

2.5.1 pH

Mycobacteria can grow over a wide range of pH values. They are likely to encounter both acidic and mildly acidic pH in an environment they inhabit (Oh and Straubinger 1996, Iivanainen *et al.* 1999, Rao *et al* 2001). The growth optimal for almost all mycobacterial species is achieved in acidic pH and there is little growth at alkaline pH values above 7.5 (Falkinham 2002). Slow growing mycobacteria, with exception of *M. lepraemurium,* develop optimally between pH of 5.8 and 6.5 and all strains of rapidly growing mycobacteria with exception of *M. chelonei* develop between optimal pH range of 7.0 and 7.4 (Portaels and Pattyns 1982). Saprophytic mycobacteria grow well through the wide range of pH, whereas the pathogenic mycobacteria develop within a very narrow optimal pH range (Chapman and Bernard 1962, Portaels and Pattyns 1982). The wide pH range at which several saprophytic mycobacteria multiply may be an important factor responsible for the widespread occurrence in an environment (Portaels and Pattyns 1982). Saprophytic mycobacteria are found in acidic conditions (pH 3.5 – 4.3) often prevailing in soil and aquatic habitats of coniferous forest belt of Nordic environment (Iivanainen *et al.* 1999). Growth and tolerance to low pH gives an explanation for the high numbers of mycobacteria in the Nordic environment (Iivanainen 1999, WHO 2004). Many species can develop well on a medium at a pH below 5.0, for example *M. smegmatis* grow well at pH 3.5 (Chapman and Bernard 1962, Portaels and

Pattyns 1982). The mycobacterial multiplication and metabolism was decrease due to elevated pH (Chapman and Bernard 1962). Some mycobacterial strains exhibit full growth from pH 5.0 through pH 7.3, and the light growth continues to as high as pH 8.4 (Chapman and Bernard 1962). *M. avium* and *M. intracellulare* have acidic optimal for growth and their optimal pH range was 5.0 – 6.0 (Portaels and Pattyns 1982, George and Falkinham 1986). *M. smegmatis* and *M. bovis* were able to grow at pH values of 4.0 and 4.5 respectively, maintaining their internal pH between pH 6.1 and 7.2 when exposed to decreasing external pH (Rao *et al*. 2001). *M. smegmatis* and *M. bovis* exhibit intracellular pH homeostasis which can be crucial for the survival of these organisms at acidic pH (Rao *et al.* 2001). *M. tuberculosis* H37Rv had a narrow pH range for maximal growth between pH 6.2 and 7.3 with mark attenuation observed at pH 5.0 and 8.4 (Chapman and Bernard 1962). *M. tuberculosis* had been shown to live in the phagocytic vacuole of host macrophages where the intraphagosomal pH is mildly acidic (pH 6.1- 6.5) (Strurgill-Koszycki *et al.* 1994, Oh and Straubinger, 1996). *M. tuberculosis* complex organisms multiply with narrow temperature and pH range (Barrera *et al*. 2007). *M. avium, M. fortuitum, M. chelonae, M. marinum*, and *M. scrofulaceum*, grow at pH 6.0 in an unrestricted manner indicating that *M. tuberculosis* is unique among the mycobacteria in its extreme sensitivity to acid (Piddington *et al.* 2000). The sensitivity of *M. tuberculosis* at low pH may contribute to the control of infection in humans (Gomes *et al.* 1999). Alkaline pH is an important factor in reducing the survival of *M. paratuberculosis* in the soil and could be effective for disease control procedures (Schroen 2000). It has been reported universally that viability of micro-organisms is adversely affected by high pH values (Strauch 1981). Low pH and microaerobic conditions promote the growth of M. *genavense* (Realini *et al* 1997, 1998) and low pH and pyruvate support the growth of *M. malmoense* (Katila *et al*. 1989)

2.5.2 Temperature

The influence of temperature on mycobacteria varies according to species (Iivanainen 1999). *M. tuberculosis* complex organisms are mesophile and neutrophile its multiplication are restricted to conditions offered by warm-blooded animals and a neutral pH (Barrera *at al.* 2007). In drinking (or pipeline) water systems *M. chelonae, M. flavescens, M. fortuitum, M. gordonae, M. terrae* and *M. kansasii* have highest densities on plastic surfaces which are continuously perfused with water at temperature between 22 and 30^{o}C (Schulze-Robbeeke *et*

al. 1992). Several saprophytic mycobacteria can multiply at a temperature as low as 10 ^{o}C (Vuorio *et al.* 1999). *M. avium* and *M. scrofulaceum* can grow slowly at temperature as low as 10 ^{o}C (George *et al.* 1980). The growth of *M. flavescens* has been observed at 4 ^{o}C (Ermolenko *et al.* 1997). The resistance of mycobacteria to freezing and heat affects their survival in a particular environmental reservoir (WHO 2004). Certain thermophilic species such as *M. chelonae, M. xenopi* and *M. avium* can survive temperature above 55 ^{o}C whereas under the same conditions *M. kansasii* and *M. marinum* are quickly destroyed (Merkal and Crawford 1979, Schulze-Röbbecke and Buchholtz 1992). In water distribution system, potentially pathogenic MAC and *M. xenopi* are frequently recovered more often from hot than cold water (du Moulin *et al*. 1988, von Reyn *et al.* 1993, 1994). MAC can tolerate 60 ^{o}C (Merkal and Crawford 1979) and *M. xenopi* can tolerate even higher temperatures (Schulze-Röbbecke and Buchholtz 1992). *M avium* has been recovered from hospital hot water sites with water temperature between 52 and 57 ^{o}C (du Moulin *et al.* 1988, Schulze-Röbbecke and Buchholtz 1992, Mijs *et al.* 2002). *M. intracellulare, M. marinum* and *M. fortuitum* are equally susceptible in temperature between 55 and 60 ^{o}C (Schulze-Röbbecke and Buchholtz 1992). In the laboratory experiments with natural water filtrates, the minimum temperatures for proliferation of different serotypes of *M. intracellulare* have been varied from 17.8 to 25 ^{o}C (George *et al.* 1980). Many mycobacteria can survive freezing for prolong periods with actual proliferation after freezing e.g. -75^{o}C in nutrient broth presumably due to disaggregation of the bacterial clumps (Iivanainen 1995). *M. paratuberculosis* survived in naturally infected cows feces under long period of freezing at -18 ^{o}C and -70 ^{o}C (Raizman *et al.* 2011).

Temperature significantly affects mycobacteria in the soil (Kubalek and Komenda 1995, Donoghue *et al.* 1997). In Britain, the mean temperature for the occurrence of mycobacteria species in forest soil in different niches was 16^{o}C, in arable soil was 21.4 ^{o}C (Pavlik *et al*. 2009) and in pasture soil was 19.5^{o}C and range between 14 and 25 ^{o}C (Donoghue *et al*. 1997). One reason for the difficulty in isolation of mycobacteria from environmental samples may be that the sudden change from low temperature to 37 ^{o}C, which may either retard colony formation or kill cells because the membrane becomes too fluid (Suutari and Laakso 1993). Temperatures below 37 ^{o}C are optimal for the growth of *M. marinum* (30 ^{o}C) (Clark and Shepard 1963, Petrini 2006) and *M. haemophilum* (32 ^{o}C) (Dawson and Jennis 1980) and this is constant with their clinical appearance as skin infections in most patients. Majority of

mycobacterium species are able to grow on simple substrate with optimal growth temperatures between 30 ^{o}C and 45 ^{o}C (Metchock *et al.* 1999, Covert *et al*. 2001).

2.5.3 Moisture

Many studies have indicated that moisture (water) is an important factor influencing the survival of mycobacteria and their growth in an environment. *M. bovis* BCG survival was optimal at 37°C with moist soil (Young *et al*. 2005). The growth of mycobacteria in aerobic Sphagnum vegetation is due to the high moisture content of the sphagnum layer (Kadza 1983). The highest amounts of mycobacteria have been detected in wet soils (Beerwerth and Schürmann 1969). Most mycobacteria were recovered from pastures for cattle along streams and watering places for cattle in summer (Donoghue *et al.* 1997). Mycobacteria have been common in moisture damage building materials (Anderson *et al.* 1997, Torvinen *et al.* 2006). Several investigations revealed that lack of moisture content (dryness) inhibits the survival or growth of mycobacteria in the environment. The recovery of mycobacteria from Iran soil was very much lower due to lack of soil moisture (Ghaemi *et al.* 2006). Reduced recovery of mycobacteria has been found in topsoil in South Africa (Kleeberg and Nel 1973) and lower recovery from soil in India during June than January (Kamala *et al*. 1994).

2.6 The survival time of mycobacteria in different environmental reservoirs

The survival time of mycobacteria depends highly on the environmental conditions. *M. paratuberculosis* has been shown to have longer survival time at 5 ^{o}C (252 days) than at 15 ^{o}C (182 days) in culturable pig and cattle slurry (Jøgensen 1977). In bovine feces kept outdoors at room temperature, the survival time of *M. paratuberculosis* was between 152 - 246 days (Lovell *et al*. 1944) and in dairy cow feces stored at -18^{o}C average survival time of 540 days (Raizman *et al*. 2011). The survival time of *M. paratuberculosis* in tap or pond water in sealed bottle or water trough was between 6 to 18 months (Lovell *et al*. 1944, Larsen *et al* 1956, Whittington *et al.* 2005, Cook *et al.* 2010), for up to 48 weeks in sediment (Whittington *et al.* 2005) and about 15 months in distilled water (Collins *et al*. 2001). A diversity of mycobacteria including strains of *M. avium*, *M. marinum*, *M. xenopi*, *M. fortuitum* and *M. kansasii* have been shown to survive in ocean water for more than three months and some

other strains survive up to 12 months (Viallier et al. 1977). *M. avium* survived in tap water at 4 ^{o}C and 20 ^{o}C beyond 485 days (von Reyn *et al.* 1994).

Factors that may shorten estimated survival time of *M. paratuberculosis* in soil are dryness, pH above 7.0, exposure to sunlight and low iron content (Johnson-Ifearulundu and Kaneene, 1997, 1998, Whittington *et al*. 2004). The survival of *M. paratuberculosis* was shorter in dry alkaline soils and with no apparent effect of UV light (Collins 2003). *M. avium s*ubsp. *paratuberculosis* survived up to 55 weeks in dry fully shaded environment, until 24 weeks in grass that germinated through infected materials and up to 9 weeks on grass in 70% shade (Whittington *et al*. 2004). It has been revealed that *M. tuberculosis* survived up to 172 days when inoculated into sterilized manure at room temperature with a decimal reduction time of 27 days (Scanlon and Quinn 2000) and the mean survival time in external environment was eight months (Walther and Ewald 2004). It has been reported that, *M. bovis* survived for 176 days in liquid manure stored at 5 ^{o}C (Dokoupil 1964). The survival time *M. bovis* in vegetables stored at -20 ^{o}C and 23 ^{o}C was up to 112 days. (Palmer and Whipple 2006). The survival times of 7-28 days of *M. bovis* inside the den of brushtail possums was reported in New Zealand (Jackson *et al.* 1995) but *M. bovis* survive for < 4 days in cotton strips (Jackson *et al.* 1995). Persistence of *M. bovis* in the environment was significantly shorter in the spring/summer season, characterized by the highest average daily temperatures over the 12 month period and persisted up to 88 days in soil, 58 days in water and hay, and 43 days on corn (Fine *et al.* 2011). *M. bovis cells* survived of up to 6 weeks in inoculated soil and faeces, detected by traditional selective cultivation methods (Tanner and Michel 1999) and survived up to 21 months in wet soil (Young *et al.* 2005). The survival times of *M. bovis* and *M. tuberculosis* in human urine were over 10 days at 4 ^{o}C and below three days at 22 ^{o}C (Vinnerås *et al.* 2011).

In previous studies, chemicals have been used for the treatment of sewage sludge and animal waste contaminated with pathogenic mycobacteria. The disinfection of liquid manure contaminated with mycobacteria with a solution of ammonia had been described (Scanlon and Quinn 2000). *M. bovis* survived less than two days in cattle slurry treated with 1% of ammonium hydroxide (Scanlon and Quinn 2000). *M. paratuberculosis* was inactivated in cattle slurry in two weeks after treatment with 1.5% formalin (Genov1965). *M.*

paratuberculosis was inactivated in cattle slurry four weeks after treatment with 2% calcium cyanamide (Ley and Böhm 1993).

2.7 Composition of human urine

The kidneys are the main excretory organ in human. Each person excretes 1-1.5 litres of urine per day in 4-5 times and an adult person produces an average of 500 litres per year (Wolgast 1993). Many investigators have studied the composition of human urine and about 158 different chemical constituents are summarized from urine in the NASA Bioastronautics Data Book (Putnam 1971). Urine is a complex fluid that contains various amounts of electrolytes, urea, and other metabolic products (Chambers and Kunin 1985). Basically, human urine consists of 95% of water with organic compounds which include urea, uric acid, creatinine, carbohydrates, hormones, fatty acids, pigments and enzymes (Putnam 1971). The electrolytes contain cations K^+, Na^+, Ca^{2+}, NH_4^+, and anions, Cl^-, HCO_3^-, PO_4^{3-}, SO_4^{2-} (Putnam 1971, Kirchmann and Pettersson 1995). Human urine consists of high concentrations of sodium chloride, urea, potassium and phosphate and trace levels of sulphate, magnesium and calcium (Larsen and Gujer 1996). With urine we excrete some 88% of our daily excreted nitrogen, 67% of phosphorous and 73% of potassium (Ciba-Geigy 1977). The chemical composition of human urine varies from person to person and from region to region depending on the time of the day, diet, climate, physical activity, the volume of drinking water consumed and body size (Sullivian and Grantham 1982). The color of the human urine varies from colorless to amber but is normally pale yellow. The strong odor of human urine is due to high amount of ammonia present or the body's breakdown of asparagusic acid (Lison *et al.* 1980). During excretion the pH of human urine is typically close to neutral pH 7 (Lind *et al* 2000, Ban and Dave 2004), however, it may vary between 4.5 and 8.2 (Lentner *et al.* 1981). The low pH of urine is usually caused by unbuffered organic acids and high pH is caused by unbuffered ammonium (Putnam 1971). The content of heavy metals in human urine is low (Anderson 1977, Jönnson *et al*. 1997, Vinnerås 2001) and ranges from 0.2-30 µg/L (Kirchmann & Pettersson 1995, Heitland & Köster 2004, Chen *et al.* 2010). Most composition of human urine mentioned in different journal articles is depicted in Table 3.

Table 3. The values of different substances composition present in human urine cited in different published article

Variables	Description	References
Physical characteristics		
pH		
Fresh urine	6.32- 6.90	Ban and Dave (2004)
	5.60- 6.80	Fittschen and Hahn (1998)
Stored urine	9.00 -9.10	Landry and Bazari (2011)
	9.20	Vinnerås *et al*. (2008)
		Pradhan *et al.* (2009)
Density	1003-1035 g/L	Kirchmann & Pettersson (1995)
	1002-1030 g/L	Landry and Bazari (2011)
Conductivity	14.8-25.4 $mScm^{-1}$	Jönsson *et al*. (2000)
	13.4 – 19.0 $mScm^{-2}$	Kirchmann & Pettersson (1995)
Chemical characteristics		
Organic compounds		
Urea	9.3 g/L	Putnam (1971)
Amino acid- N	0.104-0.110 g/L	Kirchmann & Pettersson (1995)
	85% total nitrogen	Kirchmann & Pettersson (1995)
Total organic carbon	3.64-6.74 g/L	Putnam (1971)
Creatinine	0.670 g/L	Putnam (1971)
Inorganic compounds		
Nitrogen (N)	7- 9 g/L	Guyton (1986)
	1.795- 2.610 g/L	Kirchmann & Pettersson (1995)
	8.57 g/L	Pradhan *et al.* (2009)
	2.4- 3.1 g/L	Heinonen-Tanski *et al*. (2007)
Chlorine (Cl)	1.87g/L	Putnam (1971)
	2,24-2.0 g/L	Kirchmann & Pettersson (1995)
	3.30g/L	Pradhan *et al.* (2009)
Sodium (Na)	1.17g/L	Putnam (1971)
	0.94- 0.98 g/L	Kirchmann & Pettersson (1995)
	0.32 mg/L	Yoshinaga *et al*. (2000)
Calcium (Ca)	13-16 mg/L	Kirchmann & Pettersson (1995)
Magnesium (Mg)	1.5- 1.6 mg/L	Kirchmann & Pettersson (1995)

Sulphur (S)	0.17-0.22 g/L 0.817 mg/L	Kirchmann & Pettersson (1995) Yoshinaga *et al*. (2000)
Phosphorus (P)	0.20- 0.21 g/L 0.20- 3.7 g/L 0.15- 0.23 g/L 0.7 g/L	Kirchmann & Pettersson (1995) Meinzinger &Oldenbrug (2008) Pradhan *et al.* (2009) Heinonen-Tanski *et al*. (2007)
Iodine (I)	105.9- 522.0 µg/L	Zhang *et al.* (2010)
Potassium (K)	0.75g/L 0.875- 1.150 g/L 0.59- 1.3 g/L 0.7- 3.3 g/L 2 g/L	Putnam (1971) Kirchmann & Pettersson (1995) Heinonen-Tanski *et al*. (2007) Meinzinger & Oldenbrug (2008) Pradhan *et al.* (2009)
Ammonium-nitrogen (NH_4^+-N)	1.117- 2.610 g/L 2.3-2.9 g/L 8.57 g/L	Kirchmann & Pettersson (1995) Heinonen-Tanski *et al*. (2007) Pradhan *et al.* (2009)
Ammonia-nitrogen (NH_3-N)	45 µg/L 0.01 g/L	Kirchmann & Pettersson (1995) Pradhan *et al.* (2009)
Phosphate-phosphorus (PO_4^{3-}-P)	161- 171 mg/L	Zhigang *et al.* (2008)
Plant Micronutrients		
Boron (B)	3.16–10.76 µg/L	Meacham *et al.* (2010)
Copper (Cu)	155 µg/L 67 µg/L	Kirchmann & Pettersson (1995) Jönsson *et al.* (2004)
Iron (Fe)	0.165- 0.205 mg/L	Kirchmann & Pettersson (1995)
Manganese (Mn)	1.05 mg/kg	Heitland & Köster (2004)
Molybednum (Mo)	0.062 µg/L	Heitland & Köster (2004
Zinc (Zn)	30 µg/L 19 -665 µg/L	Jönsson *et al*. (2004) Heitland & Köster (2004

2.8 Human urine and micro-pollutants

Micro-pollutants such as hormones and pharmaceutical residues may be excreted in urine and this can induce some limitations for the reuse of human urine as organic fertilizer. Different aspects associated with use of human urine in agricultural application, such as agronomic and

hygienic value have been extensively studied by several investigators, but little research has been done on the ecotoxicological risk of urine-fertilized crops. Around 70% of the pharmaceuticals taken in can be excreted via urine and estimated to be for 50% of the overall ecotoxicological risk (Lienert *et al.* 2007). Furthermore, urine contains micro-pollutants such as hormones, since these substances are mainly excreted via urine (Alder *et al.* 2006, Winker *et al*. 2008) and may be harmful to the ecosystems and human health. Most antibiotics used for the treatment of infection in humans and animals can be excreted via urine as parent compound (Phillips *et al*. 2004, Kumar *et al.* 2005). Nevertheless, the usage of urine is associated with a risk of transfer of pharmaceutical residues to agricultural fields (Lienert *et al.* 2007, Winker *et al.* 2008). Even low concentrations of pharmaceuticals and hormones have been shown to have the risk of negative effects to plants or human beings (Winker 2010).

Many pharmaceutical substances that are polar and hardly biodegradable can be taken up by plants and therefore possibly enter the human food chain (Winker 2010). It was reported that as much as 10% of administered Tricor (Fenofibrate) was excreted via urine without metabolism (Goodman *et al.* 2006). Pharmaceuticals can affect plant growth when in fairly high concentration in urine (Kurma *et al.* 2005, Winker *et al.* 2008, Winker 2010) and they are absorbed by plants (Boxall *et al.* 2006, Dolliver *et al.* 2007, and Winker 2010). Arsunate (anti-malaria drugs) is a natural compound, known for its phytotoxic properties, and it inhibits seed germination (Hoagland & Cutler, 2000). High concentrations of chloroquine are toxic to soybean and reduce the protozoa population of soil microbiota (Jjemba 2002) and likely to be absorbed by soil (Gemer and Sinar 2010). The individual and combined behavior of carbamazepine (CZ) and ibuprofen (IBU) was investigated by GC/MS analysis in a greenhouse experiment using ryegrass fertilized with pharmaceutical-spiked urine (Winker *et al*. 2010). The study showed that the recalcitrant CZ transported into roots and aerial plant parts of the ryegrass but ibuprofen was biologically degraded by soil bacteria and was not found in ryegrass (Winker *et al*. 2010). On the contray, Gajurel (2007) did not found any degradation of IBU, CZ and diclofenac in spike urine during one year storage period. Corn salad and winter wheat absorbed chlortetracycline from soil through their root system (Grote *et al.* 2006).

The fate and the impact of any pharmaceuticals present in urine regarding their accumulation in soil, transfer to underground water, and uptake by plants is an important focus of interest. Urine usually ends up in domestic wastewater and a lot of these pollutants are not removed in sewage treatment plants and are thus discharged into surface water bodies and can even reach the groundwater (Winker 2010). For example, Paracetamol (analgesic) has been detected in waste water (Tidåker 2003), but it has low leaching tendency (Kreuzig *et al.* 2003) and because of it fast biodegradation it has not been found in detectable concentrations in the environment (Kümmerer 2004, Thomas *et al.* 2007). Biodegradation is an important environmental process influenced by photolysis (solar radiation), pH, temperature, soil type and microbial activity (Gavalchin and Katz 1994). Majority of all pharmaceutical substances are derived from nature and many synthetically produced ones are found and degraded in natural environments with a diverse microbial activity (Jönsson *et al.* 2004). The biodegradation of some pharmaceuticals present in urine remains, anyhow, uncertain (Winker 2010).

Some antibiotics can be persistent in the environment (Thiele-Bruhn 2003, Estévez *et al.* 2005, Picó and Andreu 2007) and are strongly absorbed by soil particles (Picó and Andreu 2007, Kümmerer 2003). However, many antibiotics are photodegradable (Alexy 2004) and biological degradable under aerobic conditions (Ingerslev *et al.* 2001). Hormones originated from contraceptives are photodegradable (Coleman *et al.* 2004, Zuo *et al.* 2006). Diclofenac is efficiently degraded in urine stored after 6 months at different pH level (Butzen *et al*. 2005) and the potential environmental threat by diclofenac pollution from urine fertilization is considered to be low (Starkl *et al.* 2005). On the contrary, macrolides such as roxithromomycin are stable in organisms (Sun *et al.* 2005) and non-biodegradable in manure (Schlüsener and Bester 2006). Tetracycline concentrations do not decrease significantly during long storage times in liquid animal manure (Höper *et al.* 2002). Saniresch (2010) concluded that pharmaceutical residues are found in human urine after storage and this must be considered when reused in agriculture.

2.9 Human urine as plant fertilizer

Several investigators have demonstrated the use of human urine as organic fertilizer for growing vegetable crops. Human urine is a source of plant mineral nutrients analogy to that of synthetic chemical fertilizers. Human urine contains most excreted nitrogen (N) and also some phosphorus (P) and potassium (K) (Schouw *et al.* 2002) and its nutrient proportion as NPK ratios is 18:2:5 (Lindén 1997, Simons and Clemens 2003, Tidåker 2003). The nitrogen content in stored human urine exists in form of ammoniacal N with bicarbonate as the main anion (Kirchmann and Pettersson 1995). The rest of the nitrogen compounds are mainly creatinine, uric acids and amino acid (Ciba-Geigy 1977). The daily quantity of urea excreted by an adult varies between 11.8 and 23.8 g per day and the ratio urea-N: total–N is about 0.8 (Fittschen and Hahn 1998). During storage, urea undergoes chemical hydrolysis and biological degradation catalyzed by an enzymes urease, which is possessed by many microorganisms (Alexander 1977). During the hydrolysis ammonium and bicarbonate are produced and pH is increased (Hanæu *et al*. 1996, Udert *et al* 2003);

$$CO(NH_2)_2 + 3H_2O \longrightarrow 2NH_4^+ + HCO_3^- + OH^- \qquad (1)$$

Ammonium is in equilibrium with dissolved ammonia:

$$NH_4^+ + OH \longleftrightarrow NH_3(aq) + H_2O. \qquad (2)$$

The pKa value for the equilibrium is 9.3 at 25^oC.

Dissolved ammonia is in equilibrium with the gaseous ammonia:

$$NH_3(aq) \longleftrightarrow NH_3(g) \qquad (3)$$

The breakdown of urea will lead to an increase in concentration of ammonium- ammonia and there is an increase in pH of urine from around 6.0 to 9.5 and thus there will be losses of nitrogen through ammonia evaporation when contact with air (Hellström *et al.* 1999, Hotta *et al.* 2008). The formed ammonia has negative effects on human health and environment (Galloway and Cowling 2002) and toxicity to plants can occur during urine management and

application as fertilizer (Blouin 1979). The immediate introduction of urine to topsoil can reduce the loss of ammonia as soon as possible after the application (Rodhe *et al.* 2004). The loss of nitrogen as ammonia during urine storage can be reduced by low temperature and avoiding aeration above the liquid surface in storage tanks (Hellström *et al.* 1999, Karak and Bhattacharyya 2011). Moreover, high temperature, high pH, high N-NH_3 concentrated form of urine and long storage periods are favorable for hygienic purpose of urine (Höglund *et al*. 1998).

The prevention of ammonia losses during storage and after soil application is important aspect for an efficient use of human urine (Kirchmann and Pettersson 1995, Rodhe *et al.* 2004). Urine treatment might be needed to produce adequate fertilizer and might also be a suitable method to prevent environmental pollution with micro-pollutants (Udert *et al*. 2006). The recommendation storage periods for human urine before it application in agricultural field was to stored urine $\geq$ 6 months at temperature of 20^{o}C or higher is safe use with respect to pathogens (WHO 2006). Most of the nitrogen fractions in urine absorbed by plant are similar to that of urea or ammonium fertilizer (Jönsson *et al.* 2004). Urea and ammonium are the most widely used industrial N fertilizers in the world (Overdahl *et at.* 1991). In fertilizing experiments, about 90-100% of urine N is found as urea and ammonium (Kirchman and Pettersson 1995, Richert Stintzing *et al.* 2001). Ammonia can easily be recovered from the liquid phase of urine through air stripping (Basakcilardan-Kabakci *et al.* 2007) and subsequently be absorbed in sulphuric acid to form ammonium sulphate, a liquid fertilizer (Karak and Bhattacharyya 2011);

$$H_2SO_4 + 2NH_3 \longrightarrow (NH4)_2SO_4 \qquad (4)$$

Phosphorus is an essential element for commercial plant fertilizers. The world's supply of phosphorus is an important element in growing food. Phosphorus is produced from mined rock phosphate often used in mineral fertilizer when combined with sulphuric acid, potassium and nitrogen (Cordell *et al.* 2009). The existing world's phosphate rock (non renewable resource) reserves could be exhausted in the next 50-100 years (Smil 2000, Gunther 2005 Cordell *et al.* 2009). The response to phosphorus scarcity problems will include higher prices, introduction of alternatives, more efficient resource use and the recovery of the resource after use (Cordell *et al.* 2009).

Traditionally, crop production depends on natural levels of phosphorus in the soil and the addition of locally available organic matter such as human excreta and manure (Mårald 1998). Close to 100% of phosphorus eaten in food is excreted (Jönsson *et al*. 2004). Every year it was estimated that the global population excretes around 3 million tons of phosphorus in urine and feces. Almost all phosphorus in human urine occurs in inorganic form as phosphate ion (Lentner *et al*. 1981). In fresh urine at pH between 5.6 and 6.8 at 25^{o}C, phosphorus exists as $H_2PO_4^-$ and in stored urine with pH around 9 at 25^{o}C, it exists in form of HPO_4^{2-} (Ganrot 2005). Phosphorus in urine is directly available to plants (Kirchman and Pettersson 1995, Jönsson *et al*. 2004) and phosphorus loss during urine management is not high (Jönsson *et al*. 2004). The two major opportunities for increasing the life expectancy of world's phosphorus resources depends on wastes recycled from municipal and other organic wastes products and the efficient use in agriculture of both phosphate mineral fertilizer and animal manure (European Fertilizer Manufacture Association 2000). The over-fertilization of agricultural soil of high level phosphorus source contributes to the excess discharge of this element into lakes, rivers and other water bodies (Rosmarin 2004, Uusi-Kämppä and Heinonen-Tanski 2008, Cordell *et al*. 2009) and causes environmental problems like eutrophication (Cordell *et al*. 2009). During phosphorus recovery from human urine it is transformed into solid form by struvite precipitation (Harada *et al.* 2006, Kabdaşli *et al*. 2006). When human urine is treated with magnesium ion (Mg^{2+}), it can be used as a good slow release of phosphorus fertilizer (Udert *et al.* 2003 2006). The artificial struvite production of human urine plant have been done in Japan (Ueno and Fujii 2001), in Italy (Battistoni *et al*. 2005) and in the Netherlands (Giesen 1999).

Potassium (K) is a plant macronutrient occurring in human urine as free ions (K^+), which are directly available to plants and having similar fertilizing effect as supplied by chemical fertilizers (Jönsson *et al.* 2004). Potassium is a very important element needed for plant growth especially increase root growth (Mullins *et al.* 1994). Similarly, sulphur (S) is mainly excreted in the human urine in the form of sulphate ions (SO_4^{2-}) (Lentner 1981, Kirchman and Petterson 1995, Jönsson *et al.* 2004), which are directly available to plants having the same fertilizing effects to that of the chemical S fertilizers (Jönsson *et al.* 2004). Human urine also contains all micronutrients needed for plant growth. These elements usually considered as micronutrients are boron (B), iron (Fe), copper (Cu), chloride (Cl), manganese (Mn), molybdenium (Mo) and zinc (Zn) (Frausto da Silva and Williams 1997, Marschner 1997).

They are found in different composition in human urine cited in different publications (Table 3). Most micronutrients are needed for formation of different enzymes (Jönsson *et al*. 2004).

2.10 Human urine in agricultural application

Human urine has been proven by many investigators as an excellent plant nutrient. It has been proposed as interesting fertilizer products for organic farms with limited access to manure due to its high content of easily available nitrogen (Tidåker *et al.* 2007)). The fertilization value of human urine and its application as crop mineral nutrient source received tremendous attention among researchers in recent times (Morgan 2003, Jönsson *et al.* 2004, Mnkeni and Austin. 2009). It has been used since ancient times in many places around the world to improve the growth of vegetable plants and available universally as free natural resource (Jönsson *et al*. 2004). The research on human urine as fertilizer in agricultural application has been carried out all over the world. Urine fertilization has been carried out on Swiss chard in Ethiopia and the yield of fertilized crops were up to four times that of unfertilized (Sundin 1999). In Ghana during 2004 and 2005 a research project was conducted to investigate the nutrient efficiency of urine in comparison with mineral fertilizer on sorghum fertilization under local conditions. It was revealed that the fertilization with P and K enriched urine increases the yield of sorghum about 3.5 times greater than that of the mineral fertilizer under the same conditions (Germer *et al.* 2011). Trials conducted in Zimbabwe showed greatly increased yields of different maize and vegetables grown on sandy soil when applied urine as a liquid fertilizer (Morgan 2003). In pot trials conducted in South Africa the human urine fertilization of cabbage, maize, spinach and tomatoes showed that urine is considered agronomic effectiveness as ammonium or urea sources of nitrogen (Mnkeni *et al.* 2008, Mnkeni and Austin 2009). In South Africa a glasshouse experiment was conducted to evaluate the application of human feces and urine either separately or combined use and inorganic fertilizer as control for spinach (*Spinacia oleracea*). The result showed that the separately used urine fertilizer had significantly greater yield than the other fertilizer treatments (Kutu *et al.* 2011). In Nigeria, the use of human urine as an organic fertilizers in the production of okra (*Abelmoschus esculentus*) and the results showed that urine

fertilization increased growth and yield of okra plants relative to NPK fertilizers (Akpan-Idiok *et al.* 2012).

In a field trial in Sweden 2002, different application strategies for human urine as a fertilizer to leeks were conducted and the fertilizing with urine gave a three-fold yield increase (Båth 2003). In Germany, urine fertilization has been studied on barley and ley in both field trials and greenhouse and the results from the field trials revealed that the fertilizing effects of urine was higher than that of mineral fertilizer in barley production (Simons and Clemens 2004). The environmental impact of wheat production using human urine and mineral fertilizers, optimal fertilizing strategies regarding application time, technique and substitution of mineral fertilizer was demonstrated to be important for energy use, global warming and acidification (Tidåker *et al.* 2007). In Sweden, plant availability of nutrients recovered as solid from human urine was tested in climate chamber on wheat (*Triticum aestivum* L) and the tests show a slow release of nutrient (P and N) from struvite and from N-adsorbents (Ganrot *et al.* 2008).

Adamsson (2000) conducted a study on the potential use of human urine by greenhouse culturing of microalgae (*Scenedesmus acuminatus*), zooplankton (*Daphnia magna*) and tomatoes (*Lycopersicum*) and the results suggested that it would be possible to use an aquaculture approach and recycle nutrient from human urine by using a constructed food chain. Human urine obtained from separating toilets was tested as a fertilizer for the cultivation of outdoor cucumber (*Cucumis sativus* L.) in Nordic climate. The results showed that urine fertilization was similar or slightly better than the yield obtained from control row fertilized with mineral fertilizer and none of the cucumber contained enteric microorganisms (Heinonen-Tanski *et al.* 2007). In Finland, human urine was used as a fertilizer in cabbage cultivation and compared with industrial fertilizer and non-fertilizer treatments and the results showed that human urine could be used as a fertilizer for cabbage and does not pose any hygienic threats or leave any distinctive flavor in food products and urine achieved equal fertilizer value to that of industrial fertilizer (Pradhan *et al.* 2007). The fertilizer value of human urine was compared with mineral fertilizer in pumpkin (*Cucurbita maxima*) and the results from the study revealed that the production rate of urine fertilized pumpkin was somewhat lower than mineral fertilized but higher than non-fertilized pumpkins and the hygienic quality was equally good for all treatments (Pradhan *et al.* 2009b). In Finland, the results of the used of human urine and wood ash for tomatoes (*Solanum lycopersicum*)

cultivation in a greenhouse (Pradhan *et al.* 2009a) and for red beet (*Beta vulgaris*) cultivation in outdoor plot (Pradhan *et al.* 2010) showed that urine with or without ash increased the yields of tomatoes and red beet without posing any microbial or chemical risk.

In Mexico, urine has been tested as fertilizer to grown lettuce in greenhouse and the results revealed that urine gave the best yield of lettuce due to high availability of nitrogen when compared with other fertilization treatments such as urine –compost mixture and no fertilizer at all (Guadarrama *et al.* 2002). In India, human urine was used as fertilization for banana (*Musa paradisica*) cultivation with good yields (Sridevi *et al.* 2009).

Commercial fertilizers used intensively in agricultural production are produced with a high level of fossil energy and from limited mineral resources, and sometimes polluted by radioactive elements or cadmium (Hodge and Popovici 1994). Nutrient recovery and recycling from human urine can be a significant part of the eco-recycling society of the future (Lind *et al.* 2001). The new ideas of eco-cycling based on urine separation have been proposed to achieve maximum recovery and circulation of nutrients without contaminations by hazardous compounds and with reduced eutrophication in freshwater and coastal ecosystem (Lind *et al.* 2001). The human urine alone can replace 20-25% of commercial fertilizer presently used in food production for people and animal stock (Jönsson *et al.* 1994).

2.11 Human urine and mycobacteria

Most mycobacterial pathogens are excreted to urine. Mycobacteria have been cultured or isolated from clinical urine specimens (Hillemann *et al.* 2006, Chan *et al*. 2008, Alvarado-Esquivel *et al.* 2009). Infection in the kidney such as renal tuberculosis may result in the excretion of the bacteria in the human urine. *M. tuberculosis* can be excreted in the urine of humans with renal tuberculosis infection (Daher *et al.* 2007). Urine specimens from patients of extrapulmonary tuberculosis were found to have positive culture for *M. tuberculosis* (Bentz *et al.* 1975, Hillemann *et al.* 2006, Chan *et al*. 2008, Cannas *et al.* 2008, Alvarado-Esquivel *et al.* 2009). *M. fortuitum* has been associated with disseminated lesions in patients undergoing dialysis (Youmbissi *et al*. 2001) and renal tuberculosis can exist without clinical manifestation (Serra *et al.* 2007). Positive urine cultures for *M. kansasii* are found in patients

with persistent fever from hairly cell leukemia and disseminated *M. kansasii* infection (Weinstein *et al.* 1981). The resulting high frequency of nontuberculous mycobacteria in urine may indicate contamination by mycobacteria in the environment (Alvarado-Esquivel *et al.* 2009). Mycobacteria species detected in clinical human urine specimen cited from different publications is depicted in table 4.

Table 4. Different mycobacteria species isolated from clinical human urine specimen

Isolates	**Reference**
M. tuberculosis	Grange & Yates (1992), Aceti *et al*. (1999), Torrea *et al*. (2005), Rebollo *et al*. (2006), Aslan *et al*. (2007), Sener *et al.* (2008)
M. bovis	Grange & Yates (1992), Chambers *et al*. (2002)
M. avium complex	Cuervo *et al*. (2003), Hillemann *et al.* (2006), Chan *et al*. (2008)
M. africanum	Grange & Yates (1992)
M. gordonae	Hillemann *et al.* 2006, Chan *et al.* (2008), Alvarado-Esquivel *et al.* (2009)
M. marinum	Streit *et al.* (2006)
M. kansasii	Hillemann *et al.* (2006), Alvarado-Esquivel *et al.* (2009)
M. malmoense	Henriques *et al.* (1994)
M. scrofulaceum	Hsueh *et al.* (1996)
M. simae	Valero *et al.* (1995), Chan *et al.* (2008)
M. terrae	Chan *et al*. (2008)
M. fortuitum	Hillemann et al. (2006), Serra *et al.* (2007), Chan *et al.* (2008), Alvarado-Esquivel *et al.* (2009)
M. neoaurum	Zanetti *et al.* (2001)
M. hassiacum	Schröder *et al.* (1997) Tortoli *et al*. (1998)

M. smegmatis	Alvarado-Esquivel *et al*. (2009)
M. peregrinum	Alvarado-Esquivel *et al.* (2009)
Mycobacterium sp.	Alvarado-Esquivel *et al*. (2009)

2.12 Human urine and other microbes

In a healthy person, urine is sterile when it is excreted from the body without fecal contamination (Schönning 2001) but pick up pathogens that occur in the lower parts of the urinary tract (Schönning and Stenström 2004, Madigan and Brock 2009). When transported out of the body different types of dermal bacteria are picked up and freshly excreted urine normally contains <10 000 bacteria per ml (Tortora *et al.* 1992). Fresh human urine contains very few enteric microorganisms but some human microbial pathogens or eggs of helminthes can be emitted in urine (Heinonen-Tanski and Wijik-Sibesma 2005). The microbial quality of collected urine depends on the possible route of contamination which could be either by fecal contamination or the excretion of pathogens into urine by disease infected person. The pathogenic bacteria that are normally known to be excreted in urine are *Salmonella paratyphi, Leptospira interrogans* and *Salmonella typhi* (Feachem *et al*. 1983). Most urinary tract infections are caused by *E. coli* (Manges *et al*. 2001), excrete significantly higher amounts of bacteria in the human urine (Schönning and Stenström 2004, Gottesman *et al.* 2009). Microsporidia are group of protozoa implicated in human disease, mainly in HIV positive persons (McDougall *et al*. 1993, Franzen *et al.* 1995) and the infective spores of the microsporidia are shed in urine and faeces, and urine is a potential environmental transmission route (Haas *et al.* 1999). Urine may also contain *Ascaris* ova and *Schistosoma* eggs (Drangert 1998) or live trematodes, like *Schistosoma haematobium* (Feachem *et al*. 1983).

Human urine can contain pathogenic viruses (Vanchiere *et al.* 2005) and large number of enteric viruses may enter urine through fecal contamination in the sanitation system which is a major concern for environmental transmission route (Höglund *et al*. 2002). Adenovirus may also be excreted in urine, especially with hemorrhagic cystitis and transplant patients (Raboni

et al. 2003). Cytomegalovirus (CMV) is excreted in human urine, but the transmission of CMV may occur person from person and the virus is not considered to be spread by water and food (Jawetz *et al.* 1987). The human polyomaviruses, JC virus (JCV) and BK virus (BKV), are excreted into human urine from renal transplant patients (Holman *et al.* 2003, Biel *et al.* 2004). Hepatitis B is also excreted in human urine and urine was suggested as a possible route of transmission in highly endemic areas (Knutsson and Kidd-Ljunggren 2000, Kidd-Ljunggren *et al*. 2006). The isolation of measles virus from human urine was carried out by Gresser and Katz (1960).

2.13 Human urine as an antimicrobial compound

Human urine has been demonstrated as antimicrobial without regard to any pathogens during storage. The antibacterial activity of human urine was first studied by Kaye (1968) and the result of his work revealed that urea concentration was a more important for antibacterial activity than osmolality or ammonium concentration. The greater toxic effect of urea may be due to its ability to diffuse freely across cell membranes of bacteria (Chambers and Kunin 1985). During storage, the urea composition in human urine is converted to ammonium by enzymes *urease* (Kaye 1968, Alexander 1977, Hanæu *et al*. 1996, Udert *et al*. 2003 Zhigang *et al*. 2008) and lead to increase in concentration of ammonia and elevated pH in the urine (Lind *et al.* 2001, Udert *et al*. 2003). Ammonia is toxic to microbes and can lead to reduction of pathogens (Vinnerås *et al.* 2003). There is a strong relationship between pH and the fraction of urea decomposed in urine (Hellström *et al.* 1999) and the pH of stored urine stabilizes around 9.0 (Hellström *et al.* 1999, Zhigang *et al*. 2008). However a long storage time at high pH (alkaline conditions) seems to reduce the number of microorganisms (Hellström *et al*. 1999, Schönning and Stenström 2004).

The survival of various microorganisms in urine through time is affected by the storage conditions (Schönning and Stenström 2004). Suitable temperature is a reliable factor when considering urine storage. High temperature prefers urea hydrolysis; for instance temperature >20^{o}C increases urine hydrolysis (Zhigang *et al*. 2008). During storage of urine temperature and elevated pH around 9.0 in combination with ammonia have been concluded to affect the inactivation of microorganisms (Schönning and Stenström 2004). The alkaline pH value in

stored urine and high urine concentration are factors that facilitate the elimination of many pathogens (Höglund *et al.* 1998). In addition, high temperatures (20^{o}C instead of 4^{o}C) are favorable for pathogens die-off in stored urine (Höglund *et al.* 1998).

Studies have been conducted with different microorganisms added to the urine and their inactivation followed over time (Höglund 2001). Earlier studies have shown the inactivation of *Schistosoma haematobium* in urine (Feachem *et al.* 1983). The inactivation of enteric pathogens (*Salmonella enteric* subspecies 1 serovar typhimurium, *Enterococcus faecalis*) and viruses *MS2 and ϕx 174*) by urine storage at dilutions (urine: water) 1:0, 1:1 and 1:3 at temperature 4, 14, and 34^{o} was studied by Vinnerås *et al.* (2008). The results indicated that the current recommended storage time for urine, 6 months at 20^{o}C or higher, is safe for unrestricted use and could probably be shortened especially for undiluted urine. The survival of enteric bacteria (*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Enterococcus faecalis* and coliphage MS2) was studied in stored, fresh and diluted (1:1) human urine at 15 and 30^{o}C. The results revealed that the high pH and temperature were the major factors affecting the survival of the microorganisms and the complete inactivation of all micro-organisms in less than one week in stored urine at 30^{o}C (Chandran *et al.* 2009). It was recommended that a storage time of five weeks at temperature below 20^{o}C or two weeks at temperature above 20^{o}C is sufficient to prevent transmission of mycobacteria when recycling human urine (Vinnerås 2011).

Human urine is also ovicidal and *Ascaris* eggs are killed within hours (Feachem *et al.* 1983). The reduction of *Ascaris suum* in urine is minor; at 4^{o}C and 20^{o}C the reduction was 15-20% during a 21-day period (Olosson 1995). The total inactivation of viable eggs of *Ascaris suum* took place in less than 10 days in stored human urine at 34^{o}C (Nordin *et al.* 2009). Höglund (2001) reported the inactivation of *Cryptosporidium parvum* in stored urine at 90% reduction in 5 days at temperature 20^{o}C.

3 AIM OF STUDY

The overall aim of this thesis was to investigate the survival of different Mycobacterial strains in pure human urine.

The specific objectives of the present investigation were:

- to examine the survival rate of different Mycobacterial strains in different urine samples (stored human urine > 6 months and fresh human urine <1 day) at temperatures of 15^{o}C and 30^{o}C to mimic the two predominant world climatic conditions, temperate and tropical climates.

- to investigate the anti-pathogenic properties of the urine samples at different temperatures in relation to pH.

4 MATERIALS AND METHODS

4.1 Test organisms

The survival studies were conducted with four mycobacterial strains, two rapid growers *Mycobacterium aurum* DSM 43999 and *Mycobacterium fortuitum* ATCC 6841T and two slow growers *Mycobacterium avium* ATCC 15769 and a clinical *Mycobacterium bovis* BCG strain obtained from the Kuopio University Hospital.

4.2. Urine

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 eco-toilets in the area of Tampere, Finland. (ii) Fresh urine < 1 day old obtained from healthy students in Kuopio. The experiments of the test organisms were performed in parallel runs.

4.3 Preparation of Media

4.3.1 Tryptic Soy Agar (TSA)

40 g of the powder (from Oxoid Ltd, Basingstoke, Hampshire, England) was suspended in 1 L of purified water and mixed thoroughly. The mixture was heated and allowed to boil for 60 seconds in order to dissolve the powder. The dissolved powder was autoclaved at 121°C for 15 minutes. Then 20 mL of slightly cooled volume was poured into Petri dish.

4.3.2 Mycobacteria 7H11 Agar (M7H11)

21g of the powder (Becton Dickson and Company, Sparks, MD 21152, USA) was suspended in 900 mL of purified water containing 5mL of glycerol. The mixture was swirled to obtained smooth suspension. The suspension was autoclaved at 121°C for 15 minutes. The autoclaved medium was enriched aseptically with 100 mL of Middlebrook OADC (Becton Dickson and Company, Sparks, MD 21152, USA) at temperature of 55-60°C. Then slightly cooled 20 ml volume of the agar was poured into Petri dish.

4.4 Preparation of inocula

4.4.1 Fresh pure culture of the test organisms and growth conditions

M. aurum was grown on Tryptic Soy Agar (TSA) medium and incubated in an incubator (Memmert made in Germany) for 1 week at 30°C *M. fortuitum* was similarly grown on M7H11 medium. *M. bovis* and *M. avium* were grown separately on M7H11 medium and incubated for a minimum of 4 weeks week at 36°C.

4.4.2 Dense suspension of the test organisms

The test organism from the agar medium was suspended first to a small amount of sterile water in a test tube against glass wall using sterile culture loop. The mixture was then shaken in a vertical shaker (Vortex) to make even or homogeneous suspension without clumps. The duration for shaking varied 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 to make a suspension of 10^8 cfu/ml, the concentration was analysed by culture. 1 mL of the suspension was then pipetted to make a dilution series from 10^{-1} to 10^{-8} to 9 mL of sterile distilled water and then 0.1 ml of the serial dilutions from 10^{-4} to 10^{-7} was plated onto TSA or M7H11 medium. Sterile L-shaped glass rod was used to spread inocula along the surface of the medium. The plates of rapid-growing *M. aurum* and *M. fortuitum* were incubated at 30 ± 0.5^{o}C and of slow-growing *M. avium* and *M. bovis* were incubated at 36 ± 0.5^{o}C.

4.5 Preparation of samples

700 mL of fresh and stored urine were measured into two sterile 1 L bottles one for each urine sample. Then 3.5 mL of the fresh inoculums of mycobacterial strains was pipetted to each bottle and shaken carefully. The inoculated urine samples were subdivided into sterile 100 mL bottles with three replicates per experimental treatment (Figure 2).

(a) Fresh urine incubated at 15± 0.5^{0}C

(b) Fresh urine incubated at 30± 0.5^{0}C

(c) Stored urine incubated at 15± 0.5^{0}C

(d) Stored urine incubated at 30± 0.5^{0}C

Figure 2. Experimental set up for the test organism.

One bottle each for fresh urine without inoculum stored at temperatures of 15 and 30 ± 0.5^{o}C and one bottle each for stored urine without inoculum kept at temperatures of 15 and 30 ± 0.5^{o}C for each experimental set up were weekly analysed for the pH measurements using a pH meter (Ino Lab pH 720 WTN 82362 Weiiheim, Made in Germany). Dilution series of all 100 mL bottles (except those meant for pH) were used for plating of 10^{-3} to 10^{-6} onto TSA or M7H11. Plates were incubated at 30 ± 0.5^{o}C or 36 ± 0.5^{o}C depending on the mycobacterium strain (see chapter. 4.3.2). Follow-up of the samples were conducted weekly by taking 1 mL inoculums from the 3 replicate bottles kept at temperatures of 15 and 30 ± 0.5^{0}C to make serial dilutions and plating of the test organism. The follow-up were continued 8 - 9 weeks. Colonies on the media are counted weekly for 4 weeks at least. 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).

4.6 Analysis of mycobacteria from urine samples using decontamination procedure

4.6.1 Preparation of cetylpyridinium chloride (CPC) solution

0.264 g of hexadecyl pyridinium chloride monohydrate (MERCK) was weighed on a clean dish. The powder was poured into measuring cylinder glass and filled up to 250 mL of deionized water. The powder was allowed to dissolve and the solution was filter sterilized through a 0.45 µm membrane filter (Millipore EZ-Pak, diam. 47 mm). The solution was then poured into sterile bottle and stored at dark for maximum of 6 months. CPC is toxic and preparation was done with appropriate safety precautions; protective gloves and face and eye protections were worn.

4.6.2 Decontamination with CPC

The decontamination procedures were carried out only on the slow-growing mycobacteria. 1 mL of the spiked urine samples was pipetted into a sterile centrifuge tube, 1 mL of 0.1% of CPC solution was added and then shaken thoroughly. The tubes were kept at room temperature for 15 minutes with occasional shaking. The tubes were centrifuge at 8000 rpm (Jouan MR22i) for 15 minutes at 4^{o}C. All the supernatant was discarded carefully, and then 20 mL of sterile water was added to the remained sediments in the centrifuge tubes and shaken thoroughly. The sample was centrifuged again as told above. All the supernatant was poured off and then 1.5 ml of sterile water was added to the sediment and shaken well. Serial dilutions of 10^{-1} to 10^{-3} made from the decontaminated samples were plate on to M7H11 medium and incubated at temperatures of 36 ± 0.5^{o}C.

4.7 Statistical analysis

The association between the numbers (log cfu/ml) of mycobacteria (dependent variables) and pH (independent variables) was studied by Spearman rank correlation analysis (non-

parametric test) using SPSS statistical software (SPSS Inc., Chicago, IL. version 17.0). Graphpad Prism 5 software (Graphpad software Inc) was used for graphical analysis and exponential growth reduction/decay K-value (log cfu/week) of the different mycobacterial strains calculated from the survival curves of initial count of cfu/ml of viable cells in the human urine until no viable cells are detected.

5 RESULTS

5.1 Survival of *Mycobacterium fortuitum*

The initial concentration of *M. fortuitum* in fresh urine samples was 6.57 x 10^6 cfu/ml and that of the stored urine was 6.12 x 10^6 cfu/ml (Table 5). The survival of the mycobacterium strain was favored in the fresh human urine around neutral pH of 7 (Table 6) as its numbers slightly increases after the first week of the experiment at both 15 and 30°C (Figure 3). *M. fortuitum* survived for up to 4 weeks in fresh urine at 30^{o}C and survived up to 5 weeks in fresh urine at 15^{o}C (Figure 3) . In stored urine at 30^{o}C, *M. fortuitum* was inactivated rapidly within 1 week and survived up to 4 weeks in stored urine at 15^{o}C (Figure 3). There was a gradual increase in pH values of all urine samples at every week interval of the experiment (Table 6). The complete decimal reduction 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. There was a decline in cfu/ml of *M. fortuitum* in both fresh and stored human urine at 15 and 30^{o}C in relation to pH of the urine samples (Table 6) and the other test organisms showed a similar pattern of behaviour. All the urine samples exhibited negative correlations between pH and cfu/ml of *M. fortuitum* ($p \leq 0.05$) except for *M. fortuitum* in stored urine at 30^{O}C (Table 8). The exponential decay values (K-values) were negative for the survival of *M. fortuitum* in all the urine treatments (Table 7). The negative K-value means the averagely decreased concentration (log cfu/ml) of the numbers of mycobacteria per week over the study period.

Table 5. The initial concentration of the cfu/ml of the test organisms in urine samples

Test organisms	**Initial concentration (cfu/ml)**	
	Fresh urine	**Stored urine**
M. fortuitum	6.57 x 10^6	6.12 x 10^6
M. aurum	9.18 x 10^6	2.43 x 10^6
M. avium	8.90 x 10^6	8.10 x 10^6
M. bovis	3.60 x 10^5	1.80 x 10^5

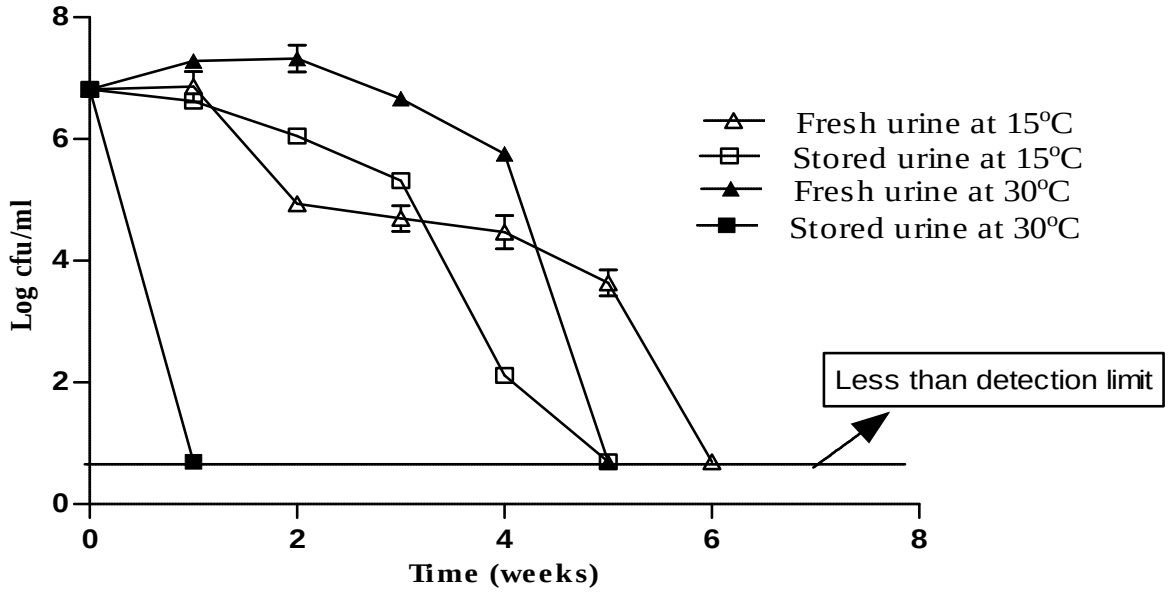

Figure 3. Survival curves of *M. fortuitum* in pure human urine (mean±SD, N = 3).

Table 6. The cfu/ml of *M. fortuitum* in pure human at different temperatures with relation to pH of the urine samples. The pH results of the other three experiments (*M. aurum, M. avium, M. bovis*) followed the same trend.

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

5.2 Survival of *Mycobacterium aurum*

The initial concentration of *M. aurum* in fresh urine was 9.18 x 10^6 cfu/ml and in stored urine it was 2.43 x 10^6 cfu/ml (Table 5). *M. aurum* declined rapidly within 1 week in stored urine at 30^{o}C and 2 weeks in fresh urine kept at 30^{o}C (Figure 4). The organism survived up to 3 weeks in fresh urine samples kept at 15^{o}C and 2 weeks in stored urine samples at 15^{o}C (Figure 4). After 6 weeks of the experiment, negative results were observed in all the spiked selective media. The numbers of *M. aurum* correlated negatively and statistically significant with pH of the urine ($p \leq 0.05$) in all samples except for the *M. aurum* in fresh urine sample at 30^{o}C (Table 8). Negative K- values were observed for all the urine treatments spiked with *M. aurum* (Table 7).

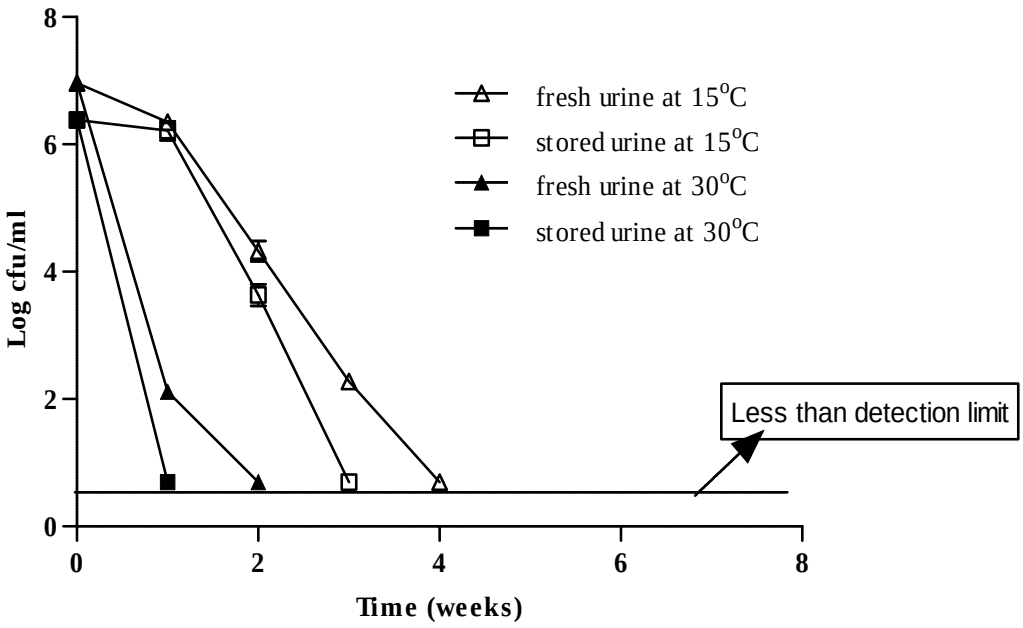

Figure 4. Survival curves of *M. aurum* in pure human urine (mean±SD, N = 3).

Table 7. The K value (log cfu/ml week^{-1}) of the exponential decay of mycobacteria survival per week in fresh and stored human urine at storage temperatures 15^{o}C and 30^{o}C over the study period

Test organism	**K- value**			
	Fresh urine at 15^{o}C	**Stored urine at 15^{o}C**	**Fresh urine at 30^{o}C**	**Stored urine at 30^{o}C**
M. fortuitum	-0.1800	-0.2355	-0.1681	-2.2780
M. aurum	-0.3662	-0.3952	-1.1780	-1.0440
M. avium	-0.1801	-0.3001	-0.2341	-0.5630
M. bovis	-0.9143	-2.0170	-0.4870	-2.0170

Table 8. Spearman rank correlation coefficients between the pH of human urine and the cfu/ ml of different mycobacterial strains survival in urine at different temperatures

Variable	**cfu/ml**	
	r	***p***
M. fortuitum in fresh urine at 15^{o}C	-0.964	<0.05
M. fortuitum in stored urine at 15^{o}C	-0.798	<0.05
M. fortuitum in fresh urine at 30^{o}C	-0.847	<0.05
M. fortuitum in stored urine at 30^{o}C	-0.635	
M. aurum in fresh urine at 15^{o}C	-0.852	<0.05
M. aurum in stored urine at 15^{o}C	-0.795	<0.05
M. aurum in fresh urine at 30^{o}C	-0.490	
M. aurum in stored urine at 30^{o}C	-0.802	<0.05
M. avium in fresh urine at 15^{o}C	-1.000	<0.05
M. avium in stored urine at 15^{o}C	-0.608	
M. avium in fresh urine at 30^{o}C	-1.000	<0.05
M. avium in stored urine at 30^{o}C	-0.949	<0.05
M. bovis in fresh urine at 15^{o}C	-0.360	Not analysed due to
M. bovis in stored urine at 15^{o}C	-0.333	lack of results*
M. bovis in fresh urine at 30^{o}C	-0.722	
M. bovis in stored urine at 30^{o}C	-0.707	

r = Spearman rank correlation coefficient ***P*** ≤ 0.05 = Statistical significant.

* The correlation could not be made because survival was so short and there were no result points.

5.3 Survival of *Mycobacterium avium*

The initial concentration of *M. avium* in fresh urine was 8.90 x 10^6 cfu/ml and in stored urine was 8.10 x 10^6 cfu/ml (Table 5). The test organisms reduced rapidly less than 1 week in the stored urine samples at 30°C and survived up to 2 weeks in the stored urine at 15°C (Figure 5). In fresh urine samples kept at temperature 15 and 30°C, the organisms had a better survival up to 4 weeks before rapid decline of their colony (Figure 5). The total reduction in the cfu/ml of *M. avium* was observed after 6 weeks for all the urine samples and there was no colony formed in any of the spiked selective media. *M. avium* 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. The correlation between pH and cfu/ml of the organism was statistically significant ($P \leq 0.05$) for fresh urine samples kept at 15 and 30°C and for stored urine samples at 30 °C (Table 8). The K-values for the survival of *M. avium* for all the urine treatments were negative.

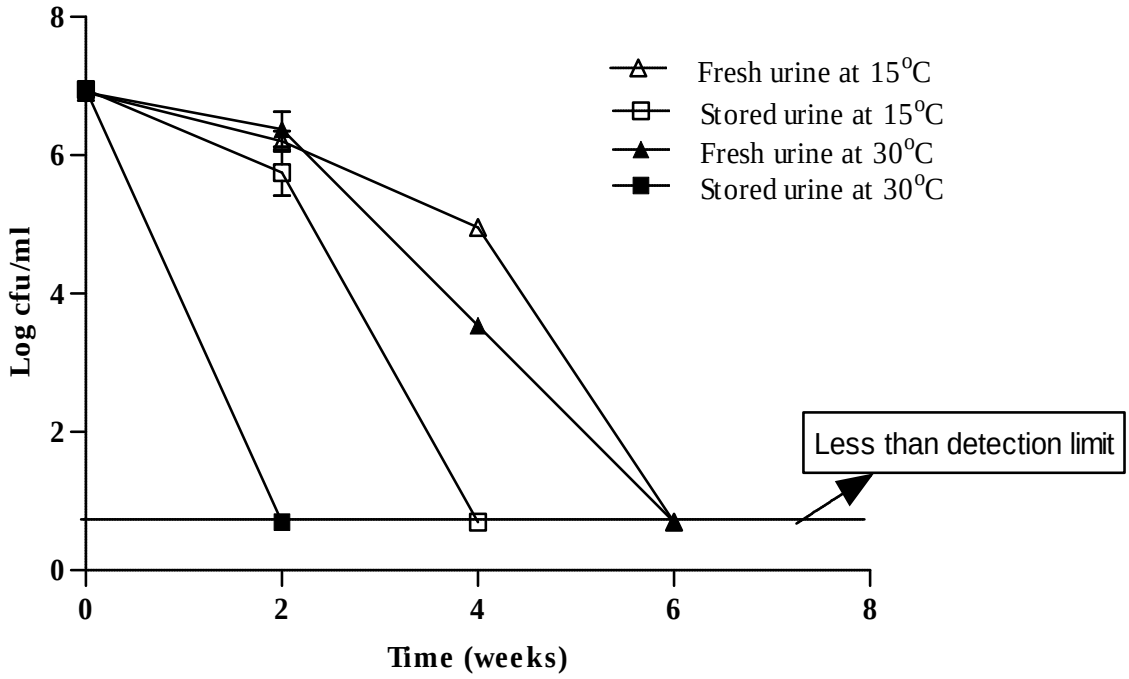

Figure 5. Survival curves of *M. avium* in pure human urine (mean±SD, N = 3).

5.4 Survival of *Mycobacterium bovis*

The initial concentration of *M. bovis* in fresh urine was 3.60 x 10^5 cfu/ml and in stored urine it was 1.8 x 10^5cfu/ml (Table 5). *M. bovis* survived up to 2 weeks in the fresh human urine samples stored at 15 and 30^{o}C but was inactivated within the first week of the experiment in the stored urine samples at 15 and 30^{o}C (Figure 6). *M. bovis* was also highly sensitive to the decontamination procedures which produced negative results for all the urine samples. The correlation coefficients for the associations between the numbers (cfu) of *M. bovis* and pH were all negative but did not reach statistical significance (Table 8). The K-values for the survival of *M. bovis* in all the urine samples were negative (Table 7).

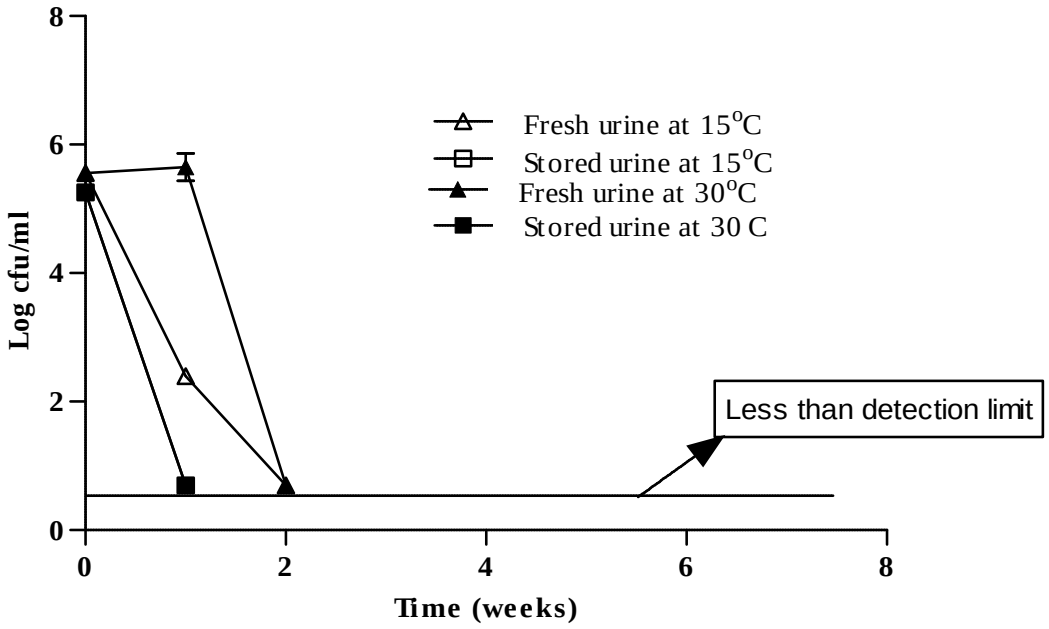

Figure 6. Survival curves of *M. bovis* in pure human urine (mean±SD, N = 3).

6 DISCUSSION

There has been little information on 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. Our findings indicate that all the test organisms studied had a low survival rate in stored human urine. They all survived < 1 week in stored human urine at 30^{o}C with pH around 9.0. Stored urine at 30^{o}C had the highest inactivation rate of 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 those of Chandran *et al.* (2009) study and slightly different to the investigation of Vinnerås *et al.* (2011). The results also revealed that mycobacteria survived better in stored urine at 15^{o}C than at 30^{o}C and the survival time varied from one to four weeks depending on the strain. The findings indicate that the survival time of mycobacteria in fresh urine at 15^{o}C varied from two to five weeks and in fresh urine at 30^{o}C, the survival time was two to four weeks. Chandran *et al.* (2009) reported that the storage time of fresh urine stored at 15^{o}C and 30^{o}C needed to inactivate enteric bacteria varied from four to seven weeks and thus survival of mycobacteria detected in this study was similar but with shorter survival time.

The longest and rather similar survival times were detected with *M. fortuitum* and *M. avium* showing that there was no difference in the survival between the rapid- and slow-growing species of mycobacteria. Fewer data points obtained with *M. avium* between weeks 4 and 6 makes it difficult to estimate the exact survival time but no survival was detected after 4 weeks. The shortest survival was detected with *M. bovis*. This was rather natural since it is a close relative to *M. tuberculosis* known to have a narrow pH range of growth, thus supporting the results of previous studies (Chapman & Bernard 1962, Portaels & Pattyn 1982, Rao *et al.* 2001, Vinnerås *et al.* 2011).

In the present study, the results on survival time of mycobacteria in human urine were promising when compared to previous survival studies conducted on mycobacteria species in different environmental reservoirs. It was reported that mycobacteria survived more than 3

months in ocean water (Viallier *et al.,* 1997). Raizman *et al* (2011) reported that the survival time of *M. paratuberculosis* in cow faeces stored at -18^{o}C was up to 540 days. von Reyn *et al.,* (1994) reported that *M. avium* survived up to 485 days in tap water stored at 4^{o}C and 20^{o}C. It was reported that *M. bovis* survived up to 176 days in liquid manure stored at 5^{o}C (Dokoupil 1964) and up to 21 months in soil (Young *et al.,* 2005). The results of the current study revealed that survival time of mycobacteria in human urine was from one week up to five 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 up to 2.3 days in human urine kept at temperature 22^{o}C and up to 28 days in human urine at 4^{o}C around pH 9.0, hence 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 (Table 6). 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 increase in the pH level of the fresh urine stored at 30^{o}C was more rapid when compared to that of the fresh urine stored at 15^{o}C. Probably this may be due to the enzyme urease was more favored at temperature 30^{o}C. Similar to our findings, other studies have shown that high temperature prefers urea hydrolysis and it was higher at temperature 30^{o}C than at temperature 15^{o}C (Zhigang *et al.* 2008). The breakdown of urea in human urine is caused by an enzyme urease to produce ammonia in the urine solution and therefore increase in 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- 8 weeks depending on the storage temperature. Similar results were also reported by Zhigang *et al.* (2008) and Chandran *et al* (2009).

The survival of the mycobacterial strains in both fresh and stored urine was dependent on the pH of the urine and the storage temperatures. In this study, these results revealed that the increase in pH of the urine lead to the progressive reduction of the colony forming units of the test organisms (Table 6). This means that the more alkaline the pH was the more negative was the effect on the survival of the test organisms. 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 findings conducted on studies on the inactivation of enteric pathogens

(*Salmonella enteric* subspecies1 serovar Typhimurium, *Enterococcus faecalis*) and viruses (*S. Typhimurium 28B, MS2 and ϕx 174*) by urine storage (Vinnerås *et al.* 2008), findings on the survival of enteric bacteria and Coliphage MS2 in pure human urine (Chandran *et al.* 2009) and the inactivation of *M. tuberculosis* and *M. bovis* in human urine (Vinnerås *et al.* 2011). The results of the present study also revealed the better tolerance of *M. fortuitum* in fresh urine samples stored at 30^{o}C and 15^{o}C at pH around 7.0 (Table 6). Thus the results are similar to those observed in previous study conducted on mycobacterium strains in relation to pH of the medium, all strain with the exception of *M. chelonei* developed optimally between pH 7.0 and 7.4 (Portaels and Pattyn 1982). Iivanainen *et al.* (1993) reported that the number of mycobacteria correlated negatively with the pH of the environment. It was also reported that low pH favors the high number of mycobacteria in the environment (WHO 2004). According to results of the present study it was indicated 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 from environmental factors affecting the occurrence of mycobacteria in brooks waters (Iivanainen *et al.* 1993).

In the current study, the pH of the urine was influenced by the storage temperatures. The results of the current study revealed that high storage temperature at 30^{o}C enhances rapid increase in the pH level of the urine compared to the urine stored at temperature 15^{o}C. Probably these are the reasons why all the test organisms had poorer survival at higher temperature than lower temperature in the studied urine samples. According to the results from previous survival study using human urine, it was reported that micro-organisms showed poor survival at high temperatures and better survival at low temperatures (Chandran *et al.,* 2009, Vinnerås *et al.,* 2011) and this was similar observations found in the present study. The results of the present study revealed the K-values of the survival of mycobacteria were negative. This implies that their growth rate decrease with storage time of the urine, thus this observation were similar to the findings of Vinnerås *et al.* (2011)

Decontamination procedures were conducted on the two slow-growing mycobacterial species BCG strains *M. bovis* and *M. avium.* CPC was used for the decontamination process during sample preparation for culture. Gao *et al.* (2005) reported that CPC decontamination resulted in a significant reduction in the number of cultured-positive milk samples. According to the results of the present study, the culture results from decontamination were all negative. Probably it is possible that this process might have killed the few viable cells due to either the

harsh decontamination steps or the sensitivity of mycobacteria to CPC, thus similar to the findings of CPC decontamination of soil samples (Raiziman *et al.* 2011) and that of bovine feces (Douarre *et al.* 2010).

The experimental work focused on both tropical and temperate climates based on the storage temperatures. Temperature of 30^{o}C proved to be most effective in the survival study. Generally, the maximum survival time of mycobacteria observed in the present study was five weeks. According to the results of the current study it was revealed that a storage period of more than five weeks will be sufficient for the inactivation of the micro-organisms in both climates. This could prevent the hygienic risk associated with human urine reuse for plant fertilization. These findings were in accordance with the recommendation on risk reduction by storage treatment of urine recycling for crop fertilization (WHO 2010, Vinnerås *et al.* 2011).

7 CONCLUSIONS

This study revealed that all the mycobacterial species tested showed lower survival in stored urine than in fresh urine. Their survival was low at 30^{o}C when compared to the storage temperature at 15^{o}C. The lowest survival time for mycobacteria was < 1 week in stored urine at 30^{o}C while the highest survival time was five weeks in fresh urine at 15^{o}C. The storage temperatures represent the two world's typical climates, temperate and tropical climate. Therefore the urine treatment work before reuse in agricultural application would be easier for most developing countries with tropical climate because high temperatures in these countries may help to facilitate the rapid destruction of pathogenic mycobacteria present in urine within a period of five weeks.

For safe agricultural application, 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^{o}C in order to prevent a new exposure route for pathogenic mycobacteria possible for the person making the application work.

The numbers of the mycobacteria correlated negatively with the increasing pH value in the fresh urine sample stored at both temperatures 15^{o}C and 30^{o}C respectively. It can be inferred that alkaline medium with pH around at 9.0 have an adverse effect on the survival of mycobacteria.

Present and previous hygienic studies on human urine for its reuse for plant fertilization are of tremendous achievements giving answers to some of the global problems such as eutrophication, food insecurity and global phosphorus depletion. Moreover, further survival studies have to be done on new pathogenic microbes present in human urine via excretion or cross contamination from human faeces.

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