Evaluation of sensitivity and specificity of ELISA against Widal test for typhoid diagnosis in endemic population of Kathmandu

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Abstract

Background: Widal test, which has poor predictive outcomes in predominant typhoid population, is not standard enough to predict accurate diagnosis. This study aims to compare the diagnostic accuracy of Widal test to ELISA using blood culture as gold standard.

Methods: The blood samples were collected in Capital Hospital, Kathmandu, Nepal from febrile patients having ≥48 h fever in 3 years study period for blood culture, Widal test and IgG-IgM ELISA.

Results: Amongst 1371 febrile cases, 237 were Salmonella typhi positive to blood culture and 71.4 % typhoid fever patient were of 46–60 years old with male to female ratio of 2:1. Blood culture confirmed patients had ≥1:40 anti-TH and anti-TO titre in 45.56 % (n = 108) and 43.88 % (n = 104) patients respectively. The sensitivity and specificity of IgG (0.96 and 0.95) and IgM (0.95 and 0.94) at 95 % confidence level were significant compared to Widal anti-TH (0.72 and 0.58) and TO (0.80 and 0.51) test (p value, 0.038) at titre level ≥1:200. Further the PPV of Widal TH and TO (0.38 and 0.23) was low compared to IgG and IgM ELISA (0.78 and 0.77) (p value, 0.045).

Conclusion: Widal test is not sensitive enough for an endemic setting like Nepal and thus should be either replaced with more accurate test like ELISA or follow an alternative diagnostic methodology.

Keywords: Typhoid Fever, Salmonella typhi, Widal test, ELISA, Sensitivity, Specificity, Nepal

Background

Typhoid fever is becoming a key health problem in developing countries. Lack of safe and clean drinking water supplies to the people and adequate sewage disposal are the major reasons [1]. The fever has been one of the leading diagnosed fever ailment among the fever related cases in most of the hospitals of Nepal [2]. It is popularly known as ‘Bisham Joro’ in local language meaning the ‘fever with poison’. Typhoid is prevalent in mid-hills, valleys and southern belts as an endemic disease with peak incidence in between April and August [3, 4].

Outbreaks are more common in summer season affecting mainly the children in Kathmandu [5–7]. Diagnosis is done by culture and immunological tests, however, isolation of the etiological agent, the Salmonella enterica (serovar typhi) from bone marrow culture is an ideal gold standard [8]. Invasive procedure and unavailability of culture facilities in rural health centers are chief limiting factors. Besides, the widely used blood culture has poor sensitivity of 40–60 % making room for false negative results to around half of the typhoid patients [9]. In addition, the requirement of 3 days time duration for diagnosis delays the line of treatment losing applicability of early diagnosis. Further, usage of the antibiotics prior to hospital admittance, which is a common practice in Nepal, cause poor sensitivity to the culture base diagnosis [10]. In spite of all, blood culture technique

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is still the gold standard in the febrile cases of typhoid. Routine Widal test is alternatively adopted second most popular choice for diagnosis as blood culture remains controversial due to its biased diagnosis [11]. Enzyme-linked immunosorbent assay (ELISA) based diagnosis has also been studied previously with good diagnostic accuracy [12–15]. This study aims to compare diagnostic accuracy of Widal test and ELISA in febrile patients taking blood culture as gold standard tool for the diagnosis of typhoid fever.

**Material and methods**

**Patients, inclusion/exclusion criteria and data analysis**

Patients attending Capital Hospital, a centrally located hospital at Kathmandu, during the months January 2011 to December 2013 with complaint of fever over 72 h without obvious focus of infection and clinical suspicion of typhoid fever (high fever, malaise, headache, constipation or diarrhoea) were prospectively enrolled in this study. Patients were divided into five age groups i.e. 1–15 (children), 16–30 (young), 31–45 (young adults), 46–60 (adults) and >60 years (olds). Pregnant women were excluded from the study. The ELISA and Widal tests were performed by a single specialized clinical researcher who was blind to the patient’s diagnosis in reference to standard throughout the study period. The patients with febrile cases were screened when found positive to reference standard (Blood Culture). The control populations with negative blood culture reports were also included in the study, so as to make self evaluation of reference standard. The sample population inclusive of blood culture positive was grouped into five different groups (Fig. 1).

**Blood collection and bacterial culture for pathogen isolation**

Blood were collected in BACTEC Peds Plus™/F culture vials and immediately (within 10 min) transferred to laboratory to be loaded in Bactec 9240 (Becton Dickinson, USA) culture instrument for growth. Blood isolates which were found culture positive were reconfirmed for *Salmonella* by slide agglutination, using monospecific antisera (Sifin, Germany) as described by the Kauffmann-White scheme [16–18].

**Quantitative Widal test**

The Widal tube agglutination test was performed using Wellcolex*® (Remel, UK) in the group A (n = 1371) as well as for group B, C D and E according to the manufacturer’s instructions parallel to the blood culture procedure of individual groups. Briefly, serum remained after keeping for blood culture was diluted in 0.86 % saline solution starting with 1:100. *Salmonella* ‘O’ and ‘H’ antigens from the standard preparation were added and the tubes

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**Fig. 1** Consort chart for patients in study. Group A: Febrile patients with suspected typhoid fever (n = 1371), Group B: Blood culture positive patients (n = 237), Group C: Post treated patients from group A (>Day 30) (n = 237), Group D: Endemic healthy population (n = 237), Group E: Other diseased patients (infection from: *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Cryptococcus neoformans*, *Vibrio cholera*, HIV-1, *Neisseria meningitidis*, *Listeria monocytogenes*, *Haemophilus influenza*, *Streptococcus pneumonia* and *Rabies virus*): Negative to *Salmonella* tests (n = 237)
were incubated at 37 °C for 1 h. After incubation, the tubes were centrifuged for 5 min, and respective agglutinations were observed. The Widal TO/TH titre were taken as the highest dilution of serum with visible agglutination. The cut-off titre set for Salmonella typhi anti O was >1:80 while it was >1:160 for anti H in this study [11].

**Enzyme Linked Immunosorbent Assay (ELISA)**

Similar to the Widal test, Anti-IgM and anti-IgG sandwich ELISA (MyBioSource, Inc. CA, USA) was done in all the groups. Briefly, 100 µl of coating antigen (1 µg/ml) diluted in antigen coating buffer (Immunochemistry, MN, USA) were dispensed in Nunc-Immuno 96 MicroWell solid plates (Thermo Fisher Scientific, USA) along with negative control (Only coating buffer) according to plan. The plates were incubated at 4 °C overnight and the wells were blocked using 1 % bovine serum albumin (BSA) prepared in phosphate buffer saline (PBS). The plates were washed by 125 µl washing buffer (0.1%BSA with Tween20). Hundred microliter of serially diluted sera (1:200 to 1:3200) in PBS-BSA was dispensed to each well and incubated at 25 °C for 4 h. After washing, 100 µl detector antibody conjugated with horse redish peroxidase (HRP) was added in dilutions (1:500 anti IgA, 1:5000 anti IgG, 1:2500 anti IgM) and incubated for 30 min at 25 °C. After that, 100 µl dimethyle benzidine (TMB) substrate was added and incubated for 15 min at dark. The reaction was stopped by addition of 1 N H2SO4 to measure optical density (OD) at 450 nm in ELISA plate reader (Bio-Rad). Cut off values were assessed following the mean ± SD of the OD from healthy endemic controls of group D which was 0.3 for IgG and 0.2 for IgM.

**Statistical analysis**

The positive predictive value is the principle finding of this study which elucidates the efficacy of current diagnostic assays. The sensitivity, specificity and predictive values were calculated as described earlier [19]. Receiver operating Characteristics (ROC) used to establish cut-off titre, was determined by plotting specificity (x-axis) versus sensitivity (y-axis) at all tested cut off titres. The highest number of true positive and the lowest false positive results were used to represent cut off values by comparing the points with maximum Youden index ($J = \max [Se(c) + Sp(c) − 1]$). Collected data were analyzed and interpreted statistically using graph-Pad prism version 6.0 and SPSS 17.0. Normal distribution of data sets were analysed by Kolmogorov-Smirnov test. All the values are expressed as mean ± SD and are analyzed using Student’s t test which is parametric as well Mann–Whitney test wherever applicable. A value (p value, <0.05) was considered significant unless stated otherwise. Sensitivity, specificity and predictive values were calculated as described elsewhere [20, 21].

**Ethical statement**

The study was approved by the Capital Hospital Ethical Review Board (CHRB). The adult participants gave written informed consent while children below 18 year were enrolled only after the written informed consent from their guardian was received.

**Results**

**Demographic and geographical distribution**

The 1371 patients, enrolled in the study held between January 2011 and December 2013, had representation from all the three geographical regions of Nepal viz. Mountain, Hill and Terai, as Kathmandu, the capital city, hosts a broad range of people from all over the country. The prevalence of typhoid cases in Terai region was highest (69 %; p value, 0.03) in comparison to mid hill regions around Kathmandu valley (30 %) and the least in mountain region (1.39 %) (Table 1). There were 400 females and 971 males who consented for this study. The infection was found more in males of 16–30 years (median age, 21.5 years) age group with male–female ratio of 2:1, among the positive cases ($n = 237$) of typhoid as confirmed by blood culture, and was followed by age groups of 1–15, 31–45, 46–60 and >60 year.

**Pathogen distribution among febrile cases**

Out of 1371 cases, only 237 samples (17.28 %) showed positive growth of *S. typhi* in the blood culture and 120 patients were admitted in the hospital for treatment. The cultures also had co-infection of Actinobacteria ($n = 3$, 2.5 %) and Klebsiella pneumoniae ($n = 2$, 1.67 %) in the admitted inpatients of the typhoid fever (Table 2). In 237 patients, Actinobacteria (0.21 %) was found only in male while Klebsiella pneumoniae (0.14 %) were found only in female. Pathogen other than Salmonella was isolated from 49 patients (Fig. 1).

**Clinical symptoms of the disease**

Different symptoms were found in the typhoid positive cases. The absolute symptom was fever (100 %; p value, 0.04) however, other major symptoms observed were

**Table 1** Distribution of Typhoid patients according to regions and sex

| Geographical Regions | Patients | Female | Total |
|----------------------|---------|--------|-------|
| Teral Region (<2297 ft) | 621 (65.92 %)  | 321 (34.07 %)  | 942 (68.71 %)* |
| Hill Region (2000–10000 ft) | 340 (82.92 %) | 70 (17.07 %) | 410 (29.91 %) |
| Mountain Region (>10000 ft) | 10 (52.63 %) | 9 (47.36 %) | 19 (1.39 %) |

*p value, <0.05 while comparing the patients from Hill region
headache (97.47 %; p value, <0.034), loss of appetite (90.3 %; p value, 0.028) and chill (74.26 %; p value, 0.034). Abdominal discomfort, myalgia, vomiting, constipation, rigor, diarrhea, and dysuria were other symptoms observed in less than half of culture confirmed typhoid patient. Hence, fever, headache and loss of appetite were significant symptoms for the fever ailment (Table 3).

Qualitative slide agglutination Widal test
Widal test used as the primary screening assay by typing O and H antigen of Salmonella showed overall positivity rate of 21.74 % and 22.68 % respectively at the titre ≥1:40. (Table 4). Among culture confirmed patients, group A (n = 237), the number of TH (n = 108) and TO (n = 104) positive at titre ≥1:40 was significantly lower (p value, 0.04) than from the culture negative patients (n = 1134) group. Though blood culture showed negative to typhoid test, Widal test was found positive to TH antigen (n = 203, 17.92 %) and TO antigen (n = 194, 17.11 %) test at same titre (Table 4).

Assessment of ELISA for the diagnosis of typhoid fever
The OD value for IgM in the culture confirmed group B (n = 237) was significantly higher (p value, 0.041) than that of control groups C, D and E. There was no significant difference between the control groups D and E (p value, 0.039). Titre value of 3200 for IgM and 200 for IgG, was observed for group B patients (193 of 237) only after ≥3 days of reported fever case, which were undetectable in initial 3 days of feverish condition. Patients of group C (n = 237), who were enrolled in medication and recovered, showed significant level of serum IgG (p value, 0.046) but not IgM, when compared to previous data from same patients before medication. For the endemic healthy group D (n = 237), the titre for IgG was 400 but with no significant IgM titre value. In case of other disease patients of group E (without Salmonella but other infections), the ELISA results were negative for both Salmonella anti IgM and IgG (Fig. 2). Serology based typhoid diagnostic tests using sera from the culture confirmed typhoid patients and

Table 2 Culture report of S.typhi positive cases and co-infection with other pathogens among inpatient and outpatient of capital hospital

|                | Inpatients | Outpatients |
|----------------|------------|-------------|
| Salmonella typhi | 115 (95.83 %) | 122 (100 %) |
| Actinobacteria  | 3 (2.5 %)  | 0 (0 %)     |
| Klebsiella pneumonia | 2 (1.67 %) | 0 (0 %)     |

Co-infection with S.typhi

Table 3 Clinical sign and symptoms distribution

| S.N | Symptom expressed by patient | Salmonella typhi (n) | Percentage |
|-----|------------------------------|----------------------|------------|
| 1   | Fever                        | 237                  | 100.00     |
| 2   | Headache                     | 231                  | 97.47      |
| 3   | Loss of appetite             | 214                  | 90.30      |
| 4   | Chills                       | 176                  | 74.26      |
| 5   | Abdominal Discomfort         | 109                  | 45.99      |
| 6   | Myalgia                      | 99                   | 41.77      |
| 7   | Vomiting                     | 89                   | 37.55      |
| 8   | Constipation                 | 64                   | 27.00      |
| 9   | Rigor                        | 55                   | 23.21      |
| 10  | Diarrhea                     | 45                   | 18.99      |
| 11  | Dysuria                      | 42                   | 17.72      |

Table 4 Number of anti TH and anti TO levels in blood isolates

| Titration Record | Total Patients (n = 137) | Culture positive patients (n = 237) | Culture negative patients (n = 1134) |
|------------------|--------------------------|------------------------------------|-------------------------------------|
| Anti TH          |                          |                                    |                                     |
| Agglutination    | 311 (22.68 %)            | 108 (45.57 %)                      | 203 (17.92 %)                      |
| ≥1:640           | 78 (25.08 %)             | 36 (33.33 %)                       | 42 (20.69 %)                       |
| 1:320            | 156 (50.16 %)            | 55 (50.93 %)                       | 101 (49.75 %)                      |
| 1:160            | 176 (56.59 %)            | 89 (82.41 %)                       | 87 (42.86 %)                       |
| 1:80             | 201 (64.63 %)            | 98 (90.74 %)                       | 103 (50.74 %)                      |
| 1:40             | 311 (100 %)              | 108 (100 %)                        | 203 (100 %)                        |
| No Agglutination| 1060 (77.32 %)           | 129 (54.43)                        | 931 (82.14 %)                      |
| Anti TO          |                          |                                    |                                     |
| Agglutination    | 298 (21.74 %)            | 104 (43.88 %)                      | 194 (17.11 %)                      |
| ≥1:640           | 71 (23.83 %)             | 29 (27.88 %)                       | 42 (21.65 %)                       |
| 1:320            | 147 (49.33 %)            | 45 (43.27 %)                       | 102 (52.58 %)                      |
| 1:160            | 164 (55.03 %)            | 84 (80.77 %)                       | 80 (41.24 %)                       |
| 1:80             | 198 (66.44 %)            | 91 (87.5 %)                        | 107 (55.15 %)                      |
| 1:40             | 298 (100 %)              | 104 (100 %)                        | 194 (100 %)                        |
| No Agglutination| 1073 (78.26 %)           | 133 (56.12)                        | 940 (82.89 %)                      |

Fig. 2 IgG and IgM occurrence in group B, C, D and E
the control subjects showed that both IgG and IgM based ELISA tests were superior to the Widal TH and TO tests. When sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of ELISA was compared among group A \( (n = 1371) \) patients against Widal test, ELISA (IgG/IgM) had higher PPV at \( \geq 1:400 \) (Table 5). The IgM/IgG titre \( \geq 1:200 \) had a high sensitivity (95.50 %/96.85 %) and specificity (94.69 %/94.95 %). The diagnostic sensitivity of Widal TO test at titre \( \geq 1/400 \) was 84.09 % and specificity was 52.65 %. There was a significant difference between case definition for suspected and probable cases of typhoid fever (Table 6).

**Discussion**

Typhoid is a major public health problem in third world countries [1,2]. In Nepal, typhoid fever is endemic and the major factors for high prevalence rate include, but are not limited to, illiteracy, poverty, poor sanitation and inadequate facilities for safe drinking water supply. The Widal test based on TO and TH titre values were observed higher in healthy subjects relating to the endemic

| Table 5 | Sensitivity, Specificity, PPV and NPV for typhoid fever of ELISA and Widal test in different cut off titers |
|---------|---------------------------------|
| Test    | Sensitivity (95 % CI) | Specificity (95 % CI) | PPV (95 % CI) | NPV (95 % CI) |
|---------|----------------------|-----------------------|---------------|---------------|
| IgM     | ≥1:200               | 95.50 %               | 94.69 %       | 77.66 %       | 99.09 %       |
|         |                      | 91.87 % to 97.82 %    | 93.23 % to 95.92 % | 72.24 % to 82.46 % | 98.33 % to 99.56 % |
|         | ≥1:400               | 81.23 %               | 98.29 %       | 91.77 %       | 95.70 %       |
|         |                      | 75.95 % to 85.78 %    | 97.34 % to 98.97 % | 87.45 % to 94.98 % | 94.36 % to 96.80 % |
|         | ≥1:800               | 61.97 %               | 96.48 %       | 78.38 %       | 92.50 %       |
|         |                      | 55.41 % to 68.21 %    | 95.24 % to 97.48 % | 71.74 % to 84.08 % | 90.85 % to 93.93 % |
|         | ≥1:1600              | 51.89 %               | 93.79 %       | 60.44 %       | 91.42 %       |
|         |                      | 44.94 % to 58.78 %    | 92.24 % to 95.11 % | 52.94 % to 67.60 % | 89.68 % to 92.95 % |
|         | ≥1:3200              | 47.44 %               | 92.73 %       | 54.84 %       | 90.46 %       |
|         |                      | 40.61 % to 54.34 %    | 91.08 % to 94.16 % | 47.39 % to 62.13 % | 88.65 % to 92.08 % |
| IgG     | ≥1:200               | 96.85 %               | 94.95 %       | 78.75 %       | 99.36 %       |
|         |                      | 93.61 % to 98.72 %    | 93.52 % to 96.14 % | 73.42 % to 83.45 % | 98.69 % to 99.74 % |
|         | ≥1:400               | 85.95 %               | 98.76 %       | 93.69 %       | 97.04 %       |
|         |                      | 80.92 % to 90.07 %    | 97.93 % to 99.32 % | 89.65 % to 96.51 % | 95.89 % to 97.94 % |
|         | ≥1:800               | 62.61 %               | 97.09 %       | 81.87 %       | 92.51 %       |
|         |                      | 56.12 % to 68.77 %    | 95.93 % to 97.99 % | 75.49 % to 87.18 % | 90.87 % to 93.95 % |
|         | ≥1:1600              | 51.89 %               | 94.48 %       | 63.22 %       | 91.48 %       |
|         |                      | 44.94 % to 58.78 %    | 93.00 % to 95.72 % | 55.59 % to 70.39 % | 89.75 % to 93.00 % |
|         | ≥1:3200              | 47.44 %               | 92.73 %       | 54.84 %       | 90.46 %       |
|         |                      | 40.61 % to 54.34 %    | 91.08 % to 94.16 % | 47.39 % to 62.13 % | 88.65 % to 92.08 % |
| TO      | ≥1:100               | 90.32 %               | 52.41 %       | 44.22 %       | 92.12 %       |
|         |                      | 87.25 % to 92.47 %    | 49.10 % to 53.91 % | 41.18 % to 50.73 % | 88.71 % to 94.83 % |
|         | ≥1:200               | 80.95 %               | 51.77 %       | 23.29 %       | 93.76 %       |
|         |                      | 74.98 % to 86.03 %    | 48.85 % to 54.68 % | 20.27 % to 26.53 % | 91.60 % to 95.50 % |
|         | ≥1:400               | 84.09 %               | 52.65 %       | 25.34 %       | 94.54 %       |
|         |                      | 78.58 % to 88.66 %    | 49.72 % to 55.57 % | 22.22 % to 28.66 % | 92.49 % to 96.17 % |
| TH      | ≥1:100               | 80.32 %               | 53.22 %       | 48.40 %       | 90.87 %       |
|         |                      | 75.28 % to 85.91 %    | 46.42 % to 55.36 % | 42.83 % to 50.27 % | 87.14 % to 92.84 % |
|         | ≥1:200               | 72.23 %               | 58.33 %       | 38.13 %       | 89.37 %       |
|         |                      | 68.24 % to 79.72 %    | 52.93 % to 64.22 % | 31.23 % to 43.10 % | 83.11 % to 94.07 % |
|         | ≥1:400               | 62.47 %               | 55.23 %       | 39.48 %       | 90.62 %       |
|         |                      | 59.23 % to 66.82 %    | 47.21 % to 59.99 % | 31.34 % to 45.83 % | 86.92 % to 95.27 % |

*Highest PPV value*
setting of typhoid fever in Nepal. Even the typhoid re-
Iam a 1:160, describing the persistent anti H in blood sample
[11] which also had higher serum IgG but lower IgM
antibodies suggesting the late class switching from IgM
to IgG in response to the infection well after elimination
of pathogen [22]. The sensitivity and specificity of the
IgG/IgM ELISA were higher than that of Widal test
showing Widal’s incompetence in accurate diagnosis.
The cut off titre is more in developing countries com-
pared to developed countries due to sanitary and en-
demic reasons [23], thus the titre value from Widal test
is not accurate enough to picture the complete diagno-
sis, since the PPV value of TO and TH were too low
(0.44 and 0.48 respectively). In developing country like
Nepal, the haphazard usage of antibiotic is common thus
providing false negative in blood culture, though there
were no patients who admitted taking antibiotic within
two weeks before enrollment in this study. The false
negative Widal test results were probably due to early
blood collection before the adequate antibody produc-
tion [24]. In case of Widal test, when compared with
the WHO case definition, didn’t perform well. The
sensitivity dropped by >40 % compared to gold standard
blood culture showing that Widal test alone can’t be
used as diagnostic approach, though is widely used in
rural setting in Nepal till date.

Conclusion
Higher titre value was observed in healthy endemic
population which showed that typhoid diagnosis is
biased when Widal test is used alone; however ELISA
had more stringent data thus resulting a clear distinc-
tion of antibody production against salmonella, and
making differential diagnosis more accurate among fe-
brile cases. This findings also suggest implementing
ELISA on daily routine diagnosis of typhoid fever espe-
cially in endemic typhoid area like Nepal.

Table 6 Sensitivity, specificity, PPV and NPV for typhoid fever of WHO case definition

| Suspected case of Typhoid fever | Sensitivity | Specificity | PPV | NPV |
|---------------------------------|------------|------------|-----|-----|
|                                 | 95 % CI    | 95 % CI    | 95 % CI | 95 % CI |
| Isolation in Blood culture of;  |
| S. typhi                        | 76.81 %    | 99.22 %    | 97.07 % | 92.71 % |
|                                 | 71.99 % to 81.16 % | 98.47 % to 99.66 % | 94.31 % to 98.73 % | 91.01 % to 94.18 % |
| Probable case of Typhoid fever  |
| TH(1:160)                       | 45.22 %    | 82.32 %    | 34.23 % | 87.83 % |
|                                 | 41.27 % to 49.17 % | 79.91 % to 87.82 % | 29.46 % to 38.96 % | 81.59 % to 91.31 % |
| TO(1:80)                        | 43.45 %    | 82.31 %    | 34.11 % | 87.14 % |
|                                 | 39.29 % to 48.97 % | 78.96 % to 84.78 % | 31.41 % to 38.85 % | 83.72 % to 92.91 % |

Abbreviations
BACTEC: Bactenecin; ELISA: Enzyme Linked Immunosorbent Assay;
Peds: Pediatrics; TH: Titer of Salmonella H- Flagellar antigen; TO: Titer of Salmonella H- Somatic antigen; BSA: Bovine Serum Albumin; PBS: Phosphate Buffer Saline; HRP: Horse Reddish Peroxidase; TMB: Trime thyl Benzidine; OD: Optical Density; SD: Standard Deviation; ROC: Receiver Operating Characteristics; CHRB: Capital Hospital Ethical Review Board; PPV: Positive Predictive Value; NPV: Negative Predictive Value; NATRC: Aayurveda Training and Research Center; UGC: University Grant Commission.

Competing interests
The authors declare that they have no competing interests.

Author contributions
BPG, AA and RR designed the experiment. BPG, AA, RR, KDM performed the research study. BPG, AA, RR, PPR & KDM analyzed the data. BPG, AA, PPR & KDM wrote the manuscript. All authors have read and approved the final version of the manuscript.

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