EVALUATION OF THE H_2S PAPER STRIP TEST – A FIELD TEST FOR ASSESSING THE MICROBIOLOGICAL QUALITY OF WATER

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ABSTRACT

Epidemics arising from drinking water contaminated with faecal matter are a global health problem. Standard methods currently used for routine microbiological testing of drinking water have many limitations such as the requirement of short transport time, need to employ specialist personnel and equipment, high costs of testing materials. Manja et al (1982) developed a simple and inexpensive field test that could be particularly useful for screening of water supplies in remote and rural areas.

The present paper describes validation of the H_2S strip method. The new method was compared with the standard microbiological tests including total coliforms, *E.coli*, heterotrophic plate count, sulphite reducing clostridia spores count, aerobic spores count and F-specific bacteriophage count. A limited number of samples were also tested in parallel for pathogenic bacteria (*Campylobacter* and *Salmonella*) and viruses (Adenoviruses, Enteroviruses, Noroviruses and Hepatitis A virus).

A total of 312 samples were studied. Different types of water samples, including both uncontaminated drinking water and contaminated environmental sources (tank waters, bore waters, surface waters, geothermal waters) were tested. Geothermal groundwater and high manganese water were specifically included to determine whether presence of hydrogen sulphide and manganese salts in water may cause interferences. Sensitivity of the test was assessed by spiking the samples with low and high levels of bacteria known to produce H_2S. Both reference and wild strains isolated from the H_2S-positive samples were used.

A range of temperatures (ambient , 20 ^oC, 25 ^oC, 30 ^oC and 35 ^oC) and incubation times (24 and 48 hrs) were tested. Although the H_2S method could be used at the temperature range from ambient to 35 ^oC, temperatures between 30-35 ^oC produced the results faster.

The results indicate that the H_2S paper strip method and the Total Coliform/*E.coli* Colilert test were equally effective in the detection of faecal contamination in water samples. Especially, excellent agreement (97.3%) was found between the H_2S method and *E.coli* by the Colilert test. The H_2S method does not detect viruses but detects microorganisms other than coliforms that are associated with faecal contamination, including *Clostridium perfringens*, *Salmonella* and bacteriophages.

The H_2S paper strip test appears to be a sensitive, simple and inexpensive procedure for screening of water supplies towards potential contamination. It eliminates complicated procedures and costly chemicals and lab equipment, including an incubator in subtropical and tropical regions.

KEYWORDS

Hydrogen sulphide paper strip test, H_2S-metabolising bacteria, drinking water standards, *E.coli*

1 INTRODUCTION

An important aspect of the protection of public health is the provision of safe and reliable drinking water. This means that the water used for domestic purposes should be free of pathogenic microorganisms and other substances that may present a health risk. It is impractical to test water supplies for all potential pathogens for a

number of reasons such as the time required to carry out tests, the cost of testing and the inability to test for some organisms. For this reason a system of testing for organisms that indicate faecal contamination (indicator organisms) is used to ensure the safety of drinking water.

Indicator organism tests, such as those for faecal coliforms and *E. coli*, have limitations. They require trained staff and expensive materials and equipment for their execution. In addition, the monitoring of water supplies in remote areas is hindered by the requirement for samples to be tested within 24 hrs of sample collection, for results to be valid. If no resources are available locally, remoteness and a lack of funds may make it impractical to adequately monitor drinking water.

The H_2S paper strip test is simple and inexpensive. This method was developed by Manja et al (1982) and it is particularly suitable for developing countries with ambient temperatures of 25-44 ^{0}C. The method has been extensively evaluated during last 20 years. Four commercially produced brands of the H_2S medium are available for customers.

The purpose of this trial is:

- To validate the H_2S paper strip method against the reference coliform/*E. coli* most probable number (MPN) method (Colilert® MPN 9223 APHA, 1998) using both naturally contaminated and spiked samples
- To test possible limitations and sources of misinterpretation in the H_2S paper strip test
- To determine the sensitivity of the H_2S paper strip method against the reference coliform/*E. coli* method
- To determine the sensitivity of the H_2S paper strip method against methods for detection/ enumeration of other faecal indicator microorganisms and pathogens (e.g. *Salmonella*, *Campylobacter*, viruses)
- To determine whether presence of sulphide-containing groundwaters and high manganese waters cause interference
- To provide cost estimates for the manufacture of the H_2S paper strip test.

2 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Samples were collected from various natural sources such as shallow and deep bore wells, roof water supplies, surface waters including rivers, dams, streams, geothermal wells and treated drinking water supplies.

A minimum 800mL of water was collected into sterile 1L bottle for each sample. For the pathogen study, larger volumes, up to 100L were collected in sterile plastic containers. For chlorinated waters, sodium thiosulphate was added to sample bottles. All samples were processed in the laboratory within 24 hours of collection and were maintained at low temperature during transport.

2.2 HYDROGEN SULPHIDE STRIP TEST METHOD

2.2.1 MEDIA (STOCK SOLUTION)

The concentrated medium (final volume of 100mL) was prepared using the composition below:

Bacteriological peptone (e.g. Difco® Bacto Peptone)	40.0g
Dipotassium hydrogen phosphate	3.0g
Ferric ammonium citrate	1.5g
Sodium thiosulphate	2.0g
Sodium dodecyl sulphate (SDS)	0.4g
Distilled water	100mL

The H_2S medium was prepared according to Manja et al (1982). In the original recipe 2mL of Teepol 610 was used as a surfactant. This product is not available anymore. Sodium dodecyl sulphate (SDS) was used as a substitute surfactant for Teepol, according to Jangi at al (2001). The concentration of 0.02% SDS after the addition of water sample gave the closest results to the Teepol preparation.

2.2.2 PREPARATION OF H_2S PAPERS

H_2S paper strips were prepared by pouring of 3.2mL aliquots of stock solution onto 1 absorbent paper pad from the membrane filtration apparatus (Sartorius®, 47mm diameter, 1.5 mm thickness). A 100mL of water sample required 3.2 mL of media (1 thick Sartorius® absorbent pad). The pads were dried in an oven at 55^0C, placed in steriliser paper bags and autoclaved for 15 minutes at 121^0C. These reagent-impregnated pads can be stored dry (in their steriliser bags) for several months – until ready to use.

When ready to conduct the test, two pads were placed into a 250mL sterile Schott® bottle (or a sterile plastic container) and a volume of 100mL of water sample was added.

2.3 TOTAL COLIFORMS AND *E.COLI*

Total coliforms and *E. coli* densities were estimated using the Colilert® MPN method (Colilert® MPN 9223 APHA, 1998). For this test, the 97-well Colilert® Quanti-trays were incubated at 35 ± 0.5^0C for 18 hours.

2.4 IDENTIFICATION OF BACTERIA

Positive cultures were streaked onto plates of Tryptose Soy agar (TSA) for aerobic bacteria and TSC agar (for *Clostridium* spp.). They were incubated at 35^0C aerobically (TSA) and anaerobically (TSC). The bacterial isolates were identified by using API® kits (API20E®, API20NE® etc).

2.5 ALTERNATIVE FAECAL INDICATORS

F-specific Bacteriophages enumeration was performed according to ISO 1075–1E (1995).

For the heterotrophic plate count, samples were plated onto Plate Count Agar and incubated at 35 ^{0}C for 48 hours (pour plate method).

Clostridia spores were enumerated by heating the 100 mL of the sample at 75^0C for 15 minutes, filtering the heat-treated sample through 0.45 μm filters and incubating the filters anaerobically on the TSC agar.

Aerobic mesophilic spores count (*Bacillus* spore count) was performed according to the British Standard 4285 (1986). Samples were heated at 75^0C for 15 minutes, plated onto Plate Count Agar with starch and incubated for 3 days at 30^0C. *Bacillus* isolates were identified with the API 50Ch® kit.

2.6 PATHOGENS

The volume of water sample used varied with the test. For *Salmonella* and *Campylobacter* enumeration, 1L volumes were filtered through membrane filters, while 100L samples were filtered through hollow fibre filters for viruses.

Salmonella analysis consisted of a number of steps: filtration through 0.45μm filters, pre-enrichment in Buffered Peptone Water, enrichment in selective broths (RVS and Mannitol Selenite broths), growth and detection on selective solid media (XLD and modified Brilliant Green agars) and confirmation of *Salmonella* isolates.

For *Campylobacter* enumeration, 1L samples were filtered through 0.22μm filters, enriched in Bolton Enrichment Broth and plated onto *Campylobacter* Isolation Blood-free agar plates. The plates were incubated in microaerophilic conditions at 41^0C. Both *Salmonella* and *Campylobacter* were enumerated using MPN methods.

For virus analysis, material retained on a hollow fibre filter was eluted and concentrated. Disposable filters (one per each sample) were used for virus concentration. A negative control (20L) was pumped through a filter before concentrating of each sample. The virus concentrates were divided into 3 portions.

Two were tested for enumeration of culturable adenoviruses and enteroviruses grown on appropriate cell lines. Enteroviruses were tested by the suspended plaque assay on the African Green Monkey kidney (BGM) cell line. Adenoviruses were tested by the MPN method using two cell lines (Hep 2 Clone B and 293 N3S) and confirmed with the Adenovirus Direct Immunofluorescent Assay kit. ESR, Wellington using the PCR method, tested the third portion for Noroviruses and Hepatitis A viruses.

3 STUDY 1: COMPARISON OF H_2S PAPER STRIP METHOD WITH THE COLILERT METHOD

3.1 PURPOSE

The purpose of this study is to compare the H_2S paper strip method with a referee method. The enzyme substrate coliform test (*E. coli*) APHA 9223B, 1998) is cited as a referee method in Drinking-water Standards for New Zealand 2000 (MoH, 2000). The Colilert® test has been used for this study.

3.2 EXPERIMENTAL PROCEDURE

All samples were tested by the H_2S paper strip test (in quintriplicate) and the Colilert® MPN method. To study the temperature range at which the H_2S paper strip method was effective, 20, 25, 30, 35^0C and ambient temperature were tested. The ambient temperature varied between 17-23^0C during the period at which the experiments were conducted.

100 mL of water sample to be tested was placed in a sterile bottle containing 2 absorbent pads impregnated with the H_2S medium, allowed to stand for 5 minutes, shaken and stored in appropriate incubators (20, 25, 30 and 35^0C). One bottle (from each sample) was left on the bench at room temperature.

All bottles were examined after one hour of incubation to check for sulphide already present in the sample (e.g. from sediments). The rapid reaction of iron with sulphide present in a water sample could result in darkening of the H_2S test almost immediately upon addition of the sample. For this reason, it is very important that the test procedure includes a visual inspection after one hour of incubation to exclude samples producing quick or early positive reactions. Any sample exhibiting such rapid discoloration indicates that it is contaminated by sulphides and should be treated as a false positive.

The bottles were incubated at various temperatures and examined after 24 hours and 48 hours to determine the extent of blackening in the bottles due to the reduction of the ferric ammonium citrate by any hydrogen sulphide gas produced by microorganisms. The date and time of observation was recorded on the report form and the observations were recorded with: (-) = no blackening, no growth, (-G) = no blackening, growth (turbid), (+) = trace of blackening, (++) = the paper strip was partially back, up to half of the bottle turned black, (+++) the strip and entire bottle was dark black.

Isolates from all positive H_2S bottles were identified.

A set of the QA/AC controls (blanks, positive and negative controls) was processed with each batch of samples. *Citrobacter freundii* and *Salmonella typhimurium* in low concentrations (5-10 cfu/100mL) were used as positive control. *E.coli* was used as negative control (growth but no blackening). Sterile distilled water was used as a blank. The blank was used as a benchmark to compare the extent of colour change in test samples and to ensure that sample bottles and H_2S paper strips had been properly sterilised prior to use.

3.3 RESULTS AND DISCUSSION

A total of 244 samples were included in the assessment of the H_2S paper strip medium. Various types of water samples, ranging from uncontaminated drinking water samples to contaminated environmental samples were collected and tested using the H_2S paper strip medium. The sample range included the following: treated water (20) surface waters (71), bore well waters (72), roof (tank) water samples (70) and geothermal well waters (11). A summary of results of tests performed on these samples are shown in Table 1.

Table 1: Comparison of the H_2S strip method and the Colilert® MPN method

Sample Type	N° of Samples Tested	Colilert® Method	H_2S Method – positive samples

		Positive samples		Negative samples	Ambient		20 °C		25 °C		30 °C		35 °C	
		TC*	E.coli		24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
Surface water	71	71	54	0	6	61	3	58	20	61	56	64	57	62
Bore Water	72	55	29	17	0	37	0	34	13	42	35	45	39	42
Tank (Roof) Water	70	50	29	20	3	17	0	14	2	30	20	40	28	35
Treated Water	20	2	0	18	0	0	0	0	0	0	0	2	0	1
Geothermal Water	11	5	1	6	0	0	0	0	0	0	5	5	5	5
TOTAL	244	183	113	61	9	115	3	106	35	133	116	156	129	145
% (all)	100	75	46	25	4	47	1	43	14	55	48	64	53	59
% (TC positive)		100	61	0	5	63	1	57	19	73	64	85	71	79
* TC = total coliforms														

Approximately 75% of the samples analysed in this study (183 samples) contained total coliforms, and nearly a third of them contained high concentrations (>100cfu/100mL). Of the 244 samples analysed, approximately half (113 samples) contained *E.coli* bacteria so the water collected from untreated water sources would not comply with the NZ Drinking Water Standards 2000 (MoH, 2000).

Out of 244 samples tested, 157 samples were positive by both the H_2S and total coliforms test and 60 samples were negative by both techniques. There were 26 samples, which were positive for total coliforms but were negative for the H_2S test and 1 sample, which was coliform-negative, but the H_2S-positive (Tables 1 and 2).

Table 2: Comparison of H_2S method and Colilert® MPN method

	Positive samples						Negative samples		Total		
	Colilert: *E.coli*	H_2S method 30-35 °C		Colilert: Total Coliforms	H_2S method 30-35 °C		Colilert	H_2S method 30-35 °C	Colilert	H_2S method 30-35 °C	
		24 hrs	48 hrs		24 hrs	48 hrs		48 hrs		24 hrs	48 hrs
N° of Samples	113	103	110	183	129	156	61	60	244	181	210
Agreement (%)		91	97		71	85		102		71	86

88 % of samples which were H_2S-negative, but total coliform-positive, contained low concentrations of coliforms (1-20 MPN/100mL) and 3 H_2S-negative samples contained 30-55 MPN/100mL, see Table 3. 86 % of the samples tested showed similar positive and negative responses in the H_2S paper strip test and the MPN Colilert® method, see Table 2. If the H_2S paper strip test was to be used alone, 84.8% of the contaminated water samples would be identified, in comparison to 99.4% if the total coliforms MPN procedure was used.

When the average total coliforms was more than 20 MPN/100mL, the H_2S test showed 98% agreement with total coliforms results. Similar results were obtained by various investigators who had tested the H_2S method in different tropical and temperate regions, including Indonesia, Peru, India, Chile, Malaysia, Nepal and South Africa (Ratto et al., 1989; Kromoredjo and Fujioka, 1991; Rijal and Fujioka, 1998; Manja et al., 1982; Manja et al., 2001; Kaspar et al., 1992; Castillo et al., 1994; Venkobachar et al., 1994; Martins et al., 1997; Genthe and Franck, 1999).

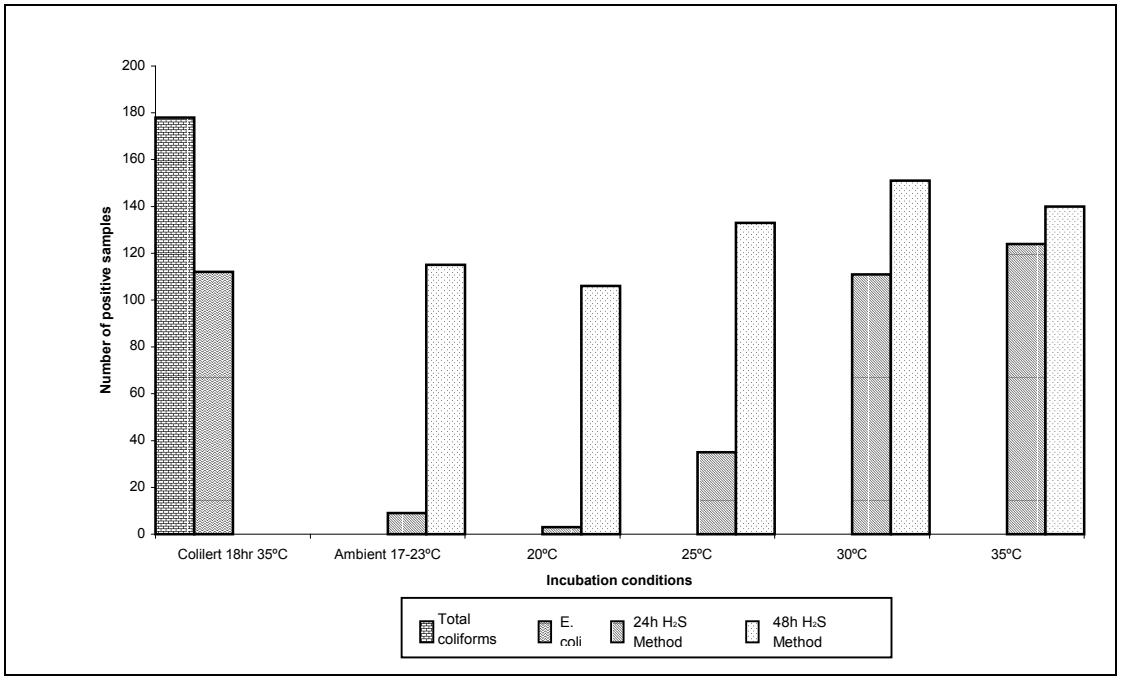
Table 3: Negative results by the H_2S method but positive by the MPN Colilert® Method

Number of Samples	Colilert® - Total Coliform Ranges (MPN/100 mL)		
	1-10	11-20	30-55
26	17	6	3
100%	65%	23%	12%

Excellent agreement (97%) was found between the H_2S test and *E. coli* MPN Colilert® method. Most of *E .coli* positive samples (91.1%) turned black in less than 24 hours incubation at 30-35 ^{0}C.

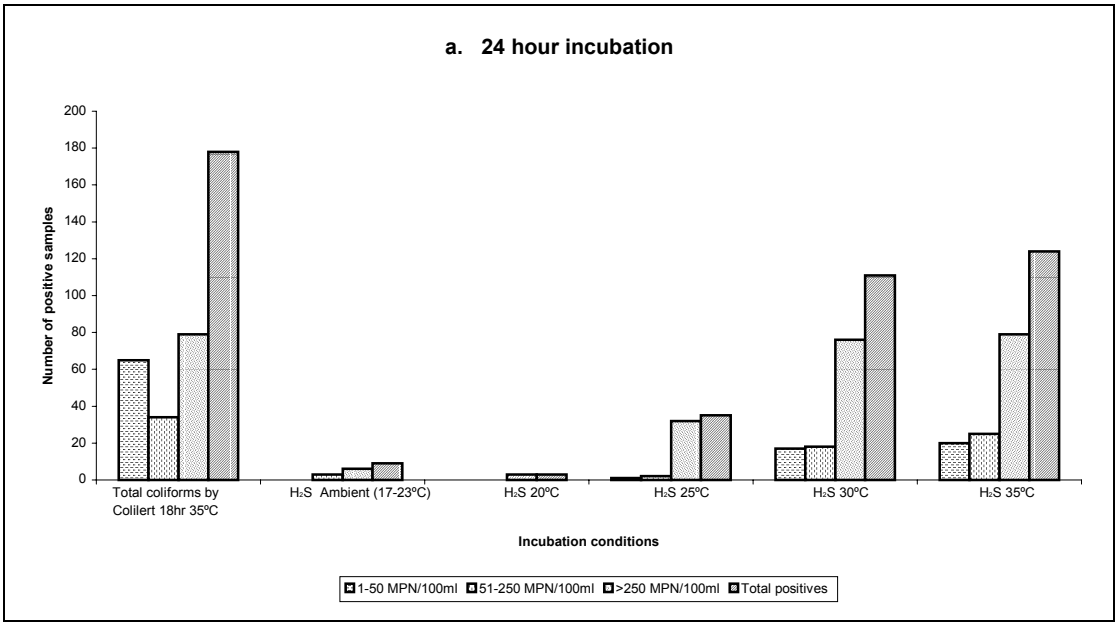
As illustrated in Figure 1, 48 hours incubation period was found to be optimal for H_2S test. After 24 hours incubation at lower ranges of temperatures (17-25 ^{0}C), only 1.7-19.6% of the contaminated samples were showing a positive H_2S reaction (usually slightly black). At higher temperatures (30-35 ^{0}C), 62.3-69.7% of the contaminated samples were H_2S-positive after 24 hours incubation.

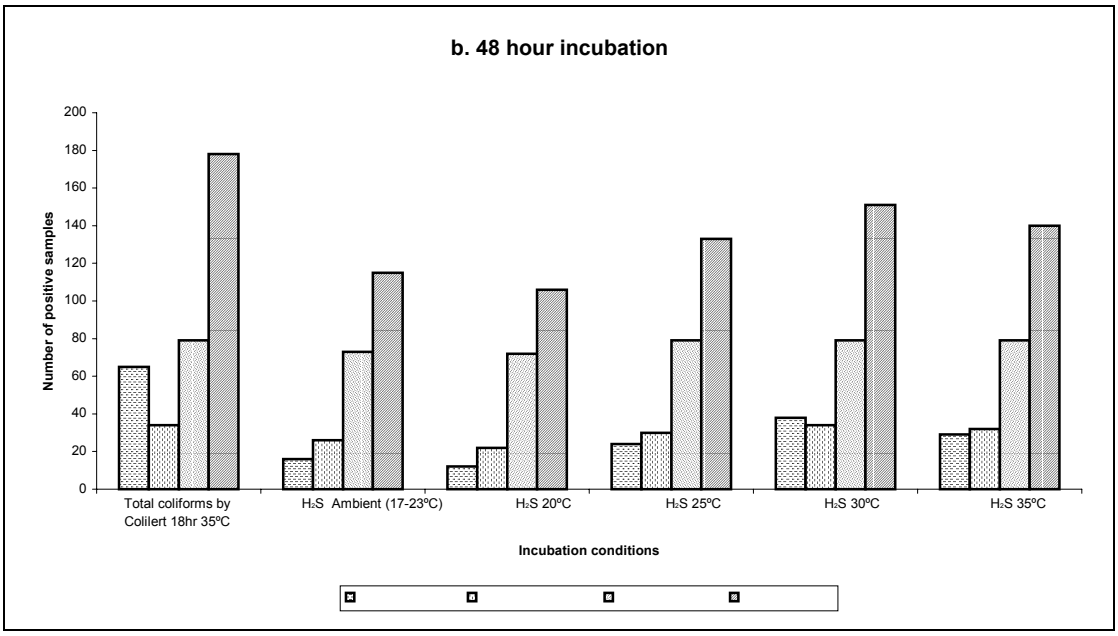
Figure 1: Comparison of numbers of positive samples for the Colilert® test and the H_2S strip test at different incubation times and temperatures



The number of H_2S positive reactions significantly increased (up to 84.8% at 30^{0}C) when the incubation period was extended to 48 hours. This indicates that at lower temperatures growth of H_2S producers was slower and therefore H_2S production was delayed. This observation was more pronounced at lower concentration of coliforms. They seemed to require a longer period to obtain H_2S positive results. At all temperatures, the incubation period required to display a positive reaction increased with a decrease in the coliform concentration (Figure 2). At lower concentrations and at lower temperatures the black colour only appeared as small patches at the bottom of the bottles and did not extend to the entire water sample. This could be due to the restricted growth of the H_2S producing bacteria because of low numbers or low temperatures.

Figure 1: Relationship between coliform concentrations and numbers of H_2S positive results





Pillai et al. (1999) noted that if the temperature was between 28-44^{0}C, blackening could be obtained within 48 hrs while at 22^{0}C it could take up to 90 hours for the same sample. Their results are in good agreement with this study. However, Genthe and Franck (1999) reported that the results of the H_2S strip test best correlated with indicator organisms when left to incubate for 48 hrs at 22^{0}C. Other workers (Castillo et al., 1994; Ratto et al., 1989 and Kasper et al., 1992) concluded that effectiveness of the method was independent of the temperature but most of them incubated their samples at higher temperatures (25-35^{0}C).

A total of 37 bacterial species were isolated from the positive H_2S bottles (Appendix 1f). Most of them belonged to the *Enterobacteriaceae* family. *E. coli* (58 isolates), *Klebsiella* spp (64), *Enterobacter* spp. (32), *Proteus* spp. (29), *Citrobacter freundii* (22) and *Serratia* spp. (23) were the most common coliforms detected.

58 of the contaminated samples (37%) were also positive for *Clostridium* spp. It would appear that clostridia might play a role equivalent to that of the coliform organisms in producing a positive H_2S paper strip reaction. More than half of *Clostridium* spp. isolates was found in surface water samples.

A small number of pathogenic bacteria such us *Salmonella* spp. (8 samples) and *Yersinia* spp. (3 samples) were isolated from the positive H_2S bottles. This indicates that the H_2S paper strip test may be an indicator of bacterial pathogen contamination.

Another significant group of bacteria isolated from the positive H_2S bottles were Gram negative oxidase positive bacteria: *Aeromonas hydrophila* (35 isolates*)* and *Pseudomonas* spp.

Several investigators (Castillo et al., 1994; Ratto et al., 1989; Nagaraju and Sastri, 1999) found a large variety of bacteria, primary *Enterobacteriaceae* and *Clostridium perfringens*, in samples giving a positive reaction in the H_2S test: *Enterobacter,* clostridia*, Citrobacter freundii, Klebsiella, Escherichia, Salmonella, Acinetobacter, Aeromonas, Morganella, Proteus, Hafnia* etc.

4 STUDY 2: COMPARISON OF H_2S PAPER STRIP TEST WITH A SELECTION OF ALTERNATIVE FAECAL INDICATORS

4.1 PURPOSE

Following a recent trend towards the use of 'alternative' indicator organisms the study was extended to evaluate a wider range of faecal indicators including sulphite reducing clostridia, aerobic spores (*Bacillus* spores), heterotrophic bacteria and F-specific bacteriophages, when compared with the H_2S paper strip method. As for Study 1, H_2S strips were incubated at various temperatures but for 48 hours only. In addition, the samples were analysed for the following tests: total coliforms and *E.coli* (MPN Colilert® method), sulphite reducing clostridia, aerobic spore count, heterotrophic plate count at 35°C and F-specific bacteriophages.

4.2 EXPERIMENTAL PROCEDURE

A total of 58 samples, comprised of surface water (15), tank water (16), bore water (17) and geothermal water samples (10) were analysed. The summary of the results is presented in Table 4. The number of chemical tests were also performed on those 10 geothermal water samples.

4.3 RESULTS AND DISCUSSION

Out of 58 samples tested, 46 were positive in both H_2S and total coliform tests and 8 were negative in both tests. There were also 4 samples that were positive for total coliforms but negative in the H_2S method. Even when the total coliforms numbers were low (1-20 MPN/100mL) the H_2S test displayed a very good correlation with the total coliform test.

Table 4: Summary of results of comparison of H_2S strip test with alternative faecal indicators

Sample Type	Result category	Surface water	Tank water	Bore water	Geothermal water	Total
Number of samples		15	16	17	10	58
H_2S test	+ve	15	13	11	7	46
	-ve	0	3	6	3	12

Total coliforms	+ve	15	15	12	8	50
MPN/100 mL	-ve	0	1	5	2	8
E.coli	+ve	11	8	7	7	33
MPN/100 mL	-ve	4	8	10	3	25
Sulphite reducing clostridia	1-50	4	5	2	4	15
Cfu/100mL	51-330	10	4	0	4	18
	<1	1	7	15	2	25
Bacillus spores cfu/100mL	1-1000	4	13	5	3	25
	1001-4900	11	3	8	7	29
	<1	0	0	4	0	4
Bacteriophages	10-100	7	6	12	5	30
Pfu/100L	101-960	8	0	0	1	9
	<10	0	10	5	4	19
Heterotrophic Plate	1-200	3	8	14	5	30
Count cfu/mL	201-26000	12	8	2	4	26
	<1	0	0	1	1	2

Excellent agreement was found between the H_2S test and *E. coli* MPN Colilert® method as well as between the H_2S test and sulphite reducing clostridia method. Nearly all *E .coli* and/or sulphite reducing clostridia-positive samples were also positive in the H_2S method. All these samples turned black after only 24 hrs of incubation at 30-35^{o}C even when the samples contained very low numbers (1-20 per 100mL) of *E. coli* or clostridia.

The H_2S test detects bacteria other than coliforms associated with faecal contamination, such as *Clostridium perfringens* which is one of the more resistant indicators of faecal contamination and can still be found when coliforms are no longer present (Sobsey and Pfaender, 2002). Grant and Ziel (1996) have found a strong agreement between the H_2S paper strip method and clostridia spore enumeration. The H_2S test produced about 10% more positive samples than the coliform test because it included samples that were positive only for clostridia (Castillo et al., 1994).

Bacillus spore count, heterotrophic plate count and F- specific bacteriophage count were found to be of no use as indicators of faecal contamination in water. Their correlation with the H_2S method was poor. A satisfactory agreement between the H_2S method and these three tests was found only for heavily contaminated samples. However, the samples testing positive also contained high numbers of total coliforms, *E.coli* and/or sulphite reducing clostridia and the positive reaction was most probably caused by these organisms. Similar results were reported by Genthe and Franck (1999). They observed poor correlations between the heterotrophic plate count and the H_2S test.

A number of samples that contained relatively high numbers of *Bacillus* spores and heterotrophic bacteria were found to be negative for total coliforms, *E .coli*, sulphite reducing clostridia and in the H_2S method. 97% of samples tested positive for heterotrophic bacteria and 93% for *Bacillus* spores while only 79% samples were found positive by the H_2S method.

A good agreement was found between F-specific bacteriophages and the H_2S method for surface water and bore water samples. A comparison with tank water was not feasible as only a few tank water samples contained bacteriophages (and at very low numbers). Most of the tank water samples that tested positive in the H_2S method were negative for bacteriophages.

The studies of Martins et al., 1997 and Castillo et al., 1994 have indicated that both the H_2S paper strip test and bacteriophage test are viable indicators of potable water quality and potable water treatment.

5 STUDY 3: COMPARISON OF H_2S PAPER STRIP METHOD WITH THE PRESENCE OF PATHOGENS

5.1 PURPOSE

The purpose of testing for indicator organisms in drinking water is to detect the risk of disease to the consumers. For this reason a limited study was carried out to relate the presence of a number of pathogens to the results from the H_2S strip test.

5.2 EXPERIMENTAL PROCEDURE

10 samples were analysed to compare the performance of the H_2S paper strip method with the presence of pathogens.

A total of 10 samples, comprised of surface water (4), tank water (3) and bore water (3) were tested tested for bacterial (*Campylobacter, Salmonella*) and viral (Enteroviruses, Adenoviruses, Noroviruses, Hepatitis A) pathogens as well as the alternative faecal indicator organisms tested in Study 2 and the H_2S paper strip method. . Only samples, which were expected to have high numbers of microorganisms, were used.

5.3 RESULTS AND DISCUSSION

All four surface water samples contained high numbers of total coliforms, *E .coli*, sulphite reducing clostridia, *Bacillus* spores, heterotrophic bacteria and bacteriophages. They were also highly positive in the H_2S method. Three of the four surface samples were found to be positive for Noroviruses, Adenoviruses, Enteroviruses, and/or *Campylobacter* and *Salmonella*. The fourth sample contained high numbers of faecal indicator bacteria but was found to be free of pathogenic organisms. All 4 samples were collected from small dams which are used as raw water supplies for producing treated drinking water for small towns.

Results of Gawthorne et al. (1996) show that H_2S test can indicate the presence of salmonellae. Their recommended length of incubation for a negative result is 48 hours at 35^{o}C to exclude the possibility of slow-growing or injured bacteria in the sample.

However, three samples that were supposed to be very "clean" because they contained very low numbers or were totally free of faecal indicator bacteria and also were negative (or weakly positive) in the H_2S test, were found to harbour pathogens. Particularly worrying was to find Noroviruses and Salmonella in bore and tank water samples that tested negative in both *E. coli* and H_2S tests. Two of these Norovirus positive samples were collected from the tanks of people who complained about chronic unexplainable diarrhoea. Both these water samples were *E. coli* free so they were meeting the criteria of NZ Drinking Water Standards.

The results of this trial confirm the known fact that high levels of faecal bacteria may increase the chances of finding pathogens but it cannot be guaranteed that water free of faecal indicator bacteria is also free of pathogens (particularly viruses).

In summary, the H_2S method has shown good potential to indicate the presence of pathogenic bacteria and viruses - six out of seven H_2S-positive samples were also found to be positive for one or more pathogens.

6 STUDY 4: EVALUATION OF POTENTIAL INTERFERENCES WITH THE H_2S STRIP TEST

6.1 PURPOSE

Geothermal waters contain hydrogen sulphide and other chemicals, which might interfere with the H2S, strip test by producing false positive or negative results. Manganese was also considered to be a possible source of interference.

6.2 EXPERIMENTAL PROCEDURE

6.2.1 INITIAL STUDY

A total of eleven samples were collected. Ten samples were collected from geothermal springs, bores, shallow geothermal pools, lakes or streams. All had a strong or very strong smell of H_2S. One treated water sample was collected from the reticulation system in a geothermal region, to serve as a control.

Five of the samples were collected from the hot springs or bores with water temperature approximately40-70^{0}C, and 6 samples from their outlets (temperature approximately 30-40^{0}C) or from shallow open pools (at ambient temperature). Additional samples were collected in separate bottles (with preservative) for sulphide analysis. Samples were tested by the H_2S paper strip test and the MPN Colilert® method.

Only samples taken straight from the hot springs contained high amount of sulphide (0.15-0.5mg/L). No sulphide was found in the samples taken from the bore outlets. Full agreement (100%) was found between total coliforms and the H_2S test results for all 11 samples. High content of sulphide did not interfere with the H_2S method and no false positive results were produced.

6.2.2 CHEMICAL CHARACTERIZATION

In a second part of the study, an additional ten geothermal water samples were collected to determine their chemical composition. Nine samples were collected from geothermal springs, bores, shallow geothermal pools, lakes or streams. All had a strong or very strong smell of H_2S. One treated water sample was collected from the reticulation system in a geothermal region to serve as a control.

Samples were analysed for total coliforms and *E.coli* (MPN Colilert method), sulphite reducing clostridia, aerobic spore count, heterotrophic plate count at 35^{o}C and F-specific bacteriophages. Chemical analyses were arsenic, boron, iron, dissolved oxygen, manganese, pH, sulphide and total dissolved solids. In the H_2S-strip test, all samples were incubated for 1 hour at appropriate temperatures and examined for early blackening.

6.2.3 MANGANESE INTERFERENCE

Raw waters with elevated manganese level are rare in New Zealand. Therefore samples with elevated levels of manganese had to be artificially created. 8 water samples were spiked with 0.5-1.5mg/L of manganese. Two samples with naturally elevated level of manganese were also tested in this trial.

6.3 RESULTS AND DISCUSSION

Most of the samples contained high levels of one or more of the following chemicals: arsenic, sulphide, boron, iron and manganese. Three samples had a very low pH (3-3.3).

In the H_2S-strip test, all samples were incubated for 1 hour at appropriate temperatures and examined for early blackening to evaluate the effect of naturally occurring H_2S. No samples changed colour to black after 1 hour of incubation.

A good comparison was found between the H_2S method and the total coliforms, *E. coli* and sulphite reducing clostridia tests in all 10 samples.

Ten samples containing high level of manganese in the water were tested by the H_2S paper strip and MPN Colilert® methods. Only two samples were collected from naturally contaminated source, the remaining samples (8) were spiked with 0.5-1.5 mg/L of manganese. Full agreement (100%) was found between total coliforms/*E. coli* tests and the H_2S paper strip method. High content of manganese did not interfere with the H_2S test results and no false positive or false negative results were produced (see Appendix 4b).

7 STUDY 1: STUDY 5: SPIKING TRIAL

7.1 PURPOSE

This study was carried out to determine the response of the H_2S strip media to known organisms in predetermined inoculum ranges. The sensitivity of the method was tested with low level inocula.

7.2 EXPERIMENTAL PROCEDURE

The following 14 reference strains of potential sulphur metabolising bacteria (common in New Zealand) were tested in this trial: *Citrobacter freundii* NZRM 982 , *Proteus vulgaris* NZRM 67, *Morganella morganii* NZRM 65, *Clostridium perfringens* NZRM 20, *Klebsiella pneumoniae spp. pneumoniae* NZRM 482, *Klebsiella ozonae* 2104, *Enterobacter aerogenes* NZRM 798, *Escherichia coli* NZRM 916, *Salmonella typhimurium* NZRM 1138, *Yersinia enterocolitica* NZRM 2603, *Campylobacter jejuni* NZRM 2397, *Acinetobacter lwoffii* NZRM 2581, *Aeromonas hydrophila* NZRM 804 and *Pseudomonas aeruginosa* NZRM 918. All bacterial reference strains were purchased from ESR, Wellington.

For the preparation of the inoculum, most of the bacteria were inoculated onto Tryptose Soy Agar (TSA). *Campylobacter jejuni* was streaked onto *Campylobacter* Isolation Blood-free Agar and *Clostridium perfringens* onto Columbia Blood Agar and incubated under microaeophilic *(Campylobacter*) and anaerobic (*Clostridium*) conditions. The TSA agar plates were incubated for 24 hours at 35 ± 0.5^0C aerobically. Next, the cells were harvested with 0.1% peptone saline diluent and then diluted with the same liquid. This procedure provided inocula of approximately 11-30 cfu/100mL (low inoculum level) and 100-210 cfu/100mL (high inoculum level) for spiking of the 100 mL aliquots of sterile distilled water.

All spiked samples were processed according to the same procedure as naturally contaminated samples.

HACH PathoScreen® Medium was used as a reference method for all spiked samples tested. The same spike levels were used for inoculation of the HACH® medium bottles. They were processed according to manufacturer's instruction, i.e. incubated at 30^0C for 48 hours.

PathoScreen® Medium is commercially produced by HACH®. Its composition is the same as the H_2S paper strip test prepared for this trial. It is dehydrated, sterilised, and packaged in powder pillows, which are added to the 100mL aliquots of water samples.

In addition to reference strain testing, a small spiking trial was conducted on 20 bacteria strains, which were isolated from positive samples. These spiked samples were processed according to the same procedure as for the reference strains.

Another spiking trial was conducted to establish the minimum number of organisms that the strip test was capable of detecting. Known concentrations (3-120cfu/100mL) of the H_2S producers: *Citrobacter freundii, Proteus vulgaris* and *Salmonella typhimurium* were added to samples used in the H_2S strip test. These spiked samples were processed according to the same procedure as for reference strains.

7.3 RESULTS AND DISCUSSION

A spiking study was conducted to determine the minimum number of cells of hydrogen sulphide-producing bacteria required to create a positive reaction in 100 mL volumes of H_2S medium. The inocula of 3-5 cells of *Salmonella typhimurium, Citrobacter freundii* and *Proteus vulgaris* produced a positive reaction in 3 out of 3 replicates by 24 hours at 30-35 ^{0}C.

The H_2S medium did not display tendency to give false positive reactions when inoculated with cultures not typically regarded as H_2S producers. When inoculated with low and high levels of *E .coli, Enterobacter aerogenes, Klebsiella pneumoniae, Acinetobacter lwoffii, Pseudomonas aeruginosa* or *Yersinia enterocolitica*, no blackening of the medium occurred during incubation for 48 hours.

A very good agreement (98%) was observed for the Watercare Laboratory Services' H_2S medium and HACH® PathoScreen H_2S medium both for samples spiked with reference cultures and for bacterial isolates from naturally contaminated samples.

8 STUDY 6: H_2S PAPER STRIP KIT PRODUCTION TRIAL

8.1 PURPOSE

This trial was to determine the cheapest and easiest way of producing the H_2S paper strip test kit. The following production issues were considered in this trial: type of the paper strip, preparation and dispensing of medium

concentrate, practical ways of drying of paper strips saturated with the H_2S medium, sterilising and packaging of dried H_2S paper strips, quality controls including sterility, and performance tests. Five types of paper strips ranging from paper tissues to microbiological filter pads were assessed for their suitability for H_2S medium concentrate saturation, drying and packaging.

8.2 EXPERIMENTAL PROCEDURE

The following issues were considered during the H_2S paper strip production trial:

- type of paper used for saturation with the H_2S medium concentrate
- preparation of medium concentrate and its dispensing
- drying of saturated paper strips at different temperatures
- packaging, sterilising and storage of produced paper strips
- costs of production of the H_2S paper strips in the lab in comparison to commercially produced kits.

Five types of paper strips including ordinary paper towels, filter papers and microbiological absorbent pads (1.5 mm thick - Sartorius® and 0.8 mm thick – Millipore®) were used for saturation with the H_2S medium concentrate. The paper strips saturated with the medium were placed onto trays and dried at room temperature, at 50^0C or at 70^0C.

After drying they were placed separately into the small paper bags and sterilised in an autoclave. They were then stored at room temperature until used.

All types of paper strips worked well but the most convenient to use were 47 mm diameter thick (1.5 mm) microbiological absorbent pads from Sartorius®. These pads were chosen and used for the whole project. They were easy to saturate with the medium and only one pad was needed per 100mL samples. The blackenings of these pads were brighter and easier to read then on other paper strips especially after the 24 hour incubation period. The most convenient method of drying was to leave the saturated paper strips on the bench overnight at room temperature. Next morning the strips were ready for packing and autoclaving. The sterilised saturated thick pads were stored at room temperature to assess their performance after different storage times. Every two months, 20 H_2S paper strips were used for testing of water samples inoculated with low level (5-20cfu) of H_2S-producing bacteria *Salmonella* and *Citrobacter freundii*. The performance of H_2S paper strips prepared in the lab was excellent. Even after 8 months, a 100% of them gave positive results and there were no problems with their sterility.

The estimated paper strip production cost in the lab was low, below $1.5 per sample. The costs included materials and labour but not the cost of preparing or buying the sterile bottle. The sterile plastic containers (120mL) can be purchased from local laboratory suppliers for 50-60 cents each.

8.3 RESULTS AND DISCUSSION

At the moment, there are at least four brands of the H_2S kit available on the market. The most often used are two modified versions of the product: HACH®' PathoScreen Medium (HACH®, 2002) and LTEK® Bacto-H_2S kit (LTEK®, 2003). In this modified version, instead of absorbing the media on a strip of paper the medium is contained in a sachet (HACH®) or a hand-breakable glass ampoule, sealed and sterilised. The manufacturers guarantee at least one year shelf life for the product when stored in a cool, dry place. Both kits are very easy to use. The HACH® medium used in this trial worked very well. Commercial kits are usually available in two versions: presence/absence per 100mL kit and MPN per 20mL kit.

In New Zealand, with the fluctuating currency exchange and very low usage of these products, they are more expensive than the good quality, reliable, rapid test kits such as Colilert® or Readycult® that are commonly used for testing of drinking water supplies for coliforms and *E .coli*.

To compete with those easy to use products a reputable local supplier that would guarantee a good quality product should produce the H_2S paper strip kit.

9 CONCLUSIONS

Data summarised above indicate that the H_2S paper strip test and coliform MPN Colilert® test were equally effective in detection of bacterial contamination in water samples.

In particular, an excellent agreement (97.3%) was found between the H_2S test and *E. coli* MPN Colilert® method.

The results of this study indicate that for the H_2S test the incubation temperature in the range of 25-35^{0}C and the incubation period of 48 hours are critical. New Zealand ambient temperatures are often lower than 20^{0}C so an incubator is essential to conduct this test.

The method does not detect viruses. However, The H_2S test detects bacteria other than coliforms that are associated with faecal contamination, including *Clostridium perfringens* and *Salmonella*.

The H_2S test is a sensitive, simple and inexpensive procedure for screening of water supplies for potential faecal contamination. However, in New Zealand conditions, it does require incubation at a controlled temperature for 48 hours to maximise performance. This means that a controlled temperature incubator with the associated temperature calibration and monitoring procedures is required. It also means that results would not be available until 30 hours later than those obtained by Colilert® 18 hour or 24 hours later than those obtained if the alternative Colilert® 24 hour test was used.

The cost of the Colilert® media is more expensive than preparation of H_2S strips and it is necessary to purchase a uv light to read *E. coli*. However, there are other enzyme substrate tests which are comparable in price or perhaps even cheaper than preparing and marketing H_2S strips. The advantage of these tests is that a confirmed result from an internationally accepted method is obtained 24 hours earlier than would be obtained from the H_2S strip method.

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REFERENCES

APHA (1998) *Standard Methods for the Examination of Water and Wastewater* Enzyme substrate coliform test. Method 9223, APHA 20th Ed., AWWA.

Castillo, G., Duarte, R., Ruiz, Z., Marucic, M.T., Honorato, B., Mercado, R., Coloma, V., Lorca, V., Martins, M.T. and Dutka, B.J. (1994) 'Evaluation of disinfected and untreated drinking water supplies in Chile by the H_2S paper strip test' *Wat. Res*. 28, 8: 1765-1770.

Desmarchelier, P., Lew, P., Caique, W., Knight, S., Toodayan, W., Isa, A.R. and Barnes, B. (1992) 'An evaluation of the hydrogen sulphide water screening test and coliform counts for water assessment in rural Malaysia' *Trans. R. Soc. Trop. Med. Hyg*. 86: 448-450.

Dutka, B.J. and El-Shaarawi, A.H. (1990) 'Use of a simple inexpensive microbial water quality test: results of three continent, eight country research project' *IDRC*, Report IDRC-MR247e, Jan 2000

Forget, G. (1994) *Community-based water quality monitoring for remote communities*. IDRC

Gawthorne, T., Gibbs, R.A., Mathew, K. and Ho, G.E. (1996) 'H_2S papers as presumptive tests for Salmonella contamination in tropical drinking water' *Wat. Sci. Tech*., 34, 7-8: 187-194.

Genthe, B. and Franck, M. (2001) 'A field test for assessing the microbial quality of water: an H_2S strip test' *Wat. Sci. Tech*., 36, 8:183-190.

Grant, M.A. and Ziel, C.A. (1996) 'Evaluation of simple screening test for faecal pollution in water' *J.Water SRT-Aqua*, 45, 1:13-18.

IDRC (2001) *Testing the waters: Split Lake Study*

HACH's Analytical Procedures (2002) Bacteria: Hydrogen Sulphide Producing. Method 10032, 1-12.

Jangi, M.S., Leong, L.C. and Ho, P.Y.C. (2000) 'Development of a simple test for the bacteriological quality of drinking water and water classification' Malaysian Centre File:3-p-83-0317-02, IDRC

Kaspar, P., Guillen, I., Rivelli, D., Valasquez, G., De Kaspar, H.M., Pozzoli ,L., Numez ,C. and Zoulek, G. (1992) 'Evaluation of the simple screening test for the quality of drinking water systems' *Tropical Medicine and Parasitology*, 43, 2: 124-127.

Kromoredjo, P. and Fujioka, R. (1991) 'Evaluating three simple methods to assess microbial quality of drinking water in Indonesia' *Environ. Tox. and Wat. Qual. J.*, 6: 259-270.

LTEK's Analytical Procedures (2003) Bacto H_2S kit.

Manja, K.S. (2001) *Report of R&D study on H_2S test for drinking water*. Rajv Gandhi National Drinking Water Mission, New Delhi – 110 003, 92pp.

Manja, K.S., Maurya, M.S. and Rao, K.M. (1982) 'A simple field test for the detection of faecal pollution in drinking water' *Bull WHO* 60:797-801.

Martins, M.T., Castillo, G. and Dutka, D.J. (1997) 'Evaluation of drinking water treatment plant efficiency in microorganisms removal by the coliphage, total coliform and H_2S paper strip tests' *Wat. Sci.Tech*., 35, 11-12:403-407.

MOH (2000) *Drinking-water standards for New Zealand 2000*. MOH:Wellington

Nagaraju, D. and Nastri, J.C.V. (1999) 'Confirmed faecal pollution to bore well waters of Mysore city' *Environmental Geology*, 38, 4: 322-326.

Pillai, J., Mathew, K., Gibbs, R. and Ho, G.E. (1999) 'H_2S paper strip method – a bacteriological test for faecal coliforms in drinking water at various temperatures' *Wat. Sci. Tech.*, 40, 2: 85-90.

RADG Projects (2003) *Bacteriological water test method: the Hydrogen Sulphide method (H_2S method)*. RADG.

Ratto, A., Dutka ,B.J., Vega, C., Lopez, C. and El-Shaarawi, A. (1989) 'Potable water safety assessed by coliphage and bacterial tests' *Water Research*, 23: 253-255.

Rijal, G.K., Fujioka R.S. and Ziel, C.A. (2000) E'valuation of the hydrogen sulphide bacteria test: A simple test to determine the hygienic quality of drinking water' *Abstracts of the General Meeting of the American Society of Microbiology*, Abstract Q354. page 622. Amer. Soc. Microbiol., Washington, DC.

Sobsey, M.D. and Pfaender, F.K. (2002) 'Evaluation of the H_2S Method for detection of faecal contamination of drinking water' *Water and Sanitation*, WHO/Sde/WSH/02.08, Geneva.

Venkobachar, C., Kumar, D., Talreja, K., Kumar, A. and Iyengar, L. (1994) 'Assessment of bacteriological water quality using a modified H_2S strip test' *J Water SRT-Aqua*, 43, 6: 311-314.