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Original Article

Pathology Section

Evaluation of Human Leukocyte Antigen-B27 Expression in Patients with Spondylopathy/ Spondylitis by Flowcytometry

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ABSTRACT

Introduction: The role of immune mediated injury in pathogenesis of Ankylosing Spondylitis (AS) is well established. HLA B27, a Major Histocompatibility Complex (MHC) class I molecule is one of the major genetic risk factors associated with the disease. Various techniques are used for testing HLA-B27 which includes Polymerase Chain Reaction (PCR) based tests, Enzyme Linked Immunosorbent Assay (ELISA) and flowcytometry. Flowcytometry has gained popularity due to ease of procedure, shortened turnaround time and cost-effectiveness.

Aim: To assess the sensitivity and specificity of flowcytometry for HLA-B27 detection, taking PCR assay as the gold standard along with its association with demographic, clinicopathological and radiological parameters.

Materials and Methods: This was a prospective study conducted in Department at Pathology of Shri Guru Ram Rai Institute of Medical and Health Sciences, Dehradun, India, for a period of 18 months from January 2020 to June 2021. The study included 51 patients for which HLA-B27 typing was done cases by flowcytometry and Sequence Specific Allele (SSA) PCR/Real time PCR on peripheral blood samples. The association of HLA-B27 with clinical features {Inflammatory Back Pain (IBP), arthritis, psoriasis, uveitis, dactylitis, Inflammatory Bowel Disease (IBD), cervicitis, urethritis, diarrhoea) along with MRI findings (sacroilitis)}, laboratory findings {C-reactive protein and Erythrocyte Sedimentation Rate (ESR)} was evaluated. The performance analysis parameters of flowcytometry were evaluated both by excluding and including the cases in grey zone taking PCR as gold standard. Statistical testing was conducted with SPSS 20.0. Chi-square test or Fisher's-exact test were used and a p-value of less than 0.05 was taken as significant.

Results: A significant association of HLA-B27 was seen only with IBP (p-value=0.001) and sacroilitis (p-value=0.03). Of the 22 (43.1%) patients positive for HLA-B27 by PCR, 18 (81.8%) patients were positive while the remaining 4 (18.1%) were in grey zone by flowcytometry. Of the 29 (56.9%) patients testing negative by PCR, 27 (93.1%) patients were negative, 1 (3.4%) was in grey zone and 1 (3.4%- false positive) tested positive for HLA-B27 by flowcytometry. Sensitivity and specificity of flowcytometry for detection of HLA-B27 was found to be 100% and 96.4%, respectively when grey zone cases were excluded.

Conclusion: The study brings to light that flowcytometry is a fairly specific and sensitive method for HLA-B27 detection with a high Negative Predictive Value (NPV) (100%) and Positive Predictive Value (PPV) (94.7%). In the Coronavirus Disease-2019 (COVID-19) era, it reiterates the importance of flowcytometry for HLA-B27 especially when PCR is overburdened.

Keywords: Diagnostic technique, Grey zone, Immune mediated, Polymerase chain reaction

INTRODUCTION

The aetiopathogenesis of AS is poorly understood but role of immune mediated injury is well established [1,2]. Genetic risk factors contribute about 80-90% to susceptibility to AS. One of the major genetic risk factor is Human Leukocyte Antigen (HLA) B27, a MHC class I molecule. HLA-B27 is found in less than 8% of the general population while 90% of patients with AS express this antigen [3]. There are 105 subtypes of B27 coded by 132 alleles. Not all alleles are associated with AS, the association strength varying with ethnicity [2].

The HLA-B27 status can neither exclude nor confirm the diagnosis of AS on its own. However, in a clinically suspicious patient with radiological sacroilitis, HLA-B27 positivity is highly suggestive of AS and hence is an important diagnostic criteria as per Assessment of Spondylo Arthritis International Society (ASAS) [3].

Various techniques are used for testing HLA-B27 which include PCR based tests, Microlymphocytotoxicity Test (MLCT), ELISA, flowcytometry and Next-Generation Sequencing (NGS). Flowcytometry has gained popularity due to ease of procedure, shortened turnaround time and cost-effectiveness as compared to established PCR based tests [4].

The objectives of the present study were to assess the sensitivity and specificity of flowcytometry for HLA-B27 detection, taking PCR assay as the gold standard along with association of HLA-B27 expression with demographic, clinicopathological and radiological parameters.

MATERIALS AND METHODS

It was a prospective study conducted in the Department of Pathology and Central Molecular Research Laboratory at Shri Guru Ram Rai Institute of Medical and Health Sciences, Dehradun, Uttarakhand, India for a period of 18 months from January 2020 to June 2021. It was approved by the Institutional Ethics Committee vide IEC no. SGRR/IEC/22/19.

Inclusion criteria: All patients referred from Surgery, Neurosurgery and Orthopaedics OPD who were clinically suspected of having Spondyloarthropathy (SpA) in the above period, were included in the study.

Exclusion criteria: Patients positive for rheumatoid factor were excluded from the study.

Study Procedure

A detailed clinical history of the patient was obtained and relevant information was entered on the predesigned proforma (Inflammatory Back Pain (IBP), arthritis, psoriasis, uveitis, dactylitis, IBD, cervicitis, urethritis, diarrhoea, sacroilitis, MRI findings along with ESR and CRP values). After obtaining an informed consent blood was collected by venepuncture observing asepsis.

The samples were subjected to PCR followed by flowcytometry in order to evaluate the sensitivity and specificity of flowcytometry. Additionally, the association of HLA-B27 expression with the above demographic, clinicopathological and radiological parameters was also assessed.

Sample collection: A 2.5 mL of EDTA whole blood was used for PCR and flowcytometry for detection of HLA-B27. PCR and flowcytometry were done within 24 and 48 hours of collection of sample respectively. All the samples were <48-hour-old in the present study.

All patients, who were clinically suspected of having SpA in the above period (18 months), were included in the study. Thus, a total of 51 cases were included in the study comprising approximately equal numbers of both HLA-B27 positive and negative cases (depending on PCR results).

HLA-B27 by PCR: SSA PCR and Real time (RT) PCR were the molecular methods used for detection of HLA-B27. A total of 28 patients were tested by sequence specific PCR (SSA) and 23 patients were tested by RT-PCR.

DNA extraction and amplification: PCR was carried out using the standard methods and was same for both the methods. Manual extraction of DNA was done followed by amplification using PCR master mix comprising reaction buffer, deoxynucleotide triphosphates, $MgCl_2$, primers, Taq DNA polymerase and nuclease free water. The amplified products were separated by agarose gel electrophoresis and visualised by staining with ethidium bromide.

Interpretation of SSA PCR: Appearance of 145 base pair (bp) specific band indicated the presence of HLA-B27 gene.

Human growth hormone, seen at 430 bp was the internal control [5,6].

Interpretation of Real time PCR: Cycle threshold (CT) value of 35 was taken as the cut-off. Samples with a CT value of 35 or more were considered as negative for HLA-B27. Samples with CT values of less than 35 were reported as positive for HLA-B27 [5,6].

HLA-B27 by flowcytometry: The blood samples were stained with Fluorescein Isothiocyanate (FITC) labelled Anti HLA-B27 clone GS 145.2 (IgG1 Kappa) and phycoerythrin (PE) labelled CD3 clone SK 7(IgG1 Kappa). Processing was done by stain-lyse-wash protocol [7].

The sample was acquired in BD FACS Canto II 8 colour flowcytometer and analysed using FACS Canto II software. Atleast 10,000 lymphocytes were selected for analysis through forward scatter/side scatter gating technique.

Sample analysis: T lymphocytes were gated in dot plots of CD3 PE versus side scatter. The T-lymphocyte population was displayed in a FITC/FL1 histogram, where Log Median Fluorescence (LMF) was calculated. Samples with LMF greater than equal to 157 (that is 10 units greater than the decision marker 147 mentioned on the reagent vial for HLA-B27 FITC/CD3 PE) were taken to be HLA-B27 positive, while samples with LMF less than the marker i.e., 147 were considered HLA-B27 negative. The grey zone was defined as 147-157 and was the 'Manufacturer Defined Equivocal Range' (MDER) [7].

STATISTICAL ANALYSIS

Statistical testing was conducted with SPSS 20.0. Continuous variables were presented as mean±SD while categorical variables were expressed as frequencies and percentages. Categorical data between the groups was compared using Chi-square test or Fisher's-exact test, as appropriate. The comparison of normally distributed continuous variables between the groups was performed using student's t-test and ANOVA applied for more than two groups/ categories comparison.

The sensitivity and specificity of HLA-B27 evaluation by flowcytometry was also done.

Sensitivity=True positive/(True positive+False negative)

Specificity=True negative/(True negative+False positive)

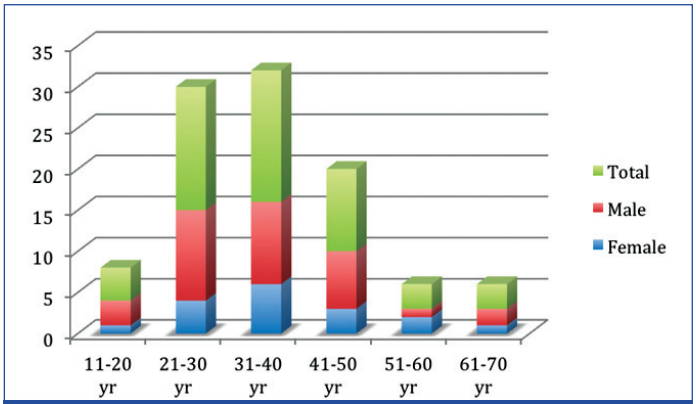
PPV=True positive/(True positive+False positive)

NPV=True negative/(True negative+False negative)

For all statistical tests, a p-value less than 0.05 were taken to be indicative of significant association.

RESULTS

Majority (16 cases-31.3%) of the patients were in the age group 31-40 years of age with a male to female ratio of 5.3:1 among the HLA-B27 positive cases [Table/Fig-1]. Forty cases (78.4 %) included in this study were less than 45 years of age, irrespective of their HLA B27 status.



[Table/Fig-1]: The age and gender distribution of the study population (n=51).

The association of HLA-B27 by flowcytometry was evaluated with clinicopathological parameters [Table/Fig-2] and a significant association (p-value=0.001) was seen only with IBP and with sacroilitis (p-value=0.03).

A total of 28 patients were tested by sequence specific PCR (SSA) and 23 patients were tested by RT-PCR. Of the 22 (43.1%) patients positive for HLA-B27 by PCR, 18 (81.8%) patients were positive, while the remaining 4 (18.1%) were in grey zone by flowcytometry [Table/Fig-3-5]. Of the 29 (56.9%) patients tested negative by PCR, 27 (93.1%) patients were negative, one (3.4%) was in grey zone and one (3.4%- false positive) tested positive for HLA-B27 by flowcytometry [Table/Fig-3-5].

The sensitivity, specificity, PPV and NPV of flowcytometry was calculated both by excluding and including the cases in the grey zone (LMF=147-157) [Table/Fig-6], taking PCR as gold standard. A higher sensitivity (100%) was observed when the grey zone cases were excluded (sensitivity of 81.8% if grey zone cases included) while specificity (96.4% vs 96.5%) was almost equivalent in both the methods. A high NPV (100%) and PPV (94.7%) were observed when grey zone cases were excluded.

DISCUSSION

In the present study, a total of 51 patients with clinical suspicion of SpA were selected and tested for the presence of HLA-B27 antigen by both PCR technique and flowcytometry. Skare TL et al., from Brazil evaluated 1424 SpA patients and noted that the mean age at disease onset was 28.56±12.34 years with 81.8% being affected before the age of 40 and another 7.5% patients between the ages of 41-45 years [8].

In the present study, MDER or grey zone was taken as 147-157 i.e., 10 channels up from the suffix mentioned on the vial (147) [7]. The authors encountered 5 (9.8%) patients in grey zone, of which 4 patients were positive for HLA-B27 and one patient was negative for HLA-B27 by PCR.

The IBP is the most common and foremost symptom of axial SpA. The severity and duration of IBP mirrors the extent of inflammation of sacroiliac joints, spine and spinal entheses [9]. In 2009, ASAS developed a new classification criteria for IBP which included: 1) Insidious onset; 2) Pain at night (with improvement

Parameters		Gender		IBP		Arthritis		Psoriasis		Uveitis		Dactylitis		IBD		Cervicitis/Urethritis/ Diarrhoea		Sacroiliitis on MRI		CRP	ESR
		M	F	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A		
HLA-B27 positive		16 (84.2%)	3 (15.2%)	14 (73.7%)	5 (26.3%)	14 (73.7%)	5 (26.3%)	4 (21.1%)	15 (78.9%)	5 (26.3%)	14 (73.7%)	1 (5.3%)	18 (94.7%)	2 (10.5%)	17 (89.5%)	2 (10.5%)	17 (89.5%)	12 (80.0%)	3 (20.0%)	12	16
HLA-B27 negative		14 (51.9%)	13 (48.1%)	3 (11.1%)	24 (88.9%)	12 (44.4%)	15 (55.6%)	1 (3.7%)	26 (96.3%)	4 (14.8%)	23 (85.2%)	0 (0.0%)	27 (100.0%)	1 (3.7%)	26 (96.3%)	3 (11.1%)	24 (88.9%)	2 (25.0%)	6 (75.0%)	18	21
HLA-B27 equivocal		4 (80%)	1 (20.0%)	4 (80.0%)	1 (20.0%)	3 (60.0%)	2 (40.0%)	0 (0.0%)	5 (100.0%)	2 (40.0%)	3 (60.0%)	0 (0.0%)	5 (100.0%)	1 (20.0%)	4 (80.0%)	0 (0.0%)	5 (100.0%)	3 (60.0%)	2 (40.0%)	4	Nil
Total		34	17	21	30	29	22	5	46	11	40	1	50	4	47	5	46	17	11	34	37
p-value				0.001		0.142		0.111		0.212		0.424		0.396		0.738		0.037		0.186	0.067

[Table/Fig-2]: Association of HLA-B27 by flowcytometry with demographic, clinicoradiological and laboratory parameters.
IBP: Inflammatory back pain; IBD: Inflammatory bowel disease; MRI: Magnetic Resonance Imaging; CRP: C-Reactive protein; ESR: Erythrocyte sedimentation rate; HLA: Human leukocyte antigen; M: Male, F: Female; P: Present, A: Absent; (Chi-square test/ Fisher-exact test)

PCR results	Flowcytometry results			Total
	Positive	Grey zone	Negative	
Positive (22)	18 (81.8%)	4 (18.1%) (False negative)	-	22
Negative (29)	1 (3.4%) (False positive)	1 (3.4%) (False positive)	27 (93.1%)	29

[Table/Fig-3]: HLA B27 results by flowcytometry and PCR (n=51).

[Table/Fig-4]: Photograph shows analysis of HLA-B27 by flowcytometry in FACS Canto II clinical software. The population of T-lymphocytes has been gated on dot plot of CD3 PE versus side scatter. T-lymphocyte population is displayed on a FITC labelled Anti HLA-B27/FL1 histogram, according to the LMF.
a) The log mean fluorescence is 169, the sample is positive for HLA-B27
b) The log mean fluorescence is 98, the sample is negative for HLA-B27
c) The log mean fluorescence is 152 which lies in grey zone

[Table/Fig-5]: a) Photograph shows HLA-B27 SSP-PCR amplification. There is simultaneous amplification and presence of Human growth hormone at 430 bp which is the internal control. Appearance of 145 base pair (bp) specific band indicates the presence of HLA-B27 gene (lane 1 and 3) and its absence indicates a negative sample for HLA-B27 (lane 2 and 4). b) Shows the graph for RT-PCR. X and Y axis depict the number of cycles and fluorescence, respectively.
The base line (threshold) is set for fluorescence
Above the threshold is the red curve (positive control) which is seen crossing the threshold in 23 cycles
Blue straight line indicates the negative control as it does not cross the threshold for fluorescence in 35 cycles
Green curve indicates the test sample, which is crosses the threshold on the 28th cycle and is positive for HLA-B27

Method	True positive	True negative	False positive	False negative	Sensitivity	Specificity	PPV	NPV
Flowcytometry (excluding grey zone cases) (n=46)	18	27	1	0	100%	96.4%	94.7%	100%
Flowcytometry (including grey zone cases) (n=51)	18	28	1	4	81.8%	96.5%	94.7%	87.5%

[Table/Fig-6]: Performance analysis for flowcytometry taking PCR method as gold standard.
PPV: Positive predictive value; NPV: Negative predictive value

upon getting up); 3) Age at onset <40 year; 4) Improvement with exercise; 5) No improvement with rest. The sensitivity of IBP for a diagnosis of axial SpA has been shown to be about 70% [3,10]. In a population-based study in Germany, >60% of patients (n=90) had symptoms suggestive of IBP, with 47% in the human leucocyte antigen B27 positive (HLA-B27+) group and 4% in the B27 negative group [11]. Similarly, 41% cases in the present study reported IBP and 66% of them tested positive for HLA-B27 by flowcytometry. A significant association was noted between IBP and HLA-B27 in the present study.

The HLA-B27 is a genetic biomarker of joint disease in psoriasis patients and is also a marker of disease expression in psoriatic arthritis (PsA) [12]. HLA-B27 was present in about 20% of the cases {Odds Ratio (OR) 3.03} in the largest study performed for the association of HLA with PsA in Canada, including 678 cases and 688 controls [13]. Contrary to published literature, in the present study no statistically significant association was noted between HLA-B27 expression and psoriasis, possibly due to small size of the study population.

Dactylitis, or "sausage-like" digit, is a typical manifestation of SpA [14]. Arevalo M, found that HLA-B27 positivity confers risk for an early disease onset and family aggregation. However, they found that cases negative for HLA-B27 had a higher propensity for peripheral arthritis, dactylitis and other extra articular manifestations [15]. In the present study, only one patient had history of dactylitis and tested positive for HLA-B27.

The prevalence of AS in patients with ulcerative colitis is 2.6% and that in Crohn's Disease is 6%, giving an overall prevalence of 3.7% in patients with IBD [16,17]. In the present study, only 5.8% cases had history of IBD and it was not significantly associated with HLA-B27 positivity.

The SpAs are long standing chronic inflammatory conditions and patients generally have elevated acute phase reactant proteins in their blood. Akassou A and Bakri Y studied phenotype association with HLA-B27 status in patients with SpA and found that patients positive for HLA-B27 presented with more severe and active disease [18]. CRP and ESR are the two most commonly used markers of disease however; they have both low sensitivity and specificity [19]. An elevated CRP is included in the ASAS Axial SpA classification criteria as a measure of disease activity. An elevated CRP or ESR is present in only about 40-50% of patients with AS, therefore, a normal ESR or CRP does not rule out AS or comprehensively capture active disease [20]. High CRP is also associated with higher incidence of radiographic changes on spine X-rays and signs of inflammation on sacroiliac MRI [21]. A recent paper mentions high sensitivity CRP to correlate better than routine CRP with clinical disease activity parameters in patients with axial SpA [22]. In the present study, no statistically significant correlation was found between CRP/ESR and HLA-B27 expression.

Involvement of sacroiliac joints is the hallmark of axial SpA with sacroilitis being the first manifestation. The most sensitive imaging technique for its detection is MRI [23]. Similar to published literature, in the present study a statistically significant association was noted between sacroilitis and HLA-B27 expression.

The intermediate LMF in 4 of the equivocal cases by flowcytometry and positive by PCR technique could be attributed either to masking effect of co-existing HLA B07 or a subtype of HLA-B27 which has lower expression when assessed by flowcytometry [24]. Other causes of decreased LMF in HLA-B27 positive cases seen by other authors include age of the sample and treatment status of patients [25].

In the present study, of the 29 patients who tested negative by PCR, 27 patients were negative for HLA-B27 by flowcytometry as well. Two cases, one equivocal by flowcytometry and one positive by flowcytometry, were found to be negative by PCR technique, which could be due to presence of cross reactive groups like HLA B37 and HLA B07. Usually, these groups cause an equivocal LMF but rarely may cause a false positive result especially with HLA B07 [26,27]. The percentage of samples in grey zone in different studies along with the clone used is summarised in [Table/Fig-7] [4,7,26,28].

Parameters	Demirel GY and Guzel O [4] (2016)	Chedda P et al., [7] (2018)	Reynolds WM et al., [26] (1996)	Bonnaud G et al., [28] (1999)	Present study
Clone used for flowcytometry	HLA-ABC-m3	GS 145.2 clone	FD 705	HLA-ABC-m3, GS145.2 and FD705 clones	GS 145.2
Definition of grey zone		138-157 LMF	10-49 channel shift	Determined by ROC curves	147-157
Percentage of samples in grey zone	13.2%	5.78% (436 cases)	5.2% (109 cases)	12% (34 cases), 8% (25 cases) and 28% (81 cases) respectively.	9.8%

[Table/Fig-7]: Comparison of percentage of samples in grey zone among various published studies [4,7,26,28].

However, Skalska U et al., in their study concluded that additional presence of HLA B07 can result in false negative results by flowcytometry using GS 145.2 monoclonal antibody, due to its cross reactivity leading to masking of coexisting HLA-B27 antigen [25].

There were five samples which tested equivocal in the present study which would definitely require confirmation by molecular testing, as per the manufacturer's guidelines. In the present study, the authors found discordance (one false positive) between PCR and flowcytometry even when the five cases in the grey zone were excluded.

The performance analysis in the present study implies that flowcytometry has a NPV of 100% though the PPV is 94.7%. [Table/Fig-8] summarises the comparison of performance analysis parameters of various studies [4,25,29,30]. Thus, the present study is in concordance with previous studies [7,25-29] and manufacturer guidelines, where further testing of equivocal cases is mandatory. Furthermore, in the present study, the authors found that cases

Parameters	Demirel GY and Guzel O [4] (2016)	Skalska U et al., [25] (2015)	Seippe MT et al., [29] (2005)	Nicknam M et al., [30] (2003)	Present study
Clone used	HLA-ABC-m3	GS 145.2	GS 145.2, FD 705, both clones combined		GS 145.2
Sensitivity	99%	99%	95.2%, 98.2%, 98.8%, respectively	100%	100%
Specificity	100%	-	88.6%, 98.6%, 97.6%, respectively	94.6%	96.4%

[Table/Fig-8]: Comparison of performance analysis parameters of flowcytometry among various published studies [4,25,29,30].

negative by flowcytometry do not require confirmation, while need for additional PCR testing for positive cases should be assessed by the clinician.

Limitation(s)

The present study was limited by non availability of allele sequencing for HLA-B27 to correlate the LMF obtained in all cases with presence of cross reactivity groups and various subtypes of HLA-B27.

CONCLUSION(S)

Despite the established efficacy of flowcytometry the issue of antibody cross reactivity persists. The concept of grey zone identifies the subset of such patients, thus subjecting them to further testing. Although the sample size was limited, the study brings to light that flowcytometry is a fairly specific and sensitive method for HLA-B27 detection with a high NPV. Taking PCR as gold standard, a higher sensitivity (100%) was observed when the grey zone cases were excluded (sensitivity of 81.8% if grey zone cases included) while specificity (96.4% vs 96.5%) was almost equivalent in both the methods. In the COVID-19 era, it reiterates the importance of flowcytometry for HLA-B27 especially when PCR is overburdened.

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