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The use of alkalinity and incubation at 9 °C for improved recovery of *Yersinia* spp. from faeces

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SUMMARY

Recovery of *Yersinia enterocolitica* and related strains from faecal samples enriched in 1% buffered peptone water (pH 7·2) and incubated at 4 °C for 17–21 days was compared with recovery from 1% peptone water buffered to pH 8·0 and incubated over the temperature range 4–26 °C. Best recovery was obtained by use of the alkaline medium incubated at 9 °C. Greatest recovery was obtained after incubation for 10–14 days, but most strains (> 75%) were recovered after 1 week.

INTRODUCTION

Enrichment in buffered 1% peptone water with incubation at 4 °C has been shown to be effective for the recovery of *Yersinia enterocolitica* and related strains from faecal specimens (Lewis & Chattopadhyay, 1986; Greenwood & Hooper, 1987). However this method requires a lengthy incubation period of 2½–3 weeks. Aulisio *et al.* (1980) observed a difference in alkalotolerance between *Y. enterocolitica* and other Gram-negative bacteria, and used a potassium hydroxide treatment to obtain rapid recovery of yersinia from foods. In an attempt to obtain rapid isolation of yersinia from faeces, Weissfeld & Sonnenwirth (1982) used an alkali treatment on faecal homogenates, and found that this allowed recovery of yersinia at concentrations of 10^3 ml. Ratnam, Looi & Patel (1983) also used an alkali treatment in an attempt to recover *Y. enterocolitica* from direct and enrichment cultures of faecal samples. These workers used a variety of enrichment broths which were either incubated overnight at 35 °C, at room temperature for 2 days, or at 4 °C for 14 days. They found that the alkali treatment did not significantly enhance the recovery rate of *Y. enterocolitica*.

This study utilizes the tolerance of yersinia to alkaline conditions by using an alkaline enrichment medium in combination with various incubation temperatures in order to produce the optimum conditions for isolation of yersinia from faecal samples.

MATERIALS AND METHODS

Media

Plate Count agar (PCA; Oxoid CM325), Cefsulodin-Irgasan-Novobiocin agar (CIN; Gibco CIN agar base no. 152-1030 plus Yersinia selective supplement

D7100), nutrient broth (Oxoid CM67), urea agar (Oxoid CM54 plus urea 40% SR20), triple sugar iron agar (Oxoid CM277) and buffered peptone water (BPW; Oxoid CM509 which contains peptone 1%, pH 7·2) were prepared according to the manufacturers' instructions. Alkaline peptone water (APW) was prepared from peptone 1%, sodium chloride 0·5%, and adjusted with sodium hydroxide to pH 8·6 without buffering. Tris-buffered peptone water (TPW) was made from peptone 1%, sodium chloride 0·5%, tris (hydroxymethyl) methylamine 1·2%, and adjusted with hydrochloric acid to pH 8·0, 8·2, 8·4 and 8·6 as required.

Faecal suspensions

These were prepared as 10^{-1} dilutions in 0·1% peptone solution. Further decimal dilutions were then made and total aerobic viable counts were performed on PCA.

Test organism

A human isolate of *Y. enterocolitica* biotype 1 serotype 0 5,27 was grown overnight in nutrient broth at 30 °C. Decimal dilutions were prepared in 0·1% peptone solution and a total viable count performed on CIN agar. An inoculum of 0·1 ml of the 10^{-5} dilution (10^2–10^3 organisms) was then added to 10 ml of each faecal suspension.

Measurement of the effect of alkalinity

A 1 ml volume of spiked faecal suspension was added to 9 ml volumes of BPW, APW, TPW (8·0), TPW (8·2), TPW (8·4) and TPW (8·6). All peptone waters were allowed to remain at ambient temperature for 24 h. Viable counts were then carried out on PCA and CIN agar. After adjustment for dilutions, the number of generations was calculated for the total aerobic flora and for *Y. enterocolitica*.

Measurement of the effect of temperature

All faecal samples received into the laboratory were inoculated directly into 10 ml volumes of BPW and TPW (8·0) or into two 10 ml volumes of TPW (8·0). The two peptone waters were incubated at either the same temperature or different temperatures in the range 4–26 °C, and were subcultured onto CIN agar at various time intervals of up to 3 weeks. Suspect colonies of yersinia were screened for urease activity, biochemical reactions in Triple Sugar Iron agar and absence of motility at 37 °C as described before (Greenwood & Hooper, 1985). Cultures confirmed as yersinia were then sent to the reference facility at Leicester Public Health Laboratory for biotyping and serotyping. Recovery of yersinia from the two peptone waters was then compared for each time/incubation temperature combination.

RESULTS

Effect of alkalinity

Nine faecal suspensions were used to measure the growth of the aerobic faecal flora and of *Y. enterocolitica* in the presence of faecal flora in peptone solutions of different alkalinity. The results are presented in Table 1. Although the

Table 1. *Growth of faecal flora and of* Y. enterocolitica *in the presence of faecal flora in peptone waters of varying pH*

	Mean no. of generations in					
	BPW	APW	TPW 8·0	TPW 8·2	TPW 8·4	TPW 8·6
Total aerobic flora	6·8	6·2	5·3	4·7	3·4	2·8
Y. enterocolitica	11·3	10·1	11·1	10·2	10·0	9·3

Table 2. *Comparison of yersinia recovery rates from faecal samples incubated in BPW at 4 °C and TPW (8·0) incubated at 9–26 °C*

Incubation temp. (°C)		Recovery of yersinia (%)		
TPW (8·0)	BPW	TPW (8·0)	BPW	Total no. isolations
22–26	4	42	89	45
18–20	4	51	83	72
14	4	70	79	77
9	4	84	61	60

Subculture times: BPW, 17–21 days; TPW (14–26 °C); 1 and 4–6 days; TPW (9 °C); 2–5 and 6–8 days.

multiplication of *Y. enterocolitica* in BPW (pH 7·2) and in TPW (8·0) was found to be similar, the numbers of competing bacteria that grew on CIN agar were dramatically reduced by using TPW (8·0). Increasing the alkalinity of the peptone water above pH 8·0 further reduced the multiplication of the faecal flora, but also reduced the growth of *Y. enterocolitica*. TPW (8·0) was therefore selected for measuring the effect of incubation temperature.

Effect of temperature

In initial investigations to assess the effect of incubation temperature, the recovery of *Yersinia* spp. from faecal specimens by the standard method of enrichment in BPW at 4 °C with subculture after 17–21 days was compared with the recovery obtained by enrichment in TPW (8·0) incubated at other temperatures for up to 8 days. The results are shown in Table 2. The recovery of yersinia from faecal specimens enriched in TPW (8·0) incubated at 9 °C for 6–8 days was found to be significantly greater ($P < 0·005$) than that obtained by enrichment in BPW at 4 °C for 17–21 days. Incubation of TPW (8·0) at higher temperatures (14–26 °C) did not enhance the recovery obtained by enrichment in BPW at 4 °C.

The next two trials involved a direct comparison of the recovery of yersinia from BPW and TPW (8·0) after incubation of both solutions at 4 °C and at 9 °C, with subculture of both solutions after 7 days and 17–21 days. Results from this comparison are shown in Table 3. Recovery of yersinia from TPW (8·0) incubated at 4 °C was not as good as from BPW incubated at this temperature. However when incubation was carried out at 9 °C, recovery was better from TPW (8·0)

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Table 3. *Comparison of yersinia recovery from BPW and TPW (8·0) incubated at 4 and 9 °C*

Incubation temp. (°C)	Recovery of yersinia (%)		Total no. isolations
	TPW (8·0)	BPW	
4	65	79	65
9	84	67	61

Subculture times: 7 days and 17–21 days.

Table 4. *Comparison of incubation at 9 and 14 °C for recovery of yersinia from faecal samples inoculated into TPW (8·0)*

Incubation temp. (°C)	Recovery of yersinia (%)					Total recovery (%)
	Incubation time (days)					
	3	7	10	14	17–21	
9	16	68	76	81	87	89
14	17	44	46	56	57	59

($P < 0·05$). Subculture after incubation at 9 °C for 7 days resulted in similar recovery from the two media, but 13 strains were detected only through TPW (8·0) and a further 12 strains detected only through BPW. Only two of the strains detected by BPW alone after 7 days were eventually isolated from TPW (8·0), but a significant number of extra strains were isolated from TPW (8·0) only after 17–21 days incubation.

In the first set of comparisons, incubation of TPW (8·0) at 14 °C was stopped after 6–8 days. A direct comparison was next carried out by inoculating each faecal sample into two bottles of TPW (8·0). One bottle was incubated at 9 °C and the other at 14 °C, and subculture from both bottles was performed at 3, 7, 10, 14 and 17–21 days. The results of this survey are shown in Table 4. Best recovery was obtained from bottles that were incubated at 9 °C ($P < 0·0005$). Although recovery was greatest when subculture was performed after 17–21 days incubation, some strains were lost after 10 days incubation. In order to determine the optimum time for subculture, TPW (8·0) enrichment cultures incubated at 9 °C were subcultured after 7, 10, 14 and 17–21 days initially and then after 3, 7 and 10–14 days. Table 5 shows the recovery obtained at each subculture time. Subculture after 3 days did not recovery many strains. Over three-quarters of strains were isolated after 7 days of incubation. Maximum recovery appeared to occur after 10 days of incubation. Although the recovery after 14 days appeared to be the same as after 10 days, two strains had been lost by extending the incubation period but two extra strains were detected. Extending the incubation period to 17–21 days resulted in further loss of strains.

In all comparisons carried out, it was found that no single method isolated all strains of yersinia. In addition, multiple subculture frequently led to the isolation of different biotypes and serotypes from the same faecal specimen. In the comparison between TPW (8·0) and BPW incubated at 9 °C, more than one strain

Table 5. *Recovery of yersinia from faecal samples after different lengths of incubation at 9 °C in TPW (8·0)*

	Recovery of yersinia (%)				Total no. strains recovered
	Length of incubation (days)				
	3	7	10–14	17–21	
Survey A	NE	82	94*	85†	34
Survey B	30	77	88†	NE	113

NE, not examined.

* Faecal samples subcultured at 10 and 14 days; recovery rate 94% for both incubation times.

† All faecal samples received in one working week were subcultured as a single batch either 10–14 days or 17–21 days after receipt in addition to earlier subculture.

was isolated from 18% of faecal specimens – 2 strains were isolated from 7 samples, 3 strains from 3 samples and 4 strains from 1 sample.

DISCUSSION

The regime combining enrichment in BPW incubated at 4 °C for 17–21 days with subculture to CIN agar has been shown to be successful for the isolation of *Y. entercolitica* and related strains from faecal samples (Lewis & Chattopadhyay, 1986; Greenwood & Hooper, 1987). Because *Y. enterocolitica* is capable of significant growth at refrigeration temperatures, use of cold enrichment helps to equalize the growth rates of *Y. enterocolitica* and the competing organisms, thus preventing the metabolic crowding seen when a faster-growing antagonistic organism reaches stationary phase density (Schiemann & Olson, 1984). However, the lengthy incubation time required for cold enrichment limits the usefulness of this technique. The main objective of this investigation was to reduce the time required for isolation of yersinia from faecal samples. The first experiments established that use of a buffered alkaline medium could reduce the growth of competing faecal flora compared with the growth obtained from a medium of neutral pH. Initial trials involving enrichment of faecal samples in TPW (8·0) were carried out at ambient temperature with subculture to CIN agar after 1 and 4–6 days. When this was found unsuccessful, the temperature of incubation was decreased and the incubation time gradually increased. Results were disappointing until incubation was carried out at 9 °C (Tables 2–4). The optimum regime for recovery of yersinia strains from faecal samples was found to be enrichment in TPW (8·0) incubated at 9 °C. This optimum temperature of incubation is lower than that suggested by Schiemann & Olsen (1984), who found that the antagonism of competing bacteria was largely eliminated at 15 °C.

The combination of enrichment in TPW (8·0) and incubation at 9 °C reduced the growth of competing flora on CIN agar to such an extent that quarter-plates were found to be adequate for subculture, and a large proportion of enrichment subcultures resulted in complete absence of growth. As so many strains could be detected after only 7 days' incubation (76–82%, Table 5), it is recommended that subculture is carried out at this time. In addition a further subculture should be

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performed, and all enrichment cultures from faecal samples received in a single working week might be subcultured as a single batch 10–14 days after receipt. The use of an alkaline peptone water medium buffered to pH 8·0 and incubated at 9 °C has enhanced the recovery of yersinia from faecal samples, and reduced the length of time needed to recover the majority of isolates.

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