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Homologous Blood Transfusion (HBT) test by cytometry for Chinese and non-Chinese population

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Abstract

Purpose: To investigate the suitability of HBT test for Chinese and non-Chinese population. Methods: Blood samples from Chinese and non-Chinese groups were tested using flow cytometry with eight antigens including C, c, E, Fy^a, Fy^b, Jk^a, Jk^b and S, which are used in our present HBT routine test. Sensitivity, specificity, robustness, precision and stability were statistically evaluated. Results: The expressing frequencies of these antigens in Chinese population were quite different from that of non-Chinese population. The statistics resulted in high possibility of that two or more people have the identical expression phenotypes in both Chinese and non-Chinese groups. Conclusion: Because of the difference of RBCs phenotypes expression for Chinese and non-Chinese, further study is necessary to investigate the blood group phenotypes of different ethnic groups in order to make this HBT method more suitable for most populations.

Introduction

Blood transfusion in athletes for non-pathological reason has been forbidden by IOC and WADA since 1988. Recent years, as a result of the development of detecting methods in erythropoietin [1] and blood substitutes [2], blood transfusion is again being utilized. [3]

In 2002 a method for detecting homologous blood transfusion was developed in Australia [4]. It was based on the genetic theories with serological and immunological approaches to detect

homologous transfusion using flow cytometry directly.

Because of the large number of blood group antigens on RBCs and their variability among individuals, it is impossible to transfuse blood completely matched from another person (except an identical twin). [5] These differences provide a possibility to distinguish the different RBC groups by using a flow cytometor with proper staining.

This study investigated the suitability of HBT test for Chinese and non-Chinese populations.

Materials and methods

Subject

In pilot study venous bloods obtained from 12 Chinese volunteers without blood transfusion during the last half year were analyzed by flow cytometor and ABO blood groups test card as single bloods. These 12 bloods were spiked in different proportion (5%, 3%, 1.5% and 0.5% minor RBCs populations) to each other with a same ABO phenotype. Then 34 blood samples, in addition to the12 original pilot study blood samples, were obtained as mixtures.

Other 59 blood samples were obtained from 59 Chinese volunteers marked as Group A and 59 blood samples from non-Chinese individuals marked as Group B. All samples listed in Table 1, were analyzed by flow cytometor.

Table 1: Sample information

Group	Chinese/non-Chinese	Single/Mixed RBCs		Count of Samples
Pilot	Chinese	Single		12
	Chinese	Mix	5%	8
	Chinese		3%	8
	Chinese		1.5%	8
	Chinese		0.5%	10
A	Chinese	Single		59
B	Non-Chinese	Single		59

RBCs staining

The blood anti-coagulated in an EDTA-K syringe was stabilized by diluting 100µl aliquots in 1ml of CellStab (DiaMed, Cressier/Morat, Switzerland) which was a low ionic RBCs

stabilization buffer known to enhance agglutination. RBCs in suspension were counted with an automatic hematological analyzer (Sysmex XE-2100) before storage at an approximate 4℃ refrigerator.

The standard RBCs (DiaMed, Cressier/Morat, Switzerland) were used to control each antibody's specificity and identification capabilities and determinate the optimum conditions to separate non-expressing from expressing RBCs groups. According to the Nelson and Giraud's protocol [6, 7, 8], RBCs suspensions were diluted with flow buffer (PBS(10mM, pH 7.4; Sigma, Switzerland) +0.1% of Bovine Serum Albumin (BSA, Sigma)+0.1% sodium azide (Sigma)) to make the RBCs concentration to 5×10^4/μl. A 0.1 ml of this suspension was washed with a centrifuge-washing machine (CentWash: DiaCentCW, DiaMed) to ensure the RBCs crowd together on the bottom of the tube. Then RBCs were labeled with 50 μl primary antibodies respectively, incubating at room temperature in the dark for 90 min and washing with CentWash, and with 50 μl second antibodies in refrigerator for 45 min. After washing with CentWash again, RBCs were diluted with 0.1 ml flow buffer and with 1ml flow buffer before analysis with flow cytometer (Cytomics FC500, Beckman Coulter). Table 2 shows the dilution of all antibodies in use.

Quality controls

For each sample, an anti-CD235a and its isotypic antibody mouse IgG1 were used as quality controls to verify that only RBCs were analyzed. A blank control was used to show the place of non expressing RBCs group in the histogram.

Table 2: Information of antibodies and quality controls

Name	Dilution factor	Isotype	Source
Primary antibodies			
DiaClon anti-C	10	Human IgM	DiaMed
DiaClon anti-c	50	Human IgM	DiaMed
DiaClon anti-E	25	Human IgM	DiaMed
Anti-Jk^a	5	Human IgM	DiaMed
Anti-Jk^b	20	Human IgM	DiaMed
Anti-Fy^a	5	Polyclonal IgG	DiaMed
Anti-Fy^b	10	Polyclonal IgG	DiaMed
Anti-S	10	Polyclonal IgG	DiaMed

Mouse IgG1-FITC	10	Mouse IgG1	DiaMed
Anti-CD235a-FITC	10	Mouse IgG1	DiaMed
Second antibodies			
Anti-human IgG-FITC	200	Goat serum	Serotec
Anti-human IgM-FITC	100	Goat serum	Serotec

Validation

Two single bloods from these 12 samples for pilot study and two samples （mixed with 3% minor RBCs populations） from these 34 mixed blood samples were stabilized for 4 weeks and analyzed by an operator before and after these 4 weeks to see the stability of these samples in 4 weeks. Other two single bloods from these 12 samples and other four samples mixed with 5%, 3%, 1.5% and 0.5% minor RBCs were analyzed respectively by three operators to evaluate the variation between operators. All these 46 samples (12 single bloods and 34 mixed blood samples) for pilot study were analyzed by the three operators, too. The results of the pilot study were used to validate the method in the aspects of stability, robustness, sensitivity, specificity and carry-over. Then all other 118 samples (59 samples from each of Group A and B respectively) were analyzed using the same validated method to compare the difference between Chinese and non-Chinese populations.

Results

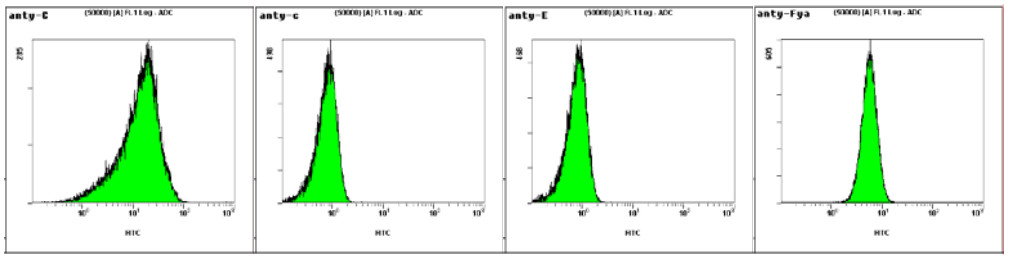
Pilot study

The stability results showed inconspicuous changes of blood samples before and after 4 weeks stored in 4℃ refrigerator. The results provided by 3 operators did not show any noticeable difference for analyzing these 6 samples containing 2 single and 4 mixed bloods (with 5%, 3%, 1.5% and 0.5% minor RBCs) in different days without any false positive or false negative. All 12 single bloods among 46 samples for pilot study (Table 1) were correctly reported as negative, so the specificity in this study is 100%. Among the 34 mixed samples from 46 samples mentioned in Table 1, 6 mixed with 0.5% minor RBCs were falsely reported as negative. So the percentage of observed positive samples among predicted positive samples

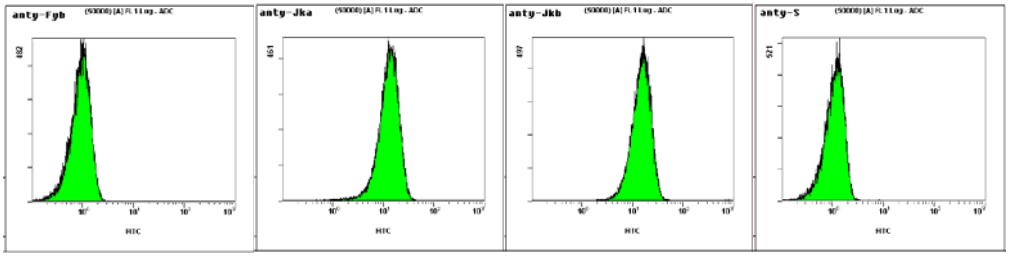
is 82.4%. In our experiment, the sensitivity was dependent on the phenotype and its antibody. Our experiment showed only lower than 1% of carry-over.

46 blood samples for pilot study were analyzed with eight RBC antigens in Table 3 by flow cytometor. 3, 3 and 2 among 12 Chinese individuals for pilot study were confirmed with same phenotypes including ABO, Rh(D) and eight RBC antigens in 3 different combinations respectively. See Table 3 and Figure 1.

a

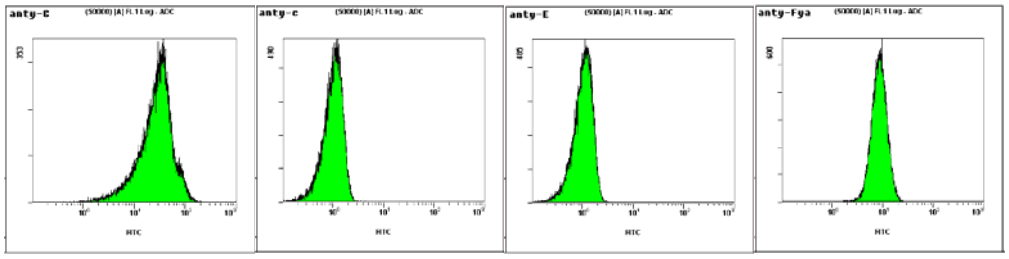


C (+) c(-) E(-) Fy^a(+)

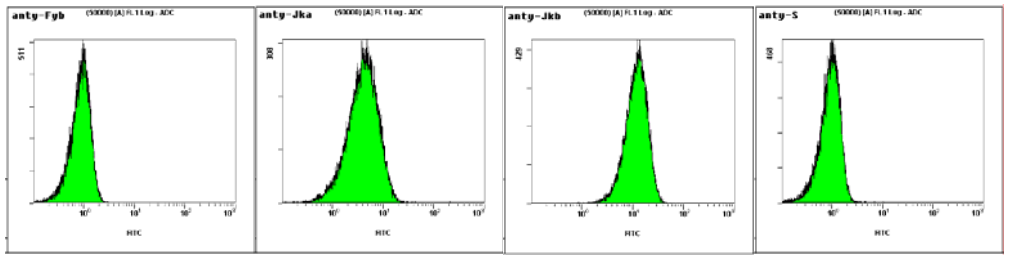


Fy^b(-) Jk^a(+) Jk^b(+) S(-)

b



C (+) c(-) E(-) Fy^a(+)



Fy^b(-) Jk^a(+) Jk^b(+) S(-)

Figure 1: Blood phenotypes of two Chinese volunteers
Fluorescence histograms of a and b showed two Chinese persons' phenotypes of eight blood group antigens. The phenotypes of a and b were entirely identical. "(+)" means expressing, "(-)" means non-expressing.

Table 3: Combinations and percentage of the repeated phenotypes in 12 Chinese
"+" means expressing of the phenotype; "-" means non-expressing of the phenotype.

Combination	C	c	E	Fya	Fyb	Jka	Jkb	S	Number of individuals	Percentage (%)
1	+	+	+	+	-	+	+	-	3	25
2	+	-	-	+	-	+	+	-	3	25
3	+	+	+	+	-	+	-	-	2	16.67
total									8	66.67

Prevalence of phenotypes expression based on Group A and Group B comparison

Table 4 showed the phenotype expressing frequency for Group A (Chinese). It could be found that the expressing frequency was very high (0.92) in Fy^a, and very low (0.05 and 0.07) in Fy^b and S. Table 5 was the phenotype expressing frequency for Group B (non-Chinese). It showed that each phenotype expressing frequency was moderate (approximately from 0.3 to 0.8). Figure 2 showed significant difference in these RBC antigens (except Jk^a and Jk^b) between Group A and B.

Table 4: Statistics of the phenotypes expressing frequency for Group A

Red cell antigens	C	C	E	Fy^a	Fy^b	Jk^a	Jk^b	S
Number of expressing sample	50	34	33	54	3	44	46	4
Number of non-expressing sample	9	25	26	5	56	15	13	55
Expressing frequency	0.85	0.58	0.56	0.92	0.05	0.75	0.78	0.07

Table 5: Statistics of the phenotypes expressing frequency for Group B

Red cell antigens	C	C	E	Fy^a	Fy^b	Jk^a	Jk^b	S
Number of expressing sample	34	46	16	35	33	39	48	28
Number of non-expressing sample	25	13	43	24	26	20	11	31
Expressing frequency	0.58	0.78	0.27	0.59	0.56	0.66	0.81	0.47

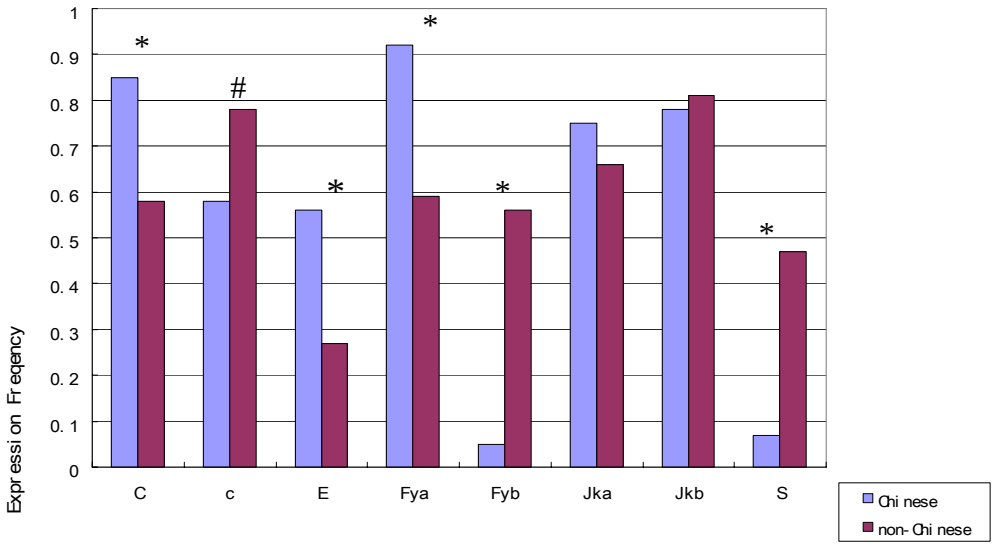


Figure 2: The expressing frequency of 8 antigens for Group A (Chinese) and Group B (non-Chinese), Symbol # indicated P<0.05, Symbol * indicated P<0.01, Chi-square Test.

Statistically, there should be maximum 256 different combinations of expressing or non-expressing the 8 antigens. Among 59 individuals in Group A only 21 combinations were found. 10 of these 21 combinations related to total 48 individuals. In other words, 48 among these 59 individuals showed total identical phenotype with, at least, other one. There were 13 of 38 combinations related to34 individuals among 59 individuals in Group B. Table 6 and 7 showed the phenotypes and number of the repeated combinations in Group A and B. Table 8 displayed the comparison of some statistical values between Group A and B.

Table 6: Repeated combinations in Group A (59 Subjects in total)
"+" means expression of the phenotype; "-" means no-expression.

Combination	C	c	E	Fya	Fyb	Jka	Jkb	S	Number of samples	Percentage (%)
1	+	-	-	+	-	+	+	-	11	18.64
2	+	+	+	+	-	+	+	-	8	13.56
3	+	-	-	+	-	+	-	-	7	11.86
4	-	+	+	+	-	-	+	-	4	6.78
5	+	+	+	+	-	-	+	-	4	6.78
6	+	+	+	+	-	+	-	-	4	6.78
7	-	+	+	+	-	+	+	-	3	5.08
8	+	-	-	+	-	-	+	-	3	5.08
9	+	+	+	+	-	+	+	+	2	3.39
10	+	-	+	+	-	+	+	-	2	3.39
total									48	81.36

Table 7: Repeated combinations in Group B (59 subjects in total)
"+" means expression of the phenotype; "-" means no-expression.

Combination	C	c	E	Fya	Fyb	Jka	Jkb	S	Number of samples	Percentage (%)
1	-	+	-	+	-	-	+	+	5	8.47
2	+	+	-	-	+	+	+	-	4	6.78
3	+	-	-	+	-	+	+	-	3	5.08
4	+	+	-	-	+	-	+	+	3	5.08
5	-	+	+	+	+	+	+	+	3	5.08
6	-	+	+	+	-	+	+	-	2	3.39
7	+	-	-	+	-	-	+	-	2	3.39
8	+	-	-	+	-	+	+	+	2	3.39
9	+	+	-	+	-	+	+	-	2	3.39
10	+	+	-	+	+	+	+	+	2	3.39
11	+	-	-	+	-	-	+	-	2	3.39
12	+	+	-	+	-	+	+	-	2	3.39
13	+	+	+	+	+	-	+	-	2	3.39
total									34	57.63

Table 8: Comparison of the combinations between Group A and B

Repeated times of one combination	Group A		Group B	
	Number of combinations	Number of samples	Number of combinations	Number of samples
11	1	11	--	--
8	1	8	--	--
7	1	7	--	--
5	--	--	1	5
4	3	12	1	4
3	2	6	3	9
2	2	4	8	16
1	11	11	25	25
total	21	59	38	59

Discussion

A flow cytometor is a useful and sensitive instrument that analyzes light emitted from cells or particles individually as they pass in a fluid stream through a beam of laser light. It has been comprehensively and practically applied in modern physic and recently in detecting blood doping. [4, 5, 6, 7, 9]

In pilot study the experiment evaluated the robustness, sensitivity, specificity, carry-over and stability of the HBT test and gained similar result with some other researchers [8]. It

displayed that the method of HBT test by flow cytometor is quite robust for different laboratories.

The same combinations existed in the pilot study aroused our attention, additional research has to be carried out to observe the difference between Chinese and non-Chinese in blood phenotypes.

The results in pilot experiment revealed that the phenotypes of the eight antigens repeated in 8 people and there were 3 people with entirely the same RBC antigen phenotypes detected. The combination type of these 3 people was C(+), c(-), E(-), Fy^a(+), Fy^b(-), Jk^a(+), Jk^b(+) and S(-), which was the most common combination in Group A(11 in 59 Chinese) and was also existent in Group B (3 in 59 non-Chinese). However, the most common combinations, such as C(-), c(+), E(-), Fy^a(+), Fy^b(-), Jk^a(-), Jk^b(+) and S(+), in 59 non-Chinese did not exist in 59 Chinese at all. These differences between Chinese and non-Chinese groups may be mainly brought by the differences of ethnics. In another word, different ethnics lead to the variety of RBCs phenotypes.

The ethnic difference also existed in expressing frequency of blood phenotypes. The phenotype expressing frequency of RBC antigens C, E and Fy^a in Chinese (0.85, 0.56 and 0.92) were much higher than that in non-Chinese (0.62, 0.29 and 0.57) ($P<0.01$), and the expressing frequency of c, Fy^b and S in Chinese (0.58, 0.05 and 0.07) were quite lower than that in non-Chinese (0.82, 0.68 and 0.51) ($P<0.01$). It indicated that the expressing frequency of red cell blood groups existed ethnic variety, and that just using these eight antigens especially Fy^a, Fy^b and S may be not suitable to effectively detect HBT for Chinese population due to their much high or low expressing frequency in Chinese.

The WADA definition of an AAF for the HBT test is that a sample presents more than one expression phenotype for a particular RBC antigen, thus indicating a mixed RBC population. [10] So it is most important to select proper RBC antigens as the targets in order to make this HBT test more sensitive to distinguish different RBC populations. At present, scientists and organizations that research and use the HBT test selected the following RBC antigens.

Table 9: The Antigens Implemented for HBT Test

Scientists / Organization / Games	RBC antigens
Nelson[4, 6, 7]	C, c, E, e, K, k, Jk^a, Jk^b, Fy^a, Fy^b, S, s and M
2004 Athens Olympics	C, c, E, Jk^a, Jk^b, Fy^a, Fy^b, M, N and S
Lausanne laboratory[8]	C, c, E, Jk^a, Jk^b, Fy^a, Fy^b and S
Patricia[5]	A, B, D, C, E, c, e, M, N, S, s, K, k, Fy^a, Fy^b, Jk^a and Jk^b
WADA educational HBT test[10]	C, c, E, e, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s and P1

Because of the importance in blood transfusion test, the antigens in Rh, MNS, Duffy and Kidd blood group systems were chosen in HBT test by most researchers and organizations from Table 9. Another reason to choose these RBC antigens was that the phenotype expressing frequencies of these antigens were not too high or too low in their racial groups. According to the two points, it was possible to detect homologous blood transfusion by observing different RBC populations in blood. Theoretically, the closer to 0.5 the RBC antigen's expressing frequency is, the more possible distinguishing the mixed bloods will be. But the reality is that it is hard to find a blood group antigen with the same moderate expressing frequency in all ethnics. It seems that the best way is increasing the number of RBC antigens to reduce the false negative frequency. However, too many antigens may waste time and lead low detection efficiency. Thus further experiments should be carried out to select and evaluate the RBC antigens for different ethnics.

Conclusion

Because of the difference of RBCs phenotypes expression for Chinese and non-Chinese, it seems that using just 8 blood antigens can not entirely distinguish mixed RBCs for different ethics, so further study is necessary to investigate the blood group phenotypes for most races.

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