Original Research Article

Monocyte monolayer assay validation in Brazilian blood donors

ABSTRACT

Aims: To validate the monocyte monolayer assay (MMA) technique concerning its suitability for blood donor screening and its cost per test performed. The MMA is an in vitro simulation of the behavior of the antibodies, demonstrating the reactions that would occur in the endothelial reticulum system after a transfusion of incompatible red blood cells, indicating the risk of a hemolytic transfusion reaction and therefore of the clinical significance of the antibodies.

Study design: Blood samples of alloimmunized patients, selected at random from a blood donation bank, were submitted to validation tests recommended by the Brazilian National Health Surveillance Agency for the approval of new testing procedures.

Place and Duration of Study: The following Brazilian institutions were involved between June 2009 and July 2010: Immunohematology laboratory of the Hematology and Hemotherapy Center of Santa Catarina state (HEMOSC) in Florianópolis, Department of Medicine (Medical Unit IV) and Department of Radiology of the Institute of Medical Sciences (SIMS), Hospital Lahore in São Paulo.

Methodology: Ninety samples of alloimmunized patients treated by HEMOSC were used. The validation tests evaluated the selectivity, linearity, precision, and accuracy of the MMA method and determined the limits of detection and quantification. External validation of the method was performed by comparing the HEMOSC results with those of an independent laboratory (SIMS) while making sure that the latter was blind to the results of the former. The coefficient of variation was used to express the MMA testing precision of 5 replicates across 5 different concentration levels. Type I error for evaluating statistical significance was set at 5%.

Results: Selectivity assessment of the impact of multiple alloantibodies on the MMA test result showed no statistically significant difference ($P>0.05$) across the titers of 64, 256, and 2048, each with three replications, thus confirming the test specificity. Homoscedasticity of the monocyte index (MI) data was not refuted by Levine's test with the F-value of 0.746, much below the value of 3.056 needed to achieve a statistical significance level of $P<0.05$. MI linearity against the logarithm of the alloantibody concentration was shown in a linear regression where the latter predicted 83% of the variation in the former, and the regression slope of 0.4 (95% confidence interval 0.32, 0.48). The limits of detection and quantification on the logarithm scale were 0.28 and 0.84, respectively. External validation found no statistically significant difference between the MMA test results from the two independent laboratories. The coefficient of variation of <15% indicated good MMA testing precision under routine laboratory conditions.

Conclusion: The assay met all validation criteria and was therefore effective in assessing the clinical significance of alloantibodies.

Keywords: Blood transfusion. Monocyte Monolayer Assay. Pre-transfusion tests. Alloimmunization.

1. INTRODUCTION

Monocyte Monolayer Assay (MMA) is an in vitro simulation of the behavior of the antibodies, thus demonstrating the reactions that would occur in the endothelial reticulum system after a transfusion of incompatible red blood cells. The test uses primary monocytes and donor red blood cells opsonized by receptor antibodies to assess the phagocytosis of these sensitized erythrocytes. In this way, it can determine the clinical significance of the antibodies and may be applied with the recipients who have alloantibodies against high-frequency antigens, multiple alloantibodies, and when it is not possible to define the specificity of these alloantibodies due to technical limitations. MMA may be applied to patients with Autoimmune Hemolytic Anemia, Drug-Induced Immune Hemolytic Anemia, and Hemolytic Disease of the Fetus and Newborn [1,2].

The effectiveness of the MMA was confirmed by multiple previous studies, which demonstrated its superiority concerning the strength of the Coombs phase reaction (after incubation at 37 ° C when Coombs IgG serum is added), in predicting the clinical relevance of alloantibodies. The results are assertive in assessing the risks of a Delayed Hemolytic Transfusion Reaction. MMA represents the best risk analysis tool when units of

compatible blood components are not available for transfusion. In these situations, the choice is between relying only on reports from the literature and/or on clinical experience and performing the MMA to complement the risk analysis of an incompatible blood transfusion. MMA has been used in various locations for decades to determine the hemolytic potential of antibodies [3].

There is a gap in the immunohematology service of the Hematology and Hemotherapy Center of Santa Catarina (acronym HEMOSC in Portuguese) when there is no compatible blood bag available, especially when rare blood is urgently needed. National Register of Rare Blood takes time to provide the blood, which the patient often cannot afford. Another problem is laboratory testing of patients with Autoimmune Hemolytic Anemia whose autoantibodies can be cold, hot, or a mixture of both, and strongly interfere with the analysis, masking pre-transfusion test results. Under these circumstances, due to the limitation of immunohematological techniques, it is not possible to ensure that this incompatibility is due only to the presence of autoantibodies, thus generating additional uncertainty about transfusion safety. The present study aims to validate the MMA technique by ensuring it meets the criteria of the guide for validation of analytical and bioanalytical methods of the National Health Surveillance Agency, Brazilian Ministry of Health [4].

2. MATERIAL AND METHODS

This is a test procedure validation study, carried out at the HEMOSC Laboratory of Immunohematology in Florianópolis, Brazil.

2.1 Sampling and testing procedures

Ninety samples from alloimmunized patients who attended the immunohematology laboratory during the year 2020, were used. The inclusion criterion was a PAI-positive (PAI is the Portuguese acronym for Irregular Antibody Research) test result, which had alloantibodies or autoantibodies identified by the red blood cells (RBC) panel test. The samples that tested positive for hot and cold autoantibodies, identified by the same panel, were excluded.

Ninety MMA test samples were selected randomly for additional testing [4] to validate the MMA. Initially, the monocytes of whole blood bags from healthy voluntary donors were separated, pouring the whole blood from the bag into a density gradient liquid (Histopaque® 1077 Sigma). The mononuclear cells, now separated, were washed with phosphate buffer solution (pH 7.4), resuspended in a culture medium (RPMI 1640 Gibco®), and subsequently distributed in the culture plate wells, where circular coverslips were added. The plate was incubated for 1 hour at 37°C, in an atmosphere of 5% CO_2. Meanwhile, red blood cells from a selected bag for the recipient were sensitized with the recipient's plasma serum after an hour-long incubation at 37°C. In parallel, the same non-sensitized red cells used in the previous step were separated as a negative control, whereas already sensitized commercial red cells served as a positive control.

After the incubation, the erythrocytes were washed three times with a phosphate buffer solution. Subsequently, an aliquot of these erythrocytes was collected and submitted to the direct antiglobulin test (acronym TAD in English). A positive result indicated that RBCs had been sensitized. Fresh blood type AB blood donor serum was added to the washed red cells, with the reaction incubated for 15 minutes at 37°C. After this time, the reaction was washed again and the sensitized erythrocytes were reconstituted in culture medium. After the incubation time of the monocyte suspension ended, the supernatant containing non-adherent lymphocytes was removed and the suspension of sensitized red blood cells was added to the plate wells and incubated for an additional hour at 37°C in an atmosphere with 5% CO . After incubation, non-adherent cells were removed and the slide was stained with hematological dye. After the end of the reaction, the circular coverslips were stained with Leishman stain according to the manufacturer's guidelines, and positioned on a slide to allow reading under a microscope at 40x magnification. The samples were evaluated by a microscopist. As a standard, 200 monocytes were counted in each coverslip, and among them, the number of monocytes that underwent phagocytosis of red blood cells. The latter was used to obtain the Monocyte Index (MI) values equal to or greater than 5%, associated with clinically significant antibodies [5].

2.2 Sample size calculation

MI from the MMA test varies between zero and 100%, with an average of around 20% in an asymmetric distribution that can be approximated with normal distribution on the logarithmic scale [1,6], with the mean, amplitude, and a standard deviation (SD) of -1.61, -15 to zero, and 2.50, respectively. MI of 5% or greater was considered clinically relevant, so the sample size calculation considered the effect size equal to the difference between the mean and this value on the logarithmic scale, with SD 2.5 on the same scale, the statistical power of 80%, and the type I error of 5%.

2.3 Statistical analysis

Descriptive statistics used means, medians, scatter, and Box plots. Simple linear regression was used to evaluate the relationship between MI and concentration levels, with a 95% confidence interval (CI) to express uncertainty. Snedecor's F-test and Student's t-test were used to evaluating the homogeneity of the variances and to compare the difference in means, respectively.
Statistical significance was set at a *P*-value of 0.05 or less.
Stata software, version 12.0, was used to calculate the sample size necessary to detect this effect which resulted in at least 26 patients.

3. RESULTS AND DISCUSSION

The present study results follow the requirements of the validation process established by the Brazilian regulatory agency [4].

3.1 Selectivity

Selectivity assesses the effect of interferences in the sample matrix, which may contain components that impair measurement performance, increasing or reducing the analyte signal. Thus, in a selectivity study, the existence of a matrix effect is verified. In the case of the present validation, two groups of test samples were prepared, one with the matrix and the other without, with the same concentration levels. Samples with multiple alloantibodies specificity were considered as interfering with the matrix. The results obtained from the study of the matrix effect by concentration level are shown in Table 1.

Table 1. Matrix effect.

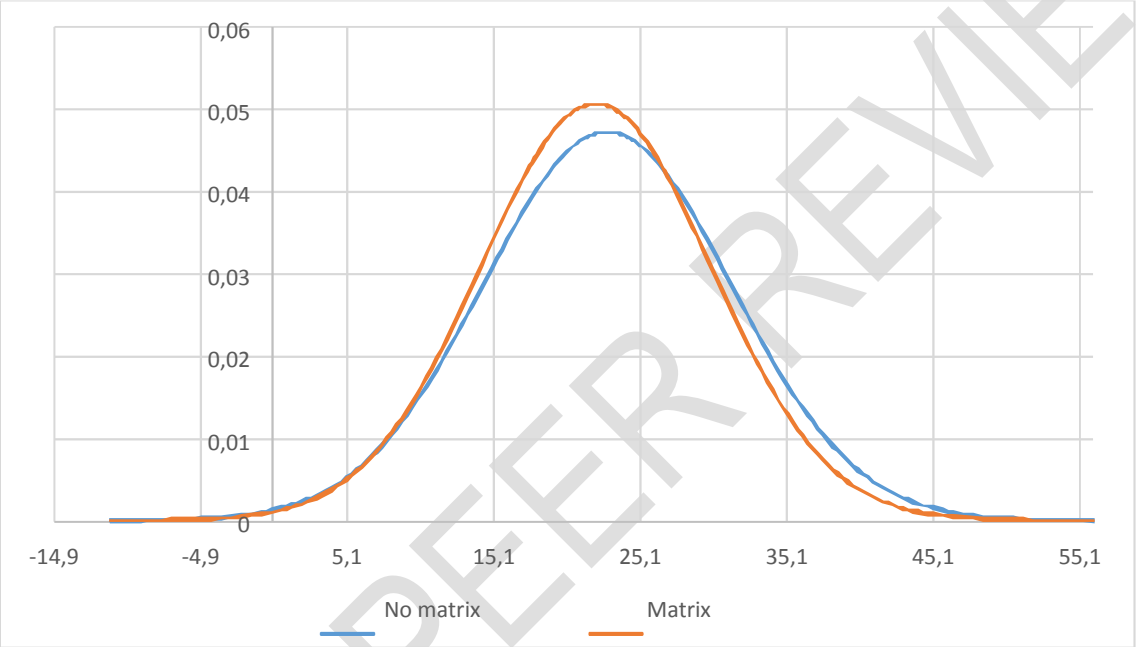
	Curve MI (%)							
	Without matrix effect			With matrix effect			*P*-value	
Titer	MI 1	MI 2	MI 3	MI 1	MI 2	MI 3	F-test	T-test
64	10,5	11	12	12	11	13	0,74	0,16
256	29,5	30	29	27,5	28	29,5	0,37	0,08
2048	27,5	26,5	27	26,5	27	28	0,60	0,38

The Snedecor's F-test for homogeneity of variances by concentration level showed no statistically significant *P*-values (>0.05). The Student's t-test for comparing means by concentration level showed statistically significant (<0.05) *P*-values and Shapiro-Wilk's test result was in line with the hypothesis of a normal distribution for the aforementioned comparison (Table 2), so the selectivity criterion was satisfied (Figure 1).

Table 2. Shapiro-Wilk's test results

Parameter	Concentration		
	64	256	2048
Sample size	6	6	6
Mean	11.67	28.55	30.55
Standard Deviation	1.21	1.06	2.99
W	0.91	0.83	0.88
P-value	0.42	0.12	0.31

Figure 1. Comparison of the matrix versus no-matrix distributions



3.2 Linearity

The linearity of a method demonstrates its ability to obtain results proportional to the concentration of the analyte in the sample within a given interval between the lowest and highest concentration, called the linear working range. To find this range, five samples were first tested at five levels of different concentrations and visually inspected to find the working range. The titer concentrations of alloantibodies were plotted against the MI percentages. Afterward, three replicates of each concentration level were tested and plotted to confirm the linearity of the selected working range.

The analysis of the presence of outliers was done by constructing the graphs with interquartile amplitude by estimating outliers with a Box plot. The homoscedasticity of the data was calculated using Levene's test. A simple linear regression was applied with the method of minimum squares method. The Durbin-Watson test was also applied to assess the auto-correlation of the residuals.

The analysis of outliers by concentration level was performed by interquartile range graphs which did not detect aberrant values (Figures 2 and 3).

Figure 2. Interquartile amplitude.

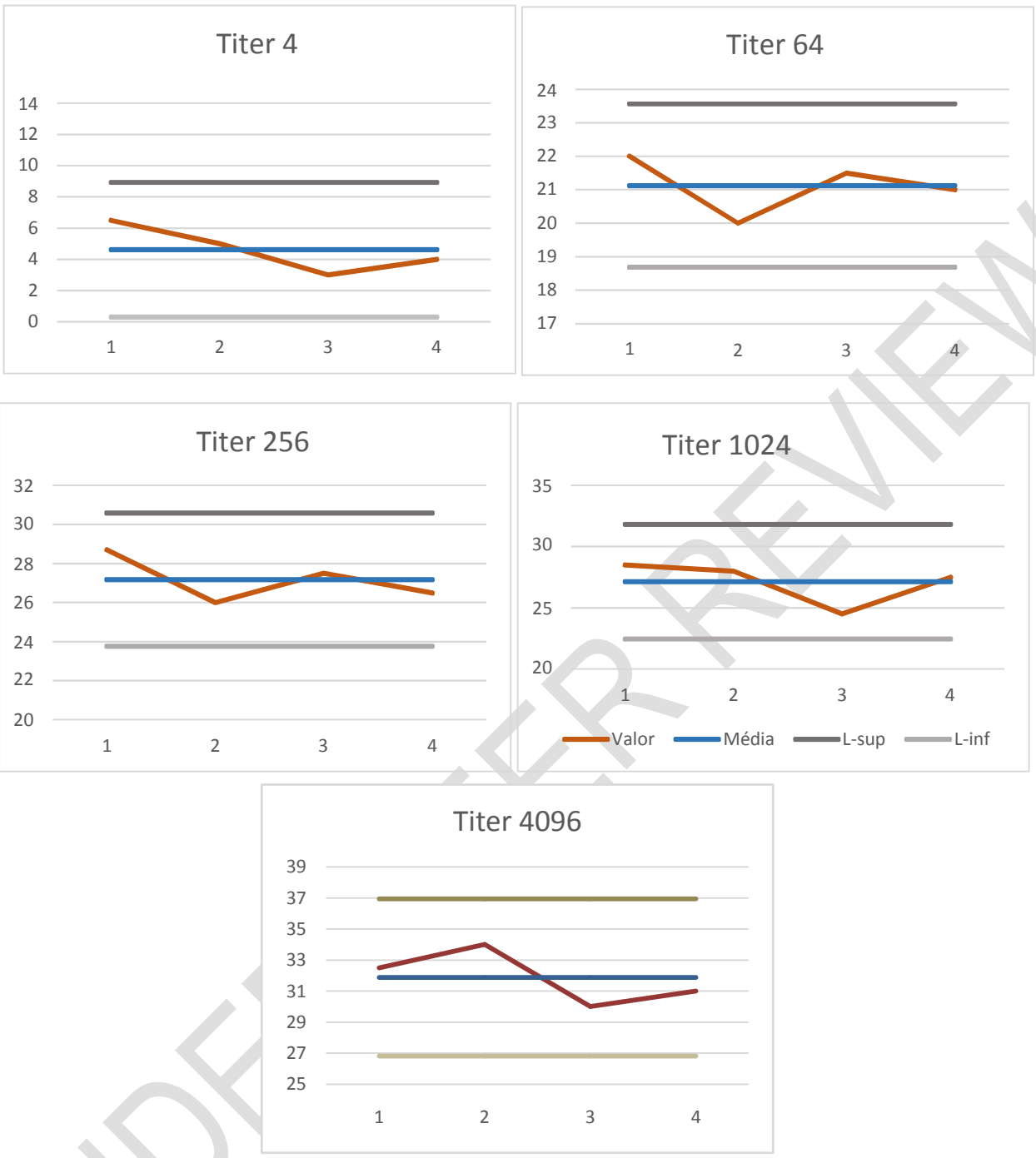
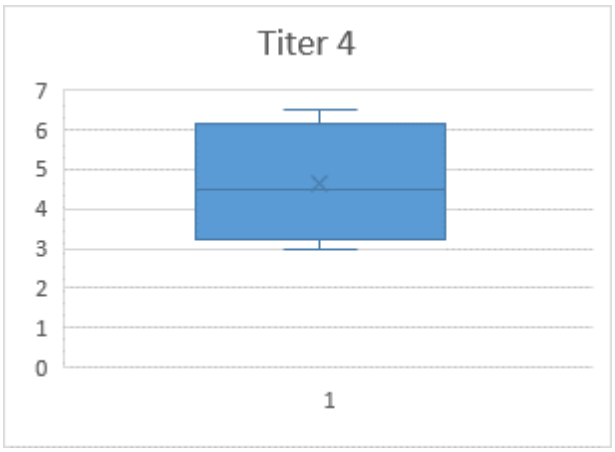
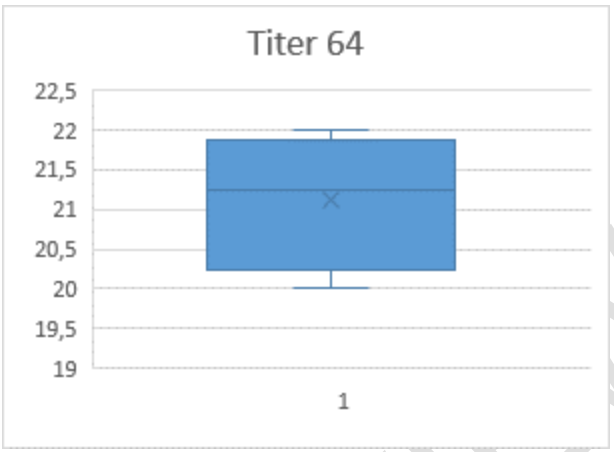
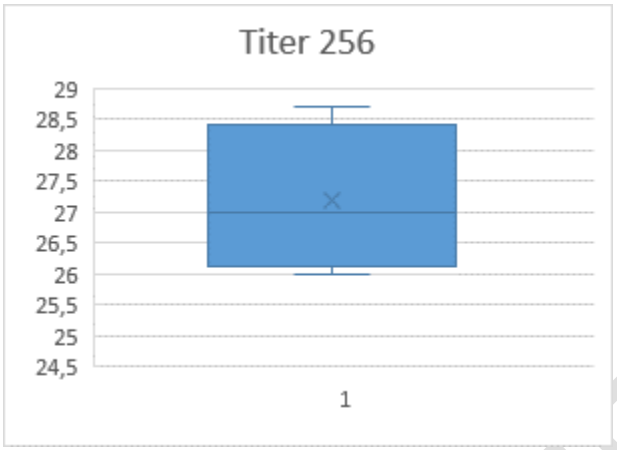
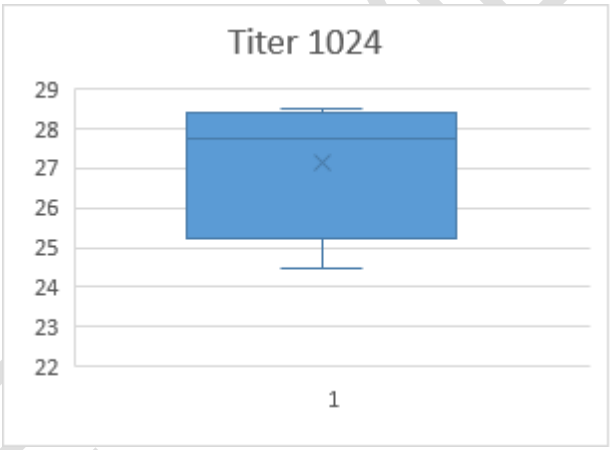


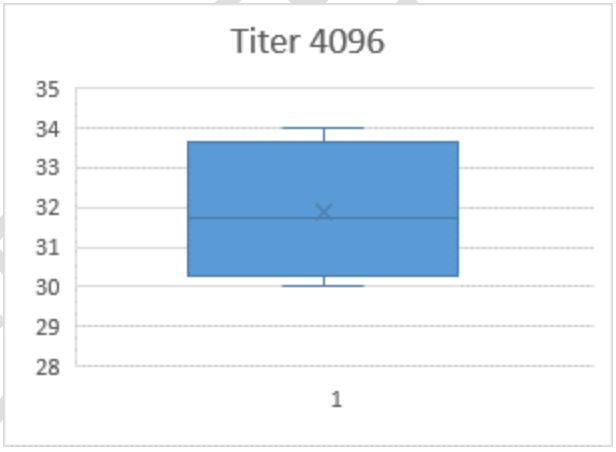
Figure 3. Box plot











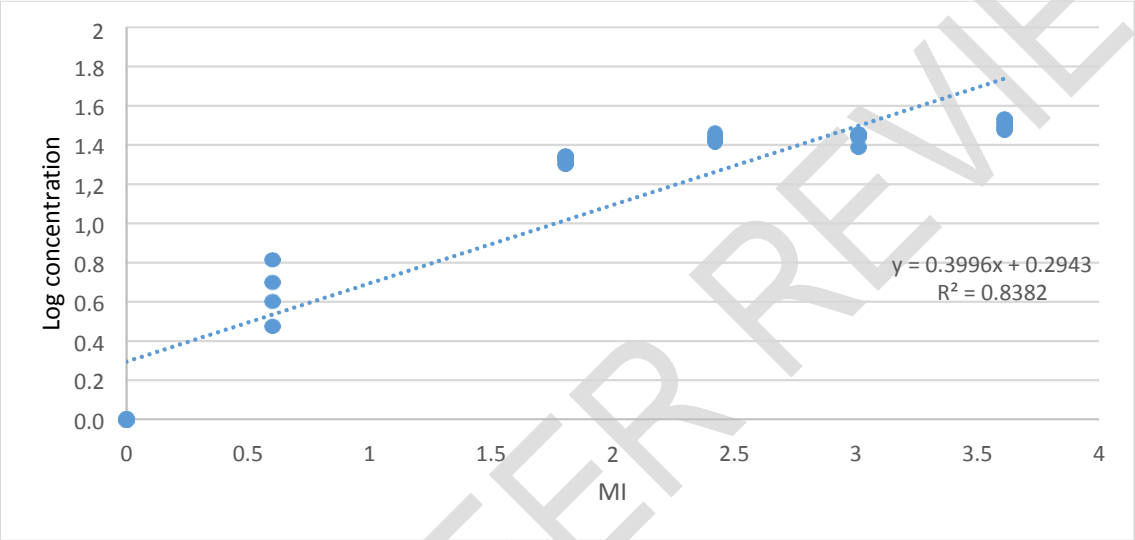
MI data homoscedasticity was also assessed by Levene's test, which showed homogeneous data, with the calculated F lower than the tabulated (0.746 vs. 3.056).

With the results approved in the previous tests, linear regression was continued as shown in Table 3 and Figure 4.

Table 3 – Linear regression MI gradient versus the logarithm of the concentration

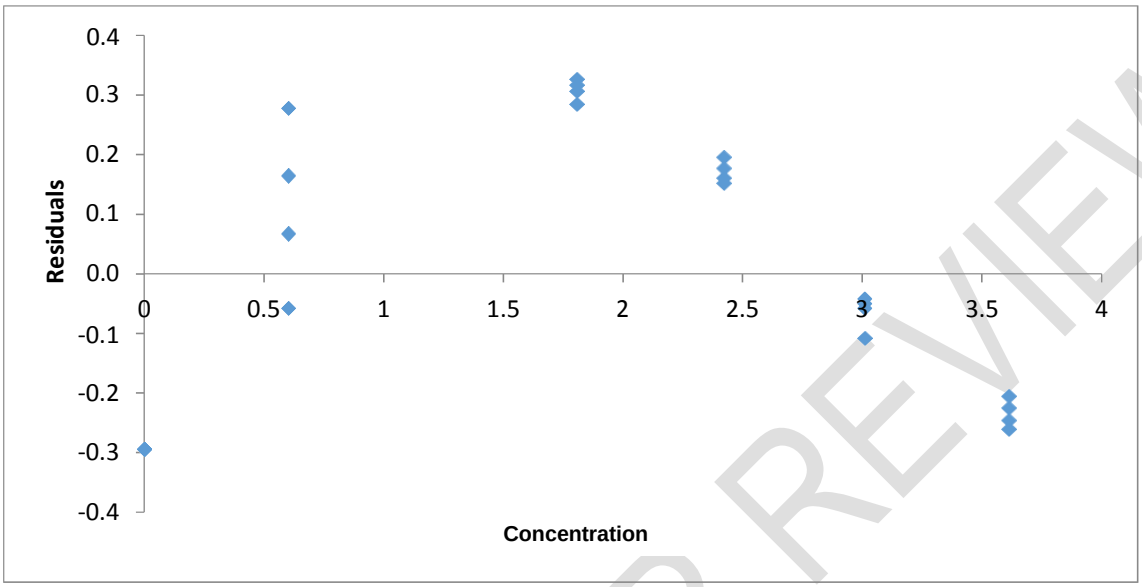
	Raw coefficient	Standard error	*P*	95% confidence interval
Intercept	0.29	0.09	<0.001	0.12 - 0.47
Gradient	0.40	0.04	<0.001	0.32 - 0.48

Figure 4. Linear regression: MI gradient versus the logarithm of concentration.



Linear regression showed that the concentration of alloantibodies predicts approximately 83% (coefficient of determination = 0.83) of the MI results. Furthermore, the slope and intercept were both statistically significant (*P*<0.001). The regression residuals (Figure 5) showed no clear pattern.

Figure 5. Linear regression residuals and gradient

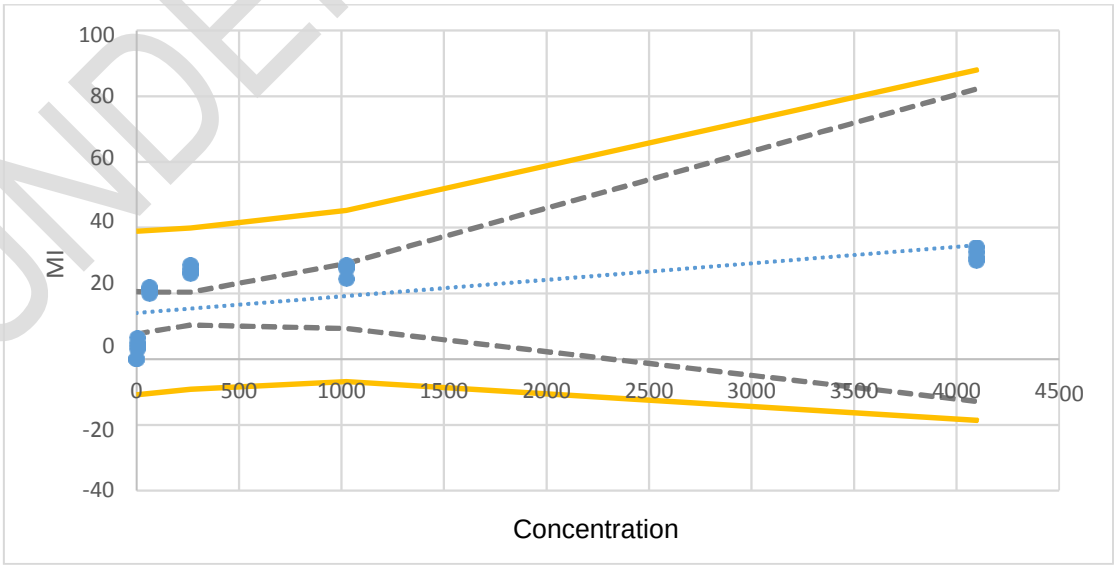


The durbin-Watson test showed no statistically significant auto-correlation between the residual values with the value of 1.776 within the interval expected for the null hypothesis. Above tests and figures all point to a linear relationship between the increase in concentration and the MI value.

3.3 Limits of detection and quantification

The limit of detection (LD) of an analytical procedure is the smallest detectable amount of the sample analyte, even if its quantity cannot be determined. The limit of quantification (LQ) is the smallest amount of the sample analyte whose quantity can be determined with acceptable precision and accuracy. The LD estimated through the standard deviation of the sample blank, was a titer of 0.28. From the LD value, 0.84 LQ was obtained.

Figure 6. Regression parameters' 95% confidence intervals



The 95% confidence intervals were 0.32 - 0.48 and 0.12 - 0.47 for the intercept and the gradient, respectively (Figure 6).

The present study also evaluated the MMA accuracy by comparing its diagnostic performance with a validated reference method. In this case, the comparison was made with the same technique already validated and used in the Immunohematology laboratory of the Pro-Sangue Foundation in the city of São Paulo.
Five representative samples were tested in the entire concentration range, both by the HEMOSC Immunohematology laboratory and by the Pro-Sangue Foundation laboratory. F-test was used to assess the homogeneity of the variances, and the Student's t-test to verify the mean difference between the two laboratories. The F-test and t-test obtained the P-values of 0.86 and 0.41, respectively, thus indicating no statistically significant difference. Of note, the number of samples used in this comparison was small due to economic limitations.

MMI precision was also tested in terms of the repeatability of test results under routine laboratory conditions, that is, the same measurement procedures, instruments, and human resources. Five runs were carried out with five replicates each, at five different concentration levels. The precision was considered satisfactory if the coefficient of variation was lower than 15% over five runs. The results obtained are shown in Table 4 and confirmed adequate precision of the MMA testing under routine laboratory conditions.

Table 4. MMA precision testing.

Replicate	**Samples**				
	1	**2**	**3**	**4**	**5**
1	24.00	25.00	29.50	11.00	28.50
2	22.00	24.50	31.00	12.00	28.00
3	23.00	25.00	29.00	12.50	29.50
4	23.00	24.00	32.00	12.00	28.00
5	24.00	24.50	30.00	13.00	27.50
Mean	23.20	24.60	30.30	12.10	28.30
Standard deviation	0.84	0.42	1.20	0.74	0.76
Coefficient of variation (%)	3.61	1.70	3.97	6.13	2.68

3. DISCUSSION

MMA has been described in the literature for almost half a century. It aims to estimate whether the immunohematological findings reflect clinically significant results before the transfusion takes place. It can serve as a risk prediction tool for preventing transfusion reactions [8]. The validation of this method is fundamental for transfusion safety. The present study results were satisfactory, but some limitations should also be noted.

The monocytes used in the analytical runs were extracted from a bag of whole blood (CPD) from healthy donors, that is, the monocytes were allogeneic. It is also possible to isolate these cells autologously, that is, through a whole blood sample from the recipient itself. There are reports that autologous monocytes have a greater capacity to induce phagocytosis and thus become a more reliable alternative in predicting clinical significance. This is because in vitro conditions would be very close to the physiological ones when using monocytes from the receptor since the antibodies of the receptor would be coming into contact with their monocytes. The use of monocytes obtained from different donors, however, would see variations in their activity. Both practices are commonly described in previous studies [9]. The use of autologous monocytes is not feasible for logistical reasons, since it would require a large volume of whole blood from the recipient. Also, transfusion recipients are often not in a clinical condition to supply blood for testing, so the use of fresh allogeneic monocytes remains a common practice. The present study testifies to the validity of this practice.

As for monocyte stability, the literature shows that heparinized whole blood can be stored at room temperature for up to 36 hours without compromising monocyte viability [10]. A later study supported this statement,

however, with a minor caveat concerning the anticoagulant used. TONG et al. [3] used an ACD anticoagulant and assessed monocyte stability in maintaining its function within 36 hours and corroborated the claim already described. Based on this premise, CPD anticoagulant was used in whole blood bags stored at room temperature, and monocytes were isolated within 24 hours after collection. Within this time interval, the phagocytic power of the cells did not change, since normally the first runs involved the samples tested for the first time, while the subsequent runs were replicates of the samples with positive MMA results. Although the anticoagulant differed from those presented in the literature so far cited, it had been successfully used in other studies [1,11].

The anticoagulant ACD is nothing more than a solution of sodium citrate, acidified with citric acid. CPD is the ACD solution with the addition of sodium bisphosphate, so both anticoagulants contain citrate, which is responsible for chelating calcium in the blood, inhibiting calcium-dependent steps in the coagulation cascade. Furthermore, both contain dextrose which serves as a substrate for the formation of adenosine triphosphate (ATP), important for the production of cellular energy. The difference between CPD and ACD is the presence of phosphate, which acts as a buffer binding to the H + ions produced during glycolysis and preventing the pH from falling, besides being a substrate for the formation of 2,3-DPG. During the study, the use of a CPD anticoagulant did not cause interference, as it is an improved version of the ACD anticoagulant. The CPD maintains the pH of the medium, which is extremely relevant because any modification can cause either the activation or a sudden reduction of the phagocytic capacity of the monocytes [12].

Due to the limited economic resources of the present study, flat-bottomed culture plates with a growth area of 9.5 cm2 were used. Inside the wells, 13 mm circular coverslips were placed, so that the reaction would take place in an easy-to-manipulate medium and take it to the microscope for reading. However, there are commercial chamber systems that consist of a glass slide coupled to a removable polystyrene chamber, used by several studies [5,3,6,9]. Although the use of culture plates with coverslips previously treated with poly-L-lysine hydrobromide solution [12] has been increasing, the use of culture plates is still considered adequate, albeit not the first option, due to its higher cost and difficulty in execution [13].
In the present study, the use of culture plates was an economically viable option. The acquisition of commercial camera systems would make it impossible to implement the MMA in the HEMOSC immunohematology laboratory. Perhaps, at first, for experimental purposes, the chambers could be purchased, but after the implementation of the routine testing, there would be a prohibitively high-cost increase.
It is also worth noting that the coverslips used did not receive chemical treatment, so they did not interfere with the monocytes' adhesion, much less with their function.

MMA is an in vitro test that aims to simulate reactions that occur in vivo. Thus, the reaction conditions should be as close to the physiological conditions as possible. Because of this, the incubation temperature is 37 ° C, as it approaches the normal average body value, and the MMA pH simulates the blood pH in the 7.2-7.4 range which optimizes monocytes' phagocytic capacity. Even partial loss of their functionality results in false negative MMA results, thus increasing the risk for transfusion recipients. To avoid this scenario, a CO^2 incubator (5% at 37°C) maintains the pH balance of the reaction [2,3].
Despite its versatility and usefulness, MMA has its limitations. As it is an "in-house" method, its results depend very much on the personal skills of a specialized professional. Reproducibility between operators and laboratories can vary, thus maintaining a quality standard challenging. Also, it is a laborious and time-consuming examination that takes about 8 hours to complete. This time is mainly related to the stage of separation of monocytes because these cells must be viable for the reaction to occur regularly. To guarantee this viability, the monocytes need to be isolated at the time of collection, which takes about 3 hours. Recent research has shown that monocytes in cryopreserved pools are not significantly affected by their phagocytic capacity and that this practice can reduce the total exam time [11] thus showing the way to optimize the MMA testing.

4. CONCLUSION

In the present study, the validation of the MMA test was carried out through tests provided for in the resolution RE N°. 899, of May 29, 2003, of the National Health Surveillance Agency. It was found that this assay met all the criteria described in this resolution and is thus effective in assessing the clinical significance of the antibodies.

CONSENT

All blood donor candidates signed an informed consent form which stated that their blood samples could be used for research purposes under the condition of ANONYMITY.

ETHICAL APPROVAL

The MMA validation study was approved by the institution's Ethics Committee under the number 18198119.1.0000.0121.

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