



Enumeration of Bacteriophages from Mmabatho Treatment Plant and Some Selected Water Sources in Ngaka Modiri Molema District of North-West Province, South Africa

Theodora Ijeoma Ekwomadu, Nomathamsanqa Patricia Sithebe

Department of Biological Sciences, Faculty of Agriculture, Science and Technology, North-West University, Mafikeng, South Africa

Email address:

23115394@nwu.ac.za (T. I. Ekwomadu), thami.sithebe@nwu.ac.za (N. P. Sithebe)

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Abstract: Bacteriophages are viruses that infect bacteria, those that specifically infect *E. coli* and other coliforms are termed coliphages. While not a threat to humans, occurrence of somatic and F-RNA coliphages has been correlated with the presence of human enteric viruses in faecal-polluted water environments. This study focused on investigating the incidence of somatic coliphages and F-RNA coliphages from Mmabatho treatment plant and some selected water sources in Ngaka Modiri Molema District of the North West province. A total of 17 water samples were analysed for the presence of somatic and F-RNA phages using the double-agar-layer plaque assay according to the standard ISO method, ISO 10705-1(1995, 2000). The physico-chemical properties of the water samples were measured before sample collection. Bottled water was used as a negative control and the phage strains ϕ X174 and MS2 as positive controls. Out of the 17 samples collected, (76%) were positive for somatic coliphages, no F-RNA coliphages were detected while 24% of the samples had no coliphages. Thus, the presence of coliphages indicates that the water sources do not meet up to national and international requirement for drinking water quality. Furthermore, monitoring water environments for possible faecal contamination is necessary to ensure public health and safety.

Keywords: Bacteriophages, Coliphages, Somatic, F-RNA, *E. Coli*, Dams, Ground Water Taps, Sewage Treatment Plant

1. Introduction

Monitoring water to assess its microbial safety has long relied on the detection of indicator organisms. Potable and recreational waters have been routinely evaluated for fecal contamination using fecal indicator bacteria [1]. Although bacteria indicators have been used over the years to detect bacterial pathogens such as *Vibrio cholerae* (which causes cholera) and *Salmonella typhi* (which causes typhoid fever) [1, 2] it has been suggested that viral pathogens are the major causative agents of waterborne illnesses [1, 3]. However, it has become increasingly clear that the traditional bacterial indicators may not be the best indicators of viral pathogens associated with fecal contamination [1, 4, 5]. This is because they respond to water treatment processes and environmental degradation processes differently from viruses. This therefore, highlights the necessity for additional standards in water quality safety. Hence, coliphages (somatic and F-RNA) and other bacteriophages, such as those that infect

Bacteroides fragilis, have been utilized as indicators in monitoring presence of viruses in faecal-contaminated water environments [6-9].

Unlike determining the viral contamination of water using methods specifically designed to detect pathogenic viruses, which are time-consuming, costly and technically difficult because of viral characteristics, methods used to detect phages are inexpensive, rapid and easy to perform and thus applicable to both industrialized and developing countries [7, 10]. Apart from that, coliphages display similarities to human enteric viruses both morphologically and behaviourally [7, 11, 12]. For example, F-RNA coliphages are morphologically similar to enteroviruses, hepatitis A and E viruses, while some somatic coliphages are similar to adenovirus [13]. Studies have also shown that coliphages are generally as resistant as enteric viruses to unfavourable environmental conditions as well as water treatment and disinfection processes [8, 14, 15]. Coliphages are persistent in the environment and have been found in feacally contaminated

waste, surface, ground waters and sand [16]. Some studies have found a correlation between the presence of coliphages and human enteric viruses [9, 17-22], while others have found no correlation between them [3, 8, 23-25]. In addition to their use as water quality indicators, FRNA coliphages may be used to help discriminate between human and non-human sources of faecal pollution in water [26].

In South Africa, the availability of clean and safe water in rural areas is still a serious problem. The majority of rural communities use water directly from available sources without any treatment and the microbial quality of these water sources is not always guaranteed [27-29]. Since coliphages typically replicate in the G. I. T of humans and warm-blooded animals, their presence in water indicates faecal pollution, potential presence of enteric viruses and possibly other pathogens [5, 7, 19, 30]. All these expose the public to water-borne illnesses. However, very little research work has been done on determining the presence of bacteriophages in water sources in Ngaka Modiri Molema district of the Northwest Province. Hence, the main goal of this study was to determine the occurrence of somatic and F-

RNA coliphages from Mmabatho treatment plant, the adjacent Setumo dam and some selected dams and taps in Ngaka Modiri Molema district of the Northwest Province of South Africa.

2. Methods

2.1. Description of Study Site

This study was carried out in the following sites: Setumo dam, Disaneng dam, Letlamoreng dam, Disaneng ground water tap, Loporong ground water tap, Mayane ground water tap, Makgobistad ground water tap, Disaneng borehole and the Mmabatho Sewage Treatment Plant, all located in Ngaka Modiri Molema District in the Northwest Province. (Figure 1). The latitude and longitude of the district is 25° 55' N and 25°50' E respectively. The dams were selected based on extensive use by people living near-by where many activities such as fishing, washing and baptism were performed. Other water sources were cited as central points commonly used by the community.

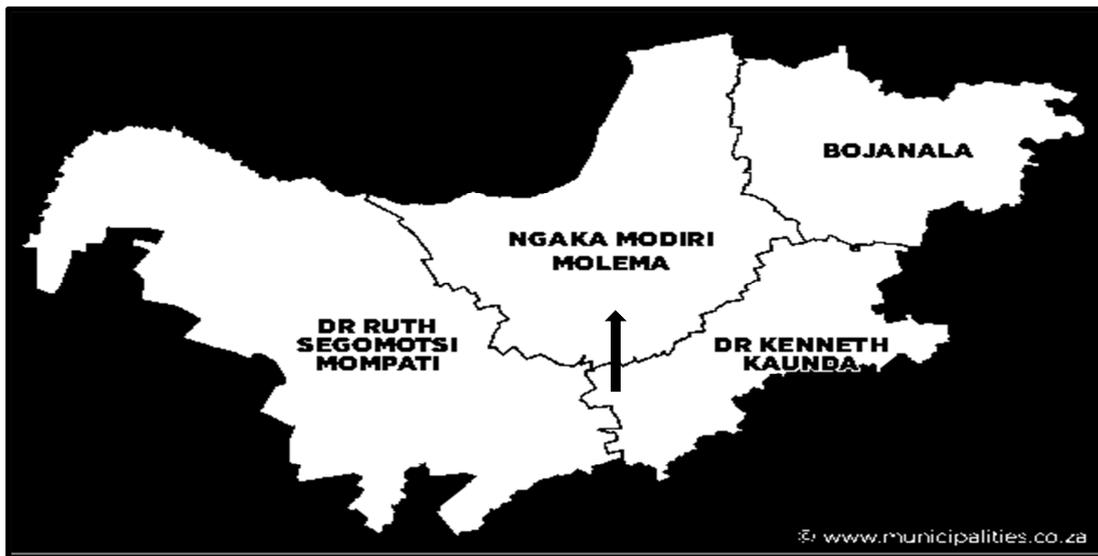


Figure 1. Ngaka Modiri Molema district where the water samples were collected.

2.2. Sample Collection

Water samples were collected in sterile, disposable, 500-mL, plastic specimen containers. Samples from the inlet, midpoint and outlet points of the dams were collected and also from the primary, secondary and tertiary treatment of the Mmabatho Sewage Treatment Plant. Water was also collected from randomly selected taps and boreholes in the district giving a total of 17 samples. Physicochemical parameters of water such as pH, temperature, total dissolved solids (TDS) and turbidity were measured on site before collection using a portable multimeter (Lasec, South Africa). The samples were transported on ice in a cooler box to the laboratory and analysed within 24 h of collection. Water samples were stored at 4°C prior to analysis.

2.3. Isolation of Somatic Phages

Isolation of somatic phages was carried out using the double-agar-layer plaque assay method according to the standard [31]. In brief, 2.5 mL aliquot of sterile, melted top agar consisting of tryptone yeast glucose-extract agar containing 1 mL of 1% nalidixic acid (Sigma-Aldrich) and 600 µL of calcium chloride (Sigma-Aldrich) with the temperature maintained at 50°C in a water bath was mixed with 1 mL of water sample. With the top-agar mixture still in water bath, the tubes were aseptically inoculated with 0.2 mL of *E. coli* WG5 host culture. The content of the tubes were gently mixed by rolling the tube briefly in palms of hands and then poured onto the bottom agar layer (tryptone yeast glucose-extract agar) of the phage agar plates in 9 mm petri

dishes, ensuring even distribution of the top agar on top of the bottom agar layer by swirling the petri dishes carefully. Samples were analysed in triplicates and phage ϕ X174 was used as a positive control strain. Plates were inverted and incubated overnight at 37°C once the molten agar has set. Plates were observed for plaque zones which appeared as clear areas around the bacteria host.

Before carrying out the actual analyses, one vial of already prepared *E. coli* WG5 host culture (stock) was thawed at room temperature and boosted by adding approximately 25 mL of sterile nutrient broth to it and was incubated for 2 h at 37°C. Phage agar plates were also at room temperature before use and bottles of top agar were melted and then cooled to 50°C.

2.4. Isolation of F_{RNA} Phage

The standard [32] double-agar-layer technique was used to isolate F_{RNA} phages. Briefly, the host culture *Salmonella typhimurium* WG49 (ATCC 700730) was grown in an incubator up to the log phase which is usually reached in 3 to 4 h while shaking (100 rpm) at 36°C. The growth rate was monitored closely every 30 mins using a spectrophotometer (Merck, Germany) against a blank reference until an absorbance of 0.76 was obtained at a wavelength of 560 nm (A560). The host suspension was removed from the incubator, placed on ice and used within 2 h.

A 2.5 mL aliquot of melted top agar (tryptone yeast glucose-extract agar) containing 100 μ L of 1% nalidixic acid (Sigma Aldrich) and 1 mL of calcium glucose (Sigma Aldrich), was pipetted into a test tube placed in test tube rack and the temperature maintained at 50°C in water bath. 1 mL of host culture was added to the top agar followed by addition of 1 mL of the test sample or an appropriate dilution of the sample. The content of the tubes were gently mixed by rolling the tube briefly in palms of hands and then poured onto the bottom agar layer (tryptone yeast glucose-extract agar) of the phage agar

plates in 9 mm petridishes, ensuring even distribution of the top agar on top of the bottom agar layer by swirling the petridishes carefully and let to solidify. Plates were inverted and incubated overnight at 37°C, and observed for plaque zones. The procedure was carried out in triplicates and bacteriophage MS2 was used as positive control phage strain. Samples were handled aseptically, since contamination with a single phage particle would lead to a false positive result. Also the workspace near the water bath was disinfected using 70% alcohol solution in order to guard against contamination.

2.5. Phage Purification

For the purpose of purification, isolated plaques were propagated serially until a single phage type was obtained according to [33] with slight modifications. Briefly, plaques were individually picked using a wire loop to carefully cut the area surrounding the plaque (excision). The plaque material was placed in 1 mL of SM buffer (Fermentas Life Science, Lithuania) containing at least 2 drops of chloroform in a 1.5 mL micro centrifuge tube. The piece of “soft agar” containing the phage was gently broken in small pieces with the wire loop and mixed briefly with a vortex mixer (Merck, Germany). It was left to stand for few minutes at room temperature to allow phage particles to diffuse out of the agar. The lysate was then stored at 4°C in SM buffer until required. From the phage stock, a volume of 100 μ L phage sample was mixed with 100 μ L of the host culture (*E. coli* WG5) in 3 mL of top agar at 50°C and poured onto freshly made bottom agar plates. The plates were incubated overnight at 37°C once the molten agar had set. This procedure was repeated until only one phage type, based on the plaque size was obtained [34]. The purified phage lysate was then stored at 4°C in SM buffer to be used for further analysis when required.

3. Results

Table 1. Some physico-chemical qualities of water samples from different sampling sites.

Water Sources	pH	Temp.(°C)	TDS(mg/l)	Turbidity(NTU)
Mmabatho treatment plant (1°)	6.40	22.1	1219	59.1
Mmabatho treatment plant (2°)	7.90	22.4	922	42.5
Mmabatho treatment plant (3°)	8.90	23.0	420	37.8
Setumo Dam (Inlet, midpoint and outlet)	9.10	17.3	254	58.9
Disaneng Dam (Inlet, midpoint and outlet)	8.95	16.6	258	50.7
Letlamoeng Dam (Inlet, midpoint and outlet)	9.18	15.8	239	48.6
Disaneng tap(GW)	8.80	32.1	296	2.9
Loporung tap(GW)	7.47	22.7	295	1.7
Mayane tap(GW)	7.65	15.9	330	26.6
Makgobistad tap (GW)	7.73	17.1	345	22.0
Disaneng borehole	7.61	23.6	301	25.9
SANS 241:2015(Standard)	≥ 5 to ≤ 9.7	None	≤ 1200	≤ 5

GW – Ground water, NTU - Nephelometric turbidity unit, SANS- South African National Standard

In physicochemical parameters of the water samples from Mmabatho treatment plant, the pH value ranges from 6.4 to 8.9, the temperature from 22.1 to 23.0°C, the TDS from 420 to 1219 mg/l and the turbidity from 37.8 to 59.1. In water samples from the dams, the pH ranges from 8.95 to 9.18, the temperature from 15.8 to 17.3°C, the TDS from 239 to 258mg/l and the turbidity

from 48.6 to 58.9. Results from the ground water taps and borehole were also recorded and all results obtained for physical parameters were compared with SANS 241:2015 standard reference values as shown in table 1. Coliphage analysis showed that out of the 17 samples analysed, (76%) were positive for somatic coliphages, no F_{RNA} coliphages were detected while

24% of the samples had no coliphages, (figures, 2-4). The somatic coliphage counts varied from 00 to 14 pfu/ml in water samples from the dams, 02-10 pfu/ml in water samples from the ground water taps and boreholes and 00-39 pfu/ml in water samples from the sewage treatment plant.

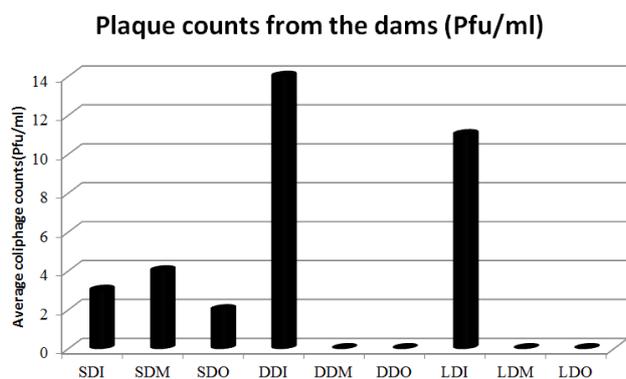


Figure 2. Graphical presentation of average plaque counts from the dams.

SDI=Setumo dam inlet point SDM=Setumo dam midpoint. SDO=Setumo dam outlet.

DDI=Disaneng dam inlet. DDM=Disaneng dam midpoint. DDO=Disaneng dam outlet.

LDI=Letlamoreng dam inlet. LDM=Letlamoreng dam midpoint. LDO=Letlamoreng dam outlet.

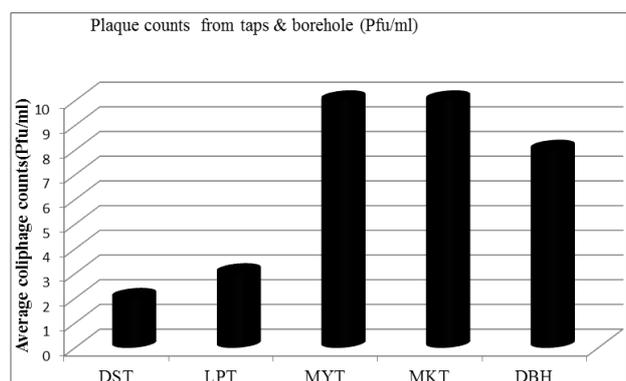


Figure 3. Graphical presentation of average plaque counts from taps and borehole.

DST=Disaneng tap (GW). LPT=Loporung tap (GW). MYT=Mayane tap (GW). MKT=Makgobistad tap (GW). DBH=Disaneng borehole.

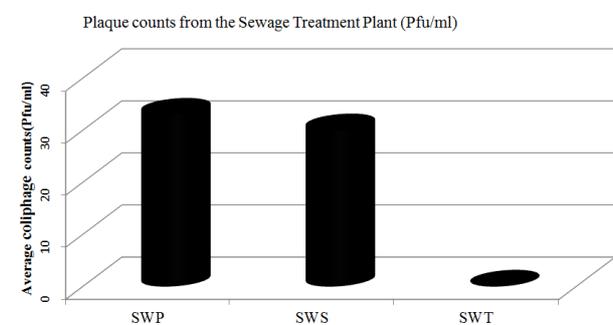


Figure 4. Graphical presentation of average plaque counts from Mmabatho Sewage Treatment plant.

SWP=Sewage primary treatment, SWS=Sewage secondary treatment, SWT=Sewage tertiary treatment.

4. Discussion

Determination of the physico-chemical properties of the water samples (which served as the starting point for this study) showed that some of the potable water samples, particularly the underground water taps, borehole and dams did not comply with the standard limits for drinking water [35]. The pH values, which give the indication of acidity and alkalinity, of the water samples are within the recommended values and therefore, the pH could be classified as suitable and safe for domestic, agricultural and recreational uses. On the contrary, the turbidity values of most of the water samples was above the required limits while only the sample from the primary treatment of the sewage exceeded the limit for TDS (Table 1). Furthermore, pH of the sewage water increased from 6.40 to 8.90 from the primary treatment to the tertiary treatment. The main reason behind this phenomenon could be the formation of ammonia and ammonium salts from nitrogen by the nitrogen-fixing bacteria present in the sludge blanket which increases the alkalinity in the water. This is in line with [36, 37] who found an increase in the alkalinity in the final effluent after anaerobic digestion. Nevertheless, the results obtained for pH measurements in the water and in the sewage samples were as expected since there are neither major industries nor mining activities in the area that could cause extreme changes in the pH of the sewage discharges or of the receiving water.

Of the 17 water samples, only 27.1% had NTU values that fell within the recommended values for drinking water. The turbidity of the water samples from the dams, most of the underground water taps and borehole was way above the recommended limits [35]. The turbidity of sewage water decrease from 59.1 to 37.8 NTU values from the primary treatment to the tertiary treatment. The decrease in turbidity could be mainly because the minute impurities and large particles get trapped in the sludge and are consumed by the microbial activities in the reactor. This was in line with the study by [38] who found that there was decrease in NTU values in the effluents from sewage. The TDS of the water samples are within the recommended values except elevated value in the primary treatment of the sewage water. The TDS also decreased from the primary treatment to the tertiary treatment in the sewage samples. The reason for the elevated TDS value in the primary treatment of the sewage water could be because of the different biological activities going on in the sludge. This is in line with [39] who reported significant reduction of TDS in the treated effluent. Temperature, although generally influences the overall quality of water (physico-chemical and biological characteristics), there are no guideline values set by SANS, but the values obtained in the present study ranged from 15.8-32.1°C which were not far from expected for the sampling period (October, 2012).

Sewage is the waste water that flows after being used for domestic, industrial and other purposes. It is well known that sewage contains pathogens, suspended solids, and other organic and inorganic pollutants [40]. Assessment of water

bodies (dams) and sewage water is very important to safeguard public health and the environment [41]. Sewage discharges are a major component of water pollution, contributing to oxygen demand and eutrophication of water bodies leading to a destabilized aquatic ecosystem [42, 43]. The problem is compounded in areas where sewage treatment systems are not efficient. However, the significant variation observed between the primary treatment and tertiary treatment of sewage samples for most physicochemical parameters (Table 1) indicated that the wastewater treatment process remarkably improved the quality of the raw wastewater influent.

However, despite the improvement on raw sewage quality, the treated effluent did not measure up to the desired target quality for turbidity and TDS with respect to domestic applications [35] and this suggests that the sewage effluent has a potential negative impact on the environment and public health. The quality of the samples from tertiary treatment was however, acceptable in terms of pH, and there was no recommended limit for temperature (Table 1). Also, results of the physico-chemical parameters from this study is an indication that the sewage wastewater is not fit for industrial, domestic and irrigation purpose, but treated final effluent of the sewage treatment plant may be recommended for use as secondary purposes like industrial cooling, gardening and irrigations.

On the other hand, detection of coliphages from the water samples is an indication of faecal pollution, potential presence of enteric viruses and possibly other pathogens [5, 7, 19, 30]. Microbes from faecal waste when ingested from water may pose short-term health effects such as diarrhoea, cramps; nausea etc. They can also pose special health risks for infants, young children, some of the elderly and people with compromised immune systems [44]. In this study, result obtained from coliphage analysis shows that no F⁺RNA phages were isolated but only somatic phages were detected in 76% of the samples while 24% of the samples had no coliphages. The result obtained from a similar study that was conducted in Brazil [45] showed a higher incidence of somatic coliphages in water environments than was obtained in the present study. This could be attributed to different methods adopted by various authors worldwide for the enumeration of phages in water environments. Thus, coliphage counts from various studies can be compared only if the same assay conditions were used. The variations that may greatly influence the final results include the growth conditions such as composition of the nutrient medium which can affect the lytic processes and hence the burst size. The age of the host culture and the type of host strain can also influence the final results [45, 46]. This latter factor is of primary importance since according to some researchers, wild-type *E. coli* strains are poor host for enumeration of coliphages in water environments since they usually have complete O-antigen that masks the majority of phage receptors on the R-core of the lipopolysaccharide.

The previous study that was conducted on Setumo dam [47] also showed highest incidence of somatic coliphages at

the inlet point of the dam, whereas in this particular study, the midpoint of the dam showed the highest incidence of the somatic phages (Figure 2). This could be attributed to the various activities taking place at the midpoint such as fishing, washing, baptism and other human activities at the site which could have contaminated the water. However, no correlation between the results of the physicochemical characterization of the areas studied and the frequency of the coliphages was observed (Table 1). Results on Figure 2 also showed that the inlet points of Disaneng and Letlamoreng dams had a very high incidence of the phages while no phages were found at the midpoints and outlets of the dams. This is in line with [47] who reported the highest count of phages at the inlet point of Setumo dam. This might have been as a result of many different activities, both from the animals and the humans occurring at those inlet points of the dams. Furthermore, the different phage counts could be attributed to the concentration of phage populations at different points of the dams. Phages were detected in all the sampled ground water taps and borehole, with Mayane tap and Disaneng tap having the highest and the lowest counts respectively (Figure 3). These findings correlate with those of [28] who reported incidence of somatic coliphages in some communal boreholes. This indicates the need for intense monitoring of these water sources, as these pose potential health risks to the rural communities, who depend on them for domestic uses including drinking. Sewage samples contain high counts of somatic coliphages, particularly the primary and secondary treatments, while no coliphages were found at the tertiary treatment (Figure 4). This correlates with the findings of [48] who isolated somatic coliphages from sewage samples and [7] also reported incidence of somatic coliphages as high as 10¹⁰/litre of sewage sample. However, absence of coliphages in the tertiary treatment could indicate the effectiveness of the treatment processes which means that presence of coliphages in the receiving water bodies might be from other sources of faecal pollution. According to [7], somatic coliphages are generally excreted by most humans and animals, whereas F⁺RNA coliphages are excreted by a variable and lower percentage of humans and animals. Also somatic coliphages have been found to outnumber F⁺RNA phages in water environments [7, 29]. While the F⁺RNA phage has a relatively low occurrence in water environments, they have been detected in high numbers from sewage samples [49]. This is quite contrary to the findings in this study where no F⁺RNA phage was detected even in sewage samples, although [50] reported that an unpublished EPA study found no F⁺RNA phage in septic tanks.

The plaques found in this research were all clear plaques (Figure 5) which indicates that they are lytic phages. This is in line with [51], who suggested that clear plaques are indicative of virulent or lytic phages and broad host range while turbid plaques indicate lysogenic phages and a narrow host range. This is also supported by [7] who defined somatic coliphages as a wide spectrum of lytic members of the families; *myoviridae*, *siphoviridae*, *podoviridae* and *microviridae* that infect *E. coli* and closely related members

of the bacteria family, *Enterobacteriaceae*. Although several other factors have been identified which can determine plaque morphology in terms of clarity [52] and these factors include, the physiological state of host, temperature and pH of media used. According to [53] tailed phages accounts for more than 95% of all the phages on the planet which shows why there is higher incidence of somatic coliphages in water environments, as was confirmed in this particular study. Apart from the inconsistency in the methods used for the recovery and enumeration of phages in water environments, which makes most of the data difficult to compare, other variables should also be considered. Many variables, such as temperature, pH, presence of organic matter (TDS), concentration and type of ions (Electrical conductivity), density of host bacteria and phages, rainfall etc., which affect the incidence, survival and behaviour of phages in water environments [54] further complicates comparison. Then, the issue of discrepancies resulting from different methods adopted by various authors worldwide is not altogether surprising. This is because work on phages and the methods for detection are still in their infancy, though international collaboration is now leading to universally accepted guidelines for the recovery of phages in water environments [4, 7].

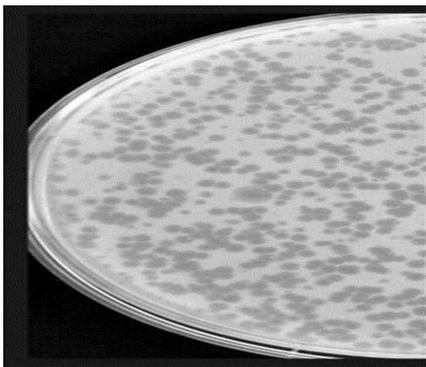


Figure 5. Phage-agar plate showing coliphage plaques.

5. Conclusion

The presence of coliphages indicates that these water sources do not meet up to SANS 241:2015 requirements for drinking water quality. The presence of phages in water is a cause for concern as it indicates faecal pollution, presence of their bacterial host and can also indicate concurrently the presence of pathogenic viruses. These can significantly pose health risks on humans either through the consumption of the water or may be through recreational exposure, posing a serious public health concern, exposing the public to water-borne illnesses. The case is even more pathetic with the rural communities where the residents do not practice proper hygiene and also have limited access to health care facilities. It may also be concluded that the primary and secondary treatment of the sewage wastewater is not fit for industrial, domestic and irrigation purposes, so it may be suggested that the tertiary effluent of the sewage treatment plant be used for

secondary purposes like industrial cooling, gardening and irrigations. Furthermore, it is hence recommended that the need for correct assessment and proper water management practices cannot be overemphasized. These should include, creating awareness in the community of the impact of human activity on water quality. There should also be hygiene education programs in order to minimize transmission of water related diseases. Surface water sources, for example, water from the dams should at least be boiled before use since microorganisms can also be destroyed by boiling. Then, frequent or routine monitoring of water sources for faecal indicator organisms to ensure microbial water safety especially the underground taps and boreholes should also be done. Wastewater needs to be treated to protect public health, to reuse the treated effluent for agriculture and also to solve social problems caused by the accumulation of wastewater.

All these will limit the health implications from water-borne pathogens associated with faecal materials and will ultimately ensure water safety.

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