The survival patterns of selected faecal bacteria in tropical fresh waters

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SUMMARY

The survival of various faecal bacteria used as indicators of the faecal contamination of water supplies has been investigated in a tropical environment (Sierra Leone). Isolates representing the thermotolerant coliform (TtC) and faecal streptococcus (FS) groups, *Clostridium perfringens* and *Salmonella* spp. were studied over a 48 h period of immersion in water from three different sources. Survival patterns varied according to source type, but some general observations were made: a portion of the TtC group was apparently capable of substantial re-growth; FS organisms died off at a faster rate than TtC organisms initially, but survived longer; vegetative cells of *C. perfringens* died off rapidly; and *Salmonella* spp. could survive for as long as the other faecal organisms tested. The implications of results for the analysis of tropical waters for faecal contamination are discussed.

INTRODUCTION

Several different organisms, or groups of organisms, have been advocated as appropriate indicators of the faecal contamination of water supplies. An appropriate indicator should be: (*a*) numerous in faeces but not other sources; (*b*) capable of being enumerated by simple and accurate means; (*c*) more resistant than pathogenic organisms to inactivating agents, and (*d*) unable to grow in conditions outside the intestine.

The coliform group was the first such indicator to be used routinely. However, studies which indicated a capacity for growth in water (Bigger, 1937) and which identified non-faecal origins for coliform organisms (Moussa, 1965) led to the widespread adoption of the 'faecal' coliform (FC) group as an alternative, or supplementary, indicator (DHSS, 1983; WHO, 1983). The term 'thermotolerant coliform' (TtC) has been proposed (DHSS, 1983) as a replacement for the more commonly used 'faecal coliform', the former representing more accurately the constituent organisms of the group, as tested for. TtC is preferred and used herein.

Faecal streptococci (FS) have also been used as indicators of faecal contamination (DHSS, 1983) and several studies have suggested that FS survival in water is nearer than that of TtC organisms to the survival of pathogenic micro-organisms, in particular enteric viruses (Keswick *et al.*, 1982; Bitton *et al.*, 1983; Fattal *et al.*, 1983).

Clostridium perfringens, because of its ability to form spores, has a potentially

long survival time in water and has been advocated as an indicator of remote faecal pollution (DHSS, 1983).

Whilst there is now considerable evidence to support the acceptability of TtC organisms, FS organisms and *C. perfringens* as appropriate indicators with respect to the criteria (*a*) and (*b*) listed above, there are far less comprehensive data relating to the other criteria. Although there have been several investigations into the relative survival characteristics of faecal bacteria in water (e.g. Allen, Pasley & Pierce, 1952; Gyllenberg, Niemelä & Sormunen, 1960; Geldreich *et al.* 1968; McFeters *et al.* 1974; Zanoni & Fleissner, 1982), these have often produced differing results. For example, Deaner & Kerri (1969) suggested that re-growth of 'non-faecal' coliforms was common in water, whereas McFeters *et al.* (1974) observed no such growth of coliform organisms in well-water. Also, whilst it has been reported that FS generally survive longer than TtC organisms (Geldreich *et al.* 1968), Evison & James (1977) suggested that at higher temperatures (> 20 °C) FS may die off rapidly.

With respect to the relative survival of faecal indicators and pathogenic organisms, *Shigella* spp. have been found to survive longer than the usual indicators, while *Salmonella* spp. survived for either a similar time or died off rapidly, according to species (McFeters *et al.* 1974).

Most of the studies on survival so far reported have been conducted in temperate countries. Under tropical conditions, survival characteristics may well differ (Evison & James, 1977). Whilst data already obtained in Sierra Leone (Wright, 1982*a*, *b*) have indicated that the indole-positive portion of the TtC group (presumptive *Escherichia coli*) conformed to the first two criteria required of an appropriate faecal indicator for tropical waters, the need remained for a study on the relative survival characteristics of faecal bacteria in such waters.

MATERIALS AND METHODS

Test organisms

Water samples were taken from each of three sources of water used for drinking by local people; a hand-dug unlined well, a shallow ephemeral stream and a river. These were tested for faecal indicator bacteria by methods previously described (Wright, 1982*b*). Two isolates were obtained from each source of each of the following: (*a*) indole-negative TtC organisms; (*b*) indole-positive TtC organisms (presumptive *Escherichia coli*); (*c*) FS which produced characteristic colonies on membrane Enterococcus agar (DHSS, 1983), but either no growth or atypical (of *Streptococcus faecalis*) colonies on tyrosine–sorbitol–thallous acetate agar (DHSS, 1983); (*d*) presumptive *S. faecalis* (organisms which produced characteristic colonies on both membrane Enterococcus agar and tyrosine-sorbitol-thallous acetate agar); and (*e*) *C. perfringens* [organisms which produced a positive reaction in differential reinforced clostridial medium (DHSS, 1983) and a 'stormy clot' reaction in Crossley milk (Oxoid CM213)].

A pure culture of each isolate was established and maintained, either in cooked meat medium (Oxoid CM439) (FS and *C. perfringens*) or on nutrient agar (Oxoid CM3) (TtC organisms).

Six isolates of *Salmonella* spp. which had been obtained during an investigation

of the seasonality of water quality in the sources described (Wright, 1986), two from each source, and which were maintained on Dorset egg (Cowan & Steel, 1965), were also included in the survival trials.

Determination of survival patterns

Each of the 36 isolates was cultured in nutrient broth (Oxoid CM1) for 24 h at 37 °C. Approx. 1 ml broth was then transferred to 9 ml membrane-filtered water which had been freshly obtained from one of the three water sources, and mixed. (The membrane filters used in this study were Millipore, 0·45 μm pore-size; they were 'washed' before use by passing water from the relevant source through them, to remove any growth-promoting or -inhibiting substance(s) associated with the filters.) Two millilitres of the suspension was then rapidly membrane-filtered and the cells retained on the filter were rinsed by passing through two successive 20 ml volumes of membrane-filtered water from the same source. The filter was transferred to a sterilized bottle containing glass beads and a further 9 ml of membrane-filtered source-water. The contents of the bottle were throughly shaken and approx. 2 ml of the suspension of washed cells was finally transferred to 18 ml of unfiltered water (unfiltered water was used to more closely reproduce conditions prevailing in the water source; account was taken of the possibility of organisms similar to the test organisms being present in this water, as described below), which had just been taken from the water source and which was contained in a sterilized screw-capped test-tube. The foregoing procedure was designed to bring the number of cells of the test organism in each tube to approx. 10^4 ml^{-1}.

The initial density of the relevant organism in each tube was determined by the methods described by Wright (1982b), except that membrane filtration using an enriched lauryl sulphate/aniline blue medium (Wright, 1982a) was used for the TtC counts and a multiple-bottle technique using Rappaport's broth (DHSS, 1969) followed by subculture to modified brilliant green agar (Oxoid CM329) was used for the determination of *Salmonella* spp. counts. Appropriate dilutions were made in quarter-strength Ringer's solution.

The final suspensions were stored either in or nearby the relevant water source in order to replicate, as closely as possible, normal light and temperature variations. At approx. 6 h intervals each suspension was shaken.

After approx. 12, 24 and 48 h (the exact time was recorded), a portion of the sample was removed from each tube and tested in the same way as used for the determination of initial densities. The same procedure was then followed for the other two water sources.

The determination of survival patterns was performed twice, at the seasonal extremes of the year in Sierra Leone: November (end of wet season) and April (end of dry season).

Results were recorded as counts ml^{-1} for each sample tube. Counts obtained from the water samples which had had no test organisms added – see below – were deducted from the sample counts and each count then converted to a percentage value of the corrected initial density. The geometric mean % survival after approx. 12, 24 and 48 h was determined for each set of six isolates belonging to each category of bacteria.

Confidence intervals of 95% were calculated for each mean, and then pairs of

means $\pm$ confidence intervals were compared for each test organism either between the two times of the trials or between two of the different source types. If the confidence intervals of a pair of means did not overlap, the means were considered significantly different at the 5% level of confidence.

Determination of water quality

The major water quality attributes of each source (pH, temperature, conductivity, 4 h permanganate value and turbidity) were determined at the times the survival trials were conducted. Faecal indicator and *Salmonella* spp. counts were also determined on a sample of water taken from each source and stored with the 'inoculated' samples, at the same times as the latter were counted.

RESULTS

Water quality in the three sources investigated was poorer at the end of the dry season than at the end of the wet season (Table 1). The stream had dried by the time survival trials were conducted in April, to the point where water only remained in pools which had been artificially deepened by local villagers. Conductivity, pH values and organic matter content (indicated by 4 h permanganate values) were generally low. All sources were contaminated with faecal bacteria.

Despite seasonal differences in water quality, no significant $(P > 0.05)$ differences were observed in the survival patterns of any of the test organisms between the two times of the trials; survival data were therefore pooled for each source type before determining the survival curves depicted in Figs. 1–3, i.e. $n = 12$ for each plotted point, rather than $n = 6$. There were, however, some significant $(P < 0.05)$ differences in survival patterns between the three source types.

For the river (Fig. 1), counts obtained at zero time and after 12 h were similar, with the exception of the *C. perfringens* count, which was markedly reduced: by 24 h *C. perfringens* could not be detected in the majority of the replicate sample tubes. Results indicated that re-growth of some of the test organisms had taken place, particularly the indole-negative TtC organisms, within the first 12 h. However, indole-negative TtC organisms then displayed the sharpest decline in survival (between 12 and 24 h).

The capacity for re-growth can be represented by the time taken for counts to reach the top of the survival curve, and also by the height of the curve. In these terms, with respect to the river, indole-negative TtC organisms, presumptive *E. coli*, FS other than *S. faecalis* and *Salmonella* spp. were all apparently capable of re-growth, in respectively decreasing amounts.

Representing survival by the time taken to achieve a 50% or 90% reduction in bacterial counts (T_{50} and T_{90} values, respectively), presumptive *E. coli* (T_{50}) and presumptive *S. faecalis* (T_{90}) displayed the highest values (Table 2) for the river.

For the stream, the survival curves were relatively similar to each other, but again with the exception of that for *C. perfringens* (Fig. 2). Both the highest T_{50} and T_{90} values were demonstrated by indole-negative TtC organisms (Table 2) although by 48 h the proportion of surviving cells of this group was lower than for all other organisms/groups except *C. perfringens*. All organisms/groups (except *C. perfringens*) were apparently capable of some re-growth.

Table 1. *Quality of water in the sources used for survival trials*

Variable	Water source					
	River		Stream		Well	
	End of wet season	End of dry season	End of wet season	End of dry season	End of wet season	End of dry season
pH	5·76	6·60	5·24	5·64	5·10	4·88
Temperature (C)	24·8	31·2	25·2	26·2	25·6	25·6
Conductivity (μS cm^{-1})	12	29	8	66	9	42
Permanganate value (mg l^{-1})	1·35	1·30	0·80	2·00	0·60	0·60
Turbidity (NTU)	8	2	2	60	2	80
Indole-negative TtC organisms*	150	150	270	8000	150	1500
Presumptive *E. coli**	90	230	60	700	150	430
FS other than pre-sumptive *S. faecalis**	8	65	10	700	70	90
Presumptive *S. faecalis**	20	12	50	900	70	40
C. perfringens spores*	90	40	150	90	90	30
Salmonella spp. organisms*	< 30	< 30	30	< 30	< 30	< 30

* Counts are recorded as either the most probable number (MPN) dl^{-1} (*C. perfringens* and *Salmonella* spp. organisms) or colony-forming units (c.f.u.) dl^{-1} (all others).
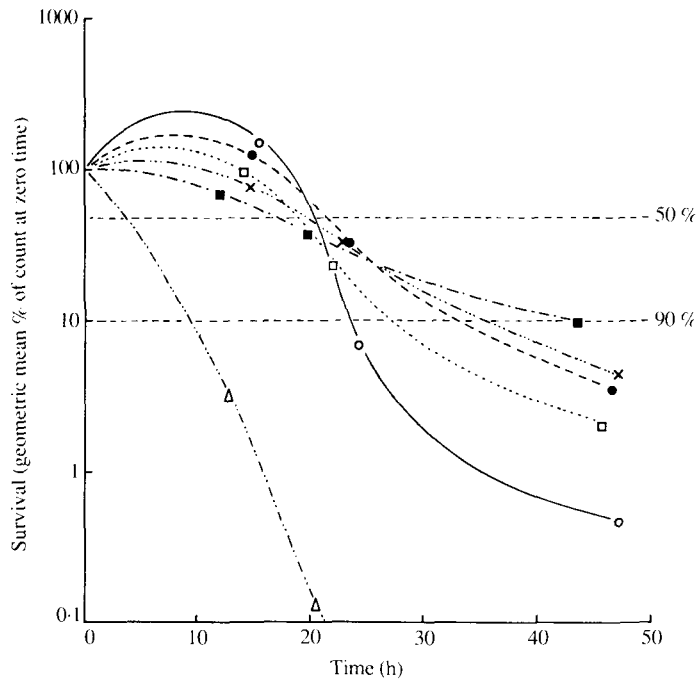

Fig. 1 Survival curves of faecal bacteria in river water ($\bigcirc$, indole-negative TtC: $\bullet$, presumptive *E. coli*: $\square$, FS other than *S. faecalis*: $\blacksquare$, presumptive *S. faecalis*: $\triangle$. *C. perfringens*: $\times$, *Salmonella* spp.). Plotted points represent the geometric mean values of 12 results.

Table 2. *Survival of faecal bacteria in water in terms of T_{50}* and T_{90}* values*

| | Water source | | | | | | | | |
| | River | | Stream | | Well | | Overall mean | | |
Organism/group	T_{50}	T_{90}	T_{50}	T_{90}	T_{50}	T_{90}	T_{50}	T_{90}	$T_{90}-T_{50}$
Indole-negative TtC organisms	20·3	23·7	24·1	32·0	20·1	23·3	21·5	26·3	4·8
Presumptive *E. coli*	21·4	32·6	20·6	29·9	17·0	21·3	19·7	27·9	8·2
FS other than presumptive *S. faecalis*	18·7	27·0	19·3	26·7	13·0	20·3	17·0	24·7	7·7
Presumptive *S. faecalis*	16·7	42·3	16·4	29·9	14·7	21·6	15·9	31·3	15·4
C. perfringens	3·7	9·7	8·5	15·9	2·9	7·7	5·0	11·1	6·1
Salmonella spp.	19·5	35·6	20·3	30·5	16·1	23·9	18·6	30·0	11·4

* Time (hours) for a 50 % or 90 % reduction, respectively, in bacterial counts to occur.

Fig. 2. Survival curves of faecal bacteria in stream water ($\bigcirc$. indole-negative TtC; $\bullet$. presumptive *E. coli*; $\square$. FS other than *S. faecalis*; $\blacksquare$. presumptive *S. faecalis*; $\triangle$. *C. perfringens*; $\times$, *Salmonella* spp.). Plotted points represent the geometric mean values of 12 results.

The well water was less conducive to the survival of the test isolates than either the stream or river water (Fig. 3). Each of the mean T_{50} and T_{90} values for this source was lower than for the other sources (Table 2). However, the apparent regrowth of indole-negative TtC organisms was greater, reaching a maximum after approx. 10 h.
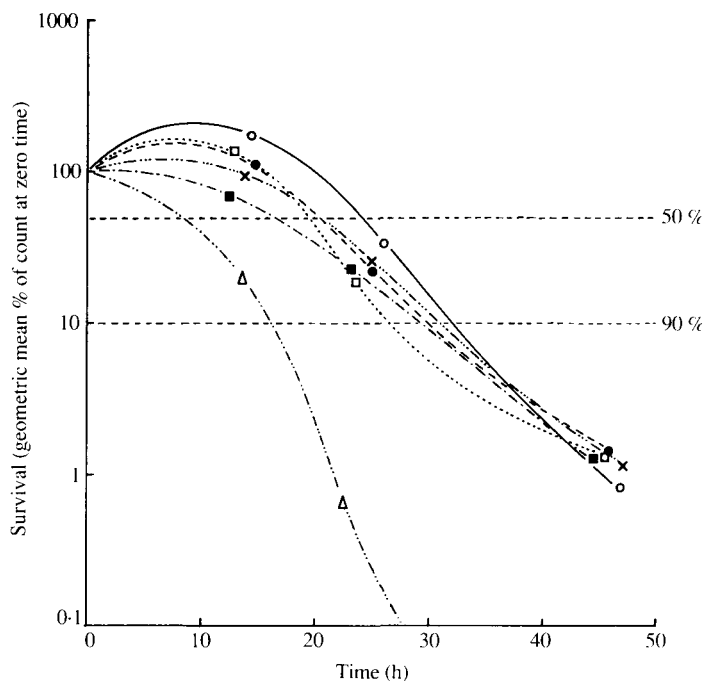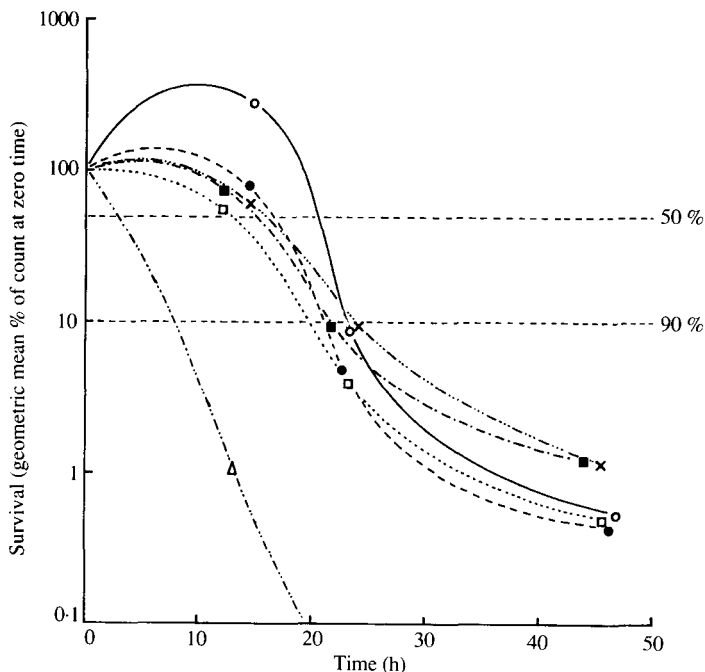
Fig. 3. Survival curves of faecal bacteria in well water (○, indole-negative TtC; ●, presumptive *E. coli*; □, FS other than *S. faecalis*; ■, presumptive *S. faecalis*; △, *C. perfringens*; ×, *Salmonella* spp.). Plotted points represent the geometric mean values of 12 results.

DISCUSSION

The survival of faecal bacteria in water can be differentiated into three phases: re-growth, die-off and resistance.

In this study, indole-negative TtC organisms displayed the greatest overall capacity for re-growth, followed by presumptive *E. coli* and FS other than *S. faecalis*; the apparent re-growth of presumptive *S. faecalis* and *Salmonella* spp. was not substantial and *C. perfringens* isolates were incapable of re-growth. The capacity for re-growth of coliform bacteria is well documented and normally associated with the 'non-faecal' portion of the group (Deaner & Kerri, 1969). However, this study indicates the re-growth potential of indole-negative TtC organisms, which suggests that some of these organisms might have a non-faecal origin. This supports the use of 'TtC' rather than 'FC' to describe those organisms isolated/enumerated by elevated temperature differential/selective coliform tests.

The die-off of faecal bacteria in water has been quantified in several ways; principal among these are the T_{50} and T_{90} values, and the rate of die-off. Each of these can present a different picture of survival, especially if taken in isolation. In particular, the re-growth and resistant phases of survival are masked when calculating die-off in these terms. McFeters *et al.* (1974), using T_{50} values, reported a greater survival potential for some *Shigella* spp. than for the commonly used faecal indicator organisms. However, the T_{50} values quoted for the *Shigella* spp. were apparently derived from the time taken for a 50% reduction in numbers from the population recorded after 24 h immersion in water, whereas the T_{50}

values for the indicators were derived from the time taken for a 50% reduction from the initial population; these should not be considered comparable. It is therefore suggested that the $(T_{90} - T_{50})$ value represents the best indication of die-off. Using $T_{90} - T_{50}$ values obtained in this study (Table 2), indole-negative TtC organisms died off most rapidly, followed by *C. perfringens*, FS other than *S. faecalis*, presumptive *E. coli*, *Salmonella* spp. and presumptive *S. faecalis*, respectively.

The resistance of faecal bacteria in water is reflected by the T_{90} values. As reported herein, the resistance of presumptive *S. faecalis* was greatest, followed by *Salmonella* spp., presumptive *E. coli*, indole-negative TtC organisms, FS other than *S. faecalis* and *C. perfringens*, respectively.

The observed differences between survival patterns in water from different source types indicated the important influence of water quality upon survival. However, no significant differences in survival were observed between water of differing physico-chemical quality from the same source. This strongly suggests that variables not quantified or controlled in this study, notably the numbers/types of microbial predators and/or competing organisms in the water source, were more important influences on survival than those which were quantified or controlled (temperature, light intensity, pH, conductivity, turbidity and organic matter concentration), at least over the range of variation of these reported in Table 1.

The generally reduced survival time of faecal bacteria in well water did not confirm the suggestion of Bitton *et al.* (1983) that survival in ground water may be more prolonged than in surface water.

Overall, these findings substantiate the use of presumptive *E. coli* as the most appropriate indicator of faecal contamination of tropical waters. However, as the mean T_{90} value for *Salmonella* spp. was slightly greater than that for presumptive *E. coli*, the possibility exists that a water source demonstrating a low or zero presumptive *E. coli* count might still contain salmonella organisms.

The FS test is considered unnecessary for the faecal analysis of tropical waters on the grounds that: (*a*) TtC organisms usually occur in greater numbers in human faecal material than FS (Wright, 1982*b*); (*b*) presumptive *E. coli* were always demonstrated in tropical water samples which were shown to contain presumptive *S. faecalis* (Wright, 1986); (*c*) methods for the enumeration/confirmation of FS are less well-established and take longer to complete than those for TtC organisms, and (*d*) no useful information could be derived from calculating a TtC/FS ratio (Wright, 1986).

The results obtained for *C. perfringens* survival indicate that the tropical water environment is inimical to the vegetative cells of this organism; moreover, the 'shock' of introduction to this environment did not induce sporulation. However, *C. perfringens* spores have been detected in local water sources (Wright, 1982*b*). Testing for these spores therefore remains a valid means of indicating remote faecal contamination where this is suspected and when TtC organism counts are zero.

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