COUNTERIMMUNOELECTROPHORESIS FOR DETECTING ANTIBODY IN THE DIAGNOSIS OF TYPHOID (ENTERIC) FEVERS: A PRELIMINARY REPORT

C Y Jad
S S Kelkar
S L Pole

SYNOPSIS

A counterimmunoelectrophoresis (CEP) technique was designed for detecting antibody in patients suspected of having typhoid (enteric) fevers. The Widal test was used as a comparison. The antigen was a soluble acid hydralysis extract of Salmonella typhi obtained after removal of flagellae. Of 50 serum-samples from clinical cases of typhoid fevers, positive by the Widal test, 49 were positive in CEP. In 50 sera from cases clinically suspected of being typhoid, 20 gave Widal titers of 30 or 15, and only 19 of these 50 were positive in CEP. In contrast, 50 sera from apyrexial healthy adults showed a single positive in CEP and two of which were positive in the Widal test. Lastly, all sera from 10 individuals immunized with TAB vaccine were positive in both the Widal and in CEP. The speed, simplicity and ease of CEP makes it a meaningful test for the detection of antibody in the diagnosis of typhoid fevers.

INTRODUCTION

Immunology has grown exponentially in the last two decades. A plethora of newer methods, particularly in the field of precipitation reactions in gels, are now available. One of these, counterimmunoelectrophoresis (CEP), has become quite popular because of its speed, ease, simplicity and specificity. Enteric fevers, which are paradoxically epidemically-endemic in our environment, continue to be diagnosed by the Widal agglutination reaction. This test, described as far back as in 1896, is laborious, time-consuming, expensive in terms of glassware needed and often difficult to interpret. Moreover, the quality of the antigens is critical. A careful survey of the literature has failed to reveal to us any reports of newer immunological methods, in the diagnosis of typhoid fevers. This, perhaps, is because they do not occur frequently in advanced countries. We describe here a comparative study of the results obtained with the Widal test and a CEP technique.

MATERIAL AND METHOD

Serum-samples

A total of 160 serum-samples were studied for the presence of antibody by the two tests. Fifty sera were from clinically-suspected cases of typhoid fevers. This group included 28 males and 22 females, had a mean age of 23.5 years, the duration of fever being

Department of Microbiology
The Grant Medical College
Bombay 400 008
Maharashtra, India

C Y Jad
Technician

S S Kelkar, MD
Professor and Head

S L Pole
Lecturer

Address for Communication:
S S Kelkar

an average of 15.4 days (minimum 6 days, maximum 90 days) and 25 had hepato-or splenomegaly. Besides clinical features this group was labelled on the criterion of a positive Widal test titer of 50 or more with at least one of the four antigens. The "titer" was the reciprocal of the highest dilution that came positive. The second group of 50 sera were from cases of pyrexia in which the Widal test was requested by a clinician but in which the titer in the Widal test was 30 or less. This group included 37 males and 13 females with a mean age of 24 years, the duration of fever being an average of 8 days (minimum 5 days maximum 15 days) and only 2 had hepato-or splenomegaly. The third, control group, comprised of sera from 50 normal healthy volunteers who were not immunised with TAB vaccine. This group included 39 males and 11 females with a mean age of 22.3 years. Lastly, ten sera were from individuals immunized sometime during the previous six months with TAB vaccine.

Antigen for Counterimmunoelectrophoresis

A heavy 18 hour growth of **Salmonella typhi** on a nutrient agar plate was scraped off into 1 ml of saline to form a thick milky turbid fluid. This was heated at 100°C for 30 minutes to detach flagellae (Cruickshank et al, 1975). The material was centrifuged at 3000 g for 30 minutes and approximately half a volume of N/5 HCl added to the visible mass of deposited organisms. After heating at 100°C for exactly 10 minutes the material was cooled and neutralised with N/5 NaOH using phenol red as indicator. The clear supernate after centrifugation, was used as antigen in the CEP test.

Counterimmunoelectrophoresis (CEP) Test

The mobility of the antigen was determined in immunoelectrophoresis using a rabbit antibody raised against the antigen. It was found to have a mobility similar to - globulins of human serum. In view of this mobility the agar for CEP was a mixture of four parts of agarose to one part of Difco Bacto agar (Kelkar & Niphadkar, 1974). Gel was prepared in 10 ml barbitone acetate buffer (pH 8.6, 0.05 M) by heat-dissolving 80 mg of agarose and 20 mg of Difco Bacto agar. Microscope slides (75 x 25 mm) kept on a levelled surface, were each layered with 1.5 ml fluid hot gel and allowed to cool. Pairs of wells 4 mm in diameter with their edges two to three mm apart were cut out. Antigen was cathodal. Electrophoresis was in a simple apparatus (Kelkar & Jad, 1976) with a continuous buffer system and a constant current of 7mA per slide for 30 minutes. Results were read on a darkground viewing box.

Widal Test (Cruickshank et al, 1975)

Serum dilutions of 1 in 15, 30, 50 and further doubling dilutions were used, each serum being titrated to its end point. Four antigens — **Salmonella typhi** 'H' and 'O' and **S. paratyphi** 'AH' and 'BH' obtained from Haffkine Biopharmaceuticals, were used. Readings were taken with the usual care and precautions.

RESULTS

Figure 1 illustrates the results in 50 clinically-evident cases of typhoid fevers. The maximum basal titer for the Widal test established in Bombay is 1 in 30. Therefore any single one or more of the four agglutinins in the Widal test at a titer of 1 in 50 or more were treated as abnormal and significant. Numbers in the symbols in Figure 1 indicate the results for the same serum sample. There was excellent correlation between CEP and Widal-positivity. The only sample which was negative in CEP was also negative for all agglutinins except **S. typhi** 'H' and that two at a titer of 50. CEP was therefore as sensitive as the Widal test.

Figure 2 illustrates the results in 50 cases of pyrexia in which the Widal test was requested by a clinician but in which the Widal test was either negative or positive at a titer of 1 in 30 or less. Because all the sera were negative for agglutination to **S. paratyphi** A & B 'H' antigen and only one each positive for **S. typhi** 'H' antigen at a titer of 1 in 15 and 1 in 30 respectively, so the figure illustrates only the results for one agglutinin, namely **S. typhi** 'O'. Twenty samples, in this lot of 50 sera were positive in CEP. Moreover nine of 35 sera negative in the Widal test were positive in CEP and 8 of 19 sera with low titers (1 in 15 or 30) were negative in CEP. The descriminatory power of CEP therefore appears comparable in this range.

In the case of the 50 sera from normal apyrexial individuals, only one sample were positive in CEP. In contrast, the results with the Widal agglutinins were: **S. typhi** 'O' all 50 negative; **S. typhi** 'H' three positive at 1 in 15 and one at 1 in 50. **S. paratyphi** A 'H' only one positive at 1 in 30, and, S. paratyphi B 'H', five positive at 1 in 30 and one at 1 in 50. CEP showed only a single false positive result with these normal controls.

Ten sera from normal healthy adults immunized with TAB vaccine in the preceeding six months, were also studied. All were positive in CEP. The detailed results for the four Widal agglutinins are illustrated in Figure 3.

DISCUSSION

Assessment of diagnostic tests is a "backwoods of clinical research" (Leading article, Lancet, 1979). It has been aptly discussed in a recent publication. (Leading article, Lancet, 1979). The variables include intra and inter-observer variation, terminology, diagnostic strategies and the very concept of disease. Disease-labelling is essentially human and, except where morbid-anatomical (autopsy) evidence exists, it rests with laboratory tests. Here, again, the evidence is often circular, the disease being diagnosed by the positive test and the test becomes established "in this dubious manner" (Leading article, Lancet, 1979). These problems are more so with enteric fevers, and, the Widal test, introduced as far back as 1896, has been the subject of extensive debate enhanced by idle questioning at examinations. Specifically, in typhoid fevers, a positive blood culture diagnosis followed by therapy disturbs the antibody-response and vitiates

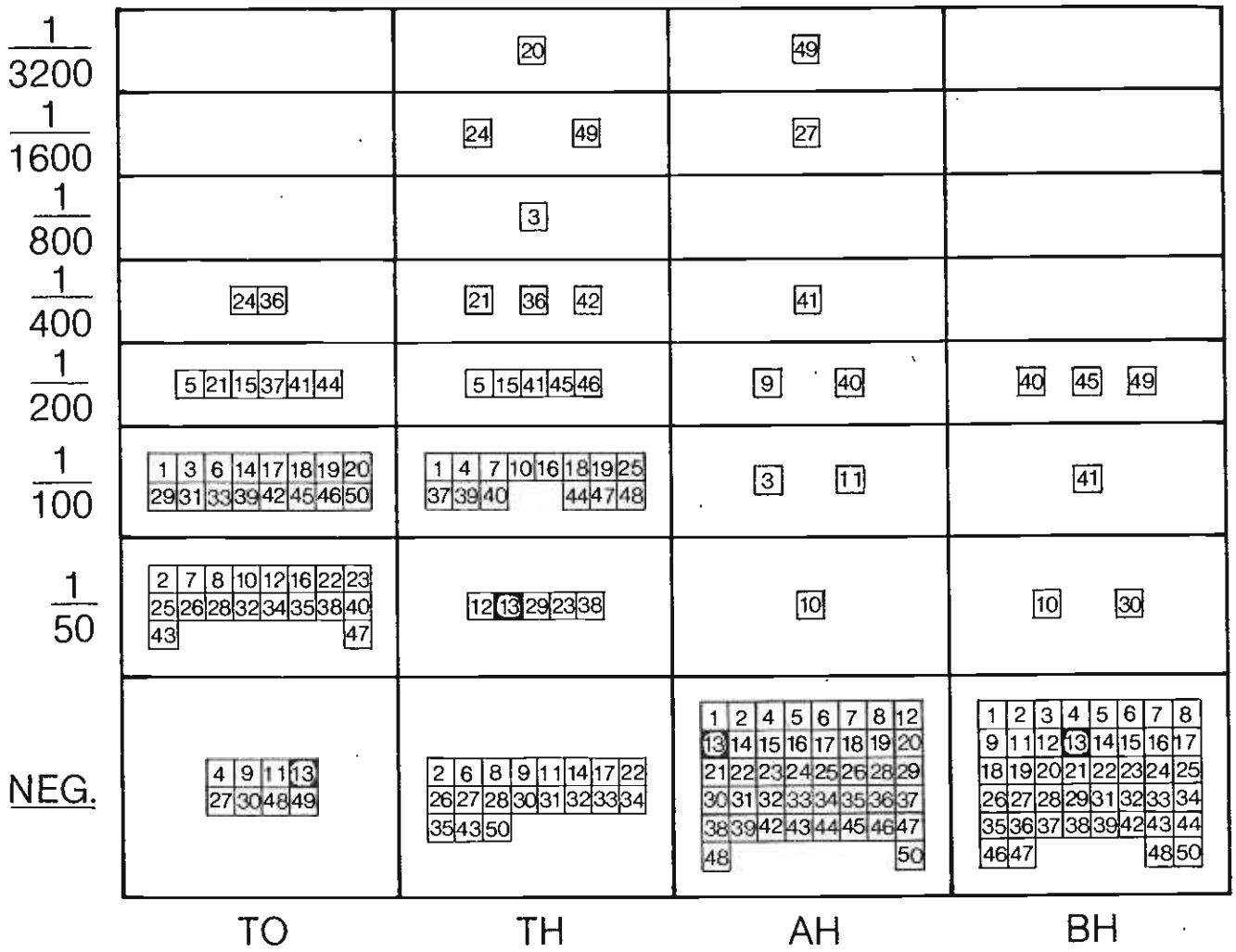

Figure 1. Details of CEP and Widal test titers in 50 Widal positive cases of typhoid fevers. Each serum was tested in CEP and with four antigens of the Widal test. Each column therefore has fifty symbols. □ = CEP positive; ◘ = CEP negative. One numbers in the figure represent the same serum sample.

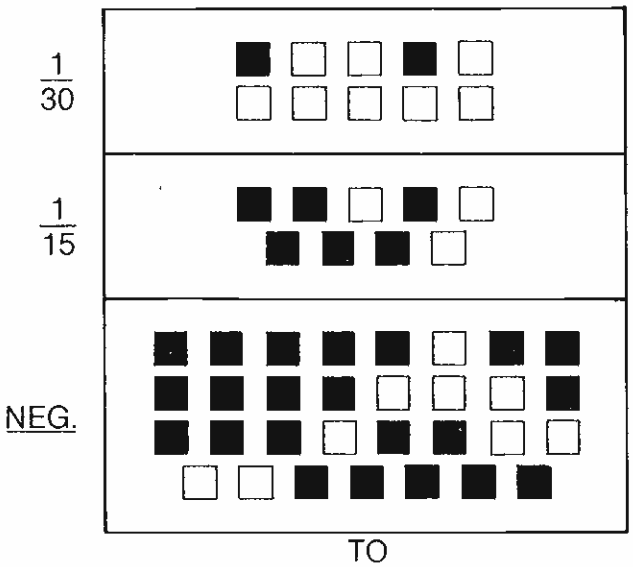

Figure 2. Details of CEP and titers of Widal test in 50 cases of fevers in which Widal titers were below 30 or negative. Only S. typhi 'O' antigen is illustrated as there was just one sample positive at 30 with S. typhi 'H' antigen the others being negative. □ = CEP positive; ■ = CEP negative.

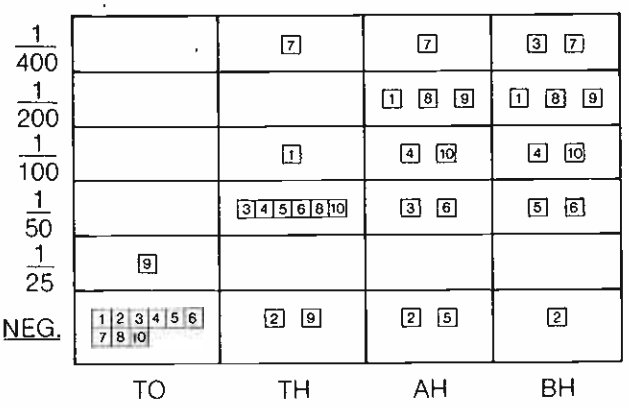

Figure 3. Results of Widal test in 10 sera from normal individuals immunized with TAB vaccine in the proceeding six months. All 10 were positive in CEP. One number in the figure indicate the same serum sample.

the study. In other cases, the diagnosis is, as mentioned above, made in a circular manner with reference to Widal agglutinin titres. Because of these limitations we have adopted conventional arbitrary parameters. Agglutinins at a level of 1 in 50 or more were taken to indicate significance. Levels of the four agglutinins of the Widal test were compared with a single soluble **S. typhi** antigen in CEP. Also, data over a wide spectrum of Widal antibody titers was obtained.

In the Widal test the **S. typhi** 'O' antigen represents the group antigen. This rises in all infections, shows less anamnestic responses and is more faithful. In contrast, the flagellar antigens reflect specificity of the infection and are notorious for an anamnestic rise. Figure 1 gives the details for the CEP test and the titers with the four Widal agglutinins. It includes all cases in which even a single agglutinin occurred at a titer of 1 in 50 or more. The CEP test could detect all except one of these serum samples. Interestingly enough out of eight serum samples with no **S. typhi** 'O' agglutinin but with significant titers of an 'H' agglutinin, seven were positive in the CEP test. The single soluble antigen we have used therefore seems to show a response whenever any of the specific flagellar agglutinins rise significantly. Figure 2 illustrates the situation with sera in the borderline group. Here again the discriminatory power of CEP appears to be as good (or as bad) as that of **S. typhi** 'O' agglutinins. With the third group of 50 normal sera there was only a single false positive while two sera showed 'H' agglutinins at a significant level.

All this evidence suggests that estimation of precipitins in CEP would be a meaningful substitute to the complicated Widal test. CEP, as a technique, has many advantages. These are, speed, ease, simplicity and the small quantities of reagents required. It must be conceded, however, that there were two shortcomings. Firstly, the test could not discriminate between the variety of the infection. We are attempting preparation of soluble flagellar antigens, so far, without success. Secondly, titers of precipitating antibody were not determined. This would be necessary to determine levels of significance and this is being attempted. Further, demonstration of a rising titer would be necessary in doubtful cases. This was done by using doubling dilutions of the sera in question and comparing end points of positivity. Another fruitful area of endeavour is demonstration of bacterial antigen in the early bacteremic phase of enteric fevers. CEP has proved its sensitivity for this purpose in bacterial meningitis (Coonrod and Rytel, 1972) and even in smallpox (Kelkar and Niphadkar, 1974) and other viral infections (James et al 1975). We were successful in demonstrating **S. typhi** antigens in two cases of enteric fever proved by blood cultures on the third and fifth days respectively of the disease.

Figure 3 illustrates the agglutinin titers in ten cases of immunized individuals. The response varied widely, and, surprisingly enough, several showed absence of some agglutinins. All these 10 sera were positive in the CEP test.

REFERENCES

1. Coonrod J D and Rytel M W: Determination of aetiology of bacterial meningitis by Counterimmunoelectrophoresis. Lancet, 1: 1154 — 1157, 1972.
2. Cruickshank R et al: The enterobacteriaceae; Salmonell. In: Medical Microbiology, 12th edd Vol. 2, Edinburg London and New York, 1975, p 408, 417.
3. James J F et al: Counterimmunoelectrophoresis for detection of viruses. Lancet, 1: 99 — 100, 1975.
4. Kelkar S S and Niphadkar K B: Agarose and counterimmunoelectrophoresis. Lancet, 2: 1394, 1974.
5. Kelkar S S and Niphadkar K B: Counterimmunoelectrophoresis for the diagnosis of smallpox. Singapore Medical Journal, 17: 104 — 105, 1976.
6. Kelkar S S and Jad C Y: A simple apparatus for counterimmunoelectrophoresis and electroimmunodiffusion. Indian J Med Res., 64: 108, 1976.
7. Leading artical. The value of diagnostic tests. Lancet, 1: 809 — 810, 1979.