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Homologous blood transfusion testing in NDTL, India: A Case study

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Introduction

Blood doping is an illegal method to increase hemoglobin level which increases the oxygen concentration of arterial blood and therefore aerobic capacity [1]. Blood transfusion methods have been banned by IOC since 1988. The analysis of RBC antigen expression patterns by flow cytometry is able to reveal the presence of mixed RBC populations in transfused athletes and hence confirms the homologous blood transfusion (HBT).

The present paper gives report of Homologous Blood Transfusion Testing carried out at NDTL, India for Singapore Youth Olympic Games (SYOG), Commonwealth Games (CWG) and 2nd Asian Beach Games (ABG) during 2010 (August to December). It also includes the case study about the blood profile of a sample otherwise tested negative for blood transfusion.

Materials and Method

The blood transfusion testing was carried out by the method of S. Giraud *et al.* [2] which was validated and included in the ISO 17025; 2005 scope of testing of NDTL. The cell stabilization buffer and Control RBCs (Diapanel) were procured from DiaMed, Cressier/Morat, Switzerland. Phosphate buffer saline tablets and bovine serum albumin were purchased from Sigma-Aldrich, St. Louis, USA and sodium azide of AR Grade from Merck, India. Same lots of primary antibodies (Diamed, Cressier/Morat, Switzerland) and Secondary antibodies (Beckman Coulter International, Nyon, Switzerland) were pooled and optimized for its sensitivity and dilution factor.

Sample Reception

Blood samples from Singapore Youth Olympic Games (SYOG) were received within 24 hours of collection by NDTL, India by DHL Courier in a box at a temperature of 2 to 8 ^{o}C. During Commonwealth Games (CWG) samples were received through persons of the Organizing committee every morning and evening in the boxes with a data logger (2 to 8 ^{o}C). The blood samples from 2nd Asian Beach Games 2010 (18 samples) were received through courier in more than 48 hours of collection. Out of 125 blood samples received from SYOG-2010, 31 samples (16 Male & 15 Female) were for blood transfusion test. Out of 187 blood samples received from CWG-2010, 65 samples (34 Male & 31 Female) were for blood transfusion. Boxes were checked for any non conformity and tested accordingly. Out of 18 whole blood samples received from 2nd Asian Beach Games 2010, only five were found fit for analysis and hence tested for blood parameter and blood transfusion. Rest of the samples were received at NDTL in more than 48 hours of collection and were found hemolyzed.

Sample preparation & Analysis

Blood samples were homogenized / mixed on a roller mixer for 15 minutes and then analyzed on Sysmex XT 2000*i* for complete blood count (CBC) and reticulocytes. 150 µL aliquot of each whole blood sample was diluted in 1500 µL cell stabilization buffer in order to stabilize the sample. The stabilized samples were analyzed for RBC and then diluted in flow buffer accordingly to achieve a final suspension of 50 million RBC per mL. 100 µL RBC suspension (5 million RBC) pipetted in sarsted tubes and centrifuged on Diacent Cell wash machine and the supernatant liquid from tubes was discarded. The primary antibody (50 µL) was added in corresponding tube for staining RBC's and then kept for incubation for 90 minutes (room temperature) with intermittent shaking. RBC's were washed with normal saline, centrifuged and then 50 µL of corresponding secondary antibodies (IgM & IgG panel) were added. All the tubes were incubated for 45 minutes in dark (2-8 ^{0}C) with intermittent shaking. In the end RBC's were washed twice with normal saline and centrifuged. In order to re-suspend the RBC's pellets 100 µL flow buffer was added in each tube and vortexed. Before analysis of C, c, E (Rh group), Jk^{a}, Jk^{b} (Kidd group), Fy^{a}, Fy^{b}, (Duffy group) and S (MNS group) antigen on Beckman Coulter FC 500 Flow Cytometer, 1mL flow buffer added and sample was vortexed vigorously.

Results and Discussion

The quality control criteria were met in all the samples (101 samples) tested which was duly verified for only RBC analyzed by using an anti-CD235a and its isotypic antibody mouse IgG1. Results of the reading of fluorescence are expressed as a histogram distribution that put in relation the number of cells analyzed. All samples tested were found negative as per the WADA definition of full adverse analytical finding (AAF) for HBT test, which states that presence of more than one expression phenotype of particular RBC antigen indicates mixed population, The expression frequency for various antigens in the samples from SYOG, CWG and 2nd ABG testing are shown in Table 1. The expression frequency for Small c antigen (Rh group) was very high for both SYOG (0.90) & CWG (0.86) testing whereas the expression frequency was very low for Rh group antigen E 0.29 (SYOG) & 0.20 (CWG). The number of samples tested for 2nd Asian Beach Games is too less (5), hence not taken into consideration for these comparisons, though the results are given in Table 1.

Table 1: Phenotype Expression Frequency (EF) for SYOG, CWG and 2nd Asian Beach Games Testing of Blood Transfusion

	SYOG			**CWG**			**2nd Asian Beach Games**		
	Number of Samples			Number of Samples			Number of Samples		
Red Cell Antigens	Expressing	Non-Expressing	EF	Expressing	Non-Expressing	EF	Expressing	Non-Expressing	EF
C	22	9	0.7	31	34	0.48	5	0	1.0
c	28	3	0.9	56	9	0.86	3	2	0.6
E	9	22	0.29	13	52	0.2	2	3	0.4
Jk^a	27	4	0.87	49	16	0.75	2	3	0.4
Jk^b	22	9	0.70	43	22	0.66	5	0	1.0
Fy^a	18	13	0.58	27	38	0.42	5	0	1.0
Fy^b	20	11	0.65	27	38	0.42	0	5	0.0
S	14	17	0.45	28	37	0.43	1	4	0.2

The expression frequency of different antigens was found identical in both the games except for the Big C and Fy^b which was found to be significantly high for SYOG in comparison to CWG. Figure 1 shows the statistical significant difference between expression frequency of RBC antigens Big C and Fy^b for SYOG and CWG($P<0.05$, chi-square Test).

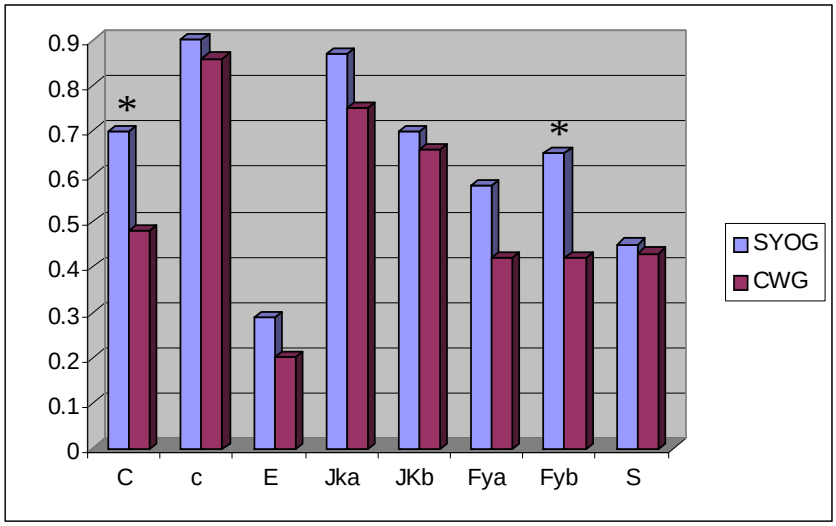

Figure1: Expression frequency of RBC antigens for SYOG and CWG testing (*indicates P<0.05, Chi Square Test)

The expression frequency among only five samples from 2nd Asian Beach Games for Big C, Jk^b and Fy^a is observed as 1.0 whereas for Fy^b it is zero. The different combinations of expressing and non-expressing antigens were evaluated in all the samples. It is observed that a total of 6 combinations were found in the 31 samples of SYOG and 11 combinations were found in 65 samples of CWG. However out of 17 repeated combinations one combination with all the eight antigens (expressing) was found common in SYOG (2 samples) and CWG (1 sample). The five samples of Asian Beach Games showed four types of combination out of which one was found in two samples of CWG.

The combination C (+), c (-), E (-), Jk^a (+), Jk^b (+), Fy^a (+), Fy^b (-), S (-) of CWG is found correlated with the 11 samples of Chinese group [3]. However the most common combination found in samples of CWG, C (+), c (-), E (-), Jk^a (+), Jk^b (+),Fy^a (+), Fy^b (+), S (+) did not exist with any of 31 samples of SYOG. The optimal frequency that allows discrimination in a mixed sample is 0.5 (50%). If an antigen is expressed at 50% within a population, this represents maximum probability that two blood samples of a particular population, mixed by chance, can be constituted by an expressing individual and a non expressing individual. Fy^a, Fy^b and Big C expression frequencies are close to the optimum value of 0.5 in CWG database and not in Singapore database, that indicates the antigens such as Fy^a, Fy^b and Big C are powerful in discriminating two individuals on regional scale but less powerful on world scale i.e. during SYOG 2010.

As a part of the blood transfusion test, all the blood sample were analyzed on Sysmex XT 2000i for hematological parameter determination. During the CWG 2010 blood testing, one of the samples for blood transfusion of a female candidate for cycling event was tested as negative, though its hematological profile showed many out of range findings (Table 2).

Table 2 Blood Parameter values of a female cyclist

Blood Parameter	1st Sample	2nd Sample
WBC, 10 E3 /µL	1.14	4.54
RBC, 10 E6 /µL	4.30	4.16
HGB, g/dL	14.1	13.6
HCT, %	46.2	46.6
MCV, fL	107.4	112.0
MCH, pg	32.8	32.7
MCHC, g/dL	30.5	29.2
PLT, 10 E3 /µL	136	185
MPV, fL	10.8	10.6
RET, %	3.60	1.30

Sample showed low white blood cell count (WBC), high values of haemoglobin (HGB), haematocrit (HCT), mean cell volume (MCV) and reticulocyte (RET) count. The red blood cell distribution was broad indicating anisocytosis. The sample was repeated many times in order to obtain consistent RET values but the majority was greater than 2.4 %. The scatter plot showed lot of platelet clumping which may have caused a high MCV value that affected other red cell parameters. Before giving weightage to the high MCV values, it was decided to get a fresh sample of the same candidate. The opinion on the out of range finding was conveyed to the testing authority.

The fresh sample was received and analyzed negative for blood transfusion. The cell counter showed a normal value for white blood cell (WBC), haemoglobin (HGB), haematocrit (HCT) and reticulocyte (RET) count followed by high mean cell volume (MCV)(Table 2). The cell counter scatter plot on RET and PLT-O shows again platelet clumps but lessened in the fresh sample. The clumping platelet may be due to EDTA anticoagulant or improper blood collection [4-5]. Improper collection and EDTA could also result in low white cell counts [6]. This pre-analytical error impacts on the CBC and RET significantly. The EDTA induced aggregation can sometimes be overcome by collecting sample in citrate, oxalate or heparin [4].

Conclusion:

It is observed that ethnic difference exist in expressing frequency of blood phenotypes which requires to be taken into account in blood transfusion detection. It is concluded that the abnormal hematological values are not always pointing towards any kind of blood manipulation. It might depend on the blood sample collection procedure, the anticoagulant used. It is proposed that the findings which are outside the preview of doping violations may also be conveyed to relevant authority in view of its clinical relevance. In such cases a fresh sample of the same athlete can be analyzed.

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