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RESEARCH ARTICLE OPEN ACCESS

A Baseline Study of Bacterial Pathogens in Greywater Samples in Jordan using Ribotyping

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Abstract

As Jordan advances in an attempt to promote greywater reuse, it is important to investigate the composition of bacteria in these new sources. To evaluate the presence of enteric pathogens in greywater, a pilot study investigating enteric pathogens in household washing machines and kitchen sink effluents from residential premises was conducted. In the culture-dependent method, bacteria were identified after using Sanger sequencing of 16S rRNA. Bacteria in the phylum *Proteobacteria* have been found to be the most abundant phyla, which may indicate that they play an important environmental role and might be representative of adaptation to different environments. *Klebsiella* sp. and *Pseudomonas* sp. were the two major genera found in this study and accounted for 78.57% of the total isolates. This is the first investigation of enteric pathogens in household washing machines and kitchen sink effluents in Jordan. To my knowledge no study has identified the microbial hazards associated with greywater reuse in Jordan yet. Additional research with more adequate methodology is needed to assist our findings.

Keywords: Enteric Pathogens, Washing Machine, Kitchen Sink, Greywater, 16S rRNA, *Klebsiella* sp., *Pseudomonas* sp.

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INTRODUCTION

Jordan is one of the most water scarce countries of the world and greywater is the best choice for water reuse due to its large quantity and low concentration of contaminants.[1] Greywater refers to wastewater produced from domestic activities such as washing machines, kitchen and wash basins, showering and bathing. It is generally defined as urban wastewater without wastewater from toilets.[2] In Jordan, the automatic washing machine or dishwasher is found to consume about 50 liters in each operating case.[3] Water resources per person in Jordan are far below the international water poverty line with less than 60 m^3/year.[4] Around 50-80% of an individual's daily water consumption in Jordan are greywater,[5,6] where washing machines account for a quarter of domestic wastewater. Thus, one of the recently undertaken strategies to enhance the water situation in Jordan is the reuse of greywater to bridge the gap and help in water balances. The reuse of greywater in Jordan is yet narrowed to rural areas where about 64% of houses are not connected to wastewater treatment plants[7] therefore, can make the most use from greywater reuse for purposes of irrigation and toilet flushing.[8] The current standard to control greywater reuse and reusing reclaimed greywater in Jordan (JS 1776/2013) permit the use of treated greywater for unrestricted irrigation.[9] Furthermore, the climate change crisis is increasing variability in the water cycle; thus inducing reducing the predictability of water availability.[10] Climate change drives increasing use of water-saving initiatives worldwide. Untreated greywater is allowed for local reuse as irrigation water in several rural areas in Jordan. Around 60 % of Amman's households and 30% of rural in Jordan reused water within their household.[11] Other countries only allow the reuse of treated greywater, while a few ban reusing it entirely due to public health concerns.[12,13]

As Jordan moves forward in an attempt to promote greywater reuse, it is important to investigate the composition of microbial communities and pathogens in these new sources. Microorganisms can be introduced into greywater by hand washing, baths, showers, laundry washing and food-handling in the kitchen.[14,15] Contaminated food and mishandling of food in the kitchen have been recognized as sources of enteric pathogens in greywater.[13,15] Enteric pathogens have also been detected in households laundry greywater during a microbial monitoring programme in Australia. The possible presence of pathogens in greywater can determine the ability to reuse it,[16] which might be transmitted to humans by direct or indirect contact.[15,17] Many studies reported the microbial contamination in home laundry operations and the risk of household microbial transmission.[18-24] Previous studies showed that microorganisms isolated from washing machines are more tolerant to chemical surfactants when compared to control strains that covered the Gram-negative and Gram-positive bacteria in addition to the yeast.[25,26] As a consequence of the self-developed matrix of extracellular polymeric substances (EPS), washing machines strains are more tolerant towards chemicals.[25] Therefore, potential drug-resistant microorganisms could emerge in the domestic environment and lead to a potential health risk because of cross-contaminations throughout the washing process.[21] The origins of greywater, the types of infrastructure and detergents used are all important environmental factors impacting greywater microbial communities.[19] According to the Centers for Disease Control and Prevention (CDC), Gram-negative bacteria cause many infections including bloodstream infections, meningitis, pneumonia, and surgical or wound site infections in healthcare settings. However, more research is still required to determine the greywater microbial communities, including bacterial pathogens.

Little has been published on enteric pathogens in domestic washing machines and kitchen sinks effluents. Therefore, this pilot study offers a view into acquiring knowledge on the potential pathogens associated with untreated greywater reuse in Jordan through characterizing the bacterial isolates of the effluent water of the domestic washing machines and kitchen sink from residential premise using a culture-dependent method followed by Sanger sequencing of 16S rRNA gene sequences. Pathogens are generally characterized by culture-dependent methods. This study will be the first to investigate enteric pathogens in household washing machines and kitchen sink effluents in Jordan.

MATERIALS AND METHODS

Sample collection and preparation

This pilot study is aimed to detect the enteric pathogens of a domestic washing machines and kitchen sink effluents that is generated at a residential premise in Aqaba City, Jordan in 2022. This study focused on the bacterial flows where the domestic washing machines and kitchen sink effluents delivered directly without treatment to storage tank through a piped distribution system. The washing machine was used twice a week. Greywater samples were collected from the storage tank during April of 2022 under aseptic condition. The tank was cleaned after each sampling to prepare for the next sampling. Samples that were collected using 1L sterile bottles were kept on ice and used within 1 hour after collection. Owner voluntarily and anonymously allowed us to use their washing machine and kitchen sink effluents. All members of the residential premises were aged between 28 and 44 years. The washing machine worked at 30°C. Washing machines were loaded with regular detergent without fabric softener (detergent name not provided by the residential premises). While kitchen sink was used on daily basis.

Microbial enumeration and identification

Bacterial numbers in greywater were estimated using surface plate techniques.[27] The microorganisms from raw washing machine and kitchen sink effluents were collected by centrifugation for 10 minutes at 12,000 ×g using sterile technique. After centrifugation, the supernatant was discarded, and the cells were resuspended in nutrient broth and incubated overnight at 37°C. The broth was streaked using sterile glass spreader onto Xylose Lysine Desoxycholate agar (DIFCO, FRANCE) and incubated overnight at 37°C. XLD agar was used to enumerate enteric pathogens using the spread plate method.[28] Colonies were identified based on morphological tests for initial identification that were further identified later by 16S rRNA gene sequencing. Therefore, for molecular identification, bacterial DNA was extracted using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research /USA) following the manufacturer's instructions. The integrity and size of genomic DNA was checked using gel electrophoresis (1% agarose), and DNA concentrations were determined using microplate spectrophotometer system (BioTek Instruments, Inc.).

The molecular-based works were carried out for microbial diversity of 16 extracts. The 16S rRNA gene for each sample was amplified using universal 16S rRNA bacterial primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3').[29] Polymerase chain reaction (PCR) mixture (25 µl) contained 2 µl of DNA template, 2U Taq DNA Polymerase, 1x PCR buffer, 1 mM Nucleotide mix, 2 mM MgCl2 and 0.2 uM of each primer. The PCR was carried out using the following protocol: one cycle at 94°C for 5 min, 30 cycles at 93°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 5 min. All PCR products were analyzed using gel electrophoresis (1% agarose).

Sequencing of amplified PCR products

A total of 16 random bacterial isolates were selected for this study and sequenced by Sanger sequencing service from Macrogen (Macrogen Inc., Korea). Sequences were edited by removing the bad quality parts from the beginning and the end of the sequences. Good quality sequences generated (n=14) were used for similarity search using BLASTn tool at the National Center for Biotechnology Information (NCBI).[30] BLASTn at NCBI was used to find similarities at the species level for this study, whereas SILVA can only go down to the genus level.[31] The 16S rRNA gene sequences of bacteria isolated in this study are uploaded in GenBank under the accession numbers (OP514804-OP514814) and (OP514817-OP514819)

RESULTS

The colonies were counted after incubation and the total count was determined as described by Speck[32] by the following equation:

cfu/ml= number of colonies × D.F/plated volume in ml

The mean total count of bacteria in greywater was $6.1x10^6$ cfu/ml. Fourteen bacterial isolates from the storage tank of domestic washing machine and kitchen sink effluents were successfully sequenced and analyzed using a 16S rRNA gene PCR (Figure) followed by Sanger sequencing. NCBI BLASTN[33] was used to find similar sequences. Identification were determined for 14 isolates identified on species level. Based on the sequencing analysis, all sequences from the storage tank of domestic washing machine and kitchen sink effluents were identified and classified as belonging to the following four genera: *Klebsiella, Citrobacter, Proteus* and *Pseudomonas* with identity percentage ranged from 95.32 to 99.93 (Table 1). The genus *Klebsiella* was predominant (Table 2). Identification at the species level was possible for all samples as they have a very close identity with sequences available on Genbank (Table 2).

Table 1. BLAST analysis of PCR amplicons from bacterial isolates according the highest similarity to 16S rRNA sequences in the GenBank nucleotide sequence database

Sample IDs	Closet organism in GenBank	NCBI accession No.	Similarity (%)	E-value	GenBank accession no.[1]
GW1	*Klebsiella grimontii*	CP091752.1	99.51%	0.00	OP514804.1
GW2	*Klebsiella oxytoca*	MT509911.1	99.50%	0.00	OP514805.1
GW3	*Klebsiella oxytoca*	MT509877.1	99.51%	0.00	OP514806.1
GW4	*Citrobacter freundii*	OQ405468.1	95.32%	0.00	OP514807.1
GW5	*Citrobacter murliniae*	NR_028688.1	97.54%	0.00	OP514808.1
GW6	*Klebsiella grimontii*	CP091752.1	99.58%	0.00	OP514809.1
GW7	*Pseudomonas aeruginosa*	FJ823152.1	99.93%	0.00	OP514810.1
GW8	*Pseudomonas aeruginosa*	KY962357.1	99.65%	0.00	OP514811.1
GW9	*Proteus vulgaris*	MN833577.1	99.51%	0.00	OP514812.1
GW10	*Klebsiella pneumoniae*	MF455200.1	99.36%	0.00	OP514813.1
GW11	*Klebsiella pneumoniae*	MZ389267.1	99.36%	0.00	OP514814.1
GW12	*Pseudomonas aeruginosa*	KC456535.1	96.06%	0.00	OP514817.1
GW13	*Pseudomonas aeruginosa*	HQ844502.1	98.73%	0.00	OP514818.1
GW14	*Klebsiella grimontii*	CP091752.1	99.58%	0.00	OP514819.1

[1]GenBank accession number of all samples used in this study

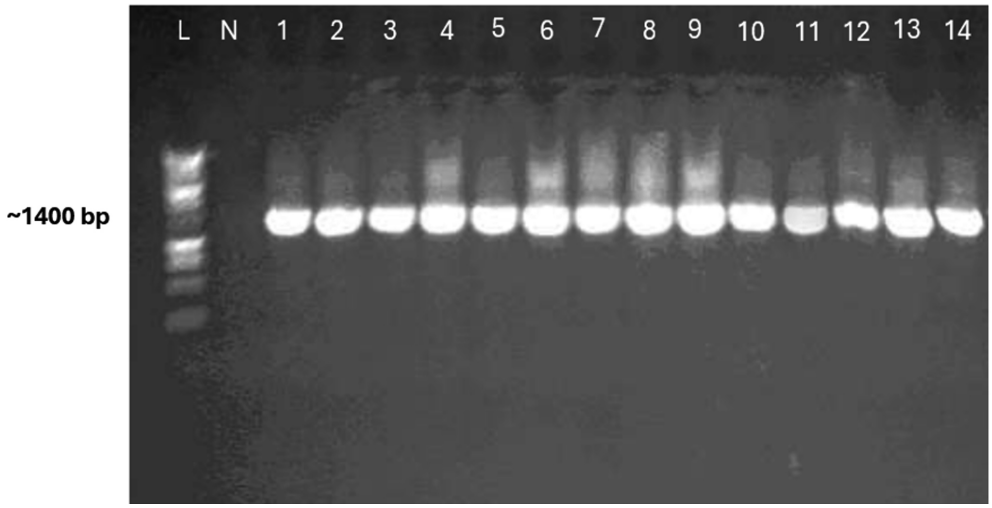

Figure. Agarose Gel Electrophoresis (1%) Showing PCR Products (~1400 bp); Lane L: 1 Kb DNA Ladder, Lane N: Negative control, and Lanes 1-14: PCR products.

Table 2. Number of bacterial isolates obtained from residential washing machine and kitchen sink storage tank and identified by PCR using amplification of 16S rRNA (Species level; n = 14) across the 14 different samples

Phylum	Class	Order	Family	Genus	Species	GenBank accession number
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Klebsiella*	*Klebsiella grimontii*	OP514804.1, OP514809.1, OP514819.1
					Klebsiella oxytoca	OP514805.1, OP514806.1
					Klebsiella pneumoniae	OP514813.1, OP514814.1
				Citrobacter	*Citrobacter freundii*	OP514807.1
					Citrobacter murliniae	OP514808.1
			Morganellaceae	*Proteus*	*Proteus vulgaris*	OP514812.1
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas aeruginosa*	OP514810.1, OP514811.1, OP514817.1, OP514818.1

DISCUSSION

Applying Sanger sequencing on the full-length bacterial 16S rRNA gene of 14 isolates obtained in this study showed a clear assignment of the full-length bacterial 16S rRNA gene sequences to assign taxonomic classification down to the species level.[31] Taxonomic identification of sequenced isolates was carried out by using BLAST to align sequences to the NCBI 16S BLAST database. Resulting hits in the present study were ranked by e-value, then the taxonomy of the highest scoring sequence was selected. Using the universal primers 27F and 1492R for a full-length bacterial 16S rRNA gene,[34] from 14 isolates obtained in this study with an expected amplicon of ~ 1400 bp, bacteria in the phylum *Proteobacteria* have been found to be the most prevalent on the items laundered. Previous studies of microbial communities in washing machines have also revealed that washing machines are mostly inhabited by the phyla *Proteobacteria, Actinobacteria, Firmicutes,* and *Bacteroidetes*.[20,21,35] In congruence with previous molecular studies,[20,35] *Proteobacteria* (93.75%), and *Firmicutes* (6.25%) also presented the most abundant phyla here.

The aim of this study was to evaluate the presence of enteric pathogens in greywater by initiating a pilot study of enteric pathogens in the effluents of domestic washing machines and kitchen sinks using the culture-dependent approach followed by using Sanger sequencing of 16S rRNA gene of all bacterial isolates. Many of the bacterial identified represent environmental bacteria, which typically found everywhere in the environment such as *K. pneumoniae* species.[36] *K. pneumoniae* have been previously isolated from washing machines.[37-39] *K. pneumoniae* is one of the species that most often involved in human infections, and frequently found in the large intestine but are also present in soil and water.[40] *K. pneumoniae* is most pathogenic to humans among all *Klebsiella* sp., followed by *K. oxytoca,* where both *K. pneumoniae* and *K. oxytoca* cause community-acquired meningitis and brain abscesses.[41] *Citrobacter* sp. occur in the environment and in the human colon, and can cause sepsis in immunocompromised patients. *Citrobacter* is predominantly spread from person-to-person,[42] it normally cause urinary tract

infections, intra abdominal sepsis, blood stream infections, pneumonia, and brain abscesses.[43] *C. freundii*, can be found in feces and the environment as well as in drinking water containing relatively high concentrations of nutrients.[44] In addition, some of the bacterial identified in this study are common biofilm formers, such as *Pseudomonas* sp., *P. aeruginosa* was found inside the detergent drawer.[35] The major route of exposure to *P. aeruginosa* is direct contact with contaminated water or skin exposure.[45] *P. aeruginosa* can also colonize on open burn wounds, causing infections, abscesses, and sepsis.[46] *K. oxytoca* was one of the opportunistic pathogens found in the washing machines as well.[37] *Proteus* sp., and *Klebsiella* sp. usually originating from food ingredients are commonly present on kitchen sponges to surfaces of stainless steel and polyethylene.[47,48] *Proteus* sp. are commonly associated with complicated urinary tract infections (UTIs), also survive well within the environment in water, soil, and sewage.[49] Where Proteus can cause uncomplicated cystitis, prostatitis, and pyelonephritis, especially in hospital-acquired cases.[50]

It should be noted that our study has limitations, which were expected as a pilot study. One limitation of this study was the population size (e.g., single household) and the small number of isolates that were sequenced which may have led to the identification of only some bacterial pathogens or non-pathogenic microflora of a domestic washing machine and kitchen sink effluents. It is anticipated that more samples collected and sequencing of more isolates could potentially reveal additional knowledge on the enteric pathogens might be present. In addition, due to the use of selective medium in this study, other groups of bacteria that may be present in the sample has not been shown. Related to the small sample size, we suggest that this study should be regarded more as a feasibility study of enteric pathogens in the effluents of domestic washing machines and kitchen sinks in Jordan. Another limitation of this study is that data generated by the current analysis does not include bacterial communities growth or decay during storage of the samples post-collection, as weekly samples for only one month were considered, and it may not reflect the actual contamination that may persist in greywater storage systems. However, this is more of a preliminary study, and additional research with more adequate methodology is needed to assist our findings. This is important since little has been known and published about bacterial pathogens in greywater in Jordan, and in domestic washing machines and kitchen sink effluents in particular. To my knowledge no study has identified the microbial hazards associated with greywater reuse in Jordan yet. Overall, the culture-dependent method can address some limitations of the possibility of interaction, and the information obtained as only certain bacteria can be isolated using culture-dependent approach. Thus, using both culture-dependent and culture-independent methods for the identification of enteric pathogens will offers wider possibilities. However, it must be taken into account that the enteric pathogens of greywater in general would vary according to many factors including the background microflora, inhibitory substances, and non-culturable cells that could be present and must be taken into consideration for further future research.

CONCLUSION

This study demonstrated the presence of enteric pathogens in the untreated greywater from residential premises. The bacteria in the phylum Proteobacteria have been found to be the most abundant phyla in this study which may indicate that they play an important environmental role and might be representative of adaptation to different environments. The search for identification of the enteric pathogens in domestic washing machines and kitchen sink effluents as described in this current study will be an important aspect considering the health risk assessment and the potential microbial hazards associated with greywater reuse in Jordan. This is the first study in Jordan to investigate enteric pathogens in household washing machines and kitchen sink effluents.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by the author.

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