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Accurate and Simple Interpretation of HLA B27 Screening by Flow Cytometry

Akan Hücre Ölçer ile HLA B27 Tarama Testinin Doğru ve Kolay Yorumu

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Objectives: This study aims to compare the results of the human leukocyte antigen (HLA) B27 screenings by flow cytometry with results obtained by the sequence-specific primer-polymerase chain reaction (SSP-PCR) HLA B27 analysis.

Patients and methods: Between January 01st, 2008 and December 31st, 2008, the flow cytometry screening results of HLA B27 analysis for 486 samples were retrospectively analyzed. The results were compared with those obtained by the SSP-PCR for the diagnostic accuracy.

Results: The sensitivity of HLA B27 screening test by flow cytometry was 99% while the specificity was 100%. For our study population, while positive predictive value was 0.99 for HLA B27 screening for cytometry; negative predictive value was 1. HLA B7 positivity is detected in 0.8% of all patients.

Conclusion: Our study results suggest that accurate flow cytometric screening test for HLA B27 is a reliable screening test with high specificity and sensitivity and can be safely used by the clinicians.

Key words: Ankylosing spondylitis; cytometry; HLA B27.

Amaç: Bu çalışmada akım sitometrisi ile insan lökosit antijeni (HLA) B27 tarama sonuçları, sekans spesifik primer-polimeraz zincir reaksiyonu (SSP-PZR) HLA B27 analizi ile elde edilen sonuçlar ile karşılaştırıldı.

Hastalar ve yöntemler: 01 Ocak 2008 - 31 Aralık 2008 tarihleri arasında 486 örneğin akım sitometrisi ile HLA B27 tarama sonuçları geriye dönük olarak incelendi. Sonuçlar, tanısal doğruluk açısından SP-PZR ile elde edilen sonuçlar ile karşılaştırıldı.

Bulgular: Akım sitometrisi ile HLA B27 tarama testinin duyarlılığı %99 iken, özgüllüğü %100 idi. Bu durumda bizim örnek popülasyonumuzda akan hücre ölçer ile HLA B27 ölçümünün pozitif kestirim gücü 0.99 iken, negative kestirim gücü 1 idi. Olguların %0.8'inde HLA B7 pozitifliği saptandı.

Sonuç: Çalışma sonuçlarımız, doğru akım sitometrisi ile HLA B27 tarama testinin yüksek duyarlılık ve özgüllüğü olan güvenilir bir tarama testi olduğunu ve hekimler tarafından güvenli bir şekilde kullanılabileceğini göstermektedir.

Anahtar sözcükler: Ankilozan spondilit; sitometri; HLA B27.

The human leukocyte antigen (HLA) system coded by the major histocompatibility complex has highly polymorphic DNA region and many HLA antigens have been recognized to date. Of these antigens, several have been associated with various diseases. The HLA B27 antigen and ankylosing spondylitis (also known as Bechterew's Disease), Reiter syndrome, and acute anterior uveitis are the most known examples of this association.[1] The frequency of the HLA B27 in Caucasians is almost 9% and almost 90% of the ankylosing spondylitis patients are HLA B27 positive.[2] The HLA B27 antigen determination for ankylosing spondylitis is used by the clinicians, mostly for exclusion of the disease. The routine HLA typing tests, whether they are the classical lymphotoxicity testing, polymerase chain reaction (PCR), or DNA sequence analysis, are time consuming and expensive. Screening by flow cytometry is quite inexpensive and easy to perform compared aforementioned tests.[3,4] However, still, there are several issues that have to be considered and taken care of.[5,6] As has been published before,[7] the antibody used is

important for reporting reliable HLA B27 results.[8-10] We have used HLA-ABC-m³/BB7.1 clone in this study. The results achieved in different laboratories with different antibodies may confuse the clinicians. To prevent the confusion and to imply on accurate interpretation of the results, a systematic approach for interpretation is applied which is necessary for diagnostic accuracy. Since the HLA B27 is a member of the large HLA B7 cross-reacting group (CREG), the inclusion of the HLA B7 to the monoclonal antibody combination provides more specific information at a tolerable higher cost.[11]

In this article, we compared the results of HLA B27 screenings by flow cytometry with results obtained by the sequence-specific primer (SSP)-PCR HLA B27 analysis.

PATIENTS AND METHODS

This retrospective analysis included list mode files of flow cytometry tests and PCR results (where applicable) of 486 samples submitted to Centro Laboratories, Istanbul, Turkey. On routine basis, all of the samples were collected in purple top Vacutainer tubes (Becton, Dickinson and Company (BD) Biosciences, New Jersey, USA) containing K2EDTA and processed within 24 hours. Samples containing aggregates with less volume than requested and hemolysis were rejected.

Flow cytometry measurements

All of the measurements were performed on FACS Calibur flow cytometry system (Becton, Dickinson and Company (BD) Biosciences, New Jersey, USA) which was a four color system with 488 nm argon ion laser. Daily quality control of the system was checked with CaliBrite beads and followed up with Levey Jennings graphics by applying Westgard's rule.[12] HLA B27- fluorescein isothiocyanate (FITC)/HLA B7-PE antibodies (Clone HLA-ABC-m³/BB7.1) were obtained from the Beckman Coulter, USA. Proficiency test results were compliant (INSTAND, Germany) for twice yearly participations for the past three years. Samples were prepared by certificated

and well trained flow cytometry technicians complying to standard operation procedures in accordance with the recommendations of the manufacturer. Briefly, cell counts were performed from all samples, when necessary, samples were diluted with PBS containing 2% bovine serum albumin (BSA) to a concentration of <10,000 cells/microliter. 100 microliter of whole blood collected into EDTA containing Vacutainer tubes was added onto 20 microliter of HLA B27/HLA B7 monoclonal antibody.
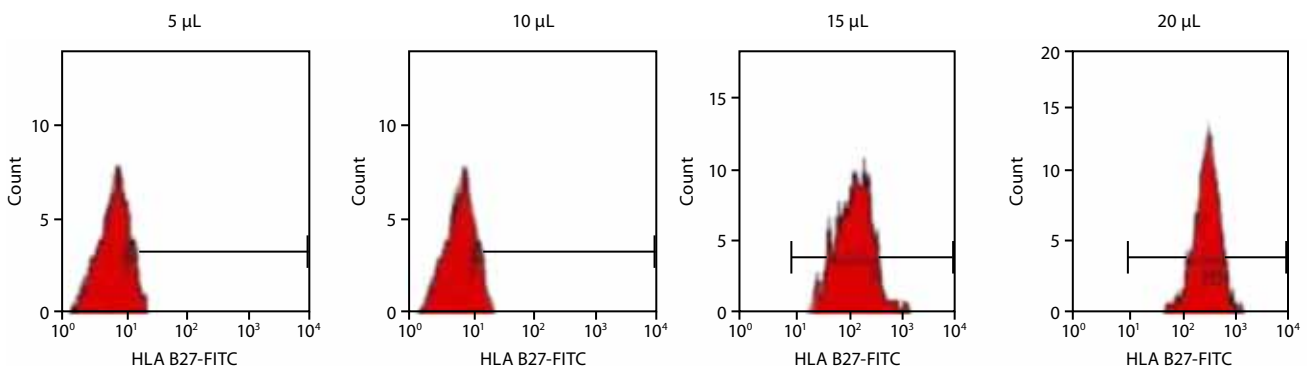
Titration of antibodies

Antibodies were titrated as described previously.[13] Briefly, a known HLA B27 positive sample was used for titration of antibodies. A serial dilution of antibodies was pipetted (20 µl, 15 µl, 10 µl, 5 µl) in four different concentrations and 100 µl whole blood was added onto each tube. Sample was processed with lyse/wash method. The mean channel changes were compared and most suitable antibody concentration (20 µl) was used for all of the sample preparations (Figure 1).

Tubes were incubated at room temperature in dark for 20 minutes and then 2 mL of lysis solution (NH4Cl2) was added to eliminate the erythrocytes from analysis. After 10 minutes of incubation cells were washed twice (400 g) and pellet was re-suspended in PBS solution to a final volume of 400 microliters. For running the samples, a protocol consisting forward scatter/side scatter (FS/SS), fluorescence 1 channel/fluorescence 2 channel (FL1/FL2) (HLA B27-FITC/HLA B7-PE) scattergrams together with FL1 and FL2 histograms were used. A lymphocyte gate on FS/SS scattergram was used to analyze the HLA B27 and B7 positive values.

We grouped the results in three categories:

1. <70% positive (negative)
2. 70-90% positive (grey zone)
3. >90% positive (positive)

Figure 1. Titration of antibodies for HLA B27. 20 µL as per the manufacturer recommendations was used.

The results in first group were evaluated as negatives and did not require any further confirmation with SSP-PCR. Those which had 70-90% positivity were always need to be confirmed with SSP-PCR and the results with >90% positivity and mean channel value >100 on a logarithmic scale of 1024 channels were evaluated and reported as HLA B27 positive.

Polymerase Chain Reaction Tests

The SSP-PCR method employs pairs of PCR primers which their 3' nucleotide or nucleotides are complementary to a polymorphic position which distinguishes an allele or allele group from other alleles. If the sample has the allele of interest, PCR will lead to a product whose size and presence will be identified by agarose gel electrophoresis. Due to the large number of allele groups in HLA-B27, about 96 reactions are necessary to perform a low-resolution HLAB27 typing for a single patient. In our study, SSP-PCR method was used for the confirmation of results obtained from flow cytometric screening of

HLA B27, when the results were in 70-90% range. HLA B27 SSP-Low resolution kit was obtained from One Lambda, (One Lambda, Canoga Park, CA, USA). Deoxyribonucleic acid (DNA) isolation of the HLA B27 samples was done with genomic DNA isolation kit (Roche Diagnostics, Basel, Switzerland). The quality assessments of the DNA isolates were performed by measurements with spectrophotometry (A260/A280). Those who complied with 25-200 ng/mL were used for PCR analysis. The PCR was performed with ABI 2700 system (Applied Biosystems, Foster City, CA, USA) by a different technician blind to result of the flow cytometry analysis. After running the PCR, the amplified DNA fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (Sigma-Aldrich Co., St. Louis, MO, USA) and exposed to the ultraviolet light. The interpretation of results was based on the presence or absence of a specific amplified DNA fragment. All of the gels obtained from the agarose gel electrophoresis were photographed.
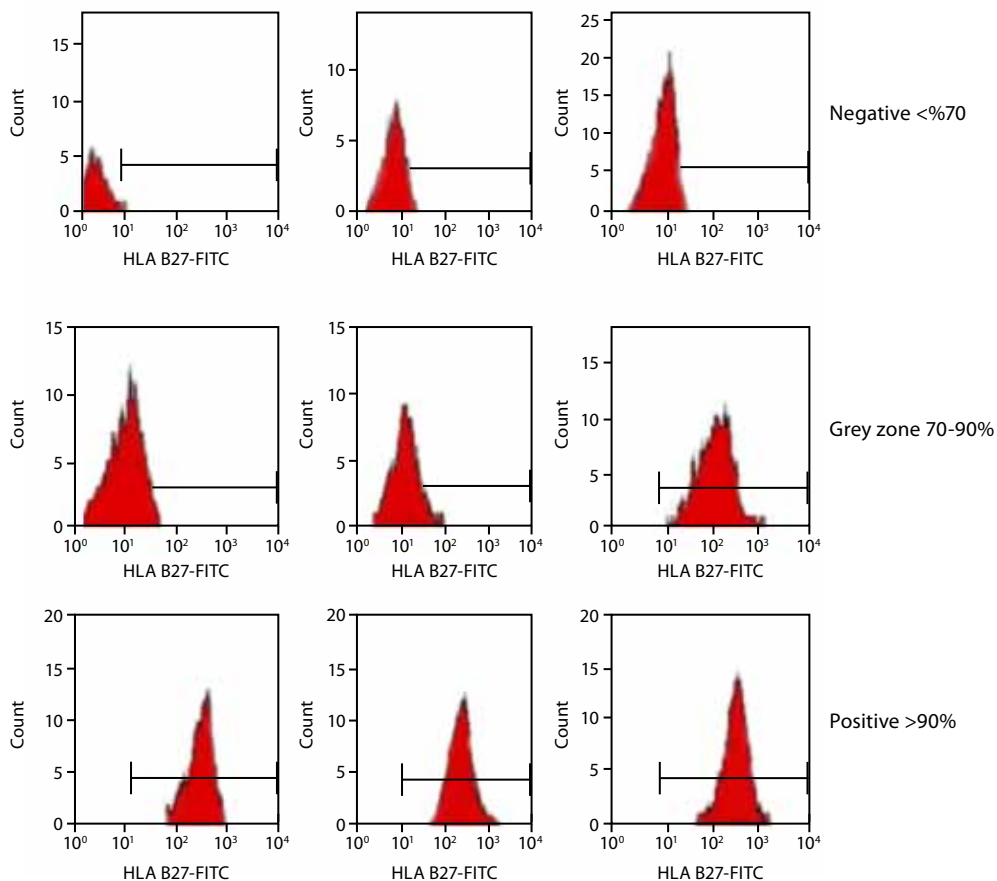
Figure 2. Variable staining patterns of HLA B27. As seen above, true positive samples always have bright staining observed after second decade, samples with 70 to 90% positivity are mostly observed between the first and second decade.

RESULTS

This retrospective study was assessed by analyzing 486 HLA B27 screening results by flow cytometry and comparisons of the immunophenotyping results with SSP-PCR from the patient samples submitted to the Centro Laboratories within one year. Age, sex, and clinical and other laboratory findings related to these samples were not in scope of this study. The samples not complying with sample inclusion criteria and samples taken more than six hours ago were excluded.

As illustrated in Figure 2, staining patterns for the HLA B27 shows variability. All of 20 randomly selected samples which were >90% positive in flow cytometric analysis were found to be positive for HLA B27 with SSP-PCR. Of the samples which had 70-90% positivity, only 2% had HLA B27 positivity by SSP-PCR (Table 1). Sequence-specific primer-polymerase chain reaction results were negative for all samples with less than 70% positivity.

By applying an evidence based laboratory medicine approach, sensitivity and specificity of the HLA B27 screening by flow cytometry is calculated.[14] The sensitivity of the HLA B27 screening by flow cytometry was 0.99 (0.97-0.99 with 95% confidence interval; CI), while the specificity was 1 (0.97-1 with 95% CI). We also calculated the prevalence for our patient group and found 0.66 (0.62-0.70 with 95% CI). Based on these values, the probability of any test result to be positive is 66%, while it is 34% for negative results. By using the SSP-PCR for those tests in 70 to 90% positivity detected by cytometry, it is possible to have a zero probability of a false positive result (0-0.14 with 95% CI). For any particular positive test result, the probability of true positivity is 1 (0.98-1 with 95% CI). Similarly, for the negative results, the probability of true negativity is 0.98 (0.95-0.99 with 95% CI), while the probability of a false negative test result is 0.012 (0.002-0.047 with 95% CI) (Table 2).

Based upon these findings, positive likelihood value calculated by conventional method is infinite, while the negative likelihood value is 0.006 (0.001-0.24 with 95% CI). If we calculate the likelihood ratios weighted by prevalence, the positive likelihood ratio is still infinite and negative likelihood ratio changes to 0.01 (0.003-0.48 with 95% CI). The positive predictive value of HLA B27 screening by flow cytometry was 0.99, while the negative predictive value was 1. Ninety-five percent CIs for the proportions are calculated according to the efficient-score method described by Nemcombe.[15] Of all samples, 0.8% was positive for HLA B7.

DISCUSSION

Based on our results, there is a need to consider several issues in flow cytometric analysis for HLA B27 and HLA B7. Titration of the antibodies is important, since the use of low concentrations may lead to false negative results. Each flow cytometry laboratory needs to evaluate the antibody concentrations by titrations. Lower amounts of antibodies without titrations should be also avoided. This approach would improve the performance of cytometry laboratory to eliminate false negative results.

TABLE 1

HLA B27 results by flow cytometry and sequence-specific primer-polymerase chain reaction

HLA B27 Range in cytometric analysis	Number of samples	%	B27 positivity with SSP-PCR
<70%	103	21.2	0
70-90%	62	12.8	2
>90%	321	66	20/20*
Total	486	100	

* Twenty randomly selected samples with >90% HLA B27 positivity were analyzed with sequence-specific primer-polymerase chain reaction

TABLE 2

Diagnostic accuracy values calculated based on the values given in this table

Test result	Present	Absent	
Positive test	True positive 321	False positive -	Total positive 321
Negative test	False negative 2	True negative 163	Total negative 165
	Total with polymorphism 323	Total without polymorphism 163	
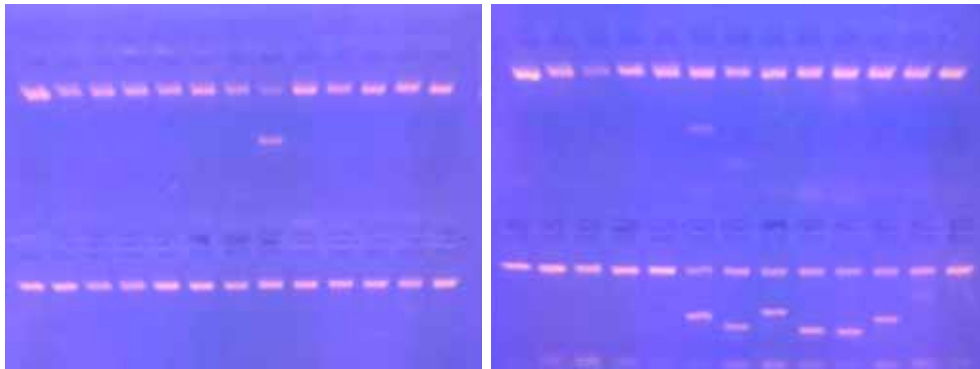
Figure 3. HLA B27 negative and positive SSP-PCR samples, each gel represents one patient sample.

The HLA B7 is the most important cross-reacting antigen for HLA-ABC-m3 antibody, using a dual color antibody recognizing both antigens on the same cell population allows the detection of the false positive results. The HLA-ABC-m3 antibody is known to fail reaction with HLA B27*03 subtype; however, this is not an issue for our study population, as it has been shown that this variant is not encountered in Caucasian population.[16]

All phases of our study complies with criteria required by the Standards for Histocompatibility Testing published by the European Federation for Immunogenetics.[17] As per protocol, the results which are interpreted with the percentage only may lead to false positive results. This can also be misleading for the physicians who do not have excessive knowledge on the interpretation of the flow cytometry results. The generally accepted cut-off value for immunophenotyping with human cells is to accept values above 2% of negative control as positive. However, this rule does not apply to HLA B27 immunophenotyping. For example, 60% may sound as a positive result for HLA B27 to a clinician; however, the same sample would be negative by HLA B27 SSP-PCR analysis. As a result, each and every flow cytometry laboratory reporting HLA B27 needs to determine a cut-off value and it should be as high as >90% when the interpretation of results is based on the percentage positivity. Furthermore, the positivity needs to be a bright one and should be observed after the second decade (>100 mean channel value) on a logarithmic scale histogram compared to the negative control. Once these requirements are met, SSP-PCR and flow cytometry results would be in full compliance with each other (Figure 3).

The quantitative analysis of fluorescence can be the choice of method for screening HLA B27 by cytometry. However, we were unable to present the quantitative data for this retrospective analysis due to fact that we did have any data for the fluorescence calibration with commercial kits for each of the days samples were run.

It would be also difficult to have consistent quantitative results due to the alterations in the calibration beads from lot to lot.

Several researchers have demonstrated that the verification of HLA-B27 positivity by an independent technique is required in case of a positive result obtained by the flow cytometry.[18,19] Our results show that the need to confirm with molecular methods such as SSP-PCR[20] may be avoided using a systematic algorithm approach. On the other hand, for those samples with 70 to 90% positivity and mean channel values <100 compared to the negative control or autofluorescence by flow cytometry screenings, we suggest to confirm these results by SSP-PCR and report results afterwards, also. For the quality and reliability of accurate HLA B27 screening by cytometry, well-calibrated systems and applications of good laboratory practice are essential.

For the utilization of a diagnostic test, clinicians use several tests as modifiers of disease probabilities to convert the pre-test probability information to post-test probability estimate of a certain target condition. For making this judgement, it is essential to know the diagnostic accuracy of that test, i.e. the sensitivities and specificities, or the odds ratios of the tests, which enable them to transform pre-test data into clinically meaningful information.[21]

In conclusion, our study results suggest that accurate flow cytometric screening test for HLA B27 is a reliable screening test with high specificity and sensitivity and can be safely used by the clinicians.

Declaration of conflicting interests

Funding

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