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Note

Gas chromatographic detection of bacteria causing enteric fever

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Enteric fever is one of the most common waterborne infections caused by *Salmonella typhi* and *Salmonella paratyphi* A, B and C. The conventional methods for the isolation of these bacteria take normally 3–4 days and for confirmation by biochemical and serological techniques a further 2–3 days. Other infectious agents that give clinical states like enteric fever are *Brucella, Plasmodium* sp. and various viral agents during the early stages of infection. To differentiate between these agents a rapid laboratory technique would be of great help. Hence there is a need for a rapid method for the detection of *Salmonella* sp., especially in the blood of patients suffering from enteric fever, and in food and water samples. Efforts have been made to reduce the detection period by adopting specific culture methods [1], fluorescent antibody techniques [2], enrichment serology [3,4] and radiometric techniques [5]. Recently, gas chromatographic (GC) techniques have attained new significance in clinical microbiology [6–9]. Reiner et al. [10] reported a pyrolysis GC method for the differentiation of *Salmonella* sp., but it is time consuming and also a pure bacterial mass is required; the method has little significance as a diagnostic procedure but it is useful for the taxonomic classification of *Salmonella*.

A simple GC method for the detection of *Salmonella* sp. is reported in this paper. It is based on the detection of ethanol as metabolic product from the fermentation of rhamnose and specific suppression of ethanol production by preservative-free *Salmonella* polyvalent H antiserum in parallel cultures. Strains of *Salmonella* consistently ferment rhamnose [11]. Dulcitol fermentation has been used [5] previously for the radiometric detection of *Salmonella* in foods but many strains of *Salmonella typhi* do not ferment it. Hence rhamnose is considered to be superior to dulcitol. *Salmonella* during fermentation splits the sugar, yielding

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ethanol [12], and the ethanol in the spent cultures can easily be detected by GC. Further, *Salmonella* polyvalent H antiserum was used for the specific suppression of *Salmonella*, which would confirm the presence of bacteria. The use of antiserum in this GC technique is a critical step because commercial antisera contain cresol or merthiolate as preservatives, which are non-specific inhibitors. Preservative-free antisera should be used for the specific suppression of growth in parallel cultures.

EXPERIMENTAL

Bacterial strains

Salmonella typhi and *Salmonella paratyphi* A, B and C were obtained from Central Research Institute, Kasauli, India, and were maintained in Mueller Hinton agar (Himedia, Bombay, India).

Equipment

A Model ROL 4 gas chromatograph (Toshniwal, New Delhi, India) was used. Dual stainless-steel columns (180 cm×0.31 cm I.D.) were packed with 15% ethylene glycol succinate on high-performance Chromosorb W (80–100 mesh). Nitrogen at a flow-rate of 40 ml/min was used as the carrier gas. The operating temperatures of the column, injection port and detector were 110, 150 and 170 °C, respectively. A Heath-Kit multi-speed servo chart recorder was operated at 1 mV sensitivity and a chart speed of 7.5 cm/min.

Procedure

Initial studies on the detection of ethanol in glucose broth gave inconsistent results because of the presence of peptone and thermally degraded products in spent cultures. Selective media with Brilliant Green at 50 to 250 μg/ml were also employed. At lower concentrations of Brilliant Green *Klebsiella* would grow in the media and at higher conentrations *Salmonella* strains would be suppressed. A salt mixture medium [13] with casamino acid, yeast extract and rhamnose was used (M-9R medium). This filter-sterilized medium on injection into the GC column did not give any peaks. A calibration graph for ethanol at various concentrations was constructed as described previously [8].

Salmonella polyvalent H antiserum was produced in rabbits according to the procedures advocated by Cruickshank et al. [14]. Serum was filter-sterilized and stored at −80 °C without any preservative. The antibody titre was established using formalin-killed flagellar strains of *Salmonella*.

Freshly grown *Salmonella* strains were inoculated on to duplicate lots of M-9R medium. One batch received 0.1 ml of *Salmonella* polyvalent H antiserum and the other an equal amount of antibody-free normal rabbit serum. At 6 and 24 h after incubation at 37 °C, spent cultures were injected directly into the GC column to determine the ethanol in both batches of spent cultures. Five strains of *E. coli* were also grown in the medium as described above and spent cultures were analysed by GC to rule out the possibility of non-specific suppression of *Salmonella* antiserum.

Sheep blood was freshly collected under aseptic conditions in preservative-free heparin (Kock-Light, Colnbrook, U.K.). The antibody titre for *Salmonella* in the plasma was determined as described previously [14]. Laboratory cultures of different *Salmonella* strains were inoculated at various concentrations into the blood and their viability count was performed on aliquots of blood. Blood samples containing *Salmonella* were inoculated into selenite broth and incubated at 40 °C overnight and then subcultured to M-9R media with and without *Salmonella* polyvalent H antiserum. Both batches were incubated at 37 °C for 6 h.

Gas chromatography

Portions (5 μl) of aqueous spent cultures of 6- and 24-h-old *Salmonella* strains in M-9R medium with and without antiserum were injected into the GC column directly to determine the amount of ethanol produced. Selenite broth cultures of blood samples subcultured on M-9R medium were also injected to detect the presence of *Salmonella* in blood. Peak heights recorded for ethanol in the media with and without antiserum were measured. Blank samples of media were also injected to ensure the quality of media lots. *E. coli* cultures and ethanol standards were also injected to record the peak for ethanol.

RESULTS AND DISCUSSION

The experiments were standardized in the initial stages using 10^5–10^6 organisms of *Salmonella* in M-9R medium grown for 6 h at 37 °C. All the strains used consistently produced ethanol in cultures. Injections of ethanol standards in the concentration range 50–900 ppm gave linear responses. The recorder response for ethanol was so sharp that the peak area could not be measured (Fig. 1), so the peak height was measured. The antibody titre of *Salmonella* polyvalent H antiserum was 2400 against *S. typhi* and *S. paratyphi* A, B and C. The plasma level of antibody titre in sheep blood samples was <40. Table I shows the peak height for ethanol produced in pure cultures of different species of *Salmonella* at 6 and 24 h. The ethanol produced by *Salmonella* from twelve blood samples grown in M-9R medium with and without *Salmonella* polyvalent H antiserum is given in Table II. *E. coli* cultures with and without antiserum gave a peak height for ethanol of 152 ± 20 mm. Different batches of blank media did not give any peaks.

It is evident from the results that ethanol can be detected in spent cultures of *Salmonella* in 6 h. The amount of ethanol produced during this period approximately corresponds to 150 ppm in the medium. The inhibition of growth of *Salmonella* was found to occur in the initial stages of the growth. This is due to the use of antiserum raised against flagellar H antigen or surface antigen, which is only part of the complex of antigens of *Salmonella*. Further antibodies, unlike antibiotics, do not penetrate the bacterial cells so as to stop the biochemical process completely. After 24 h of growth the amount of ethanol produced in the cultures with and without antiserum remained the same (Table I). A 50% reduction in the amount of ethanol produced in the medium with polyvalent H antiserum indicates that the ethanol in the other batch of parallel cultures was produced specifically by *Salmonella* causing enteric fever.

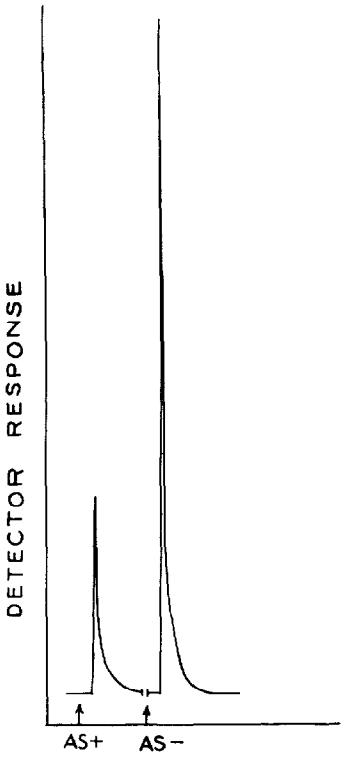

Fig. 1. GC profiles of spent culture media with (AS+) and without (AS−) polyvalent *Salmonella* H antiserum.

TABLE I

RESULTS OF GC ANALYSIS OF *SALMONELLA* IN M-9R MEDIUM

Values are means ± S.D. for five identical spent cultures

Sample No.	Bacterial strain	Organisms in the inoculum	Peak height of ethanol at 6 h (mm)		Peak height of ethanol at 24 h (mm)	
			Without AS*	With AS*	Without AS*	With AS*
1	*S typhi* KD-1	$8 \cdot 10^4$	81.8 ± 7.7	24.4 ± 6.5	192.4 ± 8.6	220.2 ± 17.3
2	*S. typhi* KD-2	$7 \cdot 10^4$	91 ± 9.8	24.4 ± 4	216.4 ± 17.2	216.4 ± 17.2
3	*S. typhi* KD-3	$9.6 \cdot 10^4$	108.4 ± 9.3	24 ± 5	223.6 ± 17.2	216.2 ± 17
4	*S. parathyphi* A	$2 \cdot 10^5$	84.2 ± 9.2	19.6 ± 6.6	180.4 ± 8	169.6 ± 18.6
5	*S paratyphi* B	$8 \cdot 10^5$	120 ± 10.8	44.4 ± 6.2	208.3 ± 18.3	216.4 ± 14.2
6	*S paratyphi* C	$7.6 \cdot 10^4$	104.2 ± 11.5	32.4 ± 5.4	216 ± 19.2	216.2 ± 15

*AS = antiserum.

TABLE II

ETHANOL PRODUCED BY BLOOD-BORNE *SALMONELLA*

Values are means ± S.D. for five identical spent cultures.

Blood sample No.	Bacterial strain	Number of bacteria in 1 ml	Peak height of ethanol at 6 h (mm)	
			Without AS*	With AS*
1	*S. typhi* KD-1	$6.1 \cdot 10^4$	112.4 ± 11.3	24.8 ± 5.8
2	*S. typhi* KD-1	$3.2 \cdot 10^4$	112.4 ± 9.6	20.2 ± 4.5
3	*S. typhi* KD-1	$0.8 \cdot 10^4$	86.4 ± 7.4	17.2 ± 3.1
4	*S. paratyphi* A	$5.8 \cdot 10^4$	106 ± 12.1	28.4 ± 6.4
5	*S. parathyphi* A	$2.8 \cdot 10^4$	102.2 ± 10.9	28.4 ± 6.4
6	*S. paratyphi* A	$0.68 \cdot 10^4$	80 ± 7.1	19 ± 3.2
7	*S. paratyphi* B	$6.6 \cdot 10^4$	112.4 ± 6.8	50.4 ± 6.9
8	*S. paratyphi* B	$3.1 \cdot 10^4$	105 ± 14	11.8 ± 3.2
9	*S. paratyphi* B	$0.9 \cdot 10^4$	55.4 ± 6	15.4 ± 4.4
10	*S. paratyphi* C	$4.9 \cdot 10^4$	105 ± 13.8	30 ± 7.7
11	*S. paratyphi* C	$1.8 \cdot 10^4$	104.6 ± 12.1	44.4 ± 6.2
12	*S. paratyphi* C	$0.7 \cdot 10^4$	70.4 ± 7.4	17.4 ± 4.3

*AS = antiserum.

Selenite broth was used for the initial inoculum of blood containing *Salmonella*. This was employed to dilute the antibody present in the blood, which would otherwise suppress the growth, and as an enrichment medium. Further, selenite broth selectively permits the growth of *Salmonella*, suppressing the other interacting bacteria. In suspected cases of enteric fever the chance of polymicrobial bacteraemia is extremely rare. Even in such cases selenite broth used in the primary culture at 40 °C inhibits other bacteria that may be present in the blood sample. Blood samples were collected with preservative-free heparin in order to avoid non-specific suppression of bacterial growth. All the species of *Salmonella* causing enteric fever could be detected in the blood by this method, but it was not possible to distinguish one species from another. In enteric fever, identification of species of *Salmonella* does not affect the course of treatment, so the technique should have good diagnostic potential. *E. coli* cultures with and without *Salmonella* polyvalent H antiserum contained ethanol at equal concentrations, indicating that the specific suppression of growth is obtained only for *Salmonella* and not for other bacteria.

The GC detection of bacteria causing enteric fever should be of great help in the early diagnosis of enteric fever during the bacteraemic stage as the result is available in 24 h. Moreover, the technique can be employed to establish the presence of viable organisms in blood, which has more relevance in cases of active disease than in detecting antigen or antibody in the blood. Antigen detection is not necessarily an indication of viable cells in the blood and a single titre of antibodies in the blood does not indicate an active infection. This is the greatest advantage of the present technique in comparison with other indirect methods of diagnosis. Although the technique is a tentative method for the identification of *Salmonella*, its utility can be further explored for the detection of other *Salmo-*

nella sp. in food and water. The sensitivity of the test can be further improved by concentrating the samples using membrane filters or disposable sorbent chromatographic columns.

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