Research/Recherche

Human African trypanosomiasis: a latex agglutination field test for quantifying IgM in cerebrospinal fluid

V. Lejon,[1] P. Büscher,[2] N.H. Sema,[3] E. Magnus,[4] & N. Van Meirvenne[2]

LATEX/IgM, a rapid agglutination test for the semi-quantitative detection of IgM in cerebrospinal fluid of patients with African trypanosomiasis, is described in this article. The lyophilized reagent has been designed for field use and remains stable at 45 °C for one year. The test has been evaluated on cerebrospinal fluid samples from trypanosome-infected and non-infected patients, by comparison with commercial latex agglutination, radial immunodiffusion, and nephelometry. All test systems yielded similar results.

Introduction

In 1968, Mattern (*1*) reported that IgM concentrations exceeding 10% of the total cerebrospinal fluid (CSF) protein concentration are a common feature of human African trypanosomiasis (HAT) in the meningoencephalitic, second stage. The phenomenon was observed in all 230 second-stage HAT patients examined, while was absent in 940 neuropsychiatric patients and in 74 of 75 neurosyphilis cases. Whittle et al. (*2*) also found high IgM levels in the CSF in 87% of second-stage patients, but not in first-stage cases. Concentrations up to 250 mg/l and 500 mg/l have been reported in *Trypanosoma brucei gambiense*-infected patients (*3*, *4*). Compared to a normal upper limit of 0.36 mg/l (*5*), CSF IgM levels in African trypanosomiasis patients are extremely high, which could be the consequence of local production of IgM in the central nervous system (*6*).

Despite its relevance to stage determination in such patients, IgM detection in CSF is not currently carried out in the field, owing to the lack of simple and robust tests. Two practical assays for IgM quantification, i.e. radial immunodiffusion (RID) and latex agglutination, are commercially available. However, RID is laborious, whereas Rapi Tex IgM (Behring, Frankfurt, Germany), originally developed for detection of IgM in the serum of neonates, has a detection limit of 33 mg/l, far above normal CSF IgM values, and the stability of the reagent is limited.

Reported here is a rapid and sensitive latex agglutination assay (LATEX/IgM), which uses a stable lyophilized reagent. Its performance was assessed on CSF samples from HAT and non-HAT patients and compared with results obtained using nephelometry, RID and Rapi Tex IgM.

Materials and methods

CSF samples from 34 untreated, parasitologically confirmed *T.b. gambiense* patients (T^+), 5 seropositive but parasitologically unconfirmed persons (T^-S^+), and 4 controls without evidence of HAT (T^-S^-) were included in the study. All CSF samples originated from Equateur Province, Democratic Republic of Congo, and were obtained from the WHO Central Serum Bank for Sleeping Sickness.

Cell counts were performed within 15 min of CSF collection using Fuchs–Rosenthal counting chambers; any trypanosomes that were found during this procedure were also recorded. CSF samples contaminated with blood were rejected. In order to remove cells, CSF was centrifuged at ambient temperature and the supernatant subsequently stored

[1] Scientist, Department of Parasitology, Institute of Tropical Medicine; and Department of Biochemistry, University of Antwerp, Antwerp, Belgium.

Requests for reprints should be sent to Dr V. Lejon, Department of Parasitology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium.

[2] Senior Scientists, Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium.

[3] Médecin régional, Bureau Central de la Trypanosomiase, Centre de Développement Intégré, Bwamanda, Democratic Republic of Congo (Deceased).

[4] Chief Technician, Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium.

Reprint No. **5895**

between −1 °C and −20 °C for a maximum of 14 days and then at −70 °C. Total protein concentration was determined using the Bio-Rad modified Coomassie brilliant blue total protein test kit (*7*). Trypanosomiasis patients with more than 5 cells per µl or protein concentrations above 300 mg/l or with trypanosomes in their CSF were considered to be in second stage.

The IgM concentration was measured by nephelometry (Behring, Frankfurt, Germany) and RID (Human IgM UL and LL Nanorid kits, the Binding Site; Birmingham, England) following the protocols prescribed by the manufacturers. The incubation time of RID was 72 h, following procedure 3 (measurement of ring diameters before completion). Quantification of IgM by means of latex agglutination was carried out with Rapi Tex IgM (Behring, Frankfurt, Germany) and LATEX/IgM following the protocols described hereafter.

Preparation of LATEX/IgM reagent. A suspension of 7.5 ml carboxyl-modified polystyrene latex (Estapor K1.08, 10% w/v suspension, particle diameter 0.9 µm) is mixed with 6 ml of water; 1.5 ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce, Rockford, IL, USA) freshly dissolved in water to a concentration of 10 mg/ml is added and the suspension is mixed and left for 15 min at room temperature. Subsequently, 3 ml of anti-human IgM (Pasteur Diagnostics, Marnes-la-Coquette, France: antisera for immunoelectrophoresis, anti-human IgM (µ) in 0.1 mol/l phosphate buffer containing 0.1% sodium azide), diluted to a protein concentration of 7.5 mg/ml in water, is added for covalent coupling. The reaction mixture is shaken gently for 1 hour at 4 °C and the reagent is washed three times with eight volumes of cold TBSA (Tris-buffered saline, 0.02 mol/l, pH 7.4, complemented with 1% bovine serum albumin, Acros Chimica, New Brunswick, NJ, USA) by centrifugation (470 *g*, 4 °C, 1 h). After the last centrifugation, the sediment is resuspended in stabilization buffer (TBSA with 10% sucrose and 0.1% sodium azide) to a total volume of 50 ml, corresponding to a 1.5% w/v latex suspension. To obtain a monodisperse suspension, the latex reagent is sonicated on ice (Vibra-cell, 6-mm probe, amplitude 80, 1 min, pulse 3 sec, 9 W output). Aliquots of latex (1 ml) are frozen at −70 °C until use.

Latex agglutination: test protocol. Twofold serial dilutions of CSF are prepared in phosphate-buffered saline (PBS, 0.013 mol/l, pH 7.2, supplemented with 0.02% sodium azide); 20 µl of latex reagent (Rapi Tex IgM or LATEX/IgM) is then applied to the reaction zone of an agglutination card (6 black zones, Murex Erembodegem, Belgium) and 40 µl of test sample is added. Both components are mixed with a plastic rod and spread over a 1.5-cm diameter zone and the card is rocked on a horizontal rotator (ITMAS type A) at 70 rpm. After 10 min, the degree of agglutination is scored macroscopically (Table 1). The end-titre of a sample is defined as the highest dilution factor still giving a reaction score of at least 2.

Table 1: **Macroscopic scoring of the latex agglutination result**

Agglutination	Result	Score
Invisible	Negative	0
Hardly visible	Negative	1
Clearly visible	Weakly positive	2
Intense	Positive	3
Maximal	Strongly positive	4

The detection limit of the agglutination tests was calculated from the results obtained with twofold serial dilutions of the positive control included in the Rapi Tex IgM kit or a Behring N/T Protein Control SY/H (human serum) for the LATEX/IgM.

Stability testing of the LATEX/IgM reagent. Aliquots (1 ml) of freshly prepared LATEX/IgM reagent were stored under different conditions: frozen at −70 °C, in suspension at 4 °C and at 45 °C and as lyophilized reagent (after snap freezing in liquid nitrogen) at 4 °C and at 45 °C. These aliquots were tested after 0, 7, 30, 98, 189, 273 and 358 days. Prior to use, lyophilized LATEX/IgM was reconstituted with 1 ml of water. Any reagent showing macroscopic or microscopic (10 × 40) autoagglutination was rejected. The reactivity of the LATEX/IgM reagent was assessed by testing twofold dilutions (1:200 etc.) of five normal human serum samples (stored at −40 °C, in aliquots sufficient for one test run with all the reagents) as described in the agglutination protocol. PBS was used as a negative control.

Results

The results are summarized in Table 2.

Number of cells, protein concentration and presence of trypanosomes in CSF. In the T^-S^- group, one CSF sample (T^-S^- no. 4) had an abnormally high cell number and protein concentration (14 cells/µl, 640 mg/ml) for unknown reasons. Cell numbers in the T^-S^+ group lay in the range 2–4 per µl and protein concentrations in the range 200–359 mg/l. According to their CSF cell number and protein concentration, four HAT patients had to be considered as first-stage cases (T^+ no. 1–4). Since the cell number in the other

Table 2: **Origin and properties of 43 CSF samples studied: sample group and number, cells, total protein concentration, finding of trypanosomes, IgM concentrations as measured by nephelometry and RID, and end-titres obtained in Rapi Tex IgM and LATEX/IgM**

Sample group	No.	No. of cells/μl	Protein concentration (mg/l)	Trypanosomes in CSF	Nephelometry (IgM mg/l)	RID (IgM mg/l)	Rapi Tex (end-titre)	LATEX/IgM (end-titre)
T^+	1	2	191	–	<5	<3.5	0	0
T^+	2	2	209	–	<5	<3.5	0	0
T^+	3	3	207	–	<5	5.92	0	2
T^+	4	3	295	–	<5	4.4	0	0
T^+	5	7	326	–	<5	<3.5	0	2
T^+	6	9	295	–	7	<3.5	0	2
T^+	7	9	330	–	5	4.4	0	2
T^+	8	10	294	–	7	11.1	0	4
T^+	9	11	387	–	<5	5.4	0	1
T^+	10	12	413	–	10	11	0	2
T^+	11	14	988	–	30	13.6	1	8
T^+	12	16	373	–	40	101	2	4
T^+	13	21	372	–	76	47	4	16
T^+	14	22	366	–	<5	<3.5	0	2
T^+	15	23	252	–	18	13.8	0	2
T^+	16	23	381	–	5	4	0	4
T^+	17	33	329	–	25	27.1	1	8
T^+	18	45	333	–	5	4.3	0	2
T^+	19	60	308	–	81	44	4	32
T^+	20	80	653	–	67	105	4	32
T^+	21	103	812	–	99	51.5	8	32
T^+	22	105	555	–	78	157	4	32
T^+	23	105	264	–	48	14.2	2	16
T^+	24	108	805	–	418	734	32	128
T^+	25	110	492	–	82	47	8	32
T^+	26	123	975	–	244	207	16	64
T^+	27	128	573	–	24	35	1	8
T^+	28	158	1142	+	349	487	32	128
T^+	29	205	391	+	287	152	16	64
T^+	30	209	799	+	208	141	16	64
T^+	31	210	247	–	24	11.1	2	8
T^+	32	304	656	–	113	232	8	32
T^+	33	580	302	+	47	22.9	2	16
T^+	34	700	794	+	124	87	8	32
T^-S^+	1	2	225	–	<5	<3.5	0	0
T^-S^+	2	2	359	–	<5	<3.5	0	0
T^-S^+	3	2	200	–	<5	<3.5	0	0
T^-S^+	4	2	237	–	<5	<3.5	0	1
T^-S^+	5	4	241	–	<5	<3.5	0	0
T^-S^-	1	0	359	–	<5	<3.5	0	0
T^-S^-	2	1	386	–	<5	<3.5	0	0
T^-S^-	3	3	239	–	<5	<3.5	0	0
T^-S^-	4	14	640	–	<5	<3.5	0	0

30 HAT patients (T^+ no. 5–34) lay in the range 7–700 cells/μl and protein concentrations between 247 and 1142 mg/l, they all had to be considered as second-stage cases. In five CSF samples trypanosomes could be detected during cell counts.

Nephelometry. All controls (T^-S^-) and all parasitologically unconfirmed, seropositive individuals (T^-S^+), including the one with 14 cells/μl and a protein concentration of 640 mg/ml (T^-S^+ no. 4), had CSF IgM concentrations <5 mg/l, the detection limit of nephelometry. First-stage HAT cases also showed IgM concentrations below the detection limit. CSF IgM was detectable in all HAT cases with >22 cells/μl or protein concentrations >387 mg/l. IgM concentrations up to 418 mg/l were found in these samples.

Radial immunodiffusion. In RID, no precipitation rings could be observed with the UL Nanorid kit designed for detection of IgM concentrations of 0.35–3.5 mg/l, not even with the calibrator and control included in the kit. All samples were there-

Fig. 1. **Regression between CSF IgM concentrations revealed by RID and nephelometry** (---: detection limit of RID (3.5 mg/l) and nephelometry (5 mg/l); ·—·—·: regression line, $y = 1.048x - 0.15$).

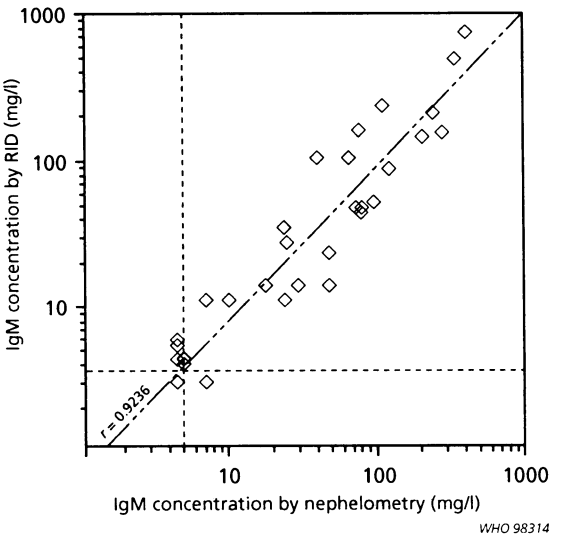
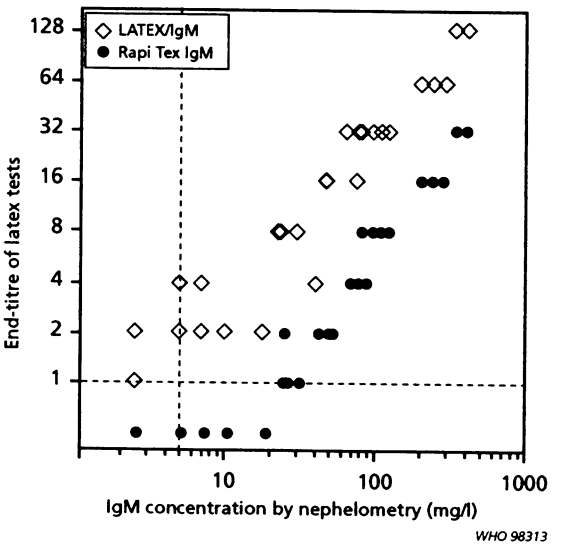


Fig. 2. **CSF end-titres obtained with Rapi Tex IgM and LATEX/IgM and IgM concentrations by nephelometry** (---: detection limit of nephelometry (5 mg/l) and of agglutination tests).



fore tested with the LL RID kit, for IgM concentrations of 3.5–35 mg/l, also with this kit, precipitation rings obtained with CSF samples were faint and hardly visible. If the IgM concentration of the CSF sample turned out to be >35 mg/l in RID, diluted CSF was retested. CSF IgM concentrations in RID compared to the results obtained using nephelometry are shown in Fig. 1.

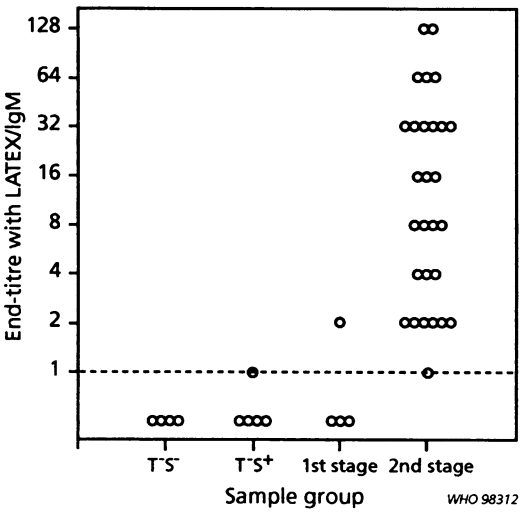
As in nephelometry, RID failed to detect IgM in CSF from the T^-S^- and T^-S^+ groups. With RID, however, CSF IgM concentrations could be measured in two out of four first-stage patients. Of the 30 second-stage patients, 27 had detectable IgM concentrations. All CSF samples with >22 cells per µl or protein concentrations >366 mg/l showed measurable IgM concentrations in RID of up to 734 mg/l.

Rapi Tex IgM. A more sensitive version of Rapi Tex IgM was introduced by modifying the ratio sample: latex volume and by increasing the reaction time from 3 to 10 min. Under these conditions, the end-titre of the control serum included in the kit became 4 instead of 1; since, according to the manufacturer, the IgM concentration of this serum is 35 mg/l, the detection threshold of the modified agglutination test was apparently lowered to approximately 9 mg/l. The relationship between the IgM concentrations measured by Rapi Tex IgM and nephelometry is shown in Fig. 2. Samples with nephelometric concentrations of <18 mg/l, remained non-reactive in the modified Rapi Tex, while visible agglutination occurred when the IgM concentration was ≥24 mg/l. The actual detection limit for the modified Rapi Tex IgM therefore seems to be between 18 and 24 mg/l of IgM in CSF.

All T^-S^-, T^-S^+ and T^+ first-stage CSF samples were non-reactive with modified Rapi Tex IgM. The CSF of an HAT patient with 45 cells/µl (T^+ no. 18) was also non-reactive and the end-titres of some other samples with high cell numbers, e.g. T^+ no. 23, 27, 31 and 33, were surprisingly low (1 or 2). The highest end-titre observed was 32.

LATEX/IgM. With LATEX/IgM, the lowest detection limit was also obtained with a sample: latex volume ratio of 40 µl:20 µl in combination with a reaction time of 10 minutes. Under these conditions, the control serum with 1800 mg/l IgM showed an end-titre of 400, suggesting a detection limit of approximately 4.5 mg/l. The relationship between LATEX/IgM titres and the IgM concentrations revealed by nephelometry is shown in Fig. 2. Even samples with IgM concentrations below the detection limit of nephelometry, thus containing <5 mg/l IgM, were reactive in LATEX/IgM, some even at a 1:2 dilution. This suggests that the detection limit of LATEX/IgM, is indeed lower than 5 mg/l.

Fig. 3. **CSF LATEX/IgM end-titres of 4 controls, 5 parasitologically unconfirmed seropositives, and 34 HAT patients** (---: detection limit of LATEX/IgM).



In LATEX/IgM, all T^-S^- CSF samples were negative, whereas 1 out of 5 T^-S^+ samples showed an end-titre of 1 (Fig. 3). Except for three non-reactive first-stage samples, all tested CSF samples of HAT patients were reactive, with end-titres up to 128.

The stability of the LATEX/IgM reagent under different storage conditions was assessed after different periods of time (Table 3). The plain reagent, stored at 4°C and 45°C, already showed autoagglutination after 7 days, and this version was rejected. The frozen reagent (−70°C) and the lyophilized reagents kept at 4°C or 45°C did not show autoagglutination after long-term storage. With these preparations the reference sera retained their original end-titres within a limit of 1 titre and the control test with PBS was always negative. In its lyophilized form LATEX/IgM reagent seems stable for at least 1 year, even at 45°C.

Discussion

For the detection of IgM in CSF, radial immunodiffusion and latex agglutination are relatively simple techniques compared to nephelometry, which can only be performed in a well-equipped laboratory. In the present study, these test systems were evaluated comparatively on a small group of CSF samples from HAT patients and controls.

With nephelometry, problems were encountered on testing lyophilized samples (unpublished observations) and such samples were therefore excluded from the present study. The nephelometric detection limit for IgM in CSF is 5 mg/l, which is far above the upper reference limit of 0.36 mg/l in healthy individuals (*5*). All CSF samples in which IgM is detected by nephelometry should therefore be considered as abnormal.

The results obtained by RID and both versions of the latex agglutination test showed a good correlation with the nephelometric results, suggesting that the same component (IgM) is being detected. According to the manufacturer, RID would allow the

Table 3: **End-titres of five reference sera obtained in LATEX/IgM, with frozen and lyophilized reagents stored for 7, 30, 98, 189, 273 and 358 days**

Storage condition and temperature	Serum	Day						
		0	7	30	98	189	273	358
Frozen: −70 °C	1	800	800	800	800	800	400	800
	2	400	400	400	400	400	400	400
	3	800	800	800	800	800	800	800
	4	800	800	800	800	800	800	800
	5	800	800	800	800	800	400	800
Lyophilized: 4 °C	1	800	800	800	800	800	400	800
	2	400	400	400	400	400	400	400
	3	400	400	800	800	800	800	800
	4	800	800	800	800	800	800	800
	5	800	400	800	800	400	400	800
Lyophilized: 45 °C	1	800	800	800	800	800	400	800
	2	400	400	400	400	400	400	400
	3	400	400	800	800	800	800	800
	4	800	800	800	800	800	800	800
	5	800	400	800	800	800	400	800

detection of IgM concentrations as low as 0.35 mg/l; in our hands, however, the detection limit was 3.5 mg/l. Because of the long incubation time and the faint precipitation rings, which are difficult to measure, implementation of RID in the field is not practical. Rapid and simple agglutination tests seem much more appropriate. There are two reasons why Rapi Tex IgM is less suited for field application. First, its detection limit in CSF was around 20 mg/l IgM, which is far above the normal upper limit. Second, the reagent has to be stored at 4 °C and has a relatively short shelf-life. The newly developed LATEX/IgM has a detection limit lower than 5 mg/l and the freeze-dried reagent proved to be stable for at least 1 year, even at 45 °C.

In conclusion, LATEX/IgM seems well suited as a field test to monitor IgM levels in the CSF of African trypanosomiasis patients before and after drug treatment; it could also be useful in other diseases with local IgM production in the central nervous system, such as neurosyphilis and human immunodeficiency virus (HIV) infection.

Acknowledgements

We are saddened by the loss of our co-author, Dr N.H. Sema, coordinator of the HAT control programme in Equateur, Democratic Republic of Congo, who died in an accident on 11 September 1997. We thank the Laboratory of Clinical Biology (Institute of Tropical Medicine, Antwerp) for performing the nephelometric assays. The technical assistance of Mrs I. Wouters and Mr N. Anné is very much appreciated.

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, from International Livestock Research Institute, Nairobi, and from the Belgian Department of Development Cooperation.

Résumé

La trypanosomiase africaine: un test d'agglutination au latex pour application sur le terrain du dosage des IgM dans le liquide céphalorachidien

La trypanosomiase humaine africaine à *Trypanosoma brucei gambiense* évolue d'un stade précoce caractérisé par la prolifération des trypanosomes dans le sang, la lymphe et d'autres tissus, vers un stade neurologique où les trypanosomes envahissent le système nerveux central. Le diagnostic différentiel de ces deux stades se fait couramment par l'évaluation de la cytorachie, de la protéinorachie et de la présence de trypanosomes dans le liquide céphalorachidien (LCR).

Le stade neurologique est également caractérisé par la présence d'IgM dans le LCR. Cependant, par manque de tests appropriés et de réactifs stables, le dosage des IgM est difficile sur le terrain.

Le test d'agglutination LATEX/IgM permet de doser de manière semi-quantitative les IgM présentes dans le LCR de patients trypanosomés au moyen d'un réactif contenant des anticorps anti-IgM humaines fixés de façon covalente sur des particules de latex en suspension. Le test LATEX/IgM a été évalué sur des échantillons de LCR de trypanosomés et de témoins non parasités en comparaison avec un test d'agglutination commercial, l'immunodiffusion radiale et la néphélémétrie. On constate une bonne corrélation des résultats avec les différentes techniques.

Sous forme lyophilisée, le réactif du test LATEX/IgM reste stable à 45°C pendant au moins une année et permet donc de doser sur le terrain les IgM présentes dans le LCR des trypanosomés.

References

1. **Mattern P.** Etat actuel et résultats des techniques immunologiques utilisées à l'Institut Pasteur de Dakar pour le diagnostic et l'étude de la trypanosomiase humaine africaine. *Bulletin of the World Health Organization*, 1968, **38**: 1–8.
2. **Whittle HC et al.** IgM and antibody measurement in the diagnosis and management of Gambian trypanosomiasis. *American journal of tropical medicine and hygiene*, 1977, **26**: 1129–1135.
3. **Lambert PH, Berney M, Kazyumba G.** Immune complexes in serum and in cerebrospinal fluid in African trypanosomiasis: correlation with polyclonal B cell activation and with intracerebral immunoglobulin synthesis. *Journal of clinical investigation*, 1981, **67**: 77–85.
4. **Knobloch J et al.** Evaluation of immunoassays for diagnosis and management of sleeping sickness in Liberia. *Tropenmedizin und Parasitologie*, 1984, **35**: 137–140.
5. **Blennow K et al.** Immunoglobulin M in cerebrospinal fluid: reference values derived from 111 healthy individuals 18–88 years of age. *European neurology (Basle)*, 1996, **36**: 201–205.
6. **Greenwood BM, Whittle HC.** Cerebrospinal-fluid IgM in patients with sleeping sickness. *Lancet*, 1973, **2**: 525–527.
7. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry (New York)*, 1976, **72**: 248–254.