Modification of the H_2S test to screen for the detection of sulphur- and sulphate-reducing bacteria of faecal origin in water

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Abstract

The H_2S test was created to assess the microbial quality of drinking-water in low-resource settings, but the original version of the H_2S test lacks sensitivity and specificity for faecal indicator bacteria. There is evidence that a modified media formula of the H_2S test may be more sensitive and specific for the faecal indicator bacterium *Escherichia coli (E. coli)* and less sensitive to organisms of non-faecal origin.

This research established the detection threshold and operational range of the H_2S test, and to increase its sensitivity and specificity for *E. coli*. A total of 20 modifications of the H_2S test, and the original test, were assayed against 20 confirmed and pure culture bacteria of faecal and non-faecal origin at varying concentrations. Additionally, some of the H_2S test modifications were evaluated against standard methods for drinking-water analysis.

Results indicate that using a modified version of the H_2S test containing L-cystine and 2-mercaptopyridine, and bile salts or penicillin G, *E. coli* will produce H_2S. In addition, this research reveals which organisms react positively to the original and modified versions of the H_2S test. The modified versions of the H_2S test can be promoted as a simple screening test for microbial drinking-water safety in low-resource settings.

Keywords:

Drinking water quality

Faecal indicator bacteria

H_2S test

Method development

Sulphur-reducing bacteria

Water quality monitoring

Highlights:

- A modified version of the H_2S test containing L-cystine and 2-mercaptopyridine is able to detect for *E. coli* in water.
- Modified H_2S tests with the addition of 2% bile salts and L-cystine produced a better performance overall at both 20°C and 37°C when compared to Manja's original H_2S test.
- The modified H_2S tests meet the specifications of standard methods for monitoring drinking water (membrane filtration).
- This study has for the first time analysed the performance of Manja's original H_2S test and its novel modifications against a range of pure-cultured bacterial strains.

- This study has identified the ability of 20 different enteric bacterial species to produce H_2S from different reduced sources of sulphur and sulphate.

Introduction

Water-related diseases are one of the major obstacles to the improvement of people's health, especially in low income countries. Worldwide, there are 785 million people without access to an improved drinking water source, and at least 2 billion people use a drinking water source contaminated with faeces (WHO, 2019). It has been estimated that 88% of all incidents of diarrhoea worldwide are caused by microbiologically unsafe water, affecting mostly children below the age of five years (WHO, 2008). The World Health Organisation (WHO) estimated that inadequate drinking-water sources and the lack of adequate sanitation and hygiene cause 842,000 diarrhoeal disease related deaths per year, especially in low income countries (WHO, 2015). The largest increase in water deterioration is expected to occur in low income countries, because of a subsequent increase in population and economic growth, which is especially the case in sub-Saharan Africa (WWAP, 2016). Therefore, SDG target 6.1 aims to achieve universal and equitable access to safe and affordable drinking water (or safely managed drinking-water water) for all by 2030 (WHO, 2017). UNEP highlights the very low density of water quality monitoring facilities and laboratories in general in low income countries, as well as the significant inconsistency between global assessment regulations and regional knowledge needs (UNEP, 2015).

Millions of people have to rely on an unimproved drinking water sources. Many low-resource settings lack adequate finances, reliable energy sources, qualified technicians, and laboratory reagents. Consequently there is a need for straightforward and affordable microbial field tests to substitute for more sophisticated laboratory procedures (McMahan *et al.*, 2011). The H_2S test has the potential to be an affordable alternative in comparison to more sophisticated laboratory-based methods (Bain *et al*., 2012).

The H_2S test was introduced by Manja *et al.* (1982) to assess the microbial quality of drinking-water in low-resource settings by testing for hydrogen sulphide (H_2S) producing bacteria. It was promoted as a promising alternative to existing technologies, but the original version lacked sensitivity and especially specificity to the faecal indicator bacteria (FIB) used in microbial drinking-water analysis (Sobsey & Pfaender 2002). Any source of H_2S or the presence of sulphur-reducing and sulphate-reducing bacteria (SRB) in the sample can lead to a false positive test result (Huang *et al.*, 2011; Sobsey *et al.*, 2002). In contrast, the presence of faecal coliforms with limited ability to reduce sulphate and the absence of related sulphate-reducing bacteria may lead to false-negative results, one of the most

concerning limitations of the H_2S test (Sobsey *et al.*, 2002; Huang *et al.*, 2011). For example, water of poor microbiological quality could be falsely classified as being of acceptable quality on the basis of the existing H_2S test. False negative results, especially when indicator densities are relatively high, are a major cause for concern (Nair *et al.*, 2001). Therefore, further research is needed in order to understand the conditions that lead to this misleading result.

The lack of available alternatives resulted in the H_2S test being promoted and used by many governmental, non-governmental and research organisations including the WHO, UNICEF, USAid, WaterAid, and ACF International, operating in rural communities or in low-resource and emergency settings (Matwewe *et al*., 2018; Peletz *et al.*, 2016; USAid, 2015; WaterAid, 2014; ACF International, 2013; IFRC, 2011; WHO, 2010 & 2009; UNICEF, 2008; Oxfam, 2006). Although alternative field tests or potable incubators for bacteriological water quality analysis are available (e.g. Colilert, CBT, DelAgua) their cost is much higher when compared to the H_2S test and access to these tests and their consumables in low-resource settings is difficult.

Previous studies investigating alternative modifications (Kejariwal *et al*., 2018; Shahryari *et al.*, 2014; Khush *et al.*, 2013; Luyt *et al*., 2012; Tambekar *et al.*, 2007; Pathak *et al.*, 2005; Pant *et al*., 2002; Manja et al., 2001; Grant & Ziel, 1996; Venkobachar *et al.*, 1994) or the microbial sensitivity and specificity of the H_2S test (Malema *et al.*, 2019; Matwewe *et al*., 2018; Tambi *et al.*, 2016; Murcott *et al.*, 2015; Weppelmann *et al.*, 2014; Yang *et al*. 2013; Khush et al., 2013; McMahan *et al*., 2012; Wright *et al*., 2012 [meta-study]; Izadi *et al.*, 2010; Gupta *et al.*, 2008; Nair *et al.*, 2001; Martins *et al.*, 1997; Castillo *et al.*, 1994; Desmarchelier *et al.*, 1992; Kromoredjo & Fujioka, 1991; Dutka & El-Shaarawi, 1990; Jacobs *et al*., 1986) missed to assess the test's performance and its level of accuracy by using a range of known concentration of confirmed and pure cultured species of organisms (confirmed to subspecies level). Further, the level of sensitivity and specificity was not performed according to accepted method validation protocols. Although, McMahan *et al*. (2012) investigated the specificity of the H_2S test by application of PCR testing, the samples were of environmental origin and not pure cultured. Most studies indicate variability in performance of the H_2S test (Wright *et al*., 2012), which justifies further investigation.

Given that the use of this method is unlikely to change in the near future and the test's simplicity and low cost makes it popular with many NGOs, efforts are required to improve the H_2S test's microbial sensitivity and specificity. Therefore, the objectives of this research were to (i) evaluate modifications to the culture media of the original H_2S test with the aim to increase its microbial sensitivity and specificity to *E. coli*; (ii) to assess the sensitivity and specificity of a range of the modifications, and (iii) to identify the specific

microorganisms that react in the H_2S test and its modifications using confirmed pure culture organisms.

Materials and methods

Study design

In order to evaluate the performance of the H_2S test and its modifications in terms of sensitivity and specificity to faecal indicator bacteria and faecal and environmental sulphate- and sulphur-reducing bacteria (SRB), the H_2S test's operational range and limit of detection (LOD) was investigated. The H_2S test and each of the 20 modifications were challenged with culture collection strains of known enteric and non-enteric sulphide-, sulphate-, sulphur-reducing, and H_2S-producing bacteria. The incubation period (time in h) to produce a positive reaction (black precipitate caused by the production and reaction of H_2S with ferric iron Fe_2O_3) for each formulation was recorded. In addition, some of the H_2S test modifications were validated against the accepted standard methods for drinking-water analysis as outlined in APHA (2012).

The H_2S test

The H_2S test culture medium was prepared according to the original description by Manja *et al.* (1982). It contains: bacteriological peptone 20 g, dipotassium hydrogen phosphate 1.5 g, ferric ammonium citrate 0.75 g, sodium thiosulphate 1.0 g, liquid detergent (Teepol) 1.0 ml, and distilled and deionised water 50 ml. All heat-resistant components were sterilized by autoclaving before use. 0.5ml of the medium was placed on a pre-cut sterile filter paper and placed into an oven at 55°C for 30 minutes to dry. Each strip was then placed in a sterile 10ml plastic culture tube.

Type of modified H_2S tests used in this study

The selective reagents considered to support or inhibit the growth of certain bacteria are shown in Table 1. Sulphur sources of different reduction stages were used, given that the process of sulphur assimilation and dissimilation by most enteric bacteria has rarely been studied nor is it properly understood (Cai *et al.*, 2019; La Faou *et al.*, 1990). All reagents were procured from Fisher Scientific UK Ltd. and Sigma-Aldrich®. Filter papers used for each of the various H_2S tests were produced by Fioroni S.A. in France. Bile salts (LP0055) and bacteriological peptone (LP0037) were manufactured by Oxoid™.

Each of the modified H_2S tests culture media formulae investigated in this study and the rationale for each culture media's modification are outlined in Table 1:

Table 1. Different H_2S test modifications used in this study incl. their formulae and reagents

Pure culture strains and their preparation

Most bacterial strains used in the testing and analysis were supplied by the National Collection of Type Cultures (NCTC) and the American Type Culture Collection (ATCC®) as frozen and freeze-dried cultures. Some have been isolated from environmental samples and the species was confirmed by bioMérieux's API® identification. This study aimed to analyse a range of commonly found and known sulphate-reducing organisms of aquatic/ environmental origin to assess to potential for false-positive and false-negative test results in comparison to test results from bacteria of enteric and faecal origin. The author realises that it is impossible to test for all relevant bacterial strains. All of the chosen bacterial strains are of relevance with regards to health-related water quality monitoring and are opportunistic or pathogenic (Percival et al. 2013).

The confirmed and pure cultured bacteria used in this research were:

- *Aeromonas hydrophila* (unknown strain)
- *Campylobacter jejuni* NC11168
- *Citrobacter freundii* ATCC® 8090™
- *Clostridium difficile* ATCC® 9689™
- *Clostridium perfringens* (unknown strain)
- *E. coli* NCEMB 10240 (ATCC® 23744™)
- *E. coli* O157:H7 NCTC 12900 (shigatoxin negative)
- *E. coli* NCTC 10418
- *E. coli* NCTC 5933
- *E. coli* (unknown strain)
- *Edwardsiella tarda* NCIMB 2056
- *Enterococcus faecalis* ATCC® 29212™
- *Klebsiella pneumoniae* (unknown strain)
- *Proteus mirabilis* ATCC® 43071
- *Salmonella enterica* serovar *enteritidis* ATCC® 13076™
- *Salmonella typhimurium* NC12023
- *Serratia marcescens* (unknown strain)
- *Staphylococcus aureus* NCTC10788
- *Vibrio cholerae* NCTC 10256 (biotype El Tor, non-toxic)
- *Yersinia enterocolitica* ATCC® 9610™

All bacterial strains used for testing the H_2S tests and its modifications were re-cultured in brain-heart infusion broth (Oxoid CM1135), and stored in 10-15% glycerol at -80°C until needed. Before each round of testing, the pure cultures were defrosted, and 0.1ml inoculated into 100ml autoclaved brain-heart infusion broth, followed by 24 hour incubation at the strain's required temperature. 0.1ml of each pure culture was used to produce dilutions of 10^{-2}, 10^{-4}, and 10^{-6} in sterile water, which when in use in the test produced final concentrations of $10^{-4}/10^{-6}/10^{-8}$. The Miles and Misra method (or surface viable count method) was subsequently used to determine the concentration of bacterial cells in the diluted inoculate (Miles *et al.*, 1938). Clostridium spp. cultures and H_2S tests containing Clostridium spp. have been cultivated in an anaerobic jar.

Performance of the H_2S test and its modifications

To perform the H_2S test, a piece of filter paper (2x8cm) inoculated with 0.5ml of the culture medium (see Figure 1) was placed in a disposable, sterile 10ml plastic culture tube (see Figure 2) to which 9.9ml of deionised, autoclaved water containing the pure cultured bacteria at the appropriate dilution. Tubes were incubated in the dark at ambient temperature (20°C) for up to 48 hours. For each version of the H_2S test, one tube contained 10ml of deionised, autoclaved water without bacteria as a blank control. If the filter paper changed colour from a light yellow to black (see Figure 3), this indicated a positive reaction for bacteria able to produce hydrogen sulphide. A change of colour change was observed between 12 and 48 hours, depending on the concentration of bacteria. The different test formulations were been examined for colour change continuously (once every hour). Tests in which the content remained light yellow in colour without any production of black precipitate after 48 hours were recorded as negative result.

It should be highlighted that Manja *et al.* (1982) used a 20ml volume glass bottle for the original H_2S test. However, research from Yang *et al.* (2013) indicates that a smaller test volume lowers the sensitivity, but raises the specificity. A smaller sample volume also reduces the price per test, and disposable culture tubes do not require sterilisation and are very cheap to procure, and are generally cost effective.

Figure 1 Filter paper stripes inoculated with H_2S culture medium

Figure 2 H_2S test tubes ready for use

Figure 3 10ml culture tube incl. positive H_2S test paper stripe

Validation of the original and modified versions of the H_2S test and statistical analysis

During the second part of this study, the H_2S test and its modifications were tested against unknown samples as suggested by the method validation protocol of EPA (2009). It was considered important to validate the original H_2S test and any modifications against accepted standard methods such as membrane-filtration with semi-selective culture medium to detect indicator bacteria. This step was also necessary to assess any differences in the performance of the original H_2S test and the modifications in different environmental and field conditions, and especially with different types of water unimproved water samples were collected from 10 sites along River Ouse in East Sussex (UK), and from one rain harvesting point also located in East Sussex.

Validation based on a sensitivity/specificity analysis was conducted on the original H_2S test and the modified versions. Membrane filtration using the semi-selective media m-FC and m-Enterococcus, as described by *Standard Methods for the Examination of Water and Wastewater* (APHA, 2012) was used as the test method. Each different H_2S test was assessed at incubation temperatures of 20°C and at 37°C, to assess any relationship between the incubation temperature and the reaction time.

The calculation of positive and negative predictive values state the probabilities that an individual (or a test) is truly positive given that it tested positive or truly negative given that it tested negative. In this study, sensitivity marks the proportion of those H_2S tests showing a positive reaction and which correctly identified a water sample containing faecal indicator bacteria. Specificity is the proportion of those H_2S tests which show no reaction (negative reaction) to a water sample which does not contain any faecal indicator bacteria (zero CFU) in a 100ml sample (Peacock & Peacock, 2013). The diagnostic sensitivity and specificity, the PPV and NPV respectively, were calculated using MedCalc® (MedCalc Software Ltd.). The PPV and NPV were defined as follows (Peacock & Peacock, 2013):

Sensitivity:	true positive / (true positive + false negative)
Specificity:	true negative / (false positive + true negative)
Positive predictive value:	true positive / (true positive + false positive)
Negative predictive value:	true negative / (false negative + true negative)
Prevalence of disease:	(true positive + false negative) / grand total

Results

Demonstration of capability: assessment of analytical specificity and sensitivity

Single-operator characteristics' and 'method detection level determination' are suggested tests in the development of new diagnostic methods by APHA (2012). The performance of each H_2S test (original and modifications) was investigated using pure cultures of selected confirmed strains of enteric and non-enteric sulphide-, sulphate-, sulphur-reducing, and H_2S-producing bacteria. Performance indicators assessed were (i) the strength of the reaction at different dilutions and (ii) the time taken to produce a positive reaction.

Specificity: testing against pure cultures of confirmed strains

20 pure cultures of bacterial strains were tested against the original H_2S test and modifications at concentrations of $10^{-4}/10^{-6}/10^{-8}$ plus one blank (no bacteria) test. This experiment was repeated twice to produce data for analysis of 3,400 H_2S tests. As shown in Table 2 & 3, the original H_2S test's positive reaction is triggered only when the sample water contains *Citrobacter freundii*, *Proteus mirabilis*, *Salmonella typhimurium*, and to a lesser extent *Salmonella enterica*. With regards to the positive reaction caused by the *Citrobacter freundii* ATCC® 8090™, *Proteus mirabilis* ATCC® 43071™, and *Salmonella typhimurium* ATCC® 14028™ strains, analysis suggests that the original H_2S test is not able to detect thermotolerant faecal coliforms or any other faecal indicator bacteria apart from *Citrobacter freundii*. This is an important finding *C. freundii* is regarded as thermotolerant, but is also ubiquitous in nature (Holt *et al.*, 1994).

Table 2 Reaction and H_2S production of pure cultures of target and non-target organisms to the original H_2S tests and its new modifications – 1st part of results

Table 3 Reaction and H_2S production of pure cultures of target and non-target organisms to the original H_2S tests and its new modifications – 2nd part of results

All of the tested *E. coli* culture collection strains plus one unclassified strain isolated from the River Ouse demonstrated the production of H_2S from sulphur sources other than thiosulphate, as used in the original H_2S test. The preferred sources of (organic) sulphur are the amino acids L-cysteine and its oxidised disulphide-bond L-cystine. When L-cysteine was provided in combination with 2-mercaptopyridine and tested in the presence of *E. coli* NCTC 5933, positive results were observed at ambient temperature after only 18 hours. Also, L-cystine produced faster (12 hours on average) results as compared to tests prepared with L-cysteine. The H_2S test with added L-cystine delivered fast and strongly readable results when tested with bacteria from the family of *Enterobacteriaceae* in particular.

The H_2S test modification containing L-glutathione showed no reaction to any organism used in this study. Also, it was not observed that a modified H_2S test containing L-cysteine or L-cystine is able to detect for the presence of *V. cholerae*.

The modified H_2S tests containing the organic sulphur compounds D-cysteine, L-methionine, L-glutathione, taurine, and pyruvate plus the tests containing the inorganic sulphur sources sodium sulphate and tetrathionate showed, mostly, either no positive result, or only reacted very weakly. The H_2S test containing L-glutathione reacted weakly in the presence of *Citrobacter freundii* ATCC® 8090™ only. However, no other organism tested was observed to be capable of reducing this amino acid to H_2S. Also, using ferrous chloride instead of ferrous citrate, as suggested by Barrett & Clark (1987), yielded no convincing results. Only *Citrobacter freundii* ATCC® 8090™, *E. coli* O157:H7 NCTC 12900, *Proteus mirabilis* ATCC® 43071™, and *Salmonella* Typhimurium NCTC 12023/ ATCC® 14028™ triggered some very weak reaction to this compound.

The modified H_2S tests containing penicillin G (benzylpenicillin) showed promising results, as it allowed for the growth of all tested *E. coli* strains and other organisms from the family of *Enterobacteriaceae*. Since penicillin G prevents the growth of Gram-positive and many Gram-negative bacteria (such as e.g. *Klebsiella pneumoniae*) but allows the growth of most other *Enterobacteriaceae* it makes the H_2S test very specific to detect for faecal contamination (Sigma Aldrich/Merck, 2017[1]). The modified H_2S tests containing gentamicin in contrast prevents the growth of Gram-negative bacteria and of Gram-positive *Staphylococcus* spp. and would theoretically allow the growth of *Enterococcus faecalis* (Sigma Aldrich/Merck, 2017[2]). However, *Enterococcus faecalis* ATCC® 29212™ showed no reaction to any of the H_2S tests.

Clostridium perfringens (River Ouse) reacted to the H_2S test which contained L-cysteine only. Positive reactions at ambient temperature were observed after between 22 and 40 hours. However, growth was very slow, and the full reaction was not completed before 72 hours of incubation time. *Klebsiella pneumoniae* (River Ouse isolate) reacted weakly after between 40 and 46 hours to the H_2S test which contained L-cysteine only. Both, *Clostridium perfringens* and *Klebsiella pneumoniae* showed no reaction to any other H_2S test modification within 48h.

A. hydrophila (River Ouse isolate), *C. perfringens* (River Ouse isolate), *C. jejuni* NCTC 11168 /ATCC® 700819™, *E. faecalis* ATCC® 29212™, *S. aureus* NCTC 10788, and *S. marcescens* didnot show a positive reaction to any of the tests, and have not been included in Tables 2 and 3.

Sensitivity: assessment of reaction time when tested with pure cultures at different dilution stages (limit of detection)

The reaction time (the time until the production of a black precipitate) of pure cultures was considerably longer (about 12h) compared to mixed cultures (undiluted raw water from the

River Ouse). Apart from *E. coli* O157, *Proteus mirabilis* ATCC® 43071™ and *Salmonella* Typhimurium ATCC® 14028™ mostly showed a positive reaction after +/- 20 hours at a low dilution (high concentration) of 1 x 10^{-4} (approx. 42,025 - 28,900 CFU, see Table 4 & 5). The other bacteria demonstrated positive reaction times of between 24 and 48 hours at ambient temperature when tested with a pure culture diluted at 1 x 10^{-4}.

At ambient temperature (20°C), the H_2S tests producing the fastest reactions are those containing L-cysteine and 2-mercaptopyridine rather than thiosulphate (28.6 hours). The original H_2S test (29 hours), that containing L-cysteine and thiosulphate (29.2 hours), and that containing L-cystine rather than thiosulphate (31.2 hours).

The H_2S test with bile salts presented stronger results as the same test prepared with liquid detergent instead, as done by Manja *et al.* (1982). Results came in even slightly faster (about one hour) when the concentration of bile salts was (in a different test modification) increased from 2% to 6%. Both H_2S tests prepared with bile salts instead of detergent yielded a positive reaction to *Citrobacter freundii* ATCC® 8090™, *Proteus mirabilis* ATCC® 43071™, and *Salmonella* Typhimurium NCTC 12023/ ATCC® 14028™ very similar to that demonstrated by the original H_2S test, but reacted additionally to most of the tested *E. coli* strains, apart from *E. coli* NCIMB 10240/ ATCC 23744 when tested with 6% bile. Overall and among all H_2S tests, *Salmonella* Typhimurium NCTC 12023/ ATCC® 14028™,*Proteus mirabilis* ATCC® 43071™ and *Citrobacter freundii* ATCC® 8090™ presented the strongest and fastest positive reactions.

Table 4 Type of reaction in comparison to organism, concentration, and time (incubation temp. 20°C) – 1st part

Table 5 Type of reaction in comparison to organism, concentration, and time (incubation temp. 20°C) – 2nd part

Validation of the original and modified versions of the H_2S test against the internationally accepted standard methods for the microbial assessment of drinking-water

This step involved evaluating the performance of the original H_2S test and its variants against water samples of unknown microbial and chemical composition from the environment. This is in compliance with ‘standard methods’ for the examination of water and wastewater (APHA, 2012; EPA, 2009). This step was necessary to assess how the original H_2S test and its new modifications function under different environmental and field conditions, and especially with different types of waters: unimproved vs. improved.

With regards to the results collected above on sensitivity, the analysis of the various H_2S test modifications has been reduced to eight H_2S tests including the original H_2S test:

- Original H_2S test
- H_2S test with L-cysteine/ no thiosulphate
- H_2S test with L-cysteine and thiosulphate
- H_2S test with 2% bile salts instead of detergent
- H_2S test with 6% bile salts instead of detergent
- H_2S test with L-cystine instead of thiosulphate
- H_2S test with penicillin G
- H_2S test with 2-mercaptopyridine

The results from the seven H_2S test modifications and the original test against the accepted standard methods (membrane-filtration with semi-selective media for presumptive thermotolerant faecal coliforms and *Enterococcus*) and by calculating and analysing the positive-predictive (PPV) and the negative-predictive value (NPV) are presented in Tables 6-7 below.

Diagnostic sensitivity versus diagnostic specificity of the H_2S tests analysed at 20°C & 37°C against membrane filtration with m-FC

The analysis of the diagnostic sensitivity and the diagnostic specificity of the eight H_2S tests, tested at an incubation temperature of 20˚C, against the membrane filtration method with m-FC culture medium (standard method), demonstrated that all H_2S test versions, including the original H_2S test, showed a diagnostic specificity of 100% and a PPV of 100% (see Table 6). Since the specificity reflects the 'true negative' value, these results reveal that all H_2S test version analysed at ambient temperature can predict the absence of any thermotolerant faecal coliforms as accurately as the membrane filtration method with m-FC culture medium. Thus, when the membrane filtration method showed a negative result, all different H_2S tests showed a negative result.

The use of 2% bile salts instead of detergent (No. 4) and L-cystine instead of thiosulphate (No. 8) produced a diagnostic sensitivity and specificity for both of 100%. The modifications were as accurate and reliable as the membrane filtration method with m-FC. The original H_2S test (No. 1), the H_2S test with L-cysteine and thiosulphate (No. 3), and the H_2S test with penicillin G (No. 6) had the second-best performance against standard methods, with a sensitivity of 97.4% and a NPV of 66.7%.

In contrast, at an incubation temperature of 37˚C, most H_2S test versions including the original H_2S test, showed a diagnostic specificity of 100% and a PPV of 100%. That

containing 6% bile salts (No. 5), presented a specificity of 50% and a PPV of 97.4 (see Table 6). Interestingly, it was observed that, compared to the H_2S tests performed at 20˚C, the diagnostic sensitivity increased considerably at 37˚C incubation temperature for most of the different H_2S test modifications. The test with 2% bile salts (No. 4) and the test with L-cystine (No. 8) demonstrated a diagnostic sensitivity and specificity of 100%. The test containing L-cysteine and the original H_2S test at 37˚C, showed a level of sensitivity of only 55.3%.

Table 6 Comparison of diagnostic sensitivity, diagnostic specificity, positive-predictive, and negative-predictive values against membrane filtration with m-FC and against different H_2S tests modifications incubated at 20˚C & 37°C

Diagnostic sensitivity versus diagnostic specificity of the H_2S tests analysed at 20°C & 37°C against membrane filtration with m-Enterococcus

The analysis of the diagnostic sensitivity and specificity of the eight H_2S tests, at 20˚C, versus the membrane filtration method with m-Enterococcus culture medium, demonstrated that all H_2S test versions showed a diagnostic sensitivity of 100% and a NPV of 100% (see Table 7). Since the sensitivity reflects the 'true positive' value, these results reveal that all H_2S test versions performed at 20˚C incubation temperature can predict the contamination with faecal enterococci as accurate as the membrane filtration method with m-Enterococcus culture medium. Therefore, when the membrane filtration method showed a positive result, all the H_2S test versions demonstrated a positive result.

Also, it was observed that, compared to the H_2S tests compared against membrane filtration with m-FC culture medium, the diagnostic sensitivity increased considerably to a value of 100%, and the diagnostic specificity declined extremely.

The specificity, which has an impact on the positive-predictive value, was generally low. The highest specificity was performed using 2-mercaptopyridine with 88.9% and a PPV of 96.6% (No.7), followed by L-cysteine instead of thiosulphate with 55.6% and a PPV of 88.6% (No. 2).

The analysis of the diagnostic sensitivity and the diagnostic specificity of the eight H_2S tests, tested at an incubation temperature of 37˚C, against the membrane filtration method with m-Enterococcus culture medium, demonstrated that most H_2S test versions showed a diagnostic sensitivity of 100% and a negative-predictive value (NPV) of 100%. The only H_2S test version which did not produce a diagnostic sensitivity of 100% was the original H_2S test at 67.7% (see Table 7). Similarly to the analysis at 20°C, it was observed that whilst the diagnostic sensitivity increased to 100%, the diagnostic specificity declined significantly.

The highest specificities was performed by the original H_2S test at 100% and a PPV of 100% (No.1), and the H_2S test containing 6% bile salts at 44.4% and a PPV of 86.1% (No. 5), and the H_2S test with L-cysteine at 33.3% and a PPV of 83.8%.

Table 7 Comparison of diagnostic sensitivity, diagnostic specificity, positive-predictive, and negative-predictive values against membrane filtration with m-Enterococcus and against different H_2S tests modifications incubated at 20˚C & 37˚C

Discussion

Testing with pure cultured strains of bacteria

Data from 20 pure culture strains used in the original H_2S test and its 20 modifications reveal that the original H_2S test's positive reaction is triggered only when the sample contains *Citrobacter freundii*, *Proteus mirabilis*, and/or *Salmonella enterica* and Typhimurium. This indicates that the original H_2S test is not able to detect thermotolerant faecal coliforms or FIB other than *Citrobacter freundii*, which also is an organism commonly found in the aquatic and soil environment. All of the tested *E. coli* strains demonstrated the production of H_2S from sulphur sources other than thiosulphate, as used in the original H_2S test (Manja *et al.* 1982). The preferred sources of sulphur were the amino acids L-cysteine and L-cystine. *E. coli* NCTC 5933 in media containing L-cysteine in combination with 2-mercaptopyridine produced results at ambient temperature (20°C) after 18 hours. These novel findings are of note because it is generally accepted that *E. coli* do not normally produce H_2S (Madigan *et al.*, 2009; Holt *et al.*, 1994).

Although the addition of L-cysteine and/or L-cystine appeared to increase the level of specificity for *E. coli*, this effect was not observed with pure cultured *V. cholerae* (el Tor strain NCTC 10256). Also, L-glutathione can be synthesised from L-cysteine, and L-cystine is the reduced form of L-cysteine, however the H_2S test modification containing L-glutathione showed no reaction to any organism used in this study. Gram *et al.* (1987) and Colwell (1970) suggested that L-cysteine would be utilised by *Vibrionaceae* to produce H_2S. Yet, this effect was not observed under the growth conditions provided by the H_2S test.

Clostridium perfringens reacted to the test which contained only L-cysteine. At ambient temperature the reaction started at between 22 and 40 hours, but growth was very slow, and the reaction was not completed before 72h. *Klebsiella pneumoniae* reacted weakly after between 40 and 46 hours to the modification containing L-cysteine only. These findings are in contrast to the findings of Martins *et al.* (1997) and Castillo *et al.* (1994) who argue, and with regards to finding *Clostridium* spp. in their analysed raw water samples, that *Clostridium* spp. could be the cause of the H_2S test's positive reaction. However these

457 analyses assessed the organisms in raw water samples, rather than pure cultures, so it is
458 not possible to infer which organism was the cause of a positive test reaction.
459 Both H_2S tests with bile salts presented stronger results as the same test prepared
460 with liquid detergent instead, as done by Manja *et al.* (1982). Results came in even slightly
461 faster (about one hour) when the concentration of bile salts was increased from 2% to 6%.
462 This is not surprising, as only few enteric bacteria including *Salmonella* spp. and *E. coli* are
463 known to tolerate such high levels of bile. Positive reactions were only observed by
464 *Citrobacter freundii* ATCC® 8090™, *Proteus mirabilis* ATCC® 43071™, and *Salmonella*
465 Typhimurium ATCC® 14028™, similar to the original H_2S test, but reacted additionally to
466 most of the tested *E. coli* strains, apart from *E. coli* ATCC 23744 when tested with 6% bile.
467 This finding contributes to the process of making the H_2S test specific to FIB, by
468 simultaneously reducing cross-reactions due to other organisms.
469 Only Grant & Ziel (1996) attempted to analyse the H_2S test's specificity by testing
470 with pure cultures. Therefore, results from other studies which used cultures with unknown
471 compositions of organisms to establish a level of specificity (Izadi *et al.*, 2010; Tambekar *et*
472 *al.*, 2008; Tambekar *et al.*, 2007a & 2007b; Gupta *et al.*, 2007) Hirulkar & Tambekar 2006;
473 Nair *et al.*, 2001; Pillai *et al.*, 1999; Martins *et al.*, 1997; were not taken into consideration.
474 The analysis of sensitivity (time-to-reaction) and the limit of detection of the original
475 H_2S test and its modifications indicate that for *E. coli,* the most sensitive results came from
476 the modified H_2S test containing penicillin G, the H_2S test containing L-cysteine and 2-
477 mercaptopyridine, and from the H_2S test containing L-cystine. Moreover, all *E. coli* strains
478 (apart from *E. coli* ATCC 23744) revealed a limit of detection as low as one CFU in the H_2S
479 tests containing penicillin or L-cysteine + 2-mercaptopyridine. It should be highlighted that
480 the sensitivity is considerably higher at 37°C than at ambient temperature (20°C).
481 Generally, the reaction time (the time until the production of a black precipitate) of
482 pure cultures was considerably (about 12 hours) longer compared to mixed cultures
483 (undiluted raw water from the River Ouse). However, this finding is not necessarily surprising
484 when considering that unimproved or unprocessed water usually contains a large number of
485 different organisms, which possibly could trigger a positive test reaction.
486 Other sensitivity related research that has been conducted with fresh water samples
487 containing unknown organisms at unknown concentrations are problematic for comparison
488 as it is very difficult to account for all viable types of bacteria and their concentrations from a
489 fresh water sample. Additionally when testing mixed cultures, it is impossible to identify
490 which organism caused what reaction. It is therefore that in method development and quality
491 assurance (QA) pure cultures are used only to establish the limit of detection of a new
492 method. It could be argued that pure strains are biased, because they don't reflect the
493 complex microbial ecology present in fresh water sources. However, this approach is against

any biological quality control and method testing protocol, which clearly state that only pure strains can be used for method testing; including standard methods (APHA, 2012).

Validation against accepted international standard methods

The analysis of the validation against membrane filtration with m-FC and m-Enterococcus medium revealed that the eight H_2S test versions, and their diagnostic sensitivity and specificity, exhibited the best correlation with me at incubation temperatures of 20˚C and 37˚C. The best correlation the majority of the H_2S test versions was observed at 37˚C. This might require the use of an incubator in the field, although ambient temperatures in tropical environments are usually above 20°C.

At 37˚C, most H_2S test versions including the original H_2S test, showed a diagnostic specificity of 100% (PPV of 100%). However, the test containing L-cysteine and the original H_2S test at 37˚C, showed a level of sensitivity of only 55.3%. Consequently, the original H_2S test seems to have a lower diagnostic sensitivity and a lower negative-predictive value (10.5%) at 37˚C when compared to membrane filtration with m-FC culture medium therefore, the lowest overall performance when compared to standard methods was demonstrated by the original H_2S test. Since the H_2S test with 2% bile salts and the H_2S test with penicillin G presented the highest analytical specificity to the five tested *E. coli* strains, these two modifications show the best overall performance, and a much better performance as compared to the original H_2S test.

The higher correlation with standard methods for thermotolerant faecal coliforms than faecal enterococci is likely explained by the finding that the majority of faecal coliforms produce H_2S depending on the type of sulphur available. In contrast, faecal enterococci do not produce H_2S. However, both types of organisms are classified as faecal indicator bacteria (FIB) (WHO, 2017[b]).

Interestingly, it was observed that, compared to the H_2S tests compared against membrane filtration with m-FC culture medium, the diagnostic sensitivity increased considerably to a value of 100%, and the diagnostic specificity declined extremely. This pattern of performance could be explained by the fact that faecal enterococci are not able to produce H_2S. Although, faecal enterococci such as *Enterococcus faecalis* indicate the contamination of water with faecal matter, same as thermotolerant faecal coliforms, it does not necessarily mean that when the membrane filtration method with m-Enterococcus detects no viable cultures, that the H_2S test method does the same, simply because the function of the H_2S test is not adapted to detect the biochemical reactions of faecal enterococci.

H_2S test modifications containing L-cysteine and thiosulphate, 2% bile salts penicillin G, 2-mercaptopyridine, and L-cystine showed 100% diagnostic sensitivity and specificity at 37°C, and were as reliable as the standard method performed with m-FC culture medium. However, only 2% bile salts, and L-cystine were as reliable as standard methods at both 20°C and 37°C, and can therefore also be performed at lower temperature without compromising its reliability. This finding is similar to the results of Gupta *et al.* (2007) using 2% bile salts producing a sensitivity of between 62% and 76%, and a specificity of 97% after 24h. Interestingly, after 48h, the sensitivity rises to between 82% and 93%, but the specificity drops to 80% compared to standard methods. After 72h the level of specificity was reduced to 58%. This could be an indication of the production of H_2S by organisms other than indicator bacteria or faecal coliforms.

Weppelmann *et al.* (2014) observed a sensitivity of 64.9% and a specificity of 93.3%, (PPV of 81.9 and a NPV of 85.3) using a 20 ml sample in the PathoScreen™ H_2S test (Hach Company). The PathoScreen™ H_2S test exhibits a much lower diagnostic sensitivity and a lower level of diagnostic specificity than the modifications discussed here. There are no data available on the validation of the operational range of the PathoScreen™ H_2S test so it is not possible to make a robust comparison of this commercially produced H_2S test against these modifications to the H_2S test.

McMahan *et al.* (2012) tested a modified protocol for the PathoScreen™ H_2S test. They used a 100ml sample volume with the MPN method, versus spread plating on a range of different selective culture media, and terminal restriction fragment length polymorphisms molecular analysis (TRFLP). They detected an average sensitivity of 100% and a specificity of 80% using their modified Pathoscreen™ H_2S test. However, the total number of natural water samples from a comparable open water source was only n=12, which may not be an adequate sample number. Also, the McMahan *et al.* (2012) study is difficult to compare to this study, since none of the methods described are internationally accepted standard methods for the examination of drinking-water.

Huang *et al.* (2011) tested the original H_2S test against membrane filtration and suggested a sensitivity of between 66% and 88%, and a specificity of between 72% and 100%. This suggests a higher level of specificity, and a lower level of sensitivity when compared to the results gathered from this study. Unfortunately, the authors only referred to testing for total coliforms incl. *E. coli*, but not the type of culture medium used, which makes a comparison to findings from this study difficult.

Sensitivity and specificity of the original H_2S test and its modifications citied in all previous studies in the literature review were much lower when compared to standard methods. However, this study demonstrates that when compared to membrane filtration on m-FC medium specific H_2S test modifications had similar sensitivity and specificity.

Following analysis of 20 modifications to the original H_2S test using pure cultures of confirmed strains, we show that all modifications tested in this study react positively only to enteric and coliform bacteria. Therefore, it can be assumed that the modified H_2S tests are as reliable as most internationally accepted methods testing for total coliforms.

Conclusion

There is generally a difference between a test's level of diagnostic sensitivity and specificity, depending on the type of water, the incubation time and temperature, and the standard method used for comparison. However, he results from this study suggest the novel H_2S test with the addition of 2% bile salts and the test with L-cystine produced a better performance overall at both 20°C and 37°C when compared to the original H_2S test. Furthermore, both modified tests meet the specifications of standard methods. These two H_2S test modifications therefore can be regarded as a methodological improvement to the analysis of the microbial quality of drinking-water in low-resource settings. The two modifications of the H_2S test needed less time for a positive reaction to take place resulting in a lower time-to-result value. Finally, this research has identified the ability of 20 different enteric bacterial species to produce H_2S from defined substrates.

Further, three modifications (2% bile salts, penicillin G, and L-cysteine and 2-mercaptopyridine) reacted positively not only with *Citrobacter freundii*, *Proteus mirabilis*, and *Salmonella* spp., but also to five different strains of *E. coli*, including pathogenic *E. coli* O157:H7. This is the first time modifications to the original H_2S test have been successfully developed for the detection of the faecal-indicator bacteria *E. coli* and one of its pathogenic strains.

These findings demonstrate the ability of *E. coli* to produce H_2S under defined conditions and the potential of a modified H_2S test to be used as a substitute for membrane-filtration with m-FC culture for water quality assessments in medium in low-resource settings.

Figures and Tables with captions

Figure 1 Filter paper stripes inoculated with H_2S culture medium

Figure 2 H_2S test tubes ready for use

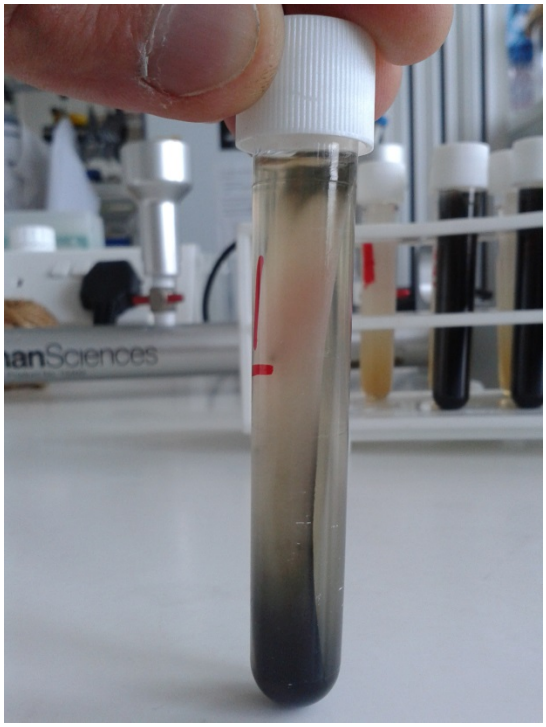

Figure 3 10ml culture tube incl. positive H_2S test paper stripe

607 **Table 1. Different types of H_2S test modifications used in this study incl. their formulae and reagents**

No.	Type of H2S test	Reagents different to original H_2S test formula	Reaction intended	Original reference for intended reaction
1	H_2S test without sodium thiosulphate, but with L-cysteine	Included: 2.0g L-cysteine Excluded: 1.0g Sodium thiosulphate	Increased bioavailability of sulphur source for many *Enterobacteriaceae*. Facilitating cysteine desulphurases and enabling H_2S production.	Hidese *et al.* (2011); Mihara & Esaki (2002)
2	H_2S test with added L-cysteine	Included: 0.25g L-cysteine	Increased bioavailability of sulphur source for many *Enterobacteriaceae*. Facilitating cysteine desulphurases and enabling H_2S production.	Shahryari *et al.* 2014; Hidese *et al.* (2011); Mihara & Esaki (2002)
3	H_2S test without detergent, but with added bile-salts (2%)	Included: 1.0g bile salts Excluded: 1ml liquid detergent	Increased selectivity through inhibiting the growth of unwanted organisms. *Clostridium perfringens*, *E. coli*, *Listeria monocytogenes*, *Salmonella* spp. are regarded as very bile tolerant.	Tambekar *et al*. (2007); Begley *et al.* (2005); Manja *et al*. (2001)
4	H_2S test without detergent, but with added bile-salts (6%)	Included: 3.0g bile salts Excluded: 1ml liquid detergent	Increased selectivity through inhibiting the growth of unwanted organisms. *Clostridium perfringens*, *E. coli*, *Listeria monocytogenes*, *Salmonella* spp. are regarded as very bile tolerant.	Begley *et al.* (2005)
5	H_2S test with added L-cysteine and citric acid	Included: 0.25g L-cysteine, 0.2g citric acid, 1.0g bile salts	Supports the growth of some Gram-negative, H_2S producing, and coliform bacteria.	Holt *et al.* (1994)
6	H_2S test with added citric acid	Included: 0.2g citric acid	Supports the growth of some Gram-negative, H_2S producing, and coliform bacteria.	Holt *et al.* (1994)

7	H_2S test with added gentamicin	Increased: 1.5g to 3.0g Dipotassium hydrogen phosphate Included: 0.005g gentamicin Excluded: 1ml liquid detergent	Gentamicin is selective for Gram-positive bacteria, and only allows Streptococci (groups A, B, C, D, and G) and *Clostridium* spp. to be cultivated.	Atlas (2010)
8	H_2S test with added penicillin G	Included: 0.25g L-cysteine, 0.05g penicillin-G Excluded: 1ml liquid detergent	Penicillin-G is selective for many Gram-negative bacteria, and inhibits most Gram-positive bacteria apart from *E. faecalis* and *E. faecium*.	Atlas (2010)
9	H_2S test with L-cysteine and 2-mercaptopyridine	Included: 0.25g L-cysteine, 0.1g 2-mercaptopyridine, 1.0g bile salts Excluded: 1ml liquid detergent	Mercaptopyridine supports sulphur transferase activity in *E. coli*.	Mikami *et al.* (2011)
10	H_2S test with added D-cysteine	Included: 0.1g D-cysteine, 0.001 biotin, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	D-cysteine is an organic sulphur source. Increased bioavailability of sulphur source for many *Enterobacteriaceae*. Facilitating D-cysteine desulphurase and enabling H_2S production.	Ellis *et al.* (1964)
11	H_2S test with added L-cystine	Included: 1.0g L-cystine, 0.001g biotin, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	L-cystine is an organic sulphur source and reduced form of L-cysteine. Increased bioavailability of sulphur source for many *Enterobacteriaceae*. Facilitating cystine desulphurase activity and enabling H_2S production.	Pathak *et al.* (2005); Pant *et al*. (2002); Venkobachar *et al.* (1994); Lautrop *et al*. (1971), Jones-Mortimer *et al.* (1968), Tanner, F.W. (1917)

12	H_2S test with ferrous chloride substituted for ferrous citrate	Included: 0.75g Ferrous chloride, 1.0g bile salts Excluded: 0.75g Ferrous citrate, 1ml liquid detergent	Ferrous chloride reacts more sensitive to hydrogen sulphide as compared to ferrous citrate.	Barrett & Clark (1987)
13	H_2S test with added L-cysteine and pyruvate	Included: 1.0g L-cysteine, 0.001g biotin, 0.5g sodium pyruvate, 1.0g bile salts Excluded: 1ml liquid detergent	Increased bioavailability of sulphur source for many *Enterobacteriaceae*, supporting H_2S production.	Artman, M. (1956), Delwiche E.A. (1951)
14	H_2S test with taurine substituted for sodium thiosulphate	Included: 0.75g Taurine, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	Increased bioavailability of sulphur source for many *Enterobacteriaceae*, enabling H_2S production.	Ellis *et al.* (1964), Tanner, F.W. (1917)
15	H_2S test with added L-methionine	Included: 1.0g L-methionine, 0.001g biotin, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	L-methionine is an organic sulphur source. Increased bioavailability of sulphur source for many *Enterobacteriaceae,* enabling H_2S production. Biotin stimulates desulphurase activity.	Ellis, R.J. (1966), Ellis *et al.* (1964), Delwiche E.A. (1951)
16	H_2S test with added L-glutathione	1.0g L-glutathione reduced, 0.5g sodium pyruvate, 0.1g 2-mercaptopyridine, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	L-glutathione is an organic sulphur source. Increased bioavailability of sulphur source for many *Enterobacteriaceae*, enabling H_2S production.	Ellis, R.J. (1966), Ellis *et al.* (1964)
17	H_2S test with added pyruvate	Included: 0.5g Sodium pyruvate, 1.0g bile salts Excluded: 1ml liquid detergent	Increased bioavailability of sulphur source for many *Enterobacteriaceae,* supporting H_2S production.	Artman, M. (1956), Delwiche E.A. (1951)

18	H_2S test with added sodium sulphate and pyruvate	Included: 1.5g Sodium sulphate, 0.05g sodium pyruvate, 1.0ml bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	Increased bioavailability of sulphur source for many *Enterobacteriaceae*, enabling H_2S production.	Ellis, R.J. (1966), Delwiche E.A. (1951)
19	H_2S test with added tetrathionate	Included: 1.0g Sodium tetrathionate, 0.001g biotin, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	Increased bioavailability of sulphur source for many *Enterobacteriaceae*. Facilitating tetrathionate reductase enabling H_2S production in *E. coli*. Biotin stimulates desulphurase activity.	Barrett & Clark (1987), Lautrop et al. (1971), Delwiche E.A. (1951)
20	H_2S test with added L-cystine and L-glutathione	Included: 1.0g L-cystine, 0.25g L-glutathione reduced, 0.001g biotin, 1.0g bile salts Excluded: 1.0g Sodium thiosulphate, 1ml liquid detergent	L-glutathione is an organic sulphur source. Increased bioavailability of sulphur source for many *Enterobacteriaceae*, enabling H_2S production. Biotin stimulates desulphurase activity.	Kredich, N.M. (1971), Jones-Mortimer *et al.* (1968), Ellis *et al.* (1964), Delwiche E.A. (1951)

Table 2. Reaction and H_2S production of pure cultures of target and non-target organisms to the original H_2S tests and its new modifications – 1st part of results

No. of H_2S test version*	-	1	2	3	4	5	6	7	8	9
Organism	Original H_2S test (Manja *et al.* 1982)	H_2S test with L-cysteine instead of thiosulphate	H_2S test with L-cysteine and thiosulphate	H_2S test with bile salts (2%) instead of detergent	H_2S test with bile salts (6%) instead of detergent	H_2S test with L-cysteine and citric acid	H_2S test with citric acid	H_2S test with gentamicin	H_2S test with penicillin G	H_2S test with L-cysteine and 2-mercaptopyridine
C. difficile ATCC® 9689™	-	-	-	-	-	-	-	(+)	(+)	-

C. freundii ATCC® 8090™	(+)	+	+	+	+	+	+	+	+	+
E. coli NCIMB 10240/ ATCC 23744	-	-	-	-	-	-	-	-	(+)	(+)
E. coli O157:H7 NCTC 12900	-	+	+	+	(+)	(+)	-	-	(+)	+
E. coli NCTC 10418	-	(+)	(+)	+	+	-	-	-	(+)	(+)
E. coli NCTC 5933	(+)	(+)	+	+	+	-	-	+	+	+
E. coli (River Ouse)	(+)	(+)	+	+	(+)	-	-	-		+
E. tarda NCIMB 2056	-	-	-	-	-	-	-	-	-	-
K. pneumoniae (River Ouse)	-	(+)	-	-	-	-	-	-	-	-
P. mirabilis ATCC® 43071™	+	-	+	+	+	+	+	+	+	+
S. enterica ATCC® 13076™	-	(+)	+	+	+	+	(+)	(+)	+	+
***S.* Typhimurium** NCTC 12023/ ATCC® 14028™	+	+	+	+	+	-	+	+	+	+
V. cholerae NCTC 10256	-	-	-	-	-	-	-	-	-	(+)

Y. enterocolitica ATCC® 9610™	-	+	-	-	-	-	-	-	-	(+)

612 + = H_2S positive, (+) = H_2S weakly positive, - = H_2S negative, organisms stated in red font are faecal indicator bacteria, * = No. of test versions corresponding
613 to Table 1

614
615 **Table 3. Reaction and H_2S production of pure cultures of target and non-target organisms to the original H_2S tests and its new modifications – 2nd**
616 **part of results**

No. of H_2S test version*	10	11	12	13	14	15	16	17	18	19	20
Organism	H_2S test with D-cysteine	H_2S test with L-cystine	H_2S test with ferrous chloride instead of ferrous citrate	H_2S test with L-cysteine and pyruvate	H_2S test with taurine instead of thiosulphate	H_2S test with L-methionine	H_2S test with L-glutathione	H_2S test with pyruvate	H_2S test with sodium sulphate and pyruvate	H_2S test with tetrathionate	H_2S test with L-cystine and L-glutathione
C. difficile ATCC® 9689™	-	-	-	-	-	-	-	-	-	(+)	-
C. freundii ATCC® 8090™	(+)	+	(+)	-	(+)	(+)	(+)	(+)	-	(+)	(+)
E. coli NCIMB 10240/ ATCC 23744	-	+	-	-	-	-	-	-	-	-	-
E. coli O157:H7 NCTC 12900	-	+	(+)	-	-	-	-	-	-	-	-
E. coli NCTC 10418	-	(+)	-	-	-	-	-	-	-	-	-
E. coli NCTC 5933	-	+	-	-	-	-	-	-	-	-	-

E. coli (River Ouse)	-	(+)	-	-	-	-	-	-	-	-	-
E. tarda NCIMB 2056	-	-	-	-	-	-	-	(+)	-	-	-
K. pneumoniae (River Ouse)	-	-	-	-	-	-	-	-	-	-	-
P. mirabilis ATCC® 43071™	(+)	+	(+)	-	(+)	(+)	(+)	+	+	(+)	-
S. enterica ATCC® 13076™	(+)	+	+	-	(+)	(+)	-	-	(+)	(+)	-
***S.* Typhimurium** NCTC 12023/ ATCC® 14028™	(+)	+	(+)	-	(+)	(+)	-	(+)	(+)	-	-
V. cholerae NCTC 10256	-	-	-	-	-	-	-	-	-	-	-
Y. enterocolitica ATCC® 9610™	-	-	-	-	-	-	-	(+)	-	-	-

+ = H_2S positive, (+) = H_2S weakly positive, - = H_2S negative, organisms stated in red font are faecal indicator bacteria, * = No. of test versions corresponding to Table 1

Table 4. Type of reaction in comparison to organism, concentration, and time (incubation temp. 20°C) – 1st part

Organism	No. of CFU	H_2S test (Manja *et al.* 1982)	H_2S test with L-cysteine instead of thiosulphate	H_2S test with L-cysteine and thiosulphate	H_2S test with bile salts (2%)	H_2S test with bile salts (6%)

		Type of reaction and time (hours)					Type of reaction and time (hours)					Type of reaction and time (hours)					Type of reaction and time (hours)					Type of reaction and time (hours)				
		< 12	12	18	24	48	< 12	12	18	24	48	< 12	12	18	24	48	< 12	12	18	24	48	< 12	12	18	24	48
E. coli ATCC 23744	4,761	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli NCTC 10418	1,225	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli NCTC 5933	250,000	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
E. coli (River Ouse)	15,625	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
	125	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli O157:H7 NCTC 12900	42,025	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
	205	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. freundii ATCC 8090	420	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+
	20	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+

	1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
P. mirabilis ATCC 43071	36,100	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+
	90	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
S. Typhimurium ATCC 13076	28,900	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	170	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

622 + = H2S positive, (+) = H2S weakly positive, - = H2S negative, organisms stated in red font are faecal indicator bacteria

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624 **Table 5. Type of reaction in comparison to organism, concentration, and time (incubation temp. 20°C) – 2nd part**

Organism	No. of CFU	H_2S test with L-cysteine and citric acid					H_2S test with penicillin					H_2S test with L-cysteine and 2-mercaptopyridine					H_2S test with D-cysteine					H_2S test with L-cystine				
		Type of reaction and time (hours)					Type of reaction and time (hours)					Type of reaction and time (hours)					Type of reaction and time (hours)					Type of reaction and time (hours)				
		< 12	12	18	24	48	< 12	12	18	24	48	< 12	12	18	24	48	< 12	12	18	24	48	< 12	12	18	24	48
E. coli ATCC 23744	4,761	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	69	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli NCTC 10418	1,225	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	35	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+

	1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli NCTC 5933	250,000	-	-	-	-	+	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+
	500	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
E. coli (River Ouse)	15,625	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	125	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
E. coli O157:H7 NCTC 12900	42,025	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	+	+	+
	205	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
C. freundii ATCC 8090	420	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+
	20	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
	1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
P. mirabilis ATCC 43071	36,100	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	90	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
	1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
S. Typhimurium ATCC 13076	28,900	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	170	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	1	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

625 + = H_2S positive, (+) = H_2S weakly positive, - = H_2S negative, organisms stated in red font are faecal indicator bacteria

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Table 6 Comparison of diagnostic sensitivity, diagnostic specificity, positive-predictive, and negative-predictive values against membrane filtration with m-FC and against different H_2S tests modifications incubated at 20˚C & 37˚C

	No.	H_2S test	Sensitivity %	Specificity %	PPV %	NPV %
20˚C	1	Original H_2S test	97.4	100	100	66.7
	2	H_2S test with L-cysteine/ no thiosulphate	92.1	100	100	40.0
	3	H_2S test with L-cysteine & thiosulphate	97.4	100	100	66.7
	4	H_2S test with bile salts 2%	100	100	100	100
	5	H_2S test with bile salts 6%	94.7	100	100	50
	6	H_2S test with penicillin G	97.4	100	100	66.7
	7	H_2S test with 2-mercaptopyridine	84.2	100	100	25.0
	8	H_2S test with L-cystine	100	100	100	100
37˚C	1	Original H_2S test	55.3	100	100	10.5
	2	H_2S test with L-cysteine/ no thiosulphate	97.4	100	100	66.7
	3	H_2S test with L-cysteine & thiosulphate	100	100	100	100
	4	H_2S test with bile salts 2%	100	100	100	100
	5	H_2S test with bile salts 6%	100	50	97.4	100
	6	H_2S test with penicillin G	100	100	100	100
	7	H_2S test with 2-mercaptopyridine	100	100	100	100
	8	H_2S test with L-cystine	100	100	100	100

Table 7 Comparison of diagnostic sensitivity, diagnostic specificity, positive-predictive, and negative-predictive values against membrane filtration with m-Enterococcus and against different H_2S tests modifications incubated at 20˚C & 37˚C

	No.	H_2S test	Sensitivity %	Specificity %	PPV %	NPV %
20˚C	1	Original H_2S test	100	33.3	83.8	100
	2	H_2S test with L-cysteine/ no thiosulphate	100	55.6	88.6	100

	3	H_2S test with L-cysteine & thiosulphate	100	33.3	83.8	100
	4	H_2S test with bile salts 2%	100	22.2	81.6	100
	5	H_2S test with bile salts 6%	100	44.4	86.1	100
	6	H_2S test with penicillin G	100	33.3	83.8	100
	7	H_2S test with 2-mercaptopyridine	100	88.9	96.9	100
	8	H_2S test with L-cystine	100	22.2	81.6	100
37˚C	1	Original H_2S test	67.7	100	100	47.4
	2	H_2S test with L-cysteine/ no thiosulphate	100	33.3	83.8	100
	3	H_2S test with L-cysteine & thiosulphate	100	22.2	81.6	100
	4	H_2S test with bile salts 2%	100	22.2	81.6	100
	5	H_2S test with bile salts 6%	100	44.4	86.1	100
	6	H_2S test with penicillin G	100	22.2	81.6	100
	7	H_2S test with 2-mercaptopyridine	100	22.2	81.6	100
	8	H_2S test with L-cystine	100	22.2	81.6	100

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Conflict of interest

The authors declare no conflict of interest.

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