Studies to distinguish between human and animal faecal pollution using F-RNA coliphages and faecal sterols

A Sundram[1]*, N Jumanlal[1] and MM Ehlers[2]
[1]*Umgeni Water, PO Box 9, Pietermaritzburg 3200, South Africa*
[2]*Department of Medical Virology, University of Pretoria/NHLS, Pretoria 0001, South Africa*

Abstract

Human enteric viral infections are considered to be predominantly associated with human wastes, as opposed to animal wastes, and a distinction between these has benefits for water quality control and risk assessment. A variety of techniques have been described to distinguish between human and animal faecal pollution of water. F-RNA (male-specific) coliphages have been classified into four sero-groups and evidence has been presented that two of these sero-groups are specific for human excreta and the other two for animal excreta. Certain chemical compounds such as the faecal sterols cholesterol and coprostanol yielded valuable results in attempts to distinguish between faecal pollution of human and animal origin.

In this study the application of F-RNA coliphages and faecal sterols to distinction between human and animal excreta has been investigated. Faecal sterols were extracted from water and analysed by gas chromatography using published methods that were adapted for the detection and quantification of cholesterol and coprostanol. Wastewater containing predominantly animal excreta was collected from cattle, pig and chicken feedlots. Wastewater containing predominantly human excreta was collected from hospitals.

Results revealed that F-RNA coliphages isolated from wastewater from four different hospitals consisted almost exclusively of genotypes 2 and 3. Only F-RNA coliphage genotypes 1 and 4 were detected in all three wastewater samples from cattle feedlots, while F-RNA coliphage genotypes 1, 3 and 4 were detected in all three chicken feedlot wastewater samples. Five wastewater samples from pig feedlots contained typically F-RNA coliphage genotypes 3 and 4.

Cholesterol and coprostanol were detected in ranges of 28 to 1 013 µg/ℓ and 19 to 1 441 µg/ℓ in wastewater, respectively. Coprostanol concentrations were more than double the cholesterol concentration in wastewaters from hospitals and pig feedlots in the five samples analysed. The opposite applied to wastewater from cattle and chicken feedlots, where cholesterol concentrations in all seven samples were higher than coprostanol concentrations. Analysis of wastewater from a poultry feedlot yielded a high cholesterol:coprostanol ratio and the presence of predominantly F-RNA coliphage genotype 4, confirming the specificity of these determinants for animal wastes.

The results of this study confirmed earlier reports on the specificity of F-RNA coliphage genotypes 1 and 4 for animal wastes, and genotypes 2 and 3 for human excreta, in a part of the world where investigations using these methods are limited. The same applies to the higher ratio of coprostanol: cholesterol in human excreta, and the higher ratio of cholesterol: coprostanol in animal excreta. The observations suggested that further optimisation of applying these indicators in combination may lead to the development of procedures for the meaningful distinction between faecal pollution of human and animal origin in quantitative terms.

Keywords: cholesterol, coprostanol, ratios, genotyping, F-RNA phages, wastewater

Introduction

The maintenance of the microbiological quality of water systems used for drinking, recreation, and the harvesting of seafood is critical, as faecal contamination of these systems can pose risks to human health as well as result in economic losses (Jagals et al., 1995; Sinton et al., 1998; Scott et al., 2002). Although limited epidemiological data are available, human faeces are generally perceived to constitute a greater human health risk than animal faeces (Sinton et al., 1998; Schaper and Jofre, 2000; Scott et al., 2002). Understanding the origin of faecal pollution is paramount in assessing associated health risks as well as the actions necessary to remedy the problem while it exists (Griffin et al., 2000; Scott et al., 2002). The identification and apportioning of human and animal faecal inputs to natural water cannot be done with great confidence by isolated methods and it has been suggested that both microbiological and chemical determinations be used to generate more reliable data (Sinton et al., 1998). Microbial determinations include using faecal streptococci: faecal coliform ratios, *Bifidobacteria*, phages infecting *Bacteroides fragilis* HP40 specific to humans, *Rhodococcus coprophilus* specific for animals and F-RNA phage subgroups (Sinton et al., 1998; Scott et al., 2002). The limited number of chemical determinations researched includes the faecal sterol profiles, caffeine known to be excreted by humans from the diet and even linear alkyl benzenes specific to humans (Sinton et al., 1998; Scott et al., 2002).

Enteric bacteriophages like the F-RNA phages are widely used as indicators of enteric viruses and research has shown that these viral indicators could be used to discriminate between human and animal faecal pollution (Havelaar and Hogeboom, 1984; Havelaar et al., 1986; Furuse, 1987; Sinton et al., 1998; Leclerc et al., 2000; Scott et al., 2002). The male-specific F-RNA phages have been classified into four groups based on their phylogenetic diversity and it has been reported that groups 1 and 4 occurred in animal wastewater while groups 2 and 3 usually predominated in human wastewater (Osawa et al., 1981; Havelaar et al., 1986; Furuse, 1987; Havelaar et al., 1990; Hsu et

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* To whom all correspondence should be addressed.
☎+2733 341-1342; fax:+2733 341-1501;
e-mail: ashogan.sundram@umgeni.co.za

al., 1995; Sinton et al., 1998; Schaper and Jofre, 2000; Schaper et al., 2002; Scott et al., 2002; Cole et al., 2003; Vinje et al., 2004). Hsu et al. (1995) and Beekwilder et al. (1996) developed gene probes for the detection of 4 F-RNA subgroups by identifying stretches of 20 to 30 nucleotides that showed complete conservation within one subgroup while being absent from other subgroups. These probes have been used in DNA-RNA hybridisations and F-RNA phages isolated in various samples were successfully typed and assigned to one of the 4 subgroups (Uys, 1999; Schaper et al., 2002).

Although F-RNA groups 2 and 3 were usually associated with human faecal matter, groups 2 and 3 F-RNA phages were isolated from pig faeces (Osawa et al., 1981; Furuse, 1987; Havelaar et al., 1990; Hsu et al., 1995; Uys, 1999; Cole et al., 2003; Vinje et al., 2004). The gastrointestinal physiology and flora of pigs are similar to those of humans and it is understandable that human specific phages could proliferate in pigs that are often exposed to human faecal wastes (Hsu et al., 1995). Thus, F-RNA group classification will not always distinguish between human and porcine faecal contamination (Osawa et al., 1981; Furuse, 1987; Havelaar et al., 1990; Hsu et al., 1995; Uys, 1999; Cole et al., 2003; Vinje et al., 2004). Sinton et al. (1998) reported that there has been reasonable evidence that F-RNA subgroups 2 and 3 predominate in human effluents, and that subgroup 1 is characteristic of non-human mammals.

Assays for the identification of faecal sterol isomers have gained considerable potential for differentiating between faecal sources (Sinton et al., 1998). On studying the sterol profiles of various warm-blooded animals it was found that each animal has its own 'sterol fingerprint' based on the content and composition of the different sterols quantified in faeces (Leeming et al., 1996; Nichols et al., 1996; Leeming et al., 1998). The faecal sterols cholesterol and more specifically coprostanol are gaining wide use as indicators of faecal pollution as these compounds are poorly soluble in water and have been detected in wastewater in concentrations exceeding 1 000 $ng \cdot \ell^{-1}$ (Leeming and Nichols, 1996).

Cholesterol occurs widely in the human body originating from the diet, intestinal epithelium and other tissues (Graham Solomons, 1980; Macdonald et al., 1983). Coprostanol in human faeces results from the biohydrogenation of cholesterol by microflora in the small intestine (Martin et al., 1973; Elhmmali et al., 2000). Research conducted by Leeming and colleagues (1996) indicated that the ratios of coprostanol: cholesterol in humans were as high as 10:1. Coprostanol concentrations are generally higher in humans than in most animals including pigs that have similar sterol profiles to humans but at concentrations about 10-fold lower (Leeming et al., 1996; Nichols et al., 1996; Scott et al., 2002). A gas chromatograph coupled to a mass spectrometer was used successfully in quantifying faecal sterols following extraction and derivatisation (Leeming and Nichols, 1996). Sterols need to be converted to their more stable ethers facilitating detection using gas chromatography and this is usually achieved by the addition of a derivitising compound such as bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Leeming et al., 1996; Nichols et al., 1996; Elhmmali et al., 2000).

This study attempted to establish whether F-RNA phage grouping and cholesterol and coprostanol analyses could be used to discriminate between animal and human faecal input. Although at least 17 faecal sterols have been identified in human and animal faeces (Leeming et al., 1996) coprostanol and to a lesser extent cholesterol, are generally present in most wastewaters in different concentrations depending on the nature of the faecal pollution. Apart from time and cost restraints, assays for only cholesterol and coprostanol were selected as literature has suggested that these two sterols may have the potential of being used to discriminate between human and animal faecal pollution (Leeming et al., 1996; Nichols et al., 1996; Scott et al., 2002).

Materials and methods

Sample collection

Wastewater samples (500 mℓ) representative of human faecal pollution were collected from two local hospitals in Pietermaritzburg. Wastewater (500 mℓ) from a cattle feedlot in the Umgeni River catchment and wastewater from a piggery in the Pietermaritzburg midlands were collected as being representative of cattle and pig faecal pollution. Chicken wash and wastewater (500 mℓ) from a poultry farm and an abattoir in the Cato Ridge area was collected as representative chicken faecal pollution.

Isolation of F-RNA phages

The standard ISO-10705–1 (1995) double-layer agar technique, using *Salmonella typhimurium* (*S. typhimurium*) WG 49, was used to isolate F-RNA phages. In short, 2.5 mℓ of top-agar containing 1% naladixic acid (Sigma Aldrich) held at 48°C, 1 mℓ of the host culture grown up to the log phase and 1 mℓ of the test sample were mixed together and poured onto the bottom agar layer that had been pre-dispensed and set in a 90 mm petri dish (Concorde Plastics). The plates were inverted before being incubated (Heraeus, SEPARATIONS SCIENTIFIC) overnight at 37±1°C. Plaques that developed were opaque and not as clear as those formed by typical somatic phages.

Transfer and fixation of F-RNA phage isolates

Plates with typical F-RNA plaques or areas of clearing were placed in the cold room at 4°C for at least 30 min. This was done to facilitate the transfer of phages by making the agar more solid and damp. The phages were transferred onto Boehringer Mannheim (Roche Diagnostics, Germany) nylon membranes. The first membrane was adsorbed to the top layer for 1 min. Up to four subsequent transfers could be obtained from one plate by increasing the time of adsorption to 2 min, 3.5 min and 5 min respectively for every additional transfer. Four membranes were prepared for each set of isolates.

Following fixation, phage RNA was released and denatured by submerging the membranes in containers with 0.05 M NaOH (Saarchem) for one min. The membranes were placed in clean containers and submerged in 0.1 M sodium acetate (Saarchem) adjusted to pH 6, for 30 s. To allow fixation of the nucleic acid, the membranes were baked in an oven (Scientific, LABOTEC) at 80°C for 2 h.

Pre-hybridisation

Each membrane was placed in hybridisation bags (Roche Diagnostics, Mannheim, Germany) with 5 mℓ of pre-hybridisation solution (University of Pretoria, Medical Virology), containing 6 X Saline Sodium Citrate (SSC); 0.1% Sodium Dodecyl Sulphate (SDS); 1 x Denhardt solution and 0.1 $mg.ml^{-1}$ salmon sperm DNA (Invitrogen, USA). The salmon sperm DNA was denatured in a Perkin Elmer GeneAmp 2400 T thermocycler using a preset cycle of 99°C for 10 min thereafter held at 4°C until used. Hybridisation bags (Roche Diagnostics) were sealed

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and air bubbles minimised. The bags were placed for 1 h at 37°C in a shaking water bath (LABOTEC) at 100 r·min^{-1}.

Hybridisation

The probes that were used in these studies were originally synthesised by Beekwilder et al. (1996). Following pre-hybridisation, the bags were carefully cut and 2.5 pmol·mℓ$^{-1}$ of each of the digoxigenin labelled probes (Sigma-Genosys, Texas, USA) were added together with (50 to 100 µℓ) pre-hybridisation solution (excluding salmon sperm DNA). The hybridisation bags were resealed and incubated at 37°C for ±18 h in the shaking water-bath (LABOTEC) at 100 r·min^{-1}. The membranes were removed from the bags and placed in clean containers and washed twice in Buffer 1 containing 0.3 X SSC and 0.1% SDS, for 15 min at 37°C in a shaking water-bath (LABOTEC) at 100 r·min^{-1}.

Detection

A DIG Wash and Block Buffer set (Roche Diagnostics) containing washing, blocking and detection solutions was used. The membranes were placed in containers together with the washing buffer for two min at 37°C. Each membrane was placed in a clean container together with 80 mℓ of Blocking Solution (Roche Diagnostics) for 15 min held at exactly 37°C in a shaking water bath (LABOTEC). Each membrane was incubated in 20 ml Blocking Solution (Roche Diagnostics) containing 1 µℓ of Anti-digoxigenin-AP Fab fragments (Roche Diagnostics) for 30 min at room temperature (±22°C). The membranes were washed twice in the Washing Buffer (Roche Diagnostics) for 15 min at room temperature. The membranes were placed in containers containing 20 ml Detection Buffer (Roche Diagnostics) agitated for 5 min at room temperature (±22°C). Fifty µℓ of the detection substrate, CPD-star (Roche Diagnostics) was diluted 1: 100 with the detection buffer and incubated with the membranes for 2.5 min. The damp membranes were sealed in new hybridisation bags with excess solution containing the CDP-Star (Roche Diagnostics) detection substrate. Membranes were placed in the Amersham hyper-cassette with Lumi film (Roche Diagnostics) placed over the membranes, all performed under dark room conditions. The cassette was sealed for 3.5-4 min followed by development of the Lumi film using the appropriate developer and fixer solutions (Axim, Durban, South Africa) to generate dark circular spots representative of F-RNA phages from the various groups.

Preparation of cholesterol and coprostanol calibration standards

A stock standard of 400 mg·ℓ$^{-1}$ was prepared by adding 0.01 g cholesterol (University of Natal) and 0.01 g of coprostanol (Sigma) into a 5 mℓ volumetric flask that was topped to the 5 mℓ mark with chloroform (Fluka). The 160 mg·ℓ$^{-1}$ calibration standard was prepared by adding 2 mℓ of both the cholesterol and coprostanol 400 mg·ℓ$^{-1}$ stock standards into a 5 mℓ volumetric flask and topping up to the 5 mℓ mark with chloroform (Fluka). This was derivatised by adding 500 µℓof BSTFA to the 160 mg·ℓ$^{-1}$ calibration standard in a clean test tube that was placed in a heating block (Bibby Stuart Scientific) and held at 60°C for 1 h. The 40 mg·ℓ$^{-1}$ and 80 mg·ℓ$^{-1}$ calibration standards were prepared by diluting the 160 mg·ℓ$^{-1}$ derivatised calibration standard with chloroform (Fluka).

Organic extraction of faecal sterols

The wastewater samples (500 mℓ) were poured into appropriate size separating flasks that were thoroughly cleaned with a household detergent, dried and rinsed with ± 10 mℓ of chloroform (Fluka). Fifty millilitres of chloroform (Fluka) was added to the flasks. The flasks were agitated vigorously for 3 min taking care to release any gas through the release valve at the bottom of the flask. The flasks were left to stand for 10 min until the organic solvent separated from the water and two clear phases could be seen. Following this, the more dense lower chloroform layer was drained into a clean 250 mℓ Erlenmeyer flask. This procedure was repeated twice by adding 50 mℓ volumes of chloroform (Fluka) to the samples. Once all three extractions were completed the combined extract was poured into a clean separating flask and agitated before being left to separate into two clear phases. The dense chloroform layer was filtered through anhydrous $MgSO_4$ (Saarchem) and allowed to drain into a clean round bottom flask. The filtrate was evaporated in a rotary evaporator (Buchi) set at 35°C and reduced pressure until approximately 5 mℓ was remaining. The filtrate was transferred to a clean 5 mℓ volumetric flask that was topped up to the mark with chloroform (Fluka). Sterols were converted into their corresponding trimethlysilyl ethers by adding 50 µℓ of BSTFA to the extract in a clean test tube that was placed in a heating block (Bibby Stuart Scientific) held at 60°C for 1 h.

Gas chromatographic analyses

Gas chromatographic analyses were performed with a Hewlett Packard 6890 GC equipped with a 30 m x 0.25 mm x 0.25 µm cross-linked methyl silicone-fused silica capillary column (Hewlett Packard), a mass selective detector (MSD Model 5973), a HP 7633a auto-injector and a split/splitless injector. Peak areas were quantified using chromatographic software (HP Chemstation) operated using a Microsoft Windows compatible personal computer. The component identification was based on comparison of retention time data with that obtained for authentic laboratory standards and mass spectra data.

Results and discussion

The F-RNA genotyping assays were done following standard protocols developed by Hsu et al. (1995) and Beekwilder et al. (1996) and adapted by Uys (1999). The chemical assays for cholesterol and coprostanol were involved and performed with assistance by the Chemical Scientist at Umgeni Water. The quantification of the sterols detected were done using the gas chromatography results (from HP Chemstation) obtained for the calibration standards that were plotted using Microsoft Excel and calculated from the calibration curve obtained for each batch of samples.

Genotyping of F-RNA phages from wastewater samples

The results in Table 1 showed that F-RNA groups 3 and 4 were the only two groups detected in pig wastewater. Unlike previous studies conducted by Hsu et al. (1995), F-RNA phage group 2 was not detected in the pig wastewater tested. Another interesting finding was that F-RNA phage groups 1 and 4 were detected along with group 3 in the chicken wastewater. Previously, groups 2 and 3 was found to be more specific to humans and by exception in pigs that were considered to have a similar gastrointesti-

TABLE 1
Molecular characterisation of F-RNA phages isolated from different wastewater samples

Sample date	Sample description	F-RNA phage groups detected
27/06/02	Pig wastewater	Groups 3 and 4
22/12/02	Pig wastewater	Groups 3 and 4
24/12/02	Pig wastewater	Group 4
06/01/03	Pig wastewater	Group 3
06/01/03	Pig wastewater	Groups 3 and 4
17/07/02	Chicken wastewater	Groups 1, 3 and 4
27/11/02	Chicken wastewater	Groups 3 and 4
31/12/02	Chicken wastewater	Group 4
02/07/02	Cattle feedlot	Group 1
06/01/03	Cattle feedlot	Groups 1 and 4
06/01/03	Cattle feedlot	Groups 1 and 4
02/09/02	Hospital wastewater discharge	Group 2
04/10/02	Hospital wastewater discharge	Group 3
29/11/02	Hospital wastewater discharge	Group 2 and 3
06/01/03	Hospital wastewater discharge	Group 3

TABLE 2
Cholesterol and coprostanol concentrations detected in different wastewater samples

Date	Sample description	Cholesterol µg·ℓ$^{-1}$	Coprostanol µg·ℓ$^{-1}$
January 2003	Local hospital	28.2	23.1
January 2003	Local hospital	160.1	337.6
January 2003	Local hospital	254.8	536.3
January 2003	Cattle feedlot	181	103.1
January 2003	Cattle feedlot	141.2	22.6
January 2003	Cattle feedlot	166.4	27.1
June 2003	Cattle feedlot	139.7	41.5
June 2003	Cattle feedlot	163.8	50.5
January 2003	Chicken wastewater	1 013.1	19
January 2003	Chicken wastewater	891.6	49.3
January 2003	Chicken wastewater	56.3	23.1
January 2003	Chicken wastewater	4 208	142.3
January 2003	Pig wastewater	345	663
January 2003	Pig wastewater	168.5	344.9
January 2003	Pig wastewater	35.3	42.7
January 2003	Pig wastewater	555	1 441

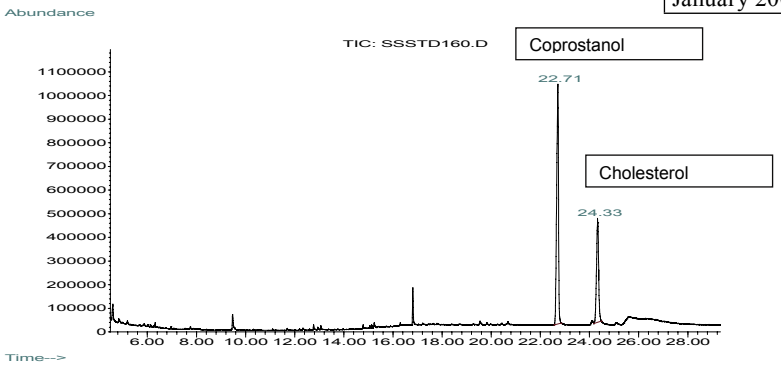

Figure 1
GC chromatogram showing retention times for coprostanol and cholesterol for the 160 mg·ℓ$^{-1}$ standard

nal physiology and flora to humans partly because of the close living conditions (Hsu et al., 1995; Schaper et al., 2002; Cole et al., 2003). It was therefore, unusual that F-RNA phage group 3 was detected in chicken wastewater on two separate occasions. It is likely that farming activities on the chicken farms could have contributed to human input in the chicken wastewater that was possibly not exclusively chicken waste. Consistent with earlier research, F-RNA phages groups 1 and 4 were the only groups found in cattle wastewater. The results also indicated the specificity of groups 2 and 3 for humans, as these were the only two F-RNA phage groups detected in all four of the hospital wastewater samples.

Faecal sterol assays on wastewater samples

After careful preparation of the 40 mg·ℓ$^{-1}$, 80 mg·ℓ$^{-1}$ and 160 mg·ℓ$^{-1}$ coprostanol and cholesterol standards, that were injected into the GC, peaks obtained after approximately 22 min were identified by the mass spectrometer as coprostanol, followed shortly by cholesterol (Fig. 1). The mass spectra of these compounds detected after 22.71 min and 24.33 min were matched to the reference library in the mass spectrometer and confirmed as coprostanol and cholesterol respectively.

The wastewater samples were very turbid and each extraction did not separate as clear chloroform/water phases. All three extracts had to be combined into a separate flask before a proper phase separation could be seen. This additional step was successful in obtaining sterol extracts with minimal trapped water remaining. Following extraction and derivatisation of extracts from the different wastewater samples, samples were injected into the GC as described above and the mass spectra of peaks obtained after 22 min where identified as coprostanol and cholesterol. These peak areas were quantified once the calibration standards were plotted and the area of abundance under each peak could be converted to the corresponding concentrations. The results presented in Table 2 indicated that there were instances when higher faecal sterol concentrations were detected (as high as 4 208 µg·ℓ$^{-1}$ cholesterol in chicken wastewater) as opposed to instances where relatively low concentrations of 56.3 µg·ℓ$^{-1}$ cholesterol was detected in chicken wastewater. These trends of varying low or high concentrations of either cholesterol or coprostanol were found in all of the wastewater samples analysed. It could be attributed to a number of factors including; samples taken over different flows, different diets, microflora in the gut and general health of the individuals.

Coprostanol was generally detected in higher concentrations (23.1 to 1 441 µg·ℓ$^{-1}$) than cholesterol (28.2 to 555 µg·ℓ$^{-1}$) in both the local hospital and pig wastewater samples (Table 2). This is possibly due to the gastrointestinal physiology of pigs that have been reported to be similar to that of humans (Scott et al., 2002). The first sample analysed from the local hospital was the only exception where 23.1 µg·ℓ$^{-1}$ of coprostanol was detected in the presence of 28.2 µg·ℓ$^{-1}$ of cholesterol. Cholesterol was consistently detected at much higher concen-

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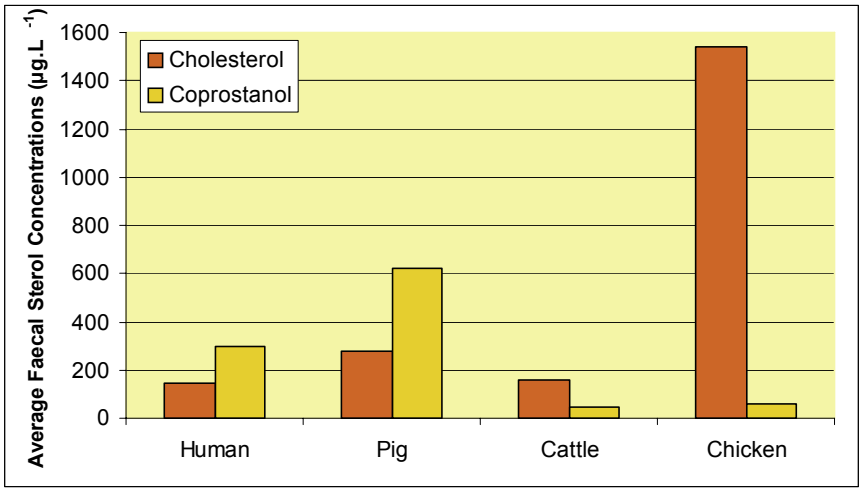

Figure 2
The relative averages of cholesterol and coprostanol concentrations detected in different wastewater samples

trations (181 to 4 208 µg·ℓ$^{-1}$) than coprostanol (22.6 to 142.3 µg·ℓ$^{-1}$) in both cattle and chicken wastewater (Table 2). This is in keeping with literature that highlighted that most animals are unable to biohydrogenate cholesterol to coprostanol due to the physiology of the animal gut including the microflora present (Elhmmali et al., 2000).

It is clear from Fig. 2 that the average concentrations of coprostanol: cholesterol were greater than 2: 1 in both human and pig wastewater. The reverse was found with cattle and chicken wastewater where the average cholesterol concentrations were higher (158 to 1 542 µg·ℓ$^{-1}$) than the average coprostanol concentrations (49 to 58 µg·ℓ$^{-1}$).

Conclusions

The study was conclusive in proving that genotyping of F-RNA phages using chemiluminescent detection techniques was easy to perform producing results that were easy to interpret. The use of a hot air oven held at 80°C was successful in fixing the viral RNA onto the positively charged membranes due to a lack of a UV Transilluminator. The least amount of background development on the X-ray film was achieved if plaques were transferred before any re-growth of bacteria occurred on plates stored in the cold room (4 to 10°C). The probes selected for the hydridisation were sensitive and no cross-reactivity between the four F-RNA phages groups occurred. The results confirmed earlier reports on the specificity of F-RNA phage genotypes 1 and 4 for animal wastes, and genotypes 2 and 3 for human excreta, in a part of the world where not much research has been done (Havelaar et al., 1986). Male specific F-RNA phage group 3 was detected in both chicken and pig wastewater indicating that this group may not be specific to humans. There is a possibility that the chicken wastewater could have been contaminated with human faecal input due to farming activities.

The GC-MS method used for the detection of cholesterol and coprostanol was successful and most positive results were obtained when faecal sterol extractions from samples were done as soon as possible after sampling. The average concentrations of coprostanol detected in human (299 µg·ℓ$^{-1}$) and pig (623 µg·ℓ$^{-1}$) wastewater was higher than their respective average cholesterol concentrations (148 µg·ℓ$^{-1}$ and 276 µg·ℓ$^{-1}$). The opposite was found with chicken and cattle wastewater where the average cholesterol concentrations (1 542 µg·ℓ$^{-1}$ and 158 µg·ℓ$^{-1}$ respectively) were higher than the coprostanol concentrations (58 µg·ℓ$^{-1}$ and 49 µg·ℓ$^{-1}$ respectively). The results for coprostanol and cholesterol are in keeping with the literature where animals in general, apart from pigs, have higher cholesterol than coprostanol concentrations because of the limitations in the gut where the flora present in most animals are unable to biohydrogenate cholesterol to coprostanol (Elhmmali et al., 2000). The analyses were unable to discriminate between human and pig faecal pollution although it has been reported that pigs would have lower concentrations of coprostanol than humans but it would be difficult to discriminate between human and pig faecal pollution in water.

Both these chemical and microbiological methods could be used to determine if pollution was of human or animal origin but more conclusive and reliable discriminatory results were obtained if used together. This was because faecal sterol assays could be used in situations when F-RNA phage genotypes could not be used to distinguish faecal pollution. Only two faecal sterols, predominant in faeces, were selected for the investigation in this study due to the cost implications and specialised chemical methods required for assays of all the sterol assays present in faeces. Analysing for all of the faecal sterols and stanols may provide more useful and conclusive data when the sensitivity of using coprostanol and cholesterol alone fails. These chemical and microbial assays are difficult, expensive and time-consuming. Despite having these shortcomings, important and useful data were obtained by applying both chemical and F-RNA phage genotyping assays.

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