SEROLOGICAL DIAGNOSIS

Low sensitivity of counter-current immuno-electrophoresis for serodiagnosis of typhoid fever

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Counter-current immuno-electrophoresis was evaluated as a diagnostic test for the serodiagnosis of typhoid fever with somatic (O), flagellar (H) and capsular polysaccharide (Vi) antigens of *Salmonella typhi* on the sera of patients who were blood culture positive (confirmed typhoid cases) or had high Widal agglutination titres, ≥ 320 , (presumptive typhoid cases). Of the 37 sera from confirmed cases, 30% showed positivity with O antigen, 24% with H antigens and 51% with Vi antigen. In patients with a presumptive diagnosis, 45% were positive for O antibody, 27% for flagellar antibody and 52% for Vi antibody. When all three antigens were combined the reactivity to any of the antigens was found to be 59% in confirmed typhoid cases, 79% in presumptive typhoid cases and 93% in patients who were simultaneously positive by blood culture and Widal agglutination. However, none of the sera from 45 controls gave a positive precipitation reaction with any of the antigens. It is concluded that counter-current immuno-electrophoresis is a rapid test with low sensitivity and high specificity with Vi antigen, a panel of antigens being most effective, and is, therefore, recommended for rapid diagnosis of typhoid fever.

Introduction

Typhoid fever is still one of the major unsolved health problems with an estimated world-wide incidence of c. 12.5 million cases annually, with $> 62\%$ of these occurring in Asia [1].

Laboratory confirmation of a clinical diagnosis of typhoid fever depends essentially on bacterial culture. This has drawbacks, as during the first week of fever, 20% of patients may be culture negative and the positivity declines further with time [2]. Serological diagnosis by Widal agglutination test is also unreliable, as demonstration of rising titres is essential [3]. In India, most patients present late to hospital and require immediate diagnosis and treatment. Therefore, it is essential to develop better methods for rapid diagnosis.

The present work was done to evaluate the usefulness of counter-current immuno-electrophoresis for detecting antibodies to somatic (O), flagellar (H) and capsular polysaccharide (Vi) antigens of *Salmonella typhi* in patients who were diagnosed as definite or

probable cases of typhoid on the basis of blood culture positivity and Widal agglutination, respectively.

Materials and methods

Patients

Sixty-six patients clinically diagnosed as having typhoid fever on the basis of fever, diarrhoea, abdominal pain, with or without perforation, were confirmed by positive blood culture for *S. typhi* or high titres (≥ 320) of Widal agglutination, or both. Blood culture positive and Widal positive patients were grouped into group 1 and group 2, respectively. Group 3 comprised patients who were simultaneously positive by blood culture and Widal agglutination. Forty-five healthy individuals with Widal agglutination titres < 80 and who were blood culture negative for *S. typhi* served as controls.

Antigens for counter-current immuno-electrophoresis

O antigen. A centrifuged ultrasonic lysate of *S. typhi* O901 strain was used as antigen as advocated by Gupta and Rao [4].

H antigen. Motile *S. typhi* of strain H901 was grown in brain heart infusion broth. Flagellar (H) antigen

Received 13 Aug. 1996; revised version received 4 April 1997; accepted 1 May 1997.

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was prepared according to the method of Ibrahim *et al.* [5].

Vi antigen. The modified method of Ewing [6] was followed to prepare the Vi antigen from a clinical isolate of *S. typhi* showing agglutination with commercially available Vi antisera. The antigen was prepared by removing the growth of a pure Vi positive *S. typhi* culture from MacConkey agar plates. It was then suspended in absolute alcohol. The bacteria were dehydrated *in vacuo* and resuspended in physiological saline. The suspension was sonicated 10 times at 20 kilocycles with bursts of 45 s each at 1-min intervals in an MSE sonicator. The sonicated fraction was used as antigen for counter-current immuno-electrophoresis.

Raising hyperimmune antisera

Antisera to O, H and Vi antigens were raised in 6–8-month-old New Zealand White rabbits as described by Ewing [6].

Counter-current immuno-electrophoresis

Counter-current immuno-electrophoresis was performed according to the method of Ouchterlony and Nilsson [7] with some modifications. The slides were prepared with noble agar 0.9% dissolved in 0.05 M barbitone buffer (pH 8.6). The antigens (O, H and Vi antigens of *S. typhi*) were placed in the cathodal wells and patients' sera diluted (1 in 200) in phosphate-buffered saline (0.02 M, pH 7.4) in the anodal wells. A constant current was applied along the slide for 45 min at a rate of 5–6 mA/cm. The samples showing the characteristic precipitation line were interpreted as positive. Antisera raised in rabbits served as positive controls. Normal rabbit serum was also included to eliminate false positive reactions for *S. typhi*.

Widal agglutination

The method given by Gupta and Rao [4] was followed for conducting the Widal agglutination test.

Results

In group 1, blood culture was positive in 37 of 66 patients over a variable time after the onset of fever as shown in Table 1. Peak blood culture positivity (40%)

was found in the second week of fever. However, 19% continued to be positive for up to nearly 1 month. In group 2, 34% were Widal positive in the second as well as the third week. Group 3, where both blood culture and Widal agglutination were simultaneously positive, comprised 15 patients, of whom 40% were positive in the second week of fever.

The rates of detection of antibodies by counter-current immuno-electrophoresis with three different antigens are shown in Fig. 1. Eleven (30%) of 37 patients of the blood culture positive group reacted with somatic O antigen. On the other hand, 13 (45%) of 29 patients in the second group were positive for O antibody. Positivity with O antigen was found in four samples from the third group.

Flagellar H antigen yielded precipitation lines in only nine (24%) of 37 patients with confirmed typhoid fever. Eight (27%) of 29 patients of the second group were positive for flagellar antibody by counter-current immuno-electrophoresis. Only two cases in group 3 were positive with H antigen.

Antibodies to Vi antigen were detected in 19 (51%) of 37 patients of the first group. In the second group 15 (52%) of 29 patients were positive for Vi antibody. However, this antigen gave a positive reaction with 12 sera from patients in the third group. When all three

Fig. 1. Detection of antibodies to O, H and Vi antigens by counter-current immuno-electrophoresis in patients with typhoid: ■ controls; ▨ blood-culture positive; □ Widal positive; ▩ blood-culture positive and Widal positive.

Table 1. Diagnosis of typhoid fever by blood culture and Widal agglutination over a variable period of infection

Method	Total number of cases	Number (% of total cases) of patients with fever for			
		0–7 days (1st week)	8–14 days (2nd week)	15–12 days (3rd week)	22–28 days (4th week)
Blood culture positive	37	7 (19%)	15 (40%)	8 (22%)	7 (20%)
Widal positive	29	5 (17%)	10 (34%)	10 (34%)	4 (14%)
Both (blood culture positive and Widal positive)	15	2 (13%)	6 (40%)	5 (33%)	2 (13%)

antigens were considered together as a panel and the positivity rates to at least one of the antigens measured, group 1 showed positivity of 59% and group 2 79%; however, group 3 showed a positivity rate of 93%.

Discussion

Typhoid fever continues to be a major endemic disease in India [8]. Patients presenting with fever and signs raising high clinical suspicion require early diagnosis with tests that should be specific, sensitive, inexpensive and rapid. The two main tests that are routinely done at present are blood culture and Widal reaction; neither of these tests fulfils all the above criteria.

Isolation of *S. typhi* from blood culture remains the gold standard, but requires at least 48 h for confirmation. Parker [9] reviewed studies conducted in the pre-antibiotic era and reported blood culture positivity in 90% of cases in the first week of illness, which declined to 70% in the second week. By the end of the third week it was positive in 45% of cases. After the fourth week the isolation of *S. typhi* from the blood of a patient was infrequent. Similarly, Stuart and Pullen [2] reported 80% positivity in the first week which declined to 30% by the end of the third week of illness. The incidence of positive isolation varied greatly in recent studies, ranging from 48% to 75% [10]. It is documented that culture may be negative in the presence of low grade bacteraemia [10] possibly because the bacteria are intracellular in the mononuclear cells of the blood and inhibitory serum factors such as antibodies or complement, may be present. Fifty-six percent of the patients in the present study were positive by blood culture, which is comparable with the earlier reports. However, positivity was found to be highest (40%) in the second week after the onset of fever and 19% of patients were positive up to the fourth week of fever.

The second most widely used test is the agglutination reaction popularly known as the Widal test. The serum of a proportion of patients in endemic countries contains antibodies capable of reacting to a variable titre in the Widal test, but usually the titres are low [9]. Even those individuals vaccinated with typhoid-paratyphoid (TAB) vaccine show H antibodies for a long period. Reactions to H antigens are more reliable than those to O antigen. The value of the Widal agglutination test for the serological diagnosis of *Salmonella* infection is often questioned [11]. Akoh [12] reported 44% Widal positivity, whereas Duthie and French [13] showed 75.5% positivity by Widal agglutination. In the present study only 40% of the blood culture-confirmed typhoid patients gave positive agglutination reactions with a titre of 320 or more in the second week of infection, 33% of those in the third week of infection, but only 13% were positive by agglutination in the first and fourth week of fever.

Demonstration of rising titres is recommended for the Widal test. However, often a single test has to be relied on for the diagnosis of typhoid. The present study included a group of patients who were blood culture negative but had high titres (≥ 320) of Widal positivity; these were considered presumptive typhoid cases. It has also been reported that culture positive cases may be Widal negative throughout the illness. The Widal test is considered to have low specificity and low sensitivity and this was corroborated by the present study.

The study by Gupta and Rao [4] established counter-current immuno-electrophoresis as a highly specific test with no false positive results. They used an ultrasonic lysate of *S. typhi* strain O901 as antigen and could detect antibody to this antigen in all the convalescent cases of typhoid fever, whereas only one of 26 acute typhoid cases gave a positive reaction. On the other hand, Sundararaj *et al.* [14] reported no false positivity by counter-current immuno-electrophoresis, but could detect only 25.2% of positive samples by this method. Tsang and Chau [15] conducted counter-current immuno-electrophoresis with three different antigens, i.e., Veronal buffer extract, ultrasonic lysate and protein antigen. They detected 98% positive and 10% false positive results with Veronal buffer extract, 96% positive and 32% false positive by ultrasonic lysate and 98% positive and 5% false positive by protein antigen.

Although serotyping is defined mainly by O and H antigens, other capsular antigens such as A, B, M and Vi have also been identified. In contrast to earlier studies the present study used relatively purified O, H and Vi antigens to detect antibodies by counter-current immuno-electrophoresis in the patients' sera with confirmed and presumptive diagnosis to assess the sensitivity of this test.

Although no false positives were detected by O, H and Vi antigens in the present study, positivity to O, H and Vi antigens by counter-current immuno-electrophoresis was low. These results indicate high specificity but low sensitivity for counter-current immuno-electrophoresis. However, counter-current immuno-electrophoresis with Vi antigen was found to be more sensitive than with other antigens, as it could detect 50% of the patients in both the first and second groups. On the other hand, H antigen was found to be least sensitive as it was able to detect only 35% of the blood culture positive patients and, thus, appears to have little significant value in diagnosis. In patients where blood culture was positive, the positivity rate with any of the three antigens was only 59%.

The failure to detect antibodies by counter-current immuno-electrophoresis and Widal agglutination in the blood culture positive typhoid patients can be due to the antibiotic treatment that most patients receive

before coming to hospital for laboratory diagnosis. Pang and Puthuchearry [16] also observed that antibiotic use may prevent the development of a rise in antibody titre.

Thus, the present findings indicate the need to develop a more sensitive, rapid and cost-effective method for the early diagnosis of typhoid fever. Although counter-current immuno-electrophoresis with O, H and Vi antigens of *S. typhi* has been found to be a highly specific and a quick test, because of its low sensitivity, either Vi antigen alone or a panel of three antigens could be used as an adjunct to diagnosis. Other methods such as DNA probes, or DNA or RNA amplification may be used in future for rapid and early diagnosis of the disease, but cost will remain a limiting factor. Therefore, there is an urgent need to develop cheaper methods such as second and third generation ELISAs which may be more suitable for routine testing.

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