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Evaluation of Novel Polymerase Chain Reaction Method for the Diagnosis of Typhoid Fever in Suspected Cases

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ABSTRACT

Objective: To evaluate viGen Q multiplex polymerase chain reaction (PCR) kit for typhoid fever using blood culture as the reference method in clinically suspected cases

Study Design: Diagnostic Accuracy Study

Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology Rawalpindi, Pakistan from Nov 2023 to Apr 2024.

Methodology: Venous samples of clinically suspected patients were collected in Ethylene Diamine Tetra Acetic Acid (EDTA) containing tube and blood culture bottle. On receipt in the Microbiology department of AFIP, a blood culture was performed and typhoidal salmonellae were identified to the species level by biochemical tests and serology. From the sample collected in the EDTA tube, deoxyribonucleic acid (DNA) was extracted using an automated DNA extractor (bioPerfectus), followed by amplification as per protocol of viGen Q multiplex PCR kit. Results were noted and data was analyzed using a statistical package for social sciences (SPSS) version 26.

Results: Blood samples of a total of 131 patients were tested by both blood culture and PCR methods for the detection of typhoidal salmonellae. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of viGen Q multiplex PCR kit were 92.7%, 94.4%, 88.4%, 96.6%, and 94% respectively. The Cohen's kappa value (κ) was approximately 0.86, indicating strong agreement.

Conclusions: The viGen Q multiplex PCR kit has good sensitivity and specificity in comparison with blood culture and has the potential to expedite diagnosis and improve patient outcomes.

Keywords: Blood culture, Diagnostic accuracy, Multiplex PCR, Enteric fever, Typhoid fever

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INTRODUCTION

Typhoid fever, caused by Salmonella Typhi and Salmonella Paratyphi A, B, and C, remains a major health issue worldwide, especially in areas with inadequate sanitation, infrastructure, and limited availability of clean water sources.[1,2] The disease manifests a spectrum of symptoms ranging from mild gastrointestinal discomfort to severe systemic complications, making it a public health challenge.3 About 27 million cases of typhoid fever occur annually worldwide, with a mortality rate of 1%.[3] The heaviest burden of typhoid is borne by Asian countries, notably Pakistan, Bangladesh, and India.[4]

The rise of typhoidal salmonella spp especially those of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains, has heightened the importance of timely diagnosis. Widal and Typhi-dot which have been used to screen typhoid for many years, have been declared unreliable because of limited sensitivity and specificity.[4] Blood culture, the reference method for diagnosing typhoid fever, is labour-intensive and time-consuming.[5] The extended time it takes to receive culture results adds to the delay in diagnosing and treating patients, which can worsen their condition and increase the risk of transmission.[6]

Developments in molecular diagnostics, like PCR, offer a better way of diagnosing typhoid fever compared to traditional methods.[7] Various PCR-based technologies are commercially available for the diagnosis of typhoid fever. Recently viGen-Q Fever Panel Multiplex PCR kit, designed as a qualitative, multiplexed, nucleic acid-based in vitro diagnostic test has been introduced. It is used to detect the DNA of Salmonella spp., Plasmodium spp., and the RNA of Dengue virus (DENV), with endogenous internal control, in clinical samples using the Real-Time PCR technique. Using this technology, we can quickly identify the cause of typhoid fever. Currently, there is no available data from Pakistan regarding the evaluation of this PCR kit. The study aims to evaluate

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the viGen-Q Fever Panel Multiplex PCR Kit for typhoid fever using blood culture as the reference method in clinically suspected cases.

METHODOLOGY

This diagnostic accuracy study was conducted in the Department of Microbiology, AFIP Rawalpindi, Pakistan from November 2023 to April 2024. Approval from Institutional Review Board (MIC22-18/READ-IRB/24/2712) was taken before commencing the study.

Inclusion Criteria: Patients of both genders and any age group were included when they present with a documented fever of 38°C or higher that has persisted for at least three days. Patients who had not used any antimicrobials before sampling were considered eligible for the study.

Exclusion Criteria: Patients with any other known focus of infection that could explain the fever were excluded from my study.

Non-probability consecutive sampling was performed and Beuderer's formula was used to calculate the sample size. Taking a 95% confidence level, 5% precision, 93% sensitivity, and a population proportion of 22%, the sample size was calculated to be 131.[8,9]

Demographic data on patients' age, gender, and occupation was collected using a pre-formed questionnaire. A total of 15 ml of venous blood was collected using an aseptic technique. About 10 ml of blood was transferred to an aerobic blood culture bottle and 5 ml into an EDTA-containing tube. The aerobic blood culture bottles were processed using the BacT/ALERT™ automated culture system.[10] After a positive signal, the samples were sub-cultured on appropriate culture media including blood and MacConkey agar. The typhoidal Salmonella spp were identified at the species level by routine microbiological tests including catalase, oxidase, motility tests, analytical profile index (API-10S) and serology. The antimicrobial susceptibility testing (AST) for the isolates was performed by a modified Kirby Bauer disc diffusion method.[11] The results were interpreted as per Clinical and Laboratory Institute (CLSI) guidelines.[11] American type culture collection (ATCC) 25922 Escherichia coli strain was used as a control.

To perform PCR, 5 milliliters (ml) of whole blood collected in 0.5 M EDTA was enriched in 5 ml tryptic soy broth (TSB) medium (Oxoid, UK) for 5 hours at 37OC under aerobic conditions.

To extract deoxyribonucleic acid (DNA), 10 ml of the culture medium was centrifuged at 12,000 rpm for 5 minutes to concentrate the cells. The supernatant was discarded, and the pellet was washed with Phosphate buffer. The pellet was then resuspended in 1 ml of Phosphate buffer. From this suspension, 200 µl was used for extraction. The sample was vortexed to ensure complete homogenization, breaking up cell clumps for efficient DNA extraction. This homogenized sample was then processed using an automated DNA extractor (bioPerfectus) following programmed protocol, which completed the extraction in 45 minutes.[12]

After extraction, the PCR mix was prepared by combining 15 µl of the reaction mix with 10 µl of the extracted nucleic acid sample in a 0.2 ml PCR tube to make up a final volume of 25 µl. The initial denaturation was done at 94 OC for 10 min, followed by subsequent denaturation at 95 OC for 45 seconds, annealing at 57 OC for 45 seconds, and extension of the primer at 72 OC for 1 min. At the end, the reaction mixture was incubated at 72 OC for 10 min. The amplification reaction was carried out for a total of 40 cycles. The PCR system detected fluorescence signals from the reporter dyes FAM (for Salmonella spp.) and TEXAS RED (for internal control). The limit of detection (LoD) for Salmonella spp. was 10^3 copies per milliliter. The expected cycle threshold (Ct) value for Salmonella spp. detection in the FAM/GREEN channel was 22 ± 4. Interpretation of results was based on a Ct cutoff of 35 for accurate analysis. This approach ensured systematic and standardized detection of typhoidal salmonellae using real-time PCR technology to achieve accuracy and reliability.

The data was analysed using Statistical Package for the Social Sciences (SPSS) version 26. Mean and standard deviation (SD) were calculated for quantitative variables such as age. Frequency and percentages were calculated for qualitative variables like gender and grade of fever. To determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the PCR kit with blood culture as the gold standard, a 2x2 table was created. The Cohen's kappa value (κ) was calculated to assess inter-rater agreement. Additionally, the diagnostic accuracy was calculated to evaluate the performance of the diagnostic test.

RESULTS

Out of 131 patients, 79(60.3%) were males and 52(39.7%) were females. The mean age of patients was 37.11±20.12 years. The documented body temperature of participants, at the time of presentation, ranged from 99.5 OF to 103 OF with a mean of 101.06 of ± 1.09 OF. Among 131 patients, 53(40.46) had a high-grade fever, while 78(59.54%) had a low-grade fever. Blood culture was found positive in 41 patients and Salmonella Typhi was found to be the most common isolate (Figure-1).The isolated typhoidal salmonella spp were found mostly sensitive to Meropenem and Azithromycin but least sensitive to Ciprofloxacin (Figure 2).

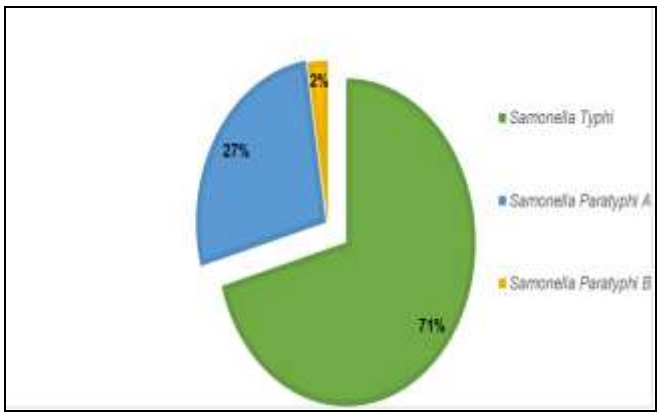

Figure-1: Percentage Distribution of Isolates in Test Samples (n=41)

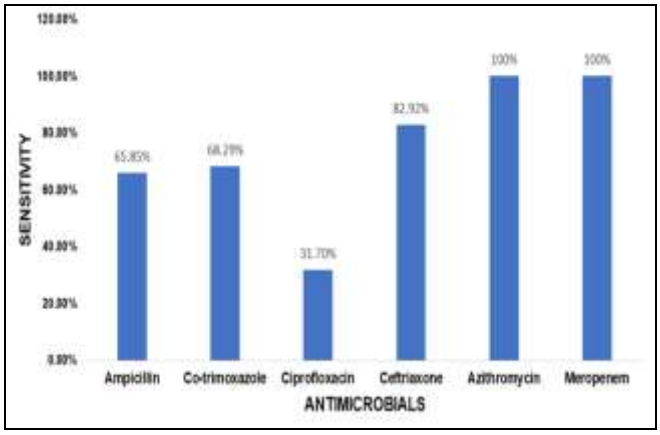

Figure-2: Percentage Sensitivity Pattern of Isolated Salmonella Species (n=41)

In thirty-eight patients, both blood culture and PCR were positive while both tests were found negative in eighty-five patients (Table-I). The sensitivity, specificity, PPV, NPV, and accuracy of the viGen-Q fever panel multiplex PCR kit for diagnosis of typhoidal salmonella spp was found to be 92.7%, 94.4%, 88.4%, 96.6% and 94%.

Table-I Contingency table for Comparison of viGen-Q fever Panel Multiplex PCR kit and blood Culture Results (n=131)

Multiplex PCR Kit	Positive	Negative	Total
Blood Culture Positive	38 (92.7%)	3 (7.3%)	41
Blood Culture Negative	5 (5.6%)	85 (94.4%)	90
Total	43	88	131

Sensitivity: True Positive / (True Positive + False Negative) = 38 / (38 + 3) = 92.7%
Specificity: True Negative / (True Negative + False Positive) = 85 / (85 + 5) = 94.4%
Positive Predictive Value (PPV): True Positive / (True Positive + False Positive) = 38 / (38 + 5) = 88.4%
Negative Predictive Value (NPV): True Negative / (True Negative + False Negative) = 85 / (85 + 3) = 96.6%
Diagnostic Accuracy: (True Positive + True Negative) / Total Patients = (38 + 85) / 131 = 93.9%

DISCUSSION

Our study revealed that compared to blood culture, viGen Q multiplex PCR has 92.7% sensitivity and 94.4% specificity. A review of published literature revealed similar results. In the era of increasing MDR and XDR typhoid and endemicity of typhoid in Pakistan, there was a dire need to look for testing options that could diagnose typhoid early and reliably.[14] Blood culture can diagnose typhoid reliably but the turnaround time is in many days.[6] Current study has evaluated viGen Q multiplex PCR against blood culture for diagnosis of typhoid fever. The results of this study revealed that compared to blood culture, viGen Q multiplex PCR has 92.7% sensitivity and 94.4% specificity. The results of our study are similar to published literature. Particularly, a study by Enany SA *et al*. found that PCR had a sensitivity of 92.86% and a specificity of 98.97%.[15] Another study by Khanum et al emphasized PCR's heightened sensitivity and specificity, showing its efficacy in precisely diagnosing typhoid fever.[16]

The kappa value (κ) of 0.86 in our study indicates a strong agreement between the viGen Q multiplex PCR and blood culture results. Moreover, 88.4% PPV and 96.6% NPV further underpin the clinical utility of PCR in identifying true positive and true negative cases, respectively. These metrics suggest that PCR can significantly reduce the likelihood of misdiagnosis and unnecessary treatments, which had been common issues associated with traditional diagnostic tests like widal and typhidot.[17]

A multicentre study was conducted by Al-emran et al. as part of the Typhoid Fever Surveillance Africa Program (TSAP) by enrolling 1674 patients. Among

these, typhoid was diagnosed by blood culture in 18 (1.1) while by PCR in 44(14.8%) cases.[18] In our study, blood culture was found positive for typhoidal salmonellae in 41(31%) and PCR in 43(32.8%) cases. The difference between the two tests in our study is much less as compared to the study conducted by Al-emran *et al.* One reason of this difference can be the inclusion criteria of the two studies. We have included only the patients who had not taken any antibiotic in this episode of fever while in the study by Al-emran *et al,* they did not exclude the patients with prior antimicrobial use which could have reduced the sensitivity of blood culture.[18]

Despite the overall effectiveness of the multiplex PCR kit, there were three instances(2.3%) where it failed to detect typhoidal Salmonella spp. Comparing our findings with published literature, we find that a study conducted in Bangladesh by Pouzol *et al.* revealed that out of 680 suspected typhoid cases, seven samples that tested positive in culture were not detected by PCR indicating a 1% failure rate in identifying these pathogens.[19] The higher failure rate in our study compared to that of Pouzol et al could be due to errors in following the PCR protocol, reduced sample volume, or low bacterial load.

Our study also focuses on important insights into the antimicrobial resistance patterns of 41 typhoidal Salmonella isolates. The 82.9% sensitivity against Ceftriaxone in our study, highlights its continued effectiveness. Moderate resistance against Ampicillin and Co-trimoxazole (34.14% and 31.70%, respectively) is a reflection of their historical misuse.[20] However, high resistance against Ciprofloxacin (68.29%), underscores the need for alternative treatment options. The high Ciprofloxacin resistance rate in our study is in concordance with a two-year study (2017-2018), on emerging resistant strains of typhoidal Salmonella spp., conducted by Fatima et al, in Karachi. In her study, the isolates exhibited high-level resistance to Ciprofloxacin at 59% and 89%.[15] The variation in exact percentages may be due to population variation, exposure to antibiotics, and disease prevalence. Azithromycin and Meropenem both exhibited 100% sensitivity, making them highly effective options against these strains. The significant resistance to Fluoroquinolones and moderate resistance to older antibiotics highlight the urgent need for continued surveillance and updated treatment guidelines.

Despite its reliability, the limitations of PCR are firstly, its inability to give a clue about the AST of the isolate. For which we still will have to resort to the blood culture. Secondly, approximately double the cost of PCR as compared to blood culture presents a challenge for its widespread implementation. However, the benefit of early diagnosis of typhoid by PCR can not only reduce mortality and morbidity of patients but also reduce the overall cost of patient management and hospitalization.

Overall, our study corroborates the body of evidence supporting the use of PCR in the rapid and accurate diagnosis of typhoid fever. The variations in performance metrics across different studies can be attributed to differences in PCR protocols, sample sizes, and study populations. Nevertheless, the high sensitivity, specificity and predictive values underscore the viGen Q multiplex PCR kit's potential for the correct diagnosis. Thus, facilitating early management of typhoid fever, ensuring better patient outcomes and more efficient use of healthcare resources.

LIMITATION OF STUDY

Our study had certain limitations. Firstly, it relied on data from a single center, which may restrict the generalizability of the findings to the broader population. Secondly, the duration of the study was relatively short, potentially impacting the results. Therefore, we recommend a larger scale, multicentre study in future for further evidence to ensure the reliability of the viGen Q multiplex PCR kit for the diagnosis of typhoid fever.

CONCLUSION

Compared to blood culture, the viGen Q multiplex PCR kit was found to have good sensitivity, specificity, PPV, NPV, accuracy and agreement. This makes viGen Q multiplex PCR kit a valuable diagnostic tool for the rapid and accurate detection of typhoid fever.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

AA & MMG: Data acquisition, data analysis, critical review, approval of the final version to be published.

SHN & AHG: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

RS & MAK: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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