Isolation and Genus identification of bacteria from urine contaminated soils of Windhoek

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Abstract

Public urination is a common sight in the vicinity of drinking establishments in Katutura, as well as other open spaces around Windhoek. A study was conducted to determine if there was a shift in the balance of the microflora in urine contaminated soils. It was hypothesized that, given soils from the same geographic region but under distinct urine conditions, the identity and counts of the pathogenic and actinobacteria communities would vary. For the purpose of this study, pathogenic microorganisms were limited to those known to cause infections of the urinary tract. Soil samples from 4 contaminated locations (Ara-Dorado, Hakahana, Eveline and Greenwell Matongo suburbs) and controls were qualitatively tested for urease activity. Selective media was used for the isolation and enumeration of pathogenic and beneficial actinobacterial colonies. Dorado showed the greatest extend of urease activity>>>Hakahana and Eveline>>Green well matongo. There is a significant difference between the colony counts at a contaminated and non-contaminated (control) site, $p = 0.019(\alpha = 0.05)$. The results indicated that public urination introduces pathogenic bacteria, causing a shift in the balance of the normal flora.

Keywords: public urination, urine contaminated, colony count, pathogenic bacteria, actinobacteria, soil microflora

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1 Introduction

Public urination is the act of openly urinating outdoors in a public place. Urine is generally sterile, except in instances where it has come into contact with bacteria associated with the urethra, genitals(Karak & Bhattacharyya, 2011), fecal material and the environment (Muratani & Matsumoto, 2006). They ascend through the urethra or the insertion sites of catheters to cause urinary tract infections (UTI) (Muratani & Matsumoto, 2006), these infections are also commonly nosocomial (Fox-Moon & Shirtliff, 2015; Gheldre et al., 1997). This bacterial infections range from asymptomatic to severe sepsis. *Enterobacteriaceae* are gram-negative bacteria abundantly found in the soil, they are considered to be primary pathogens as they are generally associated with diseases when isolated from clinical samples (Muratani & Matsumoto, 2006). Gram-negative organisms, particularly *E.coli* is the predominant causal agent of UTI (Gul, Mujahid & Ahmad, 2004; World Health Organization, 2005). Other organisms that have been reported as causal agents include *Staphyloccocus aureus* (Muder et al., 2006), *Klebsiella, Providencia* (Mobley & Hausinger, 1989), *Serratia* (Jacobsen, Stickler, Mobley & Shirtliff, 2008), *Enterobacter* (Gheldre et al., 1997), *Pseudomonas* and the gram positive *S. saprophticus*, *E. faecalis*, *S. agalactiae*, *S. pyogenes* and *Bacillus subtilis* (Gul et al., 2004). *Proteus mirabillis* commonly occurs in people with abnormal urinary tracts and in complicated UTI cases (Chen et al., 2012)

Urinary tract infections are classified as either mono or polymicrobial in nature (Saverino et al., 2011). Depending on the part of the urinary tract colonized, UTIs are classified as cystitis or pylenophritis. Depending on the absence or presence of underlying disease, UTIs are further classified as simple or complicated respectively (Muratani & Matsumoto, 2006; Srivastava & Vasudev, 2011).

Soils have been subjected to various environmental conditions, these variations have caused modifications in the taxonomic diversity and functioning of the indigenous microbial communities(Maron, Mougel & Ranjard, 2011). Actinobacteria are found in diverse habitats, which include the soil, rhizosphere, marine and freshwater. Actinobacteria play an important role in the environment and medicine, i.e. breakdown of a wide range of plant and animal debris; production of a range of bioactive compounds such as antibiotics, enzymes etc (Ul-Hassan & Wellington, 2009).

Post-infection human pathogenic organisms may be excreted in large numbers in biological specimens such as urine. These organisms maybe transmitted directly (surface-to-mouth contamination) or indirectly (hand-to-mouth) and via other routes, such as the eyes, nose, and abraded skin (Reynolds, Watt, Boone & Gerba, 2005).

It is therefore imperative that studies that determine the biogeography of pathogenic organisms are contacted, due to their potential to identify organisms that may survive for

extended periods and thus prevent the spread of human diseases (Flores et al., 2011).

Figure 1: Street Map of Windhoek, showing the 4 sampling locations.

2 Materials and Methods

Soil samples were collected from 4 contaminated locations in Hakahana, Wanahenda, Greenwell Matongo and Dorado Park (Ara). Contaminated areas were identified visually by the presence of urine. Four control sites were selected in the same vicinity based on the visual absence of urine in the soil. Soil samples were collected in separated 50 ml falcon tubes. The soil samples were scooped up with a disposable plastic spoon and the tubes were capped to avoid contamination. Fifty grams of soil was dissolved in an Erlenmeyer flask with distilled water. From which serial dilutions were prepared. Qualitative test for the presence/absence of urease activity was done by using the urea base agar slant test, the slants were incubated for 24hrs. Microorganisms were isolated and enumerated using various selective media after a 24 hour incubation. These media were: Triple Sugar Iron (TSI), Cysteine Lactose Electrolyte Deficient (CLED), Soil Extract Agar (SEA) and Zhang Starch Soil Extract Agar

(ZSSE). The former two were selective for enterobacteria and the latter for actinobacteria. Sub-culturing was done to obtain pure isolates, these isolates were characterized morphologically and biochemically using the protocols outlined in the *Bergeys manual of Determinate Bacteriology*. The genus of the colony was identified after being subjected to a series of reactions, this reactions include the gram stain, acid fast stain, simple and spore formation stain; catalase test, thioglycolate media and motility test.

3 Data Analysis

For every Dilution Factor (10^{-3} - 10^{-6}) colony counts were done in triplicates. The mean of the counts was then recorded for every dilution. The data was checked for normality using the Shapiro Wilk test in R (version 3.2.0). Levene's test for the equality of variance was performed using IBM SPSS Version 20. Analysis of Variance (ANOVA) was then done to test for the difference in mean counts between contaminated and uncontaminated sites at 5% level of significance.

4 Results and Discussion

The degree of urease activity was measured and qualitatively described by assigning a triple, double, single positive or a negative sign, depending on the percentage of the colour change from orange to beige pink in the tube after inoculation and overnight incubation. Dorado was triple (+++) positive, Greenwell Matongo and Hakahana were double (++), and Wanaheda was single (+) positive. All control (non-contaminated) samples were negative or neglible (Table 1). Urease or urea amidohydrolase is the key enzyme associated with the bioavailability of nitrogen (Zhang, Wan, Kang & Feng, 2014), it is also an extracellular enzyme associated with viable cells (Sinsabaugh, 1994). Urease activity (UA) increases in vegetative soils and decreases in barren soils. In an agricultural setting, the enhanced UA can be an early indicator for improved soil fertility (Zhang et al., 2014). This study was carried out in the suburbs of Windhoek, the soils in these suburbs are not used for agricultural purposes and urease activity is therefore expected to be minimal in non-contaminated areas due to a lack of Nitrogen. However, in urine contaminated areas an increase in urease activity was expected. In a study by Seshabala & Mukkanti (2013) looking at urease activity between garden and urinated soil, a colour change of UBA from green to pink served as a direct indicator for the presence of urease rich bacteria. Seshabala & Mukkanti (2013) also reported on the effect of pH, enzyme activity, substrate concentration and temperature on microbial urease activity. Microbial urease is therefore used as a qualitative measure

for urea, locations which were presumptively contaminated with urine were confirmed as contaminated by this assay.

Table 1: Qualitative Urea Base test for urease activity at the 4 various sampling sites.

Suburb	Presumption	Urea Base Agar
Dorado	Contaminated	+++
	Non-contaminated	-
Greenwell Matongo	Contaminated	++
	Non-contaminated	-
Wanaheda	Contaminated	+
	Non-contaminated	-
Hakahana	Contaminated	++
	Non-contaminated	-

Pure colonies that were prepared from CLED and TSI were subjected to numerous biochemical tests. A flow chart was followed sequentially in determining the biochemical assay to be carried out, which had led to the determination of the Genera by elimination. Culture based methods and biochemical characterization has long been used to evaluate the ecology of bacteria in drinking water (Hussain et al., 2013). The results of the various biochemical tests are tabulated below (Table 2). This tests allowed for the identification of 6/11 gram-negative microorganisms. Most of the isolates were facultative aerobes.

Table 2: Results of the various biochemical test and the genus identity of the isolates.

No	Origin of bacterial Colony	Colony Description	Gram	Spore stain	Simple stain (Pleoromorphism)	Acid-Fast	Catalese test	Oxygen Requirement	Motility test	Genera
1	Eveline street, Non-contaminated	Cocci, orange smooth, raised	-				-	Facultative	Motile	*Neisseria*
2	Eveline street, Contaminated	Rod, pink smooth, solid edge	+	Spore former	Regular (road shaped)	-	-	Facultative	Motile	*Bacillus*
3	Ara Bar, Contaminated	Rod, cream	+	Spore former	Regular (rod shaped)		+	Facultative	Motile	*Bacillus*
4	Ara Bar, Non-contaminated	Rod, pink	-					Facultative	Non-motile	*Shigella/ Klebsiella*
5	Single-quarters, Non-contaminated	Cocci, pink, granules	+	Non-spore former	Regular (rod shaped)	-	+	Facultative	Non-motile	*Staphylococcus/ Streptococcus*
6	Eveline, Non-contaminated	Cocci	+	Spore former					Motile	*Sporosarcina*
7	Eveline, Non-contaminated	Rod, white	-					Facultative	Motile	*Escerichichia; Enterobacteria Proteus; Salmonella*
8	Eveline, Non-contaminated	Cocci, yellow	+	Spore former	Regular (rod shaped)			Facultative	Motile	*Sporosarcina*
9	Hakahana, Non-contaminated	Red, dark green, raised	-					Facultative	Non-motile	*Shigella/ Klebsiella*
10	Single-quarters, Contaminated	Cocci, pink	-				-	Facultative	Motile	*Neisseria*
11	Ara Bar, Non-contaminated	Rod, yellow	-				+	Aerobic	Motile	*Neisseria*

The study by Hussain et al. (2013)on the biochemical characterization and identification of bacterial strains in drinking water samples has identified potential human pathogenic bacteria such as *Enterococcus* and *Staphylococcus* from different samples.

In our study, pathogenic microorganisms from 11 genera were identified: *Neisseria, Bacillus, Shigella, Klebsiella, Staphylococcus, Streptococcus, Sporosarcina, Escherichia, Enterobacter, Proteus, and Salmonella*. These are directly associated with urinary tract infections (UTI's). UTI associated microorganisms can be acquired in a hospital setting. Individuals that void may not know that they have UTIs as it can be asymptomatic. The isolation and identification of these microorganisms in this study is an indicator that urinating introduces pathogenic microorganisms to the soil. The association of these microorganisms to UTI has been discussed in the literature review section. It is also worth noting, that microorganisms such *Pseudomonas aeruginosa, Acinetobacter, Klebsiella, Serratia*, and *Aeromonas* bacteria may be naturally present in the environment. They may be able to cause disease in vulnerable subpopulations, especially those with extensive wounds. This organisms can therefore be considered as opportunistic (Soto, 2014). There is a possibility of infection, resulting from the exposure or contact with microorganisms, which will not only result in Urinary tract infections, but also other bacteria-related infections of the skin, eye, nose etc.

At Wanahenda, Hakahana and Greenwell matongo (Figure 2B, 3) suburbs of Katutura it has been found that, a contaminated site harbours more pathogenic bacteria and less beneficial actinobacteria, however, at a control site. There are more beneficial actinobacteria and less of the pathogenic UTI causing microorganisms. However, an anomaly has been found at the contaminated site in Dorado (Figure 2A), which is also the most contaminated deducing from the triple positive urease activity. At this site in Dorado, there number of beneficial actinobacteria is more than that of the pathogenic causing microorganisms. Actinomycetes are one of the predominant members of soil microbial communities and they have beneficial roles in soil nutrients cycling and agricultural productivity. Hence, at any given point, in the absence of contamination, the counts of actinobacteria are expected to be higher. Dilution plate counts were used to quantify actinomycetes numbers in different soil ecosystems for numerous studies, this method have allowed for the quantification of bacterial community size can of more than 106 cfu g^{-1} soil, which apparently cannot reflect actual numbers of actinomycetes in soil. Nonetheless, the plate count method was considered to be useful in the evaluation of the abundance of common actinomycetes in soil(Ghorbani-Nasrabadi, Greiner, Alikhani & Yakhchali, 2013). Actinobacteria were found to be generally more than that of the pathogenic bacteria in non-contaminated sites, which suggests that the soil nutrients remain undisturbed in non-contaminated soils. Since the soil in these suburbs are not being used for agricultural purposes, the effect on the soil chemistry may not be detrimental as such. From the reduction of actinobacterial communities, and flourishing of pathogenic microorganisms is another indicator of the modification of the soil chemical make-up. There increased presence of pathogenic microorganisms at contaminated sites is an indicator of the potential risk of infection that exists.

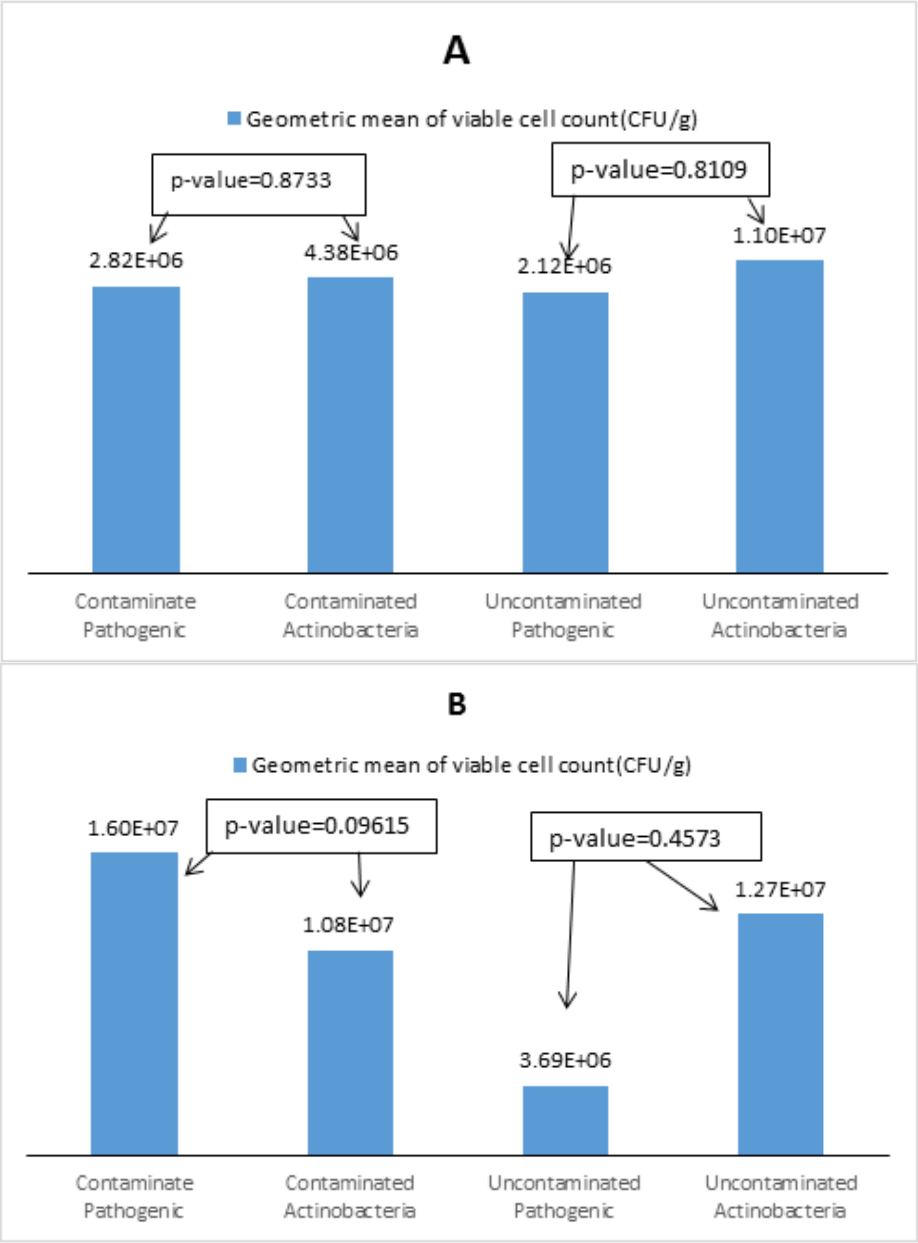

Figure 2: The graph shows the geometric mean of viable cell counts at (A) Dorado and (B) Wanaheda locations in both contaminated and non-contaminated soils. The counts of the pathogenic bacteria and actinobacteria are shown.

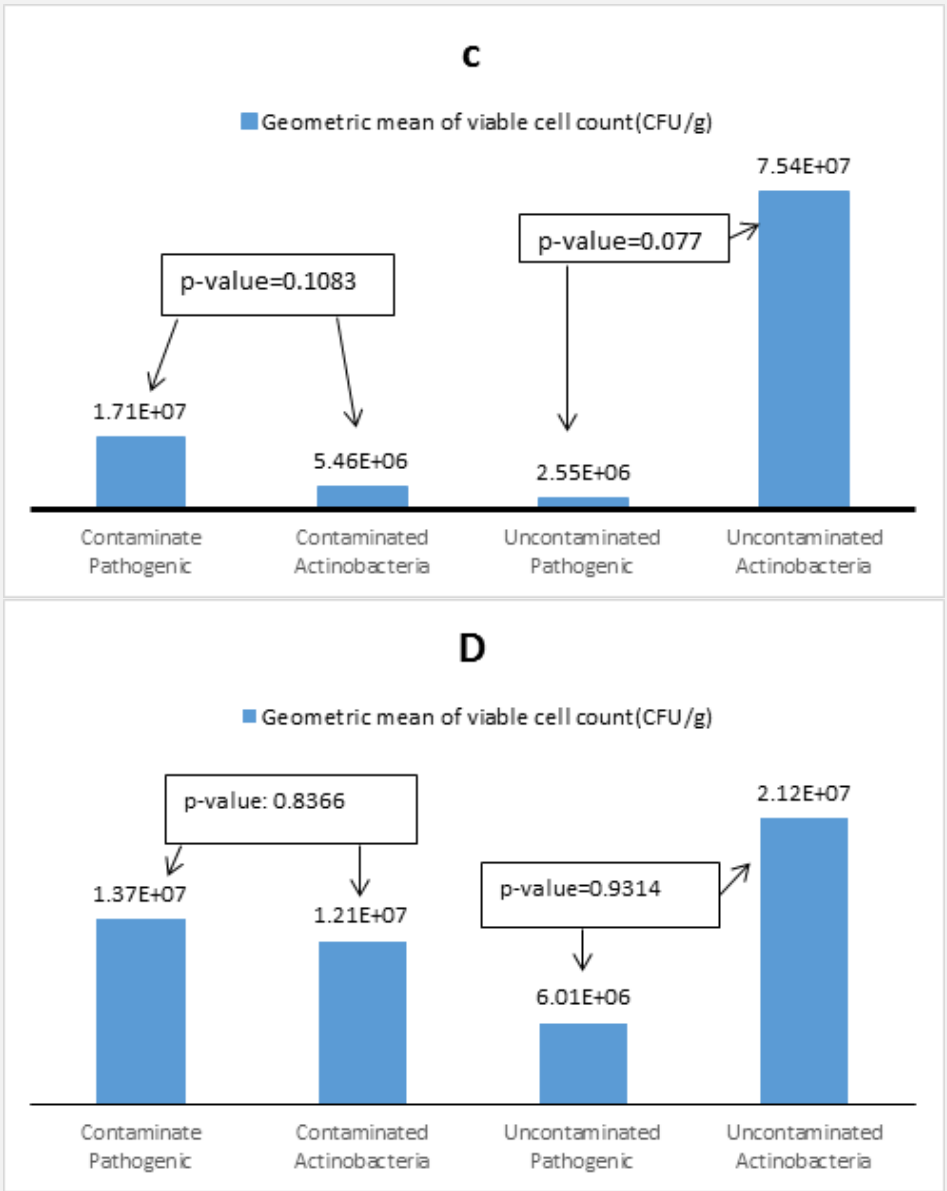

Figure 3: The graph shows the geometric mean of viable cell counts at (C) Greenwell Matongo and (D) Hakahana locations, in both contaminated and non-contaminated soils. The counts of the pathogenic bacteria and actinobacteria are shown.

5 Conclusion

It is clear that urine contamination leads to a reduction in there number of actino-bacteria which is harmful to the soil's properties. The soil properties are more important for soil bacterial composition than vegetation. Although, this study has not looked into the physico-chemical properties of the soil, the process of urine hydrolysis leads to an increase in the ammonia and carbamic acid content. The presence of urine also affects the soil pH and organic content, which affects a number of biological processes, including energy provision for microbial communities. Ablution facilities should be made available to avoid this and penalties should be put in place to discourage this behaviour. This was a preliminary study and should be followed up to determine the full molecular identification of bacteria to determine what these microorganisms are as it may give insight into the prevalence of UTI's in the demographic groups that frequent bars. A sensitivity test of the isolates to the existing regimens or antibiotics may also be necessary especially with the continued increase in drug resistance

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