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Development & standardization of an in-house IgM indirect ELISA for the detection of parvovirus B19 infections

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***Background & objectives*: Parvovirus B19 infections occur worldwide; the infection is acquired early in childhood but could occur later. B19 is reported to cause infection in childhood febrile illnesses, and arthropathies in adults and children and in end-stage renal disease (ESRD) seen in adults. This study was designed to develop an in-house IgM indirect ELISA for serological screening among patients and controls, and to compare ELISA results with those of nested polymerase chain reaction (nPCR) assay.**

***Methods*: An in-house IgM indirect ELISA was standardized using peptide sequence of VP1/VP2 region of parvovirus B19. A total of 201 children and adult with febrile illnesses, 216 individuals with non-traumatic arthropathies, 201 cases of chronic anaemia associated with ESRD and 100 healthy controls were tested. Serum was separated from the blood and subsequently used for DNA extraction. The nested polymerase chain reaction (nPCR) for the detection of B19V DNA was performed using primers targeting the overlapping region of VP1/VP2 capsid protein genes.**

***Results*: A total of 618 samples were tested for parvovirus B19 by an in-house IgM indirect ELISA. Among these samples, six were positive by in-house ELISA. The inter-rater agreement between ELISA and PCR assays was calculated using kappa coefficient analysis. The value of κ was 0.77 and the strength of agreement was 'good' (P<0.001).**

***Interpretation & conclusions*: The in-house IgM indirect ELISA was found to be simple with high sensitivity and specificity when compared with nPCR and could be used as an alternative to expensive commercial kits in resource-poor settings.**

Key words Arthropathy - end-stage renal disease - febrile illness - IgM indirect ELISA - parvovirus B19

Human parvovirus B19 belongs to the *Erythrovirus* genus of family *Parvoviridae.* Parvovirus B19 infection has a broad spectrum of clinical manifestations[1]. Parvovirus B19 is a small (26 nm), non-enveloped, single stranded (ss) DNA virus. The most important viral proteins include the major non-structural protein

NS1 and the two structural proteins VP1 and VP2[2]. The parvovirus B19 capsid is composed of two capsomere proteins, VP1 and VP2, which are encoded by overlapping reading frames. The only known host for parvovirus B19 is humans[3].

Most infections are asymptomatic, but it can cause erythema infectiosum (fifth disease) in children, and arthropathy in adults (both acute and chronic, more common in adult females). The infection may cause acute biphasic illness with fever, chills, headache and myalgia, followed subsequently by classic fifth disease symptoms in children associated with the appearance of IgM antibodies (generalized erythematous eruption and joint inflammation) indicative of immune complex formation[4].

IgM antibody develops 10-12 days post-infection, coinciding with a peak in virus level. IgM usually persists in serum samples for approximately three months but may be found for several months. IgG persists long term and is thought to convey immunity to reinfection. Infrequently, low-level B19V DNA in association with IgG may persist for months[5]. Generally, commercial ELISA kits are used for serological diagnosis of parvovirus B19 infections. However, these have not been compared with the sensitive molecular tests. The objectives of our study were (*i*) to develop an in-house IgM-indirect ELISA for antibody screening among patients and healthy controls, and (*ii*) to compare ELISA results with PCR findings.

Material & Methods

This prospective, cross-sectional pilot study was conducted in the Sri Sakthi Amma Institute of Biomedical Research, Sri Narayani Hospital and Research Centre, Vellore, India, during February 2013 to April 2015. The study was approved by the Institutional Review Board. A clinical questionnaire and written consent form were obtained for each patient. Patients admitted to the medical wards or attending the outpatient department presenting with fever with or without rash, oedema, joint pain, arthralgia/myalgia, general fatigue, mild illness of pyrexia, malaise, rash and arthralgia during the study period were included in the study. Patients or their attendants who did not give informed consent to participate and had conditions not of relevance to the proposed study were excluded. The patient groups included those presenting with fever with or without rash (n=201), rheumatoid factor negative non-traumatic arthropathy (n=216) and individuals on dialysis for end-stage renal disease (ESRD) undergoing dialysis (n=201). Blood samples (5 ml) from patients and healthy donors (n=100) were collected. Healthy volunteers included staff (n=30) and graduate students (n=70) of the institution. The age ranged from 17 to 44 yr and the median was 18 with male: female ratio of 1:1.

Clinical features observed in patient group: All patients with febrile illness had temperature ≥98.7°F. Cough and myalgia were seen in 43.28 (n=87) and 48.25 per cent (n=97) patients, respectively. None had joint pain and exanthematous fever. About 92 per cent (n=199) patients with non-traumatic arthropathy had arthralgia, 81.9 per cent (n=177) had myalgia, 26.8 per cent (n=58) had cough, headache was seen in 14.3 per cent (n=31) and features such as fever, nausea and skin rash were seen in 12 (n=26), 5.5 (n=12) and 10.6 per cent (n=23) of patients, respectively. Seven per cent had oedema around the joints. In patients with ESRD, myalgia was seen in 35.8 per cent (n=72), joint pain in 30.84 per cent (n=62) and cough in 30.84 per cent (n=62) and skin rash in 14.9 per cent (n=30). Generalized oedema was observed in 17.9 per cent (n=36) of patients, and other clinical features such as fever in 10.9 per cent (n=22).

ELISA procedure: Immunodominant epitope was predicted using BepiPred server (B-cell epitope prediction software) (*http://www.cbs.dtu.dk/services/BepiPred*). Input amino acid sequence of VP1/VP2 region of parvovirus B19 was analysed in the software to get the best single epitope of the peptide sequence among the predicted epitopes. The peptide: FSPAASSCHNSSGKEA (length=16 amino acid) was synthesized commercially from GenScript Biotech Corporation, Piscataway, NJ, USA. The assay was similar to previously published protocol[6].

The in-house IgM ELISA was performed as follows: a 96-well flat-bottomed microtitre ELISA plate (Nunc™ MaxiSorp™, Thermo Fisher Scientific Roskilde, Denmark) was coated with peptide antigens (1 µg/ml) at a concentration of 100 ng/well, incubated at 37°C for two hours and washed three times with phosphate buffered saline (PBS) *p*H 7.2. The wells were blocked with 300 µl of blocking solution to each well (1% bovine serum albumin, Sigma-Aldrich, USA, in 0.15 M PBS) and incubated at room temperature (RT) for one hour, then washed three times [Mindray MW12A (Washer), Shenzhen, China]. The test serum samples were diluted 1:200 in

PBS along with 10 µl serum sample+2000 µl diluents with 15 µl of RF absorbent (Fitzgerald, MA, USA) +250 µl of diluted serum and incubated for 15 min at RT. After incubation, 100 µl of diluted serum sample was distributed into duplicate wells. The plate was incubated at RT for one hour and then washed three times as described above. One hundred microlitres of diluted biotin-labelled detection antibody (anti-µ chain secondary antibody) (Sigma-Aldrich, USA) was added to each well at the concentration of 0.25 µg/ml, incubated at RT for one hour and then washed three times. After washing avidin-horse radish peroxidase (Sigma-Aldrich, USA) conjugate (1:70,000 dilution of 1 mg/ml stock solution) was added and incubated at RT for one hour. The plate was washed for five times. After washing, 100 µl of substrate solution: tetramethylbenzidine (Sigma-Aldrich, USA) was added and incubated for 15 min at RT in the dark. The reaction was stopped using 100 µl of stop solution (Sigma-Aldrich, USA) and colour development was detected at 450 nm on an ELISA reader (Mindray MR 96A, Shenzhen, China). Positive and negative controls were also used in each run (provided by Dr Amita Jain, KGMU, Lucknow).

All samples (n=718) were tested using an in-house developed nPCR assay for the detection of B19V DNA using primers targeting the overlapping region of VP1/VP2 capsid protein genes [Outer (F) CAAAAGCATGTGGAGTGAGG, Outer (R) CTACTAACATGCATAGGCGC, Inner (F) CCCAGAGCACCATTATAAGG, Inner (R) GTGCTG TCAGTAACCTGTAC]. The cycling conditions included denaturation at 95°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute for 30 cycles[7]. The 288 bp product size was detected by gel electrophoresis.

Statistical analysis: Sample size determination, test of significance for proportion and kappa coefficient were analyzed using Epi Info 6.04d (DOS version), a Centers for Disease Control and Prevention (CDC), USA, Public Domain Program (*https://www.cdc.gov/epiinfo/index.html*).

Results

To establish the optical density (OD) cut-off of a positive sample for patients groups, 100 individual serum samples from healthy controls were tested. Average OD was determined for two wells in which each sample was tested. The mean +3 standard deviation (SD) was 0.130. Using this cut-off, the patient groups were tested to determine the positives among them.

Of the 201 children and adult with febrile illnesses tested, two samples were found positive; of the 216 patients with non-traumatic arthropathies, four samples were found positive; none of the 201 patients of chronic anaemia associated with ESRD was found positive (Table). The mean age for the patient group (n=618) was 44.92±15.64 yr and for the control group (n=100) was 20.96±18.2 years. There were 350 males and 234 females in the patient group (data not available for 34 patients). The ELISA results were compared to nPCR findings in these groups.

Overall, there were seven nPCR-positive patients, of these five were positive at the original dilution by ELISA. Two nPCR-positive samples were ELISA positive on testing at lower serum dilutions (1:200, 1:100, 1:50 and 1:25) but not included for analysis as positives. One febrile illness patient was only IgM positive but nPCR negative (Table). All 100 healthy individuals were both nPCR and ELISA negative.

The inter-rater agreement between ELISA and nested PCR assays was calculated using kappa coefficient analysis. The value of κ was 0.77 and the strength of agreement was 'good' (P<0.001).

Among the patient groups tested, B19V PCR positivity was higher in patients with non-traumatic arthropathies (n=4; 1.9%) though the difference in B19V-positive rate among the three patient groups

Table. ELISA and polymerase chain reaction (PCR) positives in different patient groups

Patient group	Sample tested	ELISA positive, n (%) (95% CI)	PCR positive, n (%) (95% CI)	Both positive	Either/or
Febrile illness	201	2 (0.99) (0.17-3.25)	1 (0.5) (0.023-2.308)	1	1
Non-traumatic arthropathies	216	4 (1.85) (0.62-4.4)	4 (1.85) (0.62-4.4)	4	0
End-stage renal disease	201	0	2 (0.99) (0.17-3.25)	0	0
Healthy individuals	100	0	0	0	0

CI, confidence interval

was not significant. When compared to nPCR, ELISA had a sensitivity of 83.33 per cent, specificity of 99.72 per cent, positive predictive value of 71.43 per cent and negative predictive value of 99.86 per cent.

Discussion

The objective of this study was to develop and standardize an IgM ELISA and apply the same in three different patient groups and compare the results with PCR. Human parvovirus B19 infection is associated not only with erythema infectiosum (fifth disease) but also, rarely, with purpuric or petechial rashes of children[8-10]. In our study, of the 201 samples tested two (0.99%) were IgM positive among febrile illness patients.

Arthropathy may be a complication of erythema infectiosum or a primary presentation of parvovirus B19 infection. Arthralgia is more common in adolescents and adults with parvovirus B19 infection, affecting up to 60 per cent of these persons. Arthropathy affects women twice as often as men[11]. In this study, 216 samples of patients with non-traumatic arthropathies were tested; and four were found positive (2 male; 2 female).

B19 infection-associated joint symptoms occur most frequently in adults, presenting as a self-limited, acute symmetric polyarthritis affecting the small joints of the hands, wrists and knees. However, a small percentage of patients develop persistent chronic polyarthritis that mimics rheumatoid arthritis (RA) which has led to the hypothesis that B19 virus may have a role as a concomitant or precipitating factor in the pathogenesis of autoimmune conditions[12,13]. In another study[14], B19 IgM was detected in 16 of 74 patients (21.6%) with acute arthropathy compared with three of 74 (4.1%) in the healthy control group. It was shown that B19-positive patients with arthropathy were more likely to develop chronic disease and to be diagnosed as juvenile RA than the parvovirus B19 IgM-negative group with arthropathy. The authors conclude that their data support the hypothesis that parvovirus B19 infection may be associated with the onset of juvenile RA in a proportion of patients[14]. Danda *et al*[15] found no association of this virus with chronic inflammatory arthropathy in their study with a nine-year follow up.

Another study showed IgM anti-B19-specific antibodies in 24 per cent of RA patients; B19 DNA in plasma and/or peripheral blood leucocytes, synovial fluid cells in 34 per cent (34 patients); both markers were found in 14 per cent of the cases (14 patients)[16]. This study also indicated for a possible role of this viral infection in the pathogenesis of RA.

The diagnosis of B19 infection could be missed in immunosuppressed patients who may not mount an antibody response. Furthermore, in organ transplant recipients, diagnosis by serology can be confounded by administration of blood products or IgG after transplantation because these therapies may produce false-positive IgG antibody tests. Thus, identification of the viral DNA by PCR is preferred for diagnosis in these patients[17]. B19 could cause acute glomerulopathy and could also be a cause of anaemia in ESRD and kidney transplantation[18].

Cross-reactivity analysis by testing serum samples with heterologous virus infection showing IgM was not performed in our study as all the healthy controls were negative for IgM assays. The reproducibility of the assay was carried out by testing all samples in duplicate for IgM ELISA. Positive samples were tested twice for confirmation. The false positivity was ruled out since all healthy controls who were free of intercurrent infections for at least one month in our study group were negative for parvovirus B19[19,20].

In conclusion, the in-house IgM indirect ELISA assay was simple to use with high sensitivity and specificity when compared to nPCR and could be an alternative to commercial kits which are expensive for developing countries.

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***Conflicts of Interest*:** None.

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