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The en 455-3 modified lowry assay does not yield a reliable estimate of the allergenicity level of latex gloves with low total protein content

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	Summary
Background:	Estimation of the allergenicity of latex gloves by measurement of total extractable protein content with the modified Lowry method is not satisfactory. Therefore, complementary methods that are accurate, sensitive, and clinically relevant are needed. The Competitive Immunoassay for Antigenic Latex Proteins (CIALP) method described in a previous study could be a reliable complementary method for estimating the allergenicity of latex gloves.
Material/Methods:	Extracts from 62 powdered or powder-free gloves (16 surgical and 46 examination) were tested by the EN 455-3 modified Lowry assay, IgE-inhibition, and CIALP. The results were compared with those from 36 glove extracts reported in a previous study.
Results:	Significant correlations were observed between CIALP, IgE-inhibition, and the Lowry assay for the 62 glove extracts (r between 0.79 and 0.87; p<0.001), mostly rich in proteins. After inclusion of results from the previous study, significant correlations between the three methods were again observed (r between 0.76 and 0.90; p<0.001). However, there was no correlation between CIALP or IgE-inhibition and results of the Lowry assay for gloves that had a total protein content <50 µg/g of glove. Furthermore, 15/16 extracts with undetectable total proteins were positive in both the CIALP and IgE-inhibition methods.
Conclusions:	The modified Lowry assay alone is useful for estimating the allergenicity of latex gloves when the total protein content is ≥50 µg/g of glove. For gloves with a total protein content <50 µg/g of glove, complementary methods such as CIALP are necessary.
key words:	latex allergens • modified lowry assay • ELISA • IgE-inhibition • medical gloves
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BACKGROUND

The total protein content of natural rubber latex (NRL) varies from 1 to 2.8 % [1]. Some of these proteins remain in NRL products such as medical gloves. These proteins are responsible for clinical features of NRL-induced immediate hypersensitivity reactions, including contact urticaria, angioedema, conjunctivitis, rhinitis, asthma, and anaphylactic shock [2].

In order to prevent allergic reactions associated with the use of medical gloves, the protein content of these products should be low. Control of their allergenicity is therefore essential. RAST-inhibition, immunoblotting, and skin tests have been used for the assessment of the allergen content of medical gloves [3–6]. However, these methods are not applicable at the industrial level because they require either the use of sera from allergic patients or their participation in performing skin tests. At the moment, the only test approved for the indirect estimation of NRL glove allergenicity is the measurement of total extractable proteins by the modified Lowry method (ASTM D 5712-99 in the USA and EN 455-3 in the European Union) [7,8]. However, it has been demonstrated that some gloves with low total protein content could display high NRL allergen content and vice versa [3,9–11].

Measurement of antigenic latex protein using sera from rabbits immunized with NRL has been developed as an alternative method for this purpose. The LEAP and the CIALP immunoassays showed a better correlation with allergenic protein level measured by RAST-inhibition than with total protein level determined by the modified Lowry method [12,13]. These two immunoassays are based on competition between latex antigens in the test sample and immobilized ammoniated NRL in the LEAP or non-ammoniated NRL in the CIALP. In a previous study we demonstrated the lack of correlation between the extractable protein level measured by the modified Lowry method and the latex antigenic protein level measured by CIALP in medical gloves, most of which had a low protein content [12]. The present work was therefore undertaken to extend these data using NRL gloves with a wider range of total extractable proteins.

MATERIALS AND METHODS

Gloves

Sixty-two NRL powdered or powder-free gloves (16 surgical and 46 examination gloves) from different manufacturers were tested. All the gloves were extracted at the Laboratoire National d'Essais (LNE, Trappes, France) according to the EN 455-3 European procedure [8]. The extracts were divided in aliquots, stored at +4°C, and part of them were sent to the Laboratoire Universitaire de Recherche en Immuno-Allergologie (LURIA, Hôpital Tenon, Paris, France). Total protein measurements using the EN 455-3 modified Lowry method were simultaneously performed by the LNE and LURIA laboratories. NRL antigenic and allergenic proteins were assayed by both CIALP and IgE ELISA-inhibition methods as described below.

Total protein determination

The total protein measurement was performed by the modified Lowry method according to the standard EN 455-3 pro-

cedure [8]. For each sample, the proteins in 1 ml of glove extract were precipitated with 0.15% deoxycholic acid, 72% trichloroacetic acid, and 72% phosphotungstic acid and then centrifuged. The pellet was dissolved in 0.2 ml of NaOH 0.1 N. Samples were assayed in duplicate with a commercial ready-to-use total protein assay kit (Bio-Rad DC protein assay, Hercules, CA). Optical densities (OD) were read at 710 nm with a spectrophotometer (SECOMAM S 500I, Domont, France) at the LNE and at 630 nm wavelength with a microplate reader (MRX, Dynex, Issy-Les-Moulineaux, France) at LURIA. A six-point calibration curve with ovalbumin (grade V, Sigma, Saint Louis, MO) was used as the standard. The detection limit of the method was 5 µg/ml and the reporting limit, i.e. the detection limit reported to the weight of extracted glove, was 20 µg/g of glove.

NRL antigenic protein determination

The NRL antigenic proteins were determined by the CIALP method as previously described [12]. This assay is based on competition between the proteins of the test sample and NRL proteins of an *in vivo*-standardized non-ammoniated NRL extract (NRL-S, Stallergènes, Antony, France) coated on the wells of the microplate. Latex antigens are recognized by latex-specific antibodies from a rabbit previously immunized with NRL-S. In brief, microplates (MaxiSorp™, NUNC, Roskilde, Denmark) were coated with 0.4 µg/well of NRL-S in carbonate-bicarbonate buffer 0.1 M, pH 9.6 (Sigma). After overnight incubation, free binding sites were blocked with 1% BSA in PBS 0.1 M, pH 7.4 (Sigma). After 4 washes with PBS + 0.05% Tween 20 (PBS-Tween), the assay was performed by adding to each well 75 µl of the glove extracts or the standards with an equal volume of rabbit anti-latex serum diluted to 1/20000 in PBS + 0.1% BSA + 0.05% Tween 20. After overnight incubation at 4°C followed by 4 washes, 150 µl of peroxidase-labeled mouse monoclonal anti-rabbit IgG diluted to 1/10000 (Sigma) were added for 1 h incubation at 37°C. After 4 washes, revelation was performed with the o-phenylenediamine (OPD, Sigma) substrate. ODs were read at 430 nm wavelength after 30 min incubation at room temperature in the dark. An eight-point calibration curve with NRL-S was obtained with twofold serial dilutions (in duplicate) starting from 2 µg/ml. The detection limit of the method was 0.017 µg/ml and the reporting limit was 0.085 µg/g of glove.

IgE ELISA-Inhibition (IgE EI)

The method of Palosuo et al. [11], modified as follows, was used for allergenic NRL protein measurement. Microplates were coated with 100 µl/well of NRL-S diluted in carbonate-bicarbonate buffer 0.1 M, pH 9.6. After incubation for 3 hours at room temperature, then overnight at +4°C, the plates were washed with PBS-Tween and the free binding sites were blocked with 1% human serum albumin (HSA, Centre National de Transfusion Sanguine, Paris, France) in PBS for 1 hour at room temperature. Finally, the plates were washed with PBS-Tween then stored at –20°C until use. The IgE EI was performed by incubation of the samples (50 µl) with an equal volume of a pool of human sera diluted 1/5. This pool was obtained by mixing the sera from 25 well-established NRL-allergic patients. The concentration of specific IgE in this pool was 16.4 kIU/l (CAP System RAST®, Pharmacia, Upsala, Sweden). PBS + 0.1% HSA + 0.05% Tween 20 was

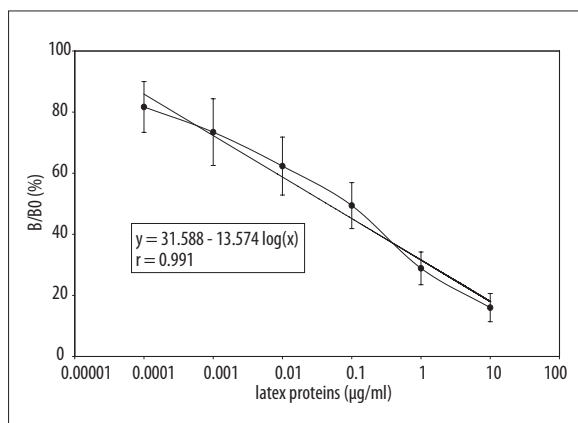


Figure 1. Calibration curve (mean of 4 different experiments $\pm$ SD) of the IgE ELISA inhibition method. A six-point calibration curve was obtained by tenfold serial dilutions of the standardized natural rubber latex extract (NRL-S, Stallergenes) starting from 10 μ g/ml. B: average OD values of standards or glove extracts. B0: mean OD value of 6 wells with no inhibiting latex proteins (diluting buffer alone).

used as the dilution buffer. Undiluted glove extracts and 3 fourfold serial dilutions of 1/4 to 1/64 were tested. After incubation overnight at +4°C, followed by 4 washes with PBS-Tween, 100 μ l of a 1/1000 dilution of goat anti-human IgE peroxidase-conjugate antibody (Sigma) was added and incubated for 1 hour at 37°C. OPD substrate was then added (100 μ l/well) after 4 washes. After 45 min of incubation at room temperature in the dark, the OD was read at 405 nm. A six-point calibration curve was obtained using tenfold dilutions of the NRL-S extract in duplicate starting from 10 μ g/ml to 0.0001 μ g/ml (Figure 1). Results obtained with this method were validated by testing 7 glove extracts by CAP RAST-inhibition. A good correlation between these methods was observed ($r=0.995$; $p<0.001$). The detection limit of the IgE EI method was 1 ng/ml and the reporting limit 5 ng/g of glove.

Statistical analysis

Calibration curves were fitted by logarithmic regression. Correlation between CIALP, Lowry, and IgE EI results were calculated using the nonparametric Spearman rank-correlation test. In order to consider all data for the correlation calculation, results of the CIALP, modified Lowry, and IgE EI methods below the detection limits were arbitrarily assigned a value equal to the threshold level of the respective method. A $p<0.05$ was considered as significant.

RESULTS

Total protein measurements

In the present study, only 9/62 (15%) gloves tested displayed a total protein content <50 μ g/g of glove, while in our previous study most of the gloves tested (23/36, 64%) had a total protein content <50 μ g/g of glove ($\chi^2=25.0$; $p<0.001$) [12]. These results are only related to the brand of gloves considered, since the results of total protein measurement obtained at LNE and LURIA for the 62 extract samples were strongly correlated ($r=0.93$; $p<0.001$).

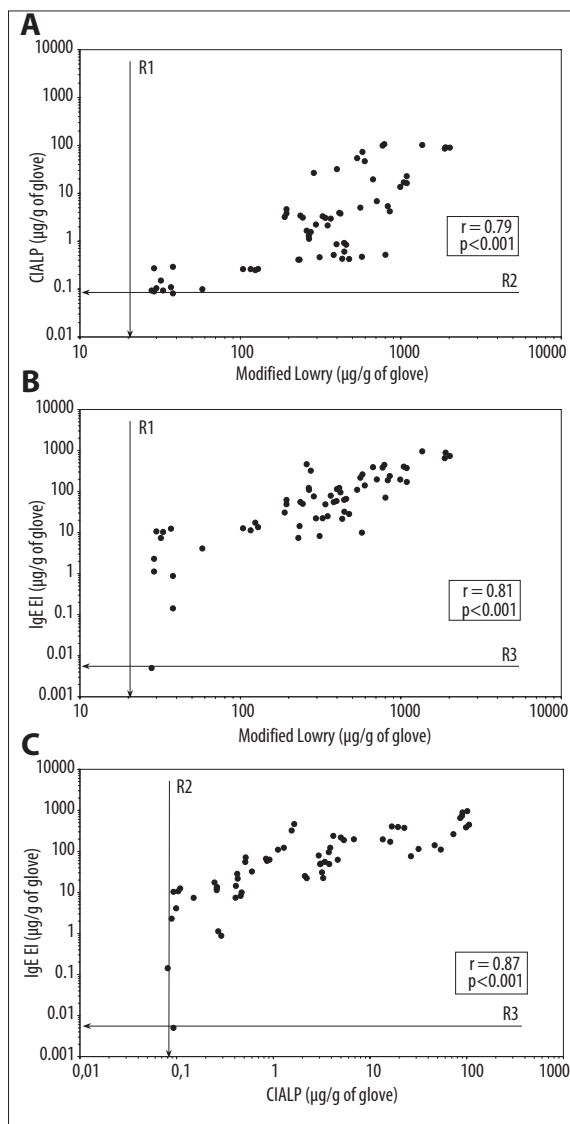


Figure 2. Correlation between modified Lowry, CIALP, and IgE EI results for 62 glove extracts calculated with the Spearman nonparametric correlation test. Results below the detection limits were arbitrarily assigned to the threshold level value of the respective method. R1, R2, and R3 are the detection limits of, respectively, the modified Lowry, CIALP and IgE EI methods. (A) comparison between the modified Lowry method and CIALP, (B) modified Lowry vs. IgE EI, and (C) IgE EI vs. CIALP.

Comparison of CIALP, IgE EI, and the modified Lowry results

Latex protein content of the 62 glove extracts are presented on Table 1. The results of the modified Lowry, CIALP, and IgE EI assays for the 62 glove extracts tested were highly correlated (Figure 2).

To enable the comparison with a wider range of extractable proteins, we added the data from the previous study, in which most of the gloves tested had a low total protein level [12]. After combining the results of the 36 glove extracts from the previous study and the 62 glove extracts from the

Table 1. Total, antigenic, and allergenic latex protein content (expressed in µg/g of glove) of 62 medical latex glove extracts, assayed by, respectively, the modified Lowry method, CIALP, and IgE inhibition assay.

Type of glove	Powder	Total protein	Antigenic protein	Allergenic protein	Type of glove	Powder	Total protein	Antigenic protein	Allergenic protein
S	PF	28	0.093	0.005	E	P	366	2.952	78.898
S	P	29	0.089	2.294	E	P	382	0.514	55.186
E	PF	29	0.270	1.123	E	P	398	0.862	58.686
S	PF	30	0.104	10.655	E	P	400	31.863	114.114
S	P	32	0.150	7.379	E	P	414	3.891	121.976
S	P	33	0.093	10.315	E	P	421	3.759	95.150
S	P	37	0.109	12.443	S	P	432	0.429	21.647
S	PF	38	0.081	0.142	E	P	444	0.915	62.763
E	PF	38	0.290	0.873	S	P	445	0.602	32.166
S	PF	58	0.099	4.095	E	P	457	0.842	66.088
E	P	104	0.260	12.682	S	P	477	0.424	28.217
E	P	116	0.260	11.293	E	P	535	54.093	110.187
E	P	124	0.247	17.346	E	P	560	5.002	216.254
E	P	129	0.262	13.509	S	P	573	0.471	9.933
E	P	189	3.191	30.738	E	P	577	72.950	260.630
E	P	194	4.643	62.365	E	P	597	46.756	140.047
E	P	194	3.797	48.858	E	P	672	19.504	391.368
S	P	230	0.408	7.397	E	P	709	6.829	196.190
S	P	234	0.411	14.353	E	P	771	98.571	383.322
E	P	237	3.416	55.372	E	P	790	105.889	444.098
E	P	245	3.096	50.105	S	P	803	0.519	70.848
E	P	259	1.650	460.143	E	P	830	5.373	187.446
E	P	267	1.288	121.986	E	P	854	4.185	238.411
E	P	268	1.119	109.443	E	P	993	13.572	195.019
E	P	275	1.551	320.264	E	P	1044	16.843	402.775
E	P	287	26.534	75.675	E	P	1089	22.670	372.494
E	P	296	2.221	22.175	E	P	1090	16.222	170.789
S	P	312	0.460	8.227	E	P	1362	101.565	952.794
E	P	326	3.288	22.235	E	P	1882	85.808	647.013
E	P	339	3.054	48.709	E	P	1903	90.699	885.702
E	P	350	2.124	24.908	E	P	2023	89.630	732.794

E – examination glove; S – surgical glove; P – powdered glove; PF – powder-free glove

current one, the correlation between the methods was calculated. Data for the 98 gloves showed that the three methods remain well correlated (Table 2).

The methods were then compared on the basis of their detection limit (Table 3). Among the 16 gloves (out of the 98) which exhibited a protein level below the detection limit by the modified Lowry assay, 15 were positive by both

the CIALP and IgE EI methods. Only one of the 16 glove extracts was negative by all three methods. The overall concordance between the CIALP or IgE EI methods and the modified Lowry assay was 80.6%, while the concordance between the CIALP and IgE EI methods reach 95.6%.

A subgroup analysis of the data showed that for gloves with a total protein level ≥ 50 µg protein/g of glove, CIALP, mo-

Table 2. Correlation between the modified Lowry, CIALP, and IgE ELISA-inhibition methods for 98 glove extracts. Results from Chabane et al. study (36 glove extracts) and the present work (62 glove extracts) were considered. Results below detection limits were arbitrarily assigned to the threshold level value of the respective method. Correlations were determined with the Spearman non-parametric rank-correlation test.

	CIALP vs. Lowry	CIALP vs. IgE EI	Lowry vs. IgE EI
All gloves (n = 98)	r=0.76 p<0.001	r=0.90 p<0.001	r=0.78 p<0.001
gloves <50 µg/g glove (n=32)	r=-0.18 NS	r=0.39 p=0.03	r=0.08 NS
gloves ≥50 µg/g glove (n=66)	r=0.66 p<0.001	r=0.88 p<0.001	r=0.65 p<0.001

CIALP – competitive immunoassay for antigenic latex proteins; IgE EI – IgE ELISA-inhibition; NS – not significant

Table 3. Concordance of the results observed between the modified Lowry, CIALP, and IgE ELISA-inhibition methods for 98 glove extracts. Results were considered as negative when below the detection limit and positive when ≥ to the detection limit of the respective method.

	Lowry +	Lowry –	Total		Lowry +	Lowry –	Total		IgE EI +	IgE EI –	Total
CIALP +	78	15	93	IgE EI +	78	15	93	CIALP +	91	2	93
CIALP –	4	1	5	IgE EI –	4	1	5	CIALP –	2	3	5
Total	82	16	98	Total	82	16	98	Total	93	5	98
Concordance: 80.6%				Concordance: 80.6%				Concordance: 95.9%			

CIALP – competitive immunoassay for antigenic latex proteins; IgE EI – IgE ELISA-inhibition

dified Lowry, and IgE EI all correlated. When gloves with a total protein level of <50 µg/g of glove were considered, then the correlation between CIALP and IgE EI was low but still significant (r=0.39, p=0.03), but neither correlated with the modified Lowry assay (Table 2).

DISCUSSION

In the present study we found a significant correlation between CIALP, modified Lowry, and IgE EI for the 62 glove extracts tested. We also observed that most of the gloves tested had a “medium” or “high” total protein level, i.e. ≥50 µg/g of glove. This cut-off value arose as a result of various regulatory groups discussing standards for analysis for total extractable proteins over the past decade [14]. In our previous work, CIALP was compared with the IgE-inhibition and modified Lowry methods in 36 gloves [12]. There was a significant correlation between CIALP and Lowry and between CIALP and IgE-inhibition, but not between IgE-inhibition and the Lowry methods. But most of the extracts tested were from surgical gloves which had a “low” total protein level, i.e. <50 µg/g of glove. To enable a comparison with a wider range of extractable proteins, the results from the previous and the present studies were combined and analyzed together (n=98 glove extracts). The comparison still showed significant correlations between CIALP, IgE EI and the modified Lowry assay. When the comparison was carried out after separating these results into “low” protein and “medium”/“high” protein groups, the correlation between CIALP and IgE-inhibition was significant for both groups. A significant correlation was also observed between the modified Lowry assay and CIALP or IgE-inhibition when the results from gloves with “medium”/“high” total protein content were analyzed, but not when only the gloves with “low” total protein content were considered.

When analyzing the results from “low” protein gloves we observed that the Lowry method failed to detect protein in 15 glove extracts, whereas antigenic proteins and allergens were respectively detected with CIALP and IgE-inhibition in all of them. Other findings support the lack of correlation between total protein and allergen content in glove extracts [3,4,13]. Here we have demonstrated that the lack of correlation is due to a lower sensitivity of the modified Lowry assay compared with the CIALP or IgE-inhibition methods for gloves with an extractable protein content less than 50 µg/g of glove.

Using a large number of gloves displaying a wide range of extractable proteins, we demonstrated that the evaluation the allergenicity of NRL gloves with the modified Lowry method is useful when the total protein level in the glove extract is ≥50 µg/g of glove. This method alone is not satisfactory for gloves with <50 µg protein/g of glove. We advise the use of complementary methods for this purpose. However, methods such as IgE ELISA-inhibition require sera from NRL allergic patients, making these methods difficult to standardize and use on an industrial scale [11]. The CIALP method, which does not require serum from allergic patients and uses an *in vivo*-standardized NRL extract for calibration, is a suitable method for the assessment of the allergenicity of NRL gloves. The LEAP assay is another method based on the use of rabbit anti-NRL IgG and ammoniated NRL proteins for calibration [13,15]. Furthermore, an assay was established in 2000 as a standard by the ASTM (ASTM D6499-00) for the evaluation of latex glove antigenicity [16]. Both the CIALP and ASTM standard methods are similar: anti-NRL rabbit antibodies are utilized for NRL determination, but the standard NRL extract is ammoniated for ASTM and non-ammoniated for CIALP. Some new

ELISA methods for quantitation of the Hev b 1, Hev b 3, Hev b 5, and Hev b 6.02 individual NRL allergens in latex devices are also available [17], but the determination of a single allergen content does not represent the entire allergenic activity.

CONCLUSIONS

The estimation of NRL glove allergenicity by measuring the total protein content using only the modified Lowry assay is useful when the protein content is ≥ 50 $\mu\text{g/g}$ of glove. For gloves with "low" total protein content, i.e. < 50 $\mu\text{g/g}$ of glove, the Lowry method is not sensitive enough. Thus, complementary methods such as CIALP are necessary. This method was shown to be sensitive, well correlated with the IgE-inhibition method, and does not require serum from allergic patients.

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