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Clinical Performance of Oxgall Pre-enrichment qPCR for Salmonella Detection in Antibiotic-Exposed Patients Suspected of Typhoid Fever

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Abstract

Introduction: *The reliability of typhoid fever diagnostics in endemic regions is frequently compromised by low circulating bacterial loads and widespread pre-hospital antibiotic use.* ***Objective:*** *This study evaluates the specific factors contributing to false-negative outcomes when using an oxgall-pre-cultured real-time polymerase chain reaction method for Salmonella typhi detection.* ***Method:*** *A cross-sectional evaluative study was conducted using thirty blood samples from Widal-positive patients. Laboratory optimization using spiked samples established a technical limit of detection. Clinical specimens were analyzed using conventional blood culture and real-time polymerase chain reaction targeting the invA gene after a six-hour enrichment in ten percent oxgall medium.* ***Result and Discussion:*** *While laboratory optimization successfully detected spiked matrices, one hundred percent of clinical samples yielded negative results via real-time polymerase chain reaction, and blood culture identified only one positive case. Evaluative analysis revealed that all subjects received antibiotic therapy prior to sampling and most were already in the second week of fever, exceeding eight days.* ***Conclusions:*** *The high rate of false-negative results is primarily due to bacterial loads dropping below the limit of detection because of antibiotic interference and late-stage sampling. Furthermore, ten percent oxgall concentration may inhibit the recovery of antibiotic-stressed bacteria.*

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Introduction

Typhoid fever, a systemic infection caused by the bacterium *Salmonella typhi*, remains a significant public health threat in numerous tropical and developing countries (Abo-Kora et al., 2026; Kouam et al., 2026). Accurate and rapid diagnosis is critical to ensure effective treatment and minimize life-threatening complications, yet non-specific clinical symptoms often hinder early identification (World Health Organization, 2023a). While blood culture is recognized as the current gold standard, it possesses major technical limitations, including low sensitivity often falling below 20% and a lengthy isolation period (Maina et al., 2022; Udaykumar et al., 2024). This low sensitivity is further exacerbated by inadequate sample volumes and the high prevalence of pre-hospital antibiotic use among patients in endemic regions (Appiah et al., 2020; Moore et al., 2025).

Molecular techniques such as Real-time PCR (qPCR) have been recommended as alternatives due to their faster processing times, broad detection ranges, and higher analytical sensitivity compared to conventional bacteriological methods (Ngan et al., 2023; Zhou et al., 2024). To enhance diagnostic performance, researchers have integrated a brief enrichment stage using selective oxgall media prior to DNA amplification to stimulate target bacterial replication (Zhou et al., 2023).

However, the operational reliability of molecular assays in routine clinical workflows remains highly susceptible to various pre-analytical and technical constraints (Nguyen et al., 2024; Park et al., 2024). False-negative outcomes in qPCR systems can be triggered by multiple factors, including insufficient blood sample volume (Maina et al., 2022), suboptimal DNA extraction quality leading to low DNA recovery (Nguyen et al., 2024), and the presence of systemic or reagent-derived PCR inhibitors such as hemoglobin or excessive bile salts from the enrichment medium (Silva et al., 2022). Additionally, pre-analytical variables like improper sample transport and storage conditions can cause significant DNA degradation prior to processing (Directo et al., 2026; Roberts et al., 2025). From a technical standpoint, limitations in primer compatibility, inadequate thermal cycling parameters, or inappropriate cycle thresholds (Ct) can further impair the detection of low-abundance target genes in clinical matrices (Carter et al., 2024; Tan et al., 2024)..

Consequently, a significant gap exists between laboratory optimization results and clinical effectiveness in real-world patients (Park et al., 2024). While laboratory spiking methods demonstrate precise detection success, clinical implementation in endemic areas frequently yields unexpected negative results (Park et al., 2025). The novelty of this study lies in its critical evaluation of risk factors underlying false-negative outcomes in molecular systems, focusing specifically on the impact of prior antibiotic interference and the duration of fever alongside these technical variables, as 73.3% of clinical subjects in this study were already in the second week of infection (Appiah et al., 2024). Understanding these external and technical variables is essential for mapping the operational boundaries of qPCR in daily medical practice. Therefore, this research aims to evaluate the factors contributing to false-negative results in oxgall-pre-cultured qPCR detection of Salmonella typhi and to compare its sensitivity against conventional blood culture methods in typhoid fever patients

Method

This research employed a comparative analytical observational study using a cross-sectional design. The primary objective was to compare the sensitivity of the oxgall pre-culture Real-Time PCR (qPCR) method against the conventional blood culture method in patients suspected of having typhoid fever. Study subjects were selected through a consecutive sampling technique at Dustira Hospital in Cimahi. The inclusion criteria consisted of male or female patients aged 18–60 years, clinically diagnosed with typhoid fever based on physical examination and positive Widal test results, and experiencing high fever for less than one week (World Health Organization, 2023b).

The research utilized 10 mL of whole blood collected from 30 patients who met the established criteria. Each blood sample was divided into two equal portions: 5 mL for bacteriological blood culture and 5 mL for molecular analysis via qPCR (Kumar et al., 2024). The procedures were conducted at multiple specialized facilities, including the Medical Laboratory Technology Service Laboratory of Poltekkes Kemenkes Bandung for DNA extraction and bacteriological work, and the West Java Provincial Health Laboratory for the qPCR execution.

The research procedure began with a technical optimization phase using spiked control isolates of Salmonella typhi, Salmonella paratyphi A, and Salmonella spp. in healthy blood to verify the performance of the reagents. For clinical specimens, 5 mL of patient blood was inoculated into 45 mL of 10% oxgall enrichment medium. These mixtures were then incubated at 37°C for a duration of 6 hours prior to DNA extraction. The choice of oxgall was based on its ability to neutralize the bactericidal activity of human blood and facilitate the release of intracellular bacteria (Rahman et al., 2025; Silva et al., 2022). Molecular materials and instruments involved the use of the G-Spin™ Total DNA Extraction kit for DNA isolation following the manufacturer's specific protocols. The extracted DNA was analyzed using a BIORAD iQ 5 machine and a Real-time PCR kit targeting the invA gene of Salmonella typhi from NEXTM Diagnostic (Malorny et al., 2022; Zhang et al., 2023). The reaction mixture totaled 20 µl, including Master Mix, primer mix, internal amplification control (IAC), and 3 µl of the DNA template. The thermal cycling protocol consisted of initial incubation at 95.00°C for 3 minutes, followed by 35 cycles of denaturation, annealing, and scanning (Carter et al., 2024).

The bacteriological blood culture served as the gold standard for comparison. Samples from the incubated oxgall media were collected using a loop and isolated on Salmonella Shigella Agar (SSA). These plates were incubated for 24 hours at 37°C. Colonies exhibiting S. typhi characteristics clear appearance with a black center underwent further biochemical confirmation using a sugar series, including TSIA, Mannitol, Peptone, and Semi-Solid media to ensure accurate identification. Data collection was based on primary data from qPCR amplification curves and bacterial growth results. Analysis techniques utilized statistical sensitivity tests to measure the diagnostic performance of each method. The results were synthesized into subject characteristic tables, melting temperature (Tm) graphs, and distribution tables. This comprehensive analysis aimed to evaluate the external factors, such as antibiotic interference and fever duration, that influenced the accuracy of the molecular detection in clinical settings.

Result and Discussion

1. Result

This study involved 30 research subjects who successfully met the established inclusion and exclusion criteria. Data were gathered through laboratory examinations conducted at TNI Dustira Hospital, Poltekkes Kemenkes Bandung, and the West Java Provincial Health Laboratory. The demographic and clinical profiles of the participants are detailed in Table 1 below:

Table 1
Characteristics of Research Subjects

No	Characteristic	Number (n)	Percentage (%)
1	Gender		
	Female	19	63.3
	Male	11	36.7
2	Age Range (Years)		
	< 20	2	6.7
	21 - 50	22	73.3
	> 51	6	20.0
3	Fever Duration (Days)		
	< 4	4	13.3
	5 - 7	4	13.3
	> 8	22	73.3
4	Antibiotic History		
	Consumed	30	100
	Did Not Consume	0	0

According to the data in Table 1, the majority of the subjects were female (63.3%) and fell within the productive age group of 21–50 years (73.3%). Notably, 100% of the participants were recorded to have received oral or injectable antibiotic therapy prior to clinical sample collection. Furthermore, a significant portion of patients (73.3%) sought laboratory testing only after experiencing a fever for more than eight days. The laboratory findings for the detection of Salmonella typhi using conventional blood culture and Real-Time PCR (qPCR) methods are summarized in Table 2:

Table 2
Distribution of S. typhi Examination Results

No	Examination Method	Positive (n)	Negative (n)	Total
1	Blood Culture	1	29	30
2	qPCR (Without Oxgall)	0	30	30
3	qPCR (With 6-Hour Oxgall)	0	30	30

As shown in Table 2, conventional blood culture identified only one positive sample (3.3%), while the qPCR method, regardless of oxgall enrichment, yielded entirely negative results (100%) for all clinical samples. To verify the diagnostic performance, a reagent optimization phase was conducted. The qPCR using the invA gene kit demonstrated high specificity, yielding two distinct peaks: the internal amplification control (IAC) at Tm 78–80°C and the Salmonella sp. target at Tm 87–89°C. The specificity and cross-reactivity analysis confirmed that the system only amplified the target DNA from the Salmonella genus, while other enteric pathogens, including

Klebsiella, E. coli, and Shigella spp., showed no amplification signals (Negative). The visualization of these optimization results is presented in Figure 1.

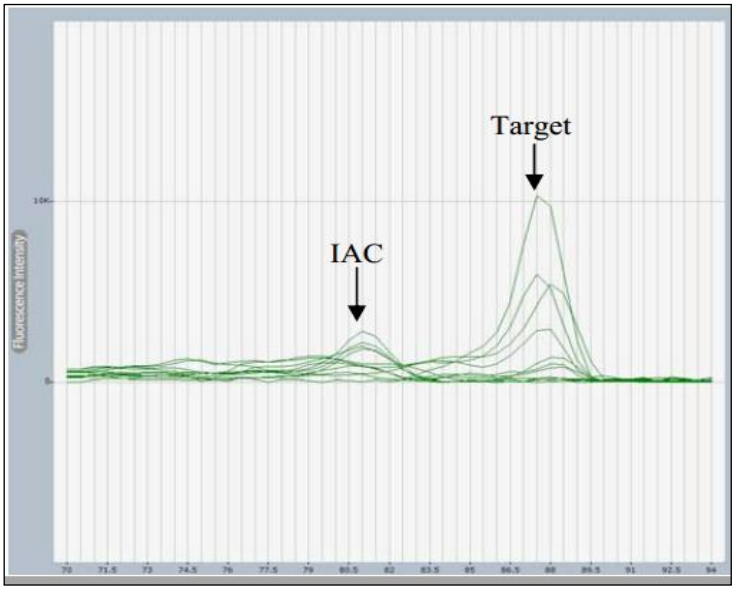

Figure 1. Tm (Melting Temperature) Curve of QPCR Optimization Results

Figure 1 displays the internal amplification control (IAC) peak at Tm 78–80°C and the target *Salmonella* sp. DNA peak at Tm 87–89°C. Sensitivity testing across concentrations from 10^3 to 10^8 CFU/mL revealed that while *S. typhi* was detectable at 10^6 CFU/mL with a DNA concentration of 0.8 ng/µL, amplification was not observed at 10^4 CFU/mL with a DNA concentration of 0.4 ng/µL. These findings verify that the reagents perform effectively on control samples but failed to detect the bacteria in clinical specimens from patients already exposed to antibiotics. Consequently, the technical Limit of Detection (LoD) for this system was established at a threshold of 10^4 CFU/mL.

Furthermore, DNA concentration analysis was performed using a NanoDrop spectrophotometer to quantify the yield from various isolates. The results showed that the minimum DNA concentration required for successful amplification was 0.4 ng/µL for S. typhi and 0.7 ng/µL for S. paratyphi A. The detailed concentration data are presented in Table 3:

Table 3
DNA Concentration of Salmonella sp. Isolates

No	Isolate and Concentration (CFU/mL)	DNA Concentration (ng/µL)
1	*Salmonella spp* 10^8	4.8
2	*Salmonella spp* 10^7	1.9
3	*Salmonella spp* 10^6	1.2
4	*Salmonella spp* 10^5	0.9
5	*Salmonella spp* 10^4	0.6
6	*Sarelmonella spp* 10^3	0.1
7	*S.typhi* 10^8	1.2
8	*S. typhi* 10^7	0.9
9	*S. typhi* 10^6	0.8
10	*S. typhi* 10^5	0.6
11	*S. typhi* 10^4	0.4
12	*S. typhi* 10^3	0.2
13	*S.paratyphi A* 10^8	5.6
14	*S. paratyphi A* 10^7	1.0
15	*S. paratyphi A* 10^6	0.8
16	*S.paratyphi A* 10^5	0.7
17	*S.paratyphi A* 10^4	0.6
18	*S.paratyphi A* 10^3	0.2

The study also evaluated the effect of pre-culturing samples in 10% oxgall medium for 6 hours. While the oxgall enrichment improved the amplification signal in spiked control samples by neutralizing blood bactericidal effects, it did not alter the diagnostic outcome for the clinical group. These findings suggest that prior antibiotic exposure and bacterial loads falling below the established technical LoD were the primary factors preventing detection in clinical specimens.

2. Discussion

The primary objective of this study was to evaluate the diagnostic performance of qPCR using the *invA* gene for detecting *Salmonella typhi* in clinical samples, specifically focusing on identifying false-negative risk factors in endemic areas where antibiotic self-medication is prevalent. This study addresses a significant diagnostic gap where molecular tools, despite their high specificity, often yield negative results due to complex pre-analytical factors (Park et al., 2024). The findings reveal a stark contrast between laboratory optimization and clinical application, where the qPCR optimization using the *invA* gene kit demonstrated high specificity yielding a distinct target peak at Tm 87–89°C without cross-reactivity from other enteric pathogens, yet the clinical results remained entirely negative (Malorny et al., 2022; Rahman et al., 2025).

This discrepancy is scientifically interpreted through the established technical Limit of Detection (LoD) identified during the optimization process, which determined that the system requires a minimum bacterial load of 10^4 CFU/mL or a DNA concentration of 0.4 ng/µL to achieve successful amplification. In clinical specimens, the bacterial density likely fell below this threshold, rendering the *invA* gene undetectable despite the reagent's proven accuracy in controlled laboratory settings (Maina et al., 2022; Nguyen et al., 2024).

The identified risk factors for these false-negative results are deeply rooted in the clinical history of the subjects, most notably the 100% antibiotic exposure rate recorded prior to sample collection. Antibiotics significantly reduce the concentration of viable bacteria and intact DNA in the bloodstream to levels far below the 10^4 CFU/mL LoD, which directly inhibits the molecular detection process (Appiah et al., 2020; Ngan et al., 2023). Furthermore, the infection timeline played a critical role, as 73.3% of subjects sought testing only after more than eight days of fever. Scientifically, the probability of isolating *Salmonella* from blood declines as bacteria migrate from the bloodstream to the reticuloendothelial system during the second week of infection, a phenomenon that is consistent with previous investigations stating that prior antimicrobial therapy and late-stage testing are the primary causes of culture-negative typhoid fever in endemic regions (Appiah et al., 2024; Hasan et al., 2023).

In an effort to mitigate these risks, this study utilized a 6-hour pre-culture in 10% oxgall medium to enhance bacterial recovery. However, the findings indicate that while oxgall enrichment can neutralize the bactericidal effects of blood and improve amplification signals in spiked control samples (Rahman et al., 2025; Silva et al., 2022), it was insufficient to overcome the severe DNA degradation or extreme low-density bacteremia present in patients already treated with antibiotics (Roberts et al., 2025). This implies that for patients with a documented history of prior antibiotic use, a 6-hour enrichment period may be insufficient to boost the bacterial load above the detection threshold.

The benefit of this study lies in its rigorous verification of why molecular tools can still yield negative results in real world clinical settings, highlighting the diagnostic gap created by self-medication. For future research, it is recommended to explore longer enrichment durations, alternative multi-copy genetic targets with a lower LoD such as the *fliC* or *H1-d* genes (Ngan et al., 2023; Tan et al., 2024), and prioritize sample collection in the early acute phase before antibiotic administration to maximize diagnostic sensitivity (Directo et al., 2026).

Conclusion

The study concludes that while the Real-Time PCR method targeting the invA gene demonstrates high technical accuracy and specificity in laboratory settings, its clinical utility is significantly limited by real world pre-analytical factors. The high rate of negative results in clinical specimens is primarily attributed to the pervasive use of antibiotics prior to sample collection and late-stage hospital presentations, both of which reduce bacterial loads to levels below the established technical Limit of Detection of 104 CFU/mL. Furthermore, although 10% oxgall enrichment provides an enhancement in amplification signals for control samples, it remains insufficient to overcome the diagnostic challenges posed by prior antimicrobial exposure in clinical subjects. These findings suggest that the effectiveness of molecular diagnostics in endemic regions is heavily dependent on early sample collection and the clinical history of the patient, highlighting a critical need for integrated diagnostic strategies that account for prior treatment interventions.

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