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Response Surface Methodology-Based Optimisation of Agarose Gel Electrophoresis for Screening and Electropherotyping of Rotavirus

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Abstract Management of rotavirus diarrhoea cases and prevention of nosocomial infection require rapid diagnostic method at the patient care level. Diagnostic tests currently available are not routinely used due to economic or sensitivity/specificity constraints. Agarose-based sieving media and running conditions were modulated by using central composite design and response surface methodology for screening and electropherotyping of rotaviruses. The electrophoretic resolution of rotavirus genome was calculated from input parameters characterising the gel matrix structure and running conditions. Resolution of rotavirus genome was calculated by densitometric analysis of the gel. The parameters at critical values were able to resolve 11 segmented rotavirus genome. Better resolution and electropherotypic variation in 11 segmented double-stranded RNA genome of rotavirus was detected at 1.96% (*w*/*v*) agarose concentration, 0.073 mol l^{-1} ionic strength of Tris base–boric acid–ethylenediamine tetraacetic acid buffer (1.4×) and 4.31 h of electrophoresis at 4.6 Vcm^{-1} electric field strength. Modified agarose gel electrophoresis can replace other methods as a simplified alternative for routine detection of rotavirus where it is not in practice.

Keywords Central composite design · Electropherotyping · Resolution · Rotavirus genome · Screening

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Introduction

The clinical symptoms associated with rotavirus are not sufficiently characteristic to distinguish between rotavirus and other causes of diarrhoea. Contact isolation for patients diagnosed with rotavirus infection is necessary for the duration of hospital stay because of sustained faecal shedding of the virus [1]. Rapid diagnosis allows for the isolation of a child to prevent nosocomial infection, which is often used as a marker of the effectiveness of precautions to control contact infection [2].

Electron microscopy and reverse transcription polymerase chain reaction are useful for elucidation of structural and genomic aspects of rotavirus but are time-consuming, highly sophisticated and require advanced technology with qualified personnel. Enzyme-linked immunosorbent assay (ELISA) has the ability for the rapid detection of rotavirus but with an inherent inefficiency to test single specimen (with multiple controls). Misleading reactions and inability to detect non-group A rotaviruses are major impediments to the use of commercial ELISA kits [3]. Rotavirus detection by polyacrylamide gel electrophoresis (PAGE) is economic and is ascendant over ELISA, by having the capacity to detect and distinguish all groups of rotaviruses. However, PAGE-based detection and electropherotyping requires more than 16 h [4] besides it being a tedious, labour-intensive technique.

Agarose gel electrophoresis (AGE)-based rotavirus screening has been used by some workers [5], but due to less resolution achieved for the distinction of individual double-stranded RNA (dsRNA) segments, it was replaced by polyacrylamide gels and silver staining [6]. Although, AGE-based rotavirus screening is less time-consuming, economic and can replace other methods as a simplified alternative for routine detection of rotavirus which is not in practice, no study has been conducted to quantitatively analyse the resolving power, nor any reliable statistical methods have been applied to quantify the factors affecting the resolution of individual dsRNA segments.

In this study, we have applied the central composite design (CCD) and response surface methodology (RSM) to enhance the capacity of AGE for the resolution of 11 segmented dsRNA genome of rotavirus. Optimisation of electrophoresis factors by the multidimensional approach not only provided optimal electrophoretic conditions for resolution but also discretely defined the role of individual factors and their interactive influences on the resolution. The RSM and CCD approach has been widely used in various process optimisation studies, but it has not been used in rotavirus research and for optimisation of agarose gel electrophoresis conditions. Snapshot image analysis was used for depiction of RNA migration and resolution. Optimised procedure was evaluated for screening and electropherotyping of rotavirus in comparison to PAGE.

Materials and Methods

Rotavirus Genome

A total of 412 faecal samples were collected between September 2004 and April 2008 of children aged ≤36 months admitted with acute watery diarrhoea to the Department of Paediatrics, Chhatrapati Shahuji Maharaj Medical University, Lucknow, UP, India, after taking institutional ethical clearance as well as written informed consent from the guardians. Each sample was separately screened for rotavirus by ELISA (RIDASCREEN® Rotavirus double sandwiched kit) and PAGE [4]. For the optimisation process, segmented dsRNA genome of a single rotavirus isolate showing long electropherotypic pattern in

PAGE was used in each trial of CCD. Optimised procedure was further validated for electropherotyping of all rotavirus-positive samples. The dsRNA genome was extracted from samples by using a standard phenol–chloroform extraction method followed by ethanol precipitation. The most simple and economic method of RNA extraction was used to facilitate routine detection and electropherotyping of rotaviruses.

Analytical Methods

The resolving power of AGE for rotavirus-segmented dsRNA genome was determined in a gel of $102\times76\times8$ mm^3 dimensions, prepared with low electroendosmosis agarose (Sigma Aldrich) containing 0.5 μg ml^{-1} ethidium bromide. Electrophoresis was performed at a field strength of 4.60 V cm^{-1} in Tris base–boric acid–ethylenediamine tetraacetic acid (TBE) buffer (0.09 mol l^{-1} Tris–borate, 0.002 mol l^{-1} EDTA 1×, Sigma Aldrich) with 0.5 μg ml^{-1} ethidium bromide and 20 μl of identical rotavirus genome (with bromophenol blue as tracking dye) in each trial of CCD. Imaging of fluorescence from ethidium bromide-stained gels was done on a transilluminator (Fotodyne USA), and RNA bands were quantified by optical density analysis using UnScanIt gel software 5.1 (Silk Scientific Co., UT, USA).

For densitometric analysis of the gel, the region of a given lane to be fit was defined and its boundaries defined the width of the lane. Each band in the lane corresponded to a peak, and a closed area of the peak was defined by dropping vertical lines at the two borders of the peak. Average pixel intensity from the region outside the selected area served as the background value [7].

The electrophoretic mobility (μ) is a linear function of gel concentration (T) at all agarose concentrations and field strengths, and the retardation coefficient ($\mathrm{d}\mu/\mathrm{d}T=K_R$) is a characteristic of nucleic acid conformation under controlled conditions. Resolving power is an objective measure of the ability of different separation methods to detect closely spaced molecular species. It is a dimensionless quantity and, hence, facilitates comparison of the performance of electrophoretic systems that operate differently [8]. Resolving power thus can be expressed as:

$$R_s = \frac{1}{4(N_2 - N_1)}\left|\frac{\Delta\mu}{\mu_{av}}\right|N_p \tag{1}$$

where, R_s is the resolving power or resolution; N_p the measure of peak width or width at half maximum (hw) of the separated band, which is a dispersion characteristic of a single molecular component; μ_{av} the average mobility; $\Delta\mu$ the measure of variability; N_2–N_1 the size difference of two closely resolved nucleic acid molecules and N the number of bases in the molecule [9].

By solving the dispersion function, resolving power for two nucleic acid molecules with differing molecular lengths were calculated using Eq. 2, which involved both the centre-to-centre distances (l_1, l_2) and the peak widths at half height (hw_1, hw_2) of the two resolved bands.

$$R_s = \frac{l_2 - l_1}{hw_1 - hw_2} \tag{2}$$

The 11 segmented dsRNA genome of the rotavirus exhibits characteristic difference in the migration of the tenth and eleventh segments, which is relatively faster in long

electropherotypes and slower in short electropherotypes [10]. Thus, the resolving power was defined as the ratio of relative electrophoretic migration of the eleventh and tenth segments and relative peak widths at half height as determined by densitometric analysis for all the trials of CCD. It is worthwhile to mention that rotaviruses belong to the *Reoviridae* family known for its characteristic segmented dsRNA genome and electropherotypes. Electropherotypic variations are not only genome-specific but can also be used to examine the species-specific variations [11].

Experimental Design

To find the optimum conditions for resolution of 11 segmented dsRNA genome of rotavirus by AGE, the response surface for resolving power as a function of the selected key factors was determined. Based on preliminary experiments, three independent factors most likely affecting the resolution of rotavirus genome were agarose concentration, TBE buffer ionic strength and running time which were executed in design, while thickness of the gel and current flow were kept constant. For each factor, five levels were ascertained (Table 1) and the correspondence between these values were obtained by

$$X = (x - x_0)/\delta x \tag{3}$$

where, X is the coded value, x the corresponding uncoded value, x_0 the uncoded value in the centre of the domain and δx the increment of x corresponding to 1 unit of X.

Table 1 The 2^3 central composite design with observed and predicted values of resolution of rotavirus genome.

Trial	Agarose concentration (X_1)		TBE (X_2)		Time of run (X_3)		Resolving power	
	Uncoded (%, *w*/*v*)	Coded	Uncoded (X)	Coded	Uncoded (h)	Coded	Observed	Predicted
1	1.50	−1	1.00	−1	3.50	−1	0.359	0.393
2	1.50	−1	1.00	−1	4.50	1	0.621	0.622
3	1.50	−1	2.00	1	3.50	−1	0.426	0.444
4	1.50	−1	2.00	1	4.50	1	0.565	0.571
5	2.50	1	1.00	−1	3.50	−1	0.504	0.517
6	2.50	1	1.00	−1	4.50	1	0.608	0.608
7	2.50	1	2.00	1	3.50	−1	0.492	0.509
8	2.50	1	2.00	1	4.50	1	0.514	0.498
9	1.16	−1.68	1.50	0	4.00	0	0.571	0.545
10	2.84	1.68	1.50	0	4.00	0	0.589	0.589
11	2.00	0	0.66	−1.68	4.00	0	0.532	0.512
12	2.00	0	2.34	1.68	4.00	0	0.469	0.463
13	2.00	0	1.50	0	3.16	−1.68	0.480	0.441
14	2.00	0	1.50	0	4.84	1.68	0.610	0.624
15[a]	2.00	0	1.50	0	4.00	0	0.677	0.676

[a] Centre points were replicated nine times

A second-order polynomial model obtained by multiple regression technique for the three independent factors by using Statistica 8.0 software was adopted to describe the response surface. The model is expressed as:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j + \varepsilon \quad (4)$$

where, Y is the measured response in each trial (resolving power), k the number of factors, β_0 the model constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient, X_i and X_j the factors in coded form and ε the error factor.

Here, the standard 2^3 central composite design with eight cube points, six star points and eight replicates of the centre point were employed [12] that indicated requirement of 23 trials for estimation of the coefficients of the model (Eq. 4). Experimental results were fitted in Statistica 8.0 to further analyse the predicted values, individual and interactive influences, optimum levels and to evaluate the contribution of each selected factor on resolving power of the gel. For validation of the optimised protocol, electropherotypic pattern of each positive sample was scored by PAGE as well as by optimised AGE method, and electropherograms were digitised for comparison (Fig. 1a, b).

Results

Optimisation of Rotavirus Genome Resolution by AGE

Out of 412 faecal specimens screened, 79 were found positive for rotavirus by ELISA and PAGE. The characteristic long electropherotypic band pattern of selected rotavirus strain was resolved in each of the 23 trials of CCD. The resolution of bands varied in each trial in response to the variation in the levels of the three independent factors and their interactive influences. Electrophoretic migration of the eleventh band was directly proportional to the resolving power of the gel, but the intensity of this band decreased as it was resolved, this may be due to the small size of this dsRNA segment (~667 bp), which can also vary in different rotavirus strains. The observed values of the resolving power of the gel and predicted values as obtained by Statistica 8.0 are depicted in Table 1.

Linear and quadratic effects of all the three factors were found to be significant at 95% confidence, also, except agarose and TBE ionic strength, all interaction coefficients were significant (Table 2). Significance of the model has been validated by ANOVA (F=56.19, $P \leq 0.001$), and it has been found that the second-order response surface model is adequate for optimisation (R^2=0.975) giving an excellent correlation between independent factors (R=0.987; Table 3).

The application of RSM yielded the following regression equation showing a relationship between the resolving power of the gel and the three independent factors.

$$\begin{aligned}\text{'Resolving power'} = {} & -6.126 + 1.282(X_1) - 0.154(X_1)^2 + 1.294(X_2) - 0.266(X_2)^2 \\ & + 2.163(X_3) - 0.203(X_3)^2 - 0.058(X_1)(X_2) - 0.138(X_1)(X_3) \\ & - 0.102(X_2)(X_3) \end{aligned} \quad (5)$$

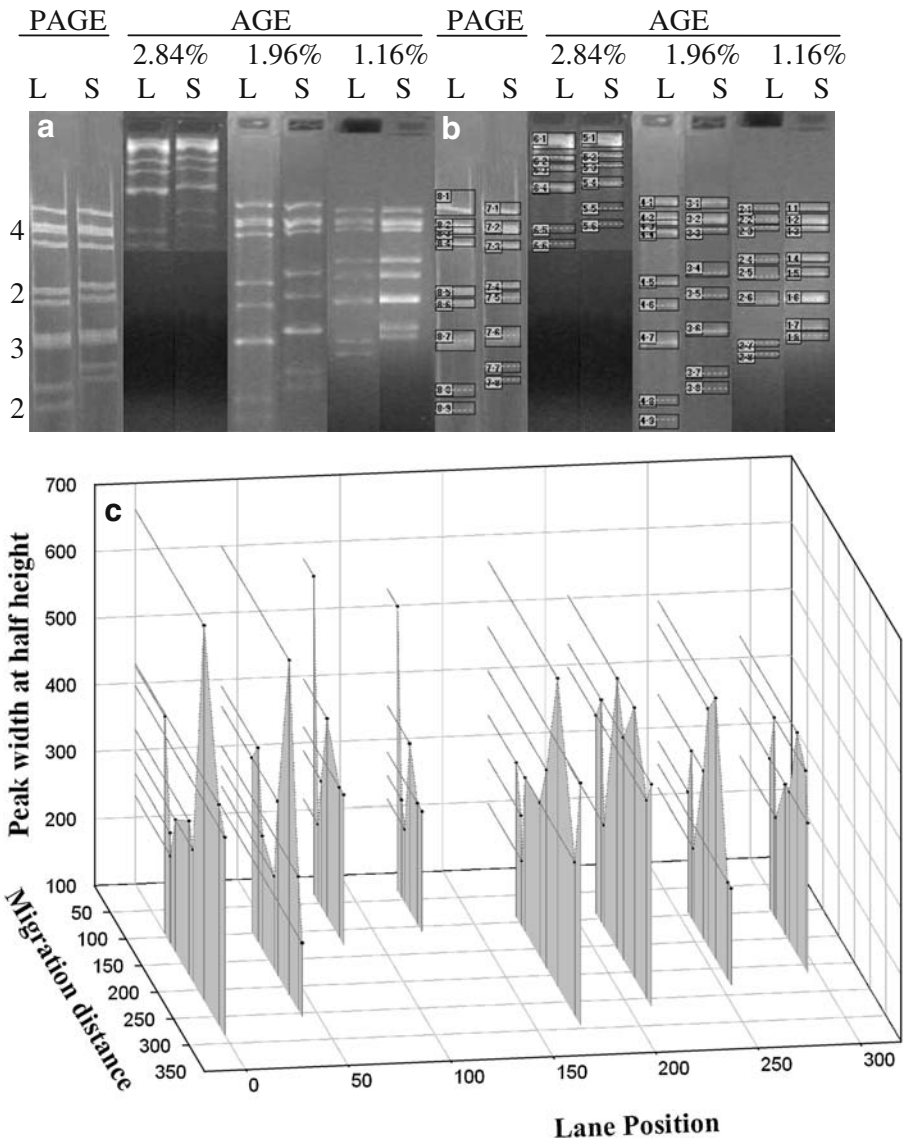

Fig. 1 Conglomerate of one long (*L*) and one short (*S*) electropherotype of 11 segmented rotavirus dsRNA genome (4–2–3–2). **a** At 10% PAGE and at 2.84%, 1.96% and 1.16% agarose, respectively (*left to right*). **b** Digitalisation of electropherograms for detection of lane position, migration distance and peak width at half height (UnScanIt gel). **c** Three-dimensional trajectory plot of lane position, migration distance and peak width at half height. At 10% PAGE and at 2.84%, 1.96% and 1.16% agarose, respectively (*left to right*)

The relationship between the resolving power and the factors were visualised in three-dimensional response surface plots by placing the values of the above equation (Fig. 2). By canonical analysis of the regression equation, the optimum values of the three factors were found to be 1.96% (*w*/*v*), 1.40× (0.073 mol l^{-1}) and 4.31 h, for agarose, ionic strength of TBE and time of run, respectively.

Table 2 Estimated regression coefficients for the main, quadratic and interaction effects of all the three factors in response to resolving power as an dependent factor.

	Coefficient	Standard error coefficient	*t* value	*p* value
Intercept	−6.13	0.44	−13.94	<0.001
Agarose	1.28	0.14	8.95	<0.001
Agarose^2	−0.15	0.02	−7.81	<0.001
TBE	1.29	0.14	9.36	<0.001
TBE^2	−0.27	0.02	−13.51	<0.001
Time of run	2.16	0.17	12.51	<0.001
Time of run^2	−0.20	0.02	−10.29	<0.001
Agarose × TBE	−0.06	0.03	−2.10	0.06
Agarose × time of run	−0.14	0.03	−4.95	<0.001
TBE × time of run	−0.10	0.03	−3.66	<0.001

Validation of the Model

Optimal conditions predicted were verified experimentally and compared with the calculated data to validate the fitness of the model. The observed resolving power of AGE at optimised conditions was 0.68 (predicted value 0.69). The verification revealed not only a high degree of model accuracy (98.0%), but also was taken a step further by comparing a short and a long form of electropherotype at all the minimum, maximum and optimal concentrations of agarose (Fig. 1a, b). The factors at critical values were able to resolve the eleven bands and their banding patterns, by providing the highest resistance to the movement of dsRNA but not so much that the higher molecular weight initial bands would not separate. A typical banding pattern of group A rotaviruses (4–2–3–2) (Fig. 1a, b), with grouping of four large segments, then two of medium size followed by a closely running triplet which occasionally resolved in PAGE and finally two small segments, was observed in all long and short electropherotypes (Fig. 1a, b). The comparative quantifications of peak widths at half height and relative migration distance of bands (Fig. 1c) have clarified that the obtained optimal conditions could be reiterated with the experimental restraints of molecular variations in the genome of rotavirus. To further consolidate the utility of AGE for combined screening and electropherotyping of rotavirus, all the 79 positive samples, previously electropherotyped by PAGE, were successfully electropherotyped by AGE.

Discussion

Rotavirus genome is highly prone to reassortment and recombination at the intra- and inter-species levels, making it difficult to use ELISA for epidemiological surveying [13]. Simple

Table 3 Analysis of variances (ANOVA) for adequacy of second-order model.

Source of variations	Sum of squares	Degree of freedom	Mean square	*F* value	Probability (*p* value)
Regression	0.20	9	0.02	56.19	<0.001
Residual	0.005	13	0.0003		

R=0.987; R^2 =0.975; R^2 (adj)=0.958

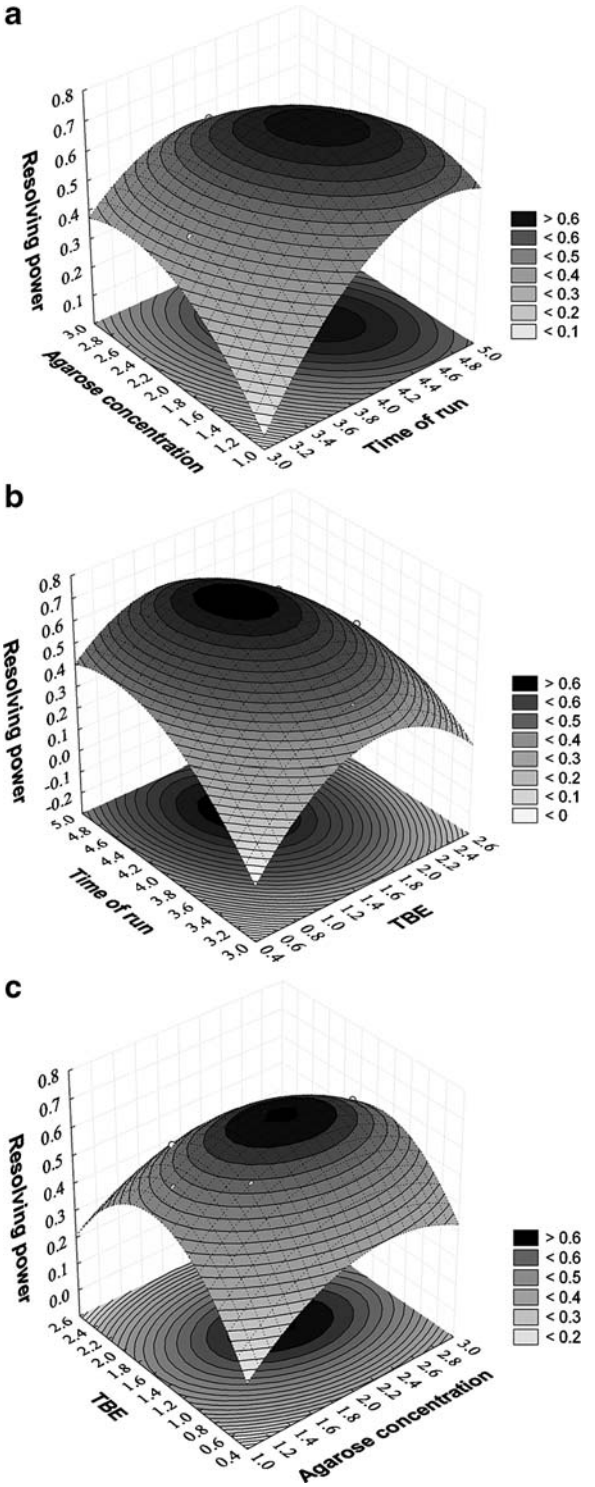

Fig. 2 **a** Response surface contour plot for concentration of agarose and time of run while keeping the ionic strength of TBE constant at centre value of 1.5× concentration. **b** Response surface contour plot for concentration of TBE and time of run while keeping the agarose concentration constant at centre value of 2% (*w*/*v*). **c** Response surface contour plot for ionic strength of TBE and agarose while keeping the time of run constant at centre value of 4 h

single dimensional genome profile analysis is an excellent tool for not only epidemiological surveying and grouping rotavirus isolates, but also detecting coinfection of different rotavirus strains. Resolution of rotavirus genome by AGE makes this epidemiological and molecular surveying approach excessively uncomplicated as compared to PAGE.

The electrophoretic mobility of nucleic acid in AGE is affected by several factors like the size and shape of the nucleic acid, pore size of the matrix, ionic strength of the electrophoresis buffer, temperature, applied voltage/field strength, and presence of intercalators (ethidium bromide) [14]. Since electropherotypic profiles of rotavirus dsRNA typically show four size classes of segments according to their molecular weight [15], the size and shape factor was excluded from CCD. The electrophoretic mobilities of linear nucleic acid fragments ≤1 kbp at ≤1.4% (*w*/*v*) agarose are independent of electric field strength from 0.6 to 4.6 $V cm^{-1}$ (corrected to a common temperature). However, mobility of larger fragments increases linearly with electric field strength at this agarose concentration. Conversely, if agarose concentration was >2% (*w*/*v*), mobility of all fragments increases with increasing electric field strength [16]. The segments of rotavirus genome range in size from 667 (segment 11) to 3,302 bp (segment 1) [10]; consequently, to resolve all the bands, a constant electric field of 4.6 $V cm^{-1}$ was selected and the factor of time of application of field strength was incorporated in CCD.

TBE buffer was selected due to higher buffering capacity as compared to Tris–acetate–EDTA and better resolution of smaller nucleic acid fragments at higher agarose concentrations, where 0.5× TBE corresponds to 0.026 mol l^{-1} ionic strength [17]. Ethidium bromide, an intercalator, was used at 0.5 μg ml^{-1} at the time of casting of gel instead of post-run staining to decrease experimental error which may affect band intensity measurements and hence decrease specificity of CCD.

So, in the central composite design, agarose concentration, ionic strength of the electrophoresis buffer and time of application of field strength were taken as independent variables affecting the resolving power of the gel, while keeping the size of gel and applied field strength constant in all the trials. The multidimensional approach in statistical designs is inherently superior to optimising one factor at a time [18]. In experiments where multiple factors influence the optimal response, single factorial optimisation can predispose towards a false optima as factors influence one another's optimum values. As a result, one can readily rationalise losses approaching an order of magnitude, even when each of several parameters has been 'optimised' by one-dimensional experiments.

Although there are reports for the use of RSM for optimisation of parameters of electrophoresis [19, 20], to the best of our knowledge, this is the first study involving statistical optimisation of AGE parameters for resolution of rotavirus-segmented dsRNA genome and for detection of miniscule variations in the electrophoretic profile of various long and short forms. In fact, the strength of the study is that the optimised conditions were validated on all the positive 79 samples, and a comparison was also done with PAGE analysis.

Appliance of a technique is conceptually more complicated to demonstrate because until a difference between two paired samples has not been shown, so the emphasis was laid upon the comparison of the two widely different electropherotypes. The comparative quantifications of peak width at half height and the relative migration distance for prediction of resolution emphasise the relevance of this method for the detection of non-group A rotaviruses, which are not detected in routine ELISA-based diagnostics. It is worthwhile to mention that the application of experimental design for optimisation of resolving power of AGE, besides its efficiency and cost effectiveness, has given an insight

on the interactions between the factors and has illustrated the significance of a well-acknowledged and extensively established method in resolving the 11 segmented genome of rotavirus.

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