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THE DEVELOPMENT OF AN ENTEROCOCCAL PHAGE ASSAY FOR WATER SAMPLES

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ABSTRACT: Preliminary work was carried out for the development of a rapid method for the detection and enumeration of enterococcal phages in natural water. Eight enterococcal phages were isolated after amplification from water obtained from 2 local ponds. These were plaque-purified and used to test the phage susceptibility of 34 strains of enterococci isolated from pristine and polluted waters. Eight strains with the widest phage susceptibility were used as hosts in a double agar layer (DAL) and a single agar layer (SAL) assay for phage enumeration without pre-assay amplification. No phages were obtained with the DAL method with all 8 hosts. Using the SAL method, 2 phages were obtained with 1 of the 2 hosts used. Phage isolation was not improved by incubation at 44.5°C. For rapid quantitative testing, the sensitivity of the assays has to be increased to enable phage isolation without the need for prior amplification. *(JUMMEC 1996 1(1):25-28)*

KEY WORDS: Enterococci, phage, enumeration

Introduction

Standard tests for faecal contamination of water are based on the enumeration of coliform bacteria which are normally found in high concentrations in the faeces of all warm-blooded animals. However, it has been shown by a number of researchers that these bacteria may not be suitable indicators for assessing the hygienic quality of tropical waters as they have been found in significant numbers in water, soil and on epiphytic plants in tropical pristine environments(1,2). Their replication in the natural environment has been postulated(3,4) and if this were true, their presence in water would not necessarily indicate faecal contamination. In addition, faecal coliforms are less resistant to disinfection than some human pathogens like human enteric viruses which have been recovered from waters considered safe by faecal coliform tests(5).

In the search for better indicator organisms, *Clostridium welchii*, faecal streptococci and coliphages were found to be the most promising. *C welchii* correlated better than faecal coliforms or faecal streptococci with wastewater contamination and behaved more like viruses in its resistance to chlorination and environmental factors(1). Coliphages appeared to be good indicators of long-term enterovirus contamination of water even in the presence of chemical pollution(6). Faecal streptococci (>80% *Enterococcus faecalis*) which are more numerous in the animal gut than in the human intestine, have been used mostly to obtain the faecal coliform (FC):faecal streptococci (FS) ratio. In appropriate situations, a FC:FS ratio of >4 is strong evidence that contamination is by human waste whereas a ratio of <0.7 indicates pollution from livestock or poultry waste. Faecal streptococci have not been as extensively studied as faecal coliforms. They will not grow in water but their die-off rates in the environment are generally unknown although some species show remarkable tolerance to unfavourable conditions. Much less is known about the phages that lysogenize these streptococci and their distribution in the natural environment.

This paper describes some preliminary work on the detection and enumeration of faecal streptococcal phages conducted with the aim of developing an assay for the study of the ecology of these phages and their potential use as an alternative indicator of faecal contamination of water. Since faecal streptococci are mostly enterococci, the latter terminology is used throughout this paper for simplicity.

Materials and Methods

Isolation of enterococcal hosts

Water samples collected from the Endau-Rompin watershed were filtered at the sampling point with bacte-

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riological field monitors (Millipore) and incubated up to 4 days at ambient temperatures on M-VFC holding medium before being transferred onto KF Streptococcus agar (Difco) for incubation at 44.5°C. Water samples from Taman Jaya and the University of Malaya were transported back to the Department of Medical Microbiology in ice and examined within 4 hours of collection by membrane filtration using 0.45mm membranes (Millipore) and KF Streptococcus medium for incubation. Typical enterococcal colonies on KF agar were identified by the API 20 STREP system (Bio Méreux, France).

Isolation and enumeration of enterococcal phages

Amplification of phages in water samples was carried out by adding 10ml of *Enterococcus* host to 10ml of concentrated (10x) basal broth and 90ml of water sample. The mixture was incubated overnight at 35°C with periodic shaking. At the end of incubation 0.1ml of the mixture was removed for titration.

Two phage assays were used, the American Public Health Association (APHA 919C) single agar layer (SAL) assay(7)and the DAL assay which was a modification of a double agar layer method for bacteriophage enumeration(8) . The medium used for the SAL method consisted of trypticase soy broth to which was added per litre, calcium chloride dihydrate (0.63g) sodium azide (0.43g) and agar (15g). The basal medium for the DAL assay was made up per litre with tryptose (10.0g), Lablemco (3.0g), sodium chloride (5.0g), sodium azide (0.2g) and calcium chloride dihydrate (0.3g). Agar plates were prepared from this basal broth with 1.5% agar, while the soft agar overlay was prepared with 0.8% agar.

Table 1. Susceptibility of 34 strains of enterococci to a panel of 8 phages

Phage	% *Enterococcus* strains susceptible
4A	21.9
4B	18.8
5A	40.6
5B	53.1
6A	42.4
6B	51.5
6C	66.7
6D	75.8

In both assays, a log phase culture of *Enterococcus* host was prepared by diluting an overnight culture 100-fold in fresh basal broth and incubating at 35°C for 5 hours. In the DAL assay, 3 ml of the overlay assay medium were melted and allowed to cool to 50°C. To this was added 1 ml of water sample, 1 ml of the host suspension and 0.1ml of a 0.4% triphenyltetrazolium chloride (TPTZ) solution in ethanol. The mixture was then poured over the prepared agar plates, allowed to set and incubated at 35°C.

In the SAL assay, 5ml of water sample, 1 ml of host suspension and 0.1ml of the 0.4% TPTZ solution were added to the basal medium. After overnight incubation, phage plaques were counted.

Phage susceptibility of enterococcal hosts

Phages were isolated from plaques and grown up in flasks with host suspensions at log phase for 24 hours. Bacterial cells and debri were then removed by centrifugation and the phage preparation treated with chloroform to kill remaining bacteria. The phages were used to screen *Enterococcus* phage susceptibility by swabbing concentrated phage preparations (10^5 to 10^7) onto agar plates and then streaking *Enterococcus* strains across the swabbed area. After incubation, an area of no growth was evident in the streaks with strains that were susceptible to the phage on the agar plate.

Results

Thirty-four strains of enterococci were isolated from the environment. Twenty-four were from pristine waters within an equatorial rain forest watershed (Endau-Rompin, Pahang), 7 from 3 sites within the same watershed that received light human faecal contamination, 2 from sewage-contaminated Taman Jaya recreational lake in Petaling Jaya and 1 from a sewage pond in Ottawa, Canada. These included 22 *E faecalis*, 4 *S bovis*, 2 *E avium*, 3 *E faecium* and 3 which were not identified to species level.

Initially, 7 of these 34 strains of enterococci were randomly selected as preliminary hosts to test water samples obtained from the Taman Jaya recreational lake and the University of Malaya pond. Phage amplification and detection was successful with 3 of the preliminary hosts (C10-10, F100-7, H100-8) and a total of 8 phages were isolated and plaque purified (4A, 4B, 5A, 5B, 6A, 6B, 6C, 6D).

These 8 phages were then used to screen all the 34 *Enterococcus* isolates to determine their phage susceptibility pattern (Table 1). Only 1 *Enterococcus* isolate (an *E. faecalis*) was not susceptible to any of the 8 panel phages. The majority (70%) were susceptible to 2-4 of the phages. All 8 phages caused lytic infection in more than 1 strain of *Enterococcus* and each exhibited a different host range which was cross-species except for phages 4A and 4B which appeared to be species-specific as they caused lytic infection only in *E. faecalis*.

Eight strains with the widest susceptibility to the panel of phages used were chosen as candidate hosts for subsequent phage assays (Table 2). Seven (all from Endau-Rompin) were selected based on their susceptibility to over 50% of the panel phages. The eighth (PJ-2) was

included for comparison as the only non-Endau-Rompin isolate.

All 8 candidate hosts were first used in the DAL assay without pre-assay amplification, on water samples collected from the Taman Jaya lake and University of Malaya pond. No phages were isolated. The samples were then tested with the SAL assay using J10-2 and H100-8 as hosts. Two plaques were obtained with host J 10-2 at 35°C incubation. In the SAL assay, 2 variations in incubation temperature were tried out : a) incubation at room temperature and b) an initial incubation at 35°C for 3 hours followed by overnight incubation at 44.5°C. In both cases, no advantage was obtained over incubation at 35°C. Besides, the *Enterococcus* host grew very poorly at the lower and higher temperatures, making the culture plates difficult to read.

Table 2. Selected hosts for phage assay and their susceptibility to a panel of 8 phages

Enterococcal Host	No. of panel phages capable of lytic infection
J10-2 J100-7	8
B100-3 H100-8 J100-8	7
G10	6
H100-1	5
PJ-2	4

To compare the phage assays with the standard (APHA) faecal coliform, enterococci and coliphage enumeration tests, all 4 examinations were carried out on 3 water samples : a) water from Taman Jaya lake b) water from the University of Malaya pond and c) faecal washing from the Faculty of Medicine's animal house. No enterococcal phages were detected in any of the samples although high counts were obtained for coliphages, *E. coli* and enterococci (Table 3).

Table 3. Enumeration of bacteria and phages in faecally-contaminated water samples

Water source	Test method	No. of organisms (CFU/100ml or PFU/100ml)
Taman Jaya lake	*Enterococcus* phage SAL	<5
	Enterococcus phage DAL	<25
	Coliphage	OG
	E coli MF	$1.1X10^5$
	Enterococcus MF	$4.0X10^4$
University Malaya pond	*Enterococcus* phage SAL	<5
	Enterococcus phage DAL	<25
	Coliphage	530
	E. coli MF	$1.1X10^4$
	Enterococcus MF	$1.2X10^4$
Animal faecal washing	*Enterococcus* phage SAL	<5
	Enterococcus phage DAL	<25
	Coliphage	$>2.0X10^4$
	E. coli MF	$3.0X10^6$
	Enterococcus MF	$2.7X10^7$

OG = overgrown, MF = membrane filtration method

Discussion

Researchers at the Faculty of Medicine, University of Malaya have shown significant correlation between membrane-faecal coliform counts and coliphage numbers in well waters located in Central Malaysia. Since enterococci have been often used as an alternative or adjunct indicator to coliform bacteria for faecal contamination of water, it was of interest to see if enterococcal phages can also play a role in the assessment of water quality.

The first requirement for a phage assay was getting a suitable host organism like *E. coli* C in the coliphage test. In the absence of reference strains for the assay of enterococcal phages, an attempt was made to identify suitable hosts from among the wild-type strains isolated from the environment. The 8 candidate hosts used in this study were susceptible to at least 4 of the 8 phages isolated from 2 local ponds. For further development of the phage assay, more enterococcal strains need to be examined to select a host with the widest possible phage susceptibility, suitable growth characteristics and genetic stability for reliable phage infection.

With a phage amplification step, 8 phages were isolated with 3 preliminary hosts without much difficulty but in subsequent assays carried out without amplification, only 2 phages were isolated from a total of 23 attempts using water from the same ponds. In particular, the failure to detect enterococcal phages in samples with high counts of *E. coli*, enterococci and coliphage was disappointing. In a study on 1,145 well water samples in various locations in Malaysia(9), it was observed that coliphage counts were usually about 2 logs lower than coliform counts. This bacteria: phage ratio was also seen with coliforms and coliphages in this study but not with enterococci and enterococcal phages. Both the SAL and DAI methods are standard tests for bacteriophage enumeration and they worked well with the coliphages. The lower rate of enterococcal phage isolation in this study could be due to the use of unoptimised hosts or to the presence of much smaller numbers of enterococcal phages in the water samples studied. At their present stage of development, both the tests used are not yet totally satisfactory for the assay of enterococcal phages.

The SAL method appeared to be more sensitive than the DAL method probably because the inoculum used for the former (5ml) was larger than that for the latter

(1ml). Using 4 replicates per assay, the limit of detection for the SAL assay was 5 PFU/100ml whereas that for the DAL assay was 25 PFU/100ml. This greater sensitivity with a simpler test should be advantageous for field work. Another modification which would facilitate field testing is room temperature incubation which would eliminate the need for a field incubator. On the other hand, incubation at 44.5°C would help to cut down on the background growth of contaminating bacteria. Unfortunately, both temperatures did not allow adequate growth of the host organism. Perhaps the culture medium could be improved to overcome this problem.

The US Environmental Protection Agency (US EPA, 1986) recommends that recreational waters should not have >126 *E coli*/100ml and >33 enterococci/100ml for fresh water. By these standards, the 2 ponds surveyed should be considered hygienically unsafe for recreational use. Since the ponds have not been known to be health hazards, could the US EPA's requirements be too stringent for local waters? Is it possible that the high counts obtained for the 2 indicator bacteria be reflective of their multiplication in the water rather than faecal contamination? The findings in this preliminary study have identified directions for further research on the extra-intestinal survival and replication of bacterial and viral indicators of faecal contamination of water and the suitability of traditional standards of water quality for local recreational waters.

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