Introduction and Evaluation of Light Transmission Platelet Aggregation Method on the Sysmex CS-series Automated Coagulation Analyzer.

Tasuku SAKAYORI [1)], Yuri WATANABE [1)], Kyoko NAKAJIMA [1)], Eri MISAWA [1)], Katsushi KOBAYASHI [2)], Hiroshi KURONO [1)], Nobuo ARAI [1)], Hidenari TAKAOKA [1)]

[1)] *Sysmex Corporation R&D group Hemostasis Product Engineering*

[2)] *HYPHEN BioMed SAS*

Light transmission aggregometry (LTA) is known as a gold standard method of platelet aggregation testing. However, it has been mainly measured by dedicated, semi-automated analyzers. The fully automated Sysmex CS-series coagulation analyzers have recently been upgraded with new software to perform platelet aggregation tests, and permits access to more accurate results.

In this report, we have evaluated performance of platelet aggregation tests on the CS-series with new software; aggregation imprecision, on-board stability of the agonists and reference intervals using the following agonists (reagents): Revohem™ ADP (2 μM), Revohem™ Collagen (2 μg/mL), Revohem™ Epinephrine (5 μM), Revohem™ Arachidonic acid (1 mM) and Revohem™ Ristocetin (1.2 mg/mL). Platelet agonists were adjusted according to the recommendations of SSC/ISTH.

Aggregation imprecision for maximal aggregation (%) was CV 5% or less in normal samples and CV 10% or less in abnormal samples. On-board stability of the agonists was until 10 hours on a CS analyzer. The reference intervals for each agonist was 60% or more.

The CS-series with new software is unique in its ability to perform both automated platelet aggregation and coagulation tests. These instruments have already been installed in clinical laboratories in different countries. The ability to perform platelet aggregation tests on this routine coagulation analyzer will allow access for more clinical laboratories to generate highly standardized platelet function test results.

Key Words Platelet Aggregation, LTA, CS-series, Revohem™

INTRODUCTION

Light transmission aggregometry (LTA), a standard platelet aggregation test method developed by Born in 1962, [1)] has been used for the diagnosis of congenital bleeding disorders, confirmation of blood clot tendencies, and confirmation of the pharmaceutical benefits of antiplatelet drugs. [2)]

Although LTA is known as the gold standard method, some issues have been reported such as difference in preparation conditions of platelet rich plasma (PRP) / platelet poor plasma (PPP) and different usage of agonists concentration. In 2013, the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (SSC/ISTH) reported a recommended measurement protocol toward the standardization of platelet aggregation tests using LTA [3)], with recommendations for sample preparation and the final concentrations of agonists to be used, which were not standardized before. However, the recommendations stated that LTA is clinically useful only for congenital bleeding disorders but not for evaluation of blood clot risk or antiplatelet drug monitoring. Thus, proceeding with further studies is imperative.

To date, semi-automated analyzers were mainly used for LTA, where aggregation detection and data analysis were performed automatically, while sample and reagent dispensing were performed manually. The platelet aggregation function was installed on the fully automated

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blood coagulation analyzer CS-series in 2015. This function allowed for automatic sample and reagent dispensing, and contributed to standardization by reducing the complexity and errors that might be caused by semi-automated analyzers (***Table 1***). In the same year, five kinds of agonists for platelet aggregation testing (Revohem™ series) were launched by Sysmex Corporation. (***Fig. 1***).

In this report, we introduced the measurement of platelet aggregation in the CS-series and described basic evaluation results of within-run reproducibility, on-board stability and reference interval at the agonist concentration recommended by SSC/ISTH in healthy subjects.

Table 1 *Comparison of platelet aggregation testing work flow between the CS-series and semi-automated analyzer (conventional analyzer)*

Work Flow	Semi-automated	CS-series
Blood sampling	Manual	Manual
Prepare(centrifuge sample) PPP and PRP	Manual	Manual
Dispense PPP and PRP to sample cuvette	Manual	Auto
Adding agonist to cuvette	Manual	Auto
Detection	Auto	Auto
Results	Auto	Auto

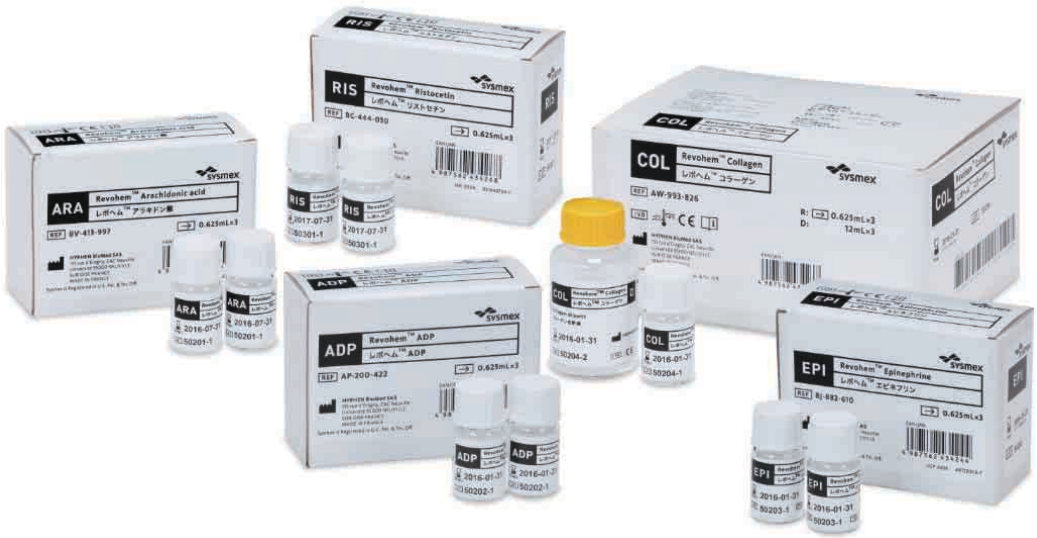


Fig. 1 *Platelet aggregation agonists (Revohem™ series)*

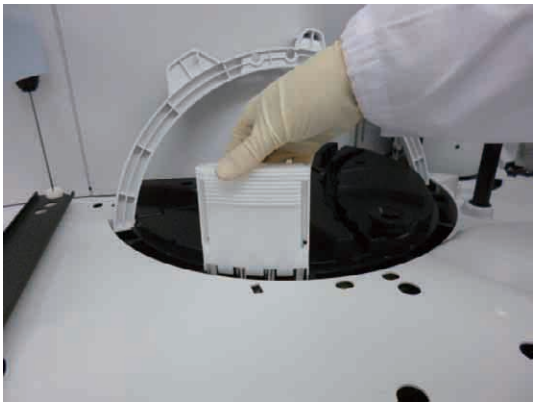
PLATELET AGGREGATION TESTING WITH THE CS-SERIES

1. Preparation of Sample Tube SB and agonists

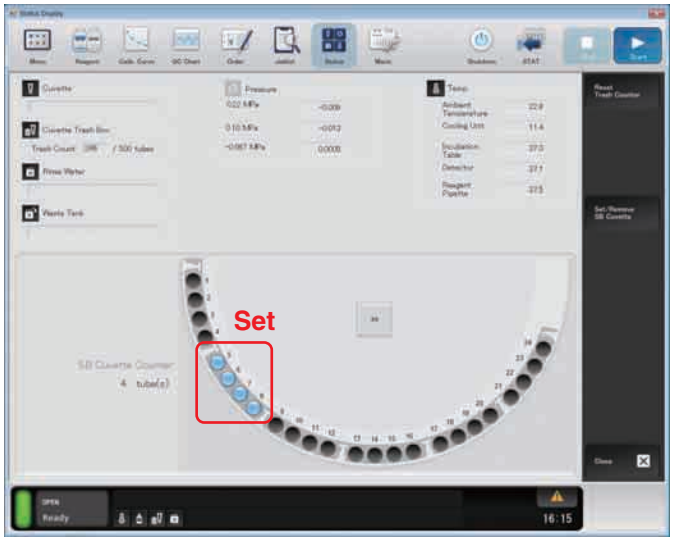
A sample tube with a stirring bar (Sample Tube SB) is required for platelet aggregation testing. Sample Tube SB was set at the designated location (dispensing table) around the reagent table.

The agonist was prepared to be 8 times the targeted final concentration and set on the reagent table in the same manner as the routine reagents (***Fig. 2***).

1) Cuvettes (Sample Tube SB) setting



2) Confirmation of Sample Tube SB setting



3) Preparation of agonists

Fig. 2 *Platelet aggregation testing with the CS-series (Preparation of cuvettes and agonists)*

2. Preparation and measurement order of PPP and PRP

PPP and PRP used for measurement were placed in positions marked with odd and even numbers, respectively, in the sample rack i.e., PPP of the first sample in Position 1 and PRP of the first sample in Position 2, PPP of the second sample in Position 3 and PRP of the second sample in Position 4 (***Fig. 3***).
For measurement order, measurement items were chosen from the measurement order screen dedicated for platelet aggregation testing. Platelet aggregation can be measured concurrently during measurement of coagulation and fibrinolysis parameters such as PT, aPTT, and D-dimer.

3. Measurement results and analysis

Measurement results were presented as a "joblist", in the order of PPP and PRP absorbance, and the maximal aggregation rate. Aggregation waveform and other analysis information (e.g., AUC, maximal acceleration) was displayed on the analysis results screen (***Fig. 4***).

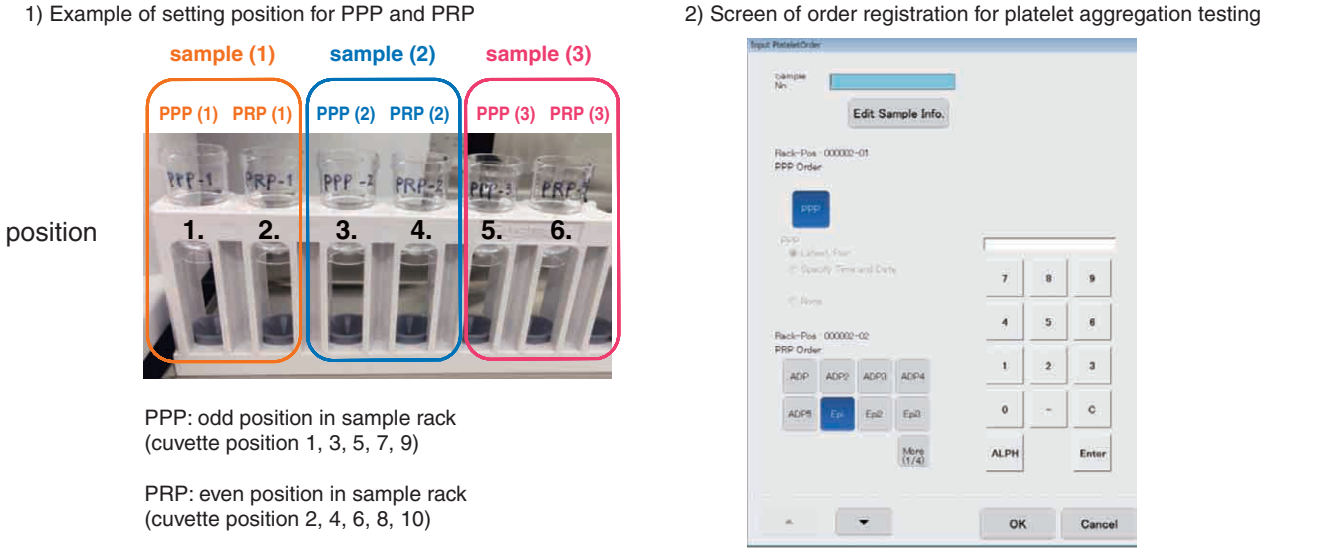


Fig. 3 Platelet aggregation testing with the CS-series (preparation of PPP and PRP and measurement order registration)

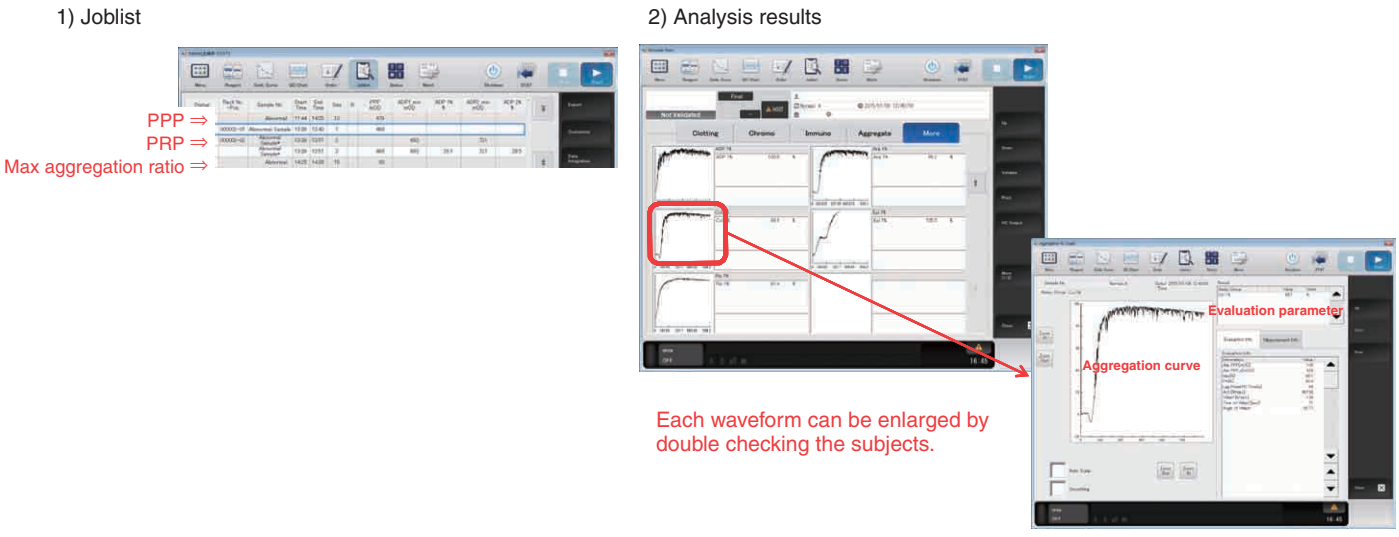


Fig. 4 Platelet aggregation testing with the CS-series (measurement results and analysis results)

MATERIALS AND METHODS

1. Subjects

Blood samples were collected using blood collection tubes with 3.2% sodium citrate from healthy employee volunteers and approved by the ethics committee of Sysmex Corporation. To obtain a normal PRP, the samples were centrifuged at 200 x g for 10 minutes or at 120 g for 15 minutes and, the supernatant was collected. The remaining blood was centrifuged at 1500 x g for 15 minutes and, the supernatant was collected and labeled as PPP.
Acetylsalicylic acid (Wako Pure Chemical Industries, Ltd.) was added to the normal sample to make a final concentration of 1 mM. The sample was then allowed to stand for 30 minutes and used as an abnormal sample.

2. Measuring equipment

Fully automated blood coagulation analyzer CS-2400 (Sysmex Corporation) was used for reproducibility and on-board stability tests.
Fully automated blood coagulation analyzers CS-5100, 2400, 2500, 2000i and 2100i (Sysmex Corporation) were used for reference interval tests.

3. Agonists for measurement

Revohem™ ADP, Revohem™ Collagen, Revohem™ Epinephrine, Revohem™ Arachidonic acid and Revohem™ Ristocetin (Sysmex Corporation) were used. The final concentrations of the agonists were used as recommended by the SSC/ISTH (***Table 2***).

4. Methods and Results

1) Within-run reproducibility
For within-run reproducibility, coefficient of variation (CV%) was obtained from the maximal aggregation rate (%) that was determined from five consecutive measurements of normal and abnormal sample (only the normal sample was measured with Revohem™ Arachidonic acid and Revohem™ Ristocetin because platelet aggregation activity reached almost 0% in the measurement using the abnormal sample with arachidonic acid and maximal aggregation activity were not decreased by acetylsalicylic acid with ristocetin).
The CV (%) of maximal aggregation (%) with ADP was 3.4 in the normal sample and 9.6 in the abnormal sample. The CV (%) of maximal aggregation (%) with collagen was 3.3 in the normal sample and 4.8 in the abnormal sample. The CV (%) of maximal aggregation (%) with epinephrine was 3.6 in the normal sample and 6.4 in the abnormal sample. The CV (%) of maximal aggregation (%) with arachidonic acid and ristocetin in the normal sample was 3.8 and 4.2, respectively (***Table 3***).

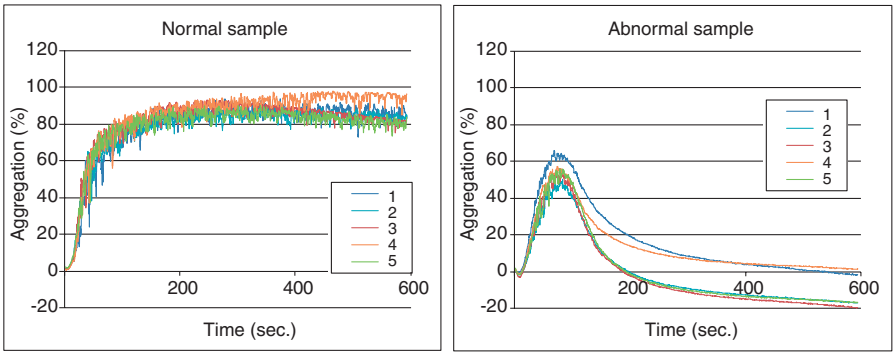
Table 2 *Reagent kits and final concentrations for measurement*

Agonist	Reagent kit	Final concentration
ADP	Revohem™ ADP	2 μM
Collagen	Revohem™ Collagen	2 μg/mL
Epinephrine	Revohem™ Epinephrine	5 μM
Arachidonic acid	Revohem™ Arachidonic acid	1 mM
Ristocetin	Revohem™ Ristocetin	1.2 mg/mL

Table 3 *Results of within-run reproducibility*

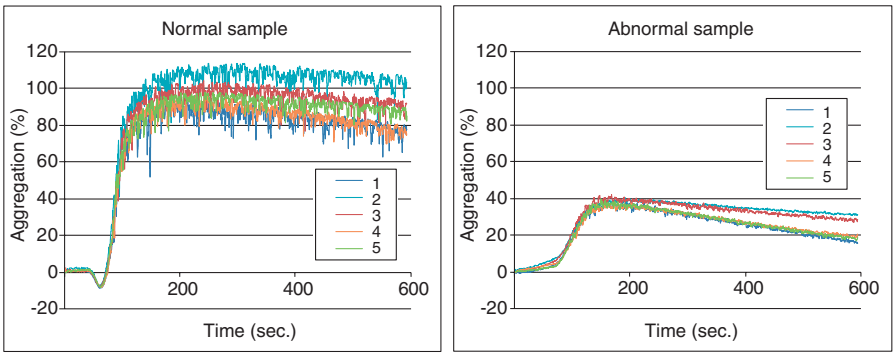
A. ADP [Maximal aggregation (%)]

	Normal sample	Abnormal sample
1	94.1	65.3
2	89.5	50.3
3	93.3	54.9
4	97.5	57.4
5	90.3	56.3
Mean	92.9	56.8
SD	3.2	5.4
CV	3.4%	9.6%



B. Collagen [Maximal aggregation (%)]

	Normal sample	Abnormal sample
1	92.5	37.8
2	100.0	40.5
3	100.0	42.1
4	95.3	37.5
5	97.5	39.7
Mean	97.1	39.5
SD	3.2	1.9
CV	3.3%	4.8%



C. Epinephrine [Maximal aggregation (%)]

	Normal sample	Abnormal sample
1	90.6	42.1
2	96.0	38.0
3	96.5	38.0
4	89.0	38.9
5	94.5	43.6
Mean	93.3	40.1
SD	3.3	2.6
CV	3.6%	6.4%

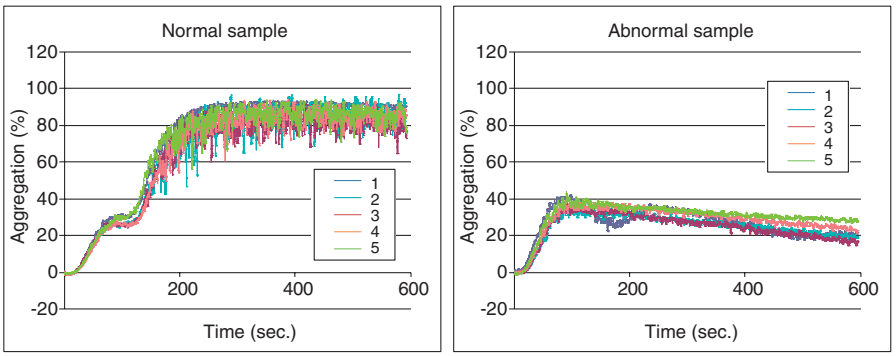
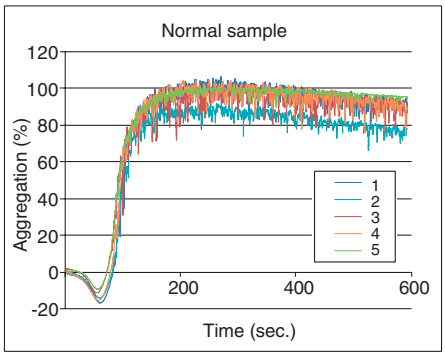


Table 3 *Results of within-run reproducibility*

D. Arachidonic acid

[Maximal aggregation (%)]

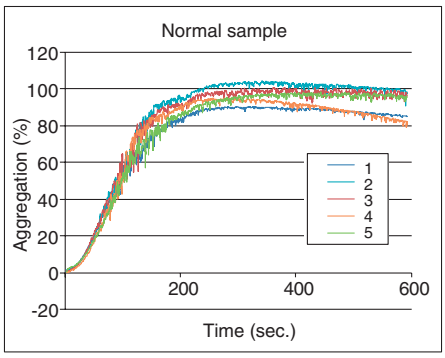
	Normal sample
1	100.0
2	91.6
3	100.0
4	100.0
5	100.0
Mean	98.3
SD	3.8
CV	3.8%



E. Ristocetin

[Maximal aggregation (%)]

	Normal sample
1	90.4
2	100.0
3	100.0
4	94.6
5	97.8
Mean	96.6
SD	4.1
CV	4.2%



2) On-board stability

On-board stability was evaluated using the maximal aggregation rate (%) of two time points at 0 and 10 hours. Since the activity of samples for platelet aggregation changed depending on the time, we prepared two sets of agonists. The first set were agonists prepared and placed on the analyzer 10 hours before measurement (agonists on-board for 10 hours). The second set were agonists prepared at the time of measurement as control (agonists on-board for 10 hour.) Both agonist sets and simultaneously measured the prepared normal and abnormal samples for evaluation.

In on-board stability evaluation, the changes in maximal aggregation rates at 0 and 10 hours were within 5% with each agonist. (***Table 4***).

Table 4 *Results of on-board stability*

A. ADP [Maximal aggregation (%)]

	Normal sample		Abnormal sample	
	0 hour	10 hour	0 hour	10 hour
1	85.6	81.8	58.2	55.6
2	82.0	85.7	51.4	47.7
Mean	83.8	83.8	54.8	51.7
Difference (0 hour - 10 hours)		0.0		3.1

B. Collagen [Maximal aggregation (%)]

	Normal sample		Abnormal sample	
	0 hour	10 hour	0 hour	10 hour
1	96.5	96.4	51.9	47.9
2	91.3	86.9	54.2	57.0
Mean	93.9	91.7	53.1	52.5
Difference (0 hour - 10 hours)		2.3		0.6

C. Epinephrine [Maximal aggregation (%)]

	Normal sample		Abnormal sample	
	0 hour	10 hour	0 hour	10 hour
1	92.1	89.5	38.2	35.3
2	86.9	92.7	43.9	38.0
Mean	89.5	91.1	41.1	36.7
Difference (0 hour - 10 hours)		-1.6		4.4

D. Arachidonic acid [Maximal aggregation (%)]

	Normal sample	
	0 hour	10 hour
1	100.0	100.0
2	99.0	100.0
Mean	99.5	100.0
Difference (0 hour - 10 hours)		-0.5

E. Ristocetin [Maximal aggregation (%)]

	Normal sample	
	0 hour	10 hour
1	81.7	73.8
2	89.5	88.6
Mean	85.6	81.2
Difference (0 hour - 10 hours)		4.4

3) Reference intervals

From the samples obtained from 125 healthy volunteers, we measured ADP-induced platelet aggregation in 96 samples, collagen-induced aggregation in 85 samples, epinephrine-induced aggregation in 82 samples, arachidonic acid-induced aggregation in 42 samples, and ristocetin-induced aggregation in 44 samples. One outlier from the mean (mean ± 2SD) was excluded, and the reference interval (95% confidence interval) was obtained using the special software Analyse-it (Analyse-it Software, Ltd.).

The reference intervals were 60% to 104% for ADP-induced aggregation (91 samples), 82% to 103% for collagen-induced aggregation (83 samples), 64% to 108% for epinephrine-induced aggregation (75 samples), 75% to 105% for arachidonic acid-induced aggregation (39 samples), and 79% to 96% for ristocetin-induced aggregation (43 samples) (***Table 5, Fig. 5***).

Table 5 *Results of reference intervals (list)*

Agonist	N	Reference intervals for % maximal aggregation
ADP	91	60 – 104
Collagen	83	82 – 103
Epinephrine	75	64 – 108
Arachidonic acid	39	75 – 105
Ristocetin	43	79 – 96

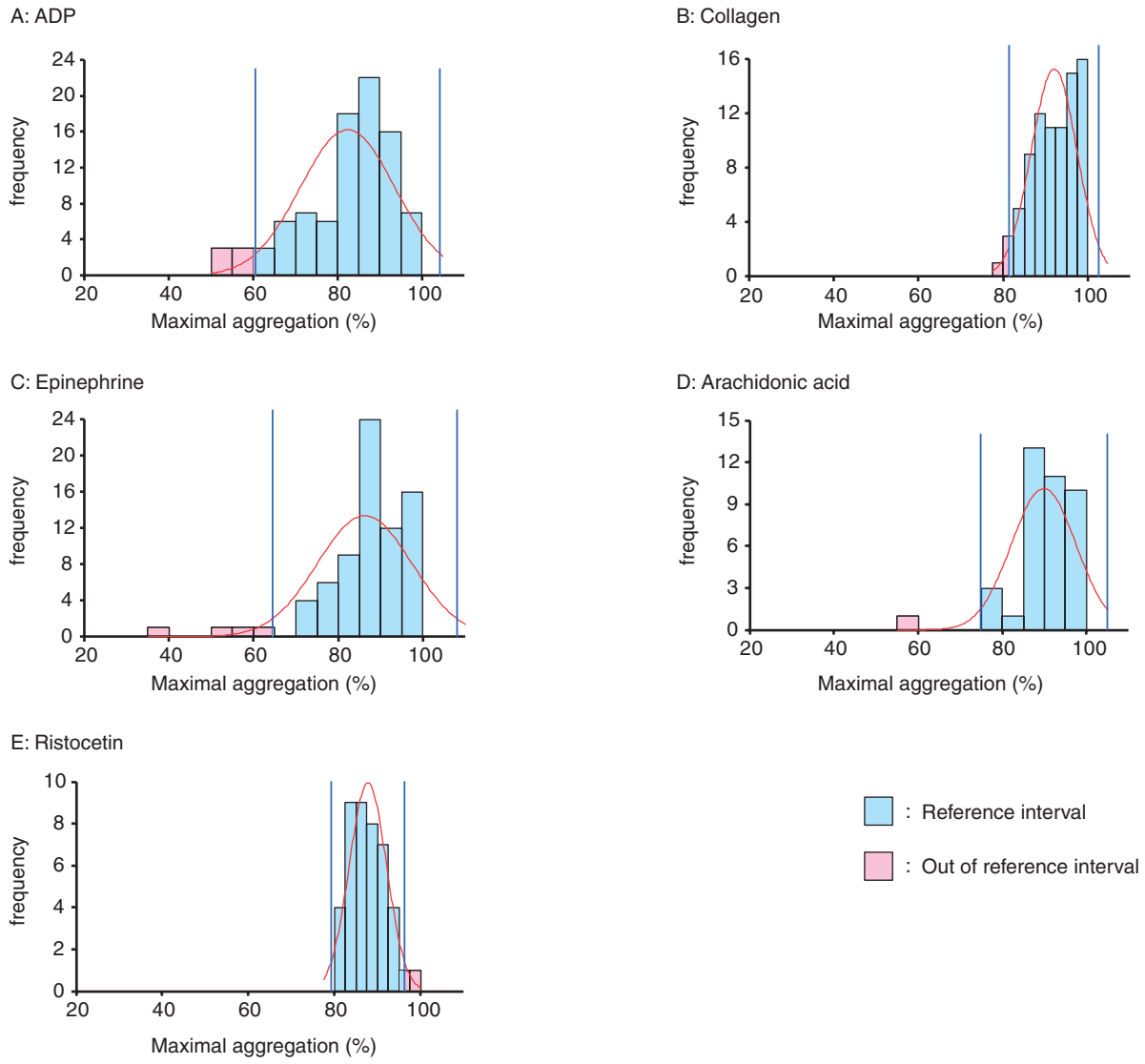


Fig. 5 *Results of reference intervals*

DISCUSSION

The within-run reproducibility at the agonist concentration recommended by SSC/ISTH was CV 5% or less for the normal sample and CV 10% or less for the abnormal sample, which showed promising results (***Table 3***). For on-board stability, all agonists were stable until 10 hours, which suggested no issues in clinical laboratory operations (***Table 4***).

In reference interval evaluation, intervals were wider for ADP and epinephrine-induced aggregation when compared with the other three agonists (***Fig. 5, A, C***). Similarly to our results, the currently published evaluation results showed wider reference intervals for ADP and epinephrine-induced aggregation than other agonists, albeit at different reagent concentrations [4]. It has also been reported that 16% of Japanese do not respond to stimulation by epinephrine for unknown cause [5]. The results of this study were presumed to have the same trend. It is recommended by SSC/ISTH to perform a confirmation test at different concentration when abnormal results are observed, since there were no clear criteria to identify the abnormal result, we propose that the reference intervals obtained in this study can be used as the criteria.

TOWARD THE STANDARDIZATION OF PLATELET AGGREGATION TESTING

Platelet aggregation testing with the CS-series has been evaluated in comparison with other existing devices, and the reported results were mostly promising. [6-9]

The recommended method of platelet aggregation test reported by SSC/ISTH is expected to facilitate the move toward the standardization of sample collection, PPP and PRP preparation methods, and concentrations of agonists in the diagnosis of congenital bleeding disorders, which were not standardized. However, there is no internationally standardized agonist concentration or method for confirmation of efficacy of antiplatelet agents frequently used in clinical practice. [2]

In addition to analyzers with LTA using PRP and PPP, there are various types of analyzers using whole blood for confirmation and monitoring of antiplatelet agent. [10] However, there are many issues toward standardization due to variation in principles, reporting units, and criteria by analyzer.

Platelet aggregation testing of the CS-series can be performed with the same analyzer as routine coagulation tests. We expect that it would contribute to standardization of antiplatelet agent efficacy confirmation in the future.

CONCLUSION

Platelet aggregation testing with the CS-series showed promising performance of within-run reproducibility and on-board stability of platelet aggregation including the benefit of automation of sample and reagent dispensing. This provides a new functionality that reduces the complexity of existing semi-automated analyzers and manual testing.

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