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ORIGINAL ARTICLE

High-throughput Luminex xMAP assay for simultaneous detection of antibodies against rabbit hemorrhagic disease virus, Sendai virus and rabbit rotavirus

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Abstract

Rabbits are widely used as models in biological research, and the pathogen status of rabbits used in studies can directly affect the results of experiments. Serological surveillance is the common monitoring method used in laboratory animals. A rapid, sensitive, and cost-effective high-throughput Luminex xMAP assay could be an attractive alternative to labor-intensive enzyme-linked immunosorbent assay (ELISA) methods. In this study, recombinant proteins from rabbit hemorrhagic disease virus and rabbit rotavirus and whole viral lysates of Sendai virus were used as coating antigens in an xMAP assay for the simultaneous detection of antibodies against these pathogens. The xMAP assay showed high specificity, with no cross-reaction with other pathogens. The coefficient of variation for intra-assay and inter-assay comparisons was less than 3% and 4%, respectively, indicating good repeatability and stability of the assay. The xMAP assay exhibited similar limits of detection for rabbit hemorrhagic virus and Sendai virus and was less sensitive for the detection of rabbit rotavirus when compared with commercial ELISA kits. A total of 52 clinical samples were tested simultaneously using both the xMAP assay and ELISA kits. The results obtained using these two methods were 100% coincident. In summary, the novel xMAP assay offers an alternative choice for rapid and sensitive high-throughput detection of antibodies in rabbit serum and can be used as a daily monitoring tool for laboratory animals.

Introduction

An appropriate animal model is crucial for mimicking disease conditions, and domestic rabbits (*Oryctolagus cuniculus*) play an important role in biological research. Since the 1980s, rabbits have been used extensively as models of human T-lymphotropic virus type 1 (HTLV-1) infection because of the consistency of viral infection and transmission in them [1]. The unique features of their lipoprotein metabolism and their sensitivity to cholesterol in their diet make rabbits a perfect model for human atherosclerosis [2] Transgenic rabbits expressing human CD4 and CCR5 homologs can be made highly susceptible to human immunodeficiency virus type 1 (HIV-1) infection and thus appropriate as models for studying disease development [3]. Rabbits are also widely used in pharmaceutical research and production of antibodies. Therefore, the quality control of rabbits is essential to guarantee the accuracy and reliability of the experiments that are performed on them.

Testing for rabbit hemorrhagic disease virus (RHDV), rabbit rotavirus (RRV), and Sendai virus (SV) is required by the national quality standards of China. Seroprevalence studies and serosurveillance are essential tools for monitoring diseases as well as vaccination efficiency [4]. Currently, the main methods for antibody detection are the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA), which are time-consuming and labor-intensive [5, 6]. Both are single-analyte technologies that fail to meet the high-throughput test requirements of routine quality

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monitoring [7]. Additionally, the enzyme-mediated amplification of signal during ELISA is not always linear and can thus skew the results [8]. It is therefore imperative to develop a rapid and sensitive high-throughput assay for simultaneous analysis of RHDV-, SV- and RRV-specific antibodies in a single biological sample.

The Luminex xMAP technology (x = analyte, MAP = multi-analyte profiling) (Luminex Crop., Austin, TX, United States) invented in the late 1990s is a high-throughput bioassay platform that enables rapid, cost-effective, and simultaneous analysis of multiple analytes of interest in one sample [9]. Changes in the concentrations of two or three dyes inside an individual bead can be recognized by the red classification laser based on its spectral signature, while the green reporter laser recognizes the fluorescent reporter bound to the captured analytes on the microsphere surface. Briefly, fluorescent microspheres that are pre-coated with specific diagnostic antigens that capture the corresponding antibodies are combined with fluorescent reporters, which are recognized by the Luminex reader, which can identify up to 500 targets in a single panel [10, 11]. In recent years, the Luminex bead system has found many applications in areas of fundamental and applied diagnostic studies [12–14].

In this study, a rapid Luminex xMAP panel was developed for simultaneous detection of specific antibodies against RHDV, SV and RRV. The performance of the xMAP assay was evaluated by comparison with a commercial ELISA kit.

Materials and methods

Virus and serum samples

Sendai virus (ATCC VR-105) was cultured in Vero cells and purified by sucrose density gradient centrifugation [15]. Negative serum from SPF rabbits and positive sera containing neutralizing antibodies against different rabbit pathogens (RHDV, SV, RRV, rabbit coronavirus [RCoV], *Encephalitozoon cuniculi*) were purchased from VRL Laboratories (Suzhou, China). Fifty-two clinical serum samples were obtained from a rabbit farm in Shandong province. All purified virus and serum samples were stored at -80 °C before use.

Expression and purification of recombinant proteins

The recombinant proteins VP60 of RHDV and VP6 of RRV were produced and purified using a prokaryotic expression system. Briefly, gene segments encoding full-length VP60 of RHDV (primers: F1, GCC*GAATTC*ATGGAGGGCAAA GCCCGTGCAGCAC; F2, GCC*GTCGAC*ATAAGAGAA ACCATTAGCTG) and a portion of VP6 of RRV (primers: F3, GCC*GAATTC*ATGGATGTCCTTTATTCTTTGACA; F4, GCC*GTCGAC*GAATGCTCAACCATTTCAGC) were amplified and cloned into the plasmid vectors vector pET28a and introduced into *Escherichia coli* BL21 strain (TransGen Biotech, Beijing, China) by transformation. Gene expression from positive clones was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the final concentration 0.1%. After 6 hours of cultivation at 37 °C, the expression products were purified from inclusion bodies, which were lysed using bacterial lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 2% Triton-X 100, pH 8.0). After sonication and centrifugation, the precipitate was re-suspended in 8 M urea and then purified by nickel column affinity chromatography following the manufacturer's instructions. Recombinant proteins were refolded in a linear gradient of 8-0 M urea and identified by SDS-PAGE.

Coupling of antigens to fluorescent beads

The recombinant proteins were desalted by gel filtration using Micro Bio-Spin 6 chromatography (Bio-Rad, California, USA) according to the manufacturer's protocol to remove sodium azide or imidazole. All antigens were quantified using a Pierce BCA Protein Quantification Kit (Thermo Scientific, Rockford, IL, USA) and then conjugated to the surface of the fluorescent magnetic beads (Luminex, USA). The coupling was carried out as described by Karanikola et al. [16]. Twenty µL of magnetic beads (about 1.25×10^6 beads) was transferred to a low-adsorption reaction tube and placed into the magnetic separator for 30 s, followed by centrifugation to remove the supernatant.

To activate the microspheres, 10 µL of 50 mg/ml N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich, Germany) and 10 µL of 50 mg/ml N-hydroxysulfosuccinimide sodium salt (S-NHS) (Sigma-Aldrich, Germany) were added to 80 µL of resuspended microspheres and agitated for 20 min at room temperature. The conjugation between antigen and activated microspheres was carried out at room temperature on a shaker for 2 hours. The coupled beads were finally resuspended in storage buffer (phosphate-buffered saline [PBS], pH 7.4, with 0.05% Tween20, 0.05% sodium azide and 1% bovine serum albumin [BSA]) and stored at 4 °C in the dark.

Luminex xMAP assay

The assay was performed in a 96-well polystyrene microplate (Thermo Scientific, Rockford, IL, USA). The coupled bead sets and the relevant positive and negative control sera were diluted with PBS containing 0.05% Tween 20 and 1% BSA (PBS-TB, pH 7.4) for the establishment of single xMAP assays to detect antibodies against RHDV, SV, and RRV respectively. Briefly, 50 µL of magnetic beads (50 beads/µL) was mixed with 50 µL of diluted serum and transferred to the wells of the plate. After 60 min of incubation on

a plate shaker (800 rpm), the plate was carefully washed 3 times with 100 µL of PBS-TB per well, followed by incubation with 100 µL of PE-conjugated goat anti-rabbit IgG per well (Sangon Biotech, Guangzhou, China) for 30 min. After the final washing step, 100 µL of assay buffer was added to each well, and the plate was shaken for approximately 10 s and then analyzed using the Luminex reader according to the manufacturer's protocol. The whole procedure was carried out at room temperature in the dark, and all of the samples were tested in triplicate.

Optimization of the assay

The recombinant VP60 and VP6 proteins and the purified virus particles of SV were coupled with magnetic microspheres at the ratios 2.5 µg, 5 µg, 10 µg, 20 µg, 30 µg, and 40 µg per 1×10^6 beads to determine the optimal antigen concentration. Each concentration was tested in triplicate, and the whole assay was carried out in duplicate.

Evaluation of specificity and sensitivity

The specificity of the test was evaluated by testing positive control antisera against RHDV, SV, RRV, RCoV, and *E. cuniculi*, and the sensitivity of the xMAP assay and that of the ELISA were compared using relevant positive virus-containing sera (twofold serial dilution from 1: 100 to 1: 25600 in PBS-TB).

Data processing

Median fluorescence intensity (MFI) values and associated standard deviations were calculated using xPONENT3.01 software. The cutoff value was the average MFI value for the negative samples plus three times the standard deviation. The cutoff value differed for each antigen, and therefore, the threshold for the limit of detection (LOD) was set based on the MFI value of the corresponding negative control serum.

Results

Selection of the optimum antigen concentration

Monoplex assays were developed to determine the optimal antigen concentration of each individual pathogen. The recombinant proteins VP60 and VP6 were tested at the ratios of 2.5 µg, 5 µg, 10 µg, 20 µg, 30 µg, and 40 µg per 1×10^6 beads, while the ratio for SV virus particles ranged from 2.5 µg to 50 µg per 1×10^6 beads. A ratio of 10 µg of VP60 per 1×10^6 beads yielded the highest positive MFI values along with the lowest negative MFI values (Table 1). For both VP6 and SV, the optimal ratio was 30 µg of antigen per 1×10^6 beads (Tables 2 and 3), indicating that the optimal coupling ratio was 10 µg per 1×10^6 beads for RHDV, and 30 µg per 1×10^6 beads for both RRV and SV.

Establishment of the multiplex xMAP assay

A multiplex assay was performed as described above using the optimal antigen concentrations. Positive sera containing the three pathogens to be tested were mixed together and tested using the newly developed assay. Each sample was tested in triplicate, and the results showed that all three targets were recognized in the same sample (Fig. 1). With a standard deviation less than 300 for positive samples and less than 50 for negative samples, the assay possessed good repeatability between duplicate wells.

Table 1 The MFI values for different coating concentrations of RHDV

Sample type	Serum number or dilution	Antigen concentration (µg /1×10^6 beads)					
		2.5	5	10	20	30	40
Positive control serum	1:100	11845	13687	15032	14864	14954	1432
	1:400	9964	10554	11465	11075	11764	10056
	1:800	8011	8084	8243	7942	8213	8321
	1:1600	6216	6344	6784	6384	6583	6534
	1:3200	2864	3054	3298	3086	3174	3285
	1:6400	2106	2321	2321	2165	2275	2203
Blank control	PBS	107	116	185	195	118	224
Negative control serum	1	229	264	237	226	289	263
	2	286	374	249	216	345	294
	3	225	241	204	256	275	232
	4	196	205	224	254	216	217
	5	187	185	164	186	337	165
Negative control average		224.6	253.8	215.6	227.6	292.4	234.2

Table 2 The MFI values for different coating concentrations of SV

Sample type	Serum number or dilution	Antigen concentration (μg /1 × 10^6 beads)					
		2.5	5	15	30	40	50
Positive control serum	1:100	15007	17564	21146	24928	24033	22067
	1:400	9884	11534	12089	16417	15336	15694
	1:800	7843	9773	12199	12789	10916	8105
	1:1600	6867	7315	7974	9567	8042	6783
	1:3200	4521	4966	6963	7544	6161	4885
	1:6400	2485	3043	3908	4698	3960	3068
	1:12800	1104	1204	2013	2213	1194	1326
Blank control	PBS	89	109	114	121	154	173
Negative control serum	1	132	150	175	139	185	184
	2	112	232	123	152	216	302
	3	134	247	185	95	162	174
	4	85	79	114	89	184	185
	5	159	158	188	101	174	184
Negative control average		124.4	173.1	157	115.2	184.2	205.8

Table 3 The MFI values for different coating concentrations of RRV

Sample type	Serum number or dilution	Antigen concentration (μg /1 × 10^6 beads)					
		2.5	5	10	20	30	40
Positive control serum	1:100	13282	14674	16483	17535	18736	18324
	1:400	8754	9964	12194	12343	13542	12965
	1:800	7643	8084	10563	10321	11043	10764
	1:1600	6496	6984	7554	7756	8352	8143
	1:3200	3569	3947	4576	4776	5213	5027
	1:6400	2435	2984	3654	3854	4034	3886
Blank control	PBS	138	156	189	205	158	232
Negative control serum	1	158	184	174	146	175	224
	2	121	158	112	253	124	253
	3	206	252	194	196	164	194
	4	227	243	221	275	205	206
	5	236	194	139	205	169	217
Negative control average		189.6	206.2	168	214.9	167.4	218.8

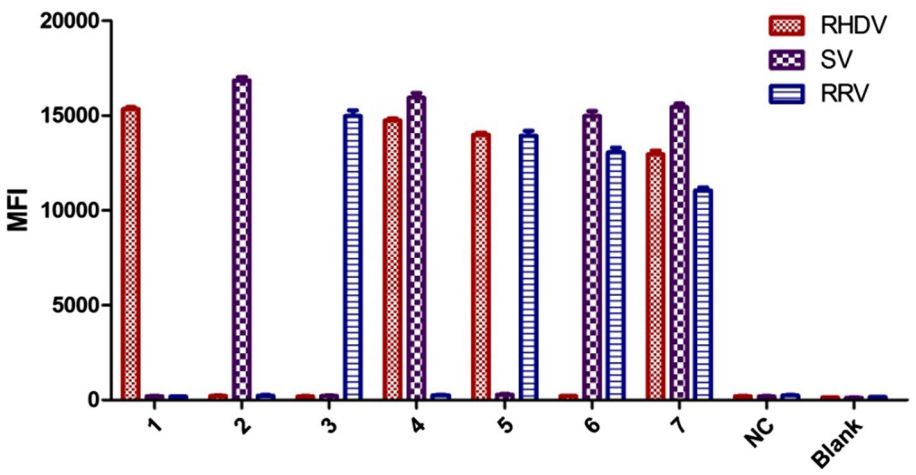

Fig. 1 Establishment of the multiplex xMAP assay. 1:RHDV positive serum; 2:SV positive serum; 3: RRV positive serum; 4: RHDV and SV positive serum; 5: RHDV and RRV positive serum; 6: SV and RRV positive serum; 7: triple positive serum; NC: negative control serum; Blank: blank control

Specificity of the multiplex xMAP assay

Positive virus-containing sera were used to evaluate the specificity of the xMAP assay. Each sample was tested in triplicate, and significant specific signals were observed only with the positive samples and no obvious cross-reactions were observed with irrelevant samples (Fig. 2).

Determination of cutoff values

The cutoff value was determined by testing a large number of negative samples. Specifically, the average MFI value of the negative samples plus three times the standard deviation was considered to be the threshold for the target. Forty SPF negative samples were tested to calculate the cutoff value. As shown in Table 4, the cutoff value for RHDV, RRV and SV was 626.22, 387.08 and 436.52, respectively.

Repeatability of the xMAP assay

To assess the reproducibility of the test results, parallel tests were carried out using diluted serum. The coefficient of variation (CV) for these three pathogens was less than 3% for intra-assay comparisons and no more than 4% for inter-assay comparisons (Table 5), demonstrating the high repeatability and good stability of the xMAP assay.

Comparison of the xMAP assay and ELISA

A comparison with commercial ELISA kits (VRL Laboratories, SuZhou, China) was carried out using serial dilutions of serum from 1:100 to 1:51200. The results indicated that the sensitivity of the newly developed xMAP assay was similar to that of the commercial ELISA kits for antibodies detection (Table 6).

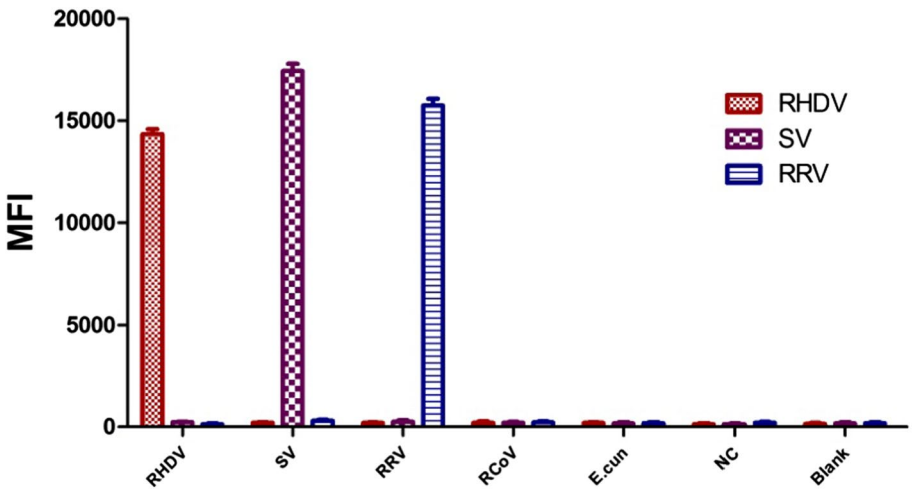

Fig. 2 The specificity of the multiplex xMAP asay. RHDV: rabbit hemorrhagic virus positive serum; SV: sendai virus positive serum; RRV: rabbit rotavirus positive serum; RCoV: rabbit coronavirus positive serum; E.cun: encephalitozoon cuniculi positive serum; NC: negative control; Blank: blank control

Table 4 The detection results obtained with 40 SPF negative serum samples

Sample	MFI value														
	RHDV					SV					RRV				
SPF negative serum	210	317	221	107	173	58	264	110	160	43	468	195	111	148	138
	242	319	264	121	185	152	173	150	173	93	82	143	132	205	77
	234	145	183	199	207	185	140	220	75	184	201	75	202	98	154
	137	126	258	174	188	123.5	186	322	107	203	174	207	285	118	221
	331	305	272	128	145	234	137	139	93	67	164	185	148	79	79
	285	48	194	102	128	150	63	218	143	184	89	94	189	121	172
	178	139	112	148	167	232	302	134	92	253	221	287	112	68	201
	143	154	166	176	123	225	113	88	372	243	264	195	94	331	198
Average	186.34					163.32					172.63				
SD	67.21					76.41					87.96				
Cutoff	626.22					387.08					436.52				

Table 5 The repeatability of the xMAP assay at different serum concentrations

Pathogen	Concentration	Intra-assay/MFI			CV (%)	Inter-assay/MFI			CV (%)
		1	2	3		1	2	3	
RHDV	1:400	11409	11206	11613	2.54	11871	11435	11312	3.3
	1:3200	3472	3271	3071	2.8	3286	3465	3217	3.5
SV	1:400	11536	11348	10997	2.6	12174	11853	12053	3.2
	1:3200	3114	3167	3072	2.73	3295	3134	3184	2.6
RRV	1:400	14587	14324	14097	1.8	14184	13996	14233	2.2
	1:3200	2564	2465	2532	2.15	2651	2438	2364	2.72

Table 6 Comparison of xMAP and ELISA sensitivity

Sample	Serum concentration	RHDV		SV		RRV	
		xMPA (MFI)	ELISA (OD)	xMPA (MFI)	ELISA (OD)	xMPA (MFI)	ELISA (OD)
Positive serum	1:100	15076	2.7056	15334	3.1220	21980	2.3301
	1:400	11410	2.1496	11563	2.6446	14098	1.7876
	1:800	8015	1.6192	8009	2.2449	8565	1.5623
	1:1600	6740	1.2326	5220	1.8729	5347	1.4383
	1:3200	3272	0.7784	3114	1.3109	2465	1.1679
	1:6400	2178	0.5674	2034	0.8275	1544	1.0443
	1:12800	805	0.3301	1084	0.4556	1053	0.8495
	1:25600	363	0.2064	232	0.2684	498	0.5588
	1:51200	–	–	–	–	354	0.3254
Negative serum	—	149	0.1512	118	0.1658	201	0.2134
Blank	—	126	0.1145	124	0.1212	165	0.2122
Cutoff	—	626.22	0.3000	387.08	0.3000	436.5	0.3000

Table 7 Comparison of xMAP and ELISA for testing of clinical samples

Pathogen	Method	Positive samples	Negative samples	Total coincidence
RHDV	xMAP	52	0	100%
	ELISA	52	0	
SV	xMAP	18	34	100%
	ELISA	18	34	
RRV	xMAP	47	5	100%
	ELISA	47	5	

Testing of clinical samples

Fifty-two clinical samples were tested using both the xMAP assay and ELISA kits. All the samples that were tested were positive for RHDV by both methods, while only 18 samples were positive for SV and 47 samples were positive for RRV. The two methods shared 100% coincidence when used for testing clinical samples (Table 7).

Discussion

ELISA is widely used in pathogen diagnosis, and commercial ELISA kits are available for detection of antibodies against RHDV, SV and RRV. Despite the fact that ELISA is an ultrasensitive method due to the use of enzymes and chemiluminescent substrates, it is labor-intensive, especially when dealing with a large number of samples with more than one detection target. The Luminex microsphere system was invented to meet analytical needs for rapid and sensitive high-throughput detection. By changing the staining ratio of the microspheres, hundreds of targets can be identified in a single reaction. In the last few years, there have been several reports describing the application of Luminex technology (xMAP/xTAG) in veterinary science [17–19]. However, most of the applications were for the diagnosis of pathogens in birds or laboratory animals, but not rabbits. Although a Luminex xTAG assay has been developed for detection of nucleic acids of pathogens in rabbits [20], there is a strong demand for an xMAP assay for antibody detection in rabbits because antibody surveillance is vital to the control of diseases.

In this study, a sensitive and specific xMAP assay was developed for surveillance of antibodies against RHDV, SV and RRV. Typically, the use of whole viral lysates results in a higher level of sensitivity [21]. However, the lack of a suitable cell line for RHDV and the inability to isolate RRV from clinical samples make it difficult to produce viral lysates [22]. Therefore, diagnostic antigens VP60 of RHDV and VP6 of RRV were expressed *in vitro* and purified for use in this study.

The approximate ratio of antigen to beads recommended by the manufacturer for the coating step is 5 μg per 1×10^6 beads. In this study, different ratios were tested, and the one yielding the highest positive MFI value accompanied by the lowest negative MFI value was defined as the optimal antigen concentration. The optimal ratio was 10 μg per1×10^6 beads for RHDV and 30 μg per 1×10^6 beads for both SV and RRV.

When compared with commercial ELISA kits, both RHDV and SV showed a similar detection capacity. With RRV, the limit of detection (LOD) was 1:25600, which was two times higher than that of ELISA (1:51200). The diagnostic antigen used in these two methods might contribute to subtle difference in the results. As discussed previously, the main advantage of using whole viral lysates is the large number of protein targets that can be detected, thus allowing a high level of sensitivity. Accordingly, the commercial ELISA kits coated with whole viral lysates showed higher sensitivity than the xMAP assay. However, high sensitivity can also increase the risk of false positive results. To avoid a false negative/positive result, samples should be rechecked to ensure the accuracy of the assay when the sample's MFI is near the cutoff value. ELISA or nucleic acid detection should be done when necessary.

Our data show that the multiplex xMAP assay is highly specific, and we did not observe any cross-reaction with irrelevant targets. The coefficient of variation for intra-assay and inter-assay comparisons was less than 4%, indicating the good repeatability and stability of the xMAP assay.

Although the LOD for RRV is somewhat higher with the xMAP assay than with the commercial ELISA kit, it is sufficient for testing clinical samples and 100% coincidence was observed when testing 52 samples. Taking all of these considerations into account, the newly developed multiplex xMAP assay is a viable alternative for conventional monitoring of antibodies in rabbits. The primary advantage of xMAP is that the coating microspheres do not interfere with each other, potentially allowing the existing xMAP assay to be expanded further as needed.

In summary, a novel multiplex xMAP assay for simultaneous detection of antibodies against RHDV, SV and RRV was established and evaluated. This assay is cost-effective and labor-saving and is therefore a promising tool for monitoring of antibodies in rabbits.

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Compliance with ethical standards

Conflict of interest All the authors declare that they do not have conflicts of interest.

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