H_2S PAPER STRIP METHOD - A BACTERIOLOGICAL TEST FOR FAECAL COLIFORMS IN DRINKING WATER AT VARIOUS TEMPERATURES

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ABSTRACT

Epidemics arising from waterborne diseases are a global health problem. Faecal contamination of drinking water is the main cause of these outbreaks. According to WHO (1996) for drinking water to be safe, a 100mL sample should not contain any coliform bacteria. The standard methods currently used for routine testing have many limitations especially when applied in remote areas. The H_2S Method has been developed as an on-site, inexpensive and easy to use method to test drinking water for remote and rural areas.

The present work analyses the reliability of the H_2S method for detecting faecal contamination in drinking water. The minimum level of faecal coliforms that could be detected and the incubation period required at various levels of contamination were studied. The range of temperatures at which the method was effective and the incubation period required at various temperatures were also determined. The H_2S method was found to be able to detect contamination down to a level of 1 CFU/100mL of coliform bacteria. Although the H_2S method could be used at a temperature range of 20 to 44^{o}C, temperatures between 28 to 37^{o}C gave faster results. An incubation period of only 24 hours was required at 37^{o}C, which was found to be the most suitable incubation temperature. The incubation period increased with a decrease or increase in temperature.

KEYWORDS

Drinking water; faecal coliforms; H_2S Method; incubation period; temperature; contamination level.

INTRODUCTION

Unpolluted safe drinking water is one of the primary requisites for healthy human life. The health hazards from polluted water are evident from the fact that about 80% of infectious diseases throughout the world are water related (World Development Report, 1992). Since most of these diseases are transmitted through human faeces the condition is more serious in densely populated areas with inadequate sanitation and sewerage facilities. Unfortunately this situation is more common in developing countries where the facilities to control a disease outbreak are limited. According to the WHO (1996), drinking water should be free from *Salmonella sp.* and thermotolerant coliforms. To ensure this, drinking water should be tested often for any possible contamination. However the standard methods for detecting coliforms currently used, such as the Most Probable Number Method (MPN) and Membrane Filtration Technique (MFT), are expensive, time consuming and require laboratory and technical support. Therefore regular monitoring of drinking water quality is a huge effort particularly for the developing countries.

The on-site testing methods such as Colilert and Colisure which are commercially available, although very convenient, are expensive to use for routine analysis. The chemicals have to be stored at 4˚C and have a short shelf life. Further the bottles have to be incubated at 35˚C. These on-site testing kits therefore

require a refrigerator and incubator. These are not available in many areas. An alternate method for testing the microbial quality of drinking water which would overcome the above problems is highly essential.

Manja *et al*. (1982) developed an on-site microbial water testing method based on the detection of hydrogen sulphide producing bacteria. Human faeces contain high concentrations of sulphate reducing bacteria, which can be as high as to 10^{10}/g (Levett, 1993). The hydrogen sulphide paper strip method (H_2S Method) was developed for testing water.in case of an emergency when frequent testing of large numbers of samples becomes essential. The method has the advantage that it is less expensive, can give a faster result, could be carried out by a local person after being trained and was found to have a good correlation with the standard methods when tested in different parts of the world (Castillo *et al*., 1994; Grant and Ziel, 1996; Hewison *et al*., 1988; Kromoredjo and Fujioka, 1991; Ratto *et al*., 1989; Sivaborvorn and Dutka, 1989). A higher percentage of positive samples noted with the H_2S method was suggested to be due to non-Enterobacteriacea such as *Clostridium perfringens*. It was further noted that H_2S production was also associated with many *Salmonella sp.* which have been isolated from the H_2S positive bottles (Castillo *et al*., 1994, Manja *et al*., 1982). Most of the previous studies were conducted using natural samples, which may contain H_2S producing bacteria other than those of faecal origin. Therefore in the present work inoculums of a faecal sample from a healthy person who was not under any treatment were used. Although it has been reported that the H_2S method could detect faecal contamination in drinking water, more details are still required as to the influence of temperature on the incubation period, whether the contamination level has any influence on the incubation period, and whether the medium could be improved to decrease the incubation period. This paper therefore looks into the above three questions.

MATERIALS AND METHODS

Preparation of the H_2S bottles

The H_2S bottles were prepared according to Manja *et al*. (1982). The original H_2S medium was prepared by dissolving peptone(20 g), di potassium hydrogen phosphate(1.5 g), ferric ammonium citrate(0.75 g), sodium thiosulfate(1 g) and teepol (detergent)(1 mL) in 50 mL of tap water. The addition of L-cystine was reported to improve the detection rate (Venkobachar *et al*., 1994) and the addition of yeast was found to improve the rate of blackening of *Salmonella* sp. as well as reduce the variability in growth (Hu *et al*., 1995). Therefore two modifications to the original medium (M1) were also tested in this study. Medium M2 contained 0.125 g 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 15 g in 50 mL of tap water.

In the original method aliquots of 5 mL of the above medium were absorbed in folded tissue paper before sterilising. For convenience for these experiments, 5 mL of the medium was pipetted into 100 mL bottles and autoclaved. The bottles were then stored at room temperature.

Preparation of faeces dilutions

Faecal samples from a healthy person who was not under any treatment for the previous six months were used for the present experiment in order to have a natural microbial population. To indicate the concentration of faecal material at each dilution the number of faecal coliforms were counted. Initially 1g of the material was weighed into 10 mL of sterilised distilled water. Serial dilutions were prepared as shown in Table 1. During the three repetitions the concentration varied slightly at each dilution and therefore the concentration was represented as a range. Each dilution (100ml) was filtered through a membrane filter of 0.45 μm and incubated on m-FC agar plates for 22 hours at 44.5˚C. The colonies were counted and recorded as the number of Colony Forming Units (CFU) of faecal coliforms/100 mL

In the present study 100 mL of the sample was used, compared to the 20 mL used in the original H_2S method. This was to make it comparable to the standard methods that test 100 mL of water sample. The different inoculum volumes shown in Table 1 were pipetted into the H_2S bottles containing 100 mL of sterilised distilled water and mixed. They were incubated at various temperatures until the bottle turned black. Incubators with constant temperatures were used for the experiment. The bottles that did not turn black after 120 hours (5 days) were discarded. The bottles were examined after 18, 24, 36, 48, 60, 72, 90, 108 and 120 hours. The first appearance of a black colour change in the water was taken as the incubation period. The incubation periods for different concentrations at each temperature were noted.

Temperature range

To study the temperature range at which the method was effective in detecting faecal coliforms in drinking water, 0, 8, 14, 22, 28, 37, 44, 47^{o}C and room temperature were tested. The room temperature varied between 20 - 24^{o}C during the period at which the experiments were conducted. For this study only the medium M2 was used. This was because in a previous experiment with the *Salmonella typhimurium* inoculums (Pillai *et al*., 1997), M2 was found to be better than the M1 and M3. A faecal coliform concentration of 52 CFU/100 mL was tested at different temperatures ranging from 0 to 47^{o}C.

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

The influence of the incubation temperature and the concentration of faecal coliforms on incubation period were tested for the three H_2S media. All the different concentrations were incubated at 22, 28, 37, 44^{o}C and the room temperature. The bottles were incubated for a maximum of five days (120 hours).

RESULTS

Concentration of faecal coliforms in inoculums

The concentrations of faecal coliforms at various dilutions were as shown in Table 1.

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

Dilution	Inoculum volume (mL)	faecal coliforms (CFU/100 mL)
10^{-4}	0.5	>1000
	0.2	400-500
	0.1	160-246
	0.05	52-96
10^{-6}	1.0	9-11
	0.1	1-2

Effect of concentration and temperature on the incubation period of different media

The incubation period varied with temperature and the concentration of faecal coliforms in the three media. This is shown in Table 2.

Table 2. Effect of temperature and concentration on incubation period for different media

Media	Concentrations	22°C	Rm(20 - 24˚C)	28°C	37°C	44°C
M1	>1000	48	48	48	36	36
	400-500	48	48	48	36	36
	160-246	72	60	60	48	48
	52-96	-	-	60	60	60
	9 - 11	-	-	-	90	-
M2	>1000	36	36	36	18	36
	400-500	36	36	36	18	36
	160-246	36	36	36	18	36
	52-96	90	60	36	18	48
	9 -11	90	60	36	18	48
M3	>1000	48	48	48	18	36
	400-500	48	48	48	18	36
	160-246	48	60	48	36	48
	52-96	-	-	48	48	60
	9 -11	-	-	-	72	-

An increase in the incubation period was observed with the lowering of the concentrations at all temperatures. Many of the bottles in the highest dilution (0.1 mL at 10^{-6} dilution) did not turn black while some turned black at the designated incubation time. The bottles that did not turned black may have missed out on an inoculum due to the low number(1-2 CFU/100mL). Therefore the incubation period for that dilution was not recorded. It was also noticed that the black colour developed slightly at the bottom for the lowest concentrations compared to the whole bottle turning black at the higher concentrations.

Temperature Range

When a range of temperatures from 0 to 47^{o} C was tested, the bottles at 0, 8,14 and 47^{o}C did not turn black even after 5 days. It was observed that 37^{o}C required the lowest incubation period of 18 hours whereas at 28^{o}C the bottles took 36 hours to turn black. At 22˚C and at room temperature (20-24˚C), 90 and 60 hours respectively were required. At a higher temperature (44^{o}C) the bottles turned black after 48 hours of incubation.

Effect of three media on incubation period

A significant difference in the incubation period was noticed between the three media. Figure 1 shows the incubation period required for the three media at various concentrations at 37˚C. The difference in the incubation period was more pronounced at lower concentrations. M1 and M3 did not turn black at the lowest concentrations in temperatures other than 37^{o}C (Table 2). At all temperatures M2 turned black much faster than the other two media. Figure 2 shows the incubation period required by the three media at different temperatures at a concentration of 160 - 246 CFU/100 mL

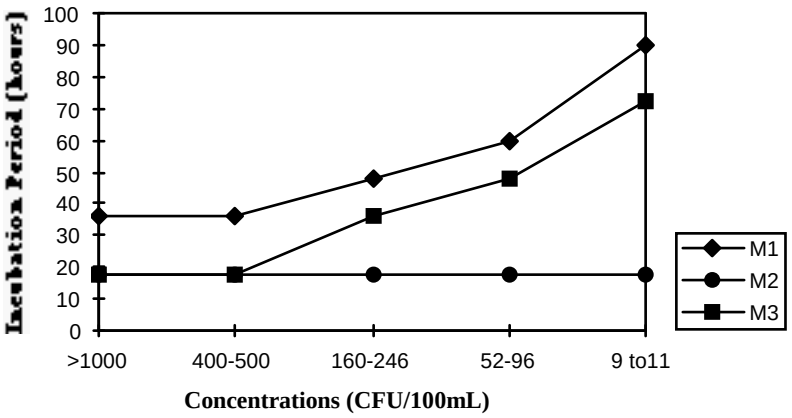
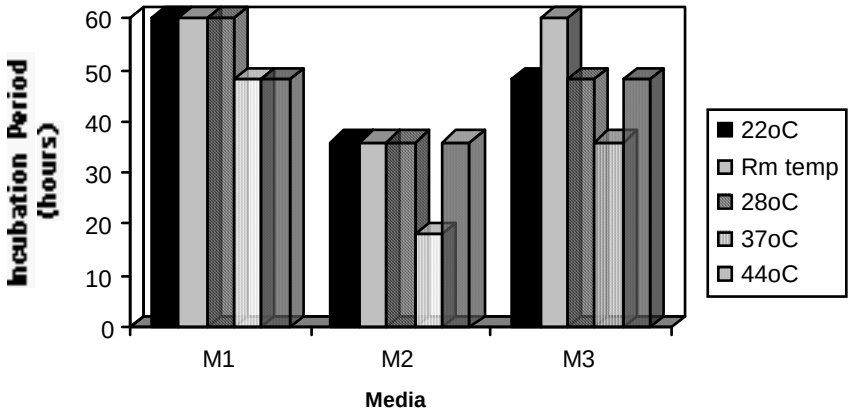


Fig. 1. Incubation period for the three media at different concentrations of faecal coliforms at 37^{o}C

Fig. 2. Incubation period for three media at different temperatures at a concentration of 160 CFU/100 mL

DISCUSSION

From the present experiment it was observed that the H_2S method was capable of detecting the presence of faecal contamination. As faecal coliforms are used as indicators of faecal contamination, the number of faecal coliforms was used to assess the contamination level. Many of the H_2S bottles having the lowest concentration of 1 CFU/100 mL turned black while some remained unchanged. It was concluded that bottles could have missed out on faecal material because of the very low number of bacteria in the inoculum. This therefore make comparison of H_2S and faecal coliform results difficult at low



concentrations.

When the influence of temperature and concentration of faecal coliforms on the incubation period were tested, only 18 hours incubation was required at 37˚C irrespective of the concentration. However at low and high temperatures, the incubation period increased with the decrease in the concentration. This showed that the growth of H_2S producers was slowed down at those temperatures and the H_2S production was delayed. Reasoner *et al*. (1979) observed that lowering of temperature from 44.5 ˚C to 41.5˚C increased the recovery rate of faecal coliforms which was supposed to be due to the less additional stress for injured bacteria enabling them to repair and grow.

The incubation period required for the H_2S bottles highly depended on the incubation temperature as shown in Fig. 1. Most of the previous studies were either done at room temperature (Manja *et al*., 1982) or at a constant incubation temperature of 37˚C. No details were available as to the temperature range at which the H_2S method would detect faecal contamination. From the present study it is evident that the faecal contamination could be detected by the H_2S method at a temperature range of 20 - 44˚C. At a still lower temperature of 14˚C the bottles required more than 120 hours (5 days) to blacken. Pillai *et al.* (1997) found that the presence of *Salmonella typhimurium* could be detected from the lowest temperature range of 14˚C. Since *S. typhimurium* is an H_2S producing species it was able to produce H_2S even at a lower temperature although at a slower rate. From Table 2 it is evident that the blackening time increased with the increase or decrease of the temperature from 37˚C especially for lower concentrations (below 96 CFU/100 mL). From the data it was also seen that the room temperature, which varied between 20- 24˚C,

required 60 hours incubation period at lower concentrations, while at 22˚C incubation at constant temperature it took 90 hours. Therefore it could be stated that incubation at constant temperature and a corresponding room temperature require a similar incubation period. Also it is clear that if the temperature is between 28 - 44˚C blackening could be obtained within 48 hours. Castillo *et al*.(1994) who incubated the 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 effectiveness of the method was independent of the incubation method or the temperature. However in the present study, it was noted that if the temperature was between 28 - 44˚C the end result could be obtained within 48 hours and a still lower temperature of 22˚C may take up to 90 hours to report the presence of faecal contamination. At lower concentrations and at lower temperatures the black colour appeared as a patch at the bottom and took about six hours for the complete bottle to turn black. In some cases the blackening was seen only at the bottom and did not colour the entire water sample. This could be due to the very restricted growth of the H_2S producing bacteria because of the low numbers or low temperature.

The experiment proved that the medium M2 was faster in the rate of blackening and also could detect faecal contamination at lower concentrations than the other media. The addition of l-cystine to the original H_2S medium could therefore definitely improve the medium. This has also been reported by Venkobachar *et al*. (1994) when he found that the percentage detection of faecal contamination in the natural samples was higher with the medium containing l-cystine. Although peptone provided a source of cystine in the original medium, it was thought that some organisms are unable to extract cystine from peptone (Smith, 1959). He also noted that the growth of *E. coli* was suppressed when peptone and not free cystine was present. With *Proteus*, the growth was reduced when peptone was used instead of free cystine. Many reports show the importance of sulphur utilisation by *Salmonella* sp when cystine was added to the medium (North and Bartram, 1953; Smith, 1959). The medium M3 containing yeast extract did not show a remarkable difference in the incubation period from the original medium. It was reported that methionine of yeast extract could annul the inhibitory effects of substances (Smith, 1959). However the original H_2S medium does not contain growth inhibitors whose effects could be annulled, the addition of yeast extract did not result in an observable improvement.

CONCLUSIONS

From the present experiment it is clear that the H_2S method could detect the presence of faecal contamination at a temperature range of 20 - 44˚C and that no constant temperature incubator is required if the room temperature is between that range. The intensity of faecal contamination influences the incubation period at a lower or higher temperature than the 37˚C. At a lower contamination level more time is required for the bottle to turn black. Another factor that was noticed in the present experiment was that the rate of blackening also depended on the concentration and the temperature. It is also clear that in order to test the water for faecal contamination to the lowest concentrations and at lower temperature addition of l-cystine is highly beneficial.

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