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Selection of a diagnostic tool for microbial water quality monitoring and management of faecal contamination of water sources in rural communities

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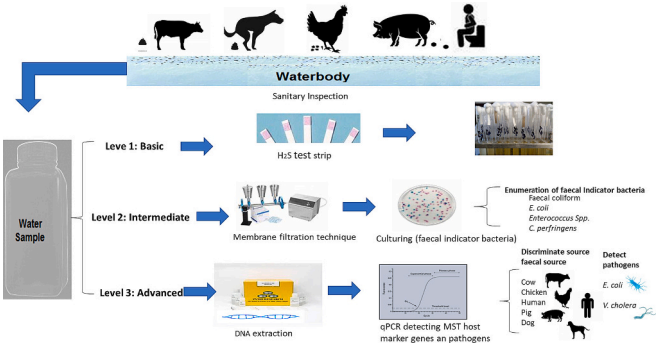
HIGHLIGHTS

- Use of faecal contaminated water from unimproved water sources
- Microbial water quality monitoring in rural communities
- Correlation between H_2S test, sanitary risk, faecal indicator bacteria, pathogens, and MST genetic markers
- H_2S test and sanitary inspections are suitable tools for monitoring water quality in rural areas at the household level.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the current study was to evaluate, validate and select microbial water quality monitoring tools to establish their suitability and feasibility for use in rural communities. The monitoring of water quality was performed at three different levels: i) basic level focusing on sanitary inspection and hydrogen sulphide (H_2S) test; ii) intermediate level via enumeration of faecal indicator bacteria (faecal coliforms, *Escherichia coli*, *Enterococcus* spp. and *Clostridium perfringens*); and iii) advanced level based on qPCR detecting host-associated genetic markers (*BacHum*, *BacCow*, *Cytb*, *Pig-2-Bac*, and *BacCan*) and pathogens (*Vibrio cholerae*, *Escherichia coli* O157:H7, and Shiga toxin-producing *Escherichia coli*). A positive correlation was recorded between sanitary risk and faecal coliforms ($r = 0.613$ and $p < 0.002$), *E. coli* ($r = 0.589$ and $p < 0.003$), and *Enterococcus* spp. ($r = 0.625$ and $p < 0.003$). The H_2S test showed positive correlations with sanitary risk score ($r = 0.623$; $p < 0.003$), faecal coliforms ($r = 0.809$; $p < 0.001$), *E. coli* ($r = 0.779$; $p < 0.001$) and *Enterococcus* spp. ($r = 0.799$; $p < 0.001$). Similar correlation patterns were also found with advanced techniques used for detecting host-associated genetic markers, excepted between *Clostridium perfringens*, and *Pig-2-Bac* (pig), *BacCan* (dog), and *V. cholerae*. The H_2S test and sanitary inspections are therefore suitable and cost-effective tools to capacitate rural areas at household level for the monitoring of faecal contamination and management of water sources.

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1. Introduction

Monitoring microbial water quality is still a major challenge in many rural communities located in sub-Saharan region. About 3.6 billion people lack access to properly maintained sanitation, and nearly 2 billion people lack safe, easily available water at home (WHO\UNICEF, 2021). By 2030, Sustainable Development Goal (SDG) 6 seeks to guarantee that everyone has access to water and sanitation systems that are managed sustainably. To accomplish the other SDG targets, which include lowering water pollution, implementing integrated water resource management, and safeguarding water-related ecosystems, the cost will play a significant role (Ajami et al., 2018). Since community members have local knowledge of potential water sources and possible sources of faecal pollution within those water sources, it is crucial to gather such data, control and prevent water pollution, and conduct microbial water quality assessments at low cost.

In most rural communities of poor countries, including South Africa (19 % of the population, an estimated 3.8 million people), residents still rely on untreated surface water and groundwater from rivers, dams, and lakes, putting their health at risk for waterborne illnesses (Verlicchi and Grillini, 2020; Pandey et al., 2014; Edokpayi et al., 2018; Donnenfeld et al., 2018). For the purpose of providing safe drinking water and enhancing water management, monitoring water quality is essential. Numerous studies have focused on identifying and quantifying the sources of faecal contamination, such as wastewater discharge, agricultural runoff, and inadequate sanitation practices (Tipton et al., 2017; Bradshaw et al., 2016; Dhundale and Tambekar, 2008; Holcomb and Stewart, 2020). Among the various contaminants, faecal contamination poses a particularly grave risk due to its association with numerous waterborne diseases. Conducting a thorough risk assessment of faecal contamination in water resources is crucial for understanding the potential hazards and implementing effective mitigation strategies. This assessment aims to inform policymakers, researchers, and communities about the magnitude of the problem and guide the development of appropriate measures to safeguard water resources.

According to Pachepsky et al. (2018), three stages may be applied in monitoring water quality. The first stage involves screening, which identifies a potential issue, and may be done using sanitary inspections and sanitary survey (by observation and using questionnaires) (WHO, 2017) and hydrogen sulphide test (H_2S paper strip) (Malema et al., 2019). The second stage involves more in-depth studies to confirm a public health risk and support management decisions through the enumeration of faecal indicator bacteria (i.e. membrane filtration technique or Colilert test) (Tok et al., 2019) and pathogen detection (culturing or PCR) (Alhamlan et al., 2015; Zhao et al., 2014). And the third stage involves studies to identify sources of faecal contamination by microbial source tracking (MST) using host-specific Bacteroidales genetic markers (qPCR) (Ahmed et al., 2019) to facilitate the reduction of the health risk through a variety of engineering and policy solutions (Pachepsky et al., 2018). A single method may be enough to assess faecal contamination; yet, when water quality testing methods are combined, it may provide effective assessment of water safety or quality (WHO, 2017). Although Yang et al. (2013) have identified the relationship between FIB and the H_2S test, no study to date has correlated the use of the above-mentioned five methods for the detection of faecal pollution in water resources. The aim of the current study was to evaluate, validate and select the microbial water quality monitoring tools (using a tiered approach to determine faecal pollution in various water resources used for drinking purposes) in order to establish their suitability and feasibility, especially for use in rural communities. The monitoring of water quality in terms of faecal contamination of water sources was performed at three different levels: i) basic level focusing on sanitary inspection and H_2S test; ii) intermediate level via the culture-based method (enumeration of faecal indicator bacteria); and iii) advanced level based on molecular biology (qPCR assays for detecting host-associated genetic markers and pathogens).

2. Materials and methods

2.1. Study site and sample collection

This study was conducted in the Vhembe District (VDM) Municipality in Limpopo Province, South Africa as previously described by Murei et al., in 2022 and 2023 (Murei et al., 2023, 2022). Briefly, water sources under the current study included the Luvuvhu River, Nandoni Dam, Phiphidi Dam, Tshakhuma Dam, Mukhase River, Mutshindudi River, springs, and boreholes. With Luvuvhu river being the largest river with a total capacity of 5941 km^2, it runs approximately 200 Km, while Mutshindudi River and Mukhase River are its tributaries. These sources collectively contribute to the water supply and agricultural development in the VDM, supporting the livelihoods of local communities and sustaining the region's ecosystems. Samples were collected between March 2021 and April 2021 for the wet season and between June 2021 and August 2021 for the dry season. In total, 3064 water samples were collected from surface water sources and groundwater sources (Table S1). Fig. 1 illustrate the study area and sampling points. Water samples were also collected from household taps (at the point of use) and water storage containers. About 64 stool samples of animals and humans were collected for validation of host-specific genetic markers in the study area. All the samples were stored in cooler boxes with ice packs and transported to Tshwane University of Technology (TUT) Water Research Unit Microbiology laboratory where analysis was performed within 24 h.

2.2. Experimental design for microbial water quality analysis for faecal pollution

Different microbial water quality monitoring tools were used to assess and evaluate faecal pollution in water resources of the VDM using a tiered approach. The methods were classified into three levels: i) basic level: sanitary inspection and H_2S test; ii) intermediate level: culture-based method (enumeration of faecal indicator bacteria); and iii) advanced level: molecular biology (qPCR assays for detecting host-associated genetic markers and pathogens). The sanitary survey and qPCR assays for detection of MST genetic markers were performed to determine the major source of faecal contamination, while faecal indicator bacterial counts combined with sanitary risk scores were used to assess the risks related to excretors.

2.3. Sanitary inspection and H_2S test

Sanitary inspection was performed in various water sources (as described by Murei et al. (2023)), and also in households to assess the water safety practice in dwellings. Human and animal activities that may result in contaminating water resources were identified. Structured questionnaires containing closed-ended questions were administered to participants in such a way to obtain responses only by YES or NO, as listed in Table S2. The YES answer indicates the presence of a risk factor, whereas the NO answer indicates the absence of this factor. The hydrogen sulphide strip test was conducted as described by Murei et al. (2023). Briefly, 1 mL of water sample was inoculated into 9 mL of Tryptone Soya Broth (Oxoid, Thermo Scientific, Johannesburg, South Africa), then the H_2S paper strip (Macherey-Nagel, Monitoring & Control Laboratories, Johannesburg, South Africa) was placed above the inoculum secured by cotton wool. Subsequently, the test tubes were then incubated for 18–24 h at 37 °C and H_2S production was observed by colour change of the paper strip from white to black.

2.4. Faecal indicator bacteria (FIB)

Faecal indicator bacteria such as *Escherichia coli*, faecal coliforms, *Enterococcus* spp. and *Clostridium perfringens* were detected in different water sources. Prior to water sample collection, dilutions using

autoclaved distilled water were made. To concentrate *E. coli*, coliform, and *Enterococcus* spp. in water samples, the membrane technique was employed (APHA, 2017). Briefly, 100 mL of water samples were filtered using a vacuum pump through a membrane filtration unit containing a sterile filter with a 47 mm diameter, the filter (Merck, Modderfontein, South Africa) was placed on the nutrient agar plate. The Chromocult® coliform agar (Merck, Modderfontein, South Africa) was used for *E. coli* and coliform bacteria. Chromocult® Enterococci agar (Merck, Modderfontein, South Africa) was used for *Enterococcus* spp. The plates were incubated at 37 °C for 24 h. Coliform bacteria, *E. coli* and *Enterococcus* spp. were characterised by mauve, dark blue to violet and mauve coloured colonies, respectively. For *Clostridium perfringens*, the pour plate method and tryptose sulphite cycloserine (TSC) agar (Thermo Fischer Scientific, Johannesburg, South Africa) were used as described elsewhere (Farhadkhani et al., 2022) Plates were then incubated anaerobically at 37 °C for 24 h. Black or grey colonies are considered as the characteristic of *C. perfringens*. Plates were always prepared in triplicate for each type of organism. For all FIB measurements, sterile distilled water was used as negative control. *Escherichia coli* ATCC 25922, *C. perfringens* ATCC 12915, and *E. faecalis* ATCC 14506 (Analytical Technology, Johannesburg, South Africa) were used as positive controls. The colonies were counted and recorded as CFU/100 mL after 24 h for the test organism.

2.5. Microbial source tracking (MST) markers and pathogen detection

2.5.1. Sample preparation

For drinking water, 300 mL of water sample was filtered using 0.45 μm membrane filters with 65 mm diameter (Merck, Modderfontein, South Africa) to concentrate microorganisms. And for stool samples, about 2–5 mg of stool samples (dry stool) was placed in test tubes containing 5 mL of Brain Heart Infusion Broth (Oxoid Limited, Basingstoke, UK) and incubated overnight to hydrate dry stool and help to revive microorganism growth.

2.5.2. Genomic DNA extraction

Membrane filtration unit was used to concentrate the microorganisms in water samples; about 300 mL were filtered through 0.22 μm cellulite membrane filters (Millipore, Merck, Modderfontein, South Africa) and the DNA was extracted from membrane filters (with trapped microbes) using ZymoBIOMICS DNA extraction kit (Zymo Research, Inqaba Biotechnical Industries, Pretoria, South Africa), following the manufacturer's instructions. Positive controls for Shiga toxin-producing *E. coli* (STEC), *E. coli* O157:H7 and *V. cholerae* were obtained from TUT Research Group Unit Microbiology Laboratory. *Escherichia coli* ATCC 43888 was purchased from Analytical technology, Johannesburg, South Africa. The DNA fragments containing genes of interest were used as positive control for host-specific *Bacteroides* markers. All colonies were isolated and purified for further molecular analysis. The NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Johannesburg, South Africa) was used to confirm the quality and quantity of extracted DNA and extracts were stored at −20 °C.

2.5.3. Quantitative polymerase chain reaction (qPCR) analyses

Quantitative PCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) for amplification of host-

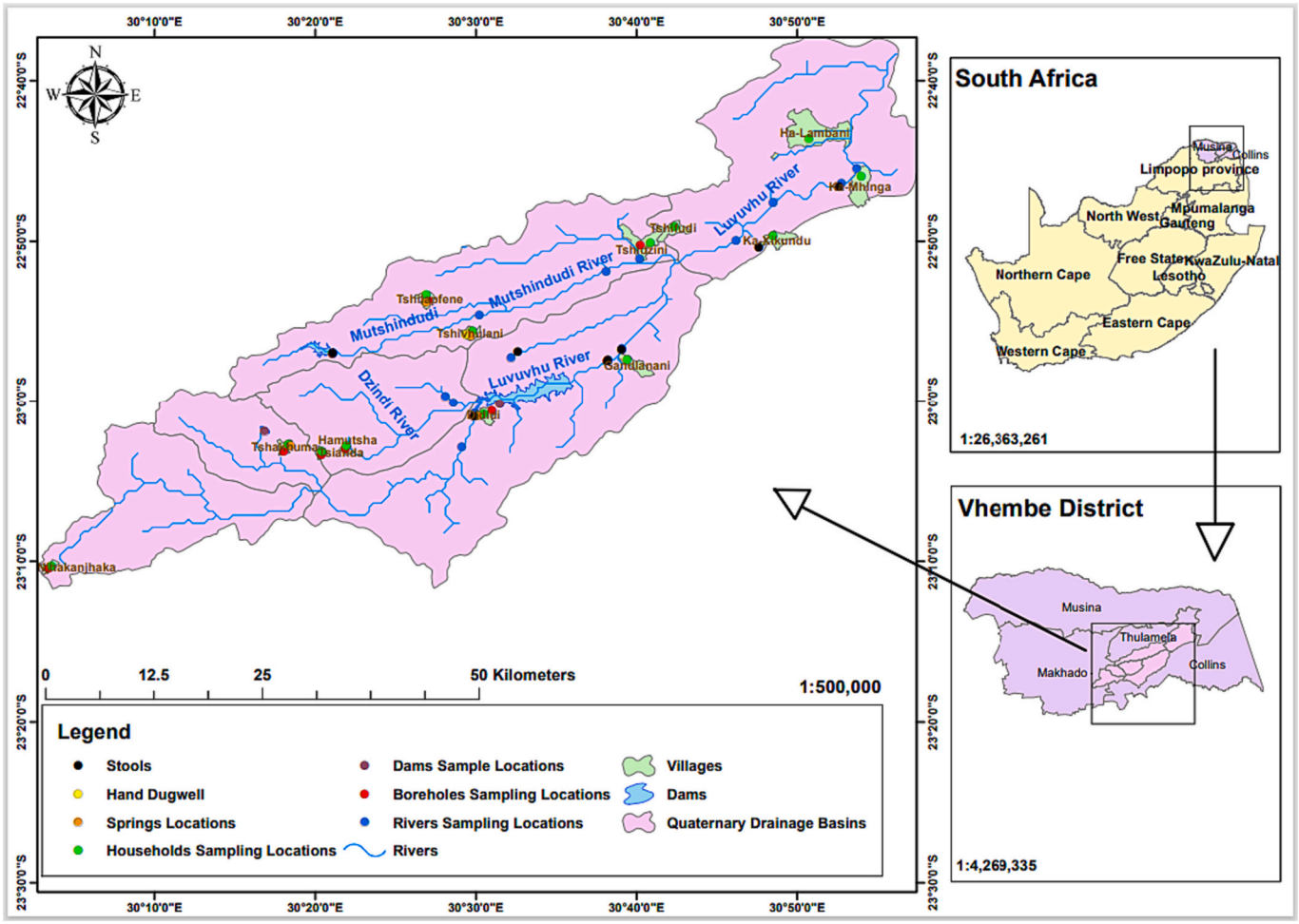

Fig. 1. Map showing study area.

specific genetic markers and the detection of bacterial pathogens. Five TaqMan assays (*BacHum*, *BacCow*, *Pig-2-Bac*, *Cytb*, and *BacCan*) were selected for the detection of human, cow, pig, chicken, and dog associated Bacteroidales in each sample. In addition, three qPCR assays targeting *stx1*, rfb_{O157}, and *OmpW* genes for Shiga toxin-producing *E. coli* (STEC), *E. coli* O157:H7, and *V. cholerae*, respectively, were selected for this study. A five-point 10-fold serially diluted recombinant plasmid DNA with a target sequence was used to generate the standard curve (range 10^1 to 10^5 copies/reaction) in each qPCR assay. The primers, probes and cycling conditions of the target genes used in this study are illustrated in Table S3. All qPCR reactions (20 μL) were performed in triplicate and TaqMan-based qPCR assays were performed with the reaction mixture including 10 μL of Luna universal probe qPCR Master Mix (New England Biolabs, Inqaba Biotechnical Industries, Pretoria, South Africa), 5 μL of template DNA, 3 μL of nuclease-free water and 0.8 μL of each primer and 0.4 μL of probes and 8 μL of the 10-fold diluted target DNA template. For each target marker, a negative control (all PCR reagents except for the template DNA) and a positive control were also added during each PCR run. Positive controls for each pathogen were used to validate whether the primers and probes, the protocol or equipment used can produce the expected results.

2.5.4. Data processing and interpretation of qPCR

All the qPCR assay results were processed based on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). In cases where the R^2 or efficiencies were not achieved by any assay, the samples were tested again. Regression analysis was performed to eliminate outliers by removing cycle threshold (Ct) values with a residual value larger than +3 or smaller than −3 (Schriewer et al., 2013). The limit of detection (LOD) was determined from the standard curve. A cycle threshold value of detected each organism was set as a cut-off point to be able to distinguish this amplification from artefacts or accidental bumps in the baseline, and the samples with a Ct value less than cut-off values were considered as positive. The lower limit of quantification (LLOQ) was determined for each assay using the lowest concentration on the standard curve where replicates of five serial dilutions were amplified (Schriewer et al., 2013). The lowest concentration of standard gene copies that could be confidently detected in all triplicates was considered as the LOD. All the qPCR results were normalised to gene copies/ng of DNA in stool samples and gene copies/100 mL in water samples and the samples considered positive if the concentrations were above the LOD. For each sample, a replicate was considered as follows: not detected (ND) when no amplification was detected by LOD; detected but not quantifiable (DNQ) when LLOQ < Ct < LOD; or detected within the range of quantification (ROQ) when Ct < LLOQ (Schriewer et al., 2013).

2.6. Combined sanitary and microbial water quality assessment and risk classification

Complementing sanitary inspection with microbial water quality monitoring was done to assess the safety of water in different sources as described by WHO (2016). Sanitary risk scores and FIB counts were combined for risk assessment as shown in the Table S4. This table was used for risk assessment for each water source and faecal indicator bacteria.

2.7. Validation of water quality monitoring tools

Validation of the water quality monitoring tools was done to assess the degree of agreement between the results obtained for all methods tested, as described by Yang et al. (2013). The H_2S test was first validated using sanitary inspection. Samples were regarded as positive when both H_2S and sanitary inspection were positive and negative when both showed negative results. Thereafter, for faecal indicator bacteria (faecal coliforms, *E. coli*, *Enterococcus* spp. and *C. perfringens*), samples were regarded as positive when the H_2S test was positive, and the FIB concentration was above the threshold value of 10,000 CFU/100 mL; and negative when the H_2S test was negative, and the FIB concentration was below the threshold value. For validation of the H_2S using the MST genetic markers and pathogen data results, stool samples should always be positive for host-specific genetic markers, and any negative result was thus considered a false negative. Water samples collected for the detection of MST host-associated genetic markers and pathogens can test either positive or negative. A sample was thus considered a negative when testing negative by all methods, whereas a sample was considered a positive if it was positive using two or more methods.

2.8. Statistical analysis

Microsoft Excel 2019 and Statistical Packages for the Social Sciences (SPSS) Version 28 were used for statistical analysis. The concentration estimates for FIB (*E. coli*, faecal coliforms, *Enterococcus* spp. and *C. perfringens*), MST markers (*BacHum*, *Pig-2-Bac*, *BacCan*, *Cytb* and *BacCow* markers), and pathogenic bacteria (*V. cholerae*, *E. coli O157:H7*, and STEC) were log transformed to reduce data skewness for linear regression, and non-detects were assigned as zero (0) (Bradshaw et al., 2016). The Two-Sample Wilcoxon Rank-Sum (Mann-Whitney) test was performed for the analysis of variance of median microbial difference between dry and wet seasons in different matrices. To assess the agreement between tested methods, the logistic regression analysis in SPSS was used. The significance was performed at a 95 % confident limit. The linear regression analysis was performed using Microsoft Excel 2019 for correctly identifying sources. The statistical significance in the abundance of host-specific markers and pathogen concentrations were determined using IBM SPSS Statistics 26.0 (IBM Corporation, Armonk, NY, United States). The relationship between results obtained from sanitary inspections, H_2S tests, FIB, MST host-associated markers and pathogenic bacteria in various water sources was analysed by Pearson's correlation. Assays were combined to help identify the risk of faecal pollution and the major contributing sources of faecal contamination.

3. Results

3.1. Identification of faecal pollution using sanitary inspection and H_2S test in household water

3.1.1. Risk assessment in household water using sanitary inspection

Identification of faecal pollution in the VDM was initially assessed in catchment areas using sanitary inspection methods. The results for sanitary risk score in water sources were reported by Murei et al. (2023),

Table 1
Characteristics of household practices that may lead to faecal pollution in household water.

Characteristics	Yes	No
Do you have any domestic animals in your household?	467 (33.64 %)	921 (66.36 %)
Does your household travel long distance (>1 km) to fetch water?	78 (72.22 %)	30 (27.78 %)
Do you use bigger sized (>120 L) container to store the water?	1473 (84.07 %)	276 (15.93 %)
Does your household clean the drinking water storage container after long time?	216 (15.56 %)	1172 (84.44 %)
Do you store water for more than a week?	564 (42.21 %)	771 (57.77 %)
Do you keep your storage containers outside?	229 (16.79 %)	1134 (83.21 %)
Do you keep your water storage container uncovered?	78 (5.70 %)	1285 (94.30 %)
Do you use utensil to withdraw water from the storage container?	834 (60.86 %)	536 (39.14 %)

which showed a high sanitary risk score in surface water while groundwater mainly exhibited low to medium risk. Table 1 lists the overall results for household sanitary practices, which result in the faecal contamination of water sources. The results revealed that 33 % of residents in the VDM have domestic animals in their households, and about 72 % travel long distances to collect drinking water. About 15.7 % of residents have large (>120 L) water storage containers. A total of 216 (15.6 %) residents indicated that they only clean their storage container after a very long time, and 1172 (84.4 %) respondents indicated that they clean their water storage containers regularly. Furthermore, 42 % of residents who stored water indicated that the water may last for more than five days in case the water supply is intermittent or there is no water supply. About 17 % of residents reported that their water storage containers remain outside, either in direct sunlight or in the shade, and 5 % of residents reported that they do not cover their container-stored water. Some residents reported that they store water in containers with taps, and 60.9 % of residents indicated that they use utensils to withdraw water from the containers.

3.1.2. Prevalence of H_2S production in various household tap water and container-stored water samples

The H_2S test results of household tap water and container-stored water samples are shown in Fig. 2. Tap water in Tshifudi showed the highest prevalence of H_2S production as 75 % of the samples tested positive, whereas H_2S production was confirmed in 50 % and 25 % of other tap water samples. Households in the VDM stored water from various water sources depending on the water availability. Taps, springs/hand-dug wells, boreholes and rivers were found to be the main water sources used for storage purposes. Prevalence of H_2S production was observed in 100 % of household container-stored water samples in Tshidzini and Tshivhulani with water collected from the rivers (HH-TDNR and HH-TVLR), and also in spring water stored in Tshivhulani and Tshakhuma (HH-TVLS and HH-TKMS2) households. No H_2S production was observed in household container-stored water that originated from tap water in Dididi (HH-DDDT), from boreholes in Tshivhulani (HH-TVLB), and in Tshilapfene (HH-TLPB). The prevalence of H_2S production was observed in 50 % or less of the container-stored water samples collected from boreholes and protected springs. Almost all household container-stored water samples were positive for H_2S production, except for those in HH-TDNT, HH-TLPB, HH-TVLB, and in HH-DDDT, in which negative H_2S test results were recorded in both dry and wet seasons. The absence of H_2S production was also observed in container-stored water samples in HH-TDNS and in HH-TDNR during wet and dry seasons, respectively. The overall results of the H_2S test showed no significant difference between dry and wet seasons.

3.2. Faecal indicator bacteria

3.2.1. Prevalence of faecal indicator bacteria in various water sources

Various water sources were assessed for faecal indicator bacteria (FIB), which included faecal coliforms, *E. coli*, *Enterococcus* and *C. perfringens* as shown in Table 2. The overall prevalence of FIB ranged from the highest to the lowest mainly in the following order: faecal coliforms > *E. coli* > *Enterococcus* > *Clostridium*. Various water sources showed a variation in the prevalence of FIB. Overall results showed the highest prevalence in river (98.2 % of samples testing positive for faecal coliforms) and dams (100 % of samples testing positive for faecal coliforms), followed by hand-dug wells (50 % of samples testing positive for faecal coliforms and *E. coli*). Household container-stored water, spring water and tap water showed a lower prevalence of FIB. The lowest prevalence of FIB among the water sources was observed in borehole water, with 4.22 % of samples testing positive for *C. perfringens*. The prevalence of *Clostridium perfringens* was the lowest across all water sources.

3.2.2. Concentration of faecal indicator bacteria in dry and wet season

The differences in loads for faecal coliforms, *E. coli*, *Enterococcus and C. perfringensh* were observed between river, spring, boreholes, and

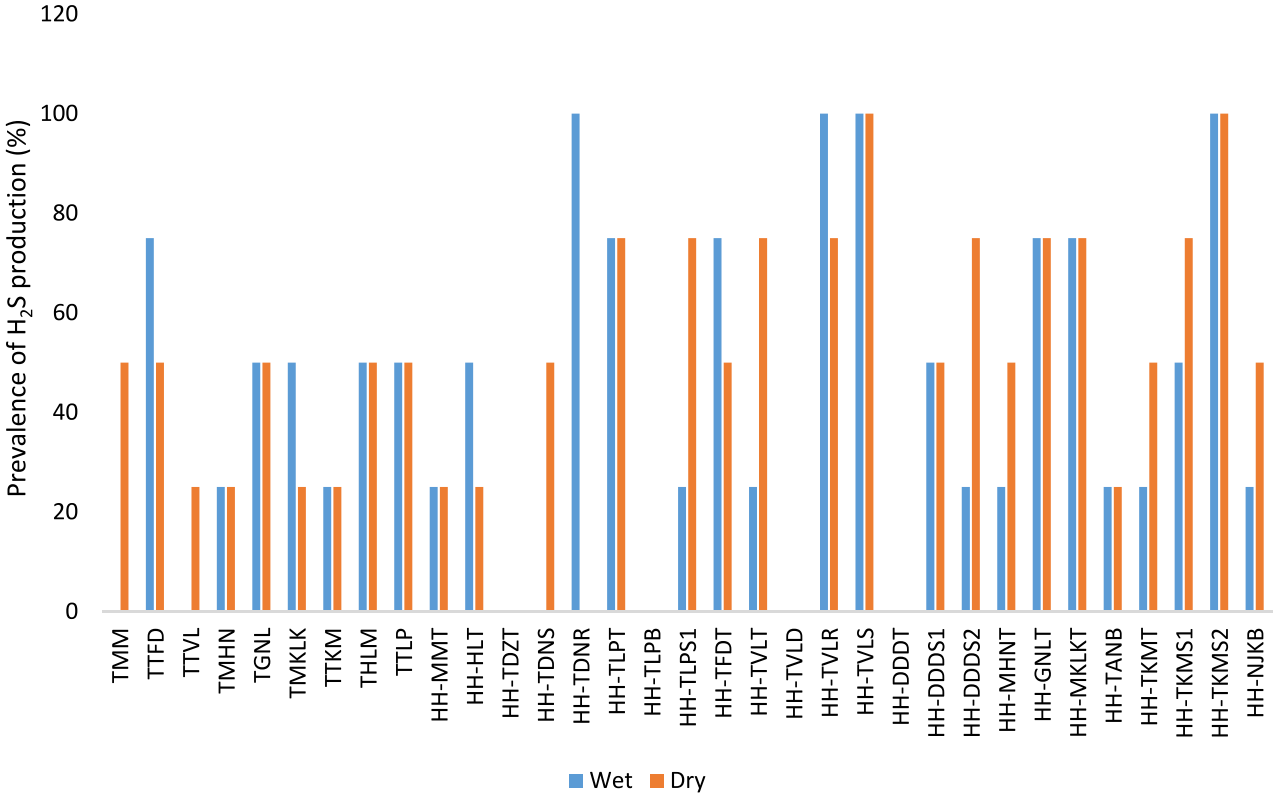

Fig. 2. Prevalence of H_2S production in household tap water and container-stored water samples in wet and dry seasons.

Table 2
Overall percentage prevalence of FIB at the sampling sites during the entire sampling period.

Sampling site	Number[a] of samples collected	Faecal indicator bacteria (number of positive samples and percentage)							
		Faecal coliforms		*E. coli*		*Enterococcus*		*C. perfringens*	
Rivers	112	110	98.21 %	106	94.64 %	89	79.46 %	58	51.79 %
Hand-dug wells	16	8	5.00 %	8	50.00 %	7	43.75 %	3	18.75 %
Dams	24	24	100 %	23	95.83 %	19	79.17 %	7	29.16 %
Springs	56	19	33.93 %	20	35.71 %	17	30.36 %	6	10.71 %
Boreholes	592	154	26.01 %	153	25.84 %	165	27.87 %	25	4.22 %
Households tap	640	242	37.81 %	191	29.84 %	120	18.75 %	45	7.03 %
Households' containers	1624	747	46.00 %	679	41.81 %	592	36.45 %	117	7.20 %

[a] The number of samples collected depended on the availability of the water during the study period.

hand-dug wells, as shown in Fig. 3. Faecal coliforms, *E. coli* and *Enterococcus* sp. counts were consistently higher than *C. perfringens* loads at all sites during the entire sampling period. Overall, surface water was found to have higher FIB concentrations ranging from 1.1 $\log_{10}$ CFU/100 mL to 4.7 $\log_{10}$ and for groundwater, FIB concentrations ranged from 0.5 $\log_{10}$ CFU/100 mL to 2.7 $\log_{10}$ CFU/100 mL. The Wilcoxon signed-rank test for FIB showed no significant changes between dry and wet seasons in faecal coliforms, *Enterococcus* spp. and *C. perfringens*, and only *E. coli* showed a significance difference between both seasons ($p = 0.028$). Seasonal variations of FIB concentrations per water source are shown in Fig. S1. The overall FIB counts were found to be higher during the wet season compared to the dry season. The highest load was for coliform bacteria in Mvudi River during the dry season with the mean concentration of 4.5 $\log_{10}$ CFU/100 mL. *Clostridium perfringens* was not detected in Tshidzini village water samples during both sampling seasons, while *Enterococcus* spp. were not detected either during the dry or wet season in the spring located at Tshilapfene village.

3.3. Evaluation of existing host-specific MST markers and bacterial pathogens

3.3.1. Characterisation of qPCR in detection of host-specific MST genetic markers and bacterial pathogens

Data on the presence and distribution of MST markers and pathogens in water samples at various sampling matrices were determined using qPCR. All of the qPCR standards were re-analysed to determine the master standard curve with standardised slope, amplification efficiencies, and correlation coefficient (R^2) values. The details of master standard curves and LOD for each tested MST assay are illustrated in Table 3. The amplification efficiencies of all MST qPCR assays tested were within the range 86–102 % and the correlation coefficient (R^2) values were ≥0.98. To precisely determine the LOD in terms of the number of genes present in 100 mL of water samples, the LOD was calculated on Microsoft Excel using linear regression. The lowest concentrations were obtained for the human-associated genetic marker *BacHum* with LOD = 0.798 $\log_{10}$ gene copies/100 mL. The LOD results showed that, with MST genetic markers and pathogen virulence genes selected in this study being present in water samples, as little as 4.9 $\log_{10}$ gene copies/100 mL of water sample should always be detected.

3.3.2. Performance characteristics of selected host-specific MST genetic markers in the Vhembe District

Human and non-human stool samples were collected in the Vhembe District to validate the selected host-specific genetic markers in terms of sensitivity and specificity for this geographical area before being tested in water samples. The MST marker was shown to yield 96–100 % of the specificity with the known human and animals stools samples (Table S5). The *BacCow* and *Pig-2-Bac* markers were highly specific (100 %) followed by *BacHum*, *Cytb* and *BacCan* (98 %, 97 % and 96 %, respectively). False negative results were mainly obtained with the dog-associated MST genetic marker (*BacCan*) where 2/9 of dog stool samples tested negative. The performance of the swine-associated assay (*Pig-2-Bac*) was evaluated with nine pig faecal samples and the target was detected in 7/9 of samples (77.78 %) at an average concentration of 1.61 ± 0.07 $\log_{10}$ copies per ng of DNA. The human-associated genetic marker *BacHum* was detected in 100 % of human faecal samples (20/20) at an average concentration of 1.49 ± 0.85 $\log_{10}$ copies per ng of DNA and showed cross-reactivity of 1/9 with dog faecal DNA samples.

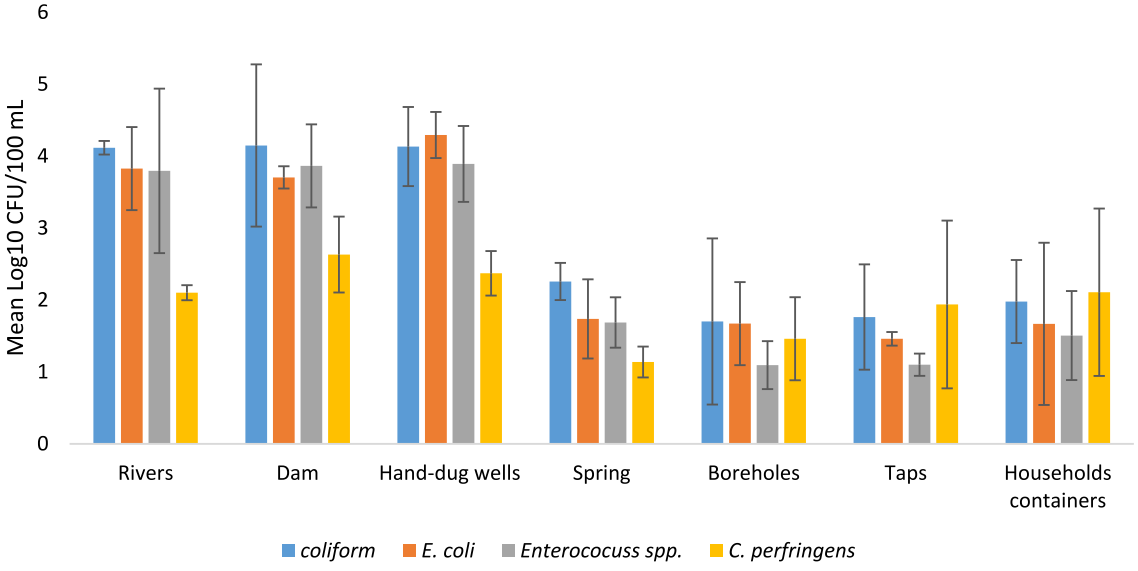

Fig. 3. Detection of faecal indicator bacteria in water sources for entire sampling period.

Table 3
Performance characteristics of qPCR method tested in various water sources (standard curve).

Host	Target gene	Slope	Y-intercept	R^2	Efficiency (%)	LOD	LOQ
Cow	*BacCow*	−3.64	42.35	0.99	88.16	0.88	2.67
Pig	*Pig-2-Bac*	−3.58	47.75	0.98	90.16	0.84	2.55
Dog	*BacCan*	−3.24	45.83	0.99	103.58	4.43	13.41
Human	*BacHum*	−3.20	43.67	0.99	105.40	0.80	2.42
Chicken	*Cytb*	−3.70	41.05	0.98	86.44	1.19	3.62
E. coli O157	rfb_{O157}	−3.28	32.88	0.99	101.95	0.94	2.82
STEC	*Stx1*	−3.30	42.29	0.98	100.89	0.81	2.46
V. cholerae	OmpW	−3.21	42.55	0.98	104.10	0.99	2.99

Note: LOD - limit of detection, LOQ - limit of quantification.

3.3.3. Prevalence of host-specific MST genetic markers in various water sources

The results for detection of host-specific MST genetic markers showed that the genetic markers are not evenly distributed in various water sources. Table 4 shows the prevalence of host-specific markers for chicken, cow, pig, human, and dog in various water sources. Among the water sources, the highest prevalence of host-specific markers was determined in rivers with human (96.43 %) and cow (91.96 %) markers, followed by dams (91.67 % of samples testing positive for cow markers). Rivers also showed the highest prevalence of 64 % ($n = 72$) for pig markers. A very low prevalence of all detected markers was observed in protected spring and borehole water samples with the lowest prevalence of 0.68 % for cow markers observed in borehole water samples. The highest prevalence in boreholes was observed for the dog-associated genetic marker *BacCan* ($n = 140$, 23.65 %). In HHs tap water, the highest host-specific genetic marker detected was *BacCan* ($n = 154$, 24.06 %) for dogs and the lowest prevalence was observed for the cow-specific *BacCow* marker ($n = 44$, 6.88 %). The highest prevalence of the chicken genetic marker (*Cytb*) was observed in 75 % ($n = 12$) of hand-dug well samples compared to the other water sources.

3.3.4. Concentration of host-specific MST genetic markers in dry and wet seasons

Fig. 4 shows the variation in mean concentration of the genetic markers from humans, cows, pigs, chickens, and dogs for both wet (A) and dry (B) seasons. In relative terms, the overall MST marker concentration was high during the wet season for all catchment sites compared to the dry season. Statistically, no significant variations were observed in the concentration of MST genetic markers during both wet season and dry season among different water sources when the Wilcoxon signed-rank test was performed. In water samples collected during the wet season, the overall mean concentration of host-associated genetic markers was 6.21 $\log_{10}$ gene copies/100 mL, with concentrations ranging from 4.77 to 7.06 $\log_{10}$ gene copies/100 mL. During the dry season, the overall mean concentration was 6.31 $\log_{10}$ gene copies/100 mL with the concentrations ranging from 5.24 $\log_{10}$ gene copies/100 mL to 6.88 $\log_{10}$ gene copies/100 mL. Based on the quantification of host-associated genetic markers, the highest level of faecal contamination was observed in water samples from DND and R-LRD, regardless of the faecal source (human versus animal species). Data on the presence and distribution of MST marker concentration per water matrix are presented in Fig. S2.

3.3.5. Overall prevalence of selected pathogenic bacteria in various water sources

The overall detection rates of all selected pathogens are shown in Table 5. The most prevalent pathogen during the entire sampling period was STEC; however, STEC was not detected in dam water samples. The highest prevalence in river water samples was observed with *V. cholerae* [$n = 102$ (91.07 %)]. Even though *E. coli* O157:H7 was detected in household tap water samples, *E. coli* O157:H7 showed the overall lowest prevalence in all water sources, except in borehole water samples where *V. cholerae* exhibited the lowest prevalence of 0.34 % ($n = 2$). The prevalence of all the tested bacterial pathogens was <10 % in groundwater samples collected from household's boreholes. Several bacterial pathogens were detected in household container-stored water samples with the highest prevalence observed for *V. cholerae* [640 (39.41 %)], followed by STEC [566 (34.85 %)].

The seasonal variation in the prevalence of each pathogen was observed but not quantifiable (DNQs) as positive samples. The most frequently detected pathogens in both seasons were STEC (64.29 % of samples in wet season and 55.36 % in dry season) followed by *V. cholerae* (58.93 % of samples in wet season and 48.21 % in dry season). The *E. coli* O157:H7 was the least detected pathogen during both seasons with 17.85 % of samples testing positive in the wet season and 5.36 % in the dry season.

3.3.6. Concentration of selected pathogenic bacteria in dry and wet season

Data on the overall abundance of the three selected bacterial pathogens are presented in Fig. 5 for both wet and dry seasons. The overall mean concentration of bacterial pathogens tested in the wet season was 6.36 $\log_{10}$ gene copies/100 mL with concentrations ranging from 5.11 to 6.85 $\log_{10}$ gene copies/100 mL (Fig. 5A). In the dry season, the overall mean concentration was 6.34 $\log_{10}$ gene copies/100 mL with the concentration ranging from 5.73 $\log_{10}$ gene copies/100 mL to 6.79 $\log_{10}$ gene copies/100 mL (Fig. 5B). The findings revealed no statistically significant association for *V. cholerae* between wet and dry seasons. The Wilcoxon signed-rank test for *E. coli* O157:H7 and STEC showed statistical differences between the dry and wet season ($p = 0.04$, and $p = 0.001$, respectively). Using the LOQ as the selection criterion, in the wet season, *V. cholerae* (*OmpW*) was present in a quantifiable range of Ct 25 to 39, equivalent to 5.35–6.73 $\log_{10}$ gene copies/100 mL of water. For

Table 4
Prevalence of host-specific genetic markers in various water sources.

Sampling Site	Number of samples collected	Number of positive samples for host-specific genetic markers (percentage)				
		Chicken	Cow	Pig	Human	Dog
Rivers	112	38 (33.93 %)	103 (91.96 %)	72 (64.29 %)	108 (96.43 %)	92 (82.14 %)
Hand-dug well	16	12 (75.00 %)	14 (87.50 %)	10 (62.50 %)	4 (25 %)	7 (43.75 %)
Dam	24	10 (41.67 %)	22 (91.67 %)	14 (58.33 %)	19 (79.17 %)	18 (75 %)
Spring	56	32 (57.14 %)	24 (42.86 %)	26 (46.43 %)	22 (39.29 %)	34 (60.71 %)
Boreholes	592	22 (3.72 %)	4 (0.68 %)	6 (1.01 %)	22 (3.72 %)	140 (23.65 %)
HHs taps	640	60 (9.38 %)	44 (6.88 %)	50 (7.81 %)	60 (9.38 %)	154 (24.06 %)
HHs container	1624	712 (43.84 %)	126 (7.76 %)	852 (52.46 %)	974 (59.96 %)	1252 (77.09 %)

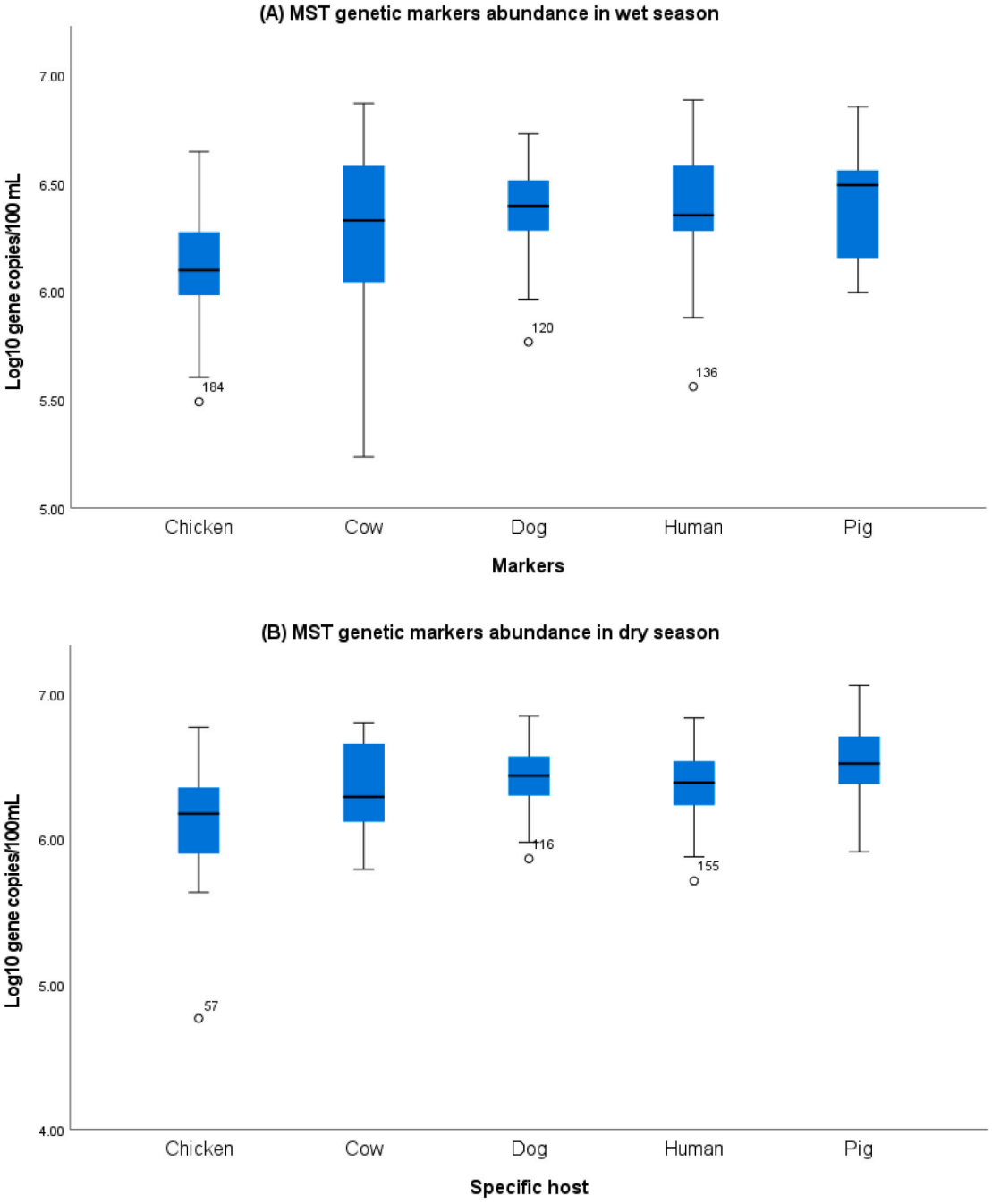

Fig. 4. Mean concentration of MST genetic markers: A) MST marker concentration/100 mL obtained in wet season; and B) MST marker concentration/100 mL obtained in dry season.

Table 5
Prevalence of pathogenic bacteria in various water sources.

Sampling site	Number of samples collected	Bacterial pathogens (number of positive samples (%))					
		V. cholerae		STEC		*E. coli* O157:H7	
Rivers	112	102	91.07 %	75	66.96 %	16	14.29 %
Hand-dug well	16	13	81.25 %	11	68.75 %	10	62.50 %
Dam	24	8	33.33 %	0	0.00 %	8	33.33 %
Spring	56	12	21.43 %	34	60.71 %	6	10.71 %
Boreholes	592	2	0.34 %	40	6.76 %	8	1.35 %
HHs taps	640	60	9.38 %	56	8.75 %	0	0.00 %
HHs containers	1624	640	39.41 %	566	34.85 %	6	0.37 %

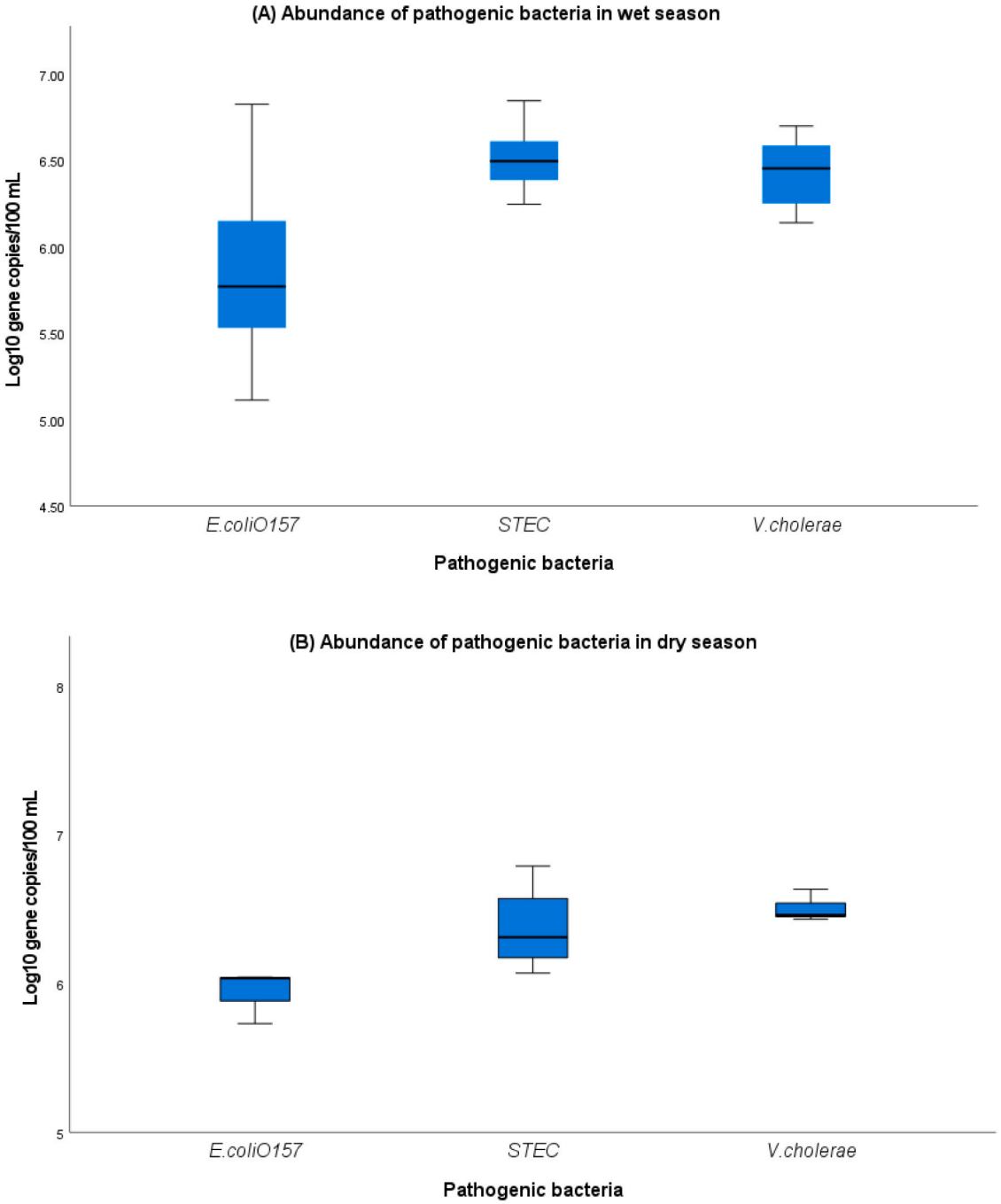

Fig. 5. Mean concentration of pathogens (*Vibrio cholerae*, *E. coli* O157:H7, and Shiga toxin-producing *Escherichia coli*) quantified in water samples at different sampling sites in the VDM: (A) mean concentration (Log_{10} gene copies/100 mL) obtained in wet season; and (B) mean concentration (Log_{10} gene copies/100 mL) obtained in dry season.

E. coli O157:H7, the range was Ct 16 to 30, corresponding to 5.11–6.83 log_{10} gene copies/100 mL of water, while STEC range was Ct 25 to 39, equivalent to 5.36–6.85 log_{10} gene copies/100 mL of water.

The variation in bacterial pathogen concentration per water matrix for wet and dry seasons is illustrated in Fig. S3. A difference in pathogen concentrations was established between the selected pathogenic bacteria (*V. cholerae*, *E. coli* O157:H7 and STEC) in various water sources in the Vhembe District. The Luvuvhu River was the most heavily contaminated source with the highest concentration of 6.69 Log_{10} gene copies/100 mL of *V. cholerae*, followed by 5.87 Log_{10} gene copies/100 mL of *E. coli* O157:H7, and 6.6 Log_{10} gene copies/100 mL of STEC. Among water samples collected from the dams, *V. cholerae* was detected only in Nandoni Dam in both seasons. No *E. coli* O157:H7 was detected in tap water samples and in only a few household storage containers in Tshivhulani village and Tsianda village in the wet season.

3.4. Combined sanitary inspection and microbial water quality assessment

The outcome of the sanitary inspection and the microbial water quality assessment was combined to determine the risk of faecal contamination associated with environmental water based on four levels (low risk, medium risk, high risk and very high risk) as can be seen in Fig. 6 (for wet season). The level of risk was found to vary with the site,

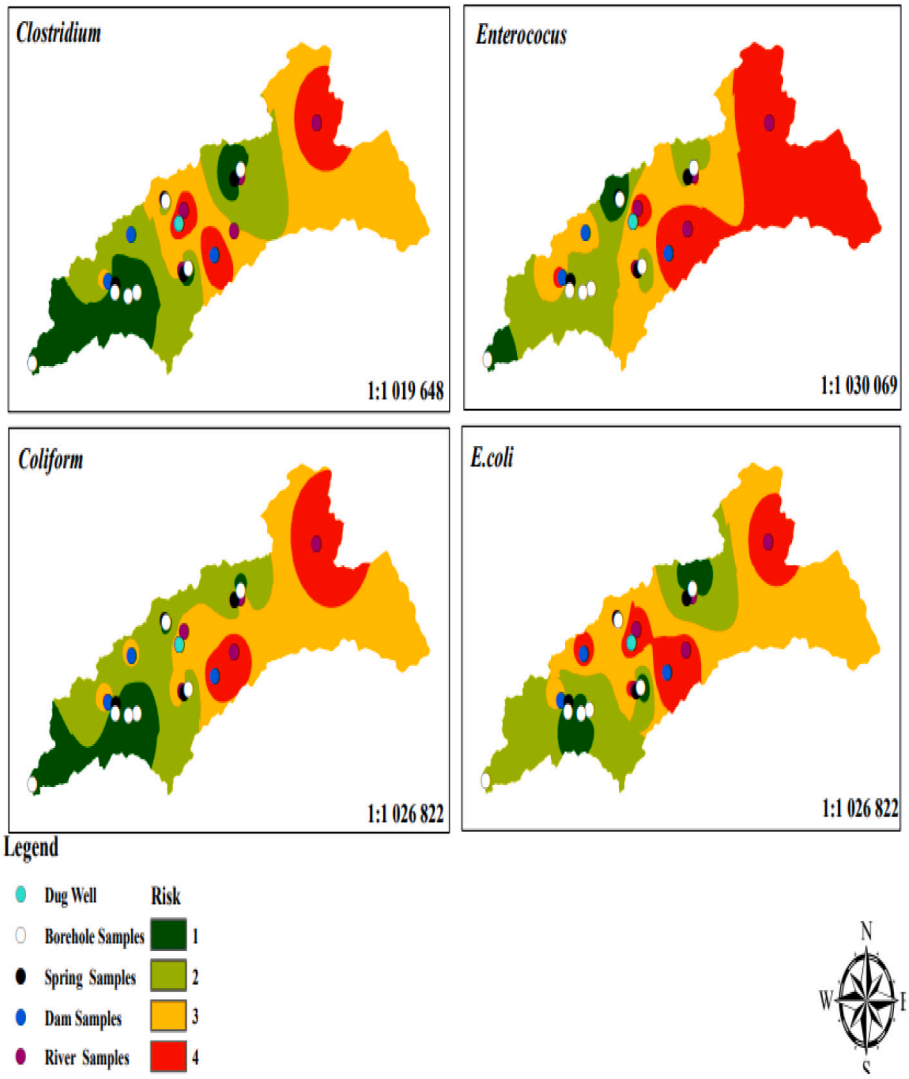

Fig. 6. Map showing risk of faecal pollution distribution across VDM during the wet season, where 1: Low risk represented by green colour; 2: medium risk represented by green light to yellow; 3: high risk represented by yellow to orange colour; and 4: very high risk represented by orange to red colour.

associated FIB tested and season. During the wet season, a very high risk was observed in Luvuvhu River, Nandoni Dam and Tshivhulani spring. Other springs and boreholes showed medium to low risk with all indicator bacteria. All rivers and dams from all catchment sites showed high risk to very high risk during the wet season. During the dry season, river water samples showed a high risk associated with all FIB in the Luvuvhu River at Mhinga site (Fig. S4); in addition, the hand-dug wells showed a high association with all FIB tested, whereas *Clostridium* showed high risk linking from Mutshindudi River in Tshivhulani village to Luvuvhu River in Gandlanani site and Mhinga site. Springs in Tshidzini, Dididi and Tshakhuma showed medium risk in association with all FIB tested. Due to the fact that any given site is affected by multiple water sources, Table S6 provides the detailed data for the risk of faecal contamination associated with FIB in each of the catchment sites.

3.5. Identification of the most contaminated source

As the performance of microbial water quality monitoring methods can vary greatly depending on the matrix, the effects of those methods were assessed in terms of detection of faecal pollution. Selected microbial water quality monitoring tools were combined to identify the most contaminated source of drinking water in the study area (Fig. S5). The river, hand-dug wells and dams showed a high prevalence of faecal pollution with the highest prevalence observed in river water, with 93.3 % of samples testing positive, while 88.9 % of hand-dug well water samples tested positive, and 73.3 % of dam water samples tested positive for faecal contamination. These natural sources were followed by household container-stored water with 67.2 % of samples testing positive. Household tap waters also showed the prevalence of faecal pollution with 60.7 % of samples testing positive. Boreholes and springs showed the lowest prevalence of faecal pollution with 53.6 % and 54.7 % of samples testing positive, respectively.

3.6. Correlation between sanitary score, H_2S, FIB, MST markers, and bacterial pathogens

All the microbial water quality monitoring methods used in this study were correlated and results are shown in Table 6. Results were considered as significant when Rho(r) and *p*-values were >0.5 and <0.05, respectively. The sanitary risk scores were statistically significant and positively correlated with total coliform bacteria detected by H_2S test ($r = 0.623$ and $p < 0.003$). Another positive correlation of sanitary risk score was observed with faecal coliforms, *E. coli* and *Enterococcus* spp. being significant at $r = 0.613$ and $p < 0.002$, $r = 0.589$ and $p < 0.003$, and $r = 0.625$ and $p < 0.003$, respectively. Production of H_2S by coliform bacteria showed a significant difference and positive

Table 6
Pearson correlation between water quality monitoring tools.

	Sanitary	H_2S	Coliform	*E. coli*	*Enterococcus*	*C. perfringens*	Human	Cow	Pig	Chicken	Dog	*V. cholerae*	*E. coli* O157	STEC
Sanitary	1	0.623**	0.613**	0.589**	0.625**	0.295215	0.015583	0.15221	−0.15054	0.375906	0.254704	−0.08625	0.673461	0.26951
H_2S		1	0.809**	0.779**	0.799**	0.488481	0.386607	−0.24864	−0.43943	0.136998	−0.20082	0.297474	0.613826	0.246857
Coliform			1	0.968**	0.960**	0.540*	0.609*	−0.0287	−0.11799	0.004313	0.172316	0.10868	0.801*	0.119006
E. coli				1	0.943**	0.494*	0.699**	−0.13197	−0.13197	−0.12497	0.151931	0.154102	0.714*	0.142131
Enterococcus					1	0.570*	0.471092	0.897**	−0.28241	0.107428	0.130913	−0.00682	0.690508	0.010516
Clostridium						1	0.168808	−0.0672	−0.13426	−0.07726	0.116302	0.56543	0.067708	−0.11433
Human							1	−0.659*	−0.11506	−0.56562	0.134549	0.155795	0.204837	0.199791
Cow								1	−0.07162	0.361555	−0.09892	−0.20174	−0.38864	−0.28219
Pig									1	−0.17994	0.500847	0.238846	0.234354	0.141711
Chicken										1	0.194647	−0.38491	0.274306	−0.3771
Dog											1	−0.05558	0.630953	0.166846
V. cholerae												1	−0.43854	0.161264
E. coli O157													1	0.776*
STEC														1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

correlation with faecal coliforms ($p < 0.001$); $r = 0.809$) and *E. coli* and $p < 0.001$; $r = 0.779$) and *Enterococcus* ($p < 0.001$; $r = 0.799$). Faecal coliforms and *E. coli* were found to have significant differences and a positive correlation ($p < 0.021$; $r = 0.609$ and $p < 0.005$; $r = 0.699$) with the *BacHum* genetic marker human faeces; they were also found to have significant differences and a positive correlation with *E. coli* O157:H7 ($p < 0.017$; $r = 0.801$). *Enterococcus* also showed a strong correlation with the *BacCow* genetic marker for cow faeces ($r = 0.897$ and $p < 0.001$). No significant correlation was observed for *Clostridium perfringens*, *Pig-2-Bac* (pig), *BacCan* (dog), and *V. cholerae* with any variable. No significant correlation was established for host-specific genetic markers and pathogen concentration with sanitary risk scores obtained from land use.

3.7. Agreement between microbial water quality monitoring tools in the detection of faecal pollution in water

Fig. 7 shows the relationship between water quality monitoring tools used in this study, from basic level to advanced level. The level of agreement between the results of the water quality monitoring tools used in this study, namely H_2S tests, faecal indicator bacteria (FIB), host-specific genetic markers, and pathogens, was then examined. The results showed that the prevalence of microorganisms detected varied from water source and sites. The results of the river water samples showed that H_2S gas production had a high prevalence of 89 %, while coliform bacteria exhibited the highest prevalence of 98 % at a 95 % confidence interval with extremely high microbial concentration (≥10,000 CFU/100 mL). The overall results for river water, dam water and hand-dug wells showed similar patterns, the microorganisms exhibited the highest prevalence when the microbial loads were categorized as high or very high. Spring water samples showed H_2S gas production with low (1–10 CFU/100 mL) to high concentration (1000–9999 CFU/100 mL) of FIB. Of the faecal indicator bacteria, only *C. perfringens* was detected showing a prevalence of 3.57 % with high concentration ranging from 1000 to 9999 CFU/100 mL and also human-associated genetic markers and pathogens tested were all positive in spring water samples. In boreholes, H_2S gas production was positive with other indicator bacteria except *C. perfringens* showing low to high concentration. However, *C. perfringens* had a prevalence of only 0.68 % and was detected at a very high concentration compared to the other indicator bacteria. In household tap water, when the H_2S test was positive, indicator bacteria were detected, and also human- and animal-associated genetic markers and pathogens were observed. Household water storage containers showed a high prevalence of human and domestic animal faecal contamination along with H_2S gas production, indicator bacteria and pathogens, and a similar pattern was observed with low to high concentration.

4. Discussion

It is crucial to identify sources of faecal contamination in order to successfully manage and eliminate future sources of contamination to safeguard public health. A site-specific assessment of sources of faecal pollution in water sources and their variability is the first step in acquiring data for estimating pathogen and indicator concentrations (WHO, 2016). In the present study, sanitary inspection was applied; this is a valuable and widely applicable tool for evaluating the risks within water supply systems. This strategy has been created and promoted as a straightforward and useful technique in areas where water testing facilities are limited (World Health Organization, 1997). Sanitary inspections may also be used at the household level to identify the most significant causes and pathways of contamination to avoid or reduce contamination (WHO, 2016). The sanitary risk inspection and survey of water sources in the study were conducted in our previous study (Murei et al., 2023); animals and agricultural activities were found to be the leading contributors of faecal contamination.

Possible pathways that may lead to water contamination at the household level may be poor hygiene practices, water collection and

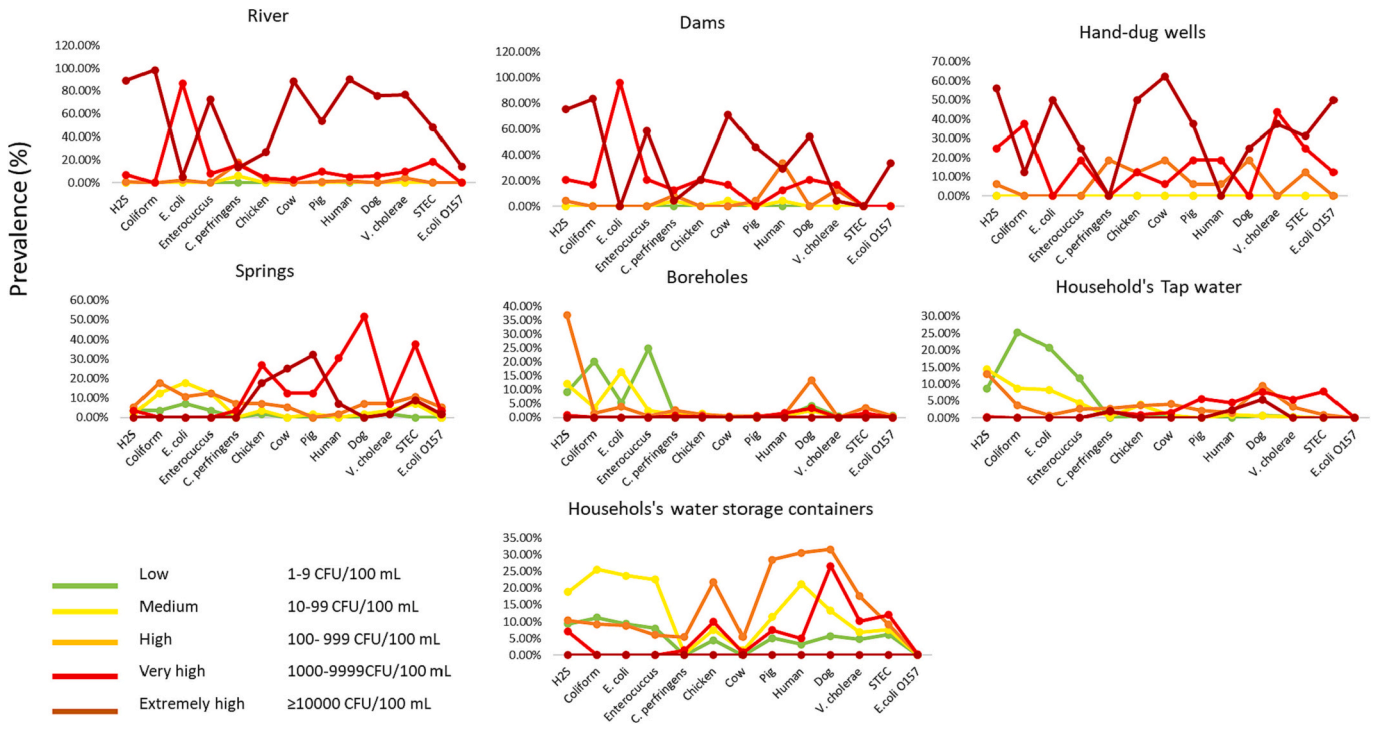

Fig. 7. Agreement between water quality monitoring tools used in this study from basic level to advanced level in various water sources used in the VDM.

storage, and the presence of domestic animals (Gundry et al., 2004). Our findings indicated that 33 % of households have domestic animals in their homes. However, a recent study by Larson et al. (2023) indicated that farm birds, and other animal waste in the kitchen are associated with thermotolerant coliforms in drinking water; this clearly shows that animals play a role in contaminating water at the point of use. Therefore, it is essential to store the water in a safe place not accessible to animals or far away from animal shelters. Furthermore, safe water storage guards against microbial contamination, maintaining water quality, and reducing diarrhoea and other waterborne illnesses. To prevent the introduction of unclean hands into the water storage container, drinking water should be kept in a container with a tight-fitting cover and a narrow mouth. Water can then be withdrawn by pouring it out or turning on a faucet (Anderson et al., 2021; Moropeng et al., 2021).

Some households (15.7 % of the population) in the study area were found to store water in large water storage tanks (>120 L) and 42 % of residents who had water stored indicated that in case of intermittent drinking water supplied by the municipalities, the water storage in dwellings remains a reliable and common practice and the stored water may last for more than five days. Large water tanks were found to have longer water storage times, which may have increased the danger of contamination and contributed to chlorine depletion (Manga et al., 2021). During the study period, 17 % of households in the selected areas were found to store their water in containers outside. For the longevity of storage tanks and cleaner water, it is crucial to have the water tanks cleaned every six to twelve months. It is also important to disinfect water prior to storage using the common treatment methods such as chlorination (which contains approximately 5–6 % sodium hypochlorite) or boiling. Furthermore, water tanks used to store water must have a lid to prevent external contaminants from entering the stored water; this lid must be properly closed. In spite of these interventions to enhance the quality of home-stored water, significant challenges are still encountered as long-standing stored water also results in recontamination of treated water. Consequently, Moropeng et al. (2021) suggest the reconsideration of hygiene education (i.e., hand washing) coupled with safe-storage condition interventions that may result in improving the microbial quality of drinking water and counteract waterborne diseases.

In this study, about 72 % of residents were found to travel long distances to collect drinking water. Similar results were observed in a study conducted in QwaQwa by Mocwagae et al. (2023) in South Africa. Travelling long distances to the water source may introduce microorganisms into the water if the container is not properly closed, thus the consumer in that household may be prone to waterborne diseases. A report by Bennion et al. (2021) in Tanzania pointed out that a reduction of 15 min in one-way walking time to the water source is associated with an average relative reduction of 41 % in diarrhoea prevalence. Therefore, provision of an alternative water source, which is closer to households may help reduce the risk of waterborne diseases. Sanitary inspection may reveal minimal risk, although microbiological water quality assessments may reveal poor water quality (Howarth et al., 2018), this may be due to unidentified sources of diffuse pollution. Hence, it is necessary to perform a microbial test such as H_2S test or culture-based methods rather than relying only on sanitary risk assessments.

The H_2S test was conducted in the household tap water and water stored in the storage containers and positive H_2S production was observed. The lowest prevalence of H_2S production (7 %) was observed in tap water samples of Tshifudi village, whereas 50 % and 25 % of other tap water samples tested positive for H_2S production. This may be due to poor water treatment at the water treatment plant or contamination that may be occurring during distribution. However, the H_2S production does not always indicate faecal contamination, because there are many different types of H_2S-producing bacteria that do not originate from faecal contamination as they are unrelated to the digestive system (Sobsey and Pfaender, 2002). Before the H_2S test is adopted as an alternative to water quality monitoring, its limitations must be addressed. Household members reported that they store water originating from various sources, and it was observed that 100 % of samples from household container-stored water that originated from the river tested positive for H_2S production. A similar observation was also noted for water from unprotected springs in a previous study conducted by Murei et al. (2023). Hence, there is a need to educate community members on the selection of their water sources in that they can use the H_2S test to assess their water quality and also to use household treatment

methods to treat water before consumption. According to Nair et al. (2001) and Pathak and Gopal (2005), the H_2S test in a liquid format is incapable of detecting low levels of contamination and this results in false negatives results. In the present study, the H_2S strip method was used and is considered as a sensitive test capable of detecting even low levels of contamination in water samples. However, when lower numbers of H_2S producing organisms were present, the results were available after a longer incubation period, with 48 h required when <10 H_2S organisms were present (Genthe and Franck, 1999). The results of this study demonstrate that the H_2S strip also accurately detects faecal contamination of drinking water at the household level, which is expected to have low levels of faecal contamination. It is also important to emphasise that 1 mL of water sample was inoculated into 9 mL of Tryptone Soya Broth prior to adding the H_2S paper strip. This modification of the method improved the ability to detect even low levels of faecal contamination.

Although no H_2S production was observed in 79.2 % of household water storage container samples, this does not mean that the water is safe for drinking. Numerous studies have evaluated the use of an H_2S test for determining the quality of water (Pachepsky et al., 2018; Nair et al., 2001; Genthe and Franck, 1999; Mcmahan et al., 2011; Shahryari et al., 2014). Due to the fact that *Clostridium perfringens* gives a positive reaction to the H_2S test, Castillo et al. (1994) discovered that the H_2S test is 10 % more sensitive to coliforms and faecal coliforms. The presence of coliforms in water is consistently linked to organisms that produce H_2S (Manja et al., 1982). This is because coliforms, which are the primary indicator organism of aquatic pollution, have been consistently found in connection with H_2S-producing bacteria in water. This study still emphasises that the H_2S test can be more effective as a surveillance and screening tool that can be conducted once a month or whenever the quality of deteriorates, but it should not replace standard monitoring techniques. Hence, culturing methods may still be conducted to confirm faecal contamination in drinking water.

It is obvious that surface water is contaminated with faecal matter since these water sources are exposed to various sources of faecal matter. In this study we also found that rivers, dams, and hand-dug wells had the highest concentrations of *E. coli*. Healthy people and warm-blooded animals have *E. coli* as a native member of their intestinal flora. Hence, the presence of *E. coli* (or thermotolerant coliforms) indicates that water is contaminated with fresh stool and the probable presence of enteric pathogens. However, groundwater (from springs or boreholes) and tap water (from municipal water treatment plant), which are regarded as clean water sources also tested positive for *E. coli* with mean concentrations of <2 $\log_{10}$ CFU/100 mL. Several studies in rural areas of South Africa have shown that high concentrations of *E. coli* can be found in drinking water (Manja et al., 1982; Mellor et al., 2013; Moropeng et al., 2018). This raises a major concern for the villagers that are using this contaminated water for drinking purposes since they are more prone to contract waterborne diseases.

The prevalence of faecal coliform counts was consistently higher than those of *E. coli*, *Enterococcus*, and *C. perfringens* at all sites during the entire sampling period (Table 2). The presence of these faecal coliforms in the water sources implies that water is polluted with faecal matter. Although 5 % of water samples from the taps, springs and boreholes were negative does not mean that water is 100 % safe. This may be due to the non-detection of slow-growing and viable but not culturable organisms. Therefore, detection time must be increased. In addition, faecal coliforms are not effective indicators of faecal pollution in drinking water due to the large number of environmental species related to plant material. The faecal coliform detection method is still used to test the quality of water produced by water treatment plants because faecal coliforms are more sensitive to chlorine and persist in the water environment for a shorter amount of time than enteric viruses and parasitic protozoa. Hence, their presence indicates recent faecal contamination (Holcomb and Stewart, 2020).

Enterococcus has been used as indicator bacteria in water due to its presence in the microflora of the gastrointestinal tract of humans and animals and due to its abundance in soil and surface water. It is therefore not surprising that *Enterococcus* spp. was detected in untreated water, either from surface water or groundwater. Furthermore, *Enterococcus* spp. were not found either during dry or wet seasons in the protected spring located at Tshilapfene village. In this study, treated water for drinking purposes in the households showed an overall prevalence of 36.5 % of *Enterococcus* spp. with a mean concentration of 1.5 $\log_{10}$ CFU/100 mL in household storage containers. This is a matter of a high concern since contamination may be due to poor hygiene practices and poor sanitary practices within those households. Hence, proper hygiene practice and sanitary practice in households must always be emphasised to protect public health.

The highest overall prevalence (51.8 %) of *C. perfringens* was observed in river water with a mean concentration of 2.1 $\log_{10}$ CFU/100 mL, followed by the dam and the hand-dug well (Fig. 3). Similar results were also obtained from various studies where surface water showed an abundance of *C. perfringens*. This is due to the fact that *C. perfringens* produces spores that can remain in the environment longer than other indicator bacteria. Hence, they can be used to determine historical faecal contamination (Davies et al., 1995; Luvhimbi et al., 2022). Furthermore, these spores are extremely resilient to both environmental stress and conventional water treatment methods as they were also found in tap water from the municipal water treatment plant. Therefore, residents in the Vhembe District who rely on this contaminated water source may be at risk of diarrhoeal diseases since the presence of *C. perfringens* is an effective indicator for *Giardia* cysts and *Cryptosporidium* oocysts (Farhadkhani et al., 2022).

The overall results of faecal indicator bacteria showed that rivers, dams, and hand-dug wells receive heavy loads of faecal contamination compared to groundwater (from springs and boreholes) and treated water (household taps and storage containers). The general microbial concentrations were always found to be higher for all indicator bacteria in the wet season compared to the dry season, although statistics showed no significant change between the dry and wet season, except for *E. coli*. Therefore, the contamination could have more of an animal faecal origin than a human faecal origin and not necessarily in one pollution event because a microorganism such as *C. perfringens*, which their spores are exceptionally resistant to unfavourable conditions and may be detected far from the source and long after a pollution event has occurred (Vierheilig et al., 2013). Residents who live close to such waterways must be warned that there is a significant health risk since the presence of this indicator bacterium represents the possibility of these natural water sources being contaminated with either human or animal faecal matter.

Various sources of faecal contamination have been identified previously using sanitary survey and sanitary risk inspection in the catchment area of the VDM (Murei et al., 2023). And the results revealed that contamination in catchment areas might come from agriculture, open defecation or other human activities that were observed in those areas. Therefore, Bacteroidales host-specific genetic markers were then used to identify whether the faecal pollution in the water resource originated from human or animal sources. Cows were found to be the main polluter of surface water with the *BacCow* marker detected in 92 % of both dam and river water samples. This is understandable since cows were observed drinking water/grazing around these sources. In addition, large amounts of cow dung were found along the rivers and around dams. Human markers were also detected in those sources with the *BacHum* marker detected in 96 % of river samples and in 79 % of dam samples. This may be the result of open defecation, the location of pit latrines next to the rivers or dams, and discharge of inadequately treated wastewater containing human faeces into rivers since *Bacteroides* spp. are abundant in both animal and human faeces.

Groundwater samples in the VDM were mainly collected from household taps either from the drilled boreholes or springs located in the mountains. Hence, overall, a low concentration of markers was observed

in groundwater samples (Table 4). Various *Bacteroides* host-specific genetic markers have been found present in various water sources; however, their detection rate varied as revealed in a study by Fremaux et al. (2009) who detected human and ruminant markers in 41 % and 14 % of the water samples, respectively, whereas the pig marker was never detected in the river water. In the present study, these water sources showed high faecal contamination linked to domestic animals such as dogs, chickens, and pigs. Due to the water scarcity in those areas, these animals might lick taps hoping to get water if not provided. It is not surprising to detect these animal markers in such water sources. In some areas of the study areas, it is common for humans and animals to share the same water source. Hence, great care must be taken to keep animals away from drinking water sources. Similarly, these animal markers were also detected in tap water supplied by the municipality and in household storage containers. Animals in the households must be provided with their own water to prevent them from roaming near water sources that are used by households for multiple purposes. However, not all known potential sources of faecal contamination were included in the current investigation, and thus other hosts may also be responsible for the contamination of water sources.

Diarrhoeal disease, intestinal infections, polio, typhoid, and cholera result from poor sanitation (Teka, 2019). In this study, the presence of pathogenic bacteria such as *V. cholerae*, *E. coli* O157:H7 and Shiga toxin-producing *E. coli* was investigated since their presence directly indicates a public health risk. All the locations with the highest rate of pathogenic organisms found in this study were along the Luvuvhu River itself. This finding is consistent with those of a previous study (Abia et al., 2015), which reported that sites along the main river course had the highest concentration of microorganisms compared to boreholes and springs with the overall lowest prevalence of those pathogenic bacteria, thus indicating that boreholes and springs are safer sources of drinking water.

Vibrio cholerae is of the most disquieting pathogens transmitted by the faecal–oral route through water (García-Aljaro et al., 2019). Its existence was detected in various water sources in the study area by detecting one of its virulence factors, the outer membrane protein W (*OmpW*). In this study, the *OmpW* gene of *V. cholerae* was detected in rivers, dams, and hand-dug well water samples, The detection of the *OmpW* gene poses a potential health risk and it demonstrated that environmental strains of *V. cholerae* may carry additional virulence factors that would enable them to cause illness (Faruque et al., 2003). Although *V. cholerae* was not detected in groundwater samples during the dry season, in the wet season, two springs in Dididi and Tshakhuma and boreholes in Tsianda showed the presence of *V. cholerae*. This may be the result of heavy rain since the residents reported that during heavy rainfall events their pit latrines become flooded when groundwater mixes with faeces inside toilets and return to their usual level after the rain. *Vibrio cholerae* was also detected in several tap water samples and household storage containers that are used for drinking purposes. Only a few studies have reported on the presence of *V. cholerae* virulence-associated genes within drinking water sources, despite numerous studies demonstrating that *V. cholerae* is present in different aquatic habitats (Abia et al., 2015).

Disease is caused only by a tiny subset of *E. coli* strains. The majority of strains are commensal bacteria that naturally inhabit the gastrointestinal tract of warm-blooded mammals (Churchill and Romanus, 2019). *Escherichia coli* O157:H7 is one of the dangerous strains that cause gastroenteritis, which can result in bloody diarrhoea, haemorrhagic colitis, and kidney failure. This strain was detected in three sample matrices, which originated from Luvuvhu River, Dididi borehole, and a hand-dug well in Tshivhulani village and was not detected in tap or stored water during the dry season (Fig. S3). However, during the wet season, this bacterium was also detected in stored river water in Tshivhulani and in stored borehole water in Tsianda. There have been reports of various prevalence rates in studies conducted by previous investigators. *Escherichia coli* O157:H7 was detected in 4.07 % of water samples, according to a recent study conducted in Pretoria, South Africa by Bolukaoto et al. (Bolukaoto et al., 2019). In the current study, the overall prevalence of *E. coli* O157:H7 across the sampling period was 10 %. However, compared to the study of Bolukaoto et al., 2019), the prevalence rates of *E. coli* O157:H7 were observed to be higher in the present investigation. *Escherichia coli* O157:H7 detected in this study might originate from cows, since they were the most commonly observed domestic animals in VDM. Studies have reported multiple environmental sources of *E. coli* O157:H7, despite the fact that it is primarily carried by healthy cattle and other ruminants (Raseala et al., 2020). In addition, it is doubtful that the prevalence (0.37 %) in household stored water samples was purely from the tap water given that no *E. coli* O157:H7 was isolated from the tap water at the point of use in households (Table 5).

Other virulence factors of *E. coli*, including *stx1* and *stx2*, which are frequently linked to severe human diseases, are involved with the pathogenesis of Shiga toxin-producing *E. coli*. Shiga toxin-producing *E. coli* (STEC) has been reported to carry the virulence genes *stx1*, *stx2*, *eaeA*, and *hlyA* in cattle, pork, human faeces, and water samples (Churchill and Romanus, 2019; Bolukaoto et al., 2019). In this study, we used the *stx1* gene for the detection of the prevalence of STEC in various water sources. The *stx1* of Shiga toxin-producing *E. coli* was detected in all types of water samples; however, the highest detection rate was observed in hand-dug well water samples used in Tshivhulani for drinking and other household chores where 68 % of samples tested positive. Hence, the danger of contracting a waterborne illness from drinking polluted water is increased by runoff from livestock farms, which may contaminate the surface water (Tanaro et al., 2014). Since VDM is populated by subsistence farmers, animals observed near water source can also affect the development and spread of STEC in cattle due to contamination of water troughs, bedding materials and dust, which can cause direct or indirect faecal contamination (Ramaite et al., 2022). Furthermore, water is a key transmission route for STEC that may survive for a long time and travel considerable distances (Amarasiri et al., 2020). Hence, it is not totally unexpected that such high detection rates were found since neighbouring areas may also be contributing to the pollution.

The above-mentioned methods for testing water quality have been used separately or combined to ascertain microbial faecal pollution of various water sources. In low-resource settings such as rural communities, the use of advanced molecular techniques may not be possible, and therefore these communities can rely on simple effective methods such as sanitary inspection combined with faecal indicator bacteria monitoring to determine public health risk. Therefore, the current study also identified the health risk in various catchment areas within the area studied. Very high risk was observed in Luvuvhu River, Nandoni Dam and Tshivhulani spring during the wet season, while during the dry season a high risk associated with all FIB was observed in the Luvuvhu River at Mhinga site. Hence, this finding confirms that local municipalities can use sanitary inspections combined with microbiological monitoring as a tool to assess the faecal pollution of individual water sources. The local municipality must therefore inform the district about risk outcomes so that control measures and actions can be taken to improve the management of water sources.

Overall, when the presence or absence of faecal pollution was assessed using all methods, rivers were found to be the most contaminated water source with a 93.3 % of samples testing positive. Similar results were also obtained from other studies; however, variations may be due to sample techniques utilised, geographic location, and culture media used (Pandey et al., 2014). Therefore, residents in the VDM who use water directly from the river must treat the water before use. For boreholes, when all methods were combined, it was found that 53.6 % of samples tested positive for faecal pollution; hence, groundwater sources could be contaminated by faeces from non-point sources such as contaminated rivers that are related to the aquifers that provide groundwater to the study sites. Considering that other human and animal surface activities like farming were observed in the VDM, these

could be the main sources of zoogenic faecal contamination in groundwater through infiltration, e.g., after manure is applied to fields. Since boreholes and springs showed a low prevalence of faecal contamination compared to other water sources and considering that they are the most reliable alternative water sources for drinking purpose, residents can monitor water quality in these sources using the H_2S test. A high prevalence of faecal pollution was established for household water storage containers with 67.2 % of samples testing positive, compared to 60.7 % for household taps. This may be due to a lack of continuous water supply in the area where stored water in households originates from different sources depending on the availability. For example, residents in Tshilapfene village lack constant water supply and rely on the storage of tap water and groundwater from boreholes and spring or storm water in the same container. Therefore, residents should practise good household hygiene and sanitary practices.

As mentioned above, the monitoring of water quality during the study period was performed at three different levels, employing five different water quality monitoring tools, namely sanitary survey, H_2S test, enumeration of FIB, qPCR for detecting host-associated genetic markers, and for detecting pathogens were used. The relationship between them was established to identify their suitability and feasibility for use in rural communities. Positive correlations between the H_2S test and sanitary inspection, between sanitary inspection and FIB, between the H_2S test and FIB, between FIB and associated host-specific Bacteroidales gene markers, and between FIB and pathogens were observed using the Pearson correlation method (Table 6). A weak positive correlation was established between the H_2S test and host-associated genetic markers. Other studies have also reported a correlation between water quality monitoring tools. Manja et al. (1982)) found agreement between H_2S and FIB techniques and in 2016; Zhang et al. (2016) found positive correlations between pathogens, FIB and MST using Pearson's correlation. In this study, no association was found between the sanitary risk score and faecal indicators. Similar results were observed in a study conducted in rural sub-Saharan Africa (in 14 countries namely, Ethiopia, Ghana, Honduras, India, Kenya, Malawi, Mali, Mozambique, Niger, Rwanda, Tanzania, Uganda, Zambia, and Zimbabwe) where no association was found between the sanitary risk score and *E. coli* (Kelly et al., 2021). The current study established a very weak positive correlation at $p = \leq 0.05$ between FIB and pathogens such as *V. cholerae* and STEC. These findings are in agreement with those of previous investigators who also did not find any correlation between FIB and pathogens (Bradshaw et al., 2016; Tanaro et al., 2014). Although widely employed, culture methods for FIB detection in water may not expressly demonstrate the presence of pathogens but rather suggest a potential danger of exposure to them. In this study, significant positive correlations were shown between human and cow markers and some FIB tested (Table 6). It is very clear that humans and cows were the primary source of faecal pollution in water sources around the Vhembe District.

Agreement was observed between the five water quality-monitoring tools at the 95 % confidence interval. The H_2S test results were generally positive for samples that were more severely contaminated (having >10 CFU/100 mL of faecal coliforms or *E. coli*) as also pointed out in another study (Gupta et al., 2008). Despite the fact that these methods had already undergone validation in the aforementioned studies, it is clear from the variations in results obtained from numerous studies carried out in various geographic locations using the same assays that the performance of assays can vary depending on the location (Mcmahan et al., 2011). However, in this study we validated the results of those methods tested if they indicated faecal contamination of water. The overall results showed a similar pattern in various water sources at different concentrations of colony-forming units in such a way that when the H_2S test was positive, one or more FIB were also positive and host-specific genetic markers and/or pathogen were also detected. And when the H_2S test was negative, very low or no FIB was detected and none of the markers and/or pathogens were detected. Although a few variations were observed, it is very important to mention that when other FIB were found to be negative in spring, household tap and in water storage container samples, *C. perfringens* was observed with very high or extremely high concentrations in those samples, and either pathogens or/and MST markers were also detected. This may indicate that contamination of those water sources is not from recent faecal matter as the presence of *C. perfringens* indicates old faecal contamination (Li et al., 2021). Hence, this study suggests the use of *C. perfringens* when monitoring water quality with FIB. Although this method has been in use for a long time, there is still a need to increase its sensitivity because membrane filtration frequently fails with samples that have low bacterial concentrations. As there are so many new developing pathogens that are causing water-related illnesses and waterborne outbreaks, pathogen indicators need to be continually updated.

5. Conclusion

Overall, this study indicates the challenge for health and water resources in rural communities of countries such as South Africa that are lacking safe drinking water supply and sanitation services. The present study reveals correlation or association between findings from the use of advanced technologies such qPCR for the detection of both sources of contaminations and pathogens and those originated from cost-effective tools such sanitary inspection and hydrogen sulphide test (H_2S). Since results obtained from the later tools are comparable to those of the advanced methods, there is, therefore, a need for the implementation of water quality monitoring at household level in rural settings without access to laboratory sources for the management of their water sources using these cost-effective tools. The present study provides to policy decision makers evidenced based parameter in enforcement of rule and regulation for safe drinking water. Local Water and Sanitation Services Authority (LWSSA) are responsible to capacitate rural communities for the monitoring of faecal contamination of water sources using both Sanitary Inspection and the H_2S test as a surveillance and screening tools that are able to provide an indication of health risks such as waterborne diseases. The success of this will also depend on the supply of the H_2S by the LWSSA to encourage municipalities to order these reagents for households to use. Furthermore, this study reveals that the main source of faecal pollution in water sources around Vhembe District Municipality is from human and domestic animals (especially cattle). However, these cannot be regarded as the main source of faecal contamination as only a few host-specific genetic markers were tested; we suggest the evaluation of other host-specific genetic markers in the VDM since faecal pollution is observed to be due to non-point source pollution. The implementation of household drinking water treatment methods [(i.e., boiling, adding chlorine (bleach) etc.] can be considered to alleviate the source of microbial contamination during water storage in dwellings.

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CRediT authorship contribution statement

Maggy NB Momba: Conceptualisation of the project, project leader, grant holder, study design and methodology, Validation. Formal analysis, Investigation, Data curation, writing original draft preparation, Writing-Review and editing, Supervision of the first author, Project administration. Arinao Murei: Study design and Methodology, Validation, Formal analysis, Investigation, Data curation, writing original draft preparation, Writing-Review and editing. Ilunga Kamika: Study design and Methodology, and Validation, Data curation, Writing original draft

preparation, Writing-Review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Data are contained within the article or supplementary material.

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Institutional review board statement

The project was conducted according to guidelines of the Declaration of Helsinki and approved by the Tshwane University of Technology Research Ethics Committee (FCRE 2019/08/003 (FCPS 03) (SCI) and 20 March 2020).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167484.

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