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Comparison of culture methods for the detection of *Salmonella* in wastewater

Nikola Roulová*, Petra Moťková, Iveta Brožková, and Marcela Pejchalová

Department of Biological and Biochemical Sciences, The University of Pardubice, CZ−532 10 Pardubice, Czech Republic

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The goal of this work was to find an effective culture method for the detection of Salmonella in wastewater, which would consider both the recommendation of ISO standard 19250 and the specific nature of the wastewater matrix. Three culture methods that differed primarily from the use of the non-selective pre-enrichment step and the processing of wastewater samples prior o the selective enrichment step and further divided into ten protocols depending on the selective enrichment conditions, have been compared with respect to the detection of Salmonella in naturally polluted municipal and hospital wastewater samples. Two selective enrichment broths (MKTTn and RVS) at two incubation temperatures (37 °C and 41.5 °C) plus four media (XLD, DC, BG, and Rambach) were used in this study. The results have shown that the selective enrichment step is the key factor in the culture protocol for detecting Salmonella in wastewater. Such conditions for enrichment (that is, the broth and incubation temperature used) had a major effect on the multiplication of Salmonella to detectable levels, suppressing competing bacteria, and on the efficiency of solid media, thus significantly influencing the isolation rates of Salmonella from wastewater. MKTTn broth performed better for Salmonella isolation from wastewater than that for RVS broth. Incubation at 41.5°C significantly increased the level of Salmonella detection by both selective enrichment broths. XLD and DC agar were the most efficient solid media in this study. The combination of MKTTn broth incubated at 41.5 °C and XLD agar constituted the best approach for isolating Salmonella from wastewater.

Keywords: Culture methods; Detection; Isolation; ISO 19250:2010; *Salmonella*; Wastewater

* Corresponding author, ✉ nikola.roulova@upce.cz

Introduction

Salmonella is a diverse genus of predominantly motile, facultative anaerobic gram-negative rods belonging to the *Enterobacteriaceae* family. Members of the *Salmonella* genus are enteric pathogens and comprise many serovars characterised by different host specificity and distribution [1–4].

Salmonella is one of the most frequently encountered pathogenic microorganisms in surface water. It has been repeatedly detected in various types of natural water, such as rivers, lakes, coastal waters, estuaries, and groundwater around the world [1,4–5]. The primary source of water contamination is faecal origin, as *Salmonella* is excreted in human and animal faeces [6–7]. Surface water pollution is typically more prevalent in the areas with intensive animal husbandry, especially poultry, cattle, and pig farming. *Salmonella* can enter the aquatic environment directly through animal faeces or indirectly through agricultural runoff, among others [1,8]. Similarly, increased pollution is common in areas where natural water is influenced by direct sewage discharge or the final effluent of municipal wastewater treatment plants (WWTPs). Although raw wastewater is polluted more heavily than biologically treated wastewater, the final effluent from municipal WWTPs is far from sterile and releases large amounts of bacteria into the receiving water body [7,9]. The conventional wastewater treatment process based on activated sludge effectively reduces the total concentration of microorganisms and can remove up to 99 % of faecal indicator bacteria. However, due to the high microbial pollution in raw wastewater, many pathogens may not be completely eliminated and can still survive in the final effluent [7,10]. Furthermore, *Salmonella* is considered a predominant pathogenic bacterium in wastewater [7,11].

ISO standard 19250 specifies the method for detecting *Salmonella* spp. in water samples and is applicable to all types of water samples [12]. However, the type of water sample plays an important role in the detection of *Salmonella*. Specifically, wastewater is a complex matrix with high levels of organic and microbial pollution. *Salmonella* is not the dominant bacteria in wastewater, and is present in relatively low concentrations amidst a considerably larger number of background microflora, especially other members of the *Enterobacteriaceae* family [13–15].

The ISO standard method includes four successive stages: pre-enrichment in a non-selective liquid medium, enrichment in a selective liquid medium, plating onto selective solid media, and confirmation of presumptive *Salmonella* colonies [12]. Pre-enrichment in the nutritious non-selective medium can effectively increase low numbers of *Salmonella* and permit the detection of injured *Salmonella*, but also lead to an increase in levels of background microflora [6,16–17]. Elimination of the non-selective enrichment step contributes to reducing the risk of *Salmonella* overgrowth by background microflora and reduces the time required to obtain negative results [18]. Furthermore, the ISO standard method recommends this approach for wastewater [12]. However, by considering the nature of wastewater,

in which a significant proportion of target bacteria can be damaged, these organisms may have difficulty in adaptation to *in vitro* conditions and may not withstand the stressful conditions imposed by culture in highly selective broths [19–20].

The sample volume to be analysed depends on the type of water sample. Volumes greater than 10 ml should be concentrated using membrane filtration prior to non-selective enrichment [12]. However, the wastewater filtration process becomes relatively complicated in actual practice, as sedimentation or floating impurities cause membranes to clog [21–22]. Although the ISO standard method recommends the addition of a sterile filter aid (diatomaceous earth) and the filtration of the water sample through a sterile absorbent pad acts as a support instead of using the membrane for turbid or polluted water, this approach can increase the labour demands on the sample preparation [12]. On the other hand, centrifugation is a simple process for the cell concentration and can be used as an alternative method [22].

The use of Xylose Lysine Deoxycholate (XLD) agar is prescribed by the ISO standard method, while the second solid selective medium is optional, and the laboratory can choose which medium is to used [12]. For example, chromogenic media allow one more specific identification of presumptive *Salmonella* colonies by eye and exhibit greater reliability and specificity than conventional media [16,23]. Additionally, the detection rate can be enhanced by using more than two selective media [16].

The isolation of *Salmonella* from wastewater samples is difficult and time-consuming. Although *Salmonella* in wastewater can be detected by non-culture methods that exhibit more rapid and accurate detection, in many cases it is crucial to obtain bacterial isolates for further characterisation. For example, the isolation of the microbial culprit is essential for typing, molecular epidemiological characterisation, or the determination of antimicrobial sensitivity [5,13,21,24–25]. For this reason, it is still necessary to improve conventional culture methods to reduce cost and labour requirements, thus achieving faster detection and identification of *Salmonella.* Therefore, this study compares modifications of the existing culture methods in an effort to find an effective approach for the detection of *Salmonella* from wastewater. To make the culture methods more applicable to wastewater samples, we considered both the recommendations of ISO standard 19250:2010 and the specific nature of the wastewater.

Materials and methods

Sample collection

The samples used for the study were raw and treated municipal wastewater, as well as raw hospital wastewater. In total, 56 wastewater samples were collected from the two sampling points in the Czech Republic. The respective sampling was performed between July 2020 and May 2022. The samples were collected during

all the seasons of the year to cover all typical weather conditions. The time interval between sampling was kept between four and six weeks. Wastewater (500 mL) was taken as a grab sample and collected by submerging a sterile glass bottle with the aid of a telescopic sampling stick. The samples were transported to the laboratory in a cooling box and analysed within two hours after collection.

Wastewater samples

Municipal wastewater samples were obtained from a municipal WWTP with conventional treatment of biological wastewater based on activated sludge. The WWTP serves a population equivalent of 40,000 and receives household, industrial, and agricultural wastewater. The amount of treated wastewater per year is $2.500 \cdot 10^3$ m^3 while household wastewater constitutes a major part of the raw influent drained into the WWTP ($1.100 \cdot 10^3$ m^3 per year). Samples were taken from the influent (samples marked as MWW INFLUENT; $n = 14$) and the final effluent (MWW EFFLUENT; $n = 14$) of the municipal WWTP.

The raw hospital wastewater was acquired from a hospital with a capacity of 1,300 beds. Wastewater samples were taken at two sampling sites and differed from composition. The first sampling site was the influent of the hospital's internal WWTP. The samples ($n = 14$) collected at this sampling site represented infectious wastewater that contained discharges generated purely by medical activities. These samples (marked as INFECT HW) consisted of wastewater from the infectious disease department, the pathology department, the research department, and the central laboratories. The wastewater from these parts of the hospital was pretreated in the internal WWTP by a conventional biological process and disinfected by chlorination prior to discharge into the municipal sewer network.

The second sampling site was located in the central inspection shaft of the hospital's sewer system. The samples ($n = 14$) collected at this sampling site were hybrid wastewater composed of discharges generated by medical and non-medical activities. These samples (marked as HW) consisted of domestic discharges from toilets and washrooms, industrial discharges generated by the kitchen and laundry, and specific discharges generated by ambulances, general wards, operating rooms, surgical departments, and radiology departments. The wastewater from these parts of the hospital is discharged directly into the public sewer network and treated in the central municipal WWTP.

Culture methods

The wastewater samples were processed using three alternative culture methods for the isolation of *Salmonella*, which differed mainly in the number of enrichment passages and the wastewater sample processing. One method included both

non-selective and selective enrichment, whereas the other two approaches used only selective enrichment and differed in the sample processing, as one method included the direct inoculation of wastewater into selective enrichment broth, and the second one used the initial concentration of wastewater by centrifugation prior to selective enrichment. All three culture methods used two selective enrichment broths and four planting media. Depending on the broth and incubation temperature used for selective enrichment, the three culture methods were further divided into individual protocols. When considering all the combinations, each wastewater sample was examined in parallel using 10 culture protocols for *Salmonella* detection. The scheme of the culture protocols used in this study is shown in Table 1. The isolation was carried out as described in the following three subsections.

Non-selective pre-enrichment and selective enrichment (Method I)

An aliquot of 10 mL of wastewater was added to 90 mL of Buffered Peptone Water (BPW) (M614; HiMedia, Mumbai, India) and incubated at 37 °C for 16 h. After incubation, 1 mL of enriched culture was transferred to 10 mL of Muller-Kauffmann Tetrathionate Novobiocin Broth (MKTTn broth) (CM1048B; Oxoid, Basingstoke, UK) and incubated at 37 °C and 41.5 °C for 21–24 hours (Table 1, culture protocols A and B). In parallel, 0.1 mL of enriched culture was transferred to 10 mL of Rappaport-Vassiliadis Soya Peptone Broth (RVS broth) (CM0866B; Oxoid) and incubated at 37 °C and 41.5 °C for 21–24 hours (Table 1, culture protocols C and D).

Selective enrichment with direct inoculation of wastewater (Method II)

1 mL of wastewater was inoculated into 10 mL of MKTTn broth and incubated at 37 °C and 41.5 °C for 21–24 hours (Table 1, culture protocols E and F). In parallel, 1 mL of wastewater was inoculated into 10 mL of RVS broth and incubated at 37 °C and 41.5 ° C for 21–24 hours (Table 1, culture protocols G and H).

Selective enrichment with initial concentration of wastewater by centrifugation (Method III)

50 mL of wastewater was centrifuged at 4,688 × *g* (ROTINA 420R, Hettich, Tuttlingen, Germany) for 30 min at 4 °C. The pellets were resuspended in 0.5 mL of saline solution (0.9%) and transferred into 10 mL of MKTTn and RVS broths and incubated at 41.5 °C for 21–24 hours (Table 1, culture protocols I and J).

Table 1 Scheme of culture protocols used in this study for the isolation of *Salmonella* from wastewater

Culture method/protocol	Non-selective pre-enrichment	Selective enrichment	Solid medium
Non-selective pre-enrichment and selective enrichment			
BPW/MKTTn 37 °C (A)	10 mL of WW/90 mL BPW 37 °C, 16 h	1 mL of BPW culture 10 mL of MKTTn broth 37 °C, 21–24 h	XLD agar DC agar BG agar Rambach agar
BPW/MKTTn 41.5 °C (B)		1 mL of BPW culture 10 mL of MKTTn broth 41.5 °C, 21–24 h	
BPW/RVS 37 °C (C)		0.1 mL of BPW culture 10 mL of RVS broth 37 °C, 21–24 h	
BPW/RVS 41.5 °C (D)		0.1 mL of BPW culture 10 mL of RVS broth 41.5 °C, 21–24 h	
Selective enrichment with direct inoculation of wastewater			
MKTTn 37 °C (E)	—	1 mL of WW 10 mL of MKTTn broth 37 °C, 21–24 h	XLD agar DC agar BG agar Rambach agar
MKTTn 41.5 °C (F)		1 mL of WW 10 mL of MKTTn broth 41.5 °C, 21–24 h	
RVS 37 °C (G)		1 mL of WW 10 mL of RVS broth 37 °C, 21–24 h	
RVS 41.5 °C (H)		1 mL of WW 10 mL of RVS broth 41.5 °C, 21–24 h	
Selective enrichment with initial concentration of wastewater by centrifugation			
CENT/MKTTn 41.5 °C (I)	—	50 mL of WW/centrifugation 10 mL of MKTTn broth 41.5 °C, 21–24 h	XLD agar DC agar BG agar Rambach agar
CENT/RVS 41.5 °C (J)		50 mL of WW/centrifugation 10 mL of RVS broth 41.5 °C, 21–24 h	

WW – wastewater; CENT – centrifugation

Plating out on solid media

At the end of the selective enrichment of each culture protocol, the enriched cultures were streaked on Xylose Lysine Deoxycholate agar (XLD agar) (M031; HiMedia), Deoxycholate Citrate agar (DC agar) (M065; HiMedia), Brilliant Green agar (BG agar) (M016A; HiMedia), and Rambach agar (1.07500, Merck Millipore, Darmstadt, Germany), and incubated at 37 °C for 24 hours. For biochemical confirmation, three to five typical colonies were selected from each positive plate, or even all colonies if fewer than three were present. The presumptive *Salmonella* colonies were sub-cultured on non-selective Blood Agar (M834; HiMedia) at 37 °C for 24 h.

Identification of *Salmonella* isolates

The identification of the presumptive *Salmonella* colonies was performed by traditional biochemical reactions. Preliminary biochemical identification was carried out on Triple Sugar Iron agar (M021; HiMedia), Christensen's Urea agar (M112; HiMedia), Lysine Decarboxylase broth (M376; HiMedia), and Simmons Citrate agar (CM0155; Oxoid). Additionally, identification of the species was performed using the ENTEROtest 24 N system (10020290; Erba Lachema, Brno, Czech Republic), according to the manufacturer's instructions. *Salmonella* isolates obtained in this study were not serotyped.

Results

In total, 40 of the 56 wastewater samples (71.4 %) examined in this study were found to be positive for *Salmonella* by at least one of the culture protocols used. However, the prevalence of *Salmonella* contamination varied according to the origin of the wastewater from 87.5 % to 35.7 % (Table 2).

Table 2 *Salmonella* isolation frequency in different types of wastewater

Type of samples	Total No. of samples	*Salmonella*-positive samples
MWW INFLUENT	14	12 (85.7 %)
MWW EFFLUENT	14	11 (78.6 %)
INFECT HW	14	5 (35.7 %)
HW	14	12 (85.7 %)
Total	56	40 (71.4 %)

All three culture methods detected a relatively similar number of *Salmonella*-positive samples when comparing culture protocols with the same selective enrichment conditions, except for protocol A (BPW/MKTTn 37 °C) and E (MKTTn 37 °C) and protocol H (RVS 41.5 °C) and J (CENT/RVS 41.5 °C). In general, selective enrichment in MKTTn broth yielded more *Salmonella*-positive wastewater samples than enrichment in RVS broth. As for the temperature of the selective enrichment, *Salmonella* was isolated more frequently after incubation at an elevated temperature of 41.5 °C, regardless of the selective enrichment broth used. However, although the protocols with selective enrichment in MKTTn broth at 37 °C performed worse than incubation at 41.5 °C, they were able to isolate *Salmonella* from wastewater. In contrast, *Salmonella* was not isolated by protocols including selective enrichment in RVS broth at 37 °C, and these selective enrichment conditions did not perform properly.

In detail, the culture protocols B (BPW/MKTTn 41.5 °C), F (MKTTn 41.5 °C), and I (CENT/MKTTn 41.5 °C) gave the highest *Salmonella* detection rate. However, they were unable to isolate *Salmonella* from all positive samples. In contrast, protocols A (BPW/MKTTn 37 °C), C (BPW/RVS 37 °C), G (RVS 37 °C) and J (CENT/RVS 41.5 °C) resulted in the lowest *Salmonella* detection rate and performed poorly compared to other culture protocols. The frequency of *Salmonella*-positive samples detected by each culture protocol is summarised in Table 3.

As for the wastewater origin, culture protocol B (BPW/MKTTn 41.5 °C) gave rise to the highest proportion of positive samples for the MWW EFFLUENT and HW samples, while protocol I (CENT/MKTTn 41.5 °C) gave the highest proportion of positive samples for MWW INFLUENT samples. Furthermore, protocols F (MKTTn 41.5 °C) and H (RVS 41.5 °C) also resulted in a high proportion of positive MWW INFLUENT samples. With INFECT HW samples, protocols B (BPW/MKTTn 41.5 °C), D (BPW/RVS 41.5 °C) and F (MKTTn 41.5 °C) provided an identical number of *Salmonella*-positive samples for this type of wastewater (Table 3).

In 11 positive samples (27.5 %, 11/40), *Salmonella* was isolated via a single-culture protocol. In all of these samples, *Salmonella* was detected using culture protocols that combined non-selective pre-enrichment and selective enrichment at a temperature of 41.5 °C. By protocol B (BPW/MKTTn 41.5 °C) eight positive samples were detected (72.7 %, 8/11), while three positive samples detected (27.3 %, 3/11) via protocol D (BPW/RVS 41.5 °C). In contrast, in 14 samples (35.0 %, 14/40) *Salmonella* was isolated using five or more culture protocols, while the maximum number of positive culture protocols in the individual sample was six, which was detected in two samples. These were municipal MWW INFLUENT samples (Table 4).

Table 3 Number of *Salmonella*-positive wastewater samples and prevalence of positive results based on culture protocol used

Culture method/protocol	MWW INFLUENT (n = 12)	MWW EFFLUENT (n = 11)	INFECT HW (n = 5)	HW (n = 12)	Total positive samples (n = 40)
Nonselective pre-enrichment and selective enrichment					
BPW/MKTTn 37 °C (A)	1 (8.3 %)	1 (9.1 %)	0 (0 %)	4 (33.3 %)	6 (15.0 %)
BPW/MKTTn 41.5 °C (B)	7 (58.3 %)	8 (72.7 %)	2 (40.0 %)	9 (75.0 %)	26 (65.0 %)
BPW/RVS 37 °C (C)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
BPW/RVS 41.5 °C (D)	5 (41.7 %)	2 (18.2 %)	2 (40.0 %)	5 (41.7 %)	14 (35.0 %)
Selective enrichment with direct inoculation of wastewater					
MKTTn 37 °C (E)	6 (50.0 %)	3 (27.3 %)	0 (0 %)	6 (50.0 %)	15 (37.5 %)
MKTTn 41.5 °C (F)	9 (75.0 %)	6 (54.5 %)	2 (40.0 %)	7 (58.3 %)	24 (60.0 %)
RVS 37 °C (G)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
RVS 41.5 °C (H)	9 (75.0 %)	2 (18.2 %)	0 (0 %)	6 (50.0 %)	17 (42.5 %)
Selective enrichment with initial concentration of wastewater by centrifugation					
CENT/MKTTn 41.5 °C (I)	10 (83.3 %)	6 (54.5 %)	0 (0 %)	7 (58.3 %)	23 (57.5 %)
CENT/RVS 41.5 °C (J)	5 (41.7 %)	2 (18.2 %)	0 (0 %)	1 (8.3 %)	8 (20.0 %)

CENT – centrifugation

Table 4 Frequency of *Salmonella*-positive wastewater samples based on the number of culture protocols positive in the individual samples

Number of culture protocols	MWW INFLUENT (n = 12)	MWW EFFLUENT (n = 11)	INFECT HW (n = 5)	HW (n = 12)	Total positive samples (n = 40)
One	0 (0 %)	5 (45.5 %)	4 (80.0 %)	2 (16.7 %)	11 (27.5 %)
Two to four	6 (50.0 %)	3 (27.3 %)	1 (20.0 %)	5 (41.7 %)	15 (37.5 %)
Five and more	6 (50.0 %)	3 (27.3 %)	0 (0 %)	5 (41.7 %)	14 (35.0 %)

Regarding the solid media used, the most effective for the isolation of *Salmonella* from wastewater was XLD agar, followed by DC agar and Rambach agar. *Salmonella* was isolated most often simultaneously by XLD agar and DC agar in many protocols, while in no culture protocol was *Salmonella* isolated only by Rambach agar. No *Salmonella* isolates were obtained from BG agar (Table 5).

Considerable differences were found between the selective enrichment steps for the same plating media. Individual selective enrichment conditions had a major effect on the presence and quantity of presumptive *Salmonella* colonies, as well as the quantity and composition of accompanying microflora on solid media (Figures 1–2). The combination of MKTTn broth incubated at 41.5 °C with XLD agar or DC agar gave the highest isolation rate. However, these combinations had also produced many false positive colonies, which was also evident on XLD agar and DC agar when combined with MKTTn broth and RVS broth incubated at 37 °C. On the other hand, a combination of RVS 41.5 °C/XLD agar had given the lowest levels of false positive colonies, but produced a high number of false negative results. Rambach agar gave the highest isolation rate when combined with MKTTn broth at 41.5 °C.

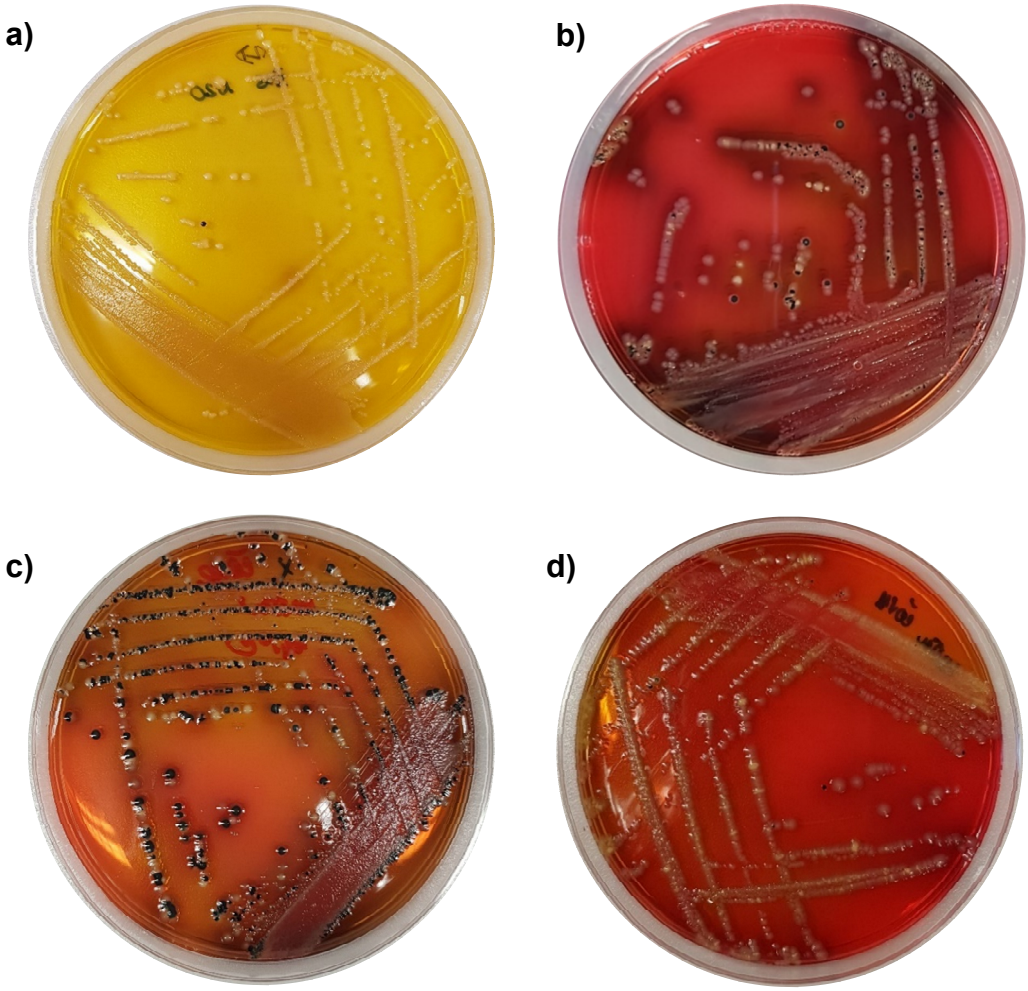

Fig. 1 Typical appearance of XLD agar plates after selective enrichment in RVS broth at 41.5 °C (a) in RVS broth at 37 °C (b) in MKTTn broth at 41.5 °C (c) and MKTTn broth at 37 °C (d)

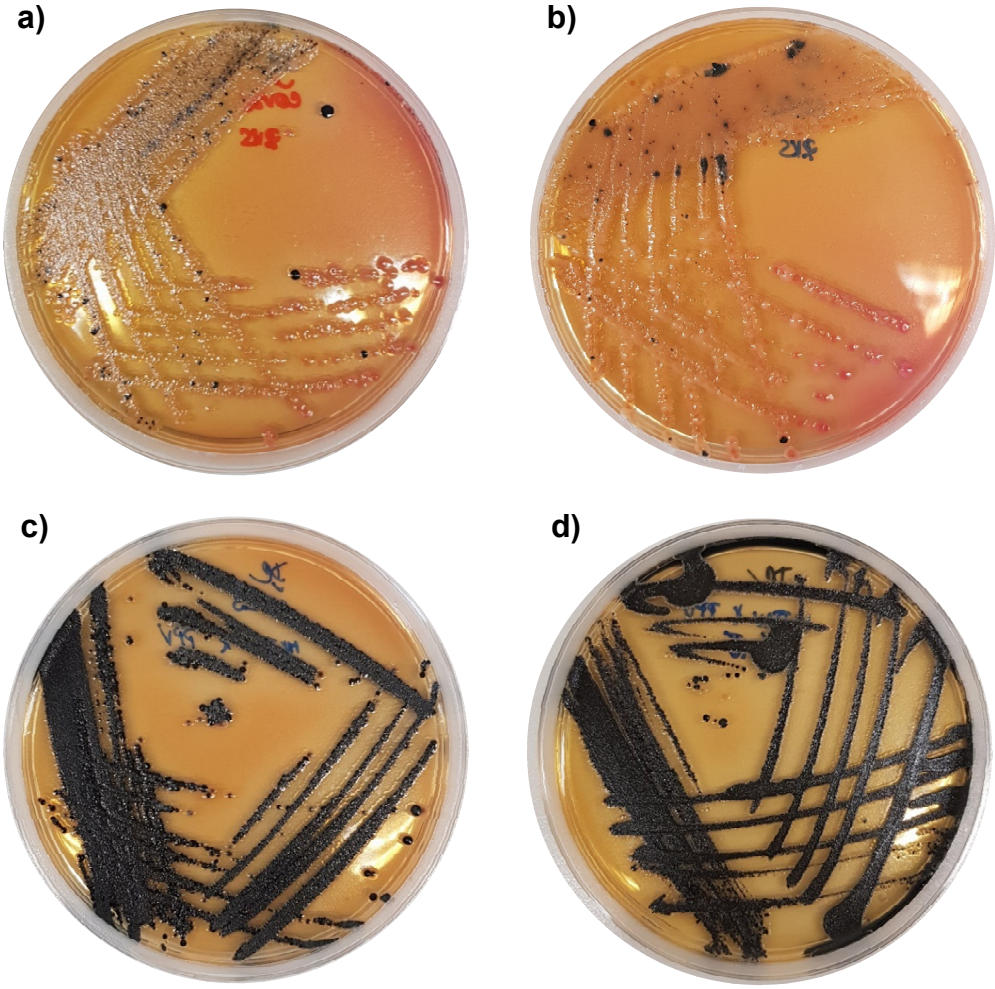

Fig. 2 Typical appearance of DC agar plates after selective enrichment in RVS broth at 41.5 °C (a) in RVS broth at 37 °C (b) in MKTTn broth at 41.5 °C (c) and MKTTn broth at 37 °C (d)

All the solid media used gave rise to false positive colonies. *Proteus* spp. were the most frequently identified bacteria that led to false positive reactions on XLD agar and DC agar. However, while on XLD agar *Salmonella* and *Proteus* colonies were relatively easily visually distinguishable, on DC agar presumptive *Salmonella* colonies were identified visually with difficulty and being indistinguishable from bacterial species that produce hydrogen sulphide, and formed colourless colonies with black centres (Figures 3–4). On the Rambach agar, *Proteus* colonies were pale orange and clearly distinguishable from presumptive *Salmonella* colonies. However, the presumptive *Salmonella* colonies on Rambach agar did not exhibit the same colour and morphology. *Citrobacter freundii* was the bacterium most frequently identified that had caused false positive reactions on Rambach agar, which was contributed to by the production of pink to red colouration in colonies on this medium (Figures 5–7). On BG agar, *Pseudomonas aeruginosa* gave the most frequent false positive results (Figure 8).

Table 5 Occurrence of presumptive colonies and *Salmonella* isolation rate from wastewater via individual culture protocols based on the solid medium used

Culture method/protocol	Solid Medium	No. of positive samples	No. of negative samples		Total No. of samples with presumptive colonies	Percentage of positively confirmed samples with presumptive colonies
			Presumptive colonies	No presumptive colonies		
Non-selective pre-enrichment and selective enrichment						
BPW/MKTTn 37 °C (A)	XLD agar	5 (8.9 %)	45 (80.4 %)	6 (10.7 %)	50 (89.3 %)	10.0 %
	DC agar	2 (3.6 %)	54 (96.4 %)	0 (0 %)	56 (100 %)	3.6 %
	BG agar	0 (0 %)	40 (71.4 %)	16 (28.6 %)	40 (71.4 %)	0 %
	Rambach agar	0 (0 %)	12 (21.4 %)	44 (78.6 %)	12 (21.4 %)	0 %
BPW/MKTTn 41.5 °C (B)	XLD agar	22 (39.3 %)	30 (53.6 %)	4 (7.1 %)	52 (92.9 %)	42.3 %
	DC agar	15 (26.8 %)	38 (67.9 %)	3 (5.4 %)	53 (94.6 %)	28.3 %
	BG agar	0 (0 %)	28 (50.0 %)	28 (50.0 %)	28 (50.0 %)	0 %
	Rambach agar	9 (16.1 %)	21 (37.5 %)	26 (46.4 %)	30 (53.6 %)	30.0 %
BPW/RVS 37 °C (C)	XLD agar	0 (0 %)	46 (82.1 %)	10 (17.9 %)	46 (82.1 %)	0 %
	DC agar	0 (0 %)	49 (87.5 %)	7 (12.5 %)	49 (87.5 %)	0 %
	BG agar	0 (0 %)	0 (0 %)	56 (100 %)	0 (0 %)	0 %
	Rambach agar	0 (0 %)	54 (96.4 %)	2 (3.6 %)	54 (96.4 %)	0 %
BPW/RVS 41.5 °C (D)	XLD agar	12 (21.4 %)	5 (8.9 %)	39 (69.6 %)	17 (30.4 %)	70.6 %
	DC agar	7 (12.5 %)	6 (10.7 %)	43 (76.8 %)	13 (23.2 %)	53.8 %
	BG agar	0 (0 %)	0 (0 %)	56 (100 %)	0 (0 %)	0 %
	Rambach agar	2 (3.6 %)	18 (32.1 %)	36 (64.3 %)	20 (35.7 %)	10.0 %
Selective enrichment with direct inoculation of wastewater						
MKTTn 37 °C (E)	XLD agar	15 (26.8 %)	19 (33.9 %)	22 (39.3 %)	34 (60.7 %)	44.1 %
	DC agar	0 (0 %)	56 (100 %)	0 (0 %)	56 (100 %)	0 %
	BG agar	0 (0 %)	20 (35.7 %)	36 (64.3 %)	20 (35.7 %)	0 %
	Rambach agar	6 (10.7 %)	40 (71.4 %)	10 (17.9 %)	46 (82.1 %)	13.0 %

Table 5 Occurrence of presumptive colonies and *Salmonella* isolation rate from wastewater via individual culture protocols based on the solid medium used (continued)

Culture method/protocol	Solid Medium	No. of positive samples	No. of negative samples		Total No. of samples with presumptive colonies	Percentage of positively confirmed samples with presumptive colonies
			Presumptive colonies	No presumptive colonies		
MKTTn 41.5 °C (F)	XLD agar	24 (42.8 %)	10 (17.9 %)	22 (39.3 %)	34 (60.7 %)	70.6 %
	DC agar	21 (37.5 %)	30 (53.6 %)	5 (8.9 %)	51 (91.1 %)	41.2 %
	BG agar	0 (0 %)	8 (14.3 %)	48 (85.7 %)	8 (14.3 %)	0 %
	Rambach agar	14 (25.0 %)	7 (12.5 %)	35 (62.5 %)	21 (37.5 %)	66.6 %
RVS 37 °C (G)	XLD agar	0 (0 %)	44 (78.6 %)	12 (21.4 %)	44 (78.6 %)	0 %
	DC agar	0 (0 %)	51 (91.1 %)	5 (8.9 %)	51 (91.1 %)	0 %
	BG agar	0 (0 %)	0 (0 %)	56 (100 %)	0 (0 %)	0 %
	Rambach agar	0 (0 %)	52 (92.9 %)	4 (7.1 %)	52 (92.9 %)	0 %
RVS 41.5 °C (H)	XLD agar	16 (28.6 %)	1 (1.8 %)	39 (69.6 %)	17 (30.4 %)	94.1 %
	DC agar	10 (17.9 %)	4 (7.1 %)	42 (75.0 %)	14 (25.0 %)	71.4 %
	BG agar	0 (0 %)	0 (0 %)	56 (100 %)	0 (0 %)	0 %
	Rambach agar	7 (12.5 %)	10 (17.9 %)	39 (69.6 %)	17 (30.4 %)	41.2 %
Selective enrichment with initial concentration of wastewater by centrifugation						
CENT/MKTTn 41.5°C (I)	XLD agar	23 (41.1 %)	33 (58.9 %)	0 (0 %)	56 (100 %)	41.1 %
	DC agar	21 (37.5 %)	35 (62.5 %)	0 (0 %)	56 (100 %)	37.5 %
	BG agar	0 (0 %)	11 (19.6 %)	45 (80.4 %)	11 (19.6 %)	0 %
	Rambach agar	23 (41.1 %)	21 (37.5 %)	12 (21.4 %)	44 (78.6 %)	52.3 %
CENT/RVS 41.5 °C (J)	XLD agar	8 (14.3 %)	2 (3.6 %)	46 (82.1 %)	10 (17.9 %)	80.0 %
	DC agar	7 (12.5 %)	4 (7.1 %)	45 (80.4 %)	11 (19.6 %)	63.6 %
	BG agar	0 (0 %)	0 (0 %)	56 (100 %)	0 (0 %)	0 %
	Rambach agar	1 (1.8 %)	13 (23.2 %)	42 (75.0 %)	14 (25.0 %)	7.1 %

CENT – centrifugation

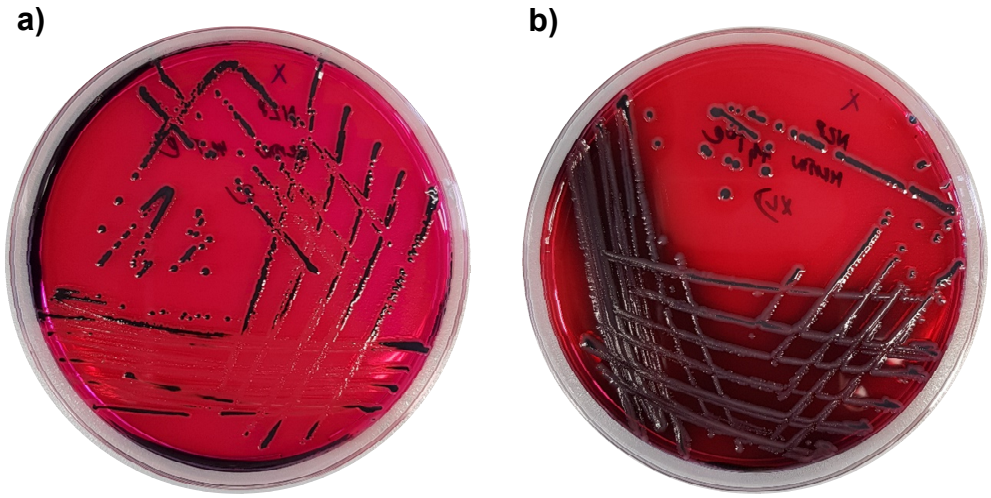

Fig. 3 Difference in colony morphology between *Salmonella* – round flat colourless colonies with a broad bright black centre (a) and *Proteus* – round slightly raised to mucoid colourless colonies with a milk-cloudy black centre (b) on XLD agar plates

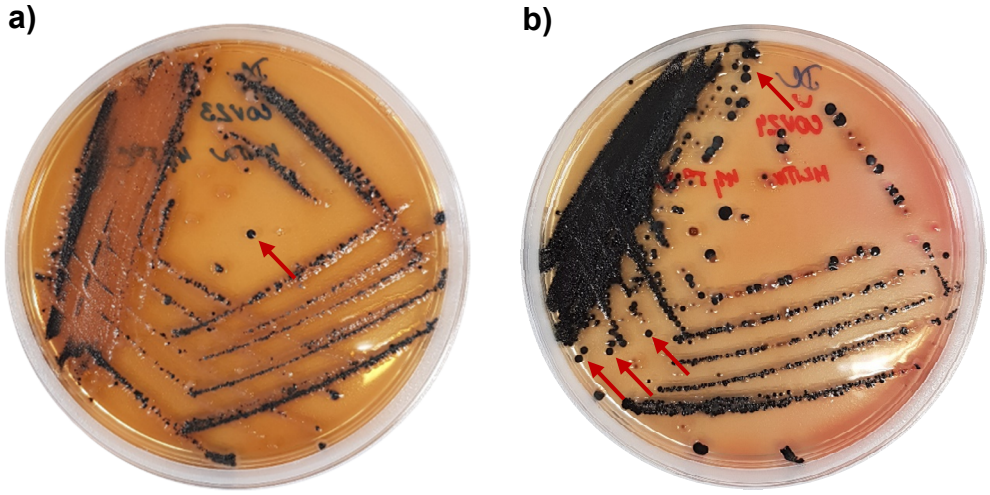

Fig. 4 Presumptive *Salmonella* colonies (marked with an arrow) on DC agar plates confirmed as *Salmonella enterica* subsp. *enterica* (a) and *Proteus* spp. (b)

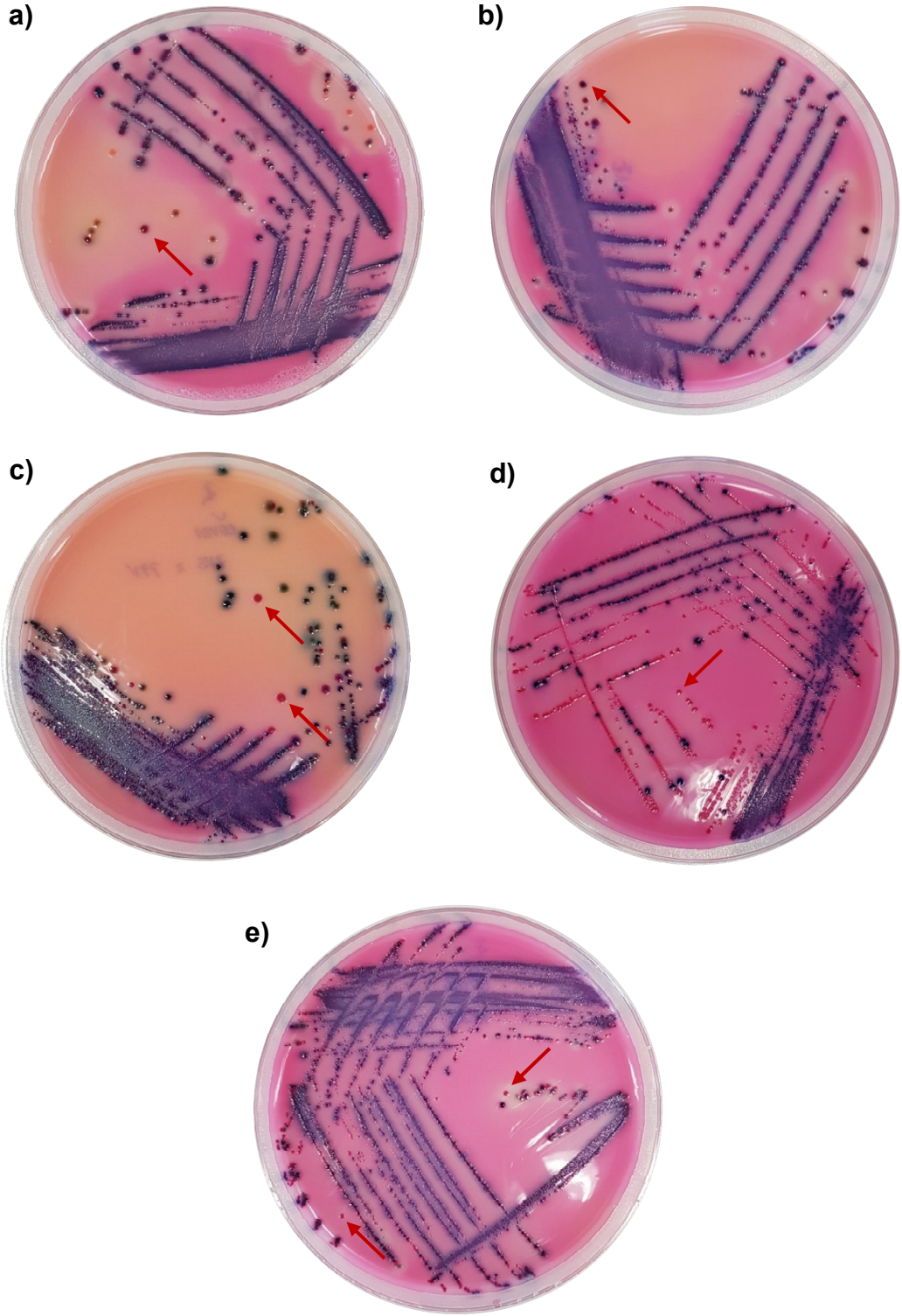

Fig. 5 Rambach agar plates with presumptive *Salmonella* colonies (marked with an arrow) non-confirmed as *Salmonella* (a–b) and confirmed as *Salmonella* (c–e)

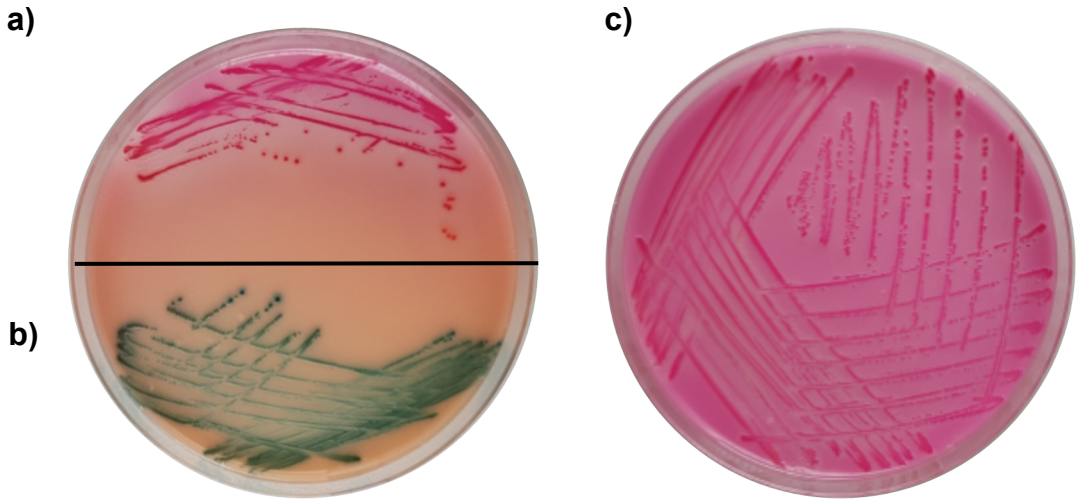

Fig. 6 Colony morphology of reference strains of *Salmonella enterica* subsp. *enterica* ATCC 13076 (a, c) and *Citrobacter freundii* ATCC 43864 (b) on Rambach agar

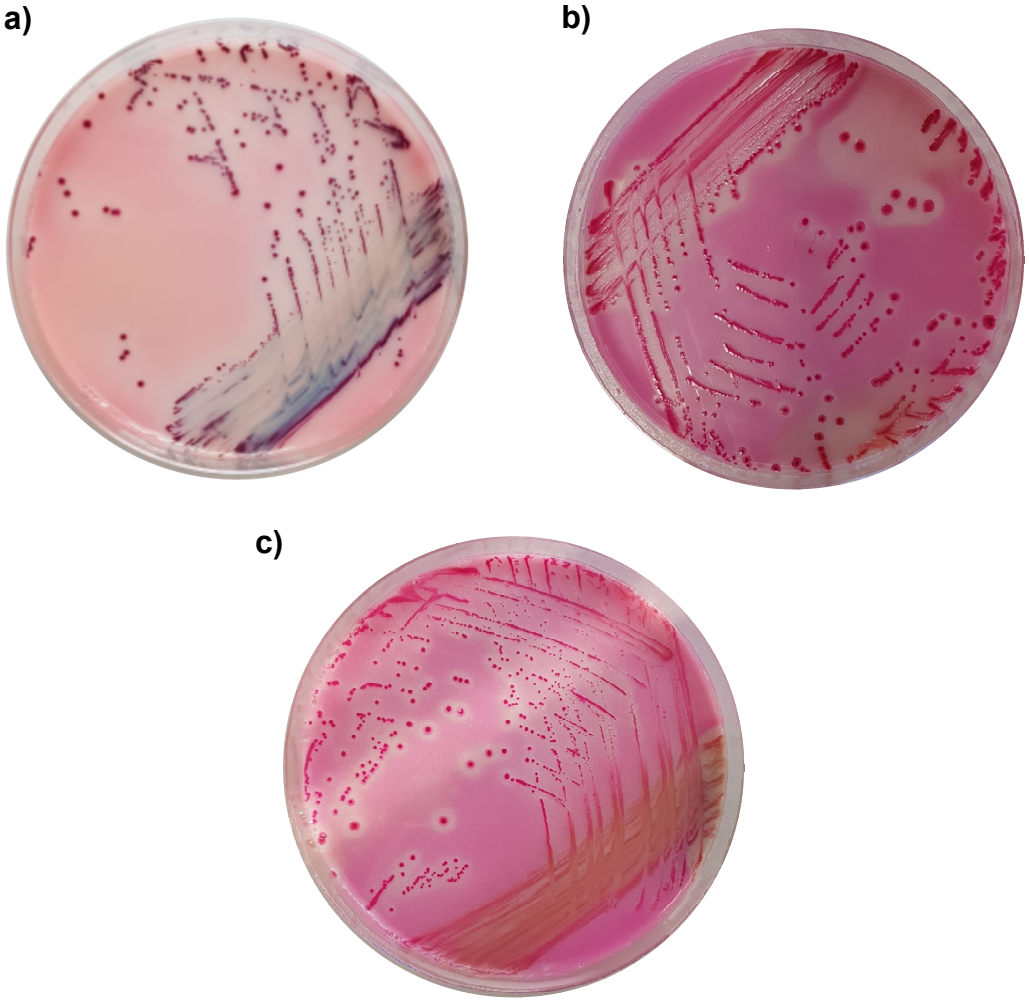

Fig. 7 Colony morphology of the *Citrobacter freundii* (a) and *Salmonella enterica* subsp. *enterica* (b–c) isolated from wastewater on Rambach agar

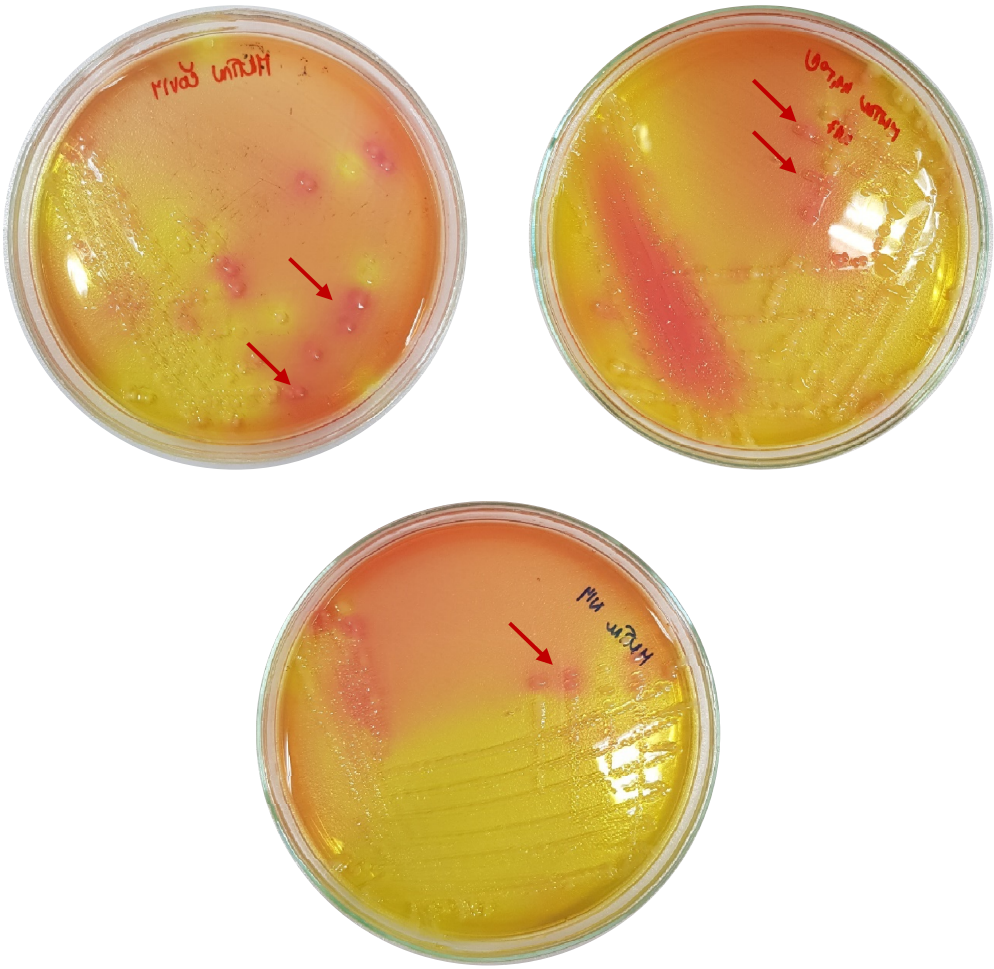

Fig. 8 Pinkish white presumptive *Salmonella* colonies (marked with an arrow) on BG agar plates identified as *Pseudomonas aeruginosa*

Discussion

In general, our study has found a high prevalence of *Salmonella* in wastewater, as *Salmonella* was present in almost 72 % of wastewater samples processed. However, it should be noted that the prevalence of *Salmonella* varied according to the origin of the wastewater, indicating that the composition and properties of the wastewater matrix have affected the number of *Salmonella* and thus the isolation rate, too. This was especially evident for INFECT HW samples. The low prevalence rate of *Salmonella* in this type of wastewater sample is probably derived from its specific nature. First, the source of pollution with *Salmonella* is limited, as wastewater is generated from a small hospital area. Second, the presence of a variety of substances with an antimicrobial effect generated by the infectious disease department and central laboratories can adversely affect the survival of *Salmonella* in wastewater.

On the other hand, the high prevalence of *Salmonella* in the second type of hospital wastewater processed in this study indicates that the composition and properties of HW samples are more like municipal wastewater. Since HW samples also contain wastewater from general services within the hospital (kitchen, laundry) and rainwater, the potential sources of *Salmonella* pollution in this type of wastewater sample are considerably more diverse compared to INFECT HW samples. Therefore, the origins of pollution include not only infected patients but also environmental sources. Additionally, the specific discharges generated by medicinal activities are diluted by domestic discharges, industrial discharges, and rainwater, contributing to a decrease in the concentration of specific pollutants that can adversely affect the survival of *Salmonella* bacteria.

The ubiquity of *Salmonella* in nature is one of the factors that contribute to the common occurrence of *Salmonella* in the MWW INFLUENT samples. *Salmonella* can enter municipal wastewater from a wide range of sources, which is also attributed to the fact that the municipal WWTP receives wastewater from vast areas. The high percentage of *Salmonella*-positive MWW EFFLUENT samples indicates that *Salmonella* commonly survives the conventional biological treatment processes based on activated sludge. As a result, *Salmonella* is released through these effluents into the receiving water. The presence of *Salmonella* in water sources poses a risk to public health, as polluted water can be a source of *Salmonella* outbreaks.

The range of variables that might be evaluated in methodological studies of *Salmonella* isolation is considerable, and it is not possible to include all of them. The volume of the water sample processed, the cell concentration approach, the number and selectivity of enrichment passages, the volume of enrichment broths, enrichment incubation conditions, and the solid medium used for *Salmonella* isolation can all play an important role [14]. The performance of the culture method is also dependent upon the relative concentrations of *Salmonella* bacteria and competing organisms in the exanimated water sample. The approach of this study was to focus on the specific nature of wastewater and modify the existing standard detection methods to be more applicable to this type of sample. This study did not use artificially spiked samples, as they may not truly reflect the physiological state of the target bacteria and the conditions in naturally polluted samples [16].

Compared to water samples with low microbial background levels, such as drinking or groundwater, in which pre-enrichment is an effective approach to increasing the low numbers of *Salmonella* bacteria, the usefulness of this step for wastewater is less clear. Pre-enrichment in a non-selective broth may be counterproductive for heavily polluted matrices [26–27]. Therefore, the direct inoculation of wastewater into BPW was evaluated as a more appropriate approach than that involving the concentration of wastewater prior to non-selective enrichment, which would lead to a further increase of the amount of background microflora. Furthermore, to ensure a sufficient dilution of the background

microflora contained in the wastewater sample and prevent the inhibition of *Salmonella* during non-selective enrichment, a higher volume of BPW was used relative to the ISO standard method. Similarly, the shortest incubation period recommended by the ISO standard method was chosen for pre-enrichment, as the use of a prolonged incubation period is more likely a cause for the overgrowth of *Salmonella* by background microflora and may reduce the likelihood of its detection [17]. Although a period of 5 to 6 h of pre-enrichment in a non-selective broth had been reported to be sufficient to resuscitate *Salmonella*, and reduction of the pre-enrichment period would have greatly accelerated sample analysis, this approach was found to yield unacceptably high number of false negative results [27–30].

However, this study has not confirmed that the omission of pre-enrichment in BPW produces a better detection rate of *Salmonella* from wastewater, as stated in the ISO standard method. Nevertheless, it must be noted that the importance of resuscitating stressed and injured *Salmonella* using pre-enrichment in BPW has been evident for wastewater samples found to be positive by a single culture protocol. The concentration of cells by centrifugation prior to selective enrichment did not lead to an increase of *Salmonella* detection. Nevertheless, our findings support the view that MKTTn broth can accept large amounts of potentially infected material without negative influence of the detection rate, unlike RVS broth. This property of MKTTn broth is advantageous for direct selective enrichment, in which a large inoculum may be necessary. On the other hand, the need for high inoculum ratios in RVS broth can be beneficial if pre-enrichment is used [26,31–32].

This study has confirmed that the incubation temperature markedly affects the productivity of selective enrichment broths [30,33]. The superiority of MKTTn broth, reflected in the number of *Salmonella*-positive wastewater samples identified, can be attributed mainly to its ability to recover *Salmonella* at different incubation temperatures, unlike RVS broth. Furthermore, our study has shown that the high-temperature incubation of both MKTTn and RVS broths is an advantageous approach for wastewater samples, although the ISO standard method recommends the incubation of MKTTn broth at 36 ± 2 °C [12]. The importance of an elevated incubation temperature as an additional selective feature of enrichment broths was demonstrated for wastewater concentration prior to selective enrichment, as the increased amount of inoculum introduced into selective broths had had no significant effect on the accompanying microflora on the solid media.

In general, the results showed that the selective enrichment step is the most critical factor for detecting *Salmonella* in wastewater. The successful detection of *Salmonella* in the subsequent plating stages depended on the ability of the individual selective conditions to suppress competing bacteria and promote the multiplication of *Salmonella*. Consequently, the selective enrichment conditions had exhibited a major effect on the efficiency of the solid media and significantly influenced the isolation rate of *Salmonella* from wastewater samples. It should be

noted that although the combination of enrichment in RVS at 41.5 °C with XLD agar gave the lowest levels of false positive colonies, the considerable selective pressure of this combination probably did not provide conditions for the effective resuscitation of stressed or injured *Salmonella*, resulting in a high number of false negative results. This observation suggests us that the use of a single selective enrichment broth can result in an unacceptable high number of false negative results. However, the choice of RV broth or modified versions of it as a single selective enrichment medium is common practice in such studies where *Salmonella* is being isolated from wastewater or sewage sludge [5,34–38]. This may be linked to the popularity of RV broth, as it is considered to be more productive for the isolation of *Salmonella* than various modified versions of tetrathionate broth, especially from highly polluted matrices [39–41]. On the other hand, although the combination of enrichment in MKTTn at 41.5 °C with XLD agar had produced the highest isolation rate, it significantly increased the need to verify false positive colonies.

Use of a chromogenic medium did not reduce the workload associated with the unnecessary examination of presumptive *Salmonella* colonies, as wastewater isolates did not always lead to the expected chromogenic colour reactions on Rambach agar. Similarly to the results observed, the pink to red colouration of the *Citrobacter freundii* colony on Rambach agar, corresponding to the typical appearance of *Salmonella*, was also reported in other studies [40,42]. In general, when taking into account that Rambach agar is more expensive than other media, there was no benefit in using it for wastewater samples in this study. The zero isolation rate of *Salmonella* from wastewater with BG agar was not surprising, and can be attributed to its high selectivity. For this reason, the manufacturer recommends that BG agar should be used in conjunction with a less inhibitory medium to increase the chances of *Salmonella* recovery [43]. In general, combining two or more solid media is advantageous, as it reduces the number of false negative results [27]. However, any improvements in detection using additional plating media results in an increase of the detection cost [23,27].

Finally, it should be noted that this study had several limitations. First, the *Salmonella* isolates obtained herein were not serotyped. However, the individual culture protocols can preferentially recover specific *Salmonella* serotypes, which can lead to a distortion of their performance based on the serotypes present in the wastewater sample given. Similarly, the performance of any plating medium is also dependent on the serovar distribution in the wastewater sample. Secondly, as this was a pilot study, the number of samples was relatively limited, especially if there were four different wastewater matrices. Furthermore, due to the almost countless methodological options and a very low number of previous studies that had compared the culture protocols for the isolation of *Salmonella* from wastewater, our findings are difficult to be confronted with other studies.

Conclusions

It can be stated that the culture protocol significantly influenced the *Salmonella* isolation rate from wastewater samples, with the selective enrichment step clearly being the key factor. In summary, the MKTTn protocols performed better than the RVS ones, and the high-temperature incubation of enrichment broths considerably increased the isolation rate of *Salmonella* from wastewater samples. The results obtained in this study showed that XLD and DC agar were the most efficient at isolating *Salmonella* from wastewater. Based on the results obtained, combining selective enrichment in MKTTn broth at an elevated incubation temperature of 41.5 ° C followed by isolation at XLD agar could constitute the best approach for detecting *Salmonella* from wastewater. However, it would be imprudent to recommend a single culture protocol for wastewater, since even in our study it was evident that the performance of the individual culture protocols was differing in a specific type of wastewater. Therefore, the inescapable conclusion is that an increased amount of diagnostic work, be it using multiple enrichment broths or plating media, will yield the increased detection rates and a reduced risk of false negative results; however, it will also increase the detection cost and workload.

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