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The Efficiency of Removal of Total Coliforms, Faecal Coliforms and Coliphages in a Wastewater Treatment Plant in Riyadh

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Abstract: Limited water resources in Saudi Arabia necessitate the re-use of treated wastewater for irrigation, industrial and recreational purposes. The large numbers of pathogenic agents present in wastewater requires continuous monitoring of the removal of such agents from water prior to re-use. In a one year study total and faecal coliforms and coliphages were estimated in monthly samples from a wastewater treatment plant in Riyadh to evaluate the removal efficiency of such indicators at various levels of wastewater treatment. The efficiency of removal of total coliform (TC) and faecal coliform (FC) following aeration and sedimentation processes ranged between (18-34%) and (17-38%) respectively and for coliphages was (4-19%). Chlorinated effluent had negligible counts of TC and FC with an efficiency of removal of (99.2-100%) and (99-100%) for TC and FC respectively whereas the efficiency of removal of coliphages ranged between (91-100%). As coliphages have been proposed as possible indicators of enteric viruses our study suggests their use as indicators of faecal pollution with traditional coliform indicators and the implementation of treatment measures more effective in virus removal in re-used wastewater. Electron microscopy of selected phage lysates showed the presence of tailed coliphages belonging to families Myoviridae, Siphoviridae and Podoviridae. Polyvalent coliphages able to infect enteric bacteria other than *E. coli* were also detected.

Key word: Total coliforms, faecal coliforms, coliphages, wastewater

Introduction

The microbiological quality of water is currently evaluated by the use of indicators of faecal contamination (total coliforms, *Escherichia coli,* and faecal streptococci in the water environment. Such faecal contamination may lead to the presence of human pathogens. Some studies have shown that these indicators do not provide adequate information about viruses, particularly in terms of their fate in the environment and their resistance to treatment (Geldenhuys and Pretorius, 1989; Havelaar *et al.,* 1993). The presence of human enteric viruses in potable water or treated wastewater for irrigation or recreation pose a serious threat to public health. More than 100 types of human pathogenic viruses may be present in faecally polluted water (Havelaar *et al.,* 1993). Hepatitis (A) virus, caliciviruses, adenoviruses, rotaviruses and enteroviruses have the greatest effect on public health. A large number of epidemics due to the presence of these viruses in the environment have been reported (Anderson and Strenstrom, 1987; Bosch *et al.,* 1991; Yao, 1989). Currently available virological techniques are rarely used for monitoring purpose as they are relatively laborious, and not well suited for routine testing (Grabow, 1986; Havelaar and During, 1988). Therefore, studies have been directed towards identifying more specific indicators of viral contamination. Coliphages have been proposed as enteric virus indicators in wastewater because of their similar behaviour and survival in the aquatic environment (Simkova and Cervenka, 1981; Stetler, 1984; Jiang, 2001).

In Saudi Arabia, due to limited water sources, treated wastewater is re-used in irrigation purposes, in industry and in recreation activities. In this paper comparative estimates of classical indicators and coliphages in a wastewater treatment plant in Riyadh at different levels of treatment were performed over a period of one year to evaluate the efficiency of removal of all 3 indicators in the wastewater ready for re-use. Electron microscopic examination and host range were also determined for selected coliphage isolates.

Materials and Methods

Wastewater samples: Monthly samples of wastewater were collected through the Riyadh Water and Sewerage Authority for a period of one year, from June 1998 until May 1999. Samples were obtained from the Northern Sewerage Treatment Plant at different levels of treatment. Raw sewage (R. S.), subjected only to physical screening; secondary effluent (S.E.), subjected to aeration and sedimentation processes and Chlorinated effluent (C.E.), subjected to gravity sand filtration and chlorination.

Estimation of total and faecal coliforms in various samples: The membrane filtration procedure (APHA, 1985) was used for the enumeration of coliforms. Suitable dilutions of water samples were filtered through millipore membranes (0.45 ìm and 0.7 ìm) for total (TC) and faecal (FC) coliforms. Each membrane was then placed on absorption pads soaked with m-ENDO MF broth (Difco Laboratories, U.S.A.) for the enumeration of TC and incubated at 37° C for 24h. Typical dark colonies with sheen were counted as TC. To enumerate FC, filters were placed on absorbent pads saturated with m-FC broth (Difco Laboratories, U.S.A.) supplemented with 1% (w/v) rosalic acid and incubated at 44.5 ± 0.5° C for 24h. All typical blue colonies were counted. TC and FC were estimated as colony forming unit/ml (cfu/ml).

Estimation of coliphages in wastewater samples: Samples were processed for coliphage enumeration using the double-agar-layer technique (DAL) as described by Rajala and Heinonen (1994). TYG-agar was used as developing medium: tryptone 10g, yeast extract 5g, glucose 2g, NaCl 5g, and $MgSO_4.7H_2O$ 0.25g supplemented with 1.2 or 0.65 % agar for hard and soft agar, respectively, in one liter of distilled water, pH was adjusted to 7.0 and the medium sterilized by autoclaving at 121 °C for 20 min. Approximately 10 ml of the wastewater sample was centrifuged at 4000 rpm for 15 min. The pellet was discarded and the supernatant was further filtered through 0.45µm pore size Millipore filters. One ml of the filtrate of suitable dilution was added to 0.2 ml of exponentially growing host culture *Escherichia coli* (ATCC 13706) then it was mixed with 2 ml of liquefied soft agar. The mixture was poured onto petri dishes containing TYG-hard agar, allowed to solidify and incubated at 37° C for 16h.

Choice of bacterial host strains: The phage sensitivity of 6 *Escherichia coli* strains, to the lytic action of coliphages was tested using the DAL method (Rajala and Heinonen, 1994) described above. The host strains were kindly supplied by R. Rajala, University of Kuopio, Finland. The selected strains were *E. coli* ATCC 13706 (Sinsheimer-C), 11125 (Bertani-C), 15597 (C-3000 of Jacob, der. Of K-12), 15669 (Hofsneider St. Hfr D, der. of K12 of Cavalli), C 600 and K 12 Hfr G6.

Isolation and purification of coliphages: Plaques of different sizes and morphology were selected for purification. The overnight culture of the host bacterium (1-2 ml) was subcultured into 50 ml of fresh liquid medium (TYG) and incubated with shaking at 37°C for about 1.5h. A well-isolated plaque was then picked with a sterile Pasteur pipette and transferred into the host culture which

was further incubated for 5-6 hr. Bacterial cells were separated from the culture by centrifugation (4000 rpm, 20 min) and by membrane filtration with 0.45µm pore size Millipore filters. The titre and purity of the phage lysate was determined by DAL-method and the purification procedure was repeated 2 - 3 times to obtain a pure one- phage lysate.

Electron microscopy: The morphology of eight selected phages was investigated using transmission electron microscope. Phage lysates were centrifuged at 40,000 rpm for 90 min. Sediments were deposited on carbon-coated copper grids and stained with 2% PTA (pH 7.2) and studied in a Joel 100-CX electron microscope.

Host range: The lytic action of 8 selected coliphage isolates was tested on different species of Enterobacteriaceae as possible hosts of the phage isolates using the DAL technique.

Results

All six *E. coli* strains tested as possible hosts of coliphages had the capacity to detect them in wastewater samples. However, strains of *E. coli* 13706 and 11125 which are known to be sensitive to somatic phages showed the highest efficiency of plating of coliphages (Table 1). *E. coli* strains sensitive to RNA phages did not differ greatly from each other in their sensitivity in coliphage detection. *E. coli* 13706 was selected as the host strain to be used in this study.

Estimates of the 3 indicators over the period of study are shown in Fig. 1(a-l). The highest counts of the 3 indicators were during the months of January and February 1999 in raw sewage samples (R.S.) as shown in (Fig. 1h). Log cfu/ml for TC, FC and pfu/ml for coliphages were 6.2, 4.8, and 2.5 respectively. Secondary effluent samples (S.E.) showed a marked decrease in counts of TC and FC. The efficiency of removal of TC and FC over the period of study ranged between (18-34%), (17-38%) respectively. The efficiency of removal of coliphages was rather lower (4-19%). Chlorinated effluent (C.E.) had negligible counts of TC and FC with an efficiency of removal of (99.2-100%) and (99-100%) respectively. However, coliphages were still detected in the chlorinated effluent in several months, the highest estimates being in the month of January (Fig. 1h and l) with log pfu/ml of 0.23.

The efficiency of removal of coliphages following chlorination for all samples ranged between 91-100%. Statistical analysis using F-test indicates significant differences between the estimates of the 3 indicators over the 12 month period of study at the 3 levels of wastewater treatment. However, there is no significant difference in the effect of each treatment on the estimates of each of the 3 indicators over the 12 months period (P = 99 % and P = 95 %). Screening of wastewater samples on sensitive *E. coli* lawns allowed the selection of eight phage isolates with different plaque size and shape which were further propagated. Upon incubation of all these phage isolates with RNase (100µg/plate), all phages selected produced plaques in the presence of enzyme, indicating that they are DNA phages. Electron microscopy of phage lysates from wastewater samples revealed several morphological patterns. All lysates examined were for tailed phages (Fig. 2a-h), however they could be further divided into different morphotypes by head shape and tail structure. The types observed were for phages with icosahedral head, collar and contracted tail in phages isolates P1, P2 and P5 (Fig. 2a,b and e), thus belonging to family Myoviridae. Phage isolate P3 with icosahedral head and short tail (Fig.1c.) characteristic of family Podoviridae. Isolates P4, P6, P7 and P8 (Fig. 2d,f,g and h), with icosahedral or spherical heads and long thin flexuous tail characteristic of family Siphoviridae.

Testing the host range of the selected isolates on other bacterial species belonging to family Enterobacteriaceae showed that 4 isolates have no lytic activity on the tested species. However, isolates P1, P2, P4 and P8 were able to propagate on enteric bacteria other than *E. coli* (Table 2) indicating that such isolates are polyvalent.

Table 1: Ability of 6 different host-bacteria to detect coliphages in wastewater.

Host strain *E. coli*		Mean pfu. ml^{-1}
Sensitive to somatic coliphages	13706	340
	11125	300
	C 600	125
Sensitive to RNA phages	15597	189
	K12HfrG6	156
	15669	170

Table 2: Host range of some phages isolated from wastewater samples

	Coliphage (pfu. ml^{-1} x 10^4)							
Bacteria	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8
Shigella sonnei	29	9	-	18	-	-	-	14
Salmonella typhimurium	6	12	-	-	-	-	-	-
Salmonella typhi	-	-	-	-	-	-	-	-
Salmonella sp.	3	1	-	9	-	-	-	-
Shigella flexneri	5	6	-	1	-	-	-	18
Yersinia enerocolitica	25	48	-	15	-	-	-	-
Yersinia pseudotuberculosis (I)*	11	31	-	3	-	-	-	3
Yersinia pseudotuberculosis (ii)	29	12	-	13	-	-	-	21
Yersinia pseudotuberculosis (iii)	18	3	-	-	-	-	-	31

* (I), (ii), (iii) : Different isolates of the same species.

Discussion

Selection of the host bacteria for the detection of coliphages in wastewater samples is essential. Using 3 different strains of *E. coli* known to be sensitive to somatic phages i.e. phages known to infect host cells via receptor molecules in the cell wall, revealed the higher sensitivity of *E. coli* 13706. Strain 13706 has been shown to be more sensitive in phage detection than other strains used by Grabow *et al.* (1984); Funderberg and Sorber (1985); Rajala and Heinonen, (1994).

The strains sensitive to RNA phages, which have the ability to transfer their DNA to other bacterial cells via the F- type conjugation system, were less sensitive.

Monthly estimates of total (TC) and faecal (FC) coliforms in raw samples collected from the wastewater treatment plant under study were found to be close to those reported in similar studies (Bellet *et al.,* 1981; Qureshi and Qureshi, 1989). Aeration tanks and sedimentation treatments appear to be more effective in the removal of coliforms than coliphages. Chlorination however, increased the removal of all 3 indicators although coliphages were still detected in some (C. E.) samples. Chlorine is known to have a limited effect on some viruses and some coliphages are resistant to it. Variations in sewage coliforms and coliphage populations were reported to occur according to the water temperature. Previous reports have indicated that the maximum number of indicators was detected during an average temperature of 19.8 °C (Iriberris *et al.,* 1987). In our study the highest estimates were in the winter months, especially in January, where the average temperature of Riyadh during the time of study was 18° C. Qureshi and Qureshi (1989) in a similar study in Bahrain, have proposed that during summer months, due to an increase in water consumption as a result of the elevated temperature, a dilution factor is created with additional pollution of soaps and detergents that could be effective in decreasing counts during that time of the year. The estimates of the 3 indicators in our study in August and September were also low. The number of coliphages significantly decreased with sewage treatment levels, although some samples from finally- treated wastewater samples still indicated their presence. The detection of coliphages in treated wastewater samples was similar to reports of Wentsel *et al. (*1982) and in potable water (El-Abagy *et al.*, 1988; El-Abagy, 2001). The persistence of viruses over bacteria in adverse condition allows for the detection of coliphages. The somatic coliphages have size, structure and survival rate in the environment similar to those of enteroviruses. A correlation between the presence of coliphages and the enteric viruses have been proposed (Gerba *et al.,* 1978; Stetler, 1984). Three types of bacteriophages have been proposed as specific indicators of viral contamination: the somatic coliphages (Morinigo *et al.,* 1992), the F-specific RNA phages (Havelaar, 1986)

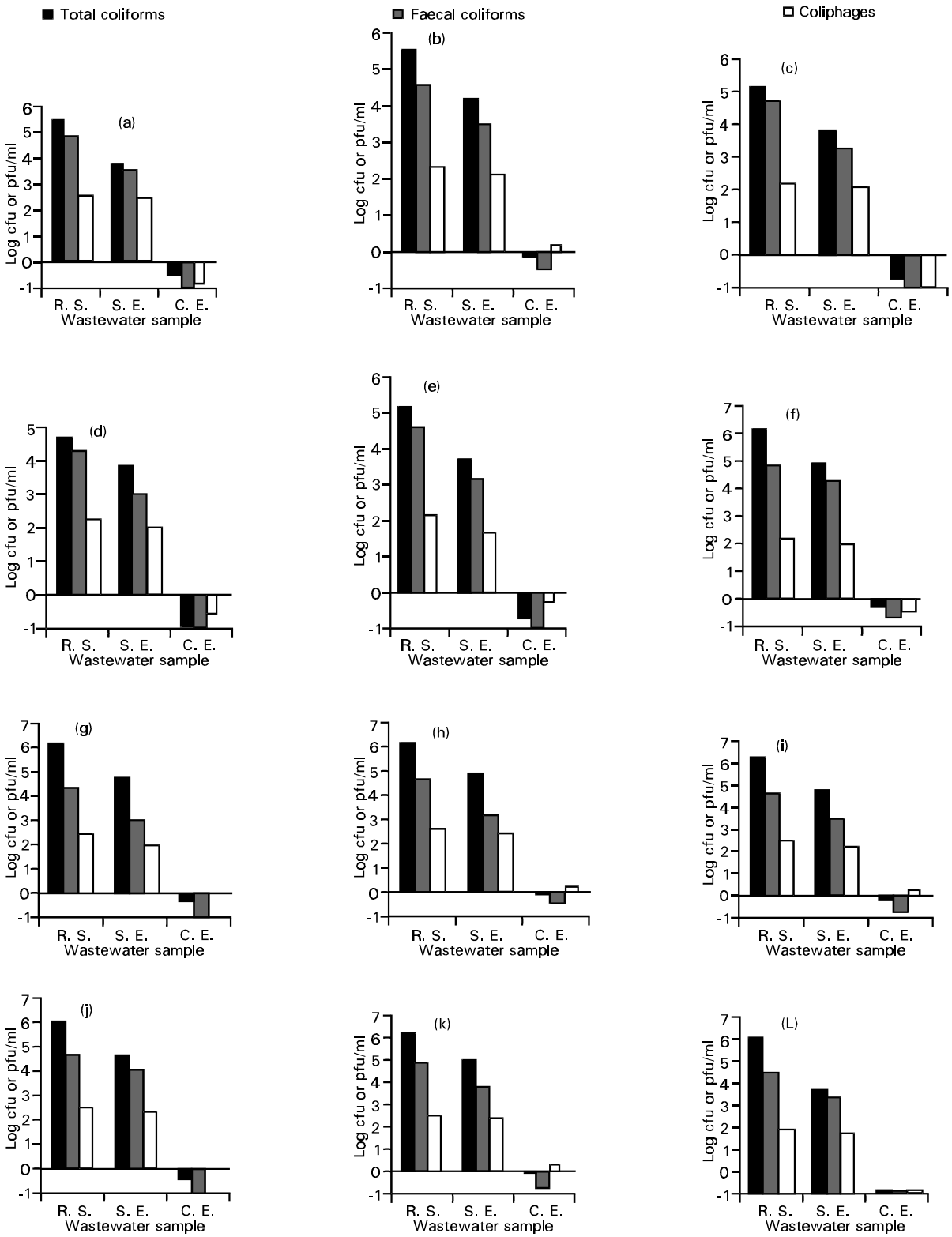

Fig. 1: Estimates of coliforms and coliphages in various samples: Raw Sewage (R.S), Secondary effluent (S.E.) and Chlorinated effluent (C.F) collection from the Northern Wastewater Treatment plant, Riyadh, in different monthly samples in 1998: (a)June; (b) July; ©August; (d) September; (e) October; (f) November; (g) December (h) January, 1999; (I) February, 1999; (j) March 1999; (k) April, 1999; (L) May, 1999.

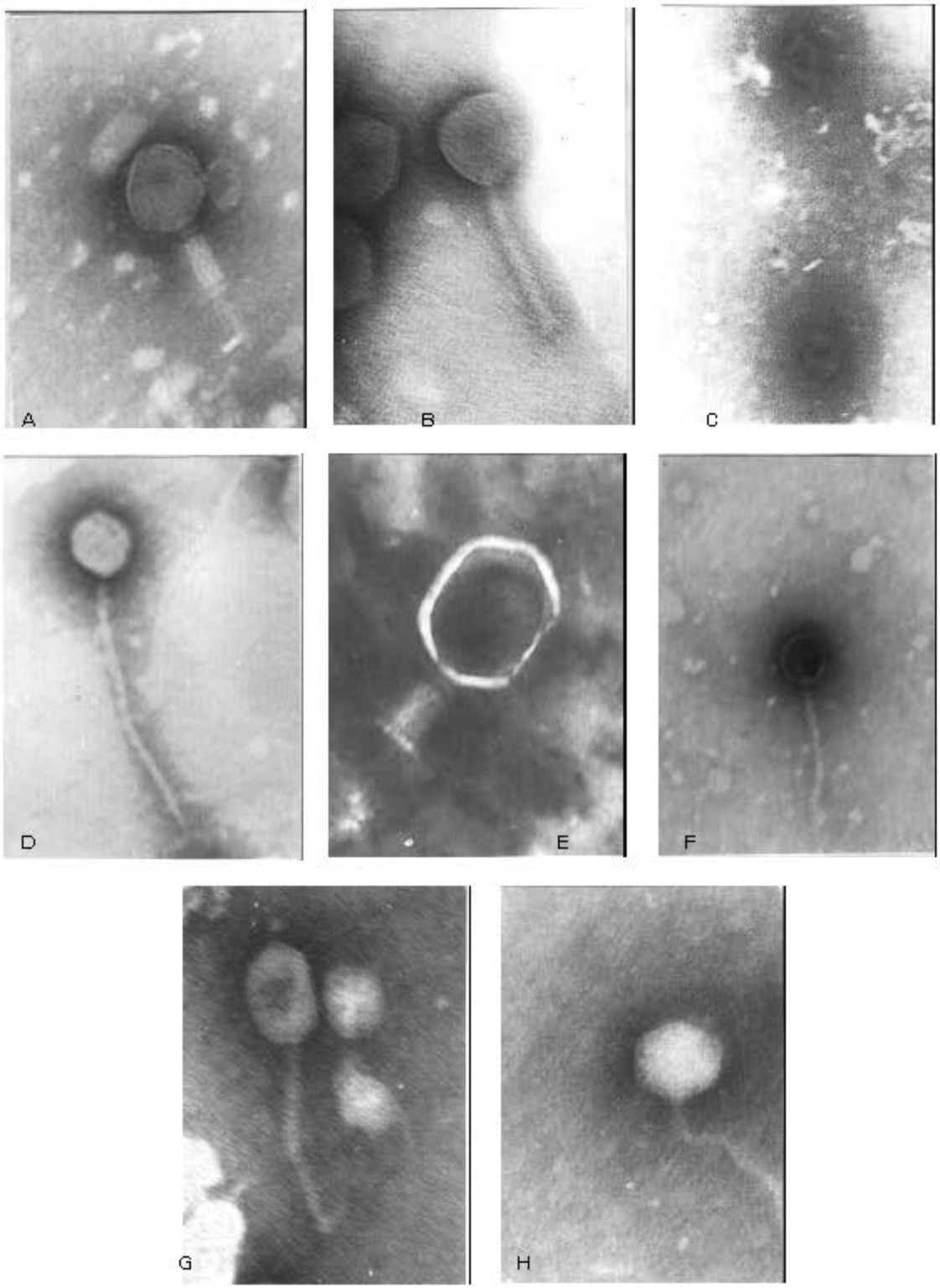

Fig. 2: Transmission electron micrographs of coliphages isolated from Riyadh wastewater
(a) P1 phage with icosahedral head, collar and contracted tail, (b) P1 phage with icosahedral head, collar and contracted tail, (c) P3 phage with isometric head and very short tail (d) P4 phage with icosahedral head and thin, elongated tail (e) P5 phage with a large icosahedral head collar and contracted tail
(f) P6 phage with isometric head and thin elongated tail
(g) P7 phage with icosahedral head and thin flexuous tail, (h) P8 phage with icosahedral head and thin very flexuous tail

and the *Bacteroides fragilis* phages (Jofre *et al.,* 1986). Longevity field studies confirmed the long-term survival of both coliphages and enteroviruses in the water (Simkova and Cervenka, 1981). This could account for their detection in C.E. chlorinated effluent. Our study suggests the use of coliphages as indicators of faecal pollution in addition to the traditionally used indicators and the implementation of treatment measures more effective in virus removal in re-used wastewater. Coliphages could initially be differentiated based on plaque size and shape though such characters are not very reliable to classification purposes. Electron microscopy is essential for studying phage ecology and for phage identification. Our study revealed the abundance of tailed somatic phages of families Myoviridae, Siphoviridae and Podoviridae in agreement with the studies of Ackermann and Nguyen (1983) and Rajala and Heinonen (1994). Podoviridae family was represented in only one phage lysate. It is obvious though, that not all morphotypes of coliphages present were detected. Cubic RNA phages and filamentous phages were not found, although RNA sensitive hosts were used in the isolation and propagation processes. It could be that some phages were possibly overgrown by others or probably none of the RNA phages sensitive host strains had grown sex pilli.

Some bacteriophages were found to infect and multiply in species of closely related genera (Qureshi *et al.,* 1988). These polyvalent phages were suspected to cause possible biological control of over whelming populations of the faecal coliforms in raw sewage prior to sewage treatments. Enteric bacteria belonging to genera *Shigella, Salmonella* and *Yersinia,* abundant in faecal contaminated water, were able to support the multiplication of some coliphage isolates tested. Such polyvalent phages might be playing a role in the ecological distribution and abundance of enteric bacteria in sewage.

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