Occurrence of bacteriophages infecting *Bacteroides* host strains (ARABA 84 and GB-124) in fecal samples of human and animal origin

David Diston and Melanie Wicki

ABSTRACT

Bacteriophage-based microbial source-tracking studies are an economical and simple way of identifying fecal sources in polluted water systems. Recently isolated *Bacteroides* spp. strains ARABA 84, and GB-124 have been shown to detect bacteriophages exclusively in aquatic systems impacted by human fecal material. To date, limited examination of the occurrence or concentration of phages capable of infecting *Bacteroides fragilis* strain GB-124 or *B. thetaiotaomicron* strain ARABA 84 in human and animal feces has been carried out. This study reports the prevalence rates and concentrations of phages infecting ARABA 84 and GB-124 host strains in human and a range of animal feces. Discrete human fecal samples ($n = 55$) and pooled animal samples ($n = 46$, representing the feces of over 230 animals) were examined for phages infecting the host strains ARABA 84, GB-124, and *E. coli* strain WG5. Both human *Bacteroides* host strains were highly specific (95% and 100% for ARABA 84 and GB-124, respectively), challenging results from previous studies. This study supports the use of *Bacteroides* strains GB-124 and ARABA 84 in fecal source tracking studies for the detection of human fecal contamination.

Key words | contamination, indicators, pollution, source tracking, water

David Diston
Federal Food Safety and Veterinary Office,
3003 Bern,
Switzerland

Melanie Wicki (corresponding author)
University Hospital Basel, CTU,
4031 Basel,
Switzerland
E-mail: *melanie.wicki@gmx.ch*

INTRODUCTION

Successful management of recreational and drinking water quality is, in part, determined by the level of understanding of the dominant microbial pollution sources. Human fecal pollution may represent a greater health risk to water users/consumers than animal fecal pollution (AWPRC Study Group on Health Related Water Microbiology 1991; Field & Samadpour 2007) and the identification of waterborne human fecal contamination is essential when assessing both the potential health risks associated with water use and the implementation of effective remediation schemes. Many microbial source tracking (MST) methods have been proposed, including antibiotic resistance analysis (Whitlock *et al.* 2002), carbon utilization patterns (Moussa & Massengale 2008), F + RNA phages (Havelaar *et al.* 1986), *Enterococcus* phages (Bonilla *et al.* 2010; Purnell *et al.* 2011), 16S rRNA sequences from *Bacteroidales* bacteria (Layton *et al.* 2006), and phages infecting *Bacteroides* spp. (Tartera & Jofre 1987; Puig *et al.* 1999; Payan *et al.* 2005). Advantages and disadvantages of these and many other MST methods have been discussed at length in review articles (Scott *et al.* 2002; Field & Samadpour 2007).

The ideal indicator of fecal material should have numerous qualities, including, but not limited to presence in host feces only, inability to replicate in natural waters, have a simple assay method not requiring a large reference strain library, and allow practical methods of sample collection and storage (Borrego *et al.* 1987; Havelaar 1993; Field *et al.* 2003). As *Bacteroides* spp. bacteriophages can be assayed using an inexpensive, standardized method (ISO 2001b), are unlikely to replicate in natural waters (Jofre *et al.* 1986), and do not require a reference library or extensive sample preparation/enrichment, bacteriophages infecting *Bacteroides* spp., host strains fulfill a number of these requirements. Moreover, the USEPA has identified research

doi: 10.2166/wh.2014.199

into *Bacteroides* bacteriophage MST tools as a 'high priority research need' (Ashbolt *et al.* 2007).

Two recently described *Bacteroides* host strains, GB-124 isolated in the UK (Payan *et al.* 2005) and ARABA 84 isolated in Switzerland (Wicki *et al.* 2011), have been proposed as indicators of human fecal pollution in aquatic systems. *B. thetaiotaomicron* strain ARABA 84 has been shown to detect phages in municipal wastewaters, surface/spring waters contaminated with human fecal material, while being absent in animal wastewaters (liquid farm manure samples and slaughterhouse wastewaters; Wicki *et al.* 2011). Data regarding sensitivity and specificity in raw fecal material are not available in the literature. Regarding phages infecting *B. fragilis* strain GB-124, conflicting results have been reported. Ebdon *et al.* (2007) demonstrated total absence of phages able to infect GB-124 in animal fecal samples, while phages were present in 100% of tested municipal wastewaters and surface waters impacted by wastewater treatment effluents. In addition, McMinn *et al.* (2014) reported the human exclusivity of phages infecting GB-124 when tested against human sewage effluent and a wide variety of animal fecal samples. However, contrary to these data, a recent study has shown that although GB-124 demonstrated high sensitivity (60.5%), it recorded low specificity (57.7%) and cross-reacted with numerous animal fecal sources from the USA (Harwood *et al.* 2013). Phages infecting GB-124 in cattle and pig feces have also been reported, albeit at very low levels, 0.17 plaque forming units (PFU)/ml and 0.33 PFU/ml, respectively, by Payan *et al.* (2005). These conflicting results reduce confidence and may hinder the deployment of GB-124 phages as a human-specific fecal indicator.

Microbial source tracking is an emerging field in Switzerland, and its use is limited. It is therefore of importance to demonstrate the usability of both the proposed Swiss strain (ARABA 84), and also one of the most promising strains (GB-124) so MST in Switzerland can be developed. At present, little is known regarding the sensitivity or specificity of phages infecting *B. fragilis* strain GB-124 or *B. thetaiotaomicron* strain ARABA 84 in fecal samples from a range of hosts in Switzerland. By undertaking an assessment of host specificity and sensitivity of both host strains, we aimed to both further validate the use of phages infecting ARABA 84, while assessing the geographical applicability of phages infecting UK *Bacteroides* strain GB-124 in Switzerland as indicators of aquatic human fecal material. This objective will be achieved by carrying out an investigation into the prevalence rates and levels of these phages in fecal samples of human and animal origin. Only after demonstrating high specificity can both *Bacteroides* indicators be employed with confidence in Swiss waterbodies for MST purposes. Furthermore, *B. fragilis* strain GB-124 is emerging as one of the most promising host strains for the detection of human fecal material globally. By undertaking a regional assessment of specificity/sensitivity, information will be provided regarding the geographical stability of these MST markers.

MATERIALS AND METHODS

Two types of sample were used in the study: (1) discrete human fecal samples and (2) pooled animal fecal samples; the details of these samples are given in full below.

Fecal sample collection and processing

Animal fecal samples (typically up to 50 g) were collected in sterile sampling pots (Sarstedt, Germany) from different farms in three Swiss Cantons (Bern, Solothurn, and Luzern), and transported to the laboratory on the same, or the next day; samples were chilled in either an insulated cool box or a refrigerator during transportation and storage, respectively. Samples were a composite of the feces of between two and seven animals (with the majority of samples being composed of the feces of five or more animals), either freshly voided or removed during rectal examination. All animal fecal samples were diluted 10-fold using peptone saline solution (pH 7.2), and mixed by magnetic stirring for 1 h. Large particles were then sedimented by low-speed centrifugation (2,000–4,000 × g) for 10 min at 4 °C, and the supernatant filtered using 0.22 μm hydrophilic polyethersulfone membranes (MillexGP, Millipore, USA). To the filtrate, glycerol was then added to a final concentration of 10% (vol/vol); samples were divided into three separate 15 ml polypropylene tubes (Sarstedt, Germany) and stored at −70 °C until analysis. Logarithmic dilutions of each fecal suspension were assayed in duplicate. For animal samples, 10^{-1} to 10^{-5} dilutions of original fecal

material were assayed for somatic coliphages, and a 10^{-1} dilution of original fecal material assayed for phages infecting *Bacteroides* spp., strains GB-124 and ARABA 84.

Discrete human fecal samples were sourced from healthy male and female subjects as part of a company's employment induction scheme. Owing to the low volume of human fecal material in some samples (33 out of 55 samples), to obtain sufficient test solution for assays it was necessary to create 100-fold dilutions; the remaining 22 human samples were processed using a 1:10 dilution of original fecal material. Human samples were then processed and stored as detailed above for the animal samples. A 10^{-1} dilution was assayed for 22 samples, and a 10^{-2} dilution was assayed for the remaining 33 samples for three phage groups (GB-1244, ARABA 84, and somatic coliphages). Where positive plates were above 200 PFU/ml in the greatest dilution, samples were repeated using new aliquots from frozen, and assayed at greater dilution factors.

Phage assays

Both human and animal fecal samples were assayed for phages infecting host strains *B. fragilis* GB-124, *B. thetaiotaomicron* ARABA 84, and *E. coli* strain WG5 (somatic coliphages; ISO 2001a). Phage assays were conducted using the double-agar method following procedure outlined in the International Standards (ISO 2001a, b). Positive controls (using appropriate reference phage) and negative controls (using sterile deionized water) were used throughout. Assays were repeated using new samples from frozen where failures in either the positive or negative controls occurred, or when further dilutions were required.

Analysis

Sensitivity [TP/(TP + FN)*100%], specificity [TN/(FP + TN) *100%], positive predictive value [PPV = TP/(TP + FP) *100%], and negative predictive value [NPV = TN/(TN + FN)*100%] of the methods applied to detect phages infecting ARABA 84 and GB-124 were determined in fecal samples and the abbreviations used in the formulas were true positive (TP), true negative (TN), false positive (FP), and false negative (FN). All statistical analyses were carried out in MSExcel (v.11).

RESULTS

Bacteriophage presence in human feces

Somatic coliphages were the most prevalent phage group detected in human feces (present in 73% of discrete samples; 40 out of 55) with a median level of 2.5×10^2 PFU/g (SD = 1.4×10^4; Table 1). Second, in prevalence were phages infecting ARABA 84 (detected in 15% of samples; 8 out of 55) with a median value of 2.5×10^2 PFU/g, the same median concentration as somatic coliphages, though ARABA 84 showed a wider range of values (SD = 6.1×10^5). Phages infecting GB-124 were detected in only 4% of samples (2 out of 55) with a median value of 5.5×10^1 PFU/g (SD = 6.4×10^1). When both phages infecting ARABA 84 and somatic coliphages were present in a fecal sample, linear regression analysis showed there was no significant relationship between the variables ($R^2 = 0.03$; $P = 0.05$; $n = 8$). Linear regression analysis was not performed for GB-124 because only two samples were positive.

Bacteriophage presence in pooled animal feces

As was observed for human samples, across all animal types somatic coliphages were the most prevalent phage group, detected in 93% of pooled samples (43 out of 46). Phages able to infect ARABA 84 were found in a single pooled pig, and a single pooled cow sample, representing 4% of total number of composite animal samples. Phages infecting GB-124 were not recorded in any animal sample.

When both were present, levels of phages infecting ARABA 84 were lower than somatic coliphages, and overall median values were 6.7×10^3 PFU/g and 2.0×10^3 PFU/g, respectively (SD = 1.6×10^7 and 9.2×10^3), though as ARABA 84 phages were only recorded in two samples, further statistical analyses could not be carried out.

Host strain sensitivity and specificity

Specificity, i.e., absence in animal samples, demonstrated for both ARABA 84 and GB-124 host strains in this study was high. The specificity of the method to detect

Table 1 | Prevalence and concentrations of phages infecting host strains GB-124, ARABA 84 and somatic coliphages in human and animal fecal material

	Bacteriophages (plaque-forming units/g)								
Sample type	GB–124 median	Range	% pos	ARABA 84 median	Range	% pos	Somatic coliphage median	Range	% pos
Human (n=55)	5.5×10^1	1.0×10^1 to 1.0×10^2	4	2.5×10^2	5 to 1.7×10^6	15	2.5×10^2	1.0×10^1 to 6.8×10^4	73
Cow (n=11)	–	–	0	1.3×10^4	nr	9	2.3×10^2	5 to 2.2×10^6	82
Horse (n=11)	–	–	0	–	–	0	2.9×10^3	1.0×10^1 to 1.0×10^8	91
Sheep (n=11)	–	–	0	–	–	0	9.0×10^2	7.5×10^1 to 1.6×10^7	100
Pig (n=10)	–	–	0	2.0×10^2	nr	10	6.8×10^5	1.0×10^1 to 4.7×10^6	100
Dog (n=1)	–	–	0	–	–	0	9.5×10^2	–	100
Donkey (n=1)	–	–	0	–	–	0	2.0×10^7	–	100
Goat (n=1)	–	–	0	–	–	0	1.0×10^1	–	100

nr = no range; one positive result only.

bacteriophages infecting ARABA 84% was 95% in pooled fecal samples. No bacteriophages were detected in animal samples using the human host strain GB-124, leading to a specificity of 100%.

The sensitivity of the methods to detect bacteriophages of the host strains ARABA 84 and GB-124 in fecal samples was 15 and 4%.

The PPV value for phages infecting GB-124 was 100%, the NPV was 46%. For phages infecting ARABA 84, the PPV was 80% and the NPV was 48%.

DISCUSSION

Both high sensitivity and high specificity are desirable when evaluating usefulness of proposed fecal indicators. Sensitivity refers to how often the assay returns a positive result when the target fecal material is present, and specificity refers to how often the result is negative in the absence of the target fecal material. PPV and NPV are useful parameters in determining the suitability of proposed MST indicators, representing the percentage of positive test results that are TPs and the percentage of test negatives that are TNs, respectively.

Specificity, sensitivity, PPV, and NPV

Specificity demonstrated for both phages infecting ARABA 84 and GB-124 in this study was high (95% and 100%, respectively), as were the PPVs (80% and 100%, respectively), indicating that both host strains may be useful indicators of human fecal material in Switzerland, showing greater specificity and with larger PPVs than other phage groups recently assessed by Harwood *et al.* (2013) (*Enterococcus* phage MB-55, *Enterococcus* phage HB-73, and F+RNA group II phages). For ARABA 84, these data confirm the results of a previous study (Wicki *et al.* 2011). However, the presence of phages infecting ARABA 84 in two pooled animal fecal samples (one cow and one pig) showed that they are not restricted to human fecal material. The positive pooled cow sample was obtained directly from rectal cavities during examination by a veterinarian, while the positive pooled pig sample was comprised of freshly voided feces. It is therefore, unlikely that the fecal samples positive for phages infecting ARABA 84 were contaminated by human fecal material. Specificity rates of >80% are considered to be acceptable for MST markers (USEPA 2005) and as ARABA 84 performed well in excess of this threshold, may be considered a human-specific marker. It is important to note that the animal test samples were a composite of an average of five animals (i.e.,

a total of approximately 230 discrete fecal samples), and therefore the specificity may be higher if discrete samples were to be assayed.

In agreement with results published by Ebdon *et al.* (2007) and McMinn *et al.* (2014), but in contrast to Harwood *et al.* (2013) and Payan *et al.* (2005), phages infecting GB-124 appear to be exclusively present in human fecal material. Harwood *et al.* (2013) recorded phages infecting GB-124 in cattle, pig, gull, dog, pigeon, horse, and chicken feces; whereas Payan *et al.* (2005) found cattle and pig fecal samples positive for GB-124 phage (albeit at very low concentrations, 0.17 PFU/ml and 0.33 PFU/ml, respectively). As phages infecting GB-124 were not detected in pooled animal fecal samples in the present study, representing over 230 animals, they may therefore be considered as a human-specific marker for use in Switzerland.

Although appearing low, the prevalence rates of phages infecting GB-124 and ARABA 84 in discrete human fecal samples recorded in this study (4% and 15%, respectively) are within the ranges recorded for *Bacteroides* host strains by other authors. *Bacteroides* phage prevalence rates in human feces of 4% in the Netherlands (Havelaar 1993), 10% in Spain (Tartera & Jofre 1987), 11% in France (Gantzer *et al.* 2002), and 13% in South Africa (Grabow *et al.* 1995) have been reported for the human-specific *B. fragilis* host strain HSP-40. For phages infecting *B. fragilis* host strain RYC-2056, used as an unspecific indicator of fecal pollution, less data were found, though Puig *et al.* (1999) recorded a prevalence rate of 30% in Spain. Owing to the low sensitivity of *B. fragilis* strain HSP-40, and low specificity of *B. fragilis* strain RYC-2056, additional human host strains *B. fragilis* GB-124 and *B. thetaiotaomicron* GA-17, were isolated (Payan *et al.* 2005) and are currently the host strains most frequently used in MST studies (Blanch *et al.* 2004; Ebdon *et al.* 2007). As environmental fecal pollution will be derived from a group, rather than individual subjects, the low prevalence rates for GB-124 and ARABA 84 are unlikely to limit the use of either host strain in MST studies. Within raw fecal material it is therefore more important for a method to demonstrate high specificity than high sensitivity.

The NPVs of phages infecting ARABA 84 and GB-124 are also low (48% and 46%, respectively), but are comparable to, or better than, other MST tools currently being developed, showing similarity to other proposed phage groups (55% for phages infecting MB-55, 42.9% for phages infecting HB-73, and 41.5% for F+ RNA phages; Harwood *et al.* 2013).

For somatic coliphages, the prevalence rates in human feces recorded by other authors vary greatly. Reported rates include 2.5% in Japan (Furuse *et al.* 1983), 68% in France (Gantzer *et al.* 2002), 71–77% in the USA (Havelaar 1993; Harwood *et al.* 2013), 54% in South Africa (Grabow *et al.* 1995), and 80% in the Netherlands (Havelaar *et al.* 1986). The data presented here, 73%, are in accordance with the prevalence rates given above, albeit at the higher end, and as reported extensively in the literature, confirms the usefulness of somatic coliphages as a general fecal indicator.

The prevalence rate of all three phage types in human feces may have been influenced by the limits of detection used in this study (10 PFU/g for 22 samples, 100 PFU/g for the remaining 33 samples). Gantzer *et al.* (2002) reported that 93% of positive human fecal samples (total number of positive samples = 21) recorded in a French study contained phages able to infect HSP-40 at levels between 1 and 10 PFU/g. It is possible that phages infecting all three studied host strains have higher prevalence rates in human feces than were measurable in the current study. It is suggested that an enrichment step, followed by qualitative spot-tests, would be useful in future studies to give exact prevalence rates.

When compared with those given for other *Bacteroides* phages in the literature, the levels of phages infecting GB-124 and ARABA 84 in human feces are within the ranges previously reported (median values 5.5×10^1 and 2.5×10^2, respectively). Mean concentrations of HSP-40 phages in human feces have been reported as being from 10^1 PFU/g (Gantzer *et al.* 2002) to 10^8 PFU/g (Tartera & Jofre 1987). As ARABA 84 (and GB-124) have been consistently recorded in Swiss wastewater (Wicki *et al.* 2011, 2014), this finding is unlikely to hinder their usage. Where recorded, mean concentrations of somatic coliphages in human feces have included 4.3×10^3 PFU/g (Gantzer *et al.* 2002) and 10^4 PFU/g in the Netherlands (Havelaar *et al.* 1986). The median value recorded in this study was 2.5×10^2, and this is at the lower range of data presented in the literature, but in line with levels suggested by Havelaar (1991), $<10^3$ PFU/g.

That somatic coliphages and phages infecting ARABA 84 in human feces had the same median level in this study is surprising. It has been previously reported that somatic

coliphages are more abundant than *Bacteroides* phages of different host strains in raw wastewaters, human stools, final effluents, and environmental waters (Havelaar 1993; Gantzer *et al*. 2002; Ebdon *et al*. 2007; Nnane *et al*. 2011). A comparison of the eight human samples where both phages infecting ARABA 84 and somatic coliphages were present indicates that in seven samples, levels of ARABA 84 phages were equal to, or greater than, somatic coliphages.

Geographical variability and recommendations for MST studies

Given the geographical variability reported for other *Bacteroides* host strains (Payan *et al*. 2005; Vijayavel *et al*. 2010), it is unsurprising that ARABA 84 had a higher prevalence rate and median concentration in human fecal samples than GB-124. ARABA 84 is a host strain local to Switzerland; whereas GB-124 was isolated in the south-east of England. Such regional variation for GB-124 has been reported previously: GB-124 phages were absent in wastewater/human feces in Hawaii (Vijayavel *et al*. 2010), but have been recorded in all human wastewater samples tested from Switzerland (Wicki *et al*. 2014), Denmark (Ebdon *et al*. 2007), France, Ireland, and Brazil (unpublished data; Ebdon 2012). Also, subsequent work has recorded bacteriophages infecting GB-124 in Hawaiian sewage, challenging previous results (unpublished data; Ebdon 2012). In comparison to ARABA 84, the lower median levels and prevalence rate of phages infecting GB-124 may indicate potential geographical limitations of bacteriophages infecting *Bacteroides*, which has been extensively discussed in literature. As has been demonstrated for other *Bacteroides* host strains, it would be useful to assess the geographical limitations of phages infecting ARABA 84 as a human-specific microbial source-tracking tool.

The findings detailed above must be placed into context with other indicators of human fecal material currently available in Switzerland, and the requirements of fecal indicators detailed in the Introduction. Although, phages infecting ARABA 84 are not limited to human feces, both ARABA 84 and GB-124 host strains show high human specificity, exceeding the 80% threshold suggested by the USEPA (2005). The specificity value of ARABA 84 was lowered by the presence of phages in two pooled samples and had discrete animal fecal samples been assayed (as was the case for human samples), it is likely specificity would have been higher. The higher sensitivity of ARABA 84 when combined with the higher specificity of GB-124 is likely to give a good indication of the human fecal pollution when environmental waters are assayed. Phages infecting GB-124 and ARABA 84 have been found in all human wastewater samples investigated from different regions in Switzerland (Wicki *et al*. 2014), showing that both strains are useful indicators of human fecal contamination for use in Switzerland. It is therefore recommended that both host strains be used in combination to detect fecal pollution of human origin in Swiss waterbodies.

CONCLUSIONS

The results given in this study show that phages infecting *Bacteroides* host strains GB-124 and ARABA 84 are highly specific with high PPVs, and are useful tools in MST studies, challenging previous studies (Payan *et al*. 2005; Harwood *et al*. 2013). Low, but comparable to other proposed host strains, sensitivity for both host strains was recorded in discrete human stool samples, though as environmental fecal pollution will be a composite of many individual sources, this is unlikely to reduce usefulness of the indicators. It is recommended that both indicators (ARABA 84 phages and phages infecting GB-124) should be used in parallel in future Swiss MST studies. This study represents an important step in both furthering MST studies in Switzerland and demonstrating the geographical stability of phages infecting GB-124 outside of the original area of isolation, thereby enhancing their international significance. To develop knowledge of phages infecting GB-124 and ARABA 84, further work should be undertaken investigating the relationship between both *Bacteroides* phage groups and enteric pathogens, an essential aspect when deploying any MST marker (Roslev & Bukh 2011).

ACKNOWLEDGEMENTS

The authors would like to acknowledge the assistance of Dr H. Hächler (University of Zürich, Switzerland) for providing access to human fecal samples, and Dr C. Boog,

K. Ruedin, M. Fankhauser, T. Habermacher, P. Hofstetter, B. Lörtscher, and A. Stettler for providing access to the animal fecal samples. Special thanks must be given to Dr Andreas Baumgartner (Federal Office of Public Health, Switzerland) for critically reviewing the manuscript prior to submission.

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First received 18 August 2014; accepted in revised form 22 November 2014. Available online 30 December 2014