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Evaluation of a PCR technique in blood samples of suspicious cases to systemic *salmonella* infections in Aliasghar children hospital, Tehran

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Abstract

Background and Objective: Typhoid fever is still a major health problem for which there are limited options for the diagnosis. Current diagnostic methods are time consuming with undesirable sensitivity. Among newer diagnostic methods, PCR is attractive and could be potentially very helpful in developing countries where systemic salmonella infections are endemic among children but needs to be investigated.

Methods: We performed a cohort study in Aliasghar Children Hospital, a teaching hospital in Tehran, between May 2012 and July 2013 to include suspicious systemic salmonella infections. Clinical and laboratory findings as well as clinical courses were followed on daily basis. Overall, 45 patients assigned into 3 groups were included into the study. For each case all appropriate cultures as well as Widal agglutination test and a blood sample for PCR of salmonella were submitted after taking informed consent from parents.

Results: Twenty one (46.7%) boys and 24 (53.3%) girls with ages ranging from 1 month of age to 17 years (mean 4.5 years) diagnosed for typhoid fever. About 88.9% of the patients had diarrhea, 84.4% had fever, 66.7% had vomiting, 28.9% had abdominal pain, 15.6% had nausea, 11.1% had rash and 2.2% had constipation. Eighteen (40%) patients have received antibiotic treatment before coming to the hospital. Group I consisted of 18(41%) patients, group II consisted of 27 patients with positive Widal test; of these, one was blood and stool cultures positive; however PCR in blood was negative in this group. Group III represented 2 patients with positive blood and stool cultures. In one case PCR in blood was positive. In another patient Widal test was negative despite positive blood culture and PCR in a leukemic kids who passed away.

Conclusion: In our setting the role of conventional PCR in blood samples of kids with suspicious systemic salmonella infections was not clearly determined. Larger sample size, preferably in a multicenter study, and using more sophisticated methods of blood cultures and novel techniques to increase the availability of organism for DNA detection is needed before determination of its role in both groups with no prior antibiotic therapy and cases with previous history of antibiotic administration.

Keywords: Salmonella, Diagnosis, PCR

Introduction

Typhoid fever is still a major health problem in many developing countries (1-3). Salmonella Typhi causes an estimated 21 million new cases of typhoid fever with >600,000 deaths annually (2-5) and is a human-specific pathogen transmitted by fecally contaminated water and food in endemic areas. Options for the diagnosis of typhoid fever are clinical signs and symptoms, serological markers, bacterial culture, antigen detection and PCR (2). The gold standard for typhoid fever is isolation of Salmonella typhi in samples including blood, bone marrow aspirates, stool, urine and rose spots (3), however blood culture is time-consuming and only identifies 45 to 70% of the cases.Serological tests are low cost and easy to perform but there is controversy regarding their sensitivity and specificity (6,7). New molecular techniques have been developed of which, PCR is sensitive and rapid test (6,8,9). Molecular techniques target the pathogen itself, so they can be useful for the early de-

tection of the disease (3). However, their sensitivity and specificity in developing countries needs further studies; this is due to high rate of previous antibiotic therapy in patients, especially for a non- specific clinical pictures as usually is seen in typhoid fever and also comparing to other gram negative bacteremia, relatively lower rate of bacteria in whole blood with even less than 1 colony forming unit/ml (10). Thus, a rapid, sensitive and specific method for the detection of S. typhi is essential for early diagnosis especially in children and also to prevent transmission by chronic typhoid carriers in countries with high rate of systemic salmonella endemicity (1,2,4,11). Inability to make an early laboratory diagnosis and empirical therapy often leads to increased morbidity and mortality in cases of typhoid fever (12). In this study, we have evaluated the results of cultures, Widal test and the PCR in patients with suspected typhoid fever.

Methods

We performed a cohort study in Aliasghar children hospital, a teaching hospital in Tehran, between May 2012 and July 2013 to include all patients who were suspicious clinically to systemic salmonella infections. Cases who were admitted in infectious diseases ward as well as those who were asked for Peds ID consult and had systemic symptoms and signs compatible with systemic salmonella infections, including toxicity, fever, malaise, diarrhoea, nausea, vomiting, abdominal pain, rash and constipation and had a suspicious recent history of travel to endemic areas and/or exposure to possibly contaminated food or water were included. An informed consent was taken from parents and study was approved by Islamic Azad University, Central Tehran Branch.

Overall, 45 patients were included into the study. All clinical information was recorded using a questionnaire. Blood samples for cultures, Widal tests and PCR, urine and stool cultures were obtained from all patients.

Laboratory tests including cell blood count (CBC), aspartate aminotransferase (AST), alanine amino transferase (ALT), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) were applied to patients. The patients' sera were tested for agglutinins against Salmonella typhi O and H antigens. Cut off titer of at least ≥1:40 for "O" or "H" antigens or both was considered positive according our previous study in Tehran.

Clinical response to therapy and final outcome were followed using daily follow up.

The total DNA from serum samples was purified using QIAamp DNA Mini (Qiagen Company). 20 pg/ml of each of the forward primer (GTA TTG TGG ATC CGA ATT AAT GA) and reverse primer (ATA TTA ACA CGT CGC ACG GAA G) were used. Material needed for salmonella PCR were as follows: 2.5 micro liter (10 mM) PCR buffer, 0.75 micro liter, $MgCl_2$ (50mMole), 0.5 micro liter dNTP (10mMole),1 micro liter forward primer (10mMole),1 micro liter reverse primer (10 mMole), 13.25 micro liter double distilled water (DDW), tag DNA polymerase 0.2 micro liter (1unit) and DNA template 5 micro liter. The temperature profile for PCR process were as follows: 2 cycles of denaturation for one min and 30 seconds at 94 c, 30 cycle of 57 c for 30 seconds, 30 seconds followed by final extension at 72 c for 5 min. Expected amplicons were visualized in 2% agarose gel after electrophoresis. Preparation of PCR mixes and electrophoresis were performed in separate laboratories to prevent cross-contamination. Specificity of our primers was checked by standard salmonella species obtained from Iran Reference Laboratory.

We finally put the case into 3 groups: group 1 with no positive Widal test and no positive culture but compatible clinical symptoms and signs and response to antimicrobial therapy. Group 2 with positive Widal test and any culture results plus compatible clinical symptoms and signs and response to antimicrobial therapy, and group 3 with positive culture of salmonella, and no positive Widal test. Data were analyzed as descriptive statistics using SPSS software v.22.

Results

Twenty-one (46.7%) boys and 24 (53.3%) girls with ages ranging from 1 month of age to 17 years (mean 4.5 years) diagnosed for typhoid fever. About 88.9% of the patients had diarrhea, 84.4 % had fever, 66.7% had vomiting, 28.9% had abdominal pain, 15.6% had nausea, 11.1% had rash and 2.2% had constipation (Figure 1).

Overall, the average serum hemoglobin level of the patients was 11.49± 2.3 g/dl [29 cases (48.3%) had various degrees of anemia]. The average white blood cell count was 9870 ± 5324/mm^3 [5 cases (8.3%) had leucopenia and 17 cases (28.3%) had mild leukocytosis].The average platelet count was 314000 ± 129538 /mm^3 [3 cases (5%) had thrombocytopenia and 10 cases (16.7%) had throbocytosis]. The mean level of AST was 45,4U/L and the mean level of ALT

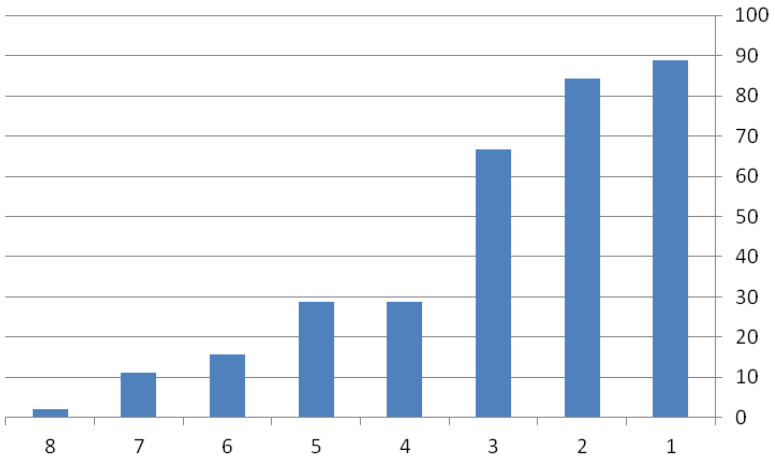

Fig 1. Clinical signs and symptoms of suspicious cases to systemic salmonella infections (1: diarrhea, 2: fever, 3: vomiting, 4: malaise, 5: abdominal pain, 6: nausea, 7: skin lesions, 8: constipation)

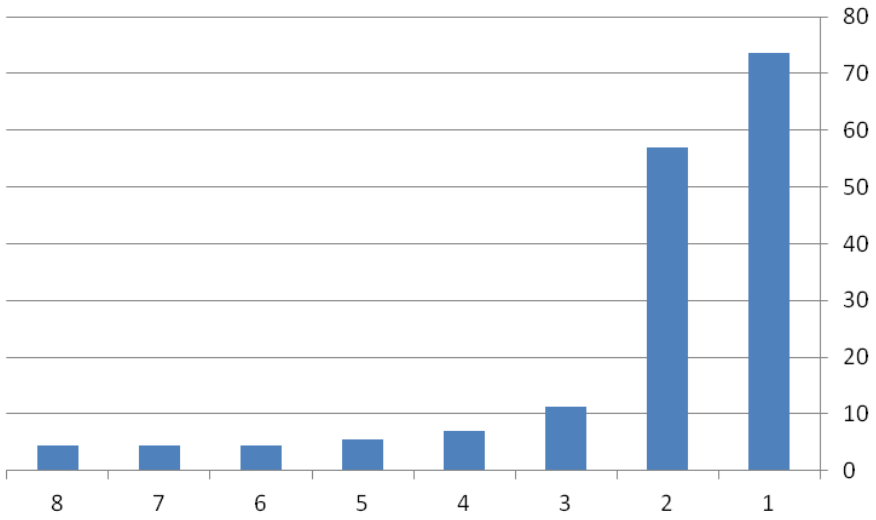

Fig 2. Previous antibiotics used before admission in suspicious cases to systemic salmonella infections (1: Ceftriaxone, 2: Cefixime, 2: Ampicillin, 4: Cefotaxime, 5: Cephalexin, 6: Clindamycin, 7: Amoxicillin, 8: Ciprofloxacin)

was 38.3U/L which was within normal level in all cases. About 30% of patients had high sedimentation rates however positive titer of CRP level was observed in 66.7% of patients on admission.

Eighteen (40%) patients had received antibiotic treatment before coming to the hospital. The most common antibiotic used before admission was 3th generation of cephalosporins; but overall, just 8.8% of cases had received antibiotics which was ineffective on salmonella spp.(figure 2)

Blood, urine and stool samples collected at first day in 15.6%, second day in 35.6% and third day in 48.9% of the patients. In total, 41(91.1%) samples were collected during antimicrobial therapy. Patients were grouped into three groups according to results of laboratory studies. Group I consisted of 18(41%) patients, group II consisted of 27 patients with positive Widal test; of these, one was blood and stool cultures positive; however PCR in blood was negative in this group. Group III represented 2 patients with positive blood and stool cultures. In one case PCR in blood was positive. In another patient Widal test was negative despite positive blood culture and PCR; this case was a leukemic kids with very fulminant septic picture who had not received any antibiotic before sampling and died rapidly with septic shock despite appropriate therapies.

Discussion

Typhoid fever is one of the most common infectious diseases in developing countries with incidence around 0.5% (13). Early and definitive diagnosis of the disease is important not only for effective therapy, but also critical in avoiding

complications (13). It also makes possible specific treatment at an early stage, which leads to the rapid elimination of the pathogen (3,14,15). Thus, a new rapid and sensitive method for diagnosis of typhoid fever is required (16). In our study 45 patients were evaluated. PCR was positive in only one patient despite the fact that two patients had positive blood and stool cultures. It means that sensitivity of our PCR technique is around 50%. On the other hand it was positive in the second case with positive cultures that shows a high specificity. Although, 26 patients had positive Widal test and compatible clinical picture but mostly had negative blood culture and PCR results. All of these cases except one responded to therapy which is a further evidence of correct diagnosis.

In group 3 with positive blood and stool culture, PCR was just positive in one case, the kid was immunocompromised who probably had higher CFU/ml of bacteria and had not taken any antibiotic before sampling.

Our results is compatible with Devrim et al study in Turkey in which just one case out of six who had positive blood culture had simultaneous positive PCR results (a sensitivity around 16.6%), but, they found one positive PCR case also in the group who had no positive culture but were clinically suspected cases and had a history of using various antibiotics before admission (3).

Our results are not compatible with several other studies in which the various targeted PCR methods have shown to be sensitive and specific for diagnosis of systemic salmonella infections (6,8,9,16). In the study done by Munir et al despite universal acceptance of positive cultures from sterile sites as gold standard of diagnosis, they had just one positive blood culture in 105 cases suspicious to typhoid fever, but had 102 positive PCR results (6). This study included both kids and adults and Widal test was positive in 25.7% if we accept that all positive PCR cases have been real typhoid cases, a finding which might be difficult to prove due to probability of false positivity of PCR methods (5,13).

It has been shown that if blood samples were first mixed with ox bile for selective lysis of human blood cells it can release human DNA which then can be digested with addition of bile resistant micrococcal nuclease. Such a technique could be useful to increase the sensitivity of PCR methods for detection of S. typhi in blood samples (5).

Other explanations of our low PCR positivity test might be prior antibiotic therapy both before admission and late requesting for consult when patient was taking a broad spectrum antibiotic (5).

Conclusion

In our setting the role of conventional PCR in blood samples of kids with suspicious systemic salmonella infections was not clearly determined. Larger sample size, preferably in a multicenter study, and using more sophisticated methods of blood cultures and novel techniques to increase the availability of organism for DNA detection is needed before determination of its role in both groups with no prior antibiotic therapy and cases with previous history of antibiotic administration.

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