Evaluation of semi-automatic image analysis tools for cerebrospinal fluid electrophoresis of IgG oligoclonal bands

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Background: IgG concentrations in cerebrospinal fluid generally range from 20 to 45 mg/L. In multiple sclerosis immune reactions lead to intrathecal synthesis of specific IgGs that can be detected in biological fluid samples both quantitatively and qualitatively by isoelectric focusing of supplementary oligoclonal IgG bands.
Method: A simple tool, using the MATLAB application, to facilitate and improve isoelectric focusing profile analysis is presented and evaluated in terms of its sensitivity, repeatability and reproducibility. A comparison between human readers and semi-automatic method has also been performed.
Results: Results from the semi-automatic method were found to be equivalent or superior to generally employed laboratory methods. Repeatability analysis for semi-automatic processing yielded coefficients of variation (CVs) in the 3–7% range, and using a sample with an estimated IgG concentration of 200 mg/L, four bands were still visible after dilution to 5 mg/L, corresponding to band concentrations of 1.1–1.6 mg/L. Discordances between visual inspection and automatic analysis only appear at threshold levels for interpretation (the gray zone).
Conclusion: The semi-automatic method has acceptable performance for routine implementation.

1. Introduction

The incidence of Multiple Sclerosis (MS) in France is one per 1000, making it the leading cause of acquired non-traumatic severe disability in young patients. MS involves inflammatory lesions of white matter in the central nervous system (CNS) that spread over time. Positive evidence of MS can be obtained with Magnetic Resonance Imaging (MRI) demonstrating the occurrence of 3 out of 4 Barkhof brain and spinal cord criteria [1]. The combination of two suggestive MRI lesions associated with a positive cerebrospinal fluid (CSF) analysis also indicates disease [1]. Detection of oligoclonal bands (OCBs) in CSF by isoelectric focusing (IEF) is a common diagnostic tool [2].

Chromogenic staining of immunoblots facilitates detection. The higher the concentration of the IgG bands, the greater the intensity of the colors and the more readable the profile, making interpretation easier. Analyzing a profile involves counting the number of visible IgG bands. A profile with at least two IgG bands in CSF which are not present in a serum sample taken at the same time is said to be oligoclonal.

Chromogen interaction with the immunoblot support determines the baseline or background noise. Other forms of noise (white and dark spots [salt and pepper], blots, stain irregularities) may appear on the profile, increasing the chance of misinterpretation for
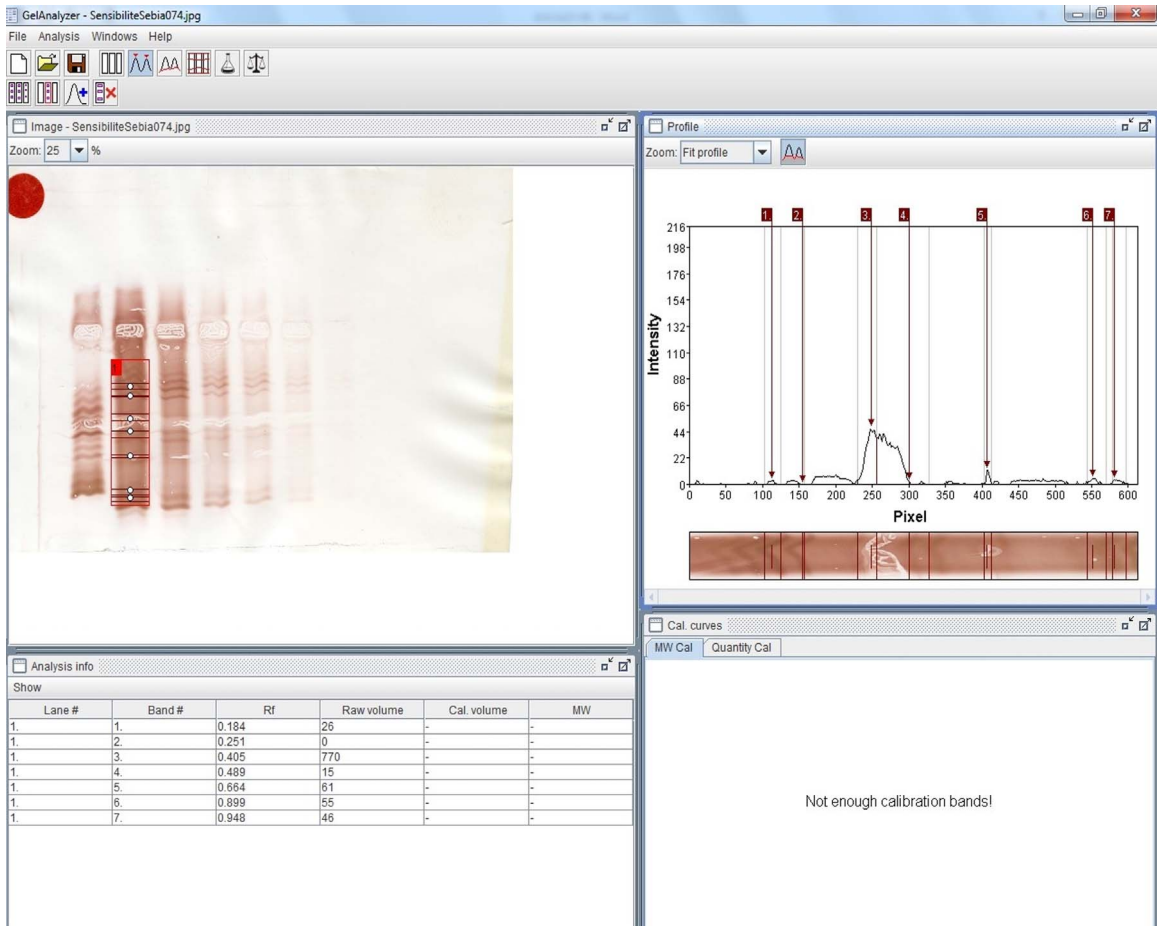

Fig. 1. Analysis of a manual ROI CSF profile by GelAnalyser software.

the operator who must distinguish peaks as band intensity versus background and noise.

Patients with MS exhibit a wide range of IgG electrophoretic profiles in CSF samples, and the skill in interpretation consists of distinguishing false-negatives and positives in samples to correctly classify the profile as oligoclonal or not in the presence of background noise. Counting bands is not an easy task and the operator frequently needs help. New technology based on computerized analysis can provide an important tool to supplement human reading. Automated analysis can also determine other properties such as surface area, band location and the intensity of the band. This information may be useful for further studies and patient follow-up.

In the past decade, methods for filtering, segmenting, and detecting gel bands, as well as rectifying geometries, have been described [3,4]. Major applications and interfaces to fine tune DNA band analysis have been developed [5,6] based on Gaussian mixture models [7] and wavelet transformation [8]

However, semi-automatic techniques require manual setting of sensitivity thresholds. Problems with grayscale intensity also occur and end-users must often adjust parameters for individual bands.

Other methods automatically filter and smooth grayscale intensities, which can cause true bands to disappear while false bands remain. Blob areas may compute as rectangular markers, decreasing reproducibility. Performance assessment of these kinds of tools and associated interfaces is, to our knowledge, absent from the literature. Use of a DNA Gel Analyzer interface for CSF profiles does not give satisfactory results (Fig. 1). Methods designed to process DNA electrophoresis need to be adapted to be relevant to IgG isoelectric focusing. OCB analysis does not require fine band tuning, but contrast enhancement and elimination of false readings and artefacts.

Existing software can, however be adapted to IgG isoelectric focusing. This article briefly describes a tool based on Gaussian adaptation (GA) that we have developed [9] to enhance peak detection. These tools were then assessed (accreditation process) according to good laboratory practice (GLP), in terms of reproducibility [10].
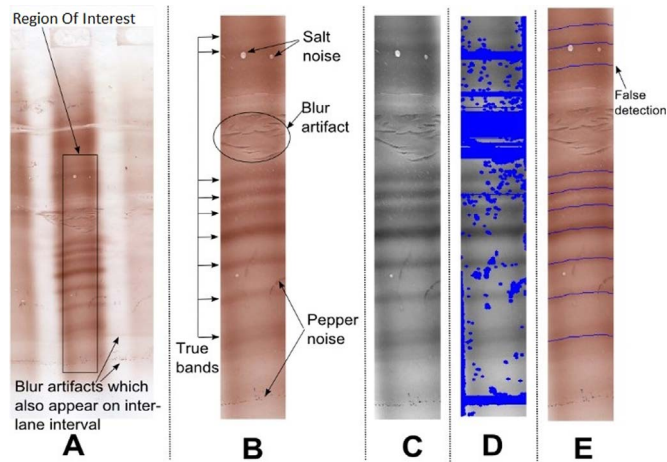
Fig. 2. Illustration of the difficulty of automatic interpretation of a 2D image.

2. Materials and methods

2.1. Semi-automatic image profile processing

IgG isoelectric focusing was performed according to the instructions of the agarose gel and immunoblot membrane (10 cm × 8 cm) supplier (Helena Biosciences Europe, Gateshead, UK). The "IgG IEF Chromogen" contains 3-amino-9-ethyl-carbazole powder that we dissolve in 50 mL of methanol. The "IgG IEF Primary Antibody" contains sheep anti-human IgG antiserum and the "IgG IEF Secondary Antibody" contains anti-sheep IgG- peroxidase conjugate.

A scanned membrane (Epson V750 PRO scanner [Epson, Levallois-Perret, France], 48 bit color, 3 color values scaled from 0 to 1, 600 dpi) contains ten profiles of different patients. From the 10 profiles you can select one that defines the Region Of Interest (ROI) that forms the basis for our experiments (Fig. 2A). The ROI corresponds to the migration zone of IgG on this type of support.

Misinterpretations described previously are due to image artefacts like salt and pepper noise (Fig. 2B), blur artefacts and false lanes (Fig. 2A, B), horizontal lane smile deformations, and baseline variations.

Automatic peak detection processing is performed in 3 stages. The first stage involves selection of a rectangular ROI as a 2D image (Fig. 2A,B). On this ROI, a grayscale conversion is performed (Fig. 2C) as well as a 2D image correction by artefact detection and straight-line realignment (Fig. 2D). Finally, the 2D signal is converted to a 1D signal (Figs. 3 and 4). The second stage involves peak detection on the 1D image (Figs. 3 and 4) and the third stage is the location of peaks on the 2D image (Fig. 2E) which can be regarded as a synthesis of the whole process. Each of these steps must be performed to improve peak detection, and we have developed tools for each step which are described in our previous work [9].

Because the existing approaches cannot achieve accurate automatic computation, in the present work, we propose an electrophoresis analysis method based on Gaussian Adaptation (GA) [11–13]. The principle is that we can find a functional Gaussian decomposition, that is to say a set of Gaussian functions whose shape will be adapted at each peak found in the 1D smoothed and
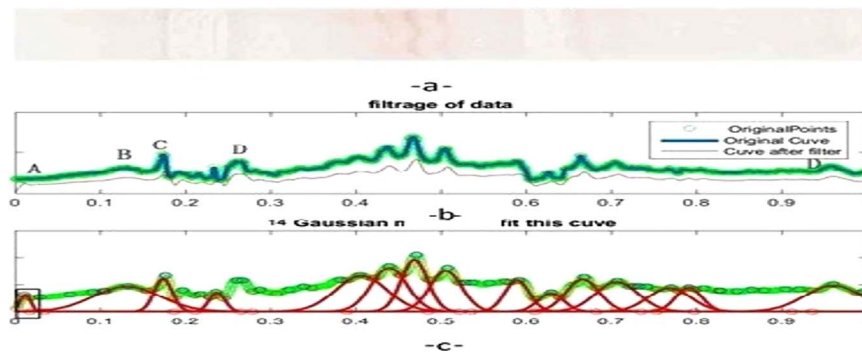

Fig. 3. Peak detection. (a) a dilute electrophoresis.(b) shows a calculated profile (blue) with a median fitness line (x) computing each column of vertical pixels. (c) red curve is the post filter median curve. Small peaks and troughs have been removed. Thus, if evolution starts at point A with a relatively small variance (the red bell curve), then evolution will take place along the red curve. The process may also yield peaks at B and C, as long as adjacent valleys to the right of these points remain. The red curve before point A is not computed because of noise. From point A, 14 Gaussian models are found. A noise zone occurs within zone D, so D is eliminated. The left peak near to D may be a fail peak (an artefact??), which would need to be evaluated by an expert. The number of Gaussian components is obtained by a simple peak detection algorithm.
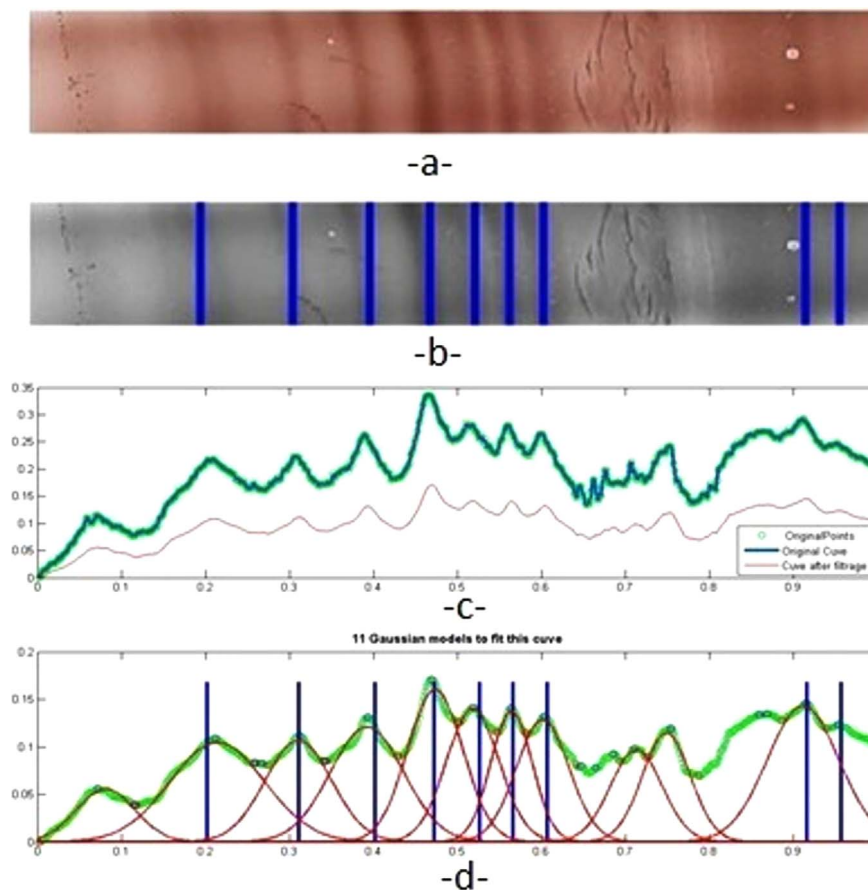
Fig. 4. a) Original scanned color image, b) gray scale image, c) filtered 1D signal, d GA representation.

filtered signal coming from the electrophoresis profile.

As the GA method is an iterative process, the initial value of the Gaussian characteristics (mean and standard deviation, location) and the stop value (precision of approximation of the shape reconstruction) must be set.

In our case, first and second statistical moment can be calculated provided that the distribution of parameters and image structure is known [Fig. 3].

To set the initial number and characteristics of the Gaussian functions, we compute the number of peaks with a classical function of peak detection available on MATLAB, 'findPeaks' (MATLAB, MathWorks, Meudon, France), with the parameter' MeanPeakdistance' equal to 25 points. The number of peaks is increased by 20%, because we assume that the error of the Matlab function is not more than 20% for a smoothed and filtered signal. The algorithm is started by using the characteristics (location and width, that is to say, mean and standard deviation) of the first detected peak with the MATLAB function. The final number of Gaussian components is obtained when the detection of two peaks shows no difference with two consecutive parameter settings, e.g. peak width. Post-processing treatment allows us to remove undesirable peaks (Fig. 4-d).

The whole process is illustrated in Fig. 4. The scanned ROI electrophoresis (Fig. 4-a), gray scale of ROI (Fig. 4-b), raw signal (Fig. 4-c, green), the rolling ball baseline and the filtered signal baseline (Fig. 4-c, red) are determined and the number of peaks is counted by the GA algorithm (Fig. 4-d) and can be compared to the original 2D image (Fig. 4a–b).

2.2. Evaluation and comparison of methods

The performance of the semi-automated tool was evaluated by visual assessment and by determining the repeatability (within one gel), detection sensitivity, reproducibility (across different gels) and the comparison between human readers and the semi-automatic method.

The electrophoretic profiles were read with and without magnification by an expert biologist and a laboratory technician. Both were blinded to the other's interpretation and to the clinical context. The sensitivity and specificity of visual assessment were determined.

2.2.1. Sensitivity analysis

According to the manufacturer, a discrete band can be detected when the CSF sample contains at least 0.28 mg/L IgG. To verify this, we started with a pure CSF sample containing 50.87 mg/L IgG according to the nephelometric assay and made 50% serial dilutions across 8 gel lanes, together with one internal quality control. For this sample 10 bands were visible before dilution.

For the computer-assisted analysis, sensitivity was tested by counting the number of peaks and their amplitude and by computing the surface area of the bands using successive dilutions of a sample with an initial IgG concentration of 200 mg/L. Mean and standard deviations were determined together with the CV for all variables.

2.2.2. Repeatability

For isoelectric focusing, we defined repeatability as the requirement for a sample to present the same number of bands of equivalent appearance when tested in the 9 wells of a single gel (with internal quality control in the 10th well). Repeatability was tested on two samples:

– a patient CSF sample containing 50.87 mg/L IgG
– a control sample (Sebia, Lisses, France) containing 201 mg/L IgG

Each sample was applied 9 times on the same gel. The gel profiles were read by two expert biologists and the software was used to count peaks from the same IgG-positive CSF sample used for the repeatability analysis. To assess the repeatability of these tools, we assessed the coefficients of variation (CVs) of the five peaks from five applications displaying the largest surface areas.

2.2.3. Reproducibility or intermediary reliability

The test was performed on successive batches, usually once or twice à day, using daily internal quality control samples. The internal quality control was tested on 30 different gels. The profiles were analyzed by two expert readers and by the semi-automatic method.

2.2.4. Visual inspection vs semi-automated inspection

31 electrophoretic profiles were compared by the two modalities. All profiles compared were from patients suspected of neurodegenerative disease, and none of the patients had detectable oligoclonal bands in the serum. We assessed the concordance between experts and compared the performance of the expert readers and the semi-automatic method.

3. Results

3.1. Manual processing

3.1.1. Repeatability

Repeatability was analyzed on a CSF sample and on a serum sample. On the CSF sample, which is the more difficult to read, we counted 8–10 bands (mean = 9 bands, CV = 9%). On the serum sample with a higher protein content, readers counted 9 times 9 bands and one time 10 bands. There was no difference in the visual reading between readers for each sample analyzed.

3.1.2. Reproducibility

On the control sample, over the 30 daily readings, readers could read either 10 or 11 bands, (CV = 4.72%) and there was no difference in the visual reading between readers for each sample analyzed.

The closeness of the repeatability and intermediary reliability results with CVs less than 10% demonstrated the robustness of the method.

3.1.3. Sensitivity analysis

In the final 1/32 dilution, corresponding to 3.15 mg/L IgG, three (or sometimes four) bands were still visible (Fig. 5). The visibility of the bands can be further improved by increasing the time of chromogen contact (20 min instead of 10), but with the risk of increasing artefact images.

3.2. Semi-automatic processing

3.2.1. Repeatability

A serum sample was used for analysis and five or more bands were visible. Repeatability was determined for the five most readily visible bands among the 10 identified in the profile. Semi-automatic processing yielded CVs in the 3–7% range, demonstrating the method's precision (Table 1)

3.2.2. Sensitivity analysis

Using a sample with an estimated IgG concentration of 200 mg/L IgG, four bands were still visible after dilution to 5 mg/L, corresponding to a relative concentration in the bands of 1.1–1.6 mg/L. Dilution proportionality remained valid up to 1/20 dilution but was no longer valid at 1/40.
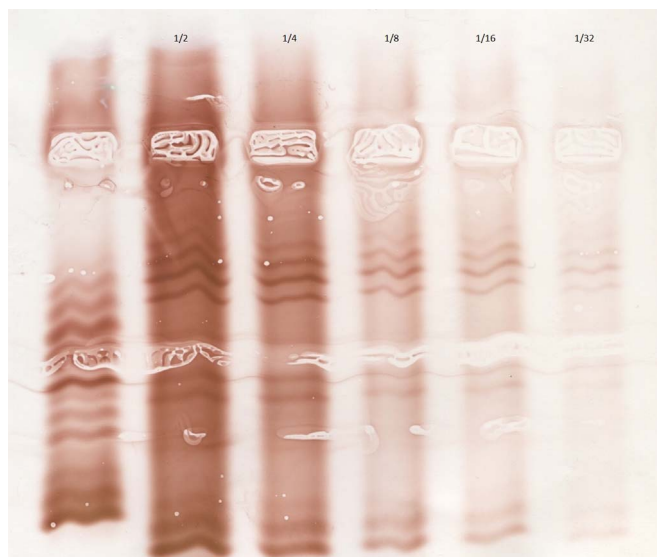
Fig. 5. Sensitivity analysis. Three bands (defining the oligoclonal profile) are still visible on the 1/32 dilution (3.5 mg/L IgG).

Table 1
Surface area of the five bands used for the repeatability test of five applications.

Band	Surface area (mm^2)							CV (%)
	Application 1	Application 2	Application 3	Application 4	Application 5	Mean	SD	
1	5.41	5.43	5.74	5.78	5.64	5.60	0.17	3.03
2	6.30	5.53	5.75	5.88	5.79	5.85	0.28	4.79
3	6.38	5.48	5.84	6.01	6.02	5.95	0.33	5.55
4	6.52	5.68	5.90	6.30	5.54	5.99	0.41	6.84
5	5.60	5.92	4.95	5.52	5.12	5.42	0.39	7.1

Like the visual analysis, four bands were also detected by semi-automatic analysis, and this method can also quantify the peak surface area for each band (Table 2). Since the background noise concentration is not taken into account in our calculations, the concentrations shown in Table 2 are partially overestimated, suggesting that the analytical sensitivity is probably better than the values stated above.

3.3. Method comparison

For the 31 electrophoretic profiles from patients, 16 of them were deemed to be oligoclonal (3 bands or more) and 15 were deemed non-oligoclonal. The concordance between the readers approaches 100%: both readers classified the same samples as oligoclonal or non-oligoclonal, with a maximum difference of 1 band (5 samples). None of the corresponding serum samples exhibited an oligoclonal profile.

The comparison between visual reading and automatic reading was: 24/31 profiles were concordant, 4 visually oligoclonal profiles were said to be said non-oligoclonal by the automatic inspection and 3 visually non-oligoclonal profiles were said to be oligoclonal by the automatic method.

For the 3 visually non oligoclonal profiles, in two cases, the experts counted 2 bands vs 3 bands for the automatic method. In one case the experts give 0 bands vs 3 for the automated method.

For the four visually oligoclonal profiles, the experts counted 3 or 4 bands vs 2 bands for the automated method.

These 7 cases are the gray area of interpretation. Some experts consider 2 or more bands in the CSF to be a positive oligoclonal pattern. If this criterion is adopted, six of the seven discordant cases become concordant.

4. Discussion

We used a serum sample to determine the reproducibility of the procedure. No target CVs or repeatability and reproducibility data are currently available in the literature for comparison [14].

The software exhibited satisfactory repeatability, yielding CVs below 10% (mean 5.46%) in all cases, illustrating the robustness of the method.

Table 2

Location and relative concentration (expressed as mg/L) of oligoclonal bands. Relative concentration was determined from the initial concentration (band surface area/total band surface area) for each dilution.

IgG concentration (mg/L)	200		100		50		20		10		5	
Bands	Location (pixels)	Relative concentration	Bands	Relative concentration	Bands	Relative concentration	Bands	Relative concentration	Bands	Relative concentration	Bands	Relative concentration
1	46	17,52	1	9,01	1	3,08	1	1,52	1	1,1	1	1,1
2	79	19,12	2	10,49	2	5,69	2	2,37	2	1,89	2	1,54
3	112	20,88	3	12,14	3	10,48	3	3,99	3	2,63	3	1,10
4	147	22,06	4	10,98	4	7,91	4	3,29	4	1,87		
5	333	21,66	5	10,7	5	4,78	5	2,31	5	1,52	5	1,24
6	364	16,28	6	7,49								
7	404	15,68	7	7,49	7	1,35	7	1,04				
8	424	15,02	8	7,41								
9	569	21,38	9	10,46	9	5,54	9	1,85	9	0,97		
10	611	26,36	10	13,79	10	11,13	10	3,58				
11	647	3,92										

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