Chapter 3
Determination of Pathogens in Greywater

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Abstract There are many methods used for isolation and the enumeration of pathogenic organisms. The direct methods depend on the culture medium and microscopic examination. However, these techniques are not effective for all pathogenic organisms in the environment since many organisms require a specific condition to grow on the culture medium. Therefore, the using of enrichment methods might exhibit more efficiency in the determination of pathogens from the wastewater samples. The main challenge in the microbiological assessment of greywater lies in finding the most effective method to detect the presence or absence of pathogens which are available in low concentrations. In this chapter, traditional methods including direct culture and enrichment methods are reviewed.

Keywords Culture methods · Greywater · Pathogens · Antibiotics resistant bacteria

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R. M. S. Radin Mohamed et al. (eds.), *Management of Greywater in Developing Countries*, Water Science and Technology Library 87,
https://doi.org/10.1007/978-3-319-90269-2_3

3.1 Introduction

Determination and isolation of pathogenic organisms from greywater and other environments require critical techniques due to the nature of greywater which has different contaminated compounds that can negatively affect the detection of these pathogens. There are two techniques which are used for the detection of microorganisms in greywater including culture-based methods and non-culture-based methods (molecular-based methods). The culture-based method depends on the detection of microorganisms based on their growth on selective and enrichment media. This method does not need highly qualified technicians but the technicians should have a very good background in isolation and identification methods using biochemical tests. In the non-culture-based method, the determination of microorganism merely on the utilization of advanced procedures such as molecular techniques which identify the microorganism based on the sequences in the 16S rRNA and require well-trained technicians. The selection of detection methods depends on the type of wastewater such as before or after the treatment process. The non-culture-based technique is efficient in the detection of microorganisms in both raw and treated wastewater. In contrast, the culture-based method is not as efficient for the detection of microorganisms in treated and disinfected wastewater due to the failure of some inactivated pathogens to grow on the culture medium while living based on its metabolic activity and energy status.

A comparison was done on the viability of advanced technology for the determination of pathogenic microorganisms between developed and developing countries. The absence of advanced cutting protein techniques in developing countries represents the main challenge in the evaluation of risk associated with the reuse of greywater for irrigation or discharge into the environment. Therefore, this chapter aims at presenting both traditional and advanced processes used for the detection of infectious pathogens in order to give the researchers from the developing countries more ideas about the techniques which can be used to give a clear indication of the risk levels of greywater, since the developing countries have strict regulations for wastewater (but not greywater). However, the main limitation lies in the absence of qualified technicians and advanced techniques which are necessary to apply those regulations.

Pathogenic bacteria and fungi in greywater are the most important among several pathogens due to their ability to multiply in the environment without the need for a host as in the case of viruses and parasites (Al-Gheethi et al. 2016). Moreover, most standard regulations for greywater require tests to gauge the concentration of pathogenic bacteria. The ability of bacteria to convert from being active to inactivate during the disinfection process of greywater or due to the presence of different chemical compounds such as xenobiotic organic compounds (XOCs) make the isolation of pathogens more difficult. Therefore, this chapter focuses on the techniques used for isolation and purification as well as the identification of bacteria and fungi from greywater or any type of wastewater. This chapter provides technicians with the basic skills required for the recovery of pathogens from greywater by using traditional

methods with the minimum requirements especially in developing countries where there are no more facilities to utilize advanced techniques. Advanced technologies have also been highlighted.

The greywater samples are collected using sterilized glass bottles (1 L or more). In order to get a more accurate evaluation of the microbiological characteristics of greywater, the samples should be collected in triplicates at different times. Some references recommend the collection of seven samples at a rate of one sample per week (U.S. EPA 2003). The obtained samples are then transported to a microbiological laboratory within an ice box and subject to analysis within 24 h.

3.2 Culture-Based Methods

The culture-based method includes procedures which depend on the observation of microbial growth in synthetic media (selective and enrichment medium) after the incubation period which ranges from 24 to 72 h at an appropriate temperature (30, 37 and 44.5 °C) and can be conducted by direct isolation on agar plates or in broth test tubes which is known as most probable numbers (MPN) method. This method might also be conducted by using membrane filters which are used for filtration of wastewater. The membrane filter will be placed on appropriate selective media to show microbial growth after the incubation period. The standard methods for isolation and determination of pathogenic organisms in wastewater are described in detail by APHA (1999). Moreover, in this section, some notes will be highlighted in order to obtain more accurate results.

3.2.1 Standard Plate Method

This technique depends on the preparation of serial dilutions of greywater samples based on the density of microorganisms which are expected to be available in the greywater. The method is appropriate for the isolation of pathogens from raw wastewaters, which are present in great numbers, since one millilitre of greywater sample may contain between 10^6 and 10^9 cells depending on the source of these wastes and microorganism species as well as other factors mentioned in Chap. 1.

The use of the standard plate method can be conducted by spreading or pouring procedure depending on the bacterial species. Pouring technique is used for the isolation of facultative anaerobic bacteria while spreading technique is used for the isolation of both obligate aerobic and facultative anaerobic bacteria. In brief; the procedure for the spreading technique is performed by preparing a culture medium one day before the isolation process. The medium is sterilized by autoclave or heating based on the manufacturing instructions for each medium. After pouring the agar, it is supposed to be left at room temperature overnight to ensure the absence of

contamination. The colonies growing on the medium are separated from each other to distinguish their characteristics and ease the purification process.

The most common indicator bacteria which are counted in greywater include FC, TC, *Enterococcus faecalis*, *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. These bacterial species can be counted by direct isolation from the appropriate selective medium while pathogenic bacteria such as *Salmonella* spp. need to be enriched by using an enrichment medium before detecting their presence or absence. The use of an enrichment medium is discussed more in Sect. 3.2.5. *Clostridium* spp. is an aerobic bacterium which has to be isolated using specific procedures using an anaerobic cabinet. The fungi most commonly detected in greywater especially that generated from the kitchen include *Aspergillus* spp. and *Penicillium* spp. The culture medium used for isolation and enumeration of indicator bacteria and pathogenic bacteria and fungi are described in APHA (1999) standard guidelines.

The dilution of greywater should be carried out by using sterilized saline solution immediately before the isolation process. Leaving the diluted sample for a long time (more than 10–15 min) before the isolation process might negatively affect the viability of the bacterial cells, and thus reduce the opportunity to determine the actual number of pathogens in the sample. The volume of the sample used to prepare the first dilution should be appropriate to represent the sample and obtain a more accurate bacteria count. For example, the dilution of 30 mL of greywater using 270 mL of sterilized saline solution or distilled water might be better than diluting 1 mL of greywater in 9 mL of sterilized saline solution. The selection of a dilution solution depends on the source of the samples. Wastewater may be diluted with distilled (or deionized) water. Moreover, the use of physiologic saline (0.85% NaCl) might be the best option to prevent any possible cell lysis due to osmotic stress. The diluted sample should be shaken well to physically separate the microorganisms to manageable levels (30 times to the front and back by hand or by shaking at 100 rpm for 5 min) but not vigorously to prevent the destruction of cells (Prescott 2002).

The usual amount of pipetted sample used for the isolation is around 0.1 mL (100 μL). The micropipette with sterilized yellow or blue tips is used to transfer the inoculums sample. The pipette is filled and emptied with the inoculums three or more times before withdrawal of the diluting solution to homogenize the bacterial cells in the solution. Thereafter, the remaining amounts of the inoculums are added to the diluting solution and mixed well. A fixed amount (1 mL) of the diluted solution is transferred to the next tube containing 9 mL of the physiologic saline solution to prepare the next dilution. This procedure is the same for the preparation of all dilutions required (Collee et al. 1989).

The spreading processes of the inoculums are carried out by using flamed-sterilized stainless-steel spreader (L-shape) or glass spreader onto the surface of the selected medium. The spreading process should be performed carefully to ensure the best distribution of the inoculums on the whole surface of the medium. For primary technicians, large Petri dishes (150 mm × 20 mm) might be used for the isolation, while the medium-sized Petri dishes (80 mm × 15 mm) are used for the purification process. The media plates are left in the laminar flow for 10 min to allow the medium to absorb the inoculum water.

The pouring method is conducted with a similar procedure, but in this technique, the media are prepared and kept at 45 °C. After the transfer of 1 mL of the greywater sample or diluted solution into the Petri dish, 20 mL of the medium is poured and then homogenized slowly by movement of the plate to the right and left and to front and back. Isolation of filamentous fungi is performed only by using the spreading method due to the nature of fungi as aerobic organisms.

The culture plates are incubated at the optimal temperature required for each organism species. Media plates used for heterotrophic bacterial counts (HBC), TC, *E. faecalis*, *S. aureus* and *P. aeruginosa* are incubated at 35–37 °C, while plates used for counting FC and *E. coli* are incubated at 44.5 °C. The media used for the isolation of fungi are incubated at 28 °C. In order to prevent the drying of the media, the plate cultures are sealed with parafilm before incubation. In regions with dry weather, a wet cotton might be placed inside the plastic page together with the culture medium to provide the moisture required for microbial growth and to prevent the drying of the medium.

The incubation period might range from 24 to 72 h for bacteria and between 5 and 7 days for fungi. The incubation period between 24 and 36 h is enough for bacteria growth and the development of the colony. However, in order to get more morphological characteristics of the colony such as colours and pigments, the medium is suggested to be incubated for more than 72 h. This is because the production of pigments take place during the late phase of bacterial growth. The production of pigments by bacteria is a response towards unfavourable conditions and to protect the cells from external factors such as low temperature. Therefore, keeping the culture medium in a fridge might accelerate the resealing of these pigments and give more distinguishing characteristics to the colony. For instance, the laboratory observations found that *S. aureus* and *Staphylococcus epidermidis* have similar colony morphology with the yellow colour on the mannitol salt agar (MSA) after 24 h. However, an extended incubation period of 48 h or keeping the culture plates at 4 °C for next 24 h improved the colony morphology for both species. *S. aureus* changed to gold with a gold colour medium while *S. epidermidis* colony appeared in yellow pink. The description of the morphological characteristics of bacterial colonies are presented in detail by Prescott (2002), Morello et al. (2003) and Benson (2005).

After the incubation period, plates with 30–300 colonies are counted. In some references, the counting colonies ranged from 20 to 200 colonies (Götz et al. 2006). The selection of the counting range is related to statistical considerations which aimed to reduce the standard division values (Collee et al. 1989). In the fungi plates, APHA (1999) recommended to count plates with 15–150 colonies. The viable numbers of the bacterial and fungal cells are expressed as CFU mL^{-1} according to Eq. (3.1).

$$\begin{aligned}&\text{Number of bacteria or fungi in the sample (CFU/mL)}\\&\quad=\text{Number of colonies grown on the culture medium}\times\frac{1}{\text{Dilution factor}}\end{aligned}\tag{3.1}$$

The standard plate method was certified by the US. EPA as a standard procedure for the environmental monitoring of wastewater plants in 1979. These standards have

been developed with more guidelines in a new version. The method has also been used for the counting of pathogenic bacteria from different wastewater samples by several authors in literature (Al-Gheethi et al. 2013, 2014). It exhibited high efficiency for the counting of bacteria in highly contaminated samples, but it was unsuccessful with the low density of bacteria in wastewater samples. The detection limit of this method is around 10 CFU/mL, where the minimum concentration might be available in 1 mL of the sample is one cell. The determination of pathogenic bacteria in greywater with a concentration of less than 10 CFU/mL requires more efficient methods such as the membrane filter method or MPN.

Götz et al. (2006) indicated that direct surface plating procedures are more accurate than MPN for detecting *S. aureus* in raw or unprocessed foods. Moreover, the sensitivity of this method might be increased with larger volumes of the tested samples (>1 ml). In contrast, the best results with a desired number of colonies might be achieved by using three replicate plates for two or more decimal dilutions.

One of the methods used for the direct isolation of pathogenic bacteria on the selective media is by using the spiral plate technique. This method is conducted by using a machine in which a known volume of the sample is disturbing a rotating agar medium. Thereafter, the total number of bacteria is counted by selecting an appropriate area of the plate. The efficiency of this method is better than the pour plate method in terms of the total number of bacteria recovered. Besides, this technique needs less time compared to the traditional direct plating procedure (Gilchrist et al. 1973). This technique has been proposed recently by a few authors who suggest it to be used for the direct isolation of *Salmonella* spp. from different sources on the selective medium (Brichta-Harhay et al. 2007).

3.2.2 Purification of Bacterial and Fungal Isolates

In order to identify the bacteria and fungi isolated from the greywater, it has to be purified as well. The morphological characteristics of the colonies grown on the culture plates might provide the primary identification of the grown microorganisms. However, the identification process should be confirmed either by phenotypic methods or molecular techniques. Moreover, the purification of the isolates represents the critical step to obtain the correct name for the pathogenic microorganism. In this section, the purification procedure for bacteria and fungi are presented.

3.2.2.1 Purification of Bacterial Isolates

The methods used for the purification of bacterial isolates have been reviewed by many manual books. However, this section provides some key notes which might contribute in obtaining a pure bacterial isolate. The streak procedure is the most common method for purifying the bacterial colony. The method is conducted by picking up a small portion of the grown colony on the isolation media and then

streaking it on the surface of a new medium. The notes discussed in this procedure include the type of medium used for the purification process and the direct streak of the picked colony on the new medium.

In terms of media used for purification, the use of nutrient agar (NA) or brain heart infusion medium (BHI) might affect the purification process. This is because the selective medium used for the isolation of pathogenic bacteria from wastewater has inhibitory substances which inhibit the growth of floral bacteria available together with the pathogenic bacteria in the wastewater sample. These inhibited bacteria on the selective medium might grow on non-selective agar such as NA or BHI. Therefore, in order to obtain pure isolates of the selected pathogenic bacteria, it should be purified on the same selective medium used for the first isolation for two times or more and then subject to purification on a non-selective medium. Second, the bacterial cells which have capsules or produce slim layers and have a mucus colony texture on the isolation medium as in the case of *Klebsiella* spp. are attached to other bacteria cells. Therefore, purification of these bacteria is quite difficult using the direct subculture on a new medium by the streak technique. Therefore, in order to overcome this problem Benson (2005) described a technique which depends on the preparation of a serial dilution for the picked colony using a nutrient broth and then subjected to the subculture on a new media. This procedure is effective for getting a pure culture of selected bacteria. However, it might not be enough to separate the bacterial cells which have very mucoid capsules or slim layers. Theatrically, the capsule and slim layer consist of polysaccharides which are more soluble in water. Therefore, the use of sterilized distilled water might be more efficient to obtain separate cells and a pure culture (Al-Gheethi et al. 2013). The purification method which depends on the use of sterilized distilled water is carried out as the following; one loopful of bacterial colony grown on an isolated medium is transported to 10 mL of sterilized distilled water to prepare bacterial suspension. The suspension is mixed well and immediately one loopful is rubbed onto the agar surface of the new medium using a sterilized glass spreader which is made in order to distribute and separate the bacterial cells as much as possible. After 24–48 h of the incubation period at 35–37 °C, a single colony is transferred to another 10 mL of sterilized distilled water, mixed well and then one loopful is transferred to the agar surface of the same medium in a similar manner. One of the developed bacteria colonies after 24 h is used for checking the purity by using Gram stain (Park et al. 2006).

3.2.2.2 Purification of Fungal Isolates (Single Spore Isolation)

In order to identify fungal isolates more successfully, the fungal isolates should be purified by using very accurate techniques. Fungal growth is quite different from that of bacteria because the production of external spores by the fungi makes their purification more difficult since the spores spread quickly and might cause contamination. There are many methods for purifying fungi. The single spore isolation technique represents the best method in terms of simple procedure and the absence of contamination yeast, bacteria and mites. The purification process

by using single spore isolation is conducted under a microscope (4–10×). The fungal colony grown on the culture medium is raised from only one spore. Hence, this method is more suitable for obtaining a pure fungi culture and also for the identification using phenotypic methods. This technique was described by Choi et al. (1999). Moreover, Noman et al. (2016) have described a modification of this method which has better performance and named it the Dr. Nagao method. In brief; the culture medium which has fungal growth from the first isolation is placed under a light microscope with a magnification of 4×. One colony is selected and picked up using a sterilized scalpel into a new culture medium. The medium should be dried at 28 °C for 24 h before use to ensure that there is no contamination. In order to get separate colonies on the culture medium, 0.1 mL of sterilized distilled water (SDW) is spread aseptically on the surface of agar by using stainless-steel or glass spreaders (L-shape). The plate is left to dry at room temperature for 10 min and then incubated at 28 °C for 16–18 h. At the end of the incubation period, the germinated spores are observed under a light microscope (4×). A small piece (0.2 × 0.2 cm) of culture medium with one germ spore is cut out aseptically by using a scalpel, transferred onto a new culture medium and then incubated at 28 °C for 5–7 days.

In order to prevent the contamination of pure cultures by mites, the stage of light microscope, equipment, laminar flow, containers, incubator, as well as the lab wares used in the purification process, should be sterilized with ethanol (70%). All plate cultures should be sealed with parafilm before the incubation, while fungal cultures should be stored in a refrigerator at 4 °C. The description of fungal culture characteristics is performed according to manual references such as Promputtha et al. (2005) and Benson (2005).

The culture characteristics of fungal colonies subcultured by using the single spore technique occurred more clearly than those subcultured directly using the needle. It might be due to the fact that in the single spore technique, the fungal colony was raised from only one spore. Therefore, the colonies grow on the medium separately. In the direct culture technique, several spores on the needle are transferred onto the culture medium, and thus the colonies imbricate. It is difficult to distinguish their characteristics. Besides that, the measurement of the colony diameter for the fungal culture purified by single spore isolation is easier than that purified by direct isolation. The fungal colony in single spore isolation possesses a circular shape while they are irregular in shape when they are cultured by direct isolation. These observations were made during work at the laboratory.

3.2.3 Identification of Bacterial and Fungal Isolates

The identification of bacteria and fungi is conducted based on phenotypic characteristics and molecular analysis of the cell genome. Phenotypic identification relies on culture characteristics of the grown colonies and cell morphology under the microscope. These characteristics differ among fungal species such as *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp., *Rhizopus* sp. and *Curvularia* sp., which have dif-

ferent shapes and sizes in their conidiophores and spores that play an important role in the identification of the species level (Emine et al. 2010). Further, the use of scanning electronic microscopy (SEM), flow cytometry as well as fluorescent microscopy provides an accurate recognition of fine structure in the conidiophore and spores (Guarro et al. 1999). The uses of SEM with high magnification reveal the slight differences in the surface spore which are quite different from one fungal species to another.

In contrast, bacterial cells have no high diversity in their morphology (cocci, bacilli and rod) and the response towards Gram staining (Gram-positive and Gram-negative). Therefore, phenotypic identification depends on biochemical tests which detect the ability of bacterial cells to produce specific enzymes such as oxidase, urease and catalase enzymes. The biochemical tests are conducted using analytical profile index (API) System which includes the biochemical API 20 test system, API 20NE and RapiD 20E that are used for different bacterial species. The API system was developed by Analytab Products of Plainview, New York. This system depends on the enzymatic reaction with the substrates available in a plastic strip with 20 separate compartments. The number of biochemical tests is different from one class to other. The bacteria suspension is prepared with physiological saline provided with these kits and then a small portion of each bacterial suspension is inoculated for each tube containing a specific medium or substrate depending on the test. The kits are incubated for 18–24 h and the results are read based on the changes in colour. In some tests, the reagents should be used, and then the identification process is performed by using computerized software which provides more than 99% accuracy (Benson 2005).

API systems might also be conducted by using VITEK® 2 Densi-CHEK Plus, which is used to determine the optical density of bacterial suspension. The instrument provides the optical density values in terms of McFarland units. This system is more commonly used in a vitro diagnostic process of pathogenic bacteria in medial units. The preparation of bacteria suspension is performed by inoculating a pure bacterial colony grown on the culture medium for 18–24 h into a test tube containing 3 mL of physiological solution. The suspension is prepared in a density of 0.5–0.63 McFarland units. APi kits inoculated with bacterial suspension are incubated in VITEK® 2 Densi-CHEK Plus is also used to read the results. The results of the API system might be read manually and the identification process is conducted by using the API software program. The recent identification for bacteria and fungi are conducted using the molecular technique which has several advantages in comparison to the traditional method in terms of accuracy and time.

3.2.4 Membrane Filtration (MF) Methods

Membrane filtration is a culture-based method with high efficiency to recover pathogenic bacteria from water and wastewater due to the high quantity of water samples filtered using a membrane filter 0.45 μm in diameter. Therefore, membrane filtration is more applicable to the recovery and assessment of the quality of drinking

water or highly treated wastewater. In some cases, the wastewater needs to be diluted before the filtration process in order to prevent clogging of the membrane pores. This method is described as a direct-plating method, where the membrane filters are transferred to a Petri dish containing selective isolation agar or an absorbent pad saturated with selective broth. Membrane filtration is more effective than the standard plate method with high limitation level (1 cell per 100 mL) depending on the level of the dilution process. In many cases, it is used as an alternative to the standard plate method if the concentration of pathogenic bacteria in polluted wastewater is less than the detection limits of the standard plate method. The protocol details for using this method are described by APHA (1999) and U.S. EPA (2006a, b).

The incubation conditions depend on the target bacterial species as in the standard plate method. However, the range of colony counted is between 20 and 80 colonies. The number of bacteria grown is reported as colony-forming units (CFU) per 100 mL which is determined according to Eq. (3.2).

$$\text{CFU } 100\ \text{mL}^{-1} = \frac{(\text{Number of colonies on agar medium})}{\text{volume (mL) of undiluted sample}} \times 100 \tag{3.2}$$

This technique has been used by many researchers for the isolation of pathogenic bacteria from wastewater before and after the disinfection process. Friedler et al. (2005) used this method for evaluating the microbiological quality of greywater onsite. In the study, *m*TEC agar was used for the isolation of FC, M-PA-C agar for *P. aeruginosa* and Staphylococcus Medium 110 agar for *S. aureus*. Plates of FC were incubated at 44.5 °C for 22–24 h and the colonies grown were confirmed as FC based on the occurrence of yellow to yellowish brown colour after placing the filter on a pad saturated with urea for 15 min. The plates containing *P. aeruginosa* were incubated at 41.5 °C for 48–72 h. The confirmation test of this bacteria included the hydrolyses casein and production of yellowish to green diffusible pigment on Milk Agar. *S. aureus* plates were incubated at 35 °C for 18–48 h. The coagulase-positive results confirmed the presence of *S. aureus.* Membrane filtration is the national standard method for enumerating *S. aureus* from the water samples regulated by the Health Protection Agency (HPA 2004) in the UK.

3.2.5 Enrichment Methods

The use of enrichment methods aims to recover pathogenic bacteria which are available in low concentrations in the tested samples or which are available in less concentrations than the detection limits of surface direct plating method (10 CFU/mL) or membrane filtrations (1 CFU/mL). Besides, the high amount of pollutants in the samples with floral bacteria makes the isolation of relevant bacteria quite difficult. Therefore, the enrichment method is necessary for the recovery of pathogenic bacteria available among all the other bacteria species. There are two types of enrichment media used, which are non-selective (pre-enrichment medium) and selective

(enrichment medium). Zhang (2005) indicated that BHI broth is the best for the pre-enrichment process while brilliant green broth (BG) is most efficient for enriching *Salmonella* from milk products. The selection of an enrichment medium plays an important role in the detection of pathogenic bacteria, regardless of the methods used. Stone et al. (1994) revealed that the Rappaport–Vassiliadis and tetrathionate broth media have inhibitory effects on the detection of *Salmonella* spp. by PCR, while BHI broth and selenite cystine broths were the best for detecting this bacterium by using the PCR procedure.

3.3 Comparison Between Direct Surface Plate Method on Different Selective Media and Enrichment Methods

A comparison between the direct surface plate method on selective medium and enrichment methods is discussed here based on the type of wastewater sample (treated or untreated). In the non-treated samples, the direct surface plate method is more effective than MPN and membrane filtrations while in the treated or disinfected samples, the efficiency of this method is negligible and the enrichment process of pathogenic bacteria is necessary before plating on the selective media. In this section, the comparison between using direct plating on different selective media as well as using enrichment methods for *Salmonella* spp. and *Shigella* spp. is done to best understand the best methods for recovering both bacteria from different sources.

Shaban and El-Taweel (1999) examined the detection of *Listeria monocytogenes* from different wastewater samples using direct surface plate counts and the MPN method. The study revealed that the direct surface plate technique detected a higher count of *L. monocytogenes* than the selective enrichment MPN technique. However, in the disinfected samples by chlorination, the variations were less than that recorded in the raw samples which were between 1 and 2 log. The study indicated that the surface plate technique is the most practical method for the determination and enumeration of *L. monocytogenes* in the wastewater samples. El-Lathy et al. (2009) studied the detection of *Salmonella* spp. in raw and disinfected wastewater by using PCR, MPN and surface plate techniques. The results revealed that *Salmonella* spp. was found to be positive in all samples when PCR was used. In contrast, it was detected in 93% of the samples using the direct surface plate technique and in 90% of the samples using the MPN technique. These findings indicated that the molecular technique is more efficient than the culture method in the isolation of bacteria from wastewater.

Nonetheless, the efficiency of surface plating depends mainly on the type of isolation medium, the use of an enrichment process and the samples tested. Below are some of the studies which investigated the isolation of pathogenic bacteria from different waste samples. King and Metzger (1968) evaluated the efficiency of Hektoen enteric agar (HE), *Salmonella* and *Shigella* agar (SS agar) and EMB Agar for the isolation of *Salmonella* spp. and *Shigella* spp. from 2855 stool specimens by direct

and indirect methods. Twice the amount of *Shigella* spp. was recovered on HE Agar than on SS Agar through both methods. Among 98 isolates, 97 isolates were obtained on HE agar, 74 on EMB agar and only 40 isolates on SS agar through the direct method. Moreover, HE agar exhibited higher efficiency than SS agar and EMB in recovering *Salmonella* spp. by direct or indirect methods. Bhat and Rajan (1975) compared the efficiency between desoxycholate citrate agar (DCA) medium and xylose lysine desoxycholate (XLD) medium in isolating *Shigella* spp. from stool specimens. The results revealed that XLD exhibited higher efficiency in recovering *Shigella* sp. than DCA, where the morphological characteristics of the colonies on XLD were clearly visible after 18–24 h. Clear characteristics on DCA appeared after 48 h. Warren (2006) reported that the DCA and XLD media are more useful as intermediate selective media for isolating *Shigella* spp. from food.

Kelly et al. (1999) estimated the cost-effective methods for the isolation of *Salmonella enterica* from clinical specimens. The study was conducted using 8717 faecal specimens. The results revealed that the primary isolation of *S. enterica* on XLD agar has enhanced the speed of the diagnostics process but with less sensitivity than that achieved with Selenite enrichment. However, the enrichment process by using selenite broth and then plating on both brilliant green and XLD agar offered no further advantage than the direct plating onto XLD without enrichment.

Nye et al. (2002) evaluated of the efficiency of XLD, mannitol lysine crystal violet brilliant green (MLCB), DCA and α-β chromogenic ABC agars for the isolation of *S. enterica* from 2409 human faeces samples by direct plating method with enrichment and non-enrichment processes. The results revealed that the 46 of the 60 possible isolates of *Salmonella* sp. were recovered by direct plating after the enrichment process via selenite enrichment while no isolates were obtained by the direct plating method without the enrichment process. MLCB recorded the high recovery rate individually (84.8%) with less amounts of competing flora (CF) which have no effect on the recognition of *Salmonella* spp. colonies. ABC was the best in terms of specificity, but CF has affected the sensitivity of this medium. DCA recognizes only 9.01% of picked positive bacteria. The study concluded that XLD and MLCB are the best media for recovering *Salmonella* spp. from faeces samples while XLD is the most effective for routine diagnostic process. Pant and Mittal (2008) claimed that the direct plating method needs less time in comparison to the conventional method based on the enrichment process. The study detected *Salmonella* sp. and *Shigella* sp. in the wastewater samples for six months and found that the isolation of both bacteria by direct methods improved by 105 and 276%, respectively, in comparison to the conventional method.

Létourneau et al. (2010) investigated the presence of TC, FC, *Enterococcus* sp., *E. coli, Y. enterocolitica* and *C. perfringens* from feed, water and manure samples of hog finishing houses using the direct plating method. The counting of bacteria in feed and manure was conducted using a dilution process where sterile sodium metaphosphate buffer (2 g/L) was used while bacterial loads in the water samples were enumerated via the membrane filtration method. The bacterial colonies grown on *m*Endo-LES agar with a metallic green sheen appearing after 18–20 h at 37 °C were counted as TC, while those grown on *m*FC agar at 44.5 °C with a distinctive indigo blue colour were counted as FC. *m*Enterococcus agar was used for the isolation of *Enterococcus*

spp. which showed a burgundy colour after 48 h at 37 °C. *E. coli* appeared as blue indicative colonies on *m*FC basal medium after 18–24 h at 44.5 °C. *E. coli* colonies exhibited yellow colour which indicated β-glucuronidase activity in the medium due to the presence of 3-bromo-4-chloro-5-indolyl-β-D-glucuronide (100 mg/L) in the medium components. *C. perfringens* have yellow colonies surrounded by a yellow halo on *m*CP agar. The exposure of the colonies to ammonium hydroxide fumes changed it to magenta. The stormy fermentation of skim milk broth inoculated with these bacteria after 24 h confirmed it as *C. perfringens*. Cefsulodin-Irgasan-Novobiocin agar was used for enumerating *Y. enterocolitica* which grew after 18 h at 30 °C as small colonies measuring 2 mm in diameter. Lysine arginine iron agar slants (LAIA) were used to confirm the identity of this bacteria which appeared as alkaline slant and acid butt without the production of gas and H_2S.

Brichta-Harhay et al. (2008) added antibiotics into the isolation medium in order to isolate *Salmonella* spp. and *E. coli* O157:H7 from hides and carcasses of culled cattle in the United States by the direct plating method, while the identification process was performed using serology tests and PCR analysis. The enumeration of *Salmonella* spp. was performed by using a spiral process for 50 μl aliquot of each 20-ml sponge sample on XLD_{tnc} medium supplied with 4.6 mL/L of tergitol (niaproof), 15 mg/L of novobiocin, and 10 mg/L of cefsulodin. The isolation media was incubated for 18–20 h at 37 °C, thereafter the plates were incubated at room temperature (23–25 °C) for 18–20 h. The most probable bacteria grown on the medium were identified as *Salmonella* spp. by using PCR. *E. coli* O157:H7. They were isolated on ntChrom-O157 agar and incubated for 18–20 h at 42 °C. The identification of *E. coli* O157:H7 was conducted by using the DrySpot agglutination test kit. The colonies with positive results for this test were subcultured inctSMac and then subjected to PCR analysis.

Nagvenkar and Ramaiah (2009) studied the abundance of TC, *E. coli*, *Vibrio cholera*, *Salmonella* spp. and *E. faecalis* from sea water contaminated with sewage. TC was isolated on McConkey Agar, *E. coli* on Thiosulphate citrate bile salts sucrose (TCBS) Agar, *Salmonella* spp. on Hi-Crome *Salmonella* Agar and *E. faecalis* on *Enterococcus* confirmatory Agar. Seawater was added to each medium to provide a salinity of 15 PSU, except for TCBS which was prepared using deionized water. The isolation procedure was conducted by using the direct plating method. The study revealed that direct isolation on the selective media exhibited efficiency in recovering pathogenic bacteria from polluted sea water.

Ongeng et al. (2011) used the surface spread plate method on selective media for the isolation of *E. coli* O157:H7 and *S. enterica* serovar *Typhimurium* from manure and manure-amended soil. The enrichment procedure was used when the concentrations of both bacteria were less than the detection limit of 2 log CFU g^{-1} to detect the presence of residual viable cells. The authors in this study used highly selective isolation media which exhibited high efficiency. These media included XLT4-Rif100-Ny50-Cy50 for *S. typhimurium* and CT-SMAC-Rif100-Ny50-Cy50 for *E. coli* O157:H7. EC broth medium containing novobiocin antibiotics was used as an enrichment media for the recovery of *E. coli*. Thompson et al. (2013) isolated *S. aureus* from hospital wastewater and sewage treatment plants using the direct method. The isolation of bacteria from raw wastewater was carried out using the

direct surface plate method, while the membrane filtration method was used for recovering the bacteria from treated samples. The isolation process was conducted on Vogel-Johnson agar and MSA containing cefoxitin antibiotic according to Broekema et al. (2009).

In some bacteria such as *Shigella* spp., it has been reported that the direct isolation on selective media is better compared to direct isolation carried out on enriched media. Dunn and Martin (1971) claimed that the best method for the isolation of *Shigella* spp. from faecal specimens is by using the direct surface plate method on the selective media. Iveson (1973) reported that the direct surface plating of *Shigella* spp. on DCA was superior to other enrichment methods. Morris et al. (1970) conducted a study to compare the effectiveness of XLD, MacConkey and SS agar for the direct isolation and transport of *Shigella* spp. from faecal specimens. The study revealed that XLD agar was better than MacConkey and SS agar for isolating *Shigella sonnei*, while SS agar and XLD were better than MacConkey agar for recovering *Shigella flexneri*. The direct plating method exhibited high efficiency for the isolation of *Shigella* spp. from faecal specimens in comparison to the transport medium or enrichment broth. The study also indicated that the use of buffered glycerol saline has improved the recovery of *Shigella* spp. by 83% within 48 h in comparison to direct plating. Finally, the study recommended XLD agar and SS agar for the direct isolation of *Shigella* spp. without the need for an enrichment process.

Pollock and Dahlgren(1974) investigated the use of the direct plating method on selective media (XLD, HE agar, MacConkey agar and SS agar) for isolating *Shigella* spp. and compared them to enrichment methods. The study was conducted for a period of one year in which 455 stool specimens were used in this period. The total number of *Shigella* spp. recovered was 53 isolates. Among them, 56% was identified as *S. sonnei* while 13% was identified as *S. flexneri*. The results showed that 90% of the isolates were recovered on XLD, 87% on HE agar, 80% on MacConkey, while only 28% was isolated on SS agar. In contrast, only 0.5% of *Shigella* spp. was recovered after the enrichment process using Selenite-F enrichment medium.

In terms of using enrichment cultures, Muniesa et al. (2005) revealed that the presence of bacteriophages was associated with *Salmonella* spp. bacteria in the enrichment culture medium. Therefore, it might negatively affect pathogenic diversity and density during the enrichment process. The study compared filtrated samples (0.45 microns) and non-filtrated samples. The results showed that the *Salmonella* biotypes recovered from the filtrated samples were changed in comparison to the non-filtrated samples.

Cassar and Cuschieri (2003) studied the isolation of *Salmonella* spp. on *Salmonella* chromogenic medium (SCM) in comparison to DCLS from 500 stool specimens by the direct plating method and the selenite enrichment method. The results found that among the 44 Salmonella-positive stool samples, the specifications for the direct plating method and the enrichment method were 82.5% versus 72.8%, respectively. For DCLS agar and SCM, the specifications were 98.5 versus 95.8%, respectively. Castillo et al. (2006) investigated the isolation of *Salmonella* spp. and *Shigella* spp. from different sources including food and clothes by direct plating, pre-enrichment process only, and pre-enrichment process followed by enrichment-

only methods. The selective media used were MacConkey agar, XLD agar, SS agar, brilliant green sulfadiazine agar (BGS) and bismuth sulfite agar (BSA) while the enrichment medium was tetrathionate broth, GN pre-enrichment broth, selenite cystine and tetrathionate broths. The direct plating on XLD, SS agar and MacConkey agar media was used for isolating the bacteria from the samples and then incubated for 24 h at 35 °C. Direct enrichment process was carried out with tetrathionate broth which was incubated for 24 h at 43 °C and then plated onto BGS agar and BSA and incubated again for 24 h at 35 °C. The pre-enrichment method was conducted using GN broth and incubated for 18 h at 35 °C. Thereafter, one loopful was streaked onto XLD, SS agar and MacConkey agar. The enrichment process was performed with selenite cystine and tetrathionate broths. After the incubation period for 24 h at 43 °C, a loopful was streaked onto BGS and BSA and incubated for 24–48 h at 35 °C. The study found that the number of positive samples increased by using the direct plating method and not by the pre-enrichment method. Moreover, the direct plating method was the best for the recovery of *Shigella* spp. in comparison to the enrichment medium.

Maddocks et al. (2002) investigated *Salmonella* spp. growth on a new formulation of CHROM agar Salmonella (CAS) medium in comparison with XLD, SS agar and HE agar. The results revealed that the efficiency of direct plating on the CAS medium for the detection of *Salmonella* spp. was 83%, while the efficiency was 55% for both XLD and SS agar. Those findings confirmed the sensitivity and specificity of the CAS medium for the direct isolation of *Salmonella* spp. Even though *Candida* spp. and *Pseudomonas* spp. have also grown on the CAS medium, their colony morphology was quite different than that of *Salmonella* spp. colonies. Based on this study, it can be indicated that the direct isolation of *Salmonella* spp. requires using a very sensitive and specific medium.

Examples of studies which have used the direct culture method for the isolation of pathogenic bacteria from different sources are presented in Table 3.1. Based on the studies reviewed above, it can be concluded that the main criteria used for the selection of the appropriate isolation method is the source of samples. Wastewater is highly polluted and contains a high concentration of pathogenic bacteria. Therefore, the direct method might be effective for the isolation and enumeration of these pathogens. In contrast, the isolation of pathogenic bacteria from food products and drinking water has to be enriched first due to the presence of chemical substances such as preservations in the foods and chlorine or ozone residues in the water which inhibit or inactivate the bacterial cells.

3.4 Antibiotic Resistance Among Pathogenic Bacteria in Greywater

Antimicrobial resistance among pathogenic bacteria in greywater needs to be assessed because bacterial resistance towards antibiotics is one of the main factors

Table 3.1 Direct plating method and membrane filter method for the isolation and enumeration of pathogenic bacteria from different sources

Bacteria	Source	Method	Reference
Shigella sp.	Faecal specimens	Direct inoculation on selective media	Morris and Fulton (1970)
Shigella sp.	Faecal specimens	Direct inoculation on selective media	Dunn and Martin (1971)
Shigella sp.	Faeces	direct inoculation on selective media	Iveson (1973)
Shigella sp.	Stool specimens	Direct plating on selective media and compared to enrichment media	Pollock and Dahlgren (1974)
TC, FC, faecal streptococci, *Staphylococcus* sp., *Klebsiella* sp., *Pseudomonas* sp.	Sewage	Membrane filtration, spread-plating directly onto appropriate selective media	Dudley et al. (1980)
Shigella isolates	Stool samples	Direct plating on selective media	Vila et al. (1994)
L. monocytogenes	wastewater	Surface plate counting with the selective media	Shaban and El-Taweel (1999)
Salmonella enterica	Faecal specimens	Direct plating method	Kelly et al. (1999)
Salmonella group	Wastewater	Direct Surface Plating Procedures	El-Taweel and Ali (2000)
Salmonella spp. *Campylobacter* spp.	Raw, whole chickens	Spread-plating	Jørgensen et al. (2002)
Salmonella enterica	faeces	Direct plating media	Nye et al. (2002)
Salmonella sp.	Freshwater and Seawater	Direct plate counts	Sugumar and Mariappan (2003)
Salmonella sp.	Stool Specimens	Direct plating	Cassar and Cuschieri (2003)
E.coli O157:H7, *S. enteric Serovar Enteritidis*	Chicken manure	Direct plating method	Erickson et al. (2004)
S. aureus	Water	Membrane filtration	HPA (2004)
FC, *P. aeruginosa, S. aureus*	Wastewater	Membrane Filter Method	Friedler et al. (2005)
Staphylococcus sp.	Water	Surface plate technique	Götz et al. (2006)
Shigella sp.	Freshly Squeezed Orange Juice, Fresh Oranges, and Wiping Cloths	Direct plating method	Castillo et al. (2006)
Salmonella *E. coli*	Compost material with cow dung	Direct counting method	Chun-ming (2007)
Salmonella sp., *E. coli* O157:H7	Ground beef, cattle carcass, hide and faecal samples	Direct plating method	Brichta-Harhay et al. (2007)
Salmonella sp.	Poultry carcass rinses	Direct plating method	
S. aureus, E.coli, *P. auroginosa*, *Proteus* sp., *Bacillus cerues, Streptococcus* sp.	Urine and swap	Direct plating method	Ogbulie et al. (2008)
Salmonella sp., *Shigella* sp.	Sewage	Direct method	Pant and Mittal (2008)

(continued)

Table 3.1 (continued)

Bacteria	Source	Method	Reference
Salmonella sp., *E. coli* O157:H7	Hides and carcasses of cull cattle	Direct plating method	Brichta-Harhay et al. (2008)
Salmonella spp., *Vibrio* spp., *Listeria* spp.	Wastewater	Surface plate technique	El-Lathy et al. (2009)
S. aureus	Milk	Direct plating method	Synnott et al. (2009)
TC, FC, Enterococci, *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*	Water and sediment samples	Direct plating method	Nagvenkar and Ramaiah (2009)
TC, FC, *E. coli*, Enterococci, *Salmonella* sp., *Shigella* sp., *Proteus* sp., *Klebsiella* sp., *Vibrio cholera*, *P. aeruginosa*	Coastal water	Direct surface plate	Patra et al. (2009)
TC, FC, *Enterococcus* sp, *E. coli*, *Y. enterocolitica*	Manure from hog finishing houses	Membrane filtration	Létourneau et al. (2010)
E. coli O157:H7, *Salmonella enterica* serovar Typhimurium	Manure and manure-amended soil	Surface spread plate	Ongeng et al. (2011)
TC, FC, *E. coli*, *Shigella* like organisms, *Vibriocholera* like organisms, *Salmonella* like organisms	Sewage	Direct plating method	Rodrigues et al. (2011)
Salmonella sp.	Organic and conventional broiler feed	Direct plating method	Petkar et al. (2011)
S. aureus	Hospital wastewaters and sewage	Membrane filtration	Thompson et al. (2013)
S. viridins, *Staphylococcus* sp., *Corynebacterium* sp., *Enterobacter* sp., *Heamophilus* sp.,	Pus specimens	Direct plating on selective medium	Chunduri et al. (2012)

which play an important role in bacterial pathogenicity to humans. However, studies on bacterial resistance towards antibiotics in non-clinical environments should be conducted in different ways and compared to those performed in hospitals or clinical environments.

The difference between the concept of antimicrobial resistance in clinical and non-clinical environments has occurred recently (Al-Gheethi et al. 2013). Walsh (2013) explained more about the differences between antimicrobial resistance tests in clinical and non-clinical environments. This term is used to define natural antibiotic resistance. Clinically, the classification of bacteria as susceptible or resistant depends on whether an infection with the bacterium responds to therapy. In contrast, in the environment, resistant microorganisms from a microbiological point of view are those that possess any kind of resistance mechanism or resistance gene.

Al-Gheethi et al. (2015a, b) investigated microbial resistance among pathogenic bacteria obtained from surface water and treated sewage effluents. The study used

two different methods. The disk diffusion technique with the minimum inhibition concentrations (MICs) of antibiotics was carried out to determine the antibiotic resistance among pathogenic bacteria from treated sewage effluents. However, the results were read as resistant, moderate and sensitive based on non-clinical applications in which the bacteria was considered resistant at 0.7–1 cm in diameter of the inhibition zone, moderately susceptible at 1.0–1.2 cm diameter of the inhibition zone and susceptible when a diameter of more than 1.2 cm of the inhibition zone was observed (Audra and Andrew 2003). This method is different from the Clinical and Laboratory Standards Institute (CLSI) guidelines which require different readings for antibiotic sensitivity based on antibiotics class and bacterial species. In this study, however, the authors used a constant range for all pathogenic bacteria and antibiotics.

In contrast, the antibiotic resistance among bacteria obtained from surface water was investigated using the culture-based technique, in which high concentrations of antibiotics (100 mg L^{-1}) were added into the culture medium after autoclaving and then the medium was used for isolating the bacteria from the surface water. The percentage of the resistance was calculated in comparison with the number of bacterial cells (CFU) in the culture medium without antibiotics (control). The authors explained the use of two different methods based on the survival period of the bacteria in the environment and the natural adaptation for antibiotic resistance. Antibiotic resistance can be due to the transmission of antibiotic gene resistance or the availability of antibiotics in the environment which leads to the development of a resistance mechanism in the bacterial cell. The authors claimed that the pathogenic bacteria isolated immediately from domestic sewage generated from households have not developed their resistance mechanism against a wide range of antibiotics and still reflect their potential to resist antibiotics which have been exposed to them in the human body. Furthermore, a constant range to classify these bacteria as either resistant, moderate or sensitive was applied because these pathogens would develop their resistance mechanism in an environment where they will be discharged along with wastewater. In time, they will be acclimatized to resist several antibiotics during their survival in the wastewater. Therefore, the antimicrobial resistance among pathogenic bacteria obtained from surface water is supposed to be tested using a culture media containing antibiotics where the bacterial cells will be exposed to high concentrations of antibiotics.

Nevertheless, the concentrations of antibiotics in surface water remain low, but the use of high concentrations aim to detect bacterial tolerance towards antibiotics which is developed during its long survival period in the environment. Bacterial resistance and tolerance are not the same. More discussion on the differences between bacterial resistance and tolerance are available in the work published by Lewis (2008) and Al-Gheethi et al. (2015b).

The most common media used for antibiotic sensitivity test is Muller Hinton agar (MHA). However, Niederstebruch and Sixt (2013) indicated that standard nutrient agar 1 (StNA1) might be the best alternative for MHA for antibiograms in developing countries. Most international method standards and guidelines for the microbiological assessment of wastewater are not applicable in developing countries due to the lack of experience among technicians as well as the absence of equipment and facilities to

meet the standard requirements. Nevertheless, MHA might be common for antibiotic susceptibility tests of pathogenic bacteria from clinical environments but not for that obtained from non-clinical environments. Wiggins (1996) used Trypticase soy agar plates to determine the antimicrobial sensitivity among faecal Streptococci isolates from natural water. Tao et al. (2007) used nutrient agar plates to study the antibiotic resistance of *Sphingomonas* sp. against cefuroxime, erythromycin, chloramphenicol, amoxicillin and tetracycline.

3.5 Conclusion

It can be concluded that different methods can be used for the determination of pathogens in greywater and the environment. The selection of specific techniques depends mainly on the aim of the isolation. The enumeration of pathogens in the raw greywater might be conducted via the culture-based method. However, pathogens such as *Salmonella* sp. need to be enriched first in order to facilitate their isolation. The enrichment method is used to detect the presence or absence the pathogenic bacteria available in a concentration less than the detection limits of the direct count plate but it cannot be used for counting their cells. This is because the pre-enrichment process would increase their number and would not reflect the actual number of the greywater samples. Moreover, in the treated samples, the use of non-culture methods might be the best alternative to detect the validity and potential of the inactivated bacteria to regrow. Molecular techniques can be used for counting the pathogenic bacteria in greywater which failed to grow on the isolation medium. However, this technique also has limitations in terms of its detection limits of bacterial cell number.

Acknowledgements The authors wish to thank the Ministry of Higher Education (MOHE) for supporting this research under FRGS vot 1574 and also the Research Management Centre (RMC) UTHM for providing grant IGSP U682 for this research.

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