RESEARCH ARTICLE

HLA-B27 testing: A journey from flow cytometry to molecular subtyping

Pratiksha Chheda¹ | Sandeep Warghade² | Jyothi Mathias² |
Tavisha Dama¹ | Sunmeet Matkar³ | Nilesh Shah⁴ | Rajesh Bendre²

¹Department of Molecular Pathology, Metropolis Healthcare Ltd, Mumbai, Maharashtra, India

²Department of Hematology, Metropolis Healthcare Ltd, Mumbai, Maharashtra, India

³Medical Communications, Metropolis Healthcare Ltd, Mumbai, Maharashtra, India

⁴Metropolis Healthcare Ltd, Mumbai, Maharashtra, India

Correspondence

Pratiksha Chheda, Department of Molecular Pathology, Metropolis Healthcare Ltd, Mumbai, Maharashtra, India.

Email: pratiksha.chheda@metropolisindia.com

Funding information

EUROIMMUN

Background: Determination of HLA-B27 status plays an important role as adjuvant in suspected cases for diagnosis of Ankylosing Spondylitis (AS). Objectives of this study were to evaluate (i) flow cytometry method in comparison with DNA microarray for HLA-B27 typing and (ii) EUROArray HLA-B27 Direct assay for HLA-B27 allele detection along with discrimination of AS/non-AS subtypes in Indian population.

Methods: A total of 7543 patients with a presumptive clinical diagnosis of AS were referred for screening of HLA-B27. All samples were initially tested by flow cytometry, and based on its findings, 1560 samples were analyzed for the presence of HLA-B27 allele by microarray technology. A subset of samples (n = 200) were further tested by DNA sequencing for identification of HLA-B27 subtypes.

Results: Screening of HLA-B27 by flow cytometry reported 1551 positive (20.56%) and 5556 negative (73.65%) cases. Remaining 436 (5.78%) samples were identified within equivocal zone. Of cases (n = 1560) analyzed by microarray method, 1333 (85.44%) and 227 (14.55%) were detected microarray positive and negative, respectively. DNA sequencing identified HLA-B*27:07 as the predominant subtype among cases showing ex2 positivity by microarray method. Of 200 cases, 20 cases (14 of HLA-B*07 and 6 of HLA-B*37) of HLA-B27 cross-reactive subtypes were also identified.

Conclusion: We recommend DNA typing as a complementary tool along with flow cytometry to accomplish successful HLA-B27 phenotype determination. This is the first study among Indian population to evaluate efficacy of EUROArray to detect B27 allele and its potential to indicate the presence of nondisease-associated alleles in Indian population.

KEYWORDS

ankylosing spondylitis, DNA microarray, DNA sequencing, flow cytometry, HLA-B27

1 | INTRODUCTION

Ankylosing spondylitis (AS) is a systemic rheumatic disease. The mean AS prevalence per 10 000 was 31.9 in North America, 23.8 in Europe, 16.7 in Asia, 10.2 in Latin America, and 7.4 in Africa.¹ HLA-B27 has a remarkable association with AS, with over 90% of AS patients with AS expressing the HLA-B27 gene. However, fewer than

5% of B27-positive individuals in the general population become affected with the disease.² The Indian population is well known for its genetic diversity and distributions of HLA antigens exhibiting a variable frequency of B27 from north regions (2.7%-29%) to south regions (0.9%-2.1%).³ HLA-B27 has a high degree of genetic polymorphism; therefore, it encompasses 105 (HLA-B*27:01 to HLA-B*27:106) known subtypes, encoded by 132 alleles that show diverse racial/

ethnic prevalence in the world.⁴ These subtypes differ by one or more amino acid substitutions in the antigenic peptide-binding groove. Not every allelic variant is disease-associated, and the strength of association varies depending on the type of spondyloarthropathy and ethnicity of carriers. HLA-B*27:06 and HLA-B*27:09 have been reported not to be associated with AS and even considered to play a protective role.^{5,6} Based on the literature available on the Indian subjects, HLA-B*27:05 is the most common subtype followed by HLA-B*27:04. Other common alleles reported from India are HLA-B*27:07, HLA-B*27:02, and HLA-B*27:10, all of which have been documented to be associated with AS.⁷⁻¹²

Serological techniques such as microcytotoxicity and flow cytometry for testing HLA-B27 require viable cells that adequately express HLA-B27 and may give false negative results if HLA-B27 is downregulated or “masked”.¹³ Flow cytometry, a widely used technique in India, is rapid, high-throughput and relatively inexpensive but has been reported to lack specificity, especially in the presence of antigens that cross-react with HLA-B27, such as HLA-B7 and HLA-B37.¹⁴ Recently, molecular typing of HLA-B27 by polymerase chain reaction (PCR) is being commonly practiced increasingly as this technique relies on the detection of the HLA-B27-specific DNA sequence for the identification of B27 allele and is not influenced by conformational antigenic changes. The EUROArray HLA-B27 Direct, a microarray-based assay, is designed to detect all currently known HLA-B*27 subtypes and also indicates whether one of the nondisease-associated alleles HLA-B*27:06 and HLA-B*27:09 may be present in the respective sample.¹⁵ Objectives of this study were (i) to evaluate flow cytometry method in comparison with DNA microarray for HLA-B27 typing and (ii) to evaluate EUROArray HLA-B27 Direct assay for HLA-B27 allele detection along with discrimination of AS/non-AS subtypes in Indian population. Our study was not aimed to show association of HLA-B27 with disease predisposition.

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 7543 patients with a presumptive clinical diagnosis of ankylosing spondylitis were referred to Metropolis Healthcare Ltd, Global Reference Laboratory (GRL), Mumbai, India. The period of the study was 6 months that is, from December 2015 to May 2016. The study protocol was approved by Independent Ethics Committee. Peripheral blood was obtained from the study population, and all the samples were initially tested by flow cytometry within 24 hours of collection. Based on flow cytometry findings, samples were analyzed for the presence of HLA-B27 allele by microarray technology and later, a subset of samples were tested by DNA sequencing for identification of HLA-B27 subtypes.

2.2 | Flow cytometry analysis

The presence of HLA-B27 was detected by GS145.2 monoclonal antibody (HLA-B27 Kit; Becton Dickinson, San Jose, CA, USA). The BD

HLA-B27 System consists of a combination of murine monoclonal antibodies Anti-HLA-B27 conjugated with fluorescein isothiocyanate (FITC) and CD3 conjugated with phycoerythrin (PE). The whole procedure was performed according to the manufacturer's instructions. Whole blood samples were stained with anti-HLA-B27 antibody conjugated with fluorescein and with anti-CD3 antibody conjugated with phycoerythrin. Samples were incubated for 20 minutes in the dark, at room temperature, and then analyzed using a FACS Calibur flow cytometer on HLA-B27 v3.1 software (Becton Dickinson).

As per the manufacturer's instructions, based on the population tested, the cross-reactive samples fall on the positive side (+10 units) of the decision marker (148 MFI). This 10-channel zone is referred to as the “gray zone” for BD FACSCalibur and BD FACSCanto systems. According to the manufacturer, samples falling within this zone should be confirmed by an alternate method. We termed this gray zone as “Manufacturer-Defined Equivocal Range” (MDER) of 148-157 MFI (mean fluorescence intensity), and all the cases falling within MDER were considered equivocal cases. To avoid misinterpretation and to obtain more clarity about the screening results, the MDER was extended on its lower side by 10 units, thereby redefining the MDER to 138-157 MFI. This extended range is termed “Laboratory-Defined Equivocal Range” (LDER) in our study.

2.3 | EUROArray HLA-B27 studies

The EUROArray HLA-B27 Direct (EUROIMMUN AG Luebeck, Germany) is an in vitro microarray-based test for detecting all currently known HLA-B*27 subtypes and indicating whether nondisease-associated alleles (B*27:06 and B*27:09) may be present in human genomic DNA. EUROIMMUN's EUROArray HLA-B27 Direct assay allows DNA analysis directly from whole blood, eliminating the step of DNA isolation.¹⁵ The test is based on amplification of both exons (ex): ex2 and ex3 of the HLA gene and detection of the labeled product by hybridization to immobilize it on the microarray probe. In our study, HLA-B27 status of 1560 cases (ie, flow cytometry equivocal cases [436] and flow cytometry positive cases [1124]) was determined from peripheral lymphocytes by microarray-based genotyping. PCR amplification, hybridization, and washing steps were performed on all the samples and later scanned by EUROIMMUN Microarray Scanner followed by analysis using EUROArray Scan Software. The results were interpreted as per manufacturer's instructions. Detection of both ex2 and ex3 or only ex3 revealed currently known AS-predisposing HLA-B*27 alleles, while detection of only ex2 indicated that the detected B27 allele is not necessarily a AS-predisposing allele but could rather be a nondisease-associated subtype (B*27:06 and B*27:09).

2.4 | DNA sequencing studies

High-resolution HLA-B typing was carried out by Sanger sequencing. Among the subset of samples which had been tested by EUROArray and flow cytometry in total, 200 samples were selected for sequencing: 99 cases showing a positive result for HLA-B27 ex 2 and ex 3 in the EUROArray analysis (disease-associated alleles present), 81 cases

showing a positive result for HLA-B*27 ex2 only in the EUROArray analysis (indication that nondisease-associated alleles may be present), and 20 cases with inconsistent results between the EUROArray and flow cytometry analysis. These 20 cases were detected negative by EUROArray test system (Figure 1). The assay was performed using the Secore IVD (1 λ) Kit using manufacturer's instructions, and capillary electrophoresis was carried out on 3730XL DNA analyzer (Applied Biosystems). The data were analyzed by uType HLA sequence analysis software, version 7.1.

3 | RESULTS

3.1 | Flow cytometry

In this study, all the test samples were subjected to preliminary screening for HLA-B*27 by flow cytometry method. Of 7543, 1551 (20.56%) and 5556 (73.65%) samples were detected as flow cytometry positive and flow cytometry negative, respectively (Table 1) (Figure 1). Remaining 436 (5.78%) samples were detected within the laboratory-defined equivocal range (138-157 MFI). These samples were termed as "Equivocal cases." Of these 436 cases, our study detected 260 cases within the MDER (148-157 MFI) and 176 cases toward lower limit of LDER (138-147 MFI). These samples falling in the equivocal range were further evaluated based on molecular findings.

3.2 | Microarray results and comparison with flow cytometry

Of the overall cases ($n = 1560$) analyzed by microarray method, 1333 (85.44%) and 227 (14.55%) were detected microarray positive and negative, respectively (Figure 1). All these cases could be unambiguously identified as HLA-B*27 as the signals obtained on the microarray were clearly distinct. Coming to the comparison between flow cytometry and microarray method, of 1124 flow cytometry positive samples, 1118 (99.46%) were positive by EUROArray thereby confirming the presence of HLA-B*27. The remaining 6 samples that were detected positive by flow cytometry turned out to be negative by microarray method indicating false positives by flow cytometry (Table 2).

From the 436 equivocal cases, 221 (50.68%) cases were detected negative by microarray method, while 215 (49.31%) cases were detected positive by microarray method. Microarray method thus assisted in resolving these inconsistent cases. These equivocal cases were further evaluated with respect to MFI values to assess the utility of LDER. Within the MDER (148-157 MFI) ($N = 260$), our study identified 188 and 72 microarray positives and negatives, respectively. Whereas, toward the lower limit of LDER (138-147 MFI, $N = 176$), our study identified 08 microarray positive cases. These 08 microarray positive cases (138-147 MFI) would have been wrongly classified as HLA-B*27 negative based on flow cytometry method if manufacturer-defined equivocal range was not expanded toward the lower side.

FIGURE 1 Flow chart: HLA-B*27 typing

Also, of 1333 EUROArray positive cases, 1227 (92.05%) cases showed the presence of both ex2 and ex3, indicating the presence of AS-associated alleles. A significant number of samples (106/1333-7.95%) showed amplification of ex2 only, indicating the possible presence of nondisease-associated subtype (B*27:06 and B*27:09).

3.3 | Sequencing for B27 subtyping

Another aim of our study was to subtype the B27 alleles by DNA sequencing of HLAB gene to evaluate the findings of EUROArray HLA-B27 Direct assay and specifically to evaluate the presence of the nondisease-associated alleles (HLA-B*27:06 and HLA-B*27:09) in our study cohort. In our study, 106 cases were indicated to potentially have nondisease-associated alleles based on microarray findings. A total of 200 cases comprising of 99 cases showing disease-associated AS alleles (EUROArray ex2/ex3 Positive), 81 cases which could potentially be positive for the nondisease-associated alleles (EUROArray ex2 Positive), and 20 EUROArray negative cases (18 flow cytometry equivocal cases and 02 flow cytometry positive cases) were sequenced to validate the microarray findings.

Among the disease-associated B27 category ($n = 99$), the prominent HLA-B27 subtype identified was HLA-B*27:05 (65 cases), followed by HLA-B*27:04 in 33 cases. A solitary case of HLA-B*27:02 was also identified. Among the possible nondisease-associated-HLA-B27 category, 80 of 81 cases were identified to be HLA-B*27:07 and a single case of HLA-B*27:19 was detected. HLA-B*27:07 was thus the predominant subtype identified among the cases that showed positivity for ex2 only, and none of these 81 cases showed the presence of B*27:06 and B*27:09 alleles (Table 3). Of the EUROArray negative cases ($n = 227$), 20 cases (18

flow cytometry equivocal cases and 2 flow cytometry positive cases) were sequenced to obtain 14 cases of HLA-B*07 and 6 cases of HLA-B*37. Both the types, HLA-B7 and HLA-B37, are known to cross-react with HLA-B27.

4 | DISCUSSION

HLA-B27 is well known for its strong association with AS, a link discovered more than 40 years ago, and prevalence of AS mirrors the HLA-B27 prevalence across the world.¹⁶⁻¹⁸ Excluding HLA-B27 virtually excludes the possibility of ankylosing spondylitis.¹³ Serological techniques for HLA antigen detection include classic microcytotoxicity and flow cytometry.¹⁹ Originally described by Terasaki and McClelland (1964), microcytotoxicity was long-established method for HLA typing, but cross-reactivity induced HLA-typing errors and labor intensiveness limited its further use.²⁰ Flow cytometry is a rapid and relatively inexpensive method for preliminary screening of HLA antigen. However, in HLA serology, cross-reactivity is well known phenomenon as some human allo-antisera to HLA-B27 cross-react with the B7 antigen. One interesting feature in flow cytometry is that the cross-reactivity produced by HLA-B7 is so strong that it can mask not only other weaker cross-reactive HLA-B molecules but also even the presence of HLA-B27.¹⁴ Besides B7, the antibody GS145.2 is known to cross-react to with HLA-B37, HLA-B44, and HLA-B55.²¹ All the 20 euroarray negative cases (18 flow equivocal cases and 02 flow positive cases) turned out to be either HLA-B7 or HLA-B37 upon sequencing. This further confirms the cross-reactivity of GS145.2 antibody with other HLA-B alleles.

Our study detected 6 false positives (all having >157 MFI) by flow cytometry. Repeat analysis with fresh blood specimens by flow cytometry gave similar results. Of these 6 false positives, 2 were sequenced to identify HLA-B7 allele in both the cases. These findings indicate that the presence of HLA-B7 subtype can not only lead to flow cytometry equivocal results but also false positives in rare instances. HLA-B27 typing by flow cytometry is more likely to yield false positive

TABLE 1 Flow cytometry results ($N = 7543$)

Flow positive cases	Flow negative cases	Flow equivocal cases
1551 (20.56%)	5556 (73.65%)	436 (5.78%)

TABLE 2 Comparison of flow cytometry and EUROArray HLA-B27 Direct ($N = 1560$)

	ex2/ex3 positive (AS-associated B27 alleles)	Only ex2 positive (non-AS-associated B27 alleles possible)	HLA-B27 negative	Total
Equivocal by flow cytometry	198	17	221	436
Positive by flow cytometry	1029	89	06	1124
Total	1227	106	227	1560

TABLE 3 DNA sequencing results ($N = 200$)

Euro (ex2/ex3) positive cases (AS-associated alleles indicated) ($N = 99$ cases)	Euro (ex 2 only) positive cases (non-AS-associated alleles indicated) ($N = 81$ cases)	Flow equivocal and Euro negative cases ($N = 20$ cases)
HLA-B*27:04 (33 cases)	HLA-B*27:07 (80 cases)	HLA-B7 (14 cases)
HLA-B*27:05 (65 cases)	HLA-B*27:19 (01 case)	HLA-B27 (06 cases)
HLA-B*27:02 (01 cases)		

TABLE 4 Identification of HLA-B27 subtypes in different studies

	Study	Nation	Subtypes of HLA-B27 identified	Techniques	Ref
GLOBAL STUDIES					
1	Martínez et al, 1999	Colombia (N = 59)	(all AS patients) B*2702 (12.8%), B*2705 (87.1%)	PCR-SSO	34
2	Zhen et al, 2009	China (N = 408)	(all AS patients) B*2704 (81.72%), B*2705 (12.9%), B*2715 (5.38%)	PCR-SSP	35
3	Nicknam et al, 2008	Iran (N = 119)	(all AS patients) B*2702 (36.6%), B*2705 (63.4%)	PCR-SSP	36
4	Ben Radhia et al, 2008	Tunisia (N = 160)	AS patients (121) B*2702 (47.1%), B*2705 (47.1%), B*2707 (3.31%) B*2704 (0.62%), B*2709 (0.62%), B*2714 (0.62%) Controls(39) B*2702 (41%), B*2705 (41%), B*2703 (NA), B*2704 (NA), B*2707 (7.6%), B*2709 (NA), B*2714 (NA)	PCR-SSP, Microlympho-cytotoxicity	27
5	Liu et al, 2010	China (N = 317)	AS patients (172) B*2704 (69.2%), B*2705 (23.8%), B*2703 (5.8%) Controls (145) B*2704 (53.8%) B*2705 (33.1%), B*2703 (8.9%)	PCR-SSP	28
6	Cipriani et al, 2003	Venezuela (N = 48)	AS patients (48) B*2702 (31.2%), B*2705 (68.8%) Controls (NA) B*2708 (NA)	PCR-SSOP PCR-SSP	29
7	Varnavidou-Nicolaidou et al, 2004	Greece (N = 102)	AS patients (42) B*2702 (73.8%), B*2705 (26.2%) Controls (60) B*2707 (16.7%), B*2702 (51.7%), B*2705 (31.7%)	PCR-SSP	37
8	Birinci et al, 2006	Turkey (N = 85)	AS patients (38) B*2705 (71.1%), B*2702 (26.3%), B*2708 (2.6%) Controls (47) B*2705 (68%), B*2702 (32%)	PCR-SSP	38
9	Meryem et al, 2013	Morocco (N = 241)	AS patients (116) B*2705 (NA) Controls (125) B*2705 (44.4%), B*2702 (29.6%), B*2703 (9.2%) B*2708 (16.7%)	PCR-SSP Microlympho-cytotoxicity	39
10	Roberts et al, 2013	New Zealand (N = 404)	AS patients (176) B*2705 (71.42%), B*2704 (21.42%) Controls (228), B*2705 (NA)	PCR-SSP	26
11	Hou et al, 2007	Taiwan (N = 385)	AS patients(314) B*2704 (NA) Controls (71) B*2705 (NA), B*2706 (NA),B*2707 (NA)	Flow cytometry PCR-SSP &	40
12	Park et al, 2009	Korea (N = 175)	AS patients (143) B*2704 (7.7%), B*2705 (90.9%), B*2710 (1.4%) Controls (32) B*2704 (34.4%), B*2705 (59.4%), B*2710 (3.1%) B*2715 (3.1%)	PCR-SSP	24
13	Lee et al, 2008	Korea (N = 267)	(All patients) B*2704 (8.23%), B*2705 (91.38%), B*2704/B*2705 (0.3%)	PEL-FREEZ kit	41
14	Ma et al, 2006	China (N = 146)	B*2704, 05,06, 07 and 24 common in patients (n = 128) & control (n = 18)	PCR-SSP	42
15	Mou et al, 2010	China (N = 1873)	AS pts (505) Ctrl (1368) High proportion of B*2704 than ctrl low proportion of B*2705 than ctrl. Only in AS pts B*2715 (1.32%) B*2702 (0.66%)	PCR-SSP and Sequencing	43

(Continues)

TABLE 4 (Continued)

	Study	Nation	Subtypes of HLA-B27 identified	Techniques	Ref
16	Yang et al, 2006	China (N = 825)	All Controls. B*2704(30.77%),B*2707(19.23%), B*2705 (23.08%), B*2711 (7.69%),B*2712(7.69%),B*2701 (3.85%), B*2713 (3.85%), B*2721 (3.85%)	PCR sequence-based typing	44
17	Grubic et al, 2001	Croatia (N = 88)	AS patients (50) B*2705 (83%), B*2702 (13.2%), B*2701 (2%), B*2704 (2%) Controls (38) B*2705 (76.3%), B*2702 (21.1%)	PCR-SSP	45
18	Fouladi et al, 2009	Iran (N = 490)	AS patients (60) B*2705 (NA), B*2702 (NA), B*2704 (NA), B*2707 (NA) Controls (430) No sig diff & same subtypes as AS pts	PCR-SSP	46
19	Liu et al, 2010	China (N = 1698)	AS patients(153) B*2704 (NA), B*2705 (NA), B*2710 (NA) Controls (1545) No sig diff & same subtypes as AS pts	PCR-SSP	47
20	Alaez et al, 2007	Israel (N = 75)	AS patients-(24) B*2702 (62.5%) B*2705 (37.5%), Ctrl (51) B*2702 (41.2%) B*2705 (50.9%), B*2707 (5.9%) B*2701 (1.9%)	PCR-SSP	48
21	Oguz et al, 2004	Turkey (N = 104)	AS patients (49) B*2702 (30.3%), B*2704 (NA), B*2705 (NA), B*2707 (7.84%), B*2708 (NA) Controls (55) B*2702 (21.5%), B*2704 (NA), B*2705 (NA), B*2707 (14.3%), B*2708 (NA)	PCR-SSP	32
22	Acar et al, 2012	Turkey (N = 101)	AS patients (66) B*2705 (65.2%), B*2702 (26.1%), B*2704 (6.5%), B*2707 (2.2%) Controls (35) B*2705 (54.3%), B*2702 (31.4%), B*2704 (2.9%) B*2703 (2.9%), B*2702/B*2705 (8.5%)	PCR-SSP	25
23	Krylov et al, 1995	Chukotka (N = 86)	(all AS patients) B*2705 (65%), B*2702 (28%)	PCR-SSP	49
24	Erdes et al, 1997	Russia (N = 219)	(all AS patients) B*2705 (99%), B*2702 (not in abstract)	Oligotyping	50
25	Baech et al, 1997	Denmark (N = 81)	AS patients (30) only B*2705 (NA) Control (51) B*2705 (90.2%), B*2702 (9.8%)	PCR-SSP	51
INDIAN STUDIES					
1	Srivastava et al, 2015	India (N = 256)	(All AS patients) B*2705, B*2704, B*2718 (not in abstract), B*2707 (7.4%),	Sequencing	9
2	Shankarkumar et al, 2003	India (N = 5129)	(All AS patients) B*2704 (34.48%), B*2705 (36.2%), B*2707 (15.51%) B*2708 (10.34%), B*2714 (3.44%)	PCR-SSP Reverse line strip	10
3	Shankarkumar et al, 2002	India (N = 51)	(All AS patients) B*2704 (34%), B*2705 (34%), B*2707 (18%), B*2708 (12%), B*2714 (2%)	PCR-SSP Reverse line strip	11
4	Chhaya et al, 2005	India (N = 1170)	(All AS patients) B*2702 (1.43%), B*2704 (14.29%), B*2705 (70%) B*2707 (12.86%), B*2718 (1.43%)	PCR-SSP	7

(Continues)

TABLE 4 (Continued)

	Study	Nation	Subtypes of HLA-B27 identified	Techniques	Ref
5	Kanga et al, 1996	India (N = 77)	AS patients (60) B*2704 (70.8%), B*2705 (73.1%), B*2702 (1.67%) B*2707 (NA) Controls(17) B*2704 (47%), B*2705 (22.2%), B*2707 (NA)	ID-IEF PCR-SSOP	8
6	Chavan et al, 2011	India (N = 100)	AS patients (81) B*2704 (28.4%), B*2705 (67.9%), B*2702 (1.23%), B*2707 (2.46%), Controls(29) B*2705 (62.07%), B*2707 (27.59%), B*2704 10.34%	PCR-SSP	31

results in the presence of HLA-B7 than is typing by molecular methods or microcytotoxicity.²²

In our total study population, flow cytometry detected 1551 (20.56%) and 5556 (73.65%) positive and negative cases, respectively, while 436 (5.78%) cases were termed as “Equivocal” as they were detected within the laboratory-defined equivocal range (LDER) (138–157 MFI) (Figure 1). Toward the lower end of LDER, our study detected 182 samples within 138–147 MFI, while other end of LDER detected 254 samples within 148–157 MFI. Of 182 cases detected upon extension of MDER, 11 cases were identified as HLA-B27 positives by microarray method. Thus, the extension of MDER into our study LDER enabled more equivocal cases to be considered for rechecking by molecular methods. It is to be noted that these equivocal cases otherwise would have been wrongly classified as flow cytometry negative for HLA-B27, thereby, failing to detect or failing to alert the clinician about an increased risk of developing into ankylosing spondylitis. Thus, in our study, extension of manufacturer-defined equivocal range helped in comprehensive screening of HLA-B27 and further molecular typing of more equivocal cases thereby significantly improving the diagnostic rationale of ankylosing spondylitis. Our study, therefore, recommends the manufacturer to redefine its equivocal range enabling incorporation of more equivocal cases for molecular studies, thereby leading to accurate typing. Also, other clinical laboratories are advised to validate flow cytometry in conjunction with molecular typing with sizeable samples to derive its own LDER to avoid incorrect typing.

Different molecular typing methods for B27 detection are PCR-SSP, PCR-SSOP, and real-time PCR methods. This is the first study on Indian Population by EUROArray HLA-B27 Direct which detects all the known HLA-B*27 subtypes and also assesses the possible presence of the nondisease-associated subtypes HLA-B*27:06 and HLA-B*27:09.¹⁵ In our study, of 1560 cases studied by microarray method, 1333 (85.44 %) were identified as positive and 227 (14.55%) as negative HLA-B27 cases. Among the positive cases analyzed by flow cytometry, 1118 of 1124 (99.46%) had consistent results by microarray method (Figure 1). EUROArray HLA-B27 Direct indicates the presences of nondisease-associated alleles, and in our study, 106 of such cases were detected.

Further, in our study, we applied DNA sequencing to identify the exact subtype in the selected cohort and to evaluate the potential of

the EUROArray to indicate the presence of nondisease-associated alleles in Indian patients. Molecular identification of HLA-B27 subtypes has been carried out by many studies in several countries, including India (Table 4). In our study, DNA sequencing of 180 HLA-B27 positives (ex2 and ex3 positive or only ex2 positive) identified HLA-B*27:05 as the most prevalent subtype (65 cases) in our study population, followed by HLA-B*27:04 (33 cases). HLA-B*27:05 has been found in most populations and is detectable in the very large majority of White B27-positive individuals. Several authors have reported HLA-B27:05 being the predominant subtype in their study populations.^{23–29} HLA-B*27:04 has been reported as the predominant subgroup in Asians.³⁰ One case of HLA-B*27:02 was also identified among the disease-associated B27 category. Low prevalence of HLA-B*27:02 subtype was also observed in the previous studies carried out on Indian population.^{7,8,31} Of 81 cases in category with a potential presence of non-AS alleles, DNA sequencing identified 80 cases of HLA-B*27:07 and 01 case of rare subtype HLA-B*27:19.

Further, sequencing identified the subtype HLA-B*27:07 for 80 of 81 cases where a nondisease-associated subtype (exon 2 only) was indicated. Several studies on different population of AS sufferers around the world have reported variable prevalence of HLA-B*27:07 ranging from 2.2%–7.84%.^{25,32} In Indian population, relatively higher prevalence (2.46%–15.51%) of HLA-B27:07 has been detected (Table 4). It is to be noted that, our study did not detect HLA-B27:06 and HLA-B27:09, which is agreement with the studies carried out by several authors on Indian population.^{7–11,31} Along with our data, these studies indicate almost nonexistence of HLA-B27:06 and HLA-B27:09 alleles in our population. Instead, HLA-B27:07 allele was identified in all the cases where HLA-B27:06 or HLA-B27:09 was expected.

HLA-B region being highly polymorphic, the presence of HLA-B*27:07 subtype may have resulted in mismatch at the primer binding site for exon 3 amplification. This would have probably led to incorrect categorization, hence, indicating the presence of non-AS alleles upon EUROArray HLA-B27 typing. Overall, our study identified the subtype HLA-B*27:07 in 7.95% of the cases, which is in line with previous findings. Many studies on association of HLA-subtypes with AS susceptibility have reported that HLA-B*27:07 has been associated with AS. Ankylosing spondylitis or related Spondyloarthritis has been described in patients with the subtypes HLA-B*27:01, HLA-B*27:02,

HLA-B*27:04, HLA-B*27:05, and HLA-B*27:07.³³ Several studies have reported the presence of HLA-B*27:06 or HLA-B*27:09 in the Italian and south-east Asian populations. Therefore, the ethnic background of patients, for whom the potential presence of the non-AS-associated alleles whenever indicated, should be identified. In our study cohort of patients from India, the 2 nondisease-associated alleles were not detected. This is consistent with previous findings (Table 4) and means that the presence of the non-AS-associated alleles can be excluded with high probability for patients originating from India and being tested positive for HLA-B27 (exon 2 only).

In summary, a clinical laboratory should define its laboratory-specific equivocal MFI range in comparison with molecular methods during initial flow cytometry method validation before using the assay for clinical testing of B27 as a primary screening method. Also, whenever the test result for a sample indicates the potential presence of a nondisease-associated allele (only exon 2 positive), it is prudent to know the ethnic background of the patient and HLA-B27 subtyping by DNA sequencing is recommended for patients belonging to non-Indian ethnic groups, specifically for patients from Italy and south-east Asia.

In conclusion, serological techniques such as microcytotoxicity and flow cytometry for testing HLA-B27 are rapid and relatively inexpensive but have been reported to lack specificity. HLA-B27 genotyping using molecular biology is a reliable but costly technique. Although EUROIMMUN's EUROArray HLA-B27 Direct assay is a cost and time-consuming molecular genetic assay, it successfully detects the presence of HLA-B27 subtype. DNA typing could be used as a complementary technique and applied to samples whose HLA-B27 phenotype cannot be determined by flow cytometry method. This approach of flow cytometry followed by DNA typing of equivocal cases is, in our experience, an economical and reliable approach in resource-limited developing countries like India.

ACKNOWLEDGMENT

We express sincere gratitude toward the management of EUROIMMUN Luebeck, Germany for funding HLAB gene sequencing studies.

ORCID

Pratiksha Chheda <http://orcid.org/0000-0002-4764-8117>

REFERENCES

- Dean LE, Jones GT, MacDonald AG, et al. Global prevalence of ankylosing spondylitis. *Rheumatology (Oxford)*. 2014;53:650-657.
- Reveille JD. Major histocompatibility genes and ankylosing spondylitis. *Best Pract Res Clin Rheumatol*. 2006;20:601-609.
- Dhurandhar PS, Shankarkumar U. HLA association in seronegative spondyloarthritis patients from Mumbai, India. *Int J Hum Genet*. 2007;7:235-239.
- Khan MA. Polymorphism of HLA-B27: 105 subtypes currently known. *Curr Rheumatol Rep*. 2013;15:362.
- Khan MA. Update on spondyloarthropathies. *Ann Intern Med*. 2002;136:896-907.
- Paladini F, Taccari E, Fiorillo MT, et al. Distribution of HLA-B27 subtypes in Sardinia and continental Italy and their association with spondylarthropathies. *Arthritis Rheum*. 2005;52:3319-3321.
- Chhaya SU. HLA-B27 polymorphism in Mumbai, Western India. *Tissue Antigens*. 2005;66:48-50.
- Kanga U, Mehra NK, Larrea CL, et al. Seronegative spondyloarthropathies and HLA-B27 subtypes: a study in Asian Indians. *Clin Rheumatol*. 1996;15:13-18.
- Srivastava R, Agnihotry S, Aggarwal R, Bajpai P, Aggarwal A. HLA-B27 subtypes in enthesitis-related arthritis category of juvenile idiopathic arthritis and ankylosing spondylitis in northern India. *Clin Exp Rheumatol*. 2015;33:931-935.
- Shankarkumar U. HLA-B27 allele diversity in Indians: impact of ethnic origin and the caste system. *Br J Biomed Sci*. 2003;60:223-226.
- Shankarkumar U, Ghosh K, Mohanty D. HLA B27 polymorphism in Western India. *Tissue Antigens*. 2002;60:98-101.
- Khan MA, Khan MK. Diagnostic value of HLA-B27 testing in ankylosing spondylitis and Reiter's syndrome. *Ann Intern Med*. 1982;96:70-76.
- Neumuller J, Schwartz DWM, Dauber E. Evaluation of four monoclonal antibodies against HLA-B27 for their reliability in HLA-B27 typing with flow cytometry (FC): comparison with the classic microlymphocytotoxic test. *Cytometry*. 1996;26:209-215.
- Cianga P, Zlei M, Rezus E, Cianga C. The flow cytometric labeling pattern in HLA-B27 detection may suggest cross reactivities. *Rev Romana Med Lab*. 2011;19:185-191.
- Euroimmun.com. EuroImmune, Inc. [April 2015]. https://www.euroimmun.com/documents/HLA-B27/MV_5110_D_UK_B.pdf. Accessed August 15, 2015.
- Schlosstein L, Terasaki PI, Bluestone R, Pearson CM. High association of an HL-A antigen, W27, with ankylosing spondylitis. *N Engl J Med*. 1973;288:704-706.
- Brewerton DA, Hart FD, Nicholls A, et al. Ankylosing spondylitis and HL-A 27. *Lancet*. 1973;28:904-907.
- Ebi.ac.uk. EMBL-European Bioinformatics Institute. Immuno Polymorphism Database. [January 27, 2015]. www.ebi.ac.uk/ipd/. Accessed May 18, 2017.
- Sharma N, Sharma V, Masood T, et al. Usage of conventional PCR technology for the detection of HLA-B27 allele: a significant molecular marker of ankylosing spondylitis. *Indian J Clin Biochem*. 2013;28:189-192.
- Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxins. *Nature*. 1964;5:998-1000.
- Levering WH, van den Beemd R, te Marvelde JG. External quality assessment of flow cytometric HLA-B27 typing. *Cytometry*. 2000;42:95-105.
- Kopko PM, Eisenbrey AB, Maha GC. College of American Pathologists HLA-B27 proficiency testing data reveals increased false positive rates for flow cytometry methods. *Hum Immunol*. 2013;74:149.
- Sampaio-Barros PD, Conde RA, Bonfiglioli R, Bértolo MB, Samara AM. Characterization and outcome of uveitis in 350 patients with spondyloarthropathies. *Rheumatol Int*. 2006;26:1143-1146.
- Park SH, Kim J, Kim SG, et al. Human leucocyte antigen-B27 subtypes in Korean patients with ankylosing spondylitis: higher B*2705 in the patient group. *Int J Rheum Dis*. 2009;12:34-38.
- Acar M, Cora T, Tunc R, Acar H. HLA-B27 subtypes in Turkish patients with ankylosing spondylitis and healthy controls. *Rheumatol Int*. 2012;32:3103-3105.
- Roberts RL, Wallace MC, Jones GT, et al. Prevalence of HLA-B27 in the New Zealand population: effect of age and ethnicity. *Arthritis Res Ther*. 2013;15:1-7.
- Ben Radhia K, Ayed-Jendoubi S, Sfar I, et al. Distribution of HLA-B*27 subtypes in Tunisians and their association with ankylosing spondylitis. *Joint Bone Spine*. 2008;75:172-175.

28. Liu X, Hu LH, Li YR, et al. The association of HLA-B*27 subtypes with ankylosing spondylitis in Wuhan population of China. *Rheumatol Int*. 2010;30:587-590.
29. Cipriani A, Rivera S, Hassanhi M, et al. HLA-B27 subtypes determination in patients with ankylosing spondylitis from Zulia, Venezuela. *Hum Immunol*. 2003;64:745-749.
30. Taurog JD. The mystery of HLA-B27: if it isn't one thing, it's another. *Arthritis Rheum*. 2007;56:2478-2481.
31. Chavan H, Samant R, Deshpande A, Mankeshwar R. Correlation of HLA B27 subtypes with clinical features of ankylosing spondylitis. *Int J Rheum Dis*. 2011;14:369-374.
32. Oguz FS, Ocal L, Diler AS, et al. HLA B-27 subtypes in Turkish patients with spondyloarthropathy and healthy controls. *Dis Markers*. 2004;20:309-312.
33. Sheehan NJ. HLA-B27: what's new? *Rheumatology (Oxford)*. 2010;49:621-631.
34. Martínez B, Caraballo L, Hernández M, et al. HLA-B27 subtypes in patients with ankylosing spondylitis (As) in Colombia. *Rev Invest Clin*. 1999;51:221-226.
35. Wu Z, Mou Y, Lin Z, et al. HLA-B27 polymorphism in Han Chinese patients with ankylosing spondylitis: a distinctive disease association for B*2715 in a multiplex family. *J Rheumatol*. 2009;36:2849-2850.
36. Nicknam MH, Mahmoudi M, Amirzargar AA, et al. Determination of HLA-B27 subtypes in Iranian patients with ankylosing spondylitis. *Iran J Allergy Asthma Immunol*. 2008;7:19-24.
37. Varnavidou-Nicolaidou A, Karpasitou K, Georgiou D, et al. HLA-B27 in the Greek Cypriot population: distribution of subtypes in patients with ankylosing spondylitis and other HLA-B27-related diseases. The possible protective role of B*2707. *Hum Immunol*. 2004;65:1451-1454.
38. Birinci A, Bilgili A, Kuru O, Durupinar B. HLA-B27 polymorphism in Turkish patients with ankylosing spondylitis. *Rheumatol Int*. 2006;26:285-287.
39. Meryem A, Noureddine B, Ouafaa MS. HLA-B27 subtypes distribution among Moroccan patients with ankylosing spondylitis. *Am J Med Biol Res*. 2013;1:28-32.
40. Hou TY, Chen HC, Chen CH, et al. Usefulness of human leucocyte antigen-B27 subtypes in predicting ankylosing spondylitis: Taiwan experience. *Intern Med J*. 2007;37:749-752.
41. Lee SH, Choi IA, Lee YA, et al. Human leukocyte antigen-B*2705 is the predominant subtype in the Korean population with ankylosing spondylitis, unlike in other Asians. *Rheumatol Int*. 2008;29:43-46.
42. Ma HJ, Hu FP. Diversity of human leukocyte antigen -B27 alleles in Han population of Hunan province, southern China. *Tissue Antigens*. 2006;68:163-166.
43. Mou Y, Wu Z, Gu J, et al. HLA-B27 polymorphism in patients with juvenile and adult-onset ankylosing spondylitis in Southern China. *Tissue Antigens*. 2010;75:56-60.
44. Yang G, Deng YJ, Yan CX, et al. Frequencies distribution of human leukocyte antigen-B27 subtypes in healthy Chinese. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*. 2006;28:240-243.
45. Grubić Z, Kerhin-Brkljacić V, Perić P, Cecuk E, Kastelan A. Variation in HLA-B27 gene subtypes and susceptibility of ankylosing spondylitis in the Croatian population. *Reumatizam*. 2001;48:7-11.
46. Fouladi S, Adib M, Salehi M, Karimzadeh H, Bakhshiani Z, Ostadi V. Distribution of HLA-B*27 alleles in patients with ankylosing spondylitis in Iran. *Iran J Immunol*. 2009;6:49-54.
47. Liu Y, Jiang L, Cai Q, et al. Predominant association of HLA-B*2704 with ankylosing spondylitis in Chinese Han patients. *Tissue Antigens*. 2010;75:61-64.
48. Alaez C, Gazit E, Ibarrola B, et al. Distribution of HLA-B27 subtypes in ankylosing spondylitis in an Israeli population. *Arch Med Res*. 2007;38:452-455.
49. Krylov MY, Reveille JD, Alexeeva LI, Erdesz S, Arnett FC, Benevolenskaya LI. HLA-B27 subtypes among the Chukotka Native groups. *Arch Immunol Ther Exp (Warsz)*. 1995;43:135-138.
50. Erdes S, Gusev IA, Krylov M, Benevolenskaia L. Spondyloarthropathies and HLA-B27 subtypes in the populations of the Russian North. *Ter Arkh*. 1997;69:41-43.
51. Baech J, Schmidt-Olsen S, Steffensen R, Varming K, Grunnet N, Jersild C. Frequency of HLA-B27 subtypes in a Danish population and in Danish patients with ankylosing spondylitis. *Tissue Antigens*. 1997;49:499-502.

How to cite this article: Chheda P, Warghade S, Mathias J, et al. HLA-B27 testing: A journey from flow cytometry to molecular subtyping. *J Clin Lab Anal*. 2018;32:e22382. <https://doi.org/10.1002/jcla.22382>