

Determination of bacterial load and antibiotic susceptibility testing of bacteria isolated from students' toilets at Sokoine University of Agriculture, Morogoro, Tanzania

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Abstract

The circulation of infectious diseases in the community settings in urban and rural areas remains to be a hectic problem. One of the sources of microbial diseases is toilets. This study aimed at isolating, identifying and establishing bacterial loads associated with public restrooms in students' hostels at Sokoine University of Agriculture in Morogoro, Tanzania. Samples were collected from a total of thirty toilets (60 samples) in different surfaces; (i) surfaces associated with toilets (toilet seats and toilet bowls), (ii) surfaces routinely touched with hands (door handles in and out of the restrooms, faucet handles and toilet flush handles) and (iii) the restroom floors. Samples were inoculated in MacConkey and Blood agar and then incubated at 37°C for 24 hours. All isolates were sub cultured and identified based on macro- and micro-morphology and Standard Biochemical Tests. The establishment of total bacteria load was done using Standard Plate Count Method. The sensitivity testing of the isolates were carried out using the Disk Diffusion Method on nutrient agar plate. The following bacteria genera and species were isolated from the students' toilets; *Staphylococcus aureus* (25.0%), *Escherichia coli* (36.7%), *Pseudomonas aeruginosa* (13.3%), *Streptococcus pyogenes* (6.7%), *Proteus mirabilis* (6.7%) and *Klebsiella pneumonia* (11.6%). The results from total bacterial count indicated that the surfaces routinely touched with hands had highest bacteria load compared to restroom floor and toilet seats. However, the differences of means among the surfaces were not statistically significant ($P = 0.6762$). Sensitivity testing of the isolates against commonly used antibiotics in the study area showed that all bacterial isolates tested were resistant and intermediate resistant to at least one antibiotic.

Keywords: Pathogenic bacteria, Students' hostels, bacteria count, antibiotic susceptibility testing.

1. Introduction

Bacteria are microscopic organism found everywhere in the Universe as pathogenic or non-pathogenic. They are found in the environment all around us and within each one of us, there are trillions and trillions of them. Majority of them are harmless to human and animals but those few which are harmful can lead to death of affected individuals (Hooper 2001; Short et al. 2007).

Public restrooms may contain a variety of dangerous bacteria, including from genus *Escherichia*, *Salmonella*, *rotavirus*, cold virus and *Staphylococcus* including Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus* (Hooper 2001; Peleg and Hooper 2010; Hooper et al. 2010; Flores et al. 2011; Adewoyin et al. 2013). They get in the restrooms via human excreta (urine and feces) (Viraraghavan et al. 2007). Improper use of the toilets, inadequate cleanliness of the toilets facilitates can transmit bacteria from the toilets to the household living rooms. Contaminated hands of toilet users can transmit the bacteria from their hands to the

flushing handles, door handles and faucets of the toilets as well as household door handles and equipment. Toilet seats and lids, the surrounding floors, and the nearby surfaces can be contaminated by toilet flush aerosols which are produced in substantial quantities during flushing (Barker and Jones 2005). The ability of the pathogen deposited to survive on the different surfaces in the toilets poses a great risk of infection to the toilet users (Boone and Gerba 2007). The time of survival depends on the type of pathogen, majority including *Shigella* species, *Escherichia* species, *Clostridium* species, severe acute respiratory syndrome (SARS) coronavirus, and norovirus which can survive on surfaces for weeks or even months (Kramer et al. 2006).

Bacteria from public restroom are of public health importance when they enter the body through hand to mouth contact or hand to food contact leading to diseases (Sabra 2013). The diseases which can be contracted through the use of restrooms and the bacteria (in brackets) include; boil and food borne diseases (*Staphylococcus aureus* and *Escherichia coli*), Urinary tract Infections (UTI) and diarrhoea (*Escherichia coli*, *Pseudomonas aeruginosa*) and sore throat (*Streptococcus pyogenes*) (Peleg and Hooper 2010; Schmidt and Brubaker 2004). To reduce the risk of bacterial infection from the toilets, regular hand washing, thorough daily washing and cleaning of public restrooms with disinfectants (at least twice daily) is particularly recommended for infection control programs (Boyce 2007). New technologies of minimizing infections from public restroom include, sensor-operated paper towel dispensers and touch-free-electric hand dryers (Agbagwa and Nwechem 2010). Also closing the toilet seat can reduce the number of microorganisms released into the air (Schmidt and Brubaker 2004).

The status of pathogenic bacteria in the public toilets of Tanzania that may lead to human infections and diseases has not been established. The number of toilets in public places is very few compared to the number of people using the toilets. This is also the case for the number of toilets in majority of the Universities in Tanzania. At Sokoine University of Agriculture (SUA) the total number of students in one hostel in 2013 was 96 making an average of 16 students per 1 toilet. The inefficient cleanliness of toilets, shortage of water at SUA also predisposes students to bacterial infections and diseases such as Urinary Tract Infections (UTIs), diarrhea, typhoid, food borne disease and skin rushes. Diagnostic reports from SUA Dispensary (personal communication) indicated that there was good number of students who were getting bacterial diseases such as UTIs and typhoid. One source of bacterial infections could be the students' toilets. Therefore, this study aimed at identifying pathogenic bacteria from students' toilets that might be the cause of some diseases to students at SUA. The information obtained in this study will help students to take hygienic precautions when using toilets and advice accordingly the management of SUA on the importance of effective cleanliness.

2. Materials and Methods

2.1 Study area

The study was conducted at the Sokoine University of Agriculture (SUA) students' hostel toilets in Morogoro, Tanzania. The laboratory processing and analysis of data were carried out in the Veterinary Microbiology laboratory at the Faculty of veterinary medicine, SUA.

2.2 Sampling frame and sample size

Samples for this study were collected at a single point in time from the students' toilets randomly. Two lists of all the toilets for the female and male students respectively were developed. There were 116 male toilets and 54 female toilets and were treated separately during calculation of sample size. A systematic random technique was employed to get the number of samples used in this study. A total of 30 toilets (15 for male and 15 for female) were sampled for bacterial isolation and identification. The sample size was calculated using a systematic random sampling method below.

$$k = N/n$$

Where,

k = sampling interval (Males 8, Female 4)

N=Total number of toilets (Males 116, Females 54)

n=sample size (Males 15, Female 15)

2.3 Sample collection

From each toilet, three samples were collected in the following areas; surfaces associated with toilets (toilet seat and toilet bowl), the restroom floor, and surfaces routinely touched with hands (door handles in and out of the restroom, faucet handles and toilet flush handles). The surfaces were sampled using sterile cotton-tipped swabs and each swab kept into a separate bottle containing sterile transport media (Stuart media). The bottles were labeled to indicate date of collection, hostel, toilet and the type of surface where the sample was collected. Then samples were transported to the laboratory located to about one kilometer from the toilets for further processing and analysis.

2.4 Bacterial isolation and identification

MacConkey and Blood agar were prepared for bacterial inoculation. Swab samples were directly inoculated onto MacConkey and Blood agar plates. The inoculated plates were incubated at 37 °C for 24 – 48 hours before being subjected to secondary culture and identification. The single colony of grown bacteria were isolated and sub-cultured on a pure solid media. Biochemical tests were conducted (CDC and WHO 2003) and included catalase, coagulase, oxidase, IMVIC, triple sugar iron (TSI) agar test and Kligler iron sugar (KIA).

Identification of bacterial isolates was carried out by using macro-morphological characteristics of the bacterial colonies grown, micro-morphology of the bacteria on Gram stain and the biochemical characteristics of bacterial isolates.

2.5 Total bacterial count from the isolates

Bacteria were counted by using Standard Plate Count Method. Samples were diluted in normal saline followed by 10-fold serial dilution with 10 dilution steps per sample. One millilitre of each dilution was added to a sterile agar plate and 15 ml of plate count agar pre-cooled to 45 °C was poured onto each plate and swirled gently to mix. After agar solidification the plates were inverted and incubated for 72 h at 30 °C. Counting the number of colony forming units (CFU) per millilitre of the sample was done by dividing the plate into four equal parts for easy counting. All the plates contained between 30 and 300 colonies for easy counting but yet represented the sample. Bacteria were grown by plating duplicates of each dilution in order to have greater accuracy.

2.6 Antibiotic susceptibility testing

A pure single colony grown overnight on blood agar was picked up using inoculating wire loop and placed in the tube containing normal saline. The mixture was then mixed thoroughly up and down using the Pasteur pipette to create a smooth suspension. The Whatman filter paper was used to prepare disks by punching using normal office two holes paper puncher. Prepared disks were placed in a sterile bottle and autoclaved for 15 minutes in 121 °C at 15 lbs. The sterilized disks were soaked in a prepared solution of different antibiotics commonly in use in Morogoro. The disks were soaked in the following antibiotics: Ciprofloxacin (Cip, 5 µg), Chloramphenicol (Chl, 30 µg), Gentamycin (Gen, 10 µg), Erythromycin (Ery, 15 µg) and Ampicillin (Amp, 10 µg) based on Clinical and Laboratory Standards Institute (CLSI, 2013) recommendations. The disks were removed from the solution after absorbing and allowed to dry in the incubator before being placed onto the inoculums.

The antibiotic susceptibility testing of the isolates were carried out using the Disk Diffusion Method on nutrient agar plate (CDC and WHO 2003). This test was employed to obtain the susceptibility of bacteria isolated from students' toilets to different antibiotic agents currently used in Morogoro Municipal hospitals.

Petri dishes with Nutrient agar were flooded with inoculums, allowed to distribute equally and to dry on the bench. Antibiotic disks were then applied on the surface of inoculated agar using sterile forceps. Finally the plates were incubated overnight at 37 °C.

Data were recorded based on the clear zones of inhibition. Zones were measured in millimeter (mm) using a ruler and compared to a standard interpretation chart based on performance standards for Antimicrobial Disk Susceptibility Tests (CLSI 2013) used to categorize the isolates as susceptible, intermediate susceptible or resistant.

2.7 Statistical analysis

Descriptive statistics of bacterial isolates from different hostels and surfaces were computed in Microsoft Excel 2013. The bacterial counts of samples were converted into logarithm of number of colony forming units per ml (log CFU/ml) for statistical analysis. Means were compared by employing analysis of variance (ANOVA built in GraphPad Prism version 6).

3. Results

3.1 Bacterial isolation and identification

The study has isolated and identified the following bacterial isolates from the different surfaces (restroom floor, toilet seat, toilet bowl, door handles in and out of the restroom, faucet handles and toilet flush handles) in the students' hostel toilets: *Staphylococcus aureus* (26.7%), *Escherichia coli* (36.7%), *Pseudomonas aeruginosa* (10.0%), *Proteus mirabilis* (6.7%), *Klebsiella pneumoniae* (11.7%) and *Streptococcus pyogenes* (6.7%). Bacteria were identified based on macro- and micro-morphological, and biochemical characteristics (Table 1). The frequencies of the bacterial isolates in the female and male toilets and in the different students' hostels are shown in Figure 1 and Table 2.

3.2 Total bacterial count of isolates from the students' toilets

Total bacteria load was done from samples collected in the different surfaces from the students' hostel toilets. Students in the New hostel were using European toilet while other hostels (Gaza and Main hostels) were using squat toilets, that is why total bacteria load in toilet seat was done only in the New hostel. The range of bacterial load in each sample source were as follows; those found on toilet seat (S) ranged from 3.2×10^4 to 2.24×10^6 CFU per ml, on the restroom floor (F) ranged from 5.8×10^4 to 2.96×10^7 CFU per ml and on door handles in and out of the restroom, faucet handles and toilet flush handle (H) ranged from 3.1×10^4 to 2.96×10^7 CFU per ml (Table 4). There was no statistical significance of bacterial load between the different surfaces sampled in male ($F = 0.3882$; $P = 0.6872$) and female ($F = 0.07407$; $P = 0.9288$) toilets at 95% level of confidence interval (Figure 2A and 2B).

3.3 Antibiotic susceptibility testing of bacterial isolates

The sensitivity tests were done using five diffusion disks containing five different antibiotics that are commonly used in Morogoro. The interpretation of results was based on the Standards for Antimicrobial Susceptibility Testing established by the Clinical and Laboratory Standards Institute (CLSI, 2013). Table 3 shows results for the sensitivity testing of bacterial isolates against antibiotics.

4. Discussion

This study aimed at isolating, identifying and establishing the bacterial load in the students' toilets at SUA in Morogoro, Tanzania. It has been reported that some people have a habit of not washing their hands after using public restroom and those who wash hands use short time with or without soap (Toshima et al. 2001). This has an implication in transmitting microorganisms from one area to another and from one individual to

another. We thought it was essential to study the type and the level of bacterial present in the students' hostel toilets. Majority of the students do not wash their hands and there is a high chance of moving microorganisms from the toilets to toilets door handles then to hostel room door handles.

The study has isolated and identified the following bacterial from the different students' hostel toilets: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Streptococcus pyogenes*. Studies by Agbagwa and Nwechem (2010) indicated *S. aureus* to be more prevalent in public toilets while studies by Adewoyin et al. (2013) indicated *Streptococcus* species (especially *Str. faecium*) to be more prevalent in the toilet-bowl of students' hostels. Both findings are contrary to this study, where *S. aureus* had the highest frequency in the toilets. The study has also showed that the frequency of bacteria isolates was higher in the new hostel (NH) followed by Hostel 1 (H1) and Gaza hostel (GH) possibly depending on the number of students in the hostels, poor sanitary conditions and lack of regular maintenance (washing and cleaning with disinfectants). The number of students and toilets were 66 and 12 in one hostel (Gaza) respectively, making an average of 6 students per toilet. The number of students and toilets in the Main hostel (Hostel 1- 7) were 96 and 6 respectively, making an average of 16 students per toilet. The New hostel had the highest frequency of isolates where the number of students in one hostel were 660 making an average of 17 students per toilet.

Possible diseases that can be caused by the isolated bacteria include Foodborne diseases (*S. aureus* and *E. coli*), Urinary tract infections (*E. coli* and *Ps. aeruginosa*), Pneumonia (*K. pneumoniae*), Sore throat (*Str. pyogenes*) and Diarrhea (*E. coli*) (Agbagwa and Nwechem 2010). *Escherichia coli* has been reported to be the most common cause of UTIs with some clones that may also be associated with gastrointestinal infections (Schönning et al. 2004).

Total bacteria load was done from samples collected in the different student hostel's toilets. The results of this study showed that the surfaces routinely touched with hands had the highest bacteria load compared to restroom floor and toilet seats. One could expect the opposite to be true. This observation could be due to cumulative contamination of door handles as result of poor sanitary conditions (not washing and cleaning hands with disinfectants after using the toilets). A study carried out on public female restrooms at Taif, Kingdom of Saudi Arabia on bacterial public health hazard (Sabra 2013) indicated similar findings. Also other studies have documented that college students are not always the most diligent of hand-washers (Anderson et al. 2008). This study has also showed that females' hostel toilets had the highest bacteria load compared to males hostel toilets (Figure 1). This observation is contrary to the previous studies where females were said to be good for keeping hygienic rules (Anderson et al. 2008). Toilet seats can act as vector for transmission of bacterial diseases especially in public toilets (in schools, hospitals, bus stands, bars and clubs). These results highlight the importance of hand-hygiene when using public restrooms since these surfaces could also be potential vehicles for the transmission of human pathogens. The use of alcohol wipes on the toilet seats prior to use of the toilets may result in a 50-fold reduction in mean daily bacterial counts and elimination of the bacteria (Giannini et al. 2009). Also cleaning the bathrooms, restrooms, toilet seats, toilet floor and toilet door handles reduces the bacterial burden tremendously (Boyce 2007; Tuladhar et al. 2012).

The determination of the antibiotic susceptibility patterns showed that all bacterial isolates tested were resistant and intermediate resistant to at least one antibiotic commonly used in Morogoro. Only *Streptococcus* species showed to be susceptible to Erythromycin. The resistance of bacteria to antibiotic commonly in use is an increasing problem in many parts of the world and especially developing world (Giannini et al. 2009). Resistance of bacteria to Ampicillin, Amoxycillin, Chloramphenicol, Streptomycin, Spectinomycin, Cotrimoxazole, Trimethoprim-Sulfisoxazole, Kanamycin, Tetracycline and Gentamycin have been documented in different parts of Africa (Kimang'a 2012). In those parts *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* have been reported to be multidrug resistant. Studies by Paryani et al. (2011) to determine the pattern and sensitivity of pathogens causing urinary tract infection at tertiary care university hospital proved that pathogens causing urinary tract infections are developing resistance against commonly used antibiotics. In developing countries like Tanzania misuse of the antibiotics is among the main factor leading to antibiotic resistance. The misuse of antibiotics accelerates the evolution of resistant strains of bacteria. The improper use of antibiotics in humans and livestock, wrong and substandard prescriptions by unqualified 'medical personnel' together with poor diagnosis or lack of it at all

have been reported to be among the main factors contributing to the development of resistant microbes in African countries (Kimang'a 2012). To fight against antibiotic resistance in Tanzania, a collaborative effort is needed as there more than one sector involved in the use of antibiotics for treating diseases. All stake holders involved (susceptible human, livestock and human medicine experts and governmental officials) in the provision and use of the drugs should be educated on the effects of indiscriminate use of antibiotics in humans and livestock.

4. Conclusion

From this study it can be noted that surfaces touched with hands (door handles in and out of the restroom, faucet handles and toilet flush handles) had the highest level of bacterial contamination. This can act as good source of transmission of pathogenic diseases to human via contaminated and improperly washed hands. The study showed that there were pathogenic bacteria from students' toilets that might be the cause of some bacterial diseases to students at SUA. Regular cleaning of the restrooms and hand washing with disinfectants is the first line of defense in preventing the spread of diseases emanating from restrooms. The cleaning of restrooms should be accompanied with the use of modern technologies available for improving hygiene and sanitary procedures.

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Table 1: Morphological and Biochemical identification of the isolates from students' toilets

Bacterial isolate	Cultural characteristics	Gram's stain reaction	Biochemical tests								
			CT	Urease	Oxidase	Indole	TSI (H ₂ S)	MR reaction	VP reaction	Catalase activity	Coagulase production
<i>E. coli</i>	White on BA, smooth, non hemolytic. LF on MC, medium colonies	Gram –ve rods in singles	-ve	-	-ve	+ve	-ve, gas	+ve	-ve	-	-
<i>K. pneumoniae</i>	Mucoid, no haemolysis on BA, LF on MC, medium colonies	Gram –ve rods in singles	+ve	-	-ve	-ve	-ve, gas	-ve	+ve	-	-
<i>Ps. aeruginosa</i>	Greeish, hemolysis on BA, raised, mucoid, cattle eye appearance, NLF on MC, medium colonies	Gram –ve rods in singles	+ve	-	+ve	-	-	-	-	-	-
<i>P. mirabilis</i>	Swarming colonies and chocking smell on BA	Gram –ve rods in singles	-	+ve	-ve	-ve	+ve	+ve	-	-	-
<i>S. aureus</i>	Golden colour, A- hemolytic on BA, small colonies	Gram +ve cocci in clusters or grapes	+ve	-	-ve	-	-	-	-	+ve	+ve
<i>Str. pyogenes</i>	Small, white, shine, raised colonies, B-hemolysis on BA	Gram +ve cocci in chains	-ve	-	-ve	-	-	+ve	-ve	-ve	-ve

- = not applicable, +ve = Positive; -ve = Negative; BA = Blood Agar; CT=Citrate, TSI= Triple Sugar Iron, VP= Voges-Proskauer, MR= Methyl Red, MC=Mac Conkey , LF= Lactose Fermenter, NLF=Non Lactose Fermenter

Table 2: Frequency (%) of bacterial isolates based on sample source and the hostels

Bacterial isolates	Sample source		Hostels		
	H(n=28)	F (n=32)	H 1(n=18)	NH(n=26)	GH(n=16)
<i>E. coli</i>	32.1	40.6	55.6	30.8	31.3
<i>S. aureus</i>	28.6	21.9	33.3	15.4	31.3
<i>Ps. aeruginosa</i>	21.4	6.3	5.6	11.5	18.8
<i>K. pneumoniae</i>	7.1	15.6	0	23.1	6.3
<i>Str. pyogenes</i>	10.7	6.3	5.6	7.7	6.3
<i>P. mirabilis</i>	0	9.4	0	11.5	6.3

Key: H - surfaces routinely touched with hands (door handles in and out of the restroom, faucet handles and toilet flush handle), F - toilet seat and restroom floor, H1-hostels in main campus, NH- new hostel and GH - Gaza hostels and n – Total number of bacteria isolate in the respective hostel.

Table 3: Inhibition zones (diameter in mm) for the sensitivity testing of bacterial isolates against antibiotics

Bacteria	Ciprofloxacin		Ampicillin	Chloramphenicol	Gentamycin	Erythromycin
<i>K. pneumoniae</i>	16	(I)	12 (I)	0 (R)	11 (R)	15 (I)
<i>S. aureus</i>	9	(R)	0 (R)	16 (I)	17.5 (I)	6 (R)
<i>Ps. aeruginosa</i>	16.5	(I)	8 (R)	6 (R)	16 (I)	15 (I)
<i>Str. pyogenes</i>	0	(R)	11 (R)	8 (R)	15.5 (I)	20 (S)
<i>E. coli</i>	15.5	(I)	13 (I)	15 (I)	15 (I)	12.5 (I)

Key: I= Intermediate; R=Resistance; S= Susceptible

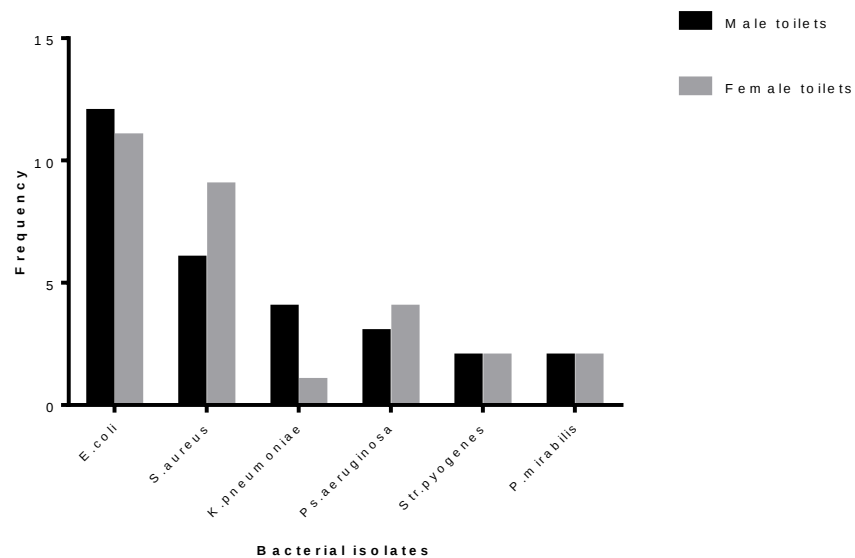


Figure 1: The frequency of bacterial isolates in the females and males students' toilets

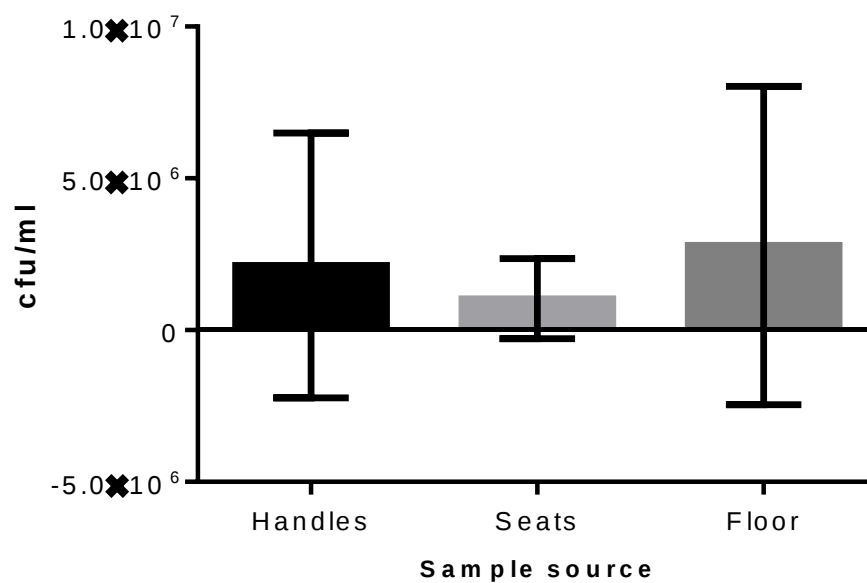


Figure 2 A Total bacteria load obtained from isolates in males' toilet based on the area sampled

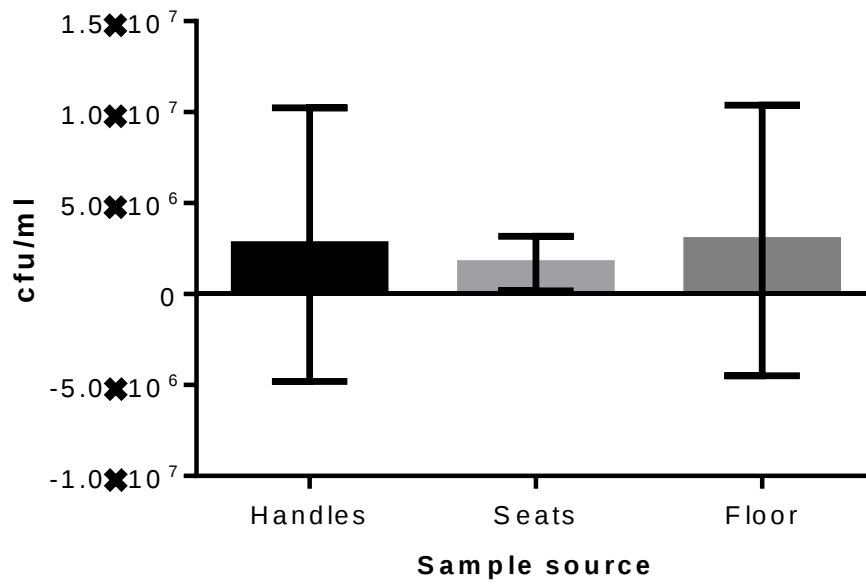


Figure 2 B Total bacteria load obtained from isolates in females' toilet based on the area sampled