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ORIGINAL ARTICLE

Childhood Brucellosis in Eastern India

Devarati Dutta[1] · Anindita Sen[2] · Debkishore Gupta[3] · Prabir Kuila[1] · Debasmita Chatterjee[4] · Sugat Sanyal[5] · Satadal Das[1]

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Abstract
Objective To investigate the presence of childhood brucellosis presenting as PUO (pyrexia of unknown origin) cases in Eastern zone of India.
Methods Blood samples were collected from PUO patients aged ≤18 y. The main diagnostic tools were STAT, RBPT, ELISA- IgM, IgG and PCR. Although mainly PUO cases were selected for the study, other associated clinical manifestations were also noted.
Results The findings revealed significantly higher percentage of infection in female children (14.3%) than in male children (10.9%). The positive results by different diagnostic tools, STAT, RBPT, ELISA- IgM, ELISA-IgG and brucella genus specific PCR were 10.6%, 7.2%, 7.2%, 0.85% and 1.3% respectively. Main associated clinical symptoms were joint pain, low backache, fatigue and night sweat.
Conclusions This hospital based study reflects a significant number of childhood brucellosis cases in Eastern zone of India, and thus emphasizes the need for further monitoring of such subjects.

✉ Satadal Das
drsatdas@hotmail.com

[1] Brucella Research Laboratory, Peerless Hospital & B.K. Roy Research Centre, 360 Panchasayar, Kolkata, West Bengal 700094, India

[2] Department of Microbiology, MGM Medical College, Kishanganj, Bihar, India

[3] Department of Microbiology & Infection Control, CMRI and BM Birla Heart Research Centre, Kolkata, India

[4] Department of Food Technology & Biochemical Engineering, Jadavpur University, Kolkata, India

[5] Department of Laboratory, Peerless Hospital & B.K. Roy Research Centre, Kolkata, India

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Introduction

Brucellosis is recognized as an important human infection in many parts of the world especially Latin America, Southern Europe, Africa and Asia including Middle East [1]. More than 500,000 human infections are recorded every year throughout the world. This figure may be low from the actual incidence of brucellosis cases, especially in endemic areas, due to underreporting in some countries [2]. It is primarily a zoonotic disease found both in domestic and wild animals [1]. Human transmission mainly occurs through direct contact with infected animals or their products such as placenta or aborted materials, and consumption of infected animal products or undercooked meat. Also, direct contact with soil, animal feces and dust contaminated with the organism is associated with a higher risk of infection [3]. Human to human transmission is rare, but has been reported in association with blood transfusion, bone marrow transplantation, transplacental or perinatal exposure, during sexual intercourse and postnatally, through breast milk. Previously it was thought that children were uncommonly affected by this disease but quite a few reports from endemic areas changed the notion by showing a higher percentage of childhood brucellosis (10–30%) [4–7]. In children, the major source of infection is consumption of unpasteurized milk products such as soft cheese, ice cream and butter. Also, the consumption of raw milk and contact with infected animals or their excretory products can be the causes of acquiring this disease. Clinical manifestations of childhood brucellosis are varied, ranging from minimal

symptoms to extreme morbidity and occasional fatality. Symptoms of childhood brucellosis vary partly because of the variable pathogenicity of different strains. Asymptomatic infections were also recorded. This hospital based study has been designed to portray a picture about the possible presence of childhood brucellosis cases in the Eastern part of India.

Material and Methods

Blood samples were collected by venipuncture from PUO (pyrexia of unknown origin) patients aged ≤18 y. In this study, PUO refers to a condition in which the patient has an elevated temperature for about 3 wk but despite investigations by a physician no explanation has been found. If a definite cause is found, it is not included under PUO. Blood was withdrawn by a single venipuncture (usually 3–5 ml but 10 ml from older children) and injected into a vacutainer tube (BD). Serum was separated by centrifugation at 2000 rpm for 15 to 20 min. The separated serum samples were then stored at −20 °C freezer before processing for further tests. The inclusion criteria of patients in this study were only children and adolescents categorized as PUO cases without any serious complications; who were not treated with any antimicrobial agent effective against brucellosis; the exclusion criteria were, patients with serious complications, or who were already treated for brucellosis. The samples were collected from different hospitals: Peerless Hospital & B.K. Roy Research Centre, a private tertiary care hospital in Kolkata, India and MGM Medical College, Kishanganj, Bihar, India. Permission for this study was taken from Institutional Ethical Committee of these hospitals. Consent for this study was taken from parents of all the children included in this study.

The duration of the study was from January, 2013 through December, 2015. A total of 236 blood samples were collected. Details of clinical findings were recorded in a typical clinical data sheet and a patient history sheet.

All the serum samples were screened by brucella specific serological tests - Standard Tube Agglutination Test (STAT; Tulip Diagnostics Pvt. Ltd., India), Rose Bengal Plate Test (RBPT; IAHVB, Bangalore, India), and ELISA (for both IgM and IgG antibodies; Immunolab GmbH, Germany).

The blood clots of serologically positive blood samples were liquefied and cultured according to Castaneda method using Castaneda's biphasic medium (Himedia, Mumbai, India). Blood was also collected directly as described earlier, in automated blood culture bottles (BACTEC 9050, BD) and was incubated up to 3 wk for isolation of the bacterium. Subcultures in case of suspected growths were done on brucella agar plates (*BD BBL* ™). The bacterial growth on agar plate was tested for identification by biochemical tests - catalase using H_2O_2 (Merck, USA), oxidase using oxidase disc (Himedia), urease using urea agar (Himedia) and H_2S production using TSI agar slant (Himedia).

The DNA was extracted from all the serologically positive serum samples using the spin column technique following the instructions provided in the QIAmp DNA Blood Mini Kit (Qiagen, Germany).

The DNA samples were tested by the brucella genus specific simplex polymerase chain reaction (PCR) using the forward and reverse *bcsp* gene primers B4 and B5 respectively. This primer pair was obtained from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. This end-point PCR was carried out in a thermal cycler (Prime, UK). The amplified product was then subjected to agarose gel electrophoresis within a gel electrophoresis trough (Peqlab, Germany). Finally the generated amplicon size of 223 bp was viewed using the gel doc apparatus (Biorad, USA). This *bcsp* gene encodes the 31 kDa brucella cell surface salt extractable protein (BCSP) and is considered as a marker gene present in all *Brucella* spp.

Quality control was done as a part of DBT (Department of Biotechnology) network project on brucellosis (Government of India) in eight different units throughout India, which includes authors' Unit BE-8. A thorough training of all the investigators and fellows, quality check of all the recommended kits, and random inter lab check of the samples were done to achieve a uniform high standard in all the units.

Results

During the period of study, altogether 236 blood samples of child PUO cases were screened for the detection of brucella infection. Out of 236 blood samples, 29 (12.3%) were denoted as brucella positive samples based on any one of the serological (STAT, RBPT, IgM ELISA, IgG ELISA) and molecular (PCR) tests. The total number of child PUO cases screened year wise during the study period and also the positive brucella cases were noted. Based on serological and molecular screening it was noted that brucella positive cases were high during 2013 and again in 2015 but somewhat low incidence was observed in 2014. The total number of child PUO cases screened each month during the study tenure and the month wise positive brucella cases were also noted. Based on this analysis it was found that the maximum brucella positive patients were identified during the months, June to September, which is the monsoon season of the year.

Results of serological tests (Table 1) revealed that 10.6% serum samples were STAT positive, 7.2% were RBPT

Table 1 Results of serological tests and molecular test among brucella positive cases

Case no.	Gender	Age (years)	Direct/Indirect animal contact	STAT	RBPT	IgM ELISA	IgG ELISA	PCR
1	M	9	Not present	+	–	–	–	–
2	M	18	Not present	–	–	–	+	–
3	F	15	Not present	–	–	–	+	–
4	M	9	Not present	+	–	+	–	–
5	M	14	Animal contact, undercooked meat consumption	+	–	–	–	–
6	M	13	Raw milk consumption	+	–	–	–	–
7	F	17	Not present	+	–	+	–	–
8	F	18	Raw milk consumption	+	+	+	–	–
9	M	17	Animal contact, raw milk & undercooked meat consumption	+	+	–	–	–
10	M	16	Animal contact, undercooked meat consumption	+	+	+	–	–
11	F	14	Not present	+	+	–	–	–
12	M	10	Not present	+	+	+	–	–
13	F	2	Not present	+	+	+	–	–
14	F	7	Raw milk consumption	+	+	+	–	+
15	F	6	Raw milk consumption	+	+	+	–	+
16	M	2	Raw milk consumption	+	+	–	–	–
17	F	2	Raw milk consumption	+	+	+	–	–
18	M	10	Raw milk consumption	+	+	+	–	–
19	M	7	Raw milk consumption	+	+	+	–	–
20	F	15	Raw milk consumption	+	+	–	–	–
21	F	12	Not present	–	–	+	–	–
22	F	16	Not present	+	+	+	–	–
23	M	15	Not present	–	–	+	–	–
24	M	12	Not present	+	+	+	–	–
25	M	15	Not present	+	–	–	–	–
26	M	15	Not present	+	–	+	–	–
27	F	18	Not present	+	–	–	–	–
28	F	14	Raw milk consumption	+	+	+	–	+
29	F	18	Animal contact, raw milk consumption	+	+	–	–	–

M Male; *F* Female; + Positive; − Negative

positive, 7.2% were IgM ELISA positive and 0.85% was IgG ELISA positive. None of the cultures showed brucella positivity. PCR test was positive in 1.3% cases (Table 1). Figure 1 indicates the three brucella positive cases obtained by PCR during the course of the study.

It was expected that Brucella positive results may be more in male children as their number was more (58.5%) in this study, but it was found that Brucella positive results were more in female child patients (14.3%) (Table 2). Chi –squared test was done in between positive results of male and females. The calculated *P* values (significance level) for differences of positive results in between male and females in relation to STAT, RBPT, IgM ELISA, IgG ELISA and PCR tests were 0.9419, 0.3488, 0.5566, 0.9600, and 0.0628 respectively. The results indicate that although there was practically no difference in STAT, RBPT, IgM ELISA, IgG ELISA test positive results, there was some significance of increased positive PCR tests in females; which also indicates there is a possibility of increased brucellosis in female child.

It was found that during the study period majority (88.6%) of the child PUO cases came from Kolkata and its adjoining districts and 11.4% were from adjoining states and Bangladesh.

Another interesting observation was that during the tenure of this study three female child patients were identified as brucella positive based on all the serological and molecular findings obtained from the laboratory except IgG ELISA. Among these three child patients, one resides in Kolkata city and the other two are in one of its adjoining district. It was also recorded that only three child patients below 5 y of age were found affected by this disease in this study. All of them were 2-y-old; one was male and the other two were females. They showed brucella positivity in all serological tests (STAT, RBPT and IgM ELISA) except IgG ELISA test.

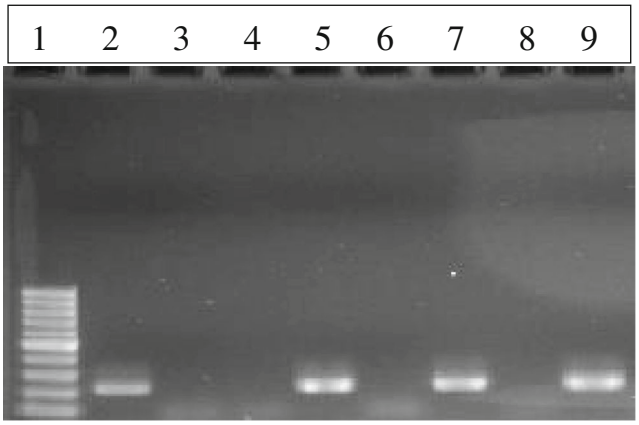

Fig. 1 Gel picture represents brucella positive cases during 2013–2015. Lane 1: 100 bp molecular size marker, Lane 2: Positive control for *bcsp* gene (*Brucella abortus*; S99), Lane 3: Negative control, Lanes 5, 7, 9: Samples positive for *bcsp* gene. The amplicon size for *bcsp* gene is 223 bp

Discussion

Brucellosis has an important public health implication and cause of economic loss in many countries, particularly in the countries of Mediterranean region and other developing countries [8]. Worldwide, reported incidence of human brucellosis in endemic disease areas varies widely, from <0.01 to >200 per 100,000 population [9]. Clinical picture of this disease is variable as it may manifest as a systemic disease. After infection, the microorganism, which exhibits multisystem involvement, proliferates in the regional lymph nodes, passes into blood stream and usually affects several tissues and multiple organs, primarily the reticuloendothelial system [10]. It is considered as an important cause of pyrexia of unknown origin (PUO) and has shown considerable mortality in endemic countries. The incidence of childhood brucellosis is usually underreported and is found to be ranging between 0.8 and 1.6% [11]. However, the rate of childhood brucellosis in endemic regions has been reported to vary from 11% [12] to 56% [13]. Brucellosis, as an infectious disease in children has been accounted rarely in India [14]. It is very uncommon under 5 y of age. The present study was conducted with child and adolescent patients aged ≤18 y and in this study three child patients aged less than 5 y were found affected by this disease. The pediatric patients included in this study presented symptoms like persistent fever, joint pain, low backache, fatigue and night sweat but fever was the most common complaint which is in accordance with previous reports [15, 16]. These symptoms were also mainly observed in the Brucella positive cases. The period of illness prior to testing and diagnosis was less than 1 mo in most of the cases, which shows consistency with previous studies [11].

Table 2 Gender distribution among PUO (aged ≤18 y) and brucella positive cases

Gender	Year	No. of PUO cases	No. of brucella positive cases (%)
M*	2013	17	5 (29.4)
	2014	30	2 (6.7)
	2015	91	8 (8.8)
M (Total)		138	15 (10.9)
F#	2013	6	1 (16.7)
	2014	32	2 (6.3)
	2015	60	11 (18.3)
F (Total)		98	14 (14.3)

* Male

Female

STAT with titres greater than 1:160 suggests active infection. STAT measures the total amount of agglutinating antibodies *i.e.*, both IgM and IgG. Thus, STAT is known to be the best diagnostic modality available. RBPT is a rapid serological screening test. It is suitable for large scale screening. In this study, several serological parameters were checked such as STAT, RBPT and ELISA. Results indicated that the majority of the positive child patients showed STAT positivity followed by RBPT and IgM ELISA.

In brucellosis endemic region, the interpretation of Brucella serological tests may be difficult due to persistent presence of Brucella specific antibodies in general population. Thus over diagnosis and unnecessary treatment against brucellosis will continue until and unless each country establishes a normal range of the titre of serological tests for the population of that country. Although rapid and low cost STAT and RBPT tests are preferred tests for brucellosis as per WHO guidelines, each positive result should be correlated with clinical symptoms and history of exposure to animals or animal products if present. This is due to the fact that the predictive value of a positive test declines as prevalence of the disease decreases in a country.

Again, serological tests vary in their ability to give a true positive result. False reaction may occur due to group cross reaction with other bacteria including *Yersinia enterocolitica* 0:9 (common major O-polysaccharide antigen), *E. coli* O116: H21, O157: H7, *Francisella tularensis*, *Salmonella* serotypes of Kauffman-White group N, *Pseudomonas maltophilia, Vibrio cholerae* and by non specific inhibitors mimicking antibodies. Specificity, sensitivity and predictive values of all serological tests should be considered during interpretation of the results. Usually specificity and sensitivity are inversely related.

The culture of brucella is difficult and usually takes a lot of time. Moreover, the culture yield remains very low.

The bacteremia condition prevails mostly in the first 2 wk of illness, when PUO diagnosis is not done. Thus as per WHO guidelines, serological tests are normally preferred for diagnosis of brucellosis in the laboratory. In this study authors could not retrieve the organism from any blood culture samples probably due to these facts. In brucellosis endemic regions, clinical symptoms associated with positive serology but without isolation of the organism are considered confirmed human cases [17].

The molecular tests like PCR are being employed for the diagnosis of brucellosis both at the genus and species levels. The technique has advantages because it is easy to perform, require a short span of time and is non-hazardous as it does not carry the risk of laboratory acquired infection. So there is a probability that this diagnostic test will be the future diagnostic modality of choice [18].

In this study ELISA (both IgM and IgG) positive results were considered true positive results, for their better sensitivity and specificity [19, 20], recent WHO guidelines also suggested that ELISA can be considered as a confirmatory test for brucellosis [21] and when compared to individual test, sensitivity was found as 82.6%, 73.1%, 90.5%, 52.8%, 54.3% with STAT, RBPT, IgM ELISA, IgG ELISA, and PCR respectively. Specificity was minimum with STAT which was 50.0%. Specificity of other tests was 100.0%. Positive predictive values of STAT, RBPT - the two commonly used tests, were 65.5% and 73.1% respectively, while negative predictive values of these two tests were 71.4% and 41.7%, respectively. Thus STAT may be used for screening Brucella infection, then the positive results may be confirmed by ELISA test results and for final diagnosis, a positive result should be correlated with clinical findings thoroughly before beginning the treatment.

During the tenure of the study, it was noticed that the incidence of positive cases of child brucellosis was significant in 2013 and 2015, with monsoon being the most favorable season for acquiring this infectious disease. This is because in this season Brucella infection is more in animals from where the disease is transmitted to human beings. Similarly, the disease was found slightly more in female children particularly in relation to PCR tests. Although it is very difficult to explain why female children are more affected in brucellosis than male children, there are several studies done previously in rats, where it was found that testosterone in male rats gives resistance to Brucella infection [22], while oestradiol in female rats decreases resistance to Brucella infection [23]. Thus similar factors may be the reason for the differences in brucella positivity among both the sexes in this study. To prevent the disease in children, drinking of unpasteurized milk, eating cheese or ice cream should be avoided and children should not come in contact with sick or dead animal. Education of general public is needed as an adjunct and not as an alternative strategy to control human brucellosis [24] as till now no vaccine is available for the prevention of brucellosis in humans.

Conclusions

It can be summarized that the present study was designed to evaluate the presence of childhood brucellosis presenting as PUO cases in Eastern zone of India. It is visualized that childhood brucellosis is prevalent in India and in PUO cases, brucellosis should also be looked for even in children below 5 y of age.

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Contributions All the authors were involved in the study. DD performed the study protocols, analyzed the data and drafted the manuscript. SD designed the study, critically reviewed, finalized the manuscript and will act as guarantor for this paper.

Compliance with Ethical Standards

Conflict of Interest None.

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References

1. Young EJ. Brucella species. In: Mandell GL, Benett JE, Dolin R, editors. Principles and Practice of Infectious Diseases. 5th ed. Philadelphia: Churchill Livingstone; 2000. p. 2386–93.
2. Young EJ. An overview of human brucellosis. Clin Infect Dis. 1995;21:283–90.
3. Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. N Engl J Med. 2005;352:2325–36.
4. Sharda DC, Lubani M. A study of brucellosis in childhood. Clin Pediatr (Phila). 1986;25:492–5.
5. Gottesman G, Vanunu D, Maayan MC, et al. Childhood brucellosis in Israel. Pediatr Infect Dis J. 1996;15:610–5.
6. Tanir G, Tufekci SB, Tuygun N. Presentation, complications and treatment outcome of brucellosis in Turkish children. Pediatr Int. 2009;51:114–9.
7. Sutcu M, Gokceer D, Akturk H, et al. Brucellosis infection in a child with progressive familial intrahepatic cholestasis type 2 who had undergone liver transplantation. Pediatr Transplant. 2015;19:E146–8.
8. Colmenero JD, Regnera JM, Martos F, et al. Complications associated with *Brucella melitensis* infection: a study of 530 cases. Medicine. 1996;75:195–211.
9. Boschiroli ML, Foulongne V, O'Callaghan D. Brucellosis: a worldwide zoonosis. Curr Opin Microbiol. 2001;4:58–64.
10. Schutze GE, Jacobs RF. Brucella. In: Kliegman RM, Stanton BF, St. Geme III JW, Schor NF, Behrman RE, editors. Nelson Textbook of Pediatrics. Philadelphia: Elsevier Saunders; 2011. p. 980–2.

11. Mantur BG, Akki AS, Mangalgi SS, Patil SV, Gobbur RH, Peerapur BV. Childhood brucellosis- a microbiological, epidemiological and clinical study. J Trop Pediatr. 2004;50:153–7.
12. Madkour MM. Childhood brucellosis. In: Madkour MM, editor. Brucellosis. Cambridge: Butterworths; 1989. p. 205–18.
13. Feiz J, Sabbaghian H, Miralai M. Brucellosis due to B. melitensis in children. Clinical and epidemiologic observations on 95 patients studied in central Iran. Clin Pediatr (Phila). 1978;17:904–7.
14. Havaldar PV, Kumar SY, Desai AS, Siddibhavi BM. Brucellosis in children. Indian Pediatr. 1987;24:995–8.
15. Mantur BG, Biradar MS, Bidri RC. Protean clinical manifestations and diagnostic challenges of human brucellosis in adults: 16 y experience in an endemic area. J Med Microbiol. 2006;55:897–903.
16. Ciftdogan DY, Bayram N, Vardar F. Brucellosis as a cause of fever of unknown origin in children admitted to a tertiary hospital in the Aegean region of Turkey. Vector-Borne Zoonotic Dis. 2011;11:1037–40.
17. Godfroid J, Cloeckaert A, Liantard JP, et al. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res. 2005;36:313–26.
18. Solera J. Update on brucellosis: therapeutic challenges. Int J Antimicrob Agents. 2010;36:S18–20.
19. Araj GF, Kaufmann AF. Determination by enzyme linked immunosorbent assay of immunoglobulin G (IgG), IgM and IgA to *Brucella melitensis* major outer membrane proteins and whole-cell heat-killed antigens in sera of patients with brucellosis. J Clin Microbiol. 1989;27:1909–12.
20. Pickering LK, Baker CJ, Kimberlin DW, Long SS. Report of the Committee on Infectious Diseases. Red Book. Elk Grove Village: American Academy of Pediatrics; 2009. p. 237–9.
21. Corbel MJ, Alton GG, Ariza J. editors. Brucellosis in humans and animals. Geneva: World Health Organization 2006.
22. Ascoli A. Immunity and receptivity to *Brucella abortus* in the albino rat. Exp Med Surg. 1947;5:107–27.
23. Pugh LH. Effect of gonadal hormones on experimental infection of rats with *Brucella abortus*. Exp Biol Med. 1948;68:591–2.
24. Memish Z. Brucellosis control in Saudi Arabia: prospects and challenges. J Chemother. 2001;13:11–7.