



## Bacteriological water testing by H<sub>2</sub>S method



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WATERBORNE DISEASES ARE a global health problem. Prevention of such diseases is highly essential, since they spread fast affecting an entire community. Regular monitoring of the microbial quality of drinking water is an essential step in ensuring safe drinking water. It has been reported that human faecal contamination is one of the main causes of waterborne diseases. WHO (1993) therefore recommends regular testing of drinking water for thermotolerant coliforms and *Salmonella sp.* to ensure their complete absence. The standard methods that are used to test the bacterial quality of drinking water are the most probable number method and the membrane filtration technique. They are procedures which are time consuming, expensive and require laboratory and technical support. Field test kits such as the Colilert and the Colisure have also been developed. Although they are easy to use methods, the short shelf life at room temperature and the high cost prevent their use in routine testing of water. The frequent testing of drinking water in remote areas, as well as in developing countries, is therefore difficult to achieve. The fact that 30 per cent of the *Salmonella sp.* isolated from drinking water in Western Australia was in the absence of coliforms, shows the necessity of routine testing of drinking water for the presence of *Salmonella sp.* (Peterson and Schorsch, 1980). A lack of correlation between coliform bacteria and *Salmonella sp.* particularly in the tropical and subtropical waters has been demonstrated by Townsend (1992). The need for separate tests for coliforms and *Salmonella sp.* is another factor that restricts testing of the bacteriological water quality. The standard methods for detecting *Salmonella sp.* are expensive and a four step procedure taking 4-5 days to give the end result. In this situation an easy to use, inexpensive method that could test for both coliforms and *Salmonella sp.* could be very beneficial.

The hydrogen sulphide paper strip method developed by Manja *et al.* (1982) is an onsite microbial water testing method based on the detection of hydrogen sulphide producing bacteria. It is less expensive than the standard tests, easy to use and could be carried out by a local person after being trained. This method was found to have good correlation with the standard methods in detecting coliforms (Castillo *et al.*, 1994; Grant and Ziel, 1996). It was further noted that H<sub>2</sub>S production was associated with *Salmonella sp.* (Cowan, 1974) and that *Salmonella sp.* have been isolated from H<sub>2</sub>S positive bottles (Castillo *et al.*, 1994; Manja *et al.*, 1982). Later Gawthorne *et al.* (1996) confirmed that *Salmonella sp.* could be detected by the H<sub>2</sub>S

method. Although it was reported that the H<sub>2</sub>S method could also report the presence of *Salmonella sp.*, more details are still required as to the influence of temperature on the incubation period and whether the contamination level has any influence on the incubation period. It is also possible that the medium could be improved to decrease the incubation period. This paper therefore looks into the above three questions.

### Methods

#### Preparation of H<sub>2</sub>S bottles

H<sub>2</sub>S bottles were prepared according to Manja *et al.* (1982). The original H<sub>2</sub>S medium was prepared by dissolving peptone (20g), dipotassium hydrogen phosphate (1.5g), ferric ammonium citrate (0.75g), sodium thiosulfate (1g) and teepol (detergent)(1mL) in 50mL of tap water.

The addition of L-cystine and yeast extract was found to reduce the variability of growth of *Salmonella sp.* (Hu *et al.*, 1995). Therefore two other modifications to the original medium (M1) were tried in this study. Medium M2 contained 0.125g of L-cystine in addition to the other ingredients whereas in medium M3, 5g of yeast extract was added and the peptone was reduced to 15g in 50ml of tap water.

Aliquots of 5mL of the above medium were absorbed in folded tissue paper which were then placed in 100mL bottles and autoclaved. The bottles were then stored at room temperature.

#### Preparation of salmonella sp. inoculums

A pure culture of *Salmonella typhimurium* was inoculated in yeast extract broth and incubated at 37°C for 22 hours before making the required dilutions. Serial dilutions were prepared with sterile distilled water. In order to count the number of colony forming units (CFU) in each dilution, 0.1mL of each dilution was spread plated on yeast extract agar and incubated at 37°C for 22 hours. The colonies were counted and calculated as the number of CFU in 100mL. The experiment was repeated three times. The number of CFU/100ml was different for the three experiments at all dilutions. Therefore the CFU/100mL was represented as a range.

In the present study 100mL of the sample was used compared to the 20mL used in the original H<sub>2</sub>S method to make it compatible to the standard methods which test 100mL of water sample. The inoculums were pipetted into 100mL of sterilised distilled water which has been added to the H<sub>2</sub>S bottles and mixed. They were then incubated at

the required temperatures until the bottle turned black for a maximum of up to 120 hours (5 days). The first appearance of a black colour in the water was taken as the incubation period. The incubation periods for different concentrations at each temperature were noted.

*Examination of temperature range*

In order to study the temperature range at which the H<sub>2</sub>S method is efficient in detecting *Salmonella sp.* in drinking water, only the original medium M1 was used. A *Salmonella typhimurium* concentration of 60 CFU/100 mL was tested at different temperatures ranging from 0 to 47°C. The temperatures tested were 0, 8, 14, 22, 28, 37, 44, 47 and the room temperature which varied between 18-24°C. The bottles were examined after 18, 24, 36, 48, 60, 72, 84, 96, 108 and 120 hours.

*Effect of temperature and concentration on incubation period for different media*

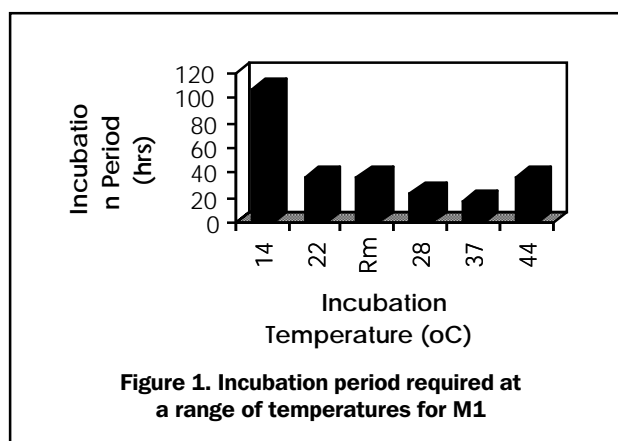
In order to study the influence of the incubation temperature and the concentration of *Salmonella sp.* on the three media, the incubation period at all the dilutions was determined at 14, 22, 28, 37, 44°C and the room temperature. The bottles were incubated for a maximum of five days (120 hours) after which the unchanged bottles were considered inefficient in detecting the presence of *Salmonella sp.*

**Results**

The range of inoculum concentrations for the inoculum volumes used in the experiments is shown in Table 1.

*Temperature range*

When a range of temperatures from 0 to 47°C were tested to study the range at which the H<sub>2</sub>S method was efficient, the bottles at 0, 8 and 47°C did not turn black even after 5 days. It was observed (Figure 1) that 37°C required the lowest incubation period of 18 hours whereas at 28°C, the bottles took 24 hours to turn black. The room temperature as well as 22 °C needed 36 hours for detecting the presence of *Salmonella typhimurium*. A higher temperature of 44°C also required 36 hours. The maximum incubation period was required at 14 °C which took 108 hours to confirm the presence of *Salmonella sp.* in the sample water.



**Figure 1. Incubation period required at a range of temperatures for M1**

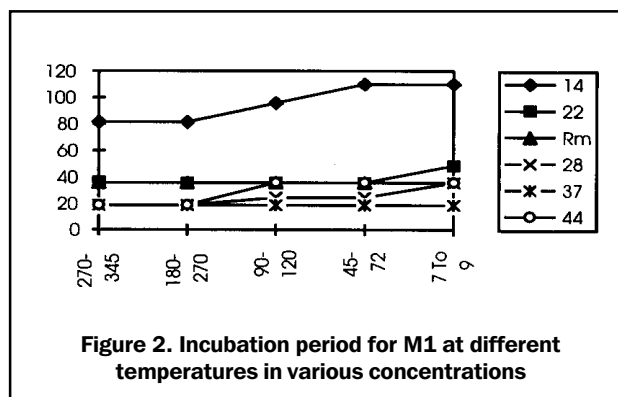
*Effect of temperature and concentration on incubation period for different media*

When the lowest dilution (2-4 CFU/100mL) was tested with the three media, many of the bottles did not turn black while some turned black at a normal time. Therefore the incubation period for the lowest dilution was not recorded. There was not much difference in the incubation period required for detecting *S. typhimurium* with the three media. However it was noticed that at extreme temperatures, the bottles with M2 turned black faster than with M3 and M1. The incubation period increased with the lowering of the inoculum concentration for all the three media at 44 and 14°C. At 44°C the maximum incubation period required at the lowest concentration for all the three media was 36 hours. The difference was noticed at the concentrations ranging from 45 - 120 CFU/100mL where M2 was faster followed by M3 and then M1. At 14°C the maximum period required for the three media was 108 hours at the lowest concentration and the minimum period, 84 hours. M2 was faster than the other two media at the concentrations of 45-120 CFU/100 mL.

At 37, 28, 22°C and the room temperature all the three media had the same incubation period for the range of inoculum concentrations. At 37°C all the media turned black at 18 hours irrespective of the concentration while at room temperature and at 22°C the incubation period required was 36 hours at all concentrations. At 28°C, all samples except the lowest concentration turned black

**Table 1. The number of CFU/100mL at different inoculum volumes**

Dilution	Inoculum volume (mL)	No. of CFU/100mL
10 <sup>-6</sup>	0.3	270-345
	0.2	180-240
	0.1	90-120
10 <sup>-8</sup>	1.0	45-72
	0.5	7-9
	0.2	2-4



**Figure 2. Incubation period for M1 at different temperatures in various concentrations**

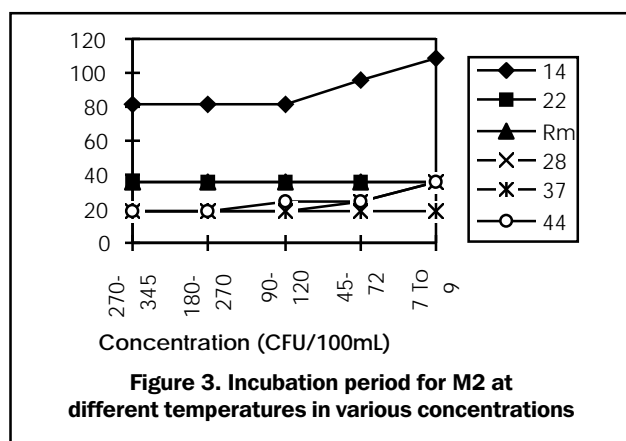


Figure 3. Incubation period for M2 at different temperatures in various concentrations

within 24 hours and the lowest concentration required 36 hours of incubation for all the three media.

The influence of concentration and temperature on the incubation period of M1, M2 and M3 are presented in Figures 2, 3 and 4

**Discussion**

The present experiments proved that the H<sub>2</sub>S method is capable of detecting the presence of *Salmonella typhimurium* in drinking water as also reported by Gawthorne *et al.* (1996). The identification of *Salmonella sp.* in the H<sub>2</sub>S positive bottles of natural samples by Castillo *et al.* (1994) and Manja *et al.* (1982) are an evidence that the H<sub>2</sub>S papers can detect the presence of *Salmonella sp.* in natural samples also. However the application of this test to salmonella detection is limited by the fact that only 92 per cent of the *Salmonella sp.* produce hydrogen sulphide (Jay and Davey, 1989). According to Gawthorne *et al.* (1996) a positive result could be obtained for the H<sub>2</sub>S test even when the inoculum concentration of *Salmonella typhimurium* was less than 5 CFU/100mL. In the present study, many of the H<sub>2</sub>S bottles having the lowest concentration of 1 CFU/100mL turned black at a normal time while some remained unchanged. It was therefore concluded that those bottles would have missed out on inoculation because of the very low number and that the H<sub>2</sub>S method was able to detect *Salmonella sp.* to the lowest

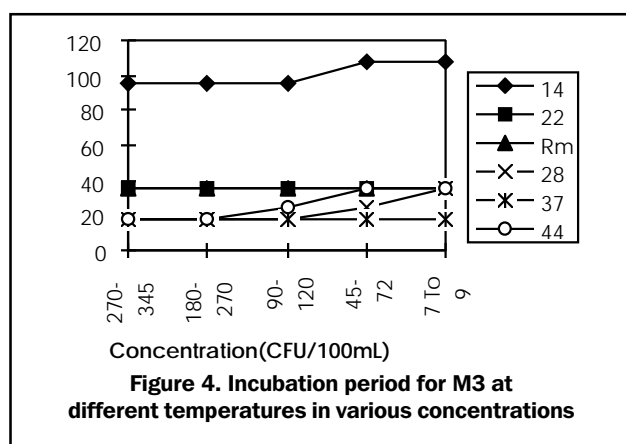


Figure 4. Incubation period for M3 at different temperatures in various concentrations

concentration of 1 CFU/100 mL. Grant and Ziel (1996) also found that a positive reaction could be obtained when *S. typhi* concentration was ~1 cell in 100 mL.

Temperature plays an important role in the growth of microorganisms. Figure 1 showed that the incubation temperature had a major influence on the blackening time of the H<sub>2</sub>S method with *Salmonella sp.* Most of the previous studies were either carried out at room temperature (Manja *et al.*, 1982) or at 37 °C. No details were available as to the temperature range at which the H<sub>2</sub>S method would be effective for detecting *S. typhimurium*. The minimum, optimum and the maximum temperature and the range of temperature over which growth is possible vary widely among bacteria. In the present study it was noted that a temperature range of 14 - 44 °C was effective for the H<sub>2</sub>S method in detecting *S. typhimurium*. The optimum temperature was 37°C where only 18 hours was required even for the lowest concentrations while at all other temperatures between 22 °C to 44 °C, the incubation period was 36 to 48 hours. The blackening time increased with the increase or decrease of the temperature from the optimum temperature. The maximum temperature for H<sub>2</sub>S production was 44°C and growth did not occur at 47°C and below 14°C. At 14°C, 108 hours was needed to report the presence of *Salmonella sp.*. It was also noted that at low and high temperatures the incubation period increased with a decrease in concentration. This showed that the growth of *Salmonella sp.* was slowed down at the extreme temperatures and that the H<sub>2</sub>S production was delayed. Gawthorne *et al.* (1996) also observed that nearly all of the bottles at 37°C in the low and lowest concentrations turned black by 24 hours, whereas at room temperatures the low and the lowest dilutions took 48 hours.

From the data it was seen that the room temperature, which varied between 18 - 24°C, had almost the same incubation period as at 22°C incubation at constant temperature. A few hours of low temperature (up to 18°C) at night time may not delay the H<sub>2</sub>S production if the bottles are exposed to higher temperatures during the day time. Therefore it could be stated that the H<sub>2</sub>S method does not require an incubation at constant temperature for detecting *Salmonella sp.* if the room temperature is between 18 - 44°C. Since there has not been much work done on the incubation period for *Salmonella sp.* comparison can be made only with the results obtained from natural waters. Castillo *et al.* (1994), who incubated the natural samples at 30 - 35°C, and Ratto *et al.* (1989) and Kasper *et al.* (1992), who incubated the natural samples at 22 and 35°C, remarked that the incubation method or the temperature were not critical in the effectiveness of the method.

The experiment also proved that the medium M2 was faster than the other media in the rate of blackening, especially at the extreme temperatures of 14°C and 44°C. Therefore it has been assumed that the addition of L-cystine to the original H<sub>2</sub>S medium could improve the medium. This concurs with the observation made by Venkobachar *et al.* (1994) that the percentage of detection

of faecal contamination in the natural samples was higher with the medium containing L-cystine. The cystine is a sulphur source for which *Salmonella sp.* have a high preference and is not used by some other competitors. Although peptone, which is a component of the H<sub>2</sub>S medium, is also a source of cystine, it was thought that some organisms are unable to extract cystine from peptone (Smith, 1959a). Hu *et al.* (1995) found that a broth that contained L-cystine reduced the variability in the growth of *Salmonella sp.*. North and Bartram (1953) reported that the addition of cystine to selenite broth enhanced the growth of *S. typhimurium*.

The medium M3 containing yeast extract was also found to be better than the original medium. In the effort of improving the isolation of *Salmonella sp.*, North and Bartram (1953) tried some modifications by adding yeast to the medium and reported that a very good growth of *Salmonella sp.* was obtained when yeast extract was incorporated into dulcitol selenite enrichment medium. It was hypothesised that yeast extract provided a source of co enzymes particularly B-complex vitamins needed for the utilisation of dulcitol. Hu *et al.* (1995) found that the variability in growth of *Salmonella sp.* population was reduced by the addition of yeast extract and that the addition of yeast extract to mannitol Selenite Enrichment broth (MSE) may improve its performance as an enrichment media for *Salmonella sp.*

## Conclusions

From the study it was evident that *Salmonella typhimurium* could be detected by the H<sub>2</sub>S method to a lowest concentration of ~ 1 CFU/100mL.

The H<sub>2</sub>S method was efficient in detecting *Salmonella sp.* at a range of temperature varying from 14-44°C although the fastest result was obtained at an incubation of 37°C. The incubation period was affected by the temperature.

The incubation period increased with the increase or decrease in the temperature from 37°C.

The concentration of the inoculum also influenced the incubation period. The incubation period increased with the decrease in the concentrations at temperatures other than 37°C.

The addition of L-cystine and yeast extract did not make a remarkable improvement in the medium for detecting *Salmonella typhimurium*, although the medium with L-cystine was found to be better than the medium without them at 14 and 44°C.

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