Microbiological Analysis of Tissue Paper Pre- and Post-Exposure in Restrooms and its effects to women's health

Sewgil S. Anwer[1], Kawther A. Muhammad Saeed[1], Muzhda Q. Qader[2], Zena B. Ali[1], Esra A. Esmail [1], Awaz I. Shekh [1], Sara S. Shakir [1]

[1]Department of Clinical Biochemistry, Hawler Medical University, Kurdistan Region, Iraq.

[2]Department of Public Health, Hawler Medical University, Kurdistan Region, Iraq.

https://doi.org/10.15218/crewh.2024.07

Abstract:

Back ground and Objectives: Toilet tissue paper are sanitary paper that comes into direct contact with the body but at the same time they are a good place for growth of microorganism. The objective of this study was to isolate, identify and evaluate the presence or absence of bacterial and fungal contaminants present on tissue paper remained in toilets.

Methods: Ten toilet tissue paper brands commercially available in Erbil City were tested for bacterial and fungal examination before placing them in toilet (zero time) and after placing them in five (5) different sites that determined within the college of health sciences building's toilets then the samples cultured on (Blood agar, Mannitol salt agar, PDA, and Nutrient agar) the microorganisms were identified based on their morphological characteristics and biochemical test (API 20E test).

Results: The study showed no bacterial growth found on the tissue paper before placing them in toilet while *Aspergillus niger* showed growth on (Gi, Al and NM) tissue paper before placing in toilet. After remaining of tissue papers in different toilet sites , *Pseudomonas fluorescens* isolated from female cafeteria (Selpak), *Serratia liquefaciens* isolated from cafeteria male (Solo) and students toilet for both gender (Gipsy), *Salmonella* spp. isolated from students toilet for both gender (Limpio), *Staphylococcus aureous* isolated from female dean (Alwazir), *Bacillus* sp. isolated from dean building (Fine) and male and female cafeteria(Papia and Selin), *Clostridium tetani* isolated from female dean (Familia and Alwazir) and dean building (No brand), and finally, *Streptococcus* spp. have isolated from male cafeteria (Solo). The fungi that have been isolated from these sites were (*Aspergillus niger, Aspergillus candidus, Rhizopus oryzae, Penicilium citrinum, Penicilium camemberti, Aternaria alternate*)

Conclusion: Different bacteria and fugal species identified after remaining the tissue papers in toilet sites that indicate transmission of bacteria present in toilet to tissue papers. contaminated toilet tissue can pose serious health risks for women, including urinary tract infections and vaginal infections due to exposure to harmful bacteria and fungi.

Key Words: Tissue paper, Isolation, Identification, Bacterial species, Fungal species, hygiene, toilets.

Introduction:

Tissue papers are very different from each other in their content and production, example: the biggest difference between toilet paper is the distinction between virgin paper products which are formed directly from chipped wood, and those made from recycled paper, Toilet paper is generally made from new or "virgin" paper which is generally a combination of approximately 70% hardwood and 30% softwood using softwood and hardwood trees. Softwood trees such as Southern pines and Douglas firs have long fibers that wrap around each other; this gives paper strength. Hardwood trees like gum, maple and oak have shorter fibers that make a softer paper[1, 2]

Paper towels are made from raw and recycled materials containing cellulose, lignin, and other nutrients suitable for the growth of microbes present in papermaking environments. The main sources of microbiological contamination in paper mills are the recycled waters, the raw materials used, parts of the machinery, and the factory environment [3]. Studies previously established that the main microbial contaminants in paper products belong to the spore-forming genera Bacillus. Indeed, because of their high resistance to a wide range of chemical and physical agents, Bacillus spores may survive the various procedures encountered in the papermaking process. It is worth mentioning that harmful toxin-producing Bacillus species were also detected in paper mills [4].

Toilet paper with highly recycled content may be inexpensive or easily obtained, such that it was reported that toilet tissue manufacturers have one of the highest recycled paper utilization rates, it is still important to note that products with recycled contents, like toilet rolls, may contain certain toxic bio-aerosols and chemicals during production. Airborne pathogenic bacteria and fungi from toilet roll dusts can be suspended air as respirable bio aerosols that can be readily inhaled. In modern usage, toilet rolls would seem to play an important role as barrier to transmission of enteric infection by fecal manual- oral route. According to some authors [5].

Bacteria in the restroom can transfer from surfaces to the human body through something called, charmingly, the fecal-oral route. It's the main way in which fecal material and its accompanying nasties can make its way into the human body. But unless human going around licking unwashed surfaces in the bathroom, probably aren't likely to develop infections from any of these bacteria, especially if human practice good hygiene and wash hands after using the restroom [6].

Different type of bacteria have been isolated from public surfaces by giving information on the relative hygiene of commonly encountered public surface, they identify the environments with contaminants and risk of exposures [7]. According to Humphrey (1994) Human carriers are the main reservoir host of infections. Both the feces and the urine may find their ways on the contact surfaces of the door handles via a hand touch since the hand is the major public serving as the vehicle of transmission of common human disease to a susceptible host. The aims of this study are to investigate microbial growth on common used toilet tissue papers in Erbil city and determine bacterial and fungal colony on plates with tissue paper that will remain in toilets.

Material and Methods:

The cultural media:

Nutrient agar, Mannitol salt agar, Blood agar, Potato dextrose agar used in the study. All the media mentioned in this study were prepared as recommended by company manufacture.

Methods:

Investigation of microbial growth on toilet tissue papers before using (zero time)

Ten toilet tissue paper brands (designated by cods), commercially available in Erbil City, were tested for microbiological examination. Approximately 5 small pieces of paper of the desired test paper were selected in the middle of the Roll, transferred with sterile forceps in a sterile medium for bacterial and fungal studies.

Isolation of microorganisms after remaining in different toilet sites:

A microbiological isolation was done according to [8, 9]. Five (5) different sites were determined within the college of health sciences building's toilets. Door handles of the college public toilets were used by remaining of tissue papers (Nutrient agar, Blood agar, Mannitol salt agar for bacterial growth and Potato Dextrose Agar (PDA) used for fungal growth each of toilet in Dean building, male cafeteria toilet, female cafeteria toilet and toilet that used by male & female students together, and female dean toilet. The samples were collected after one day remaining of tissue papers in the toilet and then used

for identification of microorganisms (Figure 1), plates with Nutrient Broth and Potato dextrose agar without tissue paper remained in lab and used as control.

Figure (1) Preparation for tissue paper culturing

Identification of isolates:

Morphological identification:

The identification of bacteria was based on Morphological and Biochemical tests. Bacterial shape and colors obtained by staining with gram stain. The small amount of colony was taken, distributed on slide to make a smear and the smear stained with gram stain, Bacteria that retain the initial Crystal violet stain (or purple) are said to be "Gram-positive" whereas those that are decolorized with Ethanol and attain red with carbolfuschin (or safranin) are said to be "Gram-negative" [9].

For fungal identification, fungal isolates were transferred to sterilized plates for purification and identification. The fungal isolates were placed on a slide, stained with Lacto phenol cotton blue to detect fungal structures covered with a cover slip, examined under microscope and identified on the basis of their morphological structure and spore characteristics [10, 11].

Biochemical tests:

API 20E test (Analytical profile index):

To identify Enterobacteriaceae Api 20E strips (BioMerieux, Hazelwood, MO) used; isolated colony (from a pure culture) picked up and add into sterile distilled water. API20E biochemical Test Strip which contains dehydrated bacterial media/bio-chemical reagents in 20 separate compartments. By using micropipette, the compartments filled up with the bacterial suspension (up to the brim). Sterile oil added into the ADH, LDC, ODC, H_2S and URE compartments, some drops of water added in the tray and putted the API test strip and the tray closed. Then incubated at 37℃ for 18-24hrs [12].

Growth on Mannitol salt agar:

To determine *Staphylococcus aerogenes* purified bacteria, cultured on Mannitol salt agar then incubated at 37 ℃ for 18-24hrs., after growth the colour of medium recorded [13].

Growth on Blood agar:

To identify strains with gram positive cocci in chain, bacterial isolates inoculated on blood agar and appearance of hemolysis will be determined [14].

Results:

Microbiological quality of tissue paper before remaining in toilet (Zero time)

Ten different toilet tissue paper rolls available in Erbil City were examined to study their microbiological quality before and after remaining in toilet for 24 hr. After culturing the papers on nutrient agar at zero time without remaining in toilets no growth of bacteria showed after incubation, while fungal growth recorded in each of the following samples (Gi, Al and NM) when cultured on potato dextrose agar as shown in table (1)

Table (1) Occurrence of microorganism (bacterial and fungal colony) on tissue paper before remaining in toilet (zero time)

Cod	Tissue paper	Bacterial colony	Fungal colony
Sel	Selpak	-	-
Fin	Fine	-	-
Pap	Pappia	-	-
Sol	Solo	-	-
Sel	Selin	-	-
Fa	Familia	-	-
Gi	Gipsy	-	+
Li	Limpio	-	-
Al	Alwazir	-	+
Nm	No-brand	-	++
control	Medium without paper	-	+

Microbiological quality of tissue papers after remaining in toilet for one day.

Growth and number of colony (bacterial and fungal colony) is shown in table (2) after remaining the toilet tissue papers for one day in (5) different toil sites within the college of Health Sciences as public toilets.

Table (2) Microbiological quality of tissue paper after remaining in toilet for one day

Cod	Tissue paper	**Bacterial colony**	**Fungal colony**	Toilet Site
Sel	Selpak	+++++++	+++	Female cafeteria
Fin	Fine	++++++	++++	Dean down for both male and female
Pap	Pappia	++++++++++	++++	Female cafeteria
Sol	Solo	+++++++	++	Male cafeteria
Sel	Selin	++++++++	+++	Male cafeteria
Fa	Familia	+++++	+	Female dean up
Gi	Gipsy	+++++++++	+++	Students up both
Li	Limpio	+++	+	Students up both
Al	Alwazir	++++++++++	+++++	Female dean up
Nm	No-brand	+++++++	+++	Down dean for both male and female
control	Medium without paper	-	+++++	

Identification of Microorganisms

Bacterial identification:

Morphological characteristics of bacteria

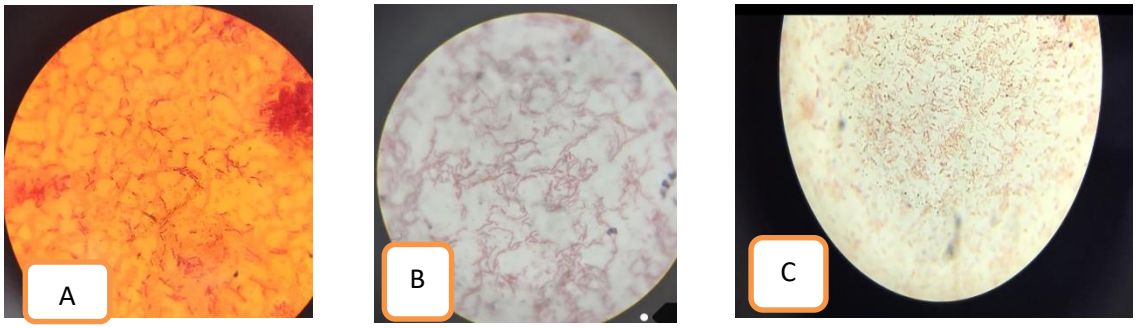
Bacterial shape and gram staining showed in tables (3 and 4) after examination under microscope. Both gram positive and gram negative were observed under microscope with different shapes.

Table (3). Gram negative and shape of bacteria from different sites of toilet

Toilet site	Gram staining	Shape
Female cafeteria (Selpak)	Negative	Rod
Male cafeteria (Solo)	Negative	Rod
Student up both (male & female) (Gipsy and Limpo)	Negative	Rod

(Table 4) Gram staining and shape of bacteria from different sites of toilet

Toilet site	Gram staining	Shape
Dean down for both male and female (Fine)	Positive	Bacilli- two and chain
Male & Female cafeteria (Pappia, Selin)	Positive	Cocci – Diplo and chain
Student up (limpo)	Positive	Cocci- diplococcus
Female dean up (familia & Alwazeer)	Positive	Cocci- Spore
Dean down for both (No-bran)	Positive	Cocci- Spore
Female up dean (Alwazeer)	Positive	Cocci- cluster



(Figure 2) Microscopic examination of gram-negative bacteria A, B and C- Rod shape.

Gram positive spore forming Rod drum stick shaped was identified as *Clostridium tetani*, spore forming rod shaped in chain identified as *Bacillus* sp. Other type of gram-positive bacteria identified by culturing on Mannitol salt agar and Blood agar.

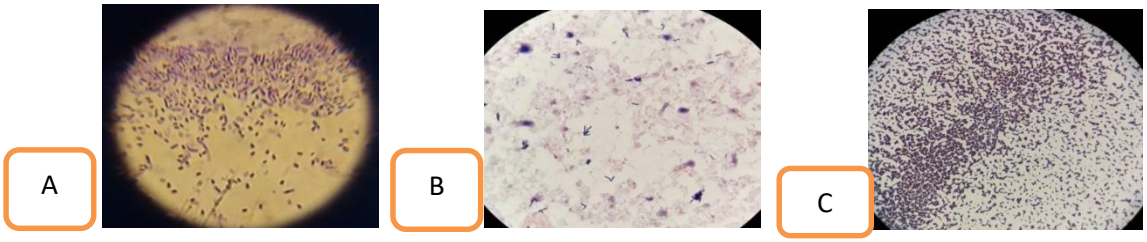


Figure (2) Microscopic examination of gram-positive Cocci A- diplococcal, B- chain, C- Cluster shape.

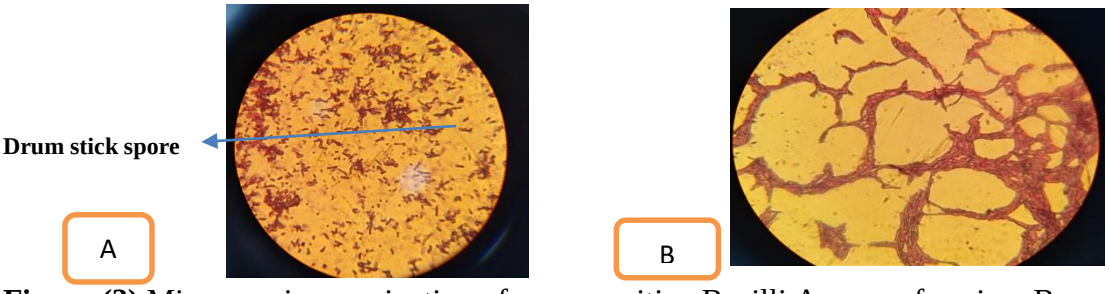


Figure (3) Microscopic examination of gram-positive Bacilli A- spore forming, B- non-spore forming bacilli

Biochemical tests API 20E tests:

Api 20 E test for gram negative bacteria

Biochemical tests for gram negative bacteria were performed to support the results using API20E test which is a rapid accurate technique for the identification of the family Enterobacteriaceae as follow; (the result of API20E for isolates of tissue papers remained in Cafeteria female was 2201004 (Selpak) and it was identified as *Pseudomonas fluorescens*, the API20E results of tissue papers remained in student up stair toilet (Gipsy) and cafeteria male toilets (Solo) were 7306763 that identified as *Serratia liquefaciens*, on the other hand the API20E results of tissue papers remained in student up stair toilet (Lipmo) stair was 6306752 that identified as *Salmonella* spp.).

Mannitol salt agar for identification of cluster shaped gram-positive bacteria

The bacteria that was gram positive cocci in cluster cultured on mannitol salt agar showed change of agar colour from red to yellow and was identified as *Staphylococcus aureous* figure (4)

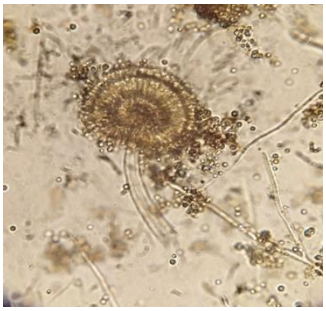
Figure 4. Mannitol salt agar for *Staphylococcus aureous*

Blood agar for identification of gram-positive cocci in chain

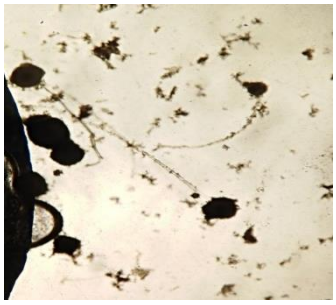
The bacteria that observed as gram positive in chain or diplococcus cultured on blood agar and hemolysis observed which was beta hemolysis clear zone around the colony.

Microscopic properties for fungal identification

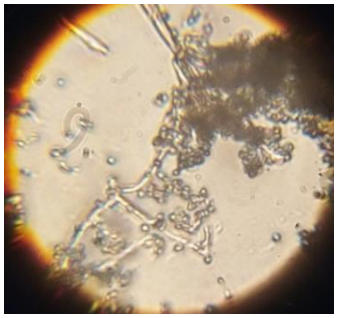
Microscopic examination for fungal isolates of tissue paper from male cafeteria toilet (Solo), female cafeteria toilet (Selpak) and toilet that used by male & female students together (Gipasy), showed dark brown to black conodiophore- conidial head radiate- with smooth walled, hyaline, Conidiophore-phialids borne on metulae and identified as Aspergillus *niger* . Fungal isolates from female dean toilet (Alwazir), toilet in Dean building (Fine), showed Colony whitish, sporangiophores arising directly from stolon without rhizoid- sporangia globules, dark brown to black colour that identified as Rhizopus *oryzae* figure (5)



Aspergillus niger

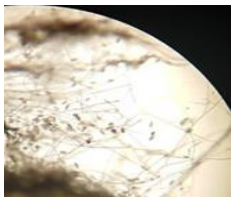


Rhizopus oryzae

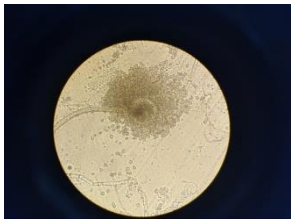


Peniciliu citrinum

Penicilium camemberti



Aternaria alternate



Aspergillus candidus

Figure (5) different Fungal isolates from toilet paper placed in bathroom

Two different species of Penicillium sp. isolated from toilet tissue paper that remained in cafeteria building (Papia) & (Selin), toilet that used by male & female students together (Limpio) with dean (male and female) (No Brand) for 24 hr, the first species showed conidiophore dark green to grey green in colour, metula long and branched identified *as Penicilium citrinum.* Conidiophore for the second species showed long 2-3 stages of branched; condiophore stipe rough walled, rarely smooth-walled, sometimes becoming ornamented. Metulae giving to phialides. Phialides flask shaped with short neck that identified as *Penicilium camemberti* and was isolated from dean (No Brand) and male & female student together toilets (Limpio)

The isolates of tissue paper remained in cafeteria building (Selpak) & (Solo) and toilet that used by male & female students together dean (male and female) (Gipsy) showed conidiophore stipe hyaline to slightly yellowish green, vesicle globule to sub globule, phialides born directly on vesicle, sue the features above the fungi identified as *Aspergillus candidus.*

Microscopic features that was used to help in identification of isolates of toilet tissue paper that remained in male & female students together (Limpio) and cafeteria toilets (Papia) & (Selin) for 24hrs. showed that conidia in long often branched chains, sometime ovoid or lipsoidal, several transvers and longitudinal depth pale to brown and can be identified as *Aternaria alternate.*

Aspergillus niger was isolated from (Gi, Al and NM) before remaining in toilet as zero time and the same isolates identified from PDA (Potato Dextrose Agar) without toilet tissue paper that used as control.

Discussion:

Public restrooms such as university are settings with microbial diversity because of the high rate of activities by individuals with different hygienic practice. The results of this study showed that out of the (10) tissue paper showed no growth of bacteria at zero time in contrast the fungal growth showed in each of (Gi, Al and NM) the reason of growth of fungi on tissue paper may be due of contamination of plates with spores during culturing the tissue papers or storage condition of papers in market or industries.

The present research was essential to determine the extent of bacterial and fungal contamination of various paper towel brands as well as to isolate and identify the bacterial and fungal community present in these commercial products, and to investigate the possible airborne and direct contact transmission of these bacterial contaminants during paper dispensing and after hand washing. The result of API20E for tissue papers remained in Cafeteria female was 2201004 (Selpak) and it was identified as *Pseudomonas fluorescens*, the API20E results of tissue papers remained in student up stair toilet (Gipsy) and cafeteria male toilets (Solo) were 7306763 that identified as *Serratia liquefaciens*, on the other hand the API20E results of tissue papers remained in student up stair toilet (Lipmo2) stair was 6306752 that identified as *Salmonella* sp, and the result for tissue paper that have remained in college dean toilet (Alwazer) was gram positive cocci in cluster cultured on mannitol salt agar showed change of agar colour from red to yellow and was identified as *Staphylococcus aureous*, the same bacterial strains isolated by [15, 16].

The tissue paper that have remained in the dean for both male and female with the tissue paper that have remained in male and female cafeteria showed gram posistive spore forming rod chain which have been identified as *Bacillus* sp, the tissue paper that have remained in female dean and the tissue paper that have remained in dean for both female and male toilet (No brand) all the three of them have been identified as gram positive spore forming rod drum stick which have been identified as *Clostridium tetani* and finally the tissue paper that have remained in cafeteria male toilet (Solo) was gram positive cocci chain then when it was cultured on blood agar it made a Beta hemolysis that identifies as *Streptococcus* sp, this study is resemble to study done by Ogunshe, Oyebajo [(8)], in Nigeria who isolated *Pseudomonas, Salmonella* spp, *Streptococcus , Bacillus, and Clostridium* from public toilets.

Another study that have been done by [3], in Canada and have been published in American Journal Of Infection Control, Observed that between 102 and 105 colony-forming units per gram of unused paper towels were isolated from the different paper towel brands. Bacteria belonging to the Bacillus genus were by far the most abundant microorganisms found (83.0%), followed by *Paenibacillus* (15.6%), *Exiguobacterium* (1.6%), and *Clostridium* (0.01%). Paper towels made from recycled fibers harbored between 100- to 1,000-fold more bacteria than the virgin wood pulp brand. Bacteria were easily transferred to disposable nitrile gloves when drying hands with paper towels. However, no evidence of bacterial airborne transmission was observed during paper towel dispensing. A study made by Ajayi and Ekozien [(17)], in Nigeria have showed Several bacterial pathogens were isolated from the floor, door-

handle and wall of toilet (rest room) in four male and four female hostels. Seven isolated bacteria were are *Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis, Enterococcus faecalis , Klebsiella pneumoniae , Escherichia coli* and *Proteus mirablis.*

Gram-negative rods isolated in this study indicate the possibility of the presence of fecal contamination on the door handles. This might be due to the fact that most people go to toilet and end up contaminating their hands with fecal and urinal material and fail to wash their hand because they take the issue of hygiene with levity, they also lack the concept of hand washing as a simple means of stopping this spread of infectious agents, this correspond with the work of [18].

In current study the fungal flora that isolated from different sites of toilets from college of Health Sciences were as follows *Aspergillus niger , Aspergillus candidus, Rhizopus oryzae, Penicilium citrinum, Penicilium camemberti, Aternaria alternate.* The most prevalence of fungi showed in toilets that used by (male and females together, Cafeteria, Dean building) the reason is that the paper towels close to the toilet seat were all contaminated the papers that remain in toilets and an increase of using the toilets by male- female rather than female or male as separate toilets enhances population of microorganisms transmission to tissue paper remained in toilets.

Ogunshe, Oyebajo (8), isolated fungal flora from the unused toilet roll and the most prevalent fungal flora were *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Botyriodiplodia, Candida and Penicillium chrysogenum species*. In another study done by [19], that clarified the factors affecting fungal contamination in bathrooms and identified species, including *Aureobasidium* sp.*, Cladosporium cladosporioides, and Fusarium* sp., Which were common both in bathrooms including the toilets.

Conclusion: Different bacterial species and fungal species isolated and identified after remaining the tissue papers in toilet sites. The toilet environment, the closeness to the toilet seat and the open garbage bins, and the quality of the paper towels might be the source of this contamination. The current study demonstrates that a diverse community of bacteria contaminates tissue paper towels and that some of these bacterial strain may be transferred from paper towels to individuals during drying. The study recommended to Placing Tissue paper out of toilet to reduce risk of contamination through allocate a place or area for tissue papers like a small cupboard away from toilet contamination.

References:1. Singh SP. Fiber from Forest and Their Importance in Modern Time. Non-Timber Forest Products: Food, Healthcare and Industrial Applications: Springer; 2021. p. 427-53. DOI: 10.1007/978-3-030-73077-2_18.
2. Snow RD. Wood properties and utilization of assorted hardwoods: Mississippi State University; 2022.
3. Gendron LM, Trudel L, Moineau S, Duchaine C. Evaluation of bacterial contaminants found on unused paper towels and possible postcontamination after handwashing: a pilot study. American journal of infection control. 2012;40(2):e5-e9. DOI: 10.1016/j.ajic.2011.12.011.
4. Harrison WA, Griffith CJ, Ayers T, Michaels B. Bacterial transfer and cross-contamination potential associated with paper-towel dispensing. American journal of infection control. 2003;31(7):387-91. DOI: 10.1016/S0196-6553(03)00079-8.
5. Jane OC, Somadina OI, Chukwuma OU, Ogonna US, Catherine ON, Chinyere N. Bacteriological Assessment of Tissue Papers Sold in Eke Awka Market in Anambra State, Nigeria.
6. Saldmann F. Wash Your Hands: Dirty Truth About Germs, Viruses and Epidemics... and the Simple Ways to Protect Yourself in a Dangerous World: Grand Central Publishing; 2008.
7. Reynolds K, Hurst C. Manual of Environmental Microbiology. 2nd Annual Public Health Association. 2005:1-9.
8. Ogunshe AA, Oyebajo OE, Odusanya OA, Ogungbesan OA. Evaluation of Physical Characteristics and Public Health Significance of Easily-Culturable and Clinically-Relevant, Inhalable Microbial Flora of Unused Toilet Rolls. Health Science Journal. 2015;9(6).
9. Bashir S, Muhammad H, Sani N, Kawo A. Isolation and identification of bacterial contaminants from door handles of public toilets in federal university Dutse, Jigawa State-Nigeria. IOSR Journal of Pharmacy and Biological Sciences. 2016;11(5):53-7.
10. Basu P. Production of chlamydospores of Phytophthora megasperma and their possible role in primary infection and survival in soil. Canadian Journal of Plant Pathology. 1980;2(2):70-5. DOI: 10.1080/07060668009501441.
11. Samson R, Hoekstra E, Lund F, Filtenborg O, Frisvad JC. Methods for the detection, isolation and characterisation of food-borne fungi. 2004.
12. Doern GV, Chapin KC. Determination of biotypes of Haemophilus influenzae and Haemophilus parainfluenzae a comparison of methods and a description of a new biotype (VIII) of H. parainfluenzae. Diagnostic microbiology and infectious disease. 1987;7(4):269-72. DOI: 10.1016/0732-8893(87)90143-X.
13. Kateete DP, Kimani CN, Katabazi FA, Okeng A, Okee MS, Nanteza A, et al. Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Annals of clinical microbiology and antimicrobials. 2010;9:1-7. DOI: 10.1186/476-0711-9-23.
14. Corry JE, Curtis G, Baird RM. Handbook of culture media for food microbiology: Elsevier; 2003.
15. Amala SE, Ade AJ, Bloomfield F. Bacteria associated with toilets and office lock handles. International Journal of Epidemiology and Infection. 2015;3(1):12-5. DOI: 0.12966/ijei.02.02.2015.
16. Ashgar SS, El-Said HM. Pathogenic bacteria associated with different public environmental sites in Mecca city. 2012:DOI: 10.4236/ojmm.2012.24020.
17. Ajayi A, Ekozien M. Sensitivity profile of bacterial flora isolated from bathroom. Elite Research Journal of Biotechnology and Microbiology. 2014;2(1):1-3.
18. Zhad P, Zhad M, Doyle J, Meng J. Development of a model for monitoring surfaces hygiene and environmental sanitation. International Journal of Infection Control. 1998;6(3):45-8.
19. Hamada N, Abe N. Physiological characteristics of 13 common fungal species in bathrooms. Mycoscience. 2009;50(6):421-9. DOI: 10.1007/s10267-009-0500-6.