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Research paper

Development of FAcE (Formulated Alhydrogel competitive ELISA) method for direct quantification of OAg present in *Shigella sonnei* GMMA-based vaccine and its optimization using Design of Experiments approach

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ABSTRACT

Many formulated vaccines, including 1790GAHB *Shigella sonnei* GMMA-based vaccine, contain Alhydrogel (aluminum hydroxide), consequently the antigen content must be determined in the formulated final vaccine product, as required by regulatory authorities. The direct quantification of antigens adsorbed on aluminum salts is difficult, and antigens may need to be extracted using laborious and often ineffective desorption procedures. To directly quantify the sugar vaccine target in the LPS of 1790GAHB, we have developed a new FAcE (Formulated Alhydrogel competitive ELISA) method. FAcE is an immunoassay based on the competition between *S. sonnei* LPS, coated on the ELISA plate, and the LPS in formulated *S. sonnei* GMMA, in binding a specific monoclonal antibody. To optimize the method, which is as easy to perform as a standard ELISA, we have applied a Design of Experiments (DOE) approach. A model was found to define the significant assay variables and to predict their impact on the output responses. Results obtained using the DOE optimized FAcE assay showed that the method is sensitive (0.02 µg/mL lower detection limit), precise, reproducible and can accurately quantify independently formulated drug products, making it a useful tool in routine tests of Alhydrogel-based vaccines. We are currently using this method to determine *S. sonnei* vaccine potency, stability and lot-to-lot variations, and are broadening its applicability to quantify active ingredients of other Alhydrogel GMMA-vaccines and in multivalent vaccines formulations.

1. Introduction

Many approved vaccines contain aluminum salts (Gupta, 1998; Singh, 2002) in different forms such as aluminum hydroxide ($Al(OH)_3$) or Alhydrogel, aluminum phosphate ($AlPO_4$), and potassium aluminum sulfate ($KAl(SO_4)_2{\cdot}12H_2O$) (Gupta, 1998; Baylor et al., 2002; Hem and Hogenesch, 2007). The development of high quality final products, as required by modern vaccinology and regulatory agencies, needs a complex set of analytical techniques to characterize each step of drug substance and drug product production. In particular, it is essential to determine the amount, as well as the identity and integrity, of antigens bound to aluminum adjuvants following formulation.

While the characterization of unformulated antigens is quite simple and can rely on several developed methods for different types of antigens (Lowry, BCA, or Bradford assays for proteins; phenol sulfuric acid assay, Size-Exclusion High Pressure Liquid Chromatography, High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection, etc. for sugars), the characterization of antigens adsorbed on aluminum salts is more difficult due to sample turbidity and viscosity, which prevents direct quantification of formulated antigens using the same set of methods. Therefore, antigen quantification typically involves methods that first extract antigen from the aluminum salts by desorption, either through aging (Burrell et al., 2000), treatment with surfactants (Rinella et al., 1998b; Zhu et al., 2012), pH (Rinella et al., 1998a), or by adding phosphate or other agents such as guanidine hydrochloride $(GnHCl)_2$ (Gupta and Rost, 2000). However, extraction procedures often result in less than 100% desorption of the antigen and may also alter its conformation, reducing the accuracy of the subsequent method used for quantification. To overcome these issues, several methods have been developed to quantify active ingredients in aluminum formulated vaccines. To determine total protein content, for example, the OPA (o-Phthalaldehyde fluorescent protein) assay has been described, which shows a moderate sensitivity (25–400 µg/mL), but lacks in specificity for protein of interest (Zhu

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et al., 2009b). Antibody-based immunoassays, on the other hand, would offer specific antigen detection. Examples of these methods are: flow cytometry, used to quantify two *Neisseria meningitidis* protein antigens (Ugozzoli et al., 2011), the DAFIA assay (Direct Alhydrogel Formulation Immunoassay), used to quantify *Plasmodium falciparum* apical membrane protein 1 (AMA1)-C1 (Zhu et al., 2009a), and Luminex-based assay, used to quantify acellular pertussis (aP) in TdaP (tetanus, diphtheria and acellular pertussis) vaccine (Agnolon et al., 2016). Although none of the above methods is widely used in vaccine development and manufacturing, immunoassays have shown promising results in terms of sensitive and specific detection of protein vaccine targets in aluminum-containing matrices.

Alhydrogel is used as an adsorbent agent to formulate our *Shigella sonnei* vaccine 1790GAHB (Gerke et al., 2015; Launay et al., 2017), which is a GMMA-based vaccine. GMMA (Generalized Modules for Membrane Antigens) are outer membrane particles shed from Gram-negative bacteria that are genetically modified to enhance the level of particle production. Similar to native outer membrane vesicles that are naturally shed by Gram-negative bacteria, GMMA consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. They are highly immunogenic in small doses in animals, as they contain several stimulators of the innate immune system, especially lipopolysaccharide (LPS) and lipidated proteins (Gerke et al., 2015).

Based on the need to directly quantify the sugar vaccine target after formulation, here we describe the development of a new FAcE (Formulated Alhydrogel competitive ELISA) method. FAcE method permits to quantify the O-antigen (OAg) present on Alhydrogel formulated *Shigella sonnei* GMMA, and it is based on the competition between *S. sonnei* LPS, coated on the ELISA plate, and the LPS on formulated *S. sonnei* GMMA (our drug product), in binding a specific anti-*S. sonnei* LPS monoclonal antibody (mAb).

To optimize the method, we have applied a Design of Experiments (DOE) approach based on Response Surface Methodology (RSM) to simultaneously study different assay's parameters. DOE is used for modeling and analyzing problems with multiple parameters, determining factor significance, developing mathematical models and optimizing system response (Montgomery, 1997). We used RSM to explore the functional relationship between controllable factors and output responses, including an assessment of their potential interaction. Different process variables, like step-fold dilution of standard curve and concentration of primary and secondary antibodies, were challenged in the experimental design and a model was found that could predict significant impact of those variables on the responses. The novelty of the study is based on using an immune-detection method to quantify OAg in formulated GMMA, by applying a DOE approach to identify the most critical parameters and their regulations to improve FAcE performance. Since its development, we have been using FAcE assay to determine vaccine lot-to-lot variations, and to follow vaccine potency and stability studies.

2. Materials and methods

2.1. Formulation of Shigella sonnei GMMA

Formulations of different lots of *Shigella sonnei* GMMA 1790GAHB were prepared by either GVGH (GSK Vaccines Institute for Global Health), or by Areta International (under GMP conditions). *Shigella sonnei* GMMA were formulated by mixing for 2 h at room temperature with Alhydrogel 2% (Brenntag Biosector, Denmark) (final concentration of 0.7 mg Al^{3+}/mL) and Tris buffer (final concentration of 10 mM Tris, 9 g/L NaCl and pH 7.4). After the incubation, a panel of analytical tests has been set up to fully characterize the formulation (pH, osmolality, visual inspection, total proteins and non-adsorbed proteins). In terms of safety of the vaccine, the most important parameter to monitor is the non-adsorbed proteins. To this end, the formulation was centrifuged and the supernatant was analyzed by SDS-PAGE Silver Staining. In these formulation conditions, GMMA adsorption on Alhydrogel was ≥97.5%.

2.2. FAcE protocol

The FAcE assay is a competitive-ELISA method, in which a specific anti-*Shigella* LPS antibody will bind to both the LPS antigen coated on the ELISA plate and to the LPS of the formulated GMMA suspension present in the ELISA wells. The more GMMA are present, the more mAb will bind to them, and the less will be available to bind to the coated antigen. Considering that the formulated GMMA with the bound mAb will be washed away during the procedure, the ELISA signal will be given by the binding of mAb to the coated LPS, therefore the lowest signal will be obtained at the highest GMMA concentrations and vice versa.

The standard curves are prepared by 10 sequential dilutions and two negative controls (Alhydrogel diluent only) of Alhydrogel formulated *Shigella sonnei* GMMA, 1790GAHB, starting from a known concentration in terms of OAg μg/mL (about 15.5 μg/mL originally quantified through a combination of NMR and HPAEC-PAD analysis (Gerke et al., 2015). To build the standard curves, freshly formulated GMMA are used. These GMMA, to be used as reference, are an ad-hoc formulation of 1790GAHB which is characterized in terms of pH, osmolality, visual inspection, total proteins and non-adsorbed proteins and is used within a week. In addition, each FAcE assay contains an internal positive control, constituted by four 4-fold dilutions in Alhydrogel diluent of the second dilution point used to build the standard curve.

The test samples are formulated GMMA, tested as undiluted and as 4-fold diluted samples.

The assay is run in independent triplicate plates (Nunc round bottom Maxisorp ELISA plates), with the standard curve in duplicate for each plate. Plates are coated overnight at 4 °C with *Shigella sonnei* LPS at 0.5 μg/mL (Gerke et al., 2015). The coating solution is removed and 200 μL of 5% fat-free milk dissolved in PBS buffer is added to the ELISA wells and incubated at 25 °C for 1 h. Plates are then washed three times with 300 μL of washing buffer (PBS with 0.05% Tween-20). Fifty microliters of each dilution point of the standard curve, and of test and control samples, are added to each ELISA plate. Immediately after this step, 50 μL of anti-LPS mAb (Inbios International, Inc. INB3192 purified mouse anti-*S. sonnei* IgG1), diluted in PBS with 1% BSA and 0.5% Tween-20, are added to the same wells and mixed. The ELISA plates, containing suspensions of formulated *S. sonnei* GMMA 1790GAHB and mAb, are incubated for 2 h at room temperature (about 21 °C) in a plate shaker (MixMate from Eppendorf) set at 600 rpm. Plates are then centrifuged for two minutes at 1500 *g* and washed three times with washing buffer. During these washing steps, formulated GMMA and the mAb bound to them are removed. One hundred μL of secondary anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich, A3438) are added to the plates and incubated for 1 h at 25 °C. After three more washes in washing buffer, 100 μL of *p*-nitrophenyl phosphate substrate solution (Sigma, N2770) are added and plates are incubated for 1 h at 25 °C. Absorbance at 405 nm and 490 nm is read and the difference between them (OD_{405nm}-OD_{409nm}) is measured. The standard curves were generated with four-parameter nonlinear regression and the amount of standards or test samples were calculated from the equation of Y = ((b/(a-Log_{10}(X)))^(d) − 1)/c, where a, b, c or d are values of the parameters, Y is the amount determined and X is the absorbance readings. The back calculation was performed by converting the observed readings of the standards to concentrations of the antigens using the four-parameter nonlinear regression equations. The percent error was calculated by the following formula: (|calculated concentration – nominal concentration| /nominal concentration) × 100. Inter-assay variation was calculated for the same samples, independently formulated, that were tested in different assays and reported as coefficient

Table 1
Independent variables with uncoded levels used in the experimental design.

Independent variables	Face-centered and central points			Star-points	
	-1	0	+1	−1.6	+1.6
Step-fold dilution of standard curve	2	2.3	2.6	1.82	2.78
Primary mAb dilution	26,000	32,000	38,000	22,400	41,600
Secondary mAb dilution	7000	10,000	13,000	5200	14,800

of variation (CV). Each assay was considered valid if the standard curve presented $R^2 > 0.9$, dynamic range > 100, background OD < 0.2 and if the OAg concentrations calculated from the internal positive control sample fell within 30% of its nominal concentration.

2.3. DOE

To evaluate what the most critical parameters are and how they should be regulated to optimize the performance of the FAcE assay, we tested three input factors at two different regulations: 1. step-fold dilutions of the standard curve (lowest regulation: 2; highest regulation: 2.6); 2. anti-*Shigella* LPS mAb dilution (lowest regulation 1: 26,000; highest regulation 1: 38,000); 3. secondary Ab dilution (lowest regulation 1:7000; highest regulation 1: 13,000) (Table 1). We used the software Design Expert (DE, version 9.0.6) and the RSM Central Composite Design (CCD) (Khuri A. I., 2010) (Montgomery, 1997) for the experimental designs and regression analysis of the experimental data, as best fit option for this study. CCD model is the modification of 2^k factorial design involving star points other than central points. We performed all the test runs in one single block, in duplicates, with 6 replicates of the central point (Table 2). Central point is used for estimating the purely experimental uncertainty variance (Raza et al., 2011). We chose 4 output response measures: 1. the curves' span, defined as the difference between the top and bottom of the curve; 2. the slope, calculated at the curves inflection point; 3. the R^2 indicating the goodness of fit of the regression curve; 4. the accuracy to estimate a value near the central point of the curve (generally corresponding to the 6th dilution point of the curve, concentration range depending on step fold dilution), or near either the lower (generally corresponding to the 9th dilution point of the curve) or the upper limit (generally corresponding to the 3rd dilution point of the curve). This accuracy was calculated by removing from the standard curve either the central point or a point near the lower/upper limit and treating them as samples. The percentage error obtained by comparing the extrapolated and the nominal values was used to measure these responses. We calculated those responses from 34 experimental runs, according to the run order obtained by DE (Table 2). Statistical analysis of the model was performed by evaluating the analysis of variance (ANOVA). The optimization was performed using response surface optimizer where desirability criteria for the responses were to maximize the span, with the slope in range.

3. Results

3.1. FAcE set up using DOE

We set up the FAcE method by applying a DOE approach. Based on some experience gained during preliminary tests, we chose a lower and a higher regulation for the three input variables we deemed mostly useful (step-fold dilution of standard curve, primary and secondary Ab dilutions). We performed all the 34 designed test trials to measure the specific outcome responses we considered mostly interesting to better define the method (Table 2). Experimental design was made considering impact of the individual variable and interaction effects with other variables, and RSM was applied to build an empirical model for describing the relation between input and output variables. ANOVA was performed through joint test of the parameters for statistical analyses.

The outcome measure R^2, which indicates the goodness of fit of the regression curve on the experimental standard curve, remained generally high (> 0.99) (Table 2) and, although it showed dependency from both the curve's dilution step and the secondary antibody, it did not result in any significant model (adjusted R^2: 0.4) (data not shown). Similarly, the accuracy to estimate a point near the central point/lower limit/ upper limit of the curve, measured in terms of % variation, was not dependent on individual factors (adjusted R^2: 0.1–0.2) and a generally higher variation was found in estimating the point near the lower limit (median values for % variation for a point near the central, upper, lower limit: 7.3%, 11.6%, 34.4%, respectively) (data not shown).

The outcome variable span ranged between 2.2 and 4.2 (Table 2) and showed a dependency from the dilutions of both the primary and the secondary antibodies, resulting in a significant model with an adjusted R^2 of 0.97 and a lack of fit *p*-value of 0.98 (indicating that lack of fit errors were due to random errors and were not considered statistically different from pure errors) (Supplementary Table S1). The model Fisher's F value of 402.15 implies that the model is significant, meaning that the model fits the reality within the investigated space. The suggested equation, relating the response and variables was:

$$\text{Span} = 2.92 - 0.053 * B - 0.78 * C + 0.26 * C^2.$$

The variable slope depended on the same two input variables (primary and secondary antibody dilutions), with an adjusted R2 of 0.95 and a lack of fit p-value of 0.27 (Fisher's F value of 149.7 for the mathematical model) (Supplementary Table S2). The suggested equation, relating the response and variables was: Slope = -1.47 + 0.046* B + 0.41* C-0.049*B2-0.15*C2. For both the span and the slope, the residuals were normally distributed (Fig. 1a and c) and equally dispersed for all predicted values, with no systematic trends observed (Fig. 1b and d) providing the evidence of constant variance. For the span, there was not strong evidence of expanding variance ("megaphone pattern < ") in this plot (Fig. 1 b), that could indicate the need for a transformation. For the slope, the residuals were marginally normally distributed. We can expect some scatter even with normal data. There was not strong evidence of definite patterns, like an "S-shaped" curve, which could indicate that a transformation of the response may provide a better analysis. Reasonable agreement was observed between adjusted and predicted R2 values (Tables S1 and S2). The response surface graphs were constructed for the two significant variables span and slope for further graphical interpretation of design space (Fig. 2).

The optimization was launched using response surface optimizer where desirability criteria for the responses were set as maximize span and slope in range. The highest desirability obtained was of 0.7 (maximum would be 1) and it was found with the following conditions: primary mAb diluted 1: 26,000 and secondary mAb diluted 1:7000, with step-fold standard curve not influent (Fig. 3).

3.2. FAcE after DOE optimization

The FAcE assay was run using the conditions found after the DOE optimization. **S**tandard curves were generated using formulated GMMA in the range from 0.009 to 15.86 μg/mL (OAg concentration), 2.3 step-fold diluted. The analysis done using four-parameter nonlinear regression showed $R^2 \geq 0.99$, slope − 2.3, and a span of 4.0, corresponding to a dynamic range of 143 (Fig. 4). Back calculation was used to confirm the reliability of the standard curves and to determine the detection range of the assay. The interval for sample extrapolation presented a typical range from 0.76 to 3.7 ELISA OD, and 0.02 μg/mL as lower limit of detection. The OAg concentrations determined by back calculation of the standard curve points agreed well with the nominal amounts of OAg on GMMA, with a percent error of 11.3% or lower,

Table 2
Experimental DOE plan.

Standard order	Run order	Input variables			Output responses					
		Curve's step-fold dilution	Dilution primary Ab	Dilution secondary Ab	R^2	Span	Slope	% variation point near central point	% variation point near upper limit	% variation point near lower limit
7	1	2.6	38,000	7000	0.999	4.115	−2.019	7.555	8.948	41.944
31	2	2.3	32,000	10,000	0.999	2.981	−1.582	3.626	9.916	37.939
19	3	2.78	32,000	10,000	0.999	2.962	−1.437	8.193	2.644	40.195
23	4	2.3	41,600	10,000	0.998	2.956	−1.557	11.574	3.937	19.208
11	5	2.6	26,000	13,000	0.999	2.477	−1.389	9.809	11.577	37.717
14	6	2	38,000	13,000	0.998	2.335	−1.127	2.432	7.286	20.020
27	7	2.3	32,000	14,800	0.999	2.403	−1.262	13.827	17.007	24.562
17	8	1.82	32,000	10,000	0.996	2.855	−1.374	10.074	6.891	13.058
8	9	2.6	38,000	7000	0.998	3.695	−2.061	6.170	6.235	22.012
26	10	2.3	32,000	5200	0.998	~~4.085~~	−2.445	8.133	2.367	18.048
2	11	2	26,000	7000	0.997	3.923	−2.339	23.509	21.407	34.484
34	12	2.3	32,000	10,000	0.999	2.830	−1.499	6.713	14.722	42.371
32	13	2.3	32,000	10,000	0.999	2.984	−1.607	1.190	14.644	41.174
25	14	2.3	32,000	5200	0.997	4.791	−2.403	13.017	48.504	50.073
24	15	2.3	41,600	10,000	0.999	2.864	−1.509	6.614	4.222	51.150
12	16	2.6	26,000	13,000	0.998	2.491	−1.297	4.783	28.768	41.501
18	17	1.82	32,000	10,000	0.999	2.970	−1.494	4.383	6.369	11.815
10	18	2	26,000	13,000	0.999	2.512	−1.314	4.026	0.627	44.390
33	19	2.3	32,000	10,000	0.999	2.930	−1.426	7.002	10.350	21.009
15	20	2.6	38,000	13,000	0.999	2.347	−1.202	3.940	12.895	45.221
9	21	2	26,000	13,000	0.997	2.388	−1.357	13.869	12.324	40.313
3	22	2.6	26,000	7000	0.998	4.053	−2.109	5.913	12.849	8.009
20	23	2.78	32,000	10,000	0.999	2.823	−1.385	2.932	7.568	36.137
30	24	2.3	32,000	10,000	0.998	2.957	−1.466	14.465	22.337	12.108
5	25	2	38,000	7000	0.997	3.735	−2.284	1.075	21.272	14.427
16	26	2.6	38,000	13,000	0.999	2.185	−1.101	1.817	16.788	43.610
1	27	2	26,000	7000	0.998	4.072	−2.145	9.091	11.693	8.589
29	28	2.3	32,000	10,000	0.998	2.791	−1.402	17.497	9.994	13.259
13	29	2	38,000	13,000	0.998	2.264	−1.261	2.925	1.645	47.749
4	30	2.6	26,000	7000	0.999	3.889	−2.066	4.945	12.763	41.887
22	31	2.3	22,400	10,000	0.999	2.990	−1.556	14.232	7.256	33.491
28	32	2.3	32,000	14,800	0.999	2.389	−1.180	8.968	33.056	33.056
21	33	2.3	22,400	10,000	0.999	3.106	−1.650	17.195	34.330	34.330
6	34	2	38,000	7000	0.998	4.172	−1.977	12.305	19.944	19.944

between a detection range of 0.021 and 3.056 μg/mL. Therefore, the standard curves, within the detection range, were useful to extrapolate OAg concentrations from formulated vaccines (Table 3). Inter-plate variation analysis of four test samples and the internal control, independently diluted and run on 3 different plates, showed coefficients of variation (CV) from 1.5% to 13.8% (Table 4) and high accuracy (% error < 15% for 4 out of 5 samples).

Moreover, specificity of such assay has been evaluated against 3 different *Shigella flexneri* serovars. By performing the FAcE assay using formulated *S. flexneri* GMMA as test samples, we did not observe any binding with *S. sonnei* mAb, therefore confirming our assay specificity (Table S3 and Supplementary Information).

4. Discussion

Antigen quantification after formulation presents some challenges and different methods have been proposed for different types of vaccines. To quantify the active OAg ingredient of our vaccine against *S. sonnei*, we developed an immune-detection based method, exploiting the principle of the competitive ELISA and the possibility to quantify formulated GMMA, with unknown OAg concentration, from a standard reference curve built using the same type of GMMA with known OAg amounts (Gerke et al., 2015). Being an immune-detection method, it is of paramount importance that the standard curve is built using material perfectly matching the samples that need to be measured, as the type of antibody-antigen interaction should not be affected by any other factor than the antigen amount. Additionally, the formulation efficiency should be known to properly attribute a nominal concentration to the material used as standard. In our case, previous formulation experiments have shown that *Shigella* 1790GAHB GMMA, in the concentration range used here, are completely adsorbed on Alhydrogel, and, moreover, the possibility to quantify total protein levels of formulated GMMA, can also allow an indirect determination of their OAg content, knowing the OAg/total protein ratio (Gerke et al., 2015). Finally, the stability of the formulated material has been also considered by performing a short-term stability study of a standard drug product. Over a range of time of 50 days, a standard formulated material has been systematically quantified by FAcE against a freshly prepared standard curve. As shown in Table S4, OAg concentrations obtained were in line with to the expected values based on protein content with a percent error of 22% or lower (Table S4).

To determine key assay parameters, we moved away from the traditional evaluation of output responses by changing parameter settings one-factor-at-a-time (OFAT). In addition to being time and resource intensive, OFAT has the major disadvantage to provide no means of determining interactions between factors. As an alternative, DOE offers an efficient opportunity of investigating the impact of changes in system parameters, of exploring factor combinations and optimizing experimental results (Montgomery, 1997; Khuri and Levy, 2010; N Politis et al., 2017). More specifically, DOE has effectively been used in assay development and optimization, including the detection of anti-drug neutralizing antibodies in human serum, the improvement of ligand-binding assay sensitivity, and the optimization of ELISAs (Joelsson et al., 2008; Chen et al., 2012; Joyce and Leung, 2013; Xie et al., 2013). In our case, the RSM showed that among the selected responses, both span and slope were significantly affected by primary and secondary antibody dilutions, with no interaction effects, while the curve's step fold dilution was uninfluenced. Span and slope appeared

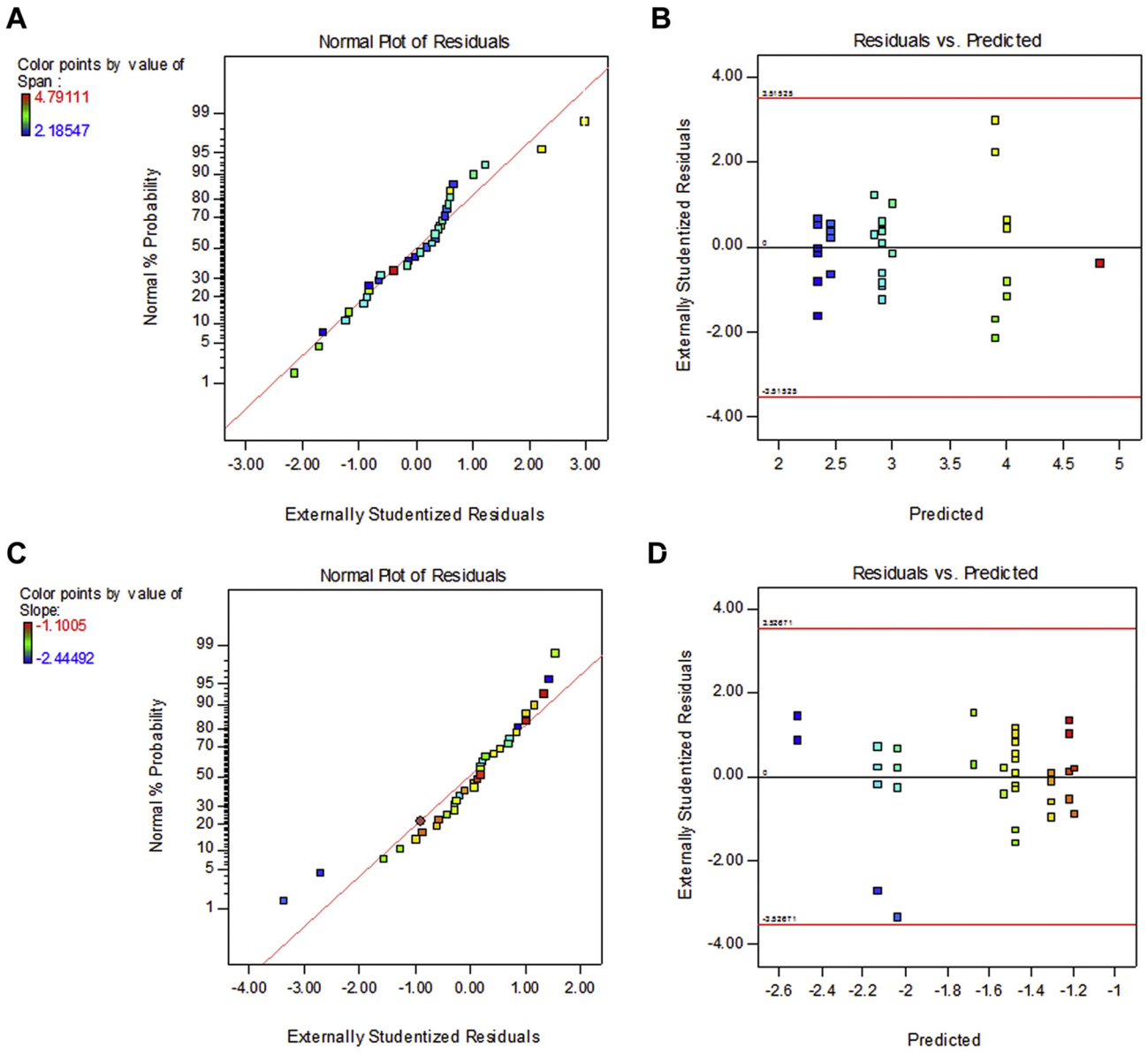

Fig. 1. Normal probability plot of residuals and plot of residuals versus predicted response values for the outcome variables span and slope. A and B normal plot of residuals and plot of residuals versus predicted values for span, respectively. C and D normal plot of residuals and plot of residuals versus predicted values for the slope, respectively. B and D, upper and lower red lines represent upper and lower limits to determine outliers within all the runs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

highly correlated. On the other hand, the % variation plotted near the central point versus either span or slope showed a lot of scatter (data not shown), hence all combinations gave a similar accuracy at the midpoint. This lack of correlation shows that the assay is very robust with similar accuracy over a wide range of parameters. FAcE improvement was therefore determined by choosing the antibodies dilutions, resulting in a larger span and a broader interpolation range. RSM provided the optimized settings of the two independent variables for the desired response with limited experimental trial runs. The results obtained using the DOE-determined conditions confirmed the method optimization in terms of increased span and dynamic range and additionally, showed that FAcE is sensitive, precise, reproducible and can accurately quantify independently formulated drug products. All these characteristics make it an ideal tool for both research and quality tests. Compared to other methods developed to quantify active ingredients in aluminum formulated vaccines, FAcE is clearly superior to OPA (Zhu et al., 2009b) in terms of sensitivity (1250 folds higher) and specificity. By more appropriately comparing it to immune-detection based methods, FAcE can detect a broader range of concentrations (2.2 to 4.5 higher depending on the method) and is more sensitive than both DAFIA (Zhu et al., 2009a) and flow-cytometry (Ugozzoli et al., 2011) (8 and 310 folds more, respectively); only the Luminex-based assay (Agnolon et al., 2016) showed a higher sensitivity (about 25-fold higher than FAcE), but a lower detection range.

At the moment, we are exploring its applications to tackle multiple questions such as efficiency of different formulation conditions, stability of formulated drug product, lot to lot variations, potency of *Shigella* 1790GAHB vaccine. Additional work is ongoing to see if it can be also used to evaluate GMMA integrity on Alhydrogel, using specific antibodies recognizing periplasmic or cytoplasmic proteins.

In summary, the use of FAcE to quantify OAg in *Shigella* 1790 GAHB eliminates the laborious OAg extraction process, preventing possible antigen loss and alterations of critical epitopes. The assay proved sensitive and accurate, while being convenient and easy to perform as a standard ELISA. We are currently setting up the same type of assay to quantify other Alhydrogel-formulated drug products such as *S.*

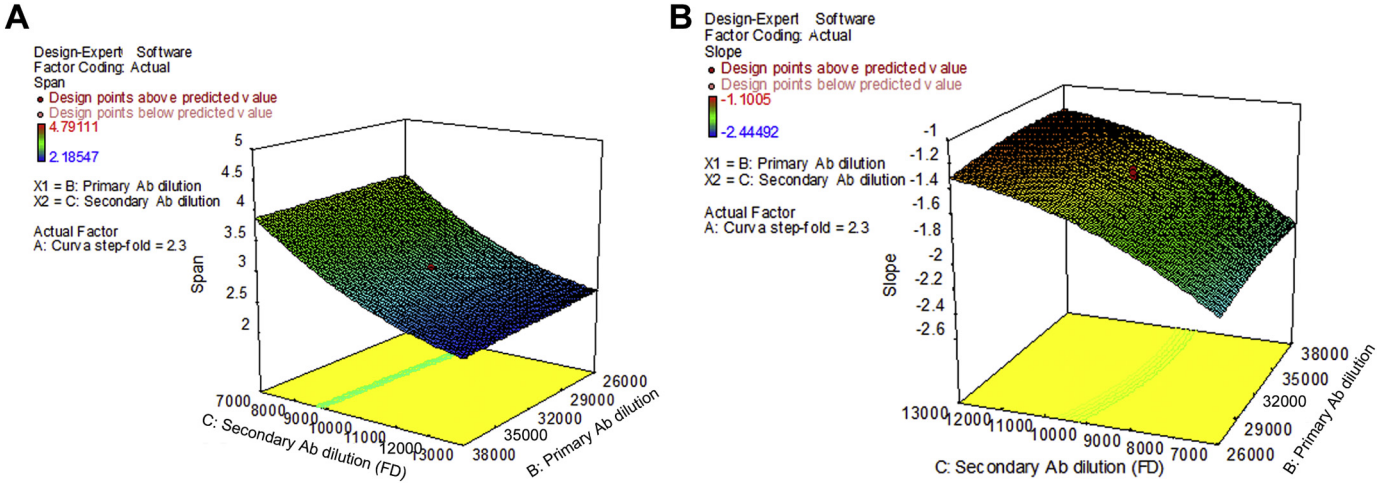

Fig. 2. Response surface graphs for span and slope. Three-dimensional response surface plots of span (A) and slope (B) versus antibodies dilutions. The other variable (dilution step) is fixed at the central point.

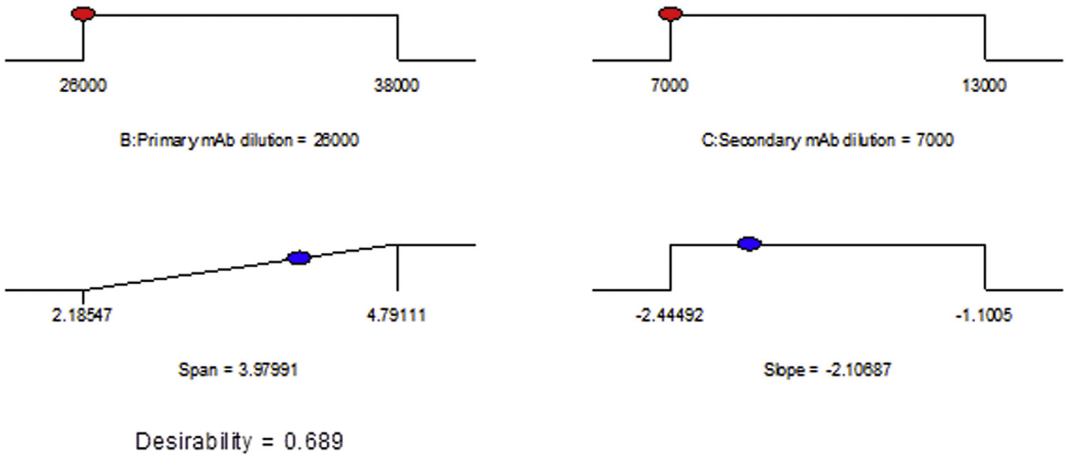

Fig. 3. Highest desirability conditions obtained by launching FAcE optimization with Design-Expert. Response surface optimizer was launched by setting maximization of span and slope in range.

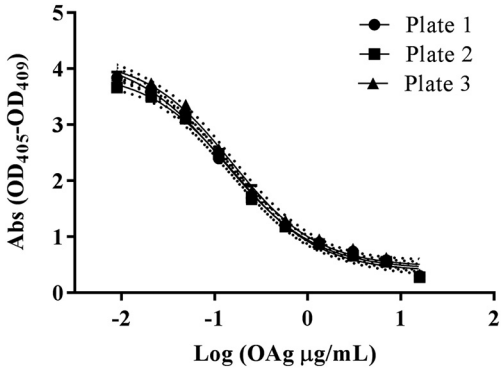

Fig. 4. FAcE standard curve for *Shigella sonnei* GMMA formulated in Alhydrogel (1790GAHB). The curve is the fitted 4-parameter nonlinear regression (R2 > 0.999), with 95% confidence intervals (dotted line) on data derived from duplicate curves from three ELISA plates. Average standard curve parameters of the standard curves were: A = 4.12, B = 1.039, C = 0.132, D = 0.575, R^2 = 0.996.

Typhimurium and *S*. Enteritidis GMMA. Moreover, we are broadening its applicability to quantify the active ingredients in multivalent vaccines formulations, using highly specific antibodies to each of the vaccine components.

Table 3
Back calculation and percent errors of standard curves.

Nominal concentration (µg/mL)[a]	Back calculated mean concentration ± SD[b]	Percent error[c]
0.000	0.001 ± 0.003	ND
0.009	0.013 ± 0.004	28.36
0.021	0.021 ± 0.002	0.49
0.049	0.046 ± 0.002	5.09
0.111	0.113 ± 0.002	1.68
0.251	0.265 ± 0.002	5.55
0.578	0.556 ± 0.005	3.91
1.331	1.181 ± 0.056	11.26
3.056	2.935 ± 0.746	3.93
6.974	27,912 ± 5.743	300.22
15.858	255,075 ± ND	1508.48

ND: Not Determined

[a] Known amount of 1790GAHB (in terms of OAg concentrations).

[b] Back calculation was performed by converting the observed readings of the standards to concentrations of the antigens using the four-parameter nonlinear regression equations. Results are means ± standard deviations (SD) from three ELISA plates with duplicate standard curves on each plate.

[c] Calculated as: (|calculated concentration – nominal concentration| /nominal concentration) × 100.

Table 4
Assay variation for 4 independently formulated samples and the internal positive control.

Sample	Nominal concentration (μg/mL)[a]	FAcE calculated concentration ± SD[b]	CV[c]	Percent error[d]
1-internal positive control	15.9	18.1 ± 2.5	13.8	13.7
2	12.5	14.3 ± 0.7	4.8	14.1
3	16.1	16.5 ± 2.0	11.8	2.9
4	12.0	15.0 ± 0.2	1.5	24.9
5	11.3	10.9 ± 1.0	9.3	3.6

[a] Known amount of control samples (in terms of OAg concentrations). All samples, but the internal positive control, were independently formulated.
[b] Results are means ± standard deviations (SD) from three ELISA plates, with duplicate standard curves on each plate. In each one of the plates, the samples were tested at five dilutions and values could be extrapolated from three to four dilution points, according with the specific plate acceptable range (ranging from max OD of 3.6 to min OD of 0.7).
[c] CV: coefficient of variation.
[d] Calculated as: (|calculated concentration – nominal concentration| /nominal concentration) × 100.

Author contributions

MC, FN, SR, AS conceived and designed the study. MC and FN performed the experiments. IP prepared and characterized the formulation samples. OR performed specificity experiment. MC, FN, SR, IP, OR and AS analyzed data. SR, FN and MC wrote the manuscript. All authors reviewed and approved the manuscript.

Conflict of interest statement

MC, FN, IP, OR, AS and SR are employees of GSK. OR reports grant from BMGF, outside the submitted work. AS reports ownership of GSK shares.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.04.012.

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