



Isolation rate of *Salmonella* species using novobiocin in different culture media

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Publication History:

Received: 20-09-2025

Revised: 08-12-2025

Accepted: 11-12-2025

Abstract

Salmonella is a leading cause of foodborne illness globally, and its detection in contaminated samples is often hampered by competing microorganisms. Novobiocin (NV), an antibiotic supplement, is known to selectively inhibit Gram-positive bacteria, potentially enhancing Salmonella recovery in culture-based diagnostics. However, the optimal point of its addition in the isolation protocol remains unclear. This study systematically evaluated the effect of novobiocin addition at different stages of the Salmonella isolation process. Using a modified ISO 6579-1 (2017) protocol, 30 known Salmonella-positive faecal samples were processed through five experimental groups in a completely randomized design. The groups tested NV addition in pre-enrichment (Buffered Peptone Water, BPW), enrichment (Muller-Kauffmann Tetrathionate, MKTT; Rappaport-Vassiliadis, RV), and plating (Xylose Lysine Deoxycholate, XLD) media, both with and without NV. Isolation rates were compared using multinomial logistic regression. The addition of novobiocin significantly improved Salmonella isolation rates ($p=0.03$). The highest isolation rate (100%) was achieved in the group using BPW followed by enrichment in MKTT supplemented with NV. This was superior to enrichment in RV with NV (90%). The addition of NV directly to the selective agar (XLD) did not yield a significant improvement in isolation rates compared to XLD without NV. Novobiocin significantly enhances Salmonella isolation when incorporated into selective enrichment broths, particularly MKTT. To improve diagnostic sensitivity for both motile and non-motile strains, a modification of the standard protocol is recommended: the parallel use of MKTT+NV and RV+NV during the enrichment step, rather than a single broth.

Keywords: Culture, Diagnostic sensitivity, Enrichment, Isolation, Novobiocin, Salmonella

Introduction

Salmonella, a Gram-negative bacterium in the *Enterobacteriaceae* family, has two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* comprises six subspecies and over 3000 serovars (Le Minor, 1994; Mare *et al.*, 2001; Tindall *et al.*, 2005; Dongyou, 2010). *Salmonella bongori* has about 22 serovars and is primarily associated with cold-blooded animals, and is less frequently implicated in human infections. It is a significant foodborne pathogen responsible for serious infections globally, mainly transmitted through contaminated foods and causing economic losses (Anon, 1995; Pang *et al.*, 1995; Batz *et al.*, 2011; Jambalang *et al.*, 2017).

Isolation efforts often face challenges due to contamination with competing microorganisms (Jambalang *et al.*, 2017), enhancing detection methods, especially through media composition, is crucial for effective control measures. Factors such as temperature, incubation duration, and media type complicate Salmonella isolation, while Buffered Peptone Water (BPW) is commonly used to resuscitate injured Salmonella cells (Andrews, 1986; Humphrey & Whitehead, 1992; Davies *et al.*, 2000; Hoorfar & Mortensen, 2000). Limited research exists on the use of selective reagents in BPW, but Malachite Green has been reported to inhibit Gram-positive bacteria, thereby promoting Salmonella growth (van Schothorst & Renaud, 1985).

Novobiocin (NV) is an aminocoumarin antibiotic that selectively suppresses Proteus and other Gram-positive bacteria, thereby enhancing Salmonella isolation in various media (Restaino *et al.*, 1977; De Smedt *et al.*, 1986; Devenish *et al.*, 1986; Tate *et al.*, 1990; Grimont & Weill, 2007; Jambalang *et al.*, 2017; NCBI, 2023). However, Jensen *et al.* (2003) noted an 80% increase in Salmonella detection when NV was added to Modified Semisolid Rappaport–Vassiliadis (MSRV) broth.

Inconsistent isolation rates have been observed in the Salmonella Research Laboratory of the National Veterinary Research Institute, Vom, Nigeria, where the ISO 6579-1 protocol (2017) is followed and NV is used at the rate of 1 vial of 10 mg/per 500 ml medium- Muller-Kauffmann Tetrathionate (MKTT) in the Salmonella selective enrichment step. While some studies show improved isolation rates with NV, others report no significant effects, leading to resource and time wastage.

There are discrepancies in the literature on the optimal timing for NV addition, whether during pre-enrichment, enrichment, or directly onto isolation

agar. This highlights the need for systematic investigation (Jensen *et al.*, 2003). This study aims to evaluate the impact of novobiocin at various stages of the isolation process to determine its effect on Salmonella isolation rates.

Materials and Methods

Study area

The study was conducted in Jos North Local Government Area (LGA) of Plateau State, Nigeria. Jos North, located at 9°55'N 8°54'E, has an area of 291 km², and there are many poultry farms within the LGA.

Sample frame

Aliquots of a total of thirty (n=30) Salmonella-positive faecal samples (originally isolated from a pilot study) were collected and utilised for the study. The test protocol was designed as outlined in Figure 1 and Table 1:

Standardized culture and isolation method (gold standard) was used for bacterial isolation which relied on four basic steps: pre-enrichment in non-selective media, enrichment in selective media, isolation on selective agar plates, and confirmation of presumptive bacterial isolates with and without the addition of novobiocin in the different growth broths to study its effect (Plates I - IV).

Isolation and identification of Salmonella

Using the ISO 6579-1 protocol (Horizontal Methods for the Detection of Salmonella, 2017), the isolation of *Salmonella* spp. was conducted in this study (ISO, 2017). The two parallel faecal samples were further divided into 5 different test groups and tested with and without the addition of NV as shown below (Figure 1, Table 1). The entire study plan and processes were conducted concurrently as a single experiment divided into 5 groups using a completely randomized design (Figure 1, Table 1). All media, except otherwise stated, which were used in this study were obtained from Oxoid Limited, Basingstoke, United Kingdom (Oxoid®), and prepared according to the manufacturer's instructions.

Briefly, approximately 10g of each sample was weighed and added to 100 ml 0.1% peptone water, and incubated overnight at 37°C. The overnight suspension was incubated and then transferred (1 ml of each) to 10 ml Rappaport-Vassiliadis (RV) broth (Oxoid® CM0669) and re-incubated overnight at 42°C. Following the incubation, samples were cultured on Xylose Lysine Deoxycholate (XLD) agar (Oxoid®) and

Table 1: Experimental Design Showing Sample Allocation Across Test Groups

Group	Pre-Enrichment	Selective Enrichment Broth	Selective Plating Agar	No. of Samples	Purpose / Description
1a	BPW without NV	–	XLD without NV	30	Control (No NV): Baseline isolation without novobiocin
1b	BPW with NV	–	XLD without NV	30	Tests effect of NV only in pre-enrichment
2a	BPW without NV	MKTT without NV	XLD without NV	30	Standard Protocol Control: ISO 6579-1 using MKTT (Gold Standard for comparison)
2b	BPW without NV	MKTT with NV	XLD without NV	30	Tests effect of NV in MKTT enrichment
3a	BPW without NV	RV without NV	XLD without NV	30	Standard Protocol Control: ISO 6579-1 using RV
3b	BPW without NV	RV with NV	XLD without NV	30	Tests effect of NV in RV enrichment
4a	BPW without NV	MKTT without NV	XLD with NV	30	Tests effect of NV only in plating agar after MKTT enrichment
4b	BPW without NV	MKTT without NV	XLD without NV	30	Control for Group 4a: Same as 4a but without NV in agar
5a	BPW without NV	RV without NV	XLD with NV	30	Tests effect of NV only in plating agar after RV enrichment
5b	BPW without NV	RV without NV	XLD without NV	30	Control for Group 5a: Same as 5a but without NV in agar
Total Samples				300	

Abbreviations: BPW = Buffered peptone water; NV = Novobiocin; XLD = Xylose Lysine Deoxycholate; MKTT = Muller-Kauffmann Tetrathionate; RV = Rappaport-Vassiliadis.

Negative Control: BPW without NV (Group 1a); Positive Control (Gold Standard): BPW + MKTT without NV (Group 2a), as per ISO 6579-1

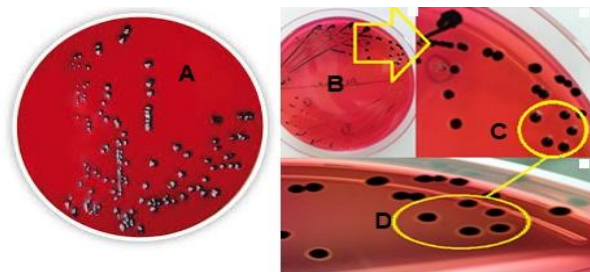


Plate I: Colonial appearance of *Salmonella* colonies on XLD agar (A and B). Close shot highlighting red/pink round colonies with black centre (C). Red/pink round colonies with black centres surrounded by red/pink ring are characteristic of *Salmonella* species

incubated again overnight at 37°C. Red colonies with a black centre were sub-cultured in nutrient agar (NA) (Oxoid® CM0003B) to perform Gram staining and biochemical tests. Colonies were Gram-stained for identification, and biochemical characterization were performed for confirmation as described below (Jambalang, 2011; ISO, 2017). Briefly, heat-fixed smears were stained with crystal violet, iodine,

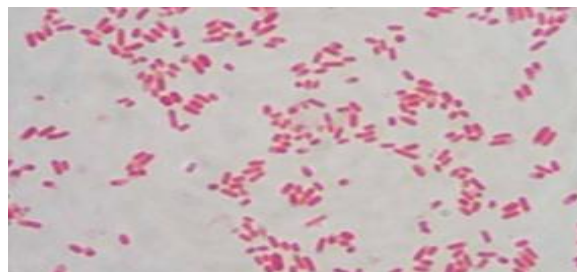


Plate II: Gram stain of typical of *Salmonella* colonies (Gram-negative pink bacilli). All 228 suspected *Salmonella* isolates showed Gram-negative bacilli with characteristic pink colour which is typical of *Salmonella* spp., after Gram staining

decolorized with acetone-alcohol, and counterstained with safranin (Jambalang, 2011; ISO, 2017). Novobiocin antibiotic was used at a concentration of 10mg/500 ml medium and was added to specific media according to the experimental design. The five main groups (1-5), each with subgroups 'a' (without NV for the variable being



Plate III: Biochemical reaction of *Salmonella* to different sugars and enzymes showing positive and negative reactions. Biochemical test on all suspected *Salmonella* samples on different sugars and enzymes showed they were oxidase negative, catalase positive, glucose positive, mannitol positive, sorbitol positive, citrate positive, lysine positive and urease negative

tested) and 'b' (with NV for that variable), were as follows: Group 1 tested NV in pre-enrichment (BPW); Group 2 tested NV in MKTT enrichment; Group 3 tested NV in RV enrichment; Group 4 tested NV in the selective plating agar (XLD) following MKTT enrichment; Group 5 tested NV in the selective plating agar (XLD) following RV enrichment. The exact media combinations for each subgroup are detailed in Figure 1 and Table 1: The whole experiment was replicated thrice.

Gram staining and Biochemical tests to identify Salmonella spp.

Gram stain was performed as described above and observed under a light microscope using X100 objectives under oil immersion. All colonies that showed typical *Salmonella* morphology were further subjected to biochemical characterization like oxidase, catalase, D-glucose, mannitol, sorbitol and triple sugar iron (TSI) to further identify *Salmonella* spp (Grimont & Weill, 2007; ISO, 2017).

Model Fitting Information and Data analysis

Data from the results of the isolation were analysed using SPSS version 21 (<https://ibm-spss-statistics.software.informer.com/21.0/>). Significance of the test was set at $P \leq 0.05$, and the odds ratios and 95% confidence intervals for the strength of association were evaluated using the multinomial logistic regression model. Bar chart was used to fit the distribution of *Salmonella*-positive samples. Comparison among groups was done using the chi-square test.

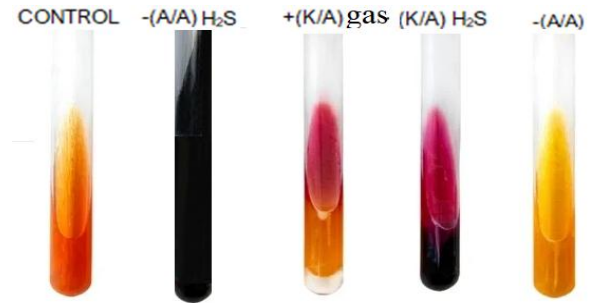


Plate IV: TSI slant reaction of *Salmonella* produced an alkaline slant (reddish colour) over an acidic butt (yellowish colour). *Salmonellae* metabolize thiosulfate in the media to produce hydrogen sulphide (H₂S), which leads to the formation of black colour. Acid over Acid (A/A) reaction is negative for *Salmonella*, while alkaline over acid (K/A) plus gas production is a positive reaction for *Salmonella*, while the first tube (no colour change) is a control for comparison

Results

Isolation rates of Salmonella across experimental groups

A total of 300 processed samples (60 per group, with and without novobiocin) were analyzed across three experimental replicates, yielding 228 confirmed *Salmonella* isolates (overall isolation rate: 76%). Pure *Salmonella* colonies appeared as red/pink round colonies with a black centre surrounded by a red/pink ring on XLD agar.

The isolation rate varied significantly depending on the medium and the stage at which novobiocin (NV) was introduced (Figure 2; Table 2).

The highest isolation rate was observed in Group 3a, which utilized Buffered Peptone Water (BPW) for pre-enrichment followed by selective enrichment in Muller-Kauffmann Tetrathionate broth supplemented with NV (MKTT+NV). This group achieved a 100% isolation rate (30/30 samples). The second-highest performance was seen in Group 4b (BPW + Rappaport-Vassiliadis broth with NV (RV+NV)), with a 90% isolation rate (27/30). Basically, the results "BPW → MKTT (No NV) → XLD (+NV)" (Group 4a) and "BPW → MKTT (No NV) → XLD only" (Group 4b) ensured the testing of the effect of NV in the plating agar after MKTT enrichment. Similarly, "BPW → RV (No NV) → XLD (+NV)" (Group 5a) and "BPW → RV (No NV) → XLD only" (Group 5b), ensured the testing of NV in the plating agar after RV enrichment.

In contrast, the lowest isolation rate (50%) was recorded in the control group without NV in any stage

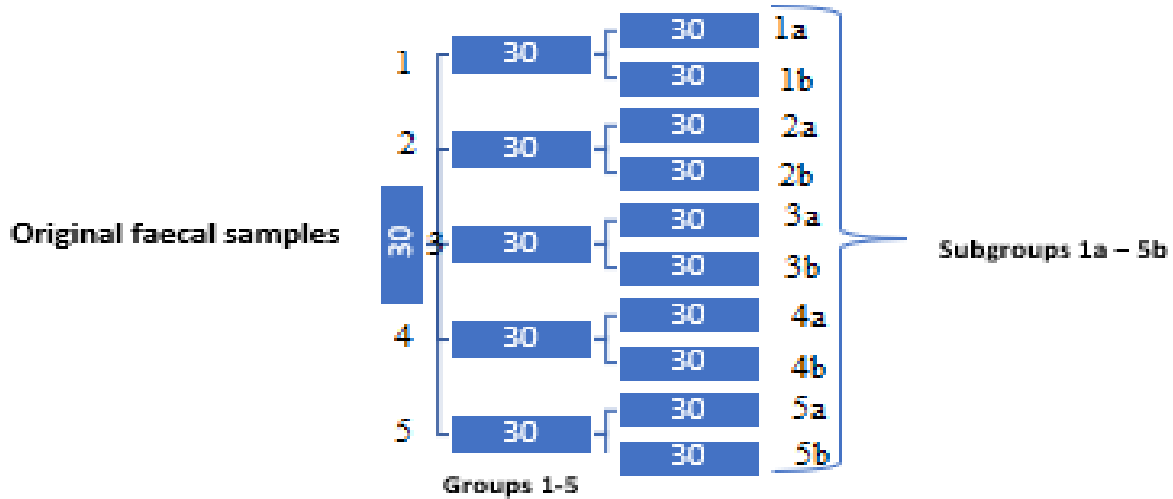


Figure 1: Schematic diagram of organization of experimental procedure

Group 1 – (Effect of NV in Pre-enrichment Broth):

Thirty (30) faecal samples were individually labelled and thoroughly mixed in 30 separate bottles containing BPW for homogeneity. For Group 1a (n = 30), NV was added to the BPW according to the manufacturer's instruction. For Group 1b (n = 30), NV was not added to the BPW. All samples (n = 60) were incubated at 37°C for 24 hours. After incubation, each broth was streaked directly onto XLD agar (without NV) and incubated for another 24 hours at 37°C. (This group tested the effect of adding NV to the pre-enrichment step only.)

Group 2 – (Effect of NV in MKTT Enrichment Broth):

Thirty (30) faecal samples were individually labelled and thoroughly mixed in 30 separate bottles containing BPW (without NV) for homogeneity (Group 2a). All samples were incubated at 37°C for 24 hours. Following pre-enrichment, 1 ml of each BPW culture was inoculated into 10 ml of MKTT broth. For Group 2a, NV was added to the MKTT broth. For Group 2b, NV was not added to the MKTT broth. All MKTT broths (n = 60) were incubated at 37°C for 24 hours, then streaked onto XLD agar (without NV) and incubated for another 24 hours at 37°C. (This group tested the effect of adding NV to the MKTT selective enrichment broth.)

Group 3 – (Effect of NV in RV Enrichment Broth):

Thirty (30) faecal samples were individually labelled and thoroughly mixed in 30 separate bottles containing BPW (without NV) for homogeneity (Group 3a). All samples were incubated at 37°C for 24 hours. Following pre-enrichment, 1 ml of each BPW culture was inoculated into 10 ml of RV broth. For Group 3a, NV was added to the RV broth. For Group 3b, NV was not added to the RV broth. All RV broths (n = 60) were incubated at 42°C for 24 hours, then streaked onto XLD agar (without NV) and incubated for another 24 hours at 37°C. (This group tested the effect of adding NV to the RV selective enrichment broth.)

Group 4 – (Effect of NV in Plating Agar after MKTT Enrichment):

Thirty (30) faecal samples were individually labelled and thoroughly mixed in 30 separate bottles containing BPW (without NV) for homogeneity (Group 4a). All samples were incubated at 37°C for 24 hours. Following pre-enrichment, 1 ml of each BPW culture was inoculated into 10 ml of MKTT broth (without NV). All MKTT broths were incubated at 37°C for 24 hours. For Group 4a, the enriched broth was streaked onto XLD agar supplemented with NV. For Group 4b, the enriched broth was streaked onto XLD agar without NV. All plates were incubated at 37°C for 24 hours. (This group tested whether adding NV directly to the selective plating agar (XLD) improved isolation rates following standard enrichment in MKTT without NV.)

Group 5 – (Effect of NV in Plating Agar after RV Enrichment):

Thirty (30) faecal samples were individually labelled and thoroughly mixed in 30 separate bottles containing BPW (without NV) for homogeneity (Group 5a). All samples were incubated at 37°C for 24 hours. Following pre-enrichment, 1 ml of each BPW culture was inoculated into 10 ml of RV broth (without NV). All RV broths were incubated at 42°C for 24 hours. For Group 5a, the enriched broth was streaked onto XLD agar supplemented with NV. For Group 5b, the enriched broth was streaked onto XLD agar without NV. All plates were incubated at 37°C for 24 hours. (This group tested whether adding NV directly to the selective plating agar (XLD) improved isolation rates following standard enrichment in RV without NV.)

Abbreviations: BPW = Buffered peptone water; NV = Novobiocin; XLD = Xylose Lysine Deoxycholate; MKTT = Muller-Kauffmann Tetrathionate; RV = Rappaport–Vassiliadis)

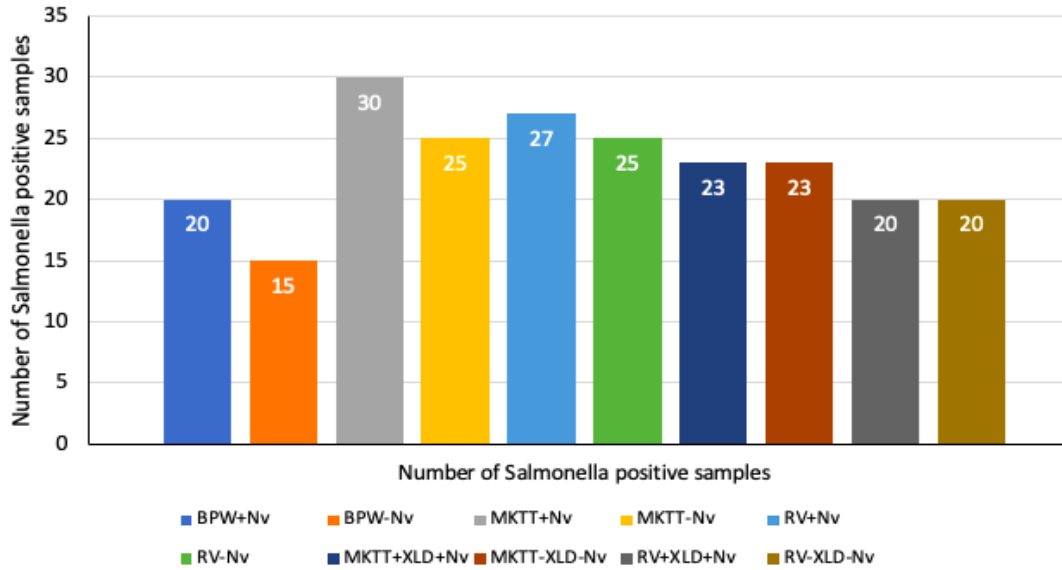


Figure 2: Bar chart showing the number of Salmonella-positive isolates from 30 samples in each tested group using media with or without novobiocin

Table 2: Distribution of Salmonella-positive Isolates Across Experimental Groups

Group	Medium Combination (Process)	No. Positive / Total	Isolation Rate (%)
1a	BPW (No NV) → XLD only	15 / 30	50
1b	BPW (+NV) → XLD only	20 / 30	67
2a	BPW → MKTT (No NV) → XLD only	25 / 30	83
2b	BPW → MKTT (+NV) → XLD only	30 / 30	100
3a	BPW → RV (No NV) → XLD only	25 / 30	83
3b	BPW → RV (+NV) → XLD only	27 / 30	90
4a	BPW → MKTT (No NV) → XLD (+NV)	23 / 30	77
4b	BPW → MKTT (No NV) → XLD only	23 / 30	77
5a	BPW → RV (No NV) → XLD (+NV)	20 / 30	67
5b	BPW → RV (No NV) → XLD only	20 / 30	67
Total		228 / 300	76

(Group 1a: BPW-NV). The addition of NV to the pre-enrichment broth alone (Group 1b: BPW+Nv) increased the isolation rate to 67%. In summary, when NV was added directly to the selective plating agar (XLD+NV) in Groups 4 and 5, no statistically significant improvement was observed compared to their counterparts using XLD only (Table 2).

Statistical analysis of novobiocin impact

The multinomial logistic regression model fitting information confirmed that the variation in isolation rates across the five groups was not random (Final Model $\chi^2 = 18.08$, $df = 9$, $p = 0.03$).

The significant model fit validates that the replicate sample size was sufficient for robust statistical inference (Table 3). Odds ratios (OR) were calculated to quantify the strength of association between NV addition and successful Salmonella isolation (Table 4).

The group with the strongest positive association was Group 3a (BPW + MKTT+NV), with an OR of 1.11 (95% CI: 1.02–1.21), indicating the highest likelihood of Salmonella recovery. The reference category (Group 5a: BPW + RV + XLD+NV) showed no change (OR=1.00). All other groups showed ORs close to 1.00, with confidence intervals crossing 1, indicating no substantial effect compared to the reference.

Overall summary of key findings

Overall, the combination of BPW (pre-enrichment) followed by MKTT broth supplemented with novobiocin yielded the highest Salmonella isolation rate (100%). Similarly, the combination of BPW followed by RV broth with novobiocin was the second most effective method (90% isolation). An ineffective application was observed by the addition of novobiocin directly to the selective agar (XLD), as it

Table 3: Model Fitting Information for evaluating *Salmonella isolation with or without novobiocin*

Model	Model Fitting Criteria		Likelihood Ratio Tests		
	-2 Log Likelihood	χ^2 value	df	Sig (<i>p</i> -value)	
Intercept Only	406.541				
Final	388.461	18.080	9	0.03	

The Intercept Only describes the model that does not control for any predictor variables and simply fits an intercept to predict the outcome variable (whether variations in the stage of novobiocin introduction in the isolation process influence *Salmonella* isolation positively). The Final describes the model that includes the specified predictor variables and has been arrived at through an iterative process that maximizes the log likelihood of the outcomes seen in the outcome variable (whether variations in the stage of novobiocin introduction in the isolation process influence *Salmonella* isolation positively). The -2 Log Likelihood is the product of -2 and the log likelihoods of the null model and the fitted 'final' model. The χ^2 is the Likelihood Ratio (LR) Chi-Square test that at least one of the predictors' regression coefficients is not equal to zero in the model. The df indicates the degrees of freedom of the chi-square distribution used to test the LR Chi-Square statistic and is defined by the number of predictors in the model (three predictors in two models). Sig. (*p*-value) is the probability of getting an LR test statistic being as extreme as, or more so, than the observed statistic under the null hypothesis; the null hypothesis is that all of the regression coefficients in the model are equal to zero.

Table 4: Output of positive samples for *Salmonella* in the different groups that were tested using the Chi-square test based on the group variance

Group	Medium combination (Process)	Sample size	No. Positive	%	P- Value (≤ 0.05)	Odd Ratio	95% C.I
1	BPW+NV	30	20	67	0.03	1.00	0.91; 1.10
	BPW-NV	30	15	50		0.94	0.84; 1.05
2	BPW + MKTT+NV	30	30	100		1.11	1.02; 1.21
	BPW + MKTT-NV	30	25	83		1.05	0.97; 1.15
3	BPW + RV+NV	30	27	90		1.08	0.99; 1.17
	BPW + RV-NV	30	25	83		1.05	0.97; 1.15
4	BPW + MKTT-NV + XLD+NV	30	23	77		1.03	0.95; 1.13
	BPW + MKTT-NV + XLD-NV	30	23	77		1.03	0.95; 1.13
5	BPW + RV + XLD+NV	30	20	67		1.00	NA
	BPW + RV + XLD-NV	30	20	67		1.00	0.91; 1.10

The reference category is Group 5a: BPW → RV (No NV) → XLD (with NV). Abbreviations: BPW = Buffered peptone water; NV = Novobiocin; XLD = Xylose Lysine Deoxycholate agar; MKTT = Muller-Kauffmann Tetrathionate broth; RV = Rappaport-Vassiliadis broth.

did not significantly improve isolation rates. The differences in isolation success across the protocols were statistically significant ($p < 0.05$), confirming that the stage and medium for NV addition are critical factors.

Discussion

This study evaluated the effect of novobiocin (NV) addition at various stages of the *Salmonella* isolation process, specifically, during pre-enrichment, enrichment, and agar plating to determine its impact on isolation rates. Novobiocin is an antibiotic that selectively inhibits the growth of *Proteus*, *Pseudomonas*, and Gram-positive bacteria, thereby promoting *Salmonella* growth indirectly.

The finding in this study indicates that the inclusion of NV in the enrichment broth significantly enhances *Salmonella* isolation rates compared to samples without NV ($p < 0.05$). This aligns with previous

studies (Restaino *et al.*, 1977; van Schothorst & Renaud, 1985; De Smedt *et al.*, 1986; Devenish *et al.*, 1986; Tate *et al.*, 1990; Jensen *et al.*, 2003; Grimont & Weill, 2007; Jambalang *et al.*, 2017). The primary benefit of adding NV is the higher isolation rate, which reduces the risk of missing weakly positive *Salmonella* isolates by suppressing competing organisms. The increase in isolation rates can be attributed to NV's antimicrobial action against Gram-positive bacteria, facilitating a more favorable environment for *Salmonella* growth. This improvement provides more suitable starting materials for sensitive tests like ELISA, MALDI-TOF MS, and real-time PCR (Sohn *et al.*, 2016; Kenmuir *et al.*, 2023).

Previous research has shown that non-*Salmonella* microorganisms compete heavily for nutrients in broth without NV (Hoben *et al.*, 1973; Jensen *et al.*,

2003; Mooijman, 2018). In this study, the absence of NV allowed these non-Salmonella organisms to dominate, leading to a reduced detection of Salmonella.

The combination of Muller-Kauffmann Tetrathionate with NV yielded the best results and had the propensity to optimally improve Salmonella isolation rate, hence its recommendation for use based on our study. Similarly, the combination of the enrichment medium RV with NV also optimized Salmonella isolation, and may be considered as an alternative.

Notably, there was no difference in Salmonella isolation rates when NV was added directly to isolation agar (XLD + NV vs. XLD - NV). This method should only be considered in the absence of both MKTT + NV and RV + NV.

There was increased diagnostic sensitivity (synergistic effect) when combining test reagents for isolation, compared to using buffered peptone water with NV alone, which predicted lower sensitivity. This corroborates findings by Hoorfar & Baggesen (1998). The missed isolates in group 1 compared to isolation rates in groups 2 and 3 may be attributed to this lower sensitivity. This study highlighted that growth broth containing novobiocin in the enrichment step significantly increased Salmonella isolation rate than those without novobiocin.

The inconsistencies observed in Salmonella isolation rates in various research laboratories, including NVRI Vom, may stem from the use of multiple protocols, mixing different manufacturers' media, inadequate sample homogenization or using samples that have been stored for a long period of time before testing. Proper inventory management is essential to ensure consistency in laboratory reagents and protocols, reducing observed discrepancies as recommended by the United States Agency for International Development (USAID, 2008). Utilizing approved in-house protocols will help ensure proper homogenization of samples and media, minimizing false results. Testing samples on the same day they arrive in the laboratory is also crucial. The insights from this study aim to assist researchers, particularly in developing countries, where challenges in Salmonella diagnosis persist.

The superior performance of NV-supplemented enrichment broths, particularly MKTT+NV, can be attributed to the synergistic selective pressure exerted by both the base medium and the antibiotic. MKTT broth itself contains tetrathionate and brilliant green, which inhibit coliforms and Gram-positive bacteria, while permitting Salmonella to metabolize tetrathionate. Novobiocin adds an additional, specific

layer of inhibition against *Proteus* spp. and other NV-sensitive competitors that might otherwise tolerate the primary selective agents (De Smedt *et al.*, 1986; Tate *et al.*, 1990). This multi-hit inhibitory strategy reduces nutrient competition more effectively than either component alone, allowing even low initial populations of stressed or sub-lethally injured Salmonella cells to resuscitate and proliferate to detectable levels. This mechanistic synergy explains why NV added directly to the non-selective BPW pre-enrichment (Group 1b) showed a moderate improvement, but the combination with a selective enrichment broth yielded the most dramatic gains. In conclusion, novobiocin significantly increased Salmonella isolation rate when used in combination with other media, and that broths containing MKTT+NV had higher Salmonella isolation rate than RV+NV alone. There was no difference in the isolation rate between XLD+NV and XLD-NV when NV was added directly to the isolation agar XLD.

It is recommended that a little modification should be made to the ISO 6579-1, 2017 protocol, especially in developing countries, in order to increase the scope and isolation rate of both motile and non-motile Salmonella strains. We suggest that two parallel broths—MKTT and RV—should be used independently (not mixed) for enrichment, allowing for the isolation of both motile and non-motile strains from the same sample. Most literatures report that RV is better in isolating motile strains of Salmonella while MKTT is better in isolating non-motile strains. The modified method if approved, will enhance the current protocol for the isolation of Salmonella in the Salmonella Research Laboratories. Furthermore, PCR should be conducted on all the isolates to fully understand their classification (motile or non-motile serovars).

Our recommendation to use parallel MKTT+NV and RV+NV broths is not merely to increase the sensitivity of raw detection, but to broaden the spectrum of recoverable Salmonella types. RV broth, with its high osmotic stress and elevated temperature (42°C), is historically recognized for favoring motile *Salmonella* serovars (Devenish *et al.*, 1986). In contrast, MKTT, incubated at 37°C, is less harsh and may better support the recovery of non-motile or slower-growing strains (ISO, 2017). By supplementing both with NV, we potentially enhance the recovery of strain types within each category that are otherwise outcompeted by NV-sensitive flora. This approach aligns with the "One Health" diagnostic challenge, where samples may contain a diversity of *Salmonella* serovars of zoonotic and clinical significance. Future

work involving serotyping or genotyping of isolates from each parallel broth would be valuable to confirm this breadth-of-recovery hypothesis.

While the proposed modification adds a second enrichment broth, the cost-benefit analysis favors its adoption in settings where false negatives carry high consequences, such as in food safety monitoring, outbreak investigation, or regulatory diagnostics. The marginal cost of an additional broth tube is offset by the significant reduction in the risk of missing a positive sample, which can lead to costly product recalls, protracted outbreaks, or misdirected public health interventions. For resource-constrained laboratories, a tiered approach could be considered: routine screening could employ the single most effective broth (MKTT+NV), while confirmatory or outbreak samples undergo parallel enrichment with both MKTT+NV and RV+NV to maximize detection certainty.

This study was conducted using archived, culture-positive faecal samples. While this provided a consistent and challenging matrix, future validation should include prospective testing of naturally contaminated, diverse sample types (e.g., food, environmental swabs) with potentially lower and more variable target pathogen loads. Furthermore, confirmation of all isolates via molecular methods (e.g., *invA* gene PCR) would provide absolute confirmation beyond biochemical profiling. Research should also explore the efficacy of NV with other emerging rapid detection platforms, such as real-time PCR or multiplex ELISA following enrichment, to determine if the improved bacterial recovery translates into improved performance of these faster, non-culture methods.

Acknowledgments

All the staff of Salmonella Research Laboratory, NVRI Vom are acknowledged for all their contribution and support during laboratory analysis of the work.

Funding

No funding was received.

Conflict of Interest

The authors declare no conflict of interest.

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