METHOD VALIDATION OF CLASSICAL AND ALTERNATIVE PATHWAY COMPLEMENT ACTIVITY IN HUMAN SERUM USING HEMOLYTIC ASSAYS

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INTRODUCTION

The complement system is a part of the immune system, and complement activation plays a vital role in identifying and cleaning pathogens, damaged cells and immune complexes. The classical and alternative pathways are two important participators involved in complement activation. Dysregulated complement activities in both pathways are found to be associated with various diseases, such as paroxysmal nocturnal hemoglobinuria, systemic lupus erythematosus and rheumatoid arthritis. Therefore, multiple anti-complement therapies under research and development in clinical trials may become promising treatment approaches for these complement-mediated disorders. Here, we developed and validated two fit-for-purpose hemolytic assays to determine the classical and alternative pathway complement activities in human serum.

METHOD

Complement activities of the classical and alternative pathways were assessed by hemolytic assays. The classical pathway complement activity is determined by the ability of complement in serum to lyse sheep red blood cells (SRBC) opsonized with rabbit anti-SRBC antibodies via the classical pathway. Similarly, the assessment of alternative pathway complement activity is based on complement-dependent lysis of rabbit red blood cells (RRBC), which cannot recruit the soluble human complement regulator Factor H, thus allowing for alternative pathway activation alone.

Specifically, to determine the classical pathway complement activity in serum samples, 60 µL of SRBC (5.0 × 108 cells/mL) suspended in Gelatin Veronal buffer containing Ca2+ and Mg2+ (GVB++) was sensitized using an equal volume of rabbit anti-SRBC antibodies (diluted 1:500 in GVB++) at 37 °C for 10 minutes. Then 180 µL of serum samples undergoing a series of dilutions in ice-cold GVB++ were added; ice-cold GVB++ and distilled water were chosen as the negative and positive control. The mixture was incubated at 37 °C for 60 minutes. After 60 minutes of incubation at 37°C and 10 minutes of centrifugation at 1000g at 4°C, 200 µL of the supernatant was transferred to a new 96-well flat bottom plate. At 412 nm, the absorbance of hemoglobin released into the supernatant was measured, corrected for the negative control, and then normalized to the corrected positive control, finally determining the hemolysis percentage. The natural logarithm (ln) of the titer, i.e., the reciprocal of the dilution factor, was plotted against the hemolysis percentage. The complement volume (mL) that causes 50% hemolysis of opsonized SRBC was defined as one CH50 Unit (U). Finally, the CH50 value was calculated as U/mL, reflecting the classical pathway complement activity of tested serum samples.

To determine the alternative pathway complement activity, 25 µL of serum samples undergoing a series of dilutions in cold BBS-G-EGTA/Mg buffer containing Ca2+ and EGTA were added to 96-well plate. Cold BBS-G-EGTA/Mg buffer and distilled water were chosen as the negative and positive control, respectively. 25 µL of RRBC (2.0×10^8 cell/mL) suspended in BBS-G-EGTA/Mg buffer was added, and the mixture was incubated at 37 °C for 60 minutes. Then 200 µL of cold BBS-G-EGTA/Mg buffer was added to the 96-well plate and mixed gently. After centrifugation at 1000g for 10 minutes at 4°C, 100 µL of supernatant was transferred to a new 96-well flat bottom plate. At 412 nm, the absorbance of hemoglobin released into the supernatant was measured, corrected for the negative control, and then normalized to the corrected positive control, finally determining the hemolysis percentage. The natural logarithm (ln) of the titer, i.e., the reciprocal of the dilution factor, was plotted against the hemolysis percentage. The complement volume (mL) that causes 50% hemolysis of opsonized RRBC was defined as one AH50 Unit (U). Finally, the AH50 value was calculated as U/mL, reflecting the alternative pathway complement activity of tested serum samples.

Figure 1. Assay design for CH50 and AH50

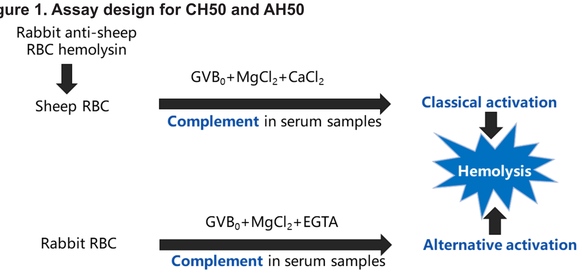
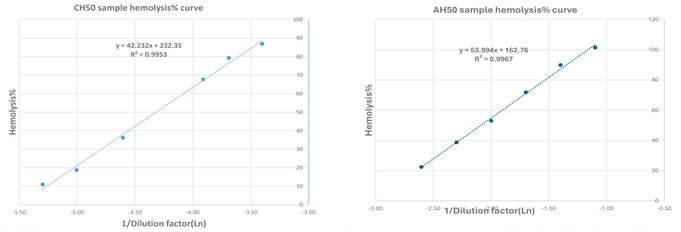


Figure 2. CH50 and AH50 sample hemolysis% curve



CH50 complement activity was measured after diluting the samples to 30, 40, 50, 100, 150, and 200 times. AH50 complement activity detection was performed after diluting the samples to 3, 4.05, 5.47, 7.38, 9.96, and 13.5 times.

RESULTS

Figure 3. Accuracy and precision data summary

	AH50(U/mL)					
	QC1(17.8)		QC2(14.1)		QC3(9.70)	
Run ID	Intra-%CV	Intra-%Bias	Intra-%CV	Intra-%Bias	Intra-%CV	Intra-%Bias
1	0.4	-15.2	0.5	-14.9	4.9	4.1
2	1.8	-5.1	0.9	-4.3	1.4	-9.5
3	1.0	11.2	2.2	9.9	0.9	3.1
4	0.5	8.4	0.7	9.9	2.4	2.5
5	0.0	-22.5	0.9	-17.7	3.6	-0.9
6	1.0	-10.1	0.8	-11.3	1.4	9.3
Inter-%CV	13.2		12.1		6.4	
Inter-%Bias	-5.6		-5.0		1.4	
Total Error	18.8		17.1		7.8	

	CH50(U/mL)					
	QC1(46.3)		QC2(50.0)		QC3(54.4)	
Run ID	Intra-%CV	Intra-%Bias	Intra-%CV	Intra-%Bias	Intra-%CV	Intra-%Bias
1	3.3	-13.0	1.4	-13.0	1.8	-13.2
2	0.7	2.2	0.4	3.6	1.7	3.9
3	0.0	10.8	1.7	9.0	1.1	9.2
4	2.4	9.7	2.2	11.4	7.0	7.2
5	1.4	9.9	1.0	16.4	2.4	12.4
6	0.5	8.4	0.8	12.0	3.5	12.1
Inter-%CV	8.4		9.3	6.6	9.1	
Inter-%Bias	4.8		6.6		5.3	
Total Error	3.6		2.7		14.4	

For the determination of CH50 and AH50: three individual human serum samples were selected as QC1, QC2, and QC3 samples. Each quality control sample was tested in triplicate, and six accuracy and precision experiments were conducted. For CH50: The intra-run CV for QC ranged from 0.0% to 7.0%, with an intra-run bias of -13.2% to 16.4%; The inter-run CV ranged from 8.4% to 9.3%, inter-run bias ranged from 4.8% to 6.6%. For AH50: The intra-run CV for QC ranged from 0.0% to 4.9%, with an intra-run bias of -22.5% to 11.2%; The inter-run CV ranged from 6.4% to 13.2%, and the inter-run bias ranged from -5.6% to 1.4%.

Figure 4. Result of interference from hyperlipidemia (10 mg/mL)

Type	Sample ID	AH50 (U/mL)	R^2	Bias(%)	Result
Lipemic sample(10 mg/mL)	Lip1	17.2	0.9959	10.3	Pass
	Lip2	16.8	0.9921	7.7	
	Lip3	17.7	0.9944	13.5	
	Neat	15.6	0.9883	NA	
Type	Sample ID	CH50 (U/mL)	R^2	Bias(%)	Result
Lipemic sample(10 mg/mL)	Lip1	85.5	0.9661	11.6	Pass
	Lip2	80.3	0.9585	4.8	
	Lip3	78.4	0.9474	2.3	
	Neat	76.6	0.9601	NA	

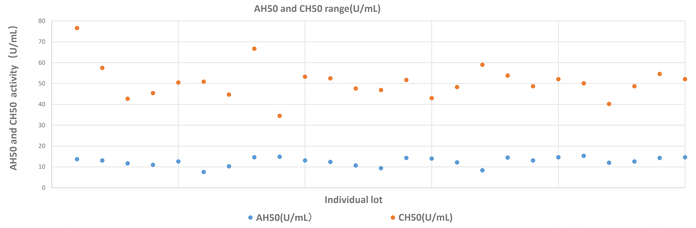
The result of interference from hyperlipidemia (10 mg/mL) for both AH50 and CH50 can meet acceptable standards.

Figure 5. CH50 and AH50 Result of Stability

Stability	CH50	AH50
RT	24h	24h
2-8°C	24h	24h
-70°C F/T	6F/T	6F/T
-20°C F/T	6F/T	6F/T
RBC	4 week	3week

The stability of sheep whole blood stored at 4°C was found to be one month and rabbit whole blood stored at 4°C was found to be 3 weeks, while serum samples remained stable at RT and 4°C for one day and were stable after being frozen and thawed six times at -20°C and -70°C for both CH50 and AH50.

Figure 6. The range for healthy human serum matrix



A total of 25 healthy human sera were tested to define the reference range for CH50 and AH50 in healthy individuals. The range for CH50 in healthy human serum matrix was 34.5-76.6 U/mL. The range for AH50 in healthy human serum matrix was 7.56-15.3 U/mL.

DISCUSSIONS

In this study, using the hemolytic assays, specific and reliable methods for assessing classical and alternative pathway complement activities in human serum were developed and validated. These validated methods provide available tools for directly and collectively reflecting the actual status of the complement system.

For bioanalysis laboratories, the validation of CH50 and AH50 detection methods lacks systematic guidance and acceptance criteria. Both CH50 and AH50 hemolytic assays are prone to several interferences, including different cell viability and number of SRBC or RRBC, affinity of rabbit anti-SRBC antibody, and thermal instability of complement in serum. Therefore, method validation and sample analysis of AH50 and CH50 in biological laboratories face great challenges. In this study, we conducted method validation according to the “fit for purpose” principles outlined in bioanalytical regulations, which including accuracy, precision, stability, interference from lipemic samples, and reference ranges for healthy individuals, achieving satisfactory performance in both assays. We also optimized the method as follows: Adjustment of the reaction system: We modified the density of blood cells and the volume of sample added to ensure method stability while reducing the volume of sample required, thereby minimizing the need for clinical blood collection to 60uL of serum for both CH50 and AH50, overcoming the disadvantages of large reactive volume and relatively low efficiency of traditional CH50 and AH50 hemolytic assays for clinical diagnosis; Blood Cell stability: By monitoring the viability of blood cells and performing cell counts, we ensured that the reaction system was more consistent and stable across experiments, leading to improved reproducibility of results. We optimized and standardized these experimental conditions, with the deviations of both CH50 and AH50 values within 20.0%.

CONCLUSION

We have successfully developed and validated stable and reliable hemolytic methods for CH50 and AH50, which can be used to support clinical investigations of complement-related candidate drugs.

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