Research Article

Diagnosis of primary immunodeficiency diseases by flow cytometry in Bangladesh

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Primary Immuno Deficiency Disorders (PIDDs) are clinically and immunologically diverse and require a wide array of clinical and laboratory modalities to make specific diagnosis. Serum immunoglobulin levels and T-B-NK cell immunophenotyping are routine laboratory investigations advised to diagnose the PIDD cases in Bangladesh. Along with T-B-NK markers, use of Naïve (CD45RA+) and memory T cell (CD45RO+), switched memory B cell (CD27+IgD-) markers, detection of intracellular Bruton Tyrosine Kinase (BTK), LRBA, DOCK8 protein expression and DHR123 (Dihydro-Rhodamine 123) assay of neutrophil can increase the PIDD cases detection in Bangladesh. The study was conducted in the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU) during the time period of August, 2021 to July, 2022. Seventy clinically suspected PIDD cases were enrolled in this study on the basis of clinical findings and peripheral venous blood was collected from all patients to perform immunophenotyping. Routine T-B-NK cell, naïve and memory T cell with switched memory B cell markers were detected by flow-cytometry. Serum immunoglobulins (IgG, IgM, IgA and IgE) were estimated by nephelometry and by chemiluminescence. Intracellular BTK, LRBA and DOCK8 protein expression was detected by flow-cytometry in suspected X-Linked Agammaglobulinemia (XLA), LRBA and DOCK8 deficiency patients respectively. DHR123 assay was performed in suspected Chronic Granulomatous Disease (CGD) patients. Among the 70 clinically suspected PIDD cases, 9 (12.9%) were diagnosed as patients of PIDDs on the basis of laboratory evidence. Five (55.55%) cases were diagnosed as Predominantly Antibody Deficiency disorders (PADs), 3 (33.33%) were patients of Combined Immuno Deficiency (CID) and 1 (11.11%) was CGD patient. Among the diagnosed PIDD cases, 2 (22.22%) were diagnosed by T-B-NK cell immunophenotyping with serum immunoglobulin levels and 7 (77.77%) cases were diagnosed by additional CD45RA, CD45RO, CD27+ and IgD- markers, BTK protein expression detection and DHR123 assay. The use of additional markers (CD45RA, CD45RO, CD27 and IgD) with BTK, LRBA, DOCK8 intracellular protein expression evaluation and DHR123 assay by flow-cytometry can increase rate of specific diagnosis of the PIDD cases in Bangladeshi paediatric population.

Keywords: PIDD, CD45RA, CD45RO, CD27, IgD-, BTK, LRBA, DOCK8, DHR123 assay, flowcytometry, Bangladesh

INTRODUCTION

Primary Immuno Deficiency Diseases (PIDDs) are heterogeneous inherited group of disorders of different components of the immune system, usually resulting from genetic defects (Madkaikar, et al., 2019). PIDDs should be considered irrespective of age with recurrent or severe infections and needed specific health care supports. An updated report in 2022, the clinical and laboratory features of inborn errors of immunity, including gene defects are listed in 10 categories (Tangye, et al., 2022). Among them combined immunodeficiencies, Predominantly Antibody Deficiencies (PADs), congenital defects of phagocytes, defects in intrinsic

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and innate immunity and disease of immune dysregulation are commonly found in different countries of Asia (Chinnabhandar, et al., 2014).

The exact prevalence of PIDDs in the general population is unknown. The prevalence may vary in different ethnic groups and countries (Aghamohammadi, et al., 2014). It is generally accepted that PIDDs are under-diagnosed and under-reported and results in suffering of the patients without specific diagnosis and management. Reports from several PIDDs registries from different countries show a prevalence of 1:8500 to 1:100000 for symptomatic patients (Abolhassani, et al., 2020). Minimum PIDDs prevalence in the United Kingdom is currently 5.90 per 100,000 UK live births and PADs are the largest group of all registered patients and recent estimates obtained were 5.93/100,000 inhabitants in France and 5.6/100,000 in Australia (Shillitoe, et al., 2018, Boyle, et al., 2007 and Al-Tamemi, et al., 2012). In Kuwait, the prevalence of PIDDs was estimated at about 12/100,000 in children (Al-Herz, et al., 2008). In 2013, a study in Sri Lanka shows that the majority (60.27%) of PIDDs are PADs; among them the commonest (28.76%) was Common Variable Immune Deficiency (CVID) followed by (20.54%) of X-Linked Agammaglobulinemia (XLA) (Sliva, et al., 2013). The prevalence of CID is about 1 in 75000-100000 live births but the true incidence is unknown because many patients die before diagnosis (Deist, et al., 2017). Among the combined immunodeficiency with syndromic features categories LRBA and DOCK8 deficiency are recently discovered very rare form of PIDDs. The worldwide prevalence of LRBA and DOCK8 deficiency is still unknown though some cases are reported in Egypt and other Middle East countries where consanguineous marriage are very common (Gámez-Díaz, et al., 2018 and Biggs, et al., 2017). Chronic Granulomatous Disease (CGD) is one of the classic primary immunodeficiencies of childhood and diagnosed in most children in the first 1 to 3 years of life. Although it varies according to ethnicity, the estimated incidence of CGD is 1 in 200000 live births. According to US birth cohort of nearly 4 million infants, approximately 20 children each year are born with CGD (Wiggs, et al., 2004). Estimates of the incidence of CGD in Europe and Asia are similar, although some populations are affected more often including the Arab population of Israel, in which the incidence is estimated to be 1.5 per 100000 live births. Males are affected more commonly than females about 2:1 because of the predominant mode of genetic transmission (Rider, et al., 2018).

A study of 2016 in a tertiary care hospital of Bangladesh showed the majority (60%) of PIDDs are diagnosed as PADs, followed by combined immune deficiency (30%) and phagocytic disorders (10%). Transient hypogammaglobulinemia of infancy (33.33%) was the commonest PADs, followed by CVID, agammaglobulinemia, selective IgM deficiency and IgA deficiency 16.66% of each (Ghosh, et al., 2016).

The increasing number of PIDD cases in the past two decades in several Asian countries such as Japan, Taiwan, Hong Kong, South Korea and Iran mainly due to presence of modern diagnostic and therapeutic facilities. India and China, the two largest countries in Asia, have shown a remarkable improvement in the care of patients with PIDDs (Pilania, et al., 2020).

Initial laboratory workup of suspected PIDD cases includes Complete Blood Counts (CBC) with Differential Counts (DC) of White Blood Cell (WBC), quantitative estimation of immunoglobulin classes, post-vaccination antibody titres and complement levels. As the spectrum of PIDDs is expanding, it is often difficult to reach the specific diagnosis of the cases based on clinical or available initial laboratory findings (Locke, et al., 2014).

Flow cytometry using monoclonal antibodies to analyse and identify cells involved in immune function is very useful in the diagnosis and management of many primary immunodeficiency disorders. It is a rapid and sensitive assay that has the advantage to evaluate several characteristics of a cell type (Nicholson, 1989).

The screening test for humoral immune deficiency is the quantitative measurement of serum immunoglobulin classes, and only immunoglobulin levels cannot specify the disease. CVID is a heterogeneous disease and one of the prevalent primary B-cell disorders shows low IgG and IgA levels and variable IgM levels and usually normal B-cell numbers in most of the cases (Locke, et al., 2014). Most of the CVID patients show defective differentiation of Naïve B cells (CD27 negative) into memory B cells (CD27 positive) and can be detected by flow-cytometry (Delmonte, et al., 2019).

In congenital agammaglobulinemia there is low IgG, IgA, IgM levels and undetectable or very low B-cell numbers (<2%). Specific antibody deficiency having normal IgG, IgM, IgA levels and normal B-cell numbers but defective antibody response to vaccination (Locke, et al., 2014). XLA is a condition caused by a mutation in Bruton Tyrosine Kinase (BTK) gene. Among the various PADs specific diagnosis of XLA is difficult without intracellular BTK protein expression detection. Absent or reduced intracellular BTK protein detection in patient's blood by flow cytometry and analysis of BTK genes can make the specific diagnosis of XLA cases (Abraham, et al., 2016).

For evaluation of suspected combined T and B cell defects, total B-cells, CD4+ T-cells, CD8+ T-cells, NK cells enumeration by flow cytometry is important. Although low T cell numbers are typically observed in the majority of defects in T-cell development, this may be masked due to transplacental transfer of maternal T lymphocytes to child. Therefore, analysis of markers of cell activation should be analysed since the transferred T cells will activate and expand in the infant. Typically, maternal T cell will display a memory (CD45RO+) phenotype, whereas healthy people have predominantly Naïve CD45RA+ T cells (Locke, et al., 2014). By analysing Naïve and memory T cell population according to ESID diagnostic criteria we can even diagnose SCID and CID other than SCID patients (ESID Registry-Working definition, 2019).

LRBA (Lipopolysaccharide Responsive Beige-like Anchor) protein deficiency presented with early onset hypogammaglobulinemia, autoimmunity and inflammatory bowel disease. LRBA deficiency having early onset hypogammaglobulinemia with reduced immunoglobulins affecting at least two isotypes (Abraham, et al., 2016). DOCK8 deficiency includes T-cell lymphopenia with decreased IgM, elevated IgE, significant eosinophilia and a variable IgG

antibody response. Absent or reduced LRBA, DOCK8 specific cellular proteins detection by flow cytometry can help to diagnose LRBA deficiency and DOCK8 deficiency respectively (Abraham, et al., 2016).

CGD is an inherited mutational defect of the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase complex leading to inability of phagocytes to perform effective respiratory burst and thus diminished killing of bacteria and fungi. Diagnosis of CGD can be done by demonstrating absent or markedly reduced oxidase activity in stimulated neutrophils by flow cytometry Di Hydro Rhodamine (DHR) 123 assay (Anjani, et al., 2020).

Flow cytometry based immunophenotyping for PIDDs that are available in Bangladesh having only T-B-NK cell markers; include B cells (CD19+), T cells (CD3+), T-helper cells (CD3+/CD4+), T-cytotoxic cells (CD3+/CD8+), Natural Killer (NK) cells (CD56+/CD16+). Only using this existing T-B-NK cell immunophenotyping have limitations for specific diagnosis of different types of PIDDs in Bangladesh.

Along with the existing flow-cytometry based immunophenotyping of T-B-NK markers, this study included CD45RA, CD45RO, CD27, IgD, BTK, LRBA, DOCK8 markers and DHR123 assay in neutrophil, that might help to make specific diagnosis of PIDD patients in Bangladesh.

MATERIALS AND METHODS

A total 70 clinically suspected PIDD patients were enrolled as cases in this cross-sectional study conducted from August, 2021 to July, 2022. All the patients were included in the study following standard criteria of the Jeffrey Modell Foundation (Modell, et al., 2017). Patients were selected by the clinicians of the Department of Paediatrics, Bangabandhu Sheikh Mujib Medical University (BSMMU) and referred to the Department of Microbiology and Immunology, BSMMU for further laboratory evaluation.

PIDD patients were diagnosed according to the European Society for Immunodeficiency (ESID) diagnostic criteria and grouped as per established criteria of International Union of Immunological Societies (IUIS) (ESID Registry-Working definition, 2019 (Tangye, et al., 2022). After obtaining informed written consent, 5 ml of peripheral venous blood was collected from each patient following universal precautions. 2 ml of collected blood was transferred into sterile BD Vacutainer EDTA coated vials (Ref-367856) for flow cytometric evaluation and residual 3 ml blood was taken into sterile plain test tube for estimation of serum immunoglobulins by nephelometry and chemiluminescence. Flow-cytometry based immunophenotyping was done by using BD FACSVerse™ (Cat. no. 651155), using monoclonal antibodies (Abcam, UK and BD Biosciences, USA). BD FACSuiteTM software was used for data acquisition and analysis.

Patients with suggestive clinical history and laboratory findings to BTK, LRBA and DOCK8 deficiencies were tested after analyzing the results of immunophenotyping of lymphocyte subsets. BTK protein expression was detected in Monocytes of XLA patients with reduces level (<2SD) of serum immunoglobulin classes than the age specific normal level

(Mayo Foundation, USA), B cells were absent or markedly reduced (<2% of circulating B cells). LRBA protein expression in B cells was tested in patients presented with early onset of hypogammaglobulinemia, autoimmunity and inflammatory bowel disease. DOCK8 protein expression in T cells was tested in patients with high serum IgE levels, reduced IgM or IgG level, eczema, eosinophilia, recurrent staphylococcal and viral skin infections. Age specific reference values of BTK, LRBA and DOCK8 proteins are yet not set down by the ESID (November, 2019) guidelines. A healthy control of same age group was adopted for each case to compare the Mean Fluorescence Intensity (MFI) of BTK, LRBA and DOCK8 proteins of suspected PIDD cases with that of healthy donor (Abraham, et al., 2016). Samples of suspected PIDD cases and healthy control for each protein were analysed simultaneously in two different test tubes and the events were compared between suspected case and healthy donor in histogram. A combination of measured fluorochrome conjugated monoclonal antibodies (Abcam, UK and BD Biosciences, USA) were added to all tubes and analysed accordingly (Gámez-Díaz, et al., 2018 and Pai, et al., 2014). DHR123 assay (ab236210 Abcam, UK) was performed in patients those naïve and memory T cells, switched memory B cells findings and serum immunoglobulin levels were nonspecific to PIDDs but having features suggestive for chronic granulomatous diseases. NADPH oxidase dependent respiratory burst was measured as MFI of rodamin 123. Here, healthy controls were adopted to compare the MFI of rodamin 123 of suspected CGD cases with that of healthy controls (Abraham, et al., 2016). Serum IgG, IgM, IgA levels were determined by an automated nephelometry analyzer and IgE by chemiluminescence auto-analyzer (SIEMENS Advia Centaur XPT; Cat. no. 1392409) as per manufacturer's instruction.

Collected data were checked, edited and analysed using SPSS software package version-27 (Strata Corporation, college station, Texas). Continuous parameters were expressed as mean ± SD/mean ± SE and categorical parameters as frequency and percentage. The independent sample t test was done to compare baseline characteristics in normally distributed data. Comparison between groups was done by Mann-Whitney test (nonparametric test for skewed data). Gender distribution was compared using Chi-square test. For all test, P value <0.05 was considered as statistically significant.

RESULTS

This cross-sectional study was conducted on 70 clinically suspected PIDD cases, among them 9 (12.9%) were diagnosed as PIDDs based on the laboratory test results evidence (Figure 1).

Table 1 shows, out of 9 confirmed PIDD cases, 5 (55.55%) patients were diagnosed as Predominantly Antibody Deficiency (PADs), 3 (33.33%) combined immunodeficiencies and 1 (11.11%) patient had phagocyte defect of neutrophils CGD. Among the 5 PADs cases common variable immunodeficiency and agammaglobulinemia consist of 2 (22.22%) cases in each clinical type. One (11.11%) patient was diagnosed as Selective IgM Deficiency (SIgMD). Among the 3 patients of combined immunodeficiency, 1 (11.11%) was diagnosed as Severe Combined Immunodeficiency (SCID) and the other 2 (22.22%) were patients of Combined Immune Deficiency (CID) other
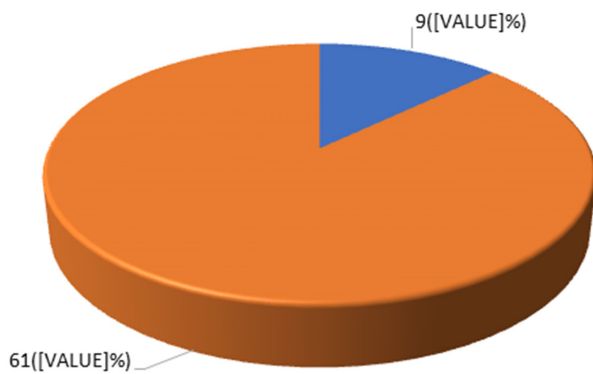
than SCID (Table 1).

Figure 1. Diagnosed primary immunodeficiency cases in suspected study population (n=70). **Note:** (■): PID; (■): Non-PID.

Table 1. Distribution of definable PIDD cases according to disease category with subtypes (n=9).

PIDD types	Number	Percentage
Combined immunodeficiencies	3	33.33%
Severe Combined Immunodeficiencies (SCID)	1	11.11%
Combined immunodeficiencies other than SCID	2	22.22%
Predominantly antibody deficiency	5	55.55%
X-Linked Agammaglobulinemia (X LA)	2	22.22%
Common Variable Immunodeficiency (CVID)	2	22.22%
Selective IgM deficiency	1	11.11%
Congenital defects of phagocytes	1	11.11%
Chronic Granulomatous Disease (CGD)	1	11.11%

Table 2 shows the different laboratory results that were performed for the diagnosis of PIDDs in suspected population. One patient of SCID (11.11%) showed very low absolute count of T (CD3+), B (CD19+) and NK (CD56+) cells with low count of T cell subsets (CD4 and CD8). Decreased percentage of Naïve CD8 cells (CD8+CD45RA+) along with decreased count of T (CD3+), B (CD19+) and NK (CD56+) cells in two patients of CID other than SCID. Two patients of XLA showed low B lymphocyte count (<2% of circulating B cells) with low switched memory B cell (CD27+IgD-) and markedly reduced (<2SD) serum IgG, IgM and IgA levels but normal distribution of T and NK cell, they also exhibited markedly reduced expression of intracellular BTK protein. Two CVID patients found to have low (<70% of age-related normal value) switched memory B cells (CD27+IgD-) with normal distribution of B-lymphocytes and markedly reduced (<2SD)

serum IgG and IgA levels with normal IgM levels. One patient of SIgMD (11.11%) was found to have IgM levels below the age specific normal range with normal IgG and IgA levels. One patient of CGD showed markedly reduced MFI of oxidative activity of neutrophil in DHR123 assay. LRBA and DOCK8 protein deficiency cases were not found (Table 2).

Characteristics of lymphocyte subsets in patients with combined immunodeficiency shows in the Table 3. One patient of SCID (11 months old) had low CD3+ T lymphocytes (<300 cells/μl) and its subsets (CD4 and CD8 cell). Naïve T cells (CD45RA) were decreased and memory T cells (CD45RO+) were increased. CD19+ B lymphocytes (8 cells/μl) and CD56+ NK cells (21 cell/μl) count were very low. Age of the two cases of CID other than SCID were 13 and 36 months and the age specific CD+3 T cells count were decreased in both cases (528 cells/μl and 1152 cells/μl respectively) along with decreased CD4 cell or CD8 cell or both. Reduced naïve (CD45RA) CD4 and/or CD8 T cells and increased memory (CD45RO) CD4 and/or CD8 T cells were found in both 2 cases (Table 3).

Among the 5 predominantly antibody deficiency patients, circulating B-lymphocytes were found <2% in the 2 patients (42 and 72 months old) of agammaglobulinemia. In the two CVID cases, with age of 84 and 108 months, both had very low (<70% of age-related normal value that were 0.1% and 0.24% respectively) switched memory B (CD27+IgD-) cells but normal B cell count. In one SIgMD patient of 10 months old, absolute B cell count was below the age specific normal range (Table 4).

The bar diagram (Figure 2) shows the distribution of switched memory B cells in CVID, other PAD and non-PIDD cases. The mean percentage of switched memory B cells is 0.15% in AG and 0.17% in CVID cases; both values are lower than SIgMD and non-PIDD cases. The Mean Fluorescence Intensity (MFI) of BTK protein expression in agammaglobulinemia patients is markedly (Figure 3) reduced (395.5) in comparison with healthy controls (1133.5) (Figures 2 and 3).

The bar diagram of Figure 4 shows the MFI of LRBA protein expression in suspected LRBA deficiency patients is 19080 with no variations in healthy controls (19367.8). The bar diagram in Figure 5 shows the MFI of DOCK8 protein expression in suspected DOCK8 deficiency patients is 2040.8 with no significant difference in healthy controls (2055.6) and none of the suspected patients were diagnosed to be DOCK8 deficient cases.

In Figure 6, the bar diagram shows MFI of oxidative activity in stimulated neutrophil is markedly reduced (55) in the diagnosed CGD patient and 21517.7 in non-PIDD patients (Figures 4-6).

Table 5 shows the demographic characteristics in suspected PIDD cases. Median ages of the patients ranged from 10 to 108 months. Median age of confirmed PIDD cases was 42 months and non-PIDD cases were 24 months. Median age of onset of symptoms was 14 months in confirmed PIDD cases and 12 months in non-PIDD cases. The male to female ratio was 1.25:1 in the confirmed PIDD cases (Table 5).

Table 2. Laboratory evaluations in the diagnosis of PIDDs among study population (n=70).

Variables	Diagnosed PIDD Cases (n=9) among study population (n=70)					
	SCID	CID	AG	CVID	SIGD	CGD
	(n=1)	(n=2)	(n=2)	(n=2)	(n=1)	(n=1)
TLC and DC of leukocytes						
Total leukocytes count (cells/μl)	1500	6240.0 ± 1357.7	7525.0 ± 2085.9	9800.0 ± 2404.2	17020	9062
Neutrophil (%)	58	79.95 ± 0.07	54.50 ± 4.95	67.50 ± 10.61	65	53.9
Lymphocytes (%)	27	14.75 ± 2.47	40.40 ± 0.57	27.50 ± 7.78	28	40.6
Eosinophil (%)	5	1.00 ± 1.41	2.00 ± 1.41	1.00 ± 1.41	3	2.9
Serum immunoglobulins						
Serum IgM (gm/l)	0.31	0.65 ± 0.69	0.17 ± 0.00	0.99 ± 0.73	0.26	1.5
Serum IgG (gm/l)	8.23	2.79 ± 0.54	2.15 ± 0.37	2.15 ± 1.15	9.2	11.81
Serum IgA (gm/l)	0.76	0.33 ± 0.10	0.26 ± 0.00	0.30 ± 0.04	1.2	1.74
Serum IgE (IU/L)	15	52.20 ± 52.47	43.50 ± 40.31	41.20 ± 37.05	5.13	88.1
Lymphocyte subset analysis						
Lymphocytes (%)	17.3	13.00 ± 4.24	41.65 ± 1.20	38.50 ± 13.44	32	40
Lymphocytes abs. count (cells/ μl)	260	840.00 ± 441.23	3217.5 ± 642.8	3611.50 ± 391.03	5446	3625
CD3+T cell (%)	79.6	80.63 ± 9.43	94.60 ± 3.39	80.30 ± 3.25	85.7	69.8
CD3+T cell absolute count (cells/μl)	207	698.50 ± 434.87	3032.50 ± 498.51	2906.00 ± 431.34	4668	2530
CD3+CD4+T cell (%)	24.2	36.89 ± 0.30	51.25 ± 11.67	49.90 ± 6.22	46.3	46.4
CD3+CD4+abs. count (cells/μl)	63	310.50 ± 164.76	1686.5 ± 704.9	1814.00 ± 420.02	2522	1682
CD3+CD8+ (%)	52.8	43.85 ± 8.27	42.55 ± 14.78	27.80 ± 3.82	37	21.5
CD3+CD8+abs. count (cells/μl)	137	387.00 ± 263.04	1321.0 ± 202.2	996.50 ± 28.99	2015	779
CD4+CD8+ratio	0.46	0.86 ± 0.16	1.33 ± 0.74	1.83 ± 0.47	1.25	2.16
CD19+B cell (%)	3	9.12 ± 10.78	0.10 ± 0.14	16.05 ± 1.34	2	22.8
CD19+B cell abs. count (cells/ μl)	8	52.50 ± 50.20	3.00 ± 4.24	577.00 ± 14.14	109	826
CD56+NK cell (%)	8	7.83 ± 1.37	3.65 ± 2.33	2.70 ± 1.70	8.3	3.4
CD56+NK cell abs. count (cells/μl)	21	68.50 ± 45.96	125.00 ± 98.99	94.00 ± 50.91	452	123
CD4+CD45RA+ (%)	42	56.50 ± 4.95	53.50 ± 9.19	69.00 ± 12.73	70	80
CD4+CD45RO+ (%)	58	43.50 ± 4.95	46.50 ± 9.19	31.00 ± 12.73	30	20
CD8+CD45RA+ (%)	40	28.50 ± 2.12	55.00 ± 16.97	78.50 ± 2.12	82	78
CD8+CD45RO+ (%)	60	71.50 ± 2.12	45.00 ± 16.97	21.50 ± 2.12	18	22
CD19+CD27+lgD- (%)	0.6	0.90 ± 0.00	0.08 ± 0.11	0.17 ± 0.10	0.5	1.54
BTK protein expression (MFI)	-	-	395.5 ± 41.7	-	-	-
LRBA protein expression (MFI)	-	-	-	-	-	-
*Deficiency not detected	-	-	-	-	-	-
DOCK8 protein expression (MFI)	-	-	-	-	-	-
*Deficiency not detected	-	-	-	-	-	-
DHR123 assay (MFI)	-	-	-	-	-	55

Note: *As the MFI of LRBA and DOCK8 protein expression are apparently same in both healthy control and suspected patients of LRBA and DOCK8 deficiency, so no LRBA and DOCK8 deficiency case is diagnosed. TLC: Total Leukocyte Count; DC: Differential Count; MFI: Mean fluorescence index; Abs: Absolute.

Table 3. Lymphocyte subset characteristics in the PIDD cases with combined immunodeficiencies (n=3).

Variables	SCID	CID other than SCID (n=2)	
	(n=1)	Patient-1	Patient-2
Age in months	11	13	36
Lymphocyte			
Absolute count (cells/µl)	260	528	1152
(Ref. values)	(3320-7006)	(3873-6141)	(2340-5028)
T cell (CD3)			
Absolute count (cells/µl)	207	391	1006
(Ref. values)	(2284-4776)	(2542-4933)	(1578-3707)
CD4 T cell			
Absolute count (cells/µl)	63	194	427
(Ref. values)	(1523-3472)	(1573-2949)	(870-2144)
CD8 T cell			
Absolute count (cells/µl)	137	201	573
(Ref. values)	(524-1583)	(656-1432)	(472-1107)
CD45RA			
%CD4 (Ref. values)	42 (58-91)	60 (58-91)	53 (47-87)
%CD8 (Ref. values)	40 (47-87)	30 (47-87)	27 (42-81)
CD45RO			
%CD4 (Ref. Values)	58 (05-18)	40 (05-18)	47 (09-26)
%CD8 (Ref. Values)	60 (01-08)	70 (01-08)	73 (04-06)
B cell (CD19)			
Absolute count (cells/µl)	8	88	17
(Ref. values)	(776-2238)	(733-1338)	(434-1274)
CD56			
Absolute count (cells/µl)	21	36	101
(Ref. values)	(203-801)	(186-724)	(155-565)

Note: SCID: Severe Combined Immunodeficiency; CID: Combined Immunodeficiency; Ref.: Reference. Reference values of lymphocyte subsets (Tosato et al., 2015). The absolute count of particular lymphocyte subset was calculated by multiplying the percentages of lymphocyte subset with the total lymphocyte count.

Table 4. Lymphocyte subset characteristics in the PIDD cases with predominantly antibody deficiency (n=5).

Variables	AG (n=2)		CVID (n=2)		SIgMD
	Patient-1	Patient-2	Patient-1	Patient -2	(n=1)
Age in month	42	72	84	108	10
Lymphocyte					
Abs. count (cells/µl)	3672	2763	3888	3335	5446
(Ref. values)	(2340-5028)	(1662-3248)	(1662-3448)	(1662-3448)	(3320-7006)
T cell (CD3)					
Abs. count (cells/µl)	3385	2680	3211	2601	4668
(Ref. values)	(1578-3707)	(1239-2611)	(1239-2611)	(1239-2611)	(2284-4776)
CD4 T cell					
Abs. count (cells/µl)	2185	1188	2111	1517	2522
(Ref. values)	(870-2144)	(646-1515)	(646-1515)	(646-1515)	(1523-3472)
CD8 T cell					
Abs. count (cells/µl)	1178	1464	976	1017	2015
(Ref. Values)	(472-1107)	(365-945)	(365-945)	(365-945)	(524-1583)
B cell (CD19)					
Abs. count (cells/µl)	0	6	587	567	109
(Ref. values)	(434-1274)	(276-640)	(276-640)	(276-640)	(776-2238)
(%)	0	0.2	15.1	17	2
(Ref. values)	(12.9-29.2)	(17-37.2)	(17-37.2)	(17-37.2)	(12.9-29.2)
CD27+IgD- (%BL)	0	0.15	0.1	0.24	0.5
(Ref. values)	(2.7-12.5)	(2.9-17.4)	(2.9-17.4)	(2.9-17.4)	(0.6-3.7)
CD56					
Abs. count (cells/µl)	195	55	58	130	452
(Ref. values)	(155-565)	(120-483)	(120-483)	(120-483)	(203-801)

Note: AG: Agammaglobulinemia; CVID: Common Variable Immunodeficiency; SIgMD: Selective IgM Deficiency; Abs. count: Absolute count; BL: B Lymphocyte; Ref.: Reference. Reference values of lymphocyte subsets (Tosato, et al., 2015).
The absolute count of particular lymphocyte subset was calculated by multiplying the percentages of lymphocyte subset with the total lymphocyte count.
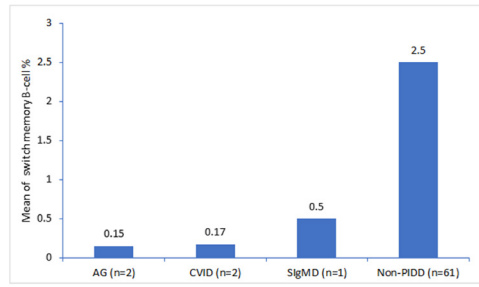
Figure 2. Distribution of switched memory B-cell percentage in CVID, other predominantly antibody deficiency PIDD and non PIDD cases.
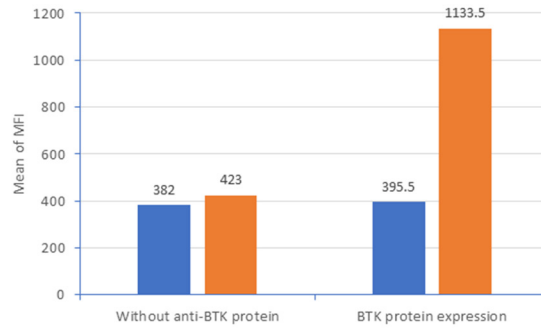

Figure 3. Distribution of intracellular BTK protein expression among suspected Agammaglobulinemia (AG) patients. **Note:** (■): AG (n=2); (■): Healthy control (n=2).
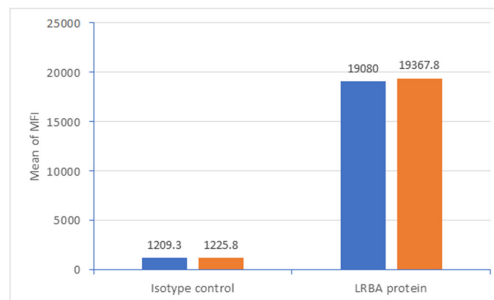

Figure 4. Distribution of intracellular LRBA protein expression among suspected LRBA deficiency patients. **Note:** (■): LRBA (n=4); (■): Healthy control (n=4).
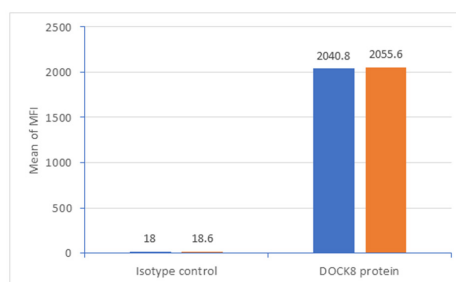
Figure 5. Distribution of intracellular DOCK8 protein expression among suspected DOCK8 deficiency patients. (■): LRBA (n=4); (■): Healthy control (n=4).
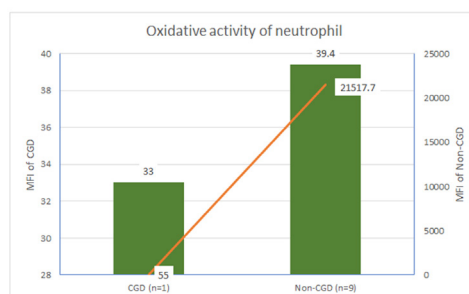

Figure 6. Estimation of oxidative activity of neutrophil among suspected CGD patients (n=10). **Note:** (—): Unstimulated; (—): Stimulated.

Table 5. Demographic characteristics of the study population in suspected PIDD patients (n=70).

Variables	PIDD (n=9)	Non-PIDD (n=61)	p-value
Gender*			
Male	5 (55.6%)	38 (62.3%)	0.698
Female	4 (44.4%)	23 (37.8%)	-
Age (months)**			
Mean ± SD	52.7 ± 38.7	40.36 ± 39.2	0.247
Median	42	24	-
Range	(10-108)	(2-132)	-
Age of onset (months)**			
Mean ± SD	17.22 ± 17.44	23.77 ± 24.73	0.667
Median	14	12	-
Range	(1-60)	(1-84)	-

Note: P-value reached by *:Chi-square test; **:Mann-Whitney test; significant:s

DISCUSSION

In this study, peripheral venous blood was collected from 70 clinically suspected PIDD cases referred by the paediatricians. Among them confirmed PIDD cases were 12.9% (9 out of 70), this finding is nearly similar to the previous study in Sri Lanka where they reported 7.75% (73 out of 942) PIDD cases (Silva, et al., 2013). In Bangladesh, in a study found 20% confirmed PIDD cases among 50 clinically suspected cases (Ghosh, et al., 2016). In a study conducted in Bangladesh reported 24% confirmed PIDD cases (Sazzad, et al., 2014). Another study in India, found 23% laboratory confirmed PIDD cases 120 out of 528 (Gupta, et al., 2012). In an Egyptian study reported a higher rate 45% of PIDDs in 204 clinically suspected patients who presented with recurrent, severe or unusual infections (Reda, et al., 2013). All these findings are higher number compare to that of our study. The similarities and dissimilarities of findings in various studies may vary according to the size of the study population, geographical location, genetic status and availability of the diagnostic facilities (Aghamohammadi, et al., 2014).

In this study among definable PIDD cases, the majority (55.55%) had predominantly antibody disorders. Nearly similar finding (60.27%) was reported by a study done in Sri Lanka (Silva, et al., 2013). In Bangladesh, in a study found that antibody disorders comprise the most common of the PIDDs representing 60% of total PIDD cases (Ghosh, et al., 2016). In a study in Mexico, 65.37% predominantly antibody deficiency was reported (Guaní-Guerra, et al., 2013). The most frequent type of PIDD was antibody deficiency (71%) in Australia (Baumgart, et al., 1997). Global statistics containing reports from major databases, including ESID (European Society for Immunodeficiencies), LASID (Latin American Society for Primary Immunodeficiency Diseases), USIDnet (US Immunodeficiency Network) as well as selected reported registries from Asia and Australia, predominantly antibody deficiencies are the most common PIDDs (Rezaei, et al., 2017).

Among predominantly antibody deficiency, CVID (22.22%) and agammaglobulinemia (22.22%) were the most common antibody deficiency disorders in this study. This result is consistent with a study in Sri Lanka where they reported CVID as the commonest (28.76%), followed by XLA that is 20.54% (Silva, et al., 2013). CVID was reported as the commonest antibody deficiency disorder in Europe representing 21.01% of PIDD cases (Gathmann, et al., 2009). Selective IgM deficiency (11.11%) was diagnosed based on age specific serum Immunoglobulin levels in this study. In Bangladesh, in a study reported 7.14% cases of SIgMD among PIDD cases which finding is lower than that of the present study (Sazzad, et al., 2014).

In the present study, 3 (33.33%) patients of combined immune deficiency were diagnosed. Similar findings (33.33%) were found in a study conducted in India (Verma, et al., 2008).

In Bangladesh, 30% patients of combined immunodeficiency found in a study (Ghosh, et al., 2016). In Egypt, 28.25% patients of combined T and B cell immunodeficiencies reported in a study (Reda, et al., 2013). In the present study among the combined immunodeficiency diseases, 1 (11.11%) patient was diagnosed as SCID and 2 (22.22%) were diagnosed as CID other than SCID. Almost similar findings of SCID patients were reported in India 14.81% and in Sri Lanka 13.69% (Verma, et al., 2008 and Silva, et al., 2013). As molecular and genetic diagnostic tests related to PIDDs were not conducted in this study, the incidence of specific subtype or variant of SCID (X-linked or autosomal) could not be detected.

In this study, congenital defect of phagocytes was diagnosed in 1 (11.11%) patient among total 9 confirmed PIDD cases. Nearly similar study finding (10%) was reported in Bangladesh (Ghosh, et al., 2016). In Sri Lanka, a study reported that 8.2% patients had phagocytic disorders among the PIDD cases (Silva, et al., 2013). In a study in Mexico, congenital defects of phagocytes number and/or function were detected in 7.69% among total PIDD cases (Guaní-Guerra, et al., 2013).

In addition to T-B-NK cell markers, some additional markers were used in this study, that included CD45RA to measure naive cells, CD45RO to detect memory cells (CD4 and CD8) and CD27+IgD- to detect Switched memory B cells. Typically, maternal T cells display a memory (CD45RO+) phenotype, whereas a healthy child should have predominantly naïve CD45RA+ T cells (Locke, et al., 2014).

In this study, 1 diagnosed SCID patient had low CD3+ T lymphocytes (<300 cells/μl) and its subsets (both CD4 and CD8

cells). Naïve cells (CD45RA) were decreased and memory cells (CD45RO) were increased. Absolute count of B lymphocytes (CD19+) and NK cells (CD56+) were very low which was similar with the ESID working definition for diagnosis.

In present study, 2 CID other than SCID patients, age specific CD+3 T cells count were decreased in both cases along with decreased CD4 cell or CD8 cell or both. CID other than SCID cases were diagnosed by using CD45RA for naive cell and CD45RO markers for memory cell in CD4 and CD8 T lymphocytes where CD45RA was decreased and CD45RO was increased, cell sorting similar markers are included in the ESID working definition for the diagnosis. Specific disease of CID group can be confirmed by genetic analysis, but it was not done in this present study. It could guide the clinician in identifying the actual defects of the immune system.

In this present study, 2 CVID patients were diagnosed based on low switched memory B cell (CD27+IgD-) with the normal distribution of B lymphocytes and markedly reduced serum IgG and IgA levels with normal IgM level which was similar with the ESID working definition for diagnosis. Similar procedures were adopted in a study in USA and in New Zealand (Locke, et al., 2014 and Ameratunga, et al., 2014). Seroconversion following immunization was also done with correlation of immunoglobulin levels and clinical findings to diagnose CVID in a study in Sri Lanka (Silva, et al., 2013). In this present cross-sectional study, seroconversion following immunization to evaluate the immunoglobulin titre for the diagnosis of CVID was not done.

In this present study, XLA were diagnosed based on low B lymphocyte count with low Switched memory B cell and markedly reduced serum IgG, IgM and IgA levels but normal distribution of T and NK cell. Then additional detection of markedly reduced intracellular BTK protein expression by flowcytometry was done for the confirm diagnosis of XLA. Similar markers along with BTK protein expression detection were described in a review article and a study in Japan for the diagnosis of XLA (Abraham, et al., 2016 and Kanegane, et al., 2018).

In this present study, SIgMD was diagnosed based on serum IgM level below the age specific normal range with normal IgG and IgA level. Similar procedure was done in a study in India and that is similar with the ESID working definition for diagnosis (Gupta, et al., 2017).

In this present study, 1 CGD patient was diagnosed by DHR123 assay where the oxidative activity of neutrophil was measured in MFI by flow cytometry in stimulated neutrophils which found markedly reduced in comparison to a healthy control. Similar diagnostic procedure was described in a review article (Abraham, et al., 2016). Nitro Blue Tetrazolium (NBT) test was done to diagnose defects of phagocytes in a study in Sri Lanka and DHR123 assay is more reliable and sensitive than NBT test recommended in a study (Silva, et al., 2013 and Anjani, et al., 2020). Among the phagocyte disorders CGD is the most common. In an Egyptian study reported that defects of individual components of NADPH oxidase system can also be diagnosed by using specific monoclonal antibody against specific components by flow cytometry (Hawary, et al., 2016).

Genetical analysis or sequencing can also be done to diagnose defect of the specific individual components of NADPH oxidase system reported in a study which was not done in the present study (Anjani, et al., 2020).

In this present study, the MFI of LRBA and DOCK8 protein expression in suspected LRBA and DOCK8 deficiency patients were as normal as the healthy control. None of the suspected patients were diagnosed to have LRBA and DOCK8 deficiency in this study as LRBA and DOCK8 deficiency are very rare and the sample size was small.

The male to female ratio was 1.25:1 in the confirmed PIDD cases in this study. Since the pattern of inheritance of some primary immunodeficiency diseases is gender related, the overall incidence of PIDDs was reported 1.4 to 2.3 times more in males than females in other studies (Gathmann, et al., 2009).

The present study showed that the predominantly antibody deficiency (55.55%) patients were in around the 6 to 9 years age group and one patient of SCID was less than 12 months of age. These findings are consistent with the study done in Sri Lanka that showed predominantly antibody deficiency disorders were predominant in 5 to <12 years age group and all SCID patients were in less than 12 months of age (Silva, et al., 2013). SCID classically present in the first few months of life (Cole, et al., 2010).

The result of the study showed that primary immunodeficiency diseases are not uncommon in our country. Children presenting with repeated infections due to usual and unusual organisms, poor response to conventional treatment and positive family history should be evaluated for PIDDs. Flow cytometry is an essential tool for the evaluation and early diagnosis of PIDD cases. While genetic or molecular analysis can detect specific diagnosis but costly and all the tests are not available always in developing countries like Bangladesh, flow cytometry plays an important role in the diagnosis of suspected patients having PIDD.

The use of additional markers (CD45RA, CD45RO, CD27 and IgD) with BTK, LRBA, DOCK8 intracellular protein expression evaluation and DHR123 assay by flow cytometry can increase the detection of PIDD cases in Bangladeshi paediatric population.

CONCLUSION

The present study results showed that predominantly antibody deficiency disorders were the most common PIDDs in the paediatric age group. CD45RA and CD45RO were used as useful markers for the detection of naïve and memory T cell subsets in the diagnosis of CID. Switched memory B cell detection by CD27 and IgD were found to be essential for the diagnosis of CVID. Intracellular BTK protein detection and DHR123 assay was found useful for the diagnosis of XLA and CGD respectively. Flow cytometry provided a useful tool for the diagnosis of PIDDs.

DATA AVAILABILITY

The clinical and laboratory data used to support the findings of this study are available from the corresponding author

(avizitk63.dmc@gmail.com) upon request.

ETHICAL APPROVAL

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Avizit Sarker, Chandan Kumar Roy, Mohammad Imnul Islam and Ismet Niger -Concept and design of study, acquisition of data, analysis of data, writing of manuscript.

Nusrat Akhter Juyee, S. M. Ali Ahmed and Tripty Chakrobortty,

Md. Eunus Ali-Acquisition of data, including immunoassays.

Susmita Karmakar Soma-Analysis of data, writing of manuscript.

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