Development and evaluation of an ELISA to detect Escherichia coli K88 (F4) fimbrial antibody levels

F. VÁZQUEZ*, E. A. GONZÁLEZ‡§, J. I. GARABAL*, SUSSET VALDERRAMA†, J. BLANCO* and S. B. BALODAS*

*Departamento de Microbiología e Parasitoloxía, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27902 Lugo, Spain, ‡Centro de Ingeniería Genética y Biotecnología (CIGB), Camagüey, PO Box 6162, La Habana, Cuba and †Department of Bacteriology, Biomedical Center (BMC), Box 583, S-751 23, Uppsala, Sweden

An enzyme-linked immunosorbent assay (ELISA) to determine IgG antibody levels against K88 (F4) fimbrial antigen from porcine enterotoxigenic Escherichia coli (ETEC) has been developed. The ELISA method was checked with serum samples obtained from rabbits and pigs, and the parameters affecting the method were also analysed. ELISA plates were optimally coated with K88 antigen 0.5 µg/ml for testing rabbit antiserum or with 1.25 µg/ml for testing pig serum. Optimal concentrations of H2O2 (0.5%) and orthophenylene-diamine (OPD) (0.125%) were chosen when a 10-min incubation period was used. The expression of antibody levels as enzyme-immunosorbent units (EIU) significantly decreased the variability of results between duplicate plates, when compared with the expression of results as direct OD values. ELISA-K88 applied to a field study with serum samples from 141 vaccinated and 52 unvaccinated sows was shown to be useful in differentiating between samples from vaccinated and unvaccinated animals.

Introduction

Enterotoxigenic Escherichia coli (ETEC) strains are commonly associated with neonatal diarrhoea in man and domestic animals [1-7]. Such diarrhoea is normally the result of a combination of two important virulence factors produced by these strains of E. coli — ability to adhere to the intestinal mucosa (which prevents the peristaltic elimination of the bacteria from the gut) and production of heat labile (LT) or heat stable (ST) enterotoxins (which disturb intestinal fluid absorption). E. coli isolates from piglets with diarrhoea may express K88 (F4), 987P (F6), K99 (F5) and F41 antigens or additional fimbrial adhesins (colonisation factors) whose structure is not yet completely determined [7,8]. Many strains isolated from both diarrhoeic and healthy piglets also express type 1 fimbriae, whose role as a virulence factor is not totally clarified [9,10]. Several studies have demonstrated that fimbrial adhesins are immunologically active [11-14] and that protection against neonatal diarrhoea caused by ETEC can be achieved by colostrum after immunisation of the pregnant female with a vaccine containing fimbrial antigens. Parenteral administration of these vaccines to the pregnant sows enhances the levels of specific antibodies in colostrum and milk and provides passive protection for the sucking piglet [15-18]. Several enzyme-linked immunosorbent assays (ELISA) have been used for the detection and quantification of antibodies against colonisation factors [12,19] and the detection of specific classes of immunoglobulins. Although IgA is recognised as the most important class of immunoglobulin involved in protection against ETEC, IgG levels in the serum of the pregnant female are representative of the efficacy of the vaccines to protect sucking piglets, as a parenteral vaccination induces both IgG and IgA [19]. This study describes the development and evaluation of an ELISA method for the detection and quantification of specific IgG antibodies against the K88 fimbrial antigen from porcine ETEC to determine the efficacy of vaccination programmes.

Materials and methods

Bacterial strains and growth conditions

Two enterotoxigenic E. coli strains, Ip102a and Ip66a, both O149 K88ac (LT+, STa+), were isolated from the faeces of piglets with neonatal diarrhoea in Spain [20,21]. Bacteria were grown in Mueller Hinton Broth (Difco) incubated at 37°C for 5 days under static conditions, until a firm pellicle was formed. From this pellicle, bacteria were inoculated on to colonisation
factor antigen (CFA) agar [22] plates and incubated at 37°C for 18 h.

**Purification of fimbriae**

Control K88ac fimbrial antigen was obtained from the Centro de Ingenieria Genética y Biotecnología (CIGB, Cuba). Fimbrial antigens were isolated from strains Ip102a and Ip66a and purified as described previously [23] with slight modifications. The growth was harvested with 50 mM phosphate buffer containing 2 M urea (pH 7.2) and the turbidity of the suspension was adjusted to MacFarland standard tube 10 (c. 3 x 10^8 cells/ml). Fimbriae were detached from bacteria by heat shock (60°C for 30 min). After heating, the bacterial suspension was centrifuged (6000 g for 30 min) and the pellet was discarded. All subsequent steps were performed at 4°C. K88 fimbriae were precipitated by slowly adding ammonium sulphate 60% w/v. Precipitated protein was collected by centrifugation (6000 g for 45 min), resuspended in 50 mM phosphate buffer (PB), pH 7.2, and dialysed for 24 h at 4°C against the same buffer with at least two changes of the buffer. Sodium deoxycholate (DOC) (Sigma) was added to the dialysate at 0.5% w/v final concentration. This suspension was dialysed against PB with DOC 0.1% w/v for a further 48 h. Insoluble material was then removed by centrifugation (6000 g, 45 min). The supernate containing the DOC-extracted K88 antigen was dialysed against PB for 24 h followed by an extensive dialysis against distilled water and kept at 4°C until use. Protein concentration was estimated by the method of Bradford et al. [24] with bovine serum albumin as standard.

**Electrophoresis in SDS-PAGE**

The purity and mol. wt. of the K88 subunit were assayed by SDS-PAGE employing the buffer system of Laemmli [25]. Stacking and separating gels of 3% and 12.5%, respectively, were prepared in a Protean II system (BioRad, USA). Electrophoresis was carried out with 25 mM Tris, 0.2 M glycine buffer containing SDS 0.1% w/v. Electrophoresis at 30 mA for 45 min was followed by a 2-h run at 35 mA. Samples were boiled for 5 min in an equal volume of 0.125 M Tris-HCl, pH 6.8, with SDS 4% w/v, glycerol 20% v/v, bromophenol blue B 0.002% v/v and mercaptoethanol 9% v/v. Proteins were stained by silver staining according to Blum et al. [26]. The presence of LPS in protein samples were detected by specific silver staining according to Tsai and Frash [27].

**Immunoblotting**

Immunoblotting was performed as described by Towbin et al. [28] with minor modifications. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Sigma) by electrophoretic blotting overnight at 150 mA in 25 mM Tris-HCl, 192 mM glycine with methanol 20% v/v. Nitrocellulose membranes were then blocked with a 3% w/v solution of bovine serum albumin in Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.4) at room temperature for 30 min, and incubated (37°C, 90 min) with anti-K88 rabbit serum diluted 1 in 200 in TBS containing Triton X-100 0.05% v/v and 5 mM EDTA. After extensive washing with distilled water, membranes were incubated (37°C for 90 min) with a 1 in 16000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma). Membranes were then washed several times with distilled water and developed with hydrogen peroxide (H_2O_2) and 4-chloronaphthol chromogen (BioRad).

**Production of antisera**

Antisera against the fimbrial antigen K88 were produced in New Zealand albino rabbits by the method of Evans et al. [29] as described previously [30]. DOC extracts containing K88ac antigens (500 µg/ml) emulsified in complete Freund’s adjuvant were injected subcutaneously in rabbits. Four weeks later the rabbits were given intravenous injections of 50 µg of purified K88 emulsified in incomplete Freund’s adjuvant at weekly intervals on three occasions and bled 2 weeks after the final injection. To remove any possible non-specific antibodies, sera were absorbed with suspensions of the homologous strain previously heat inactivated (121°C, 1.5 h) and with cultures obtained after they were grown at 18°C and checked for the lack of expression of K88. The absorbed antisera reacted with the three variants of K88 (K88ab, K88ac and K88ad) as described previously [21]. The K88 positive control serum of porcine origin was selected from a group of sows vaccinated with a commercial vaccine containing K88 antigen.

**ELISA procedures**

An indirect ELISA method for anti-K88 IgG detection was employed. Polystyrene plates (Serowell, USA) were coated with 100 µl of K88 control purified antigen diluted in 50 mM sodium carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were then washed three times with 0.1 ml of phosphate-buffered saline containing Tween 20 0.05% v/v (PBS-T). Serum samples were diluted in PBS-T containing bovine serum albumin 1% w/v (PBS-TB) and 0.1 ml of dilutions of the respective samples were added to each well in duplicate and incubated at 37°C for 1 h. The plates were then washed four times with PBS-T, and 0.1 ml of goat anti-rabbit or anti-pig IgG horseradish peroxidase conjugate (Sigma) diluted in PBS-TB was added and incubated at 37°C for 1 h. The plates were then washed four times with PBS-T, and 0.1 ml of substrate buffer (50 mM phosphate, 20 mM citric acid, pH 5.5) containing H_2O_2 0.5% v/v (6% purity) and OPD 0.1% w/v were added. After incubation at room temperature for 10 min the reaction was stopped with 2 N H_2SO_4. The optical density (OD)
at a wavelength of 492 nm was determined in an AUTO-EIA System (Labsystems, Finland). OD shown by the background controls was subtracted from the OD of each test serum sample.

K88 antigen was detected and quantified with a competitive indirect ELISA. Five two-fold dilutions of purified K88 control antigen were made in PBS-TB, and a 100-μl portion of each dilution was mixed with 100 μl of diluted K88 specific antisera. The antigen-antibody mixtures were then added to the wells of antigen-coated plates. Bound anti-K88 IgG was determined as described above. Thus, a decrease in OD by competition was used to construct a standard inhibition curve. Concentration of K88 in test samples was calculated by determining the amount of antigen that caused inhibition to the same extent as in the standard inhibition curve.

Anti-K88 IgG levels in vaccinated and unvaccinated sows

A total of 141 serum samples from vaccinated sows in 16 farms and 52 serum samples from unvaccinated sows in 11 farms were analysed by ELISA-K88 following the methodology and dilutions indicated above for porcine sera. Five commercial vaccines (VAC1–VAC5) were used. One hundred and seven sows received VAC1 (containing K88ab, K88ac, 987e K99, LT toxoid and inactivated Azjzsky disease virus), 16 received VAC2 (containing K88ab, K88ac, 987e, K99 and LT toxoid); eight received VAC3 (containing K88, 987e, K99 and common pilus type 1); three received VAC4 (containing K88, 987e, K99 and F41) and seven received VAC5 (K88, 987e, K99, F41, common pilus type 1 and LT toxoid). The parity, with an average of nine to 10 piglets/litter, and the management of the sows in the different farms was similar. The age and the number of vaccine doses received by sows vaccinated with VAC1 were controlled. Thus, 16 sows received a single dose, 22 received two doses, 28 received three doses, 15 received four doses, six received five doses and 20 received more than five doses. The age (in months) of sows vaccinated with VAC1 was as follows: 12–14 for sows receiving one dose, 15–23 for sows receiving two doses, 24–28 for sows vaccinated three times, 29–34 for sows vaccinated four times, 35–38 for sows vaccinated five times and 39–55 for sows vaccinated six or more times.

Results

Purification of K88 antigen

Electrophoresis was used to monitor all the purification stages. SDS-PAGE of the extracts containing K88ac revealed a major protein of 29.2 kDa that reacted in Western blots with specific antisera against K88 fimbriae (Fig. 1). Heat shock was shown to be an effective method for the liberation of K88 fimbriae, although repeated heat shocks did not increase the amount of antigen removed from the cells. After fractionation with DOC, the supernate contained the higher amount of K88 antigen; LPS and outer-membrane proteins were located mainly in the precipitate.

Fig. 1. a, SDS-PAGE of K88 fimbriae obtained from Ip102a strain at different steps of purification. C, marker proteins; 1, DOC-soluble material; 2, DOC-insoluble material; 3, ammonium sulphate precipitate; 4, heated suspension. The apparent mol. wts (kDa) were 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4, respectively. b, Western blot of DOC supernate containing K88 antigen obtained from strain Ip102a, developed with absorbed rabbit antiserum raised against K88 fimbriae.
Determination of the optimal concentration of K88 antigen for coating plates

Four dilutions of the purified control antigen in coating buffer were assayed to determine coating concentration of antigen. Coated plates were tested with several two-fold dilutions of rabbit or porcine antisera against K88 antigen. Optimal results for detection of K88 antigen with rabbit anti-sera were obtained at a coating antigen concentration of 0.5 μg/ml (Fig. 2a), whereas for porcine IgG, a concentration of 1.25 μg/ml (Fig. 2b) was chosen. At higher antigen concentrations, a decrease in OD was observed. A K88 antigen concentration of 0.5 μg/ml was effective in both assays in the differentiation of positive and negative sera against K88.

Determination of optimal dilution of conjugate

Several conjugate dilutions were assayed with two-fold dilutions of positive and negative sera (Fig. 3) to rule out reactions due to non-specific binding of conjugated
E. COLI
K88
ANTIBODY ELISA

Fig. 3. Determination of the optimal dilution of goat anti-rabbit IgG-peroxidase conjugate for titrating anti-K88 IgG in:
a, rabbit sera (●, 1 in 25 000; ■, 1 in 50 000; ▲, 1 in 75 000); b, porcine sera (●, 1 in 10 000; ■, 1 in 20 000; ▲, 1 in 40 000), (○, □, △, negative serum).

immunoglobulins to the plates. No variation depending on conjugate concentration was observed in ELISA results with the negative control rabbit sera. Nevertheless, OD values obtained with negative porcine sera were higher than those for the rabbit sera. For measuring IgG levels in rabbit sera, an optimal 1 in 50 000 dilution of the conjugate was selected, accounting for the high positive/negative ratio (P/N) obtained (OD serum positive 2.131; OD serum negative 0.010; P/N:21.3) (Fig. 3a). A 1 in 10 000 dilution of conjugate was optimal for assaying porcine sera (OD positive sera 1.752; OD negative sera: 0.302; P/N:5.8) (Fig. 3b).

Determination of the optimal conditions for enzymic activity

Concentration of H$_2$O$_2$. To investigate the maximum concentration of H$_2$O$_2$ for ELISA without inhibition of
the enzymic activity, several concentrations of H₂O₂ (6% purity) were tested. The results showed that concentrations of H₂O₂ > 0.5% v/v substantially decreased enzymic activity.

**Concentration of OPD.** Higher OD values were obtained at a concentration of OPD 0.125% w/v, whereas at higher concentrations a drop in OD values and an increase in background values were observed. Therefore, an optimal concentration of 0.1% was used.

**Optimal incubation time**

The kinetics of the enzymic reaction in ELISA were evaluated by measuring the absorbance at 20, 15, 10 and 5 min after addition of substrate at optimal concentrations. ELISA plates were coated with the optimal antigen concentration and 1 in 1600 dilutions of the positive and negative rabbit serum were assayed. No relevant increase in OD values of positive sera above incubation for 15 min was observed and because OD values in negative serum were slightly higher after incubation for 10 min this was used for further assays.

**Establishment of the working dilution of serum samples and expression of levels of IgG in serum**

A 1 in 1600 dilution for rabbit serum and a 1 in 200 dilution of sow serum were suitable for the standardised ELISA (Fig. 3). In addition, two expressions of IgG levels obtained after testing a single dilution of serum were analysed: direct OD value obtained at dilutions indicated above for rabbit and porcine sera and enzyme-immunosorbent units (EIU) (OD<sub>test serum</sub> - OD<sub>negative control serum</sub>) / (OD<sub>positive control serum</sub> - OD<sub>negative control serum</sub>) × 100. For this purpose, several rabbit serum samples containing high, medium and low amounts of K88 IgG antibody were assayed (Table 1). Results obtained with 30 negative rabbit serum samples at a 1 in 1600 dilution gave OD values ranging from 0 to 0.028 (mean 0.009 SD 0.007).

Results expressed by the two methods varied considerably. The coefficients of variation (CV) of the IgG levels expressed as EIU were, as expected, lower when the results of a single serum sample were compared in different plates (Table 2). Variation in

Table 1. Expression of IgG levels achieved in serum samples of rabbits inoculated with extracts containing K88 antigen or with bacteria expressing K88 antigen

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Days*</th>
<th>Titre</th>
<th>OD for serum dilution</th>
<th>EIU&lt;sup&gt;2&lt;/sup&gt; for serum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>800</td>
<td>1600</td>
</tr>
<tr>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>12800</td>
<td>0.792</td>
<td>0.359</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>1.024 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.772</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td></td>
<td>1.038 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.428</td>
</tr>
<tr>
<td>23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>25600</td>
<td>1.172</td>
<td>0.699</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>4.096 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.128</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td></td>
<td>6.553 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.354</td>
</tr>
<tr>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>6400</td>
<td>0.146</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>4.096 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.557</td>
</tr>
<tr>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>6400</td>
<td>3.277 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.849</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td></td>
<td>4.096 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.197</td>
</tr>
<tr>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>6400</td>
<td>1.638 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.245</td>
</tr>
</tbody>
</table>

*Titre serum samples obtained at days 15, 30 and 45 after the first inoculation.
1Titre expressed as the lowest dilution of serum giving an OD higher than the negative control assayed in the same plate.
2EIU calculated as (OD<sub>serum sample</sub> - OD<sub>negative control serum</sub>) / (OD<sub>positive control serum</sub> - OD<sub>negative control serum</sub>) × 100.
3Sera obtained from rabbits inoculated with DOC supernate containing K88 antigen.
4Sera obtained from rabbits inoculated with whole bacteria, expressing K88 antigen, and further absorbed.

Table 2. Variability in the ELISA for detection of specific IgG in positive rabbit control sera assayed in duplicate in two different plates

<table>
<thead>
<tr>
<th>Plate no.</th>
<th>Titre&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OD&lt;sup&gt;1&lt;/sup&gt; for serum dilution</th>
<th>EIU&lt;sup&gt;2&lt;/sup&gt; for serum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>1600</td>
</tr>
<tr>
<td>1</td>
<td>25600</td>
<td>1.032</td>
<td>0.682</td>
</tr>
<tr>
<td>2</td>
<td>25600</td>
<td>1.483</td>
<td>0.951</td>
</tr>
<tr>
<td>SD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0.309</td>
<td>0.190</td>
</tr>
<tr>
<td>CV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>25.36</td>
<td>23.29</td>
</tr>
</tbody>
</table>

*Titre calculated as the lowest dilution of serum giving an OD higher than the negative control serum, assayed in the same ELISA plate.
1Mean OD value for each serum dilution assayed in duplicate in the same plate.
2Mean EIU value for each serum dilution assayed in duplicate in the same plate.
3SD of the results obtained in the two plates.
4Coefficient of variation of the results obtained in the two plates.
results obtained with two ELISA plates did not affect the endpoint titration obtained for the serum. The higher CV obtained with results expressed as OD values was attributed to uncontrolled parameters that may affect the final results of the assay. Thus, when all the results were referred to the standard sera analysed in the same ELISA plate, the CV were considerably lower. The expression of IgG levels as EIU provided appropriate results for the assay of a large number of sera in a minimum time with low variations in the final results obtained. The result allowed the positive-negative cut-off point in rabbit sera to be established at 0.1 for direct OD and at 2 EIU.

**Application of ELISA to determine anti-K88 IgG levels in vaccinated and unvaccinated sows**

Serum samples from 141 sows vaccinated with five different vaccines (VAC1–VAC5) against porcine colibacillosis, and from 52 unvaccinated sows were analysed by K88-ELISA (Table 3). Vaccinated sows showed anti-K88 IgG levels with mean values ranging from 67.7 EIU (OD values 1.031 SD 0.290) to 115.0 EIU (OD 1.776 SD 0.112) depending on the vaccine. Thus, serum samples from sows immunised with VAC1 showed values in EIU of 23.1–135.1; with VAC2 30.5–106.9; with VAC3 98.0–138.1; with VAC4 75.5–115.7; and with VAC5 28.8–134.2. In all the 141 sows, the mean value for anti-K88 IgG levels was 84.1 EIU (OD 1.378 SD 0.314) (Table 3).

Anti-K88 IgG levels in unvaccinated sows were considerably lower, ranging from 0 to 21.8 EIU, with a mean value of 3.9 EIU (OD 0.450 SD 0.208). Distribution of OD and EIU values obtained with the 141 samples from vaccinated sows and from the 52 serum samples from unvaccinated sows are shown in Fig. 4. The results allowed us to differentiate anti-K88 IgG levels in vaccinated and unvaccinated sows and to set the positive-negative cut-off point in porcine sera at 0.800 for direct OD and at 20 EIU. The available bacteriological data on ETEC incidence in piglets born from vaccinated sows indicated that protection against ETEC diarrhoea was directly related to vaccination and that higher anti-K88 IgG levels in vaccinated sows were related to reduction of diarrhoea caused by ETEC. Thus, incidence of diarrhoea caused by ETEC was 37.5% of piglets in unvaccinated sows with a mean value of anti-K88 IgG 3.9 SD 5.8 EIU, whereas in piglets from vaccinated sows, the rate of diarrhoea caused by ETEC ranged from 14% of piglets (VAC2, 67.7 SD 24.9 EIU) to 4% of piglets (VAC3, 115.0 SD 12.0 EIU).

The influence of the vaccine dose received by sows was analysed by matching the EIU obtained with the 107 serum samples from animals immunised with VAC1 assayed at 1 in 200 dilution (Fig. 5a). EIU obtained at 1 in 200 dilution were related to the endpoint titration calculated as the lowest dilution of serum giving an EIU higher than the cut-off point established (20 EIU) (Fig. 5b). The anti-K88 IgG level was stable after the first immunisation and slowly decreased when sows received more than five immunisation doses.

**Discussion**

The use of DOC detergent for the isolation of pure fimbriae has been reported previously by Korhonen et al. [23], and shown to be useful in the purification of many different fimbriae [31–33]. Outer-membrane proteins, which are insoluble in DOC are precipitated after centrifugation. The results show that treatment with DOC is a rapid and adequate way to obtain purified K88 antigen suitable for coating ELISA plates.

ELISA methods have been used to diagnose many infectious diseases and their application to ETEC infections in calves and pigs for the detection of K99 and K88 antigens, respectively has been described previously [34–37]. However, knowledge of the appropriate conditions is necessary for optimisation of the assay and interpretation of results. Coating studies allowed the binding capacity of the K88 antigen on plate wells to be determined and the optimal concentration to be established. Clegg et al. [19] in an ELISA to detect IgG against the CFA/I antigen of ETEC from man established an optimal coating concentration of purified CFA/I antigen of 1 µg/ml. On the other hand, Payne et al. [38], without investigating the optimal coating antigen concentration in their ELISA assay for quantification of K88 antigen, described coating concentrations of purified K88 antigen as high as 100 µg/ml. In the present study, purified K88 antigen adsorbed to polystyrene plates at low concentrations, and antigen concentrations higher than the optimal decreased the OD values (Fig. 2). This effect has been reported previously, and probably results from the detachment of the antigen from the plate during the various washing steps [19, 39]. Antigen concentration used for coating plates may also affect the reproducibility of the results [40].

The optimal working dilution of each batch of enzyme

**Table 3. Mean anti-K88 IgG levels in vaccinated and unvaccinated sows**

<table>
<thead>
<tr>
<th>Vaccine (n)</th>
<th>Mean direct OD (SD)</th>
<th>Mean EIU (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAC1 (107)</td>
<td>1.407 (0.271)</td>
<td>83.5 (27.5)</td>
</tr>
<tr>
<td>VAC2 (16)</td>
<td>1.031 (0.290)</td>
<td>67.7 (24.9)</td>
</tr>
<tr>
<td>VAC3 (8)</td>
<td>1.776 (0.112)</td>
<td>115.0 (12.0)</td>
</tr>
<tr>
<td>VAC4 (3)</td>
<td>1.401 (0.269)</td>
<td>100.2 (21.6)</td>
</tr>
<tr>
<td>VAC5 (7)</td>
<td>1.253 (0.432)</td>
<td>85.6 (35.0)</td>
</tr>
<tr>
<td>Total VAC (141)</td>
<td>1.378 (0.314)</td>
<td>84.1 (28.3)</td>
</tr>
<tr>
<td>Unvaccinated (52)</td>
<td>0.450 (0.208)</td>
<td>3.9 (5.8)</td>
</tr>
</tbody>
</table>
conjugate must also be determined because of batch to batch variation [19]. OD obtained with negative rabbit sera did not increase when enzyme conjugate was used under optimal concentration, whereas OD values increased when enzyme conjugate less than the optimal concentration was used in the assay with negative sera from sows. These latter results may be due to the high prevalence of ETEC infection in pigs which possess a basal level of K88 antibodies in sera. Lower OD results obtained with positive porcine sera in comparison to OD results obtained with sera from hyperimmunised rabbits were representative of the different methods used for immunisation of the animals. Thus, sows were inoculated with a commercial vaccine which may also contain many other different antigens in addition to the K88 antigen, whereas rabbits were immunised repeatedly with a purified antigen.

The positive-negative cut-off points of the results in the ELISA to detect specific K88 antibodies in rabbit sera, and the reproducibility of the assay, were also studied. IgG level in a serum may be established as the endpoint titration calculated as the lowest dilution of serum giving an OD 2 or 3 SD higher than a negative control serum, when both sera are assayed in the same ELISA plate. Nevertheless, the choice of a single dilution of serum allows the analysis of a higher number of sera in each assay. The working dilution of serum samples was the one at which a maximal OD value for positive control serum and a minimal OD value for negative control serum were obtained, while maintaining the optimal conditions of the remaining parameters. Results from samples analysed in a single plate showed lower CV values than the results obtained when the same sample was analysed in different plates. Therefore, the IgG levels were expressed as EIU which reduced the CV. However, Martinez et al. [42] obtained higher CV when their results were expressed with reference to a standard sera. Nevertheless, the expression of results as EIU may be important primarily when comparing the results obtained by different methods. Bishop et al. [43] have expressed ELISA results as IgG levels by referring to a standard curve obtained with five dilutions of the standard serum tested in each assay plate. Furthermore, the results may also be affected by the antibody affinity [44]. Low affinity antibodies that show low OD and EIU results in ELISA may not confer protection to animals against infection. As the
Fig. 5. Distribution of anti-K88 IgG levels expressed as EIU (a) in 107 serum samples from sows vaccinated with VAC1. Vaccine dose indicates the number of times that sows received the vaccine. Bars show the serum values and SD for each dose group of serum samples. The EIU levels obtained when serum samples were assayed at a 1 in 200 dilution were related to the titre calculated as the lowest dilution of serum giving an EIU higher than the cut-off point established (20 EIU). b, Serum dilutions assayed: ○, 0; ■, 1 in 200; ▲, 1 in 400; ♦, 1 in 800; ★, 1 in 1600; ◼, 1 in 3200).

The final objective of the ELISA method is to determine the protection achieved by a particular vaccine in comparison with unvaccinated animals, the minor variation in results observed is not very critical, although methods with high reproducibility are desirable.

The K88-ELISA method has been shown to be highly specific and reproducible for the detection of K88-IgG antibodies. Preliminary results with porcine sera show that the method is useful for epidemiological studies on the potency of the vaccines against porcine colibacillosis, because higher levels of K88 IgG antibodies (expressed as EIU) were related to lower incidence of diarrhoea caused by ETEC. Previous studies showed that the incidence of diarrhoea caused by ETEC in farms ranged from around 35% of piglets [2,20], and showed that K88ac was the most important variant among K88 ETEC [21]. This study has shown that K88 IgG levels in vaccinated sows were related to a reduced incidence of diarrhoea caused by ETEC observed in piglets from vaccinated sows, although many other colonisation factor antigens in addition to K88 are involved in porcine diarrhoea in Spain [2]. A high variation in the levels of IgG against K88 antigen between sows vaccinated with the same vaccine was demonstrated. This variation may be attributed to variations in vaccine batches, to the
different intervals of time between the last inoculation and the collection of the serum samples, and to differing responses in individual animals. Thus, although the ELISA method was useful for demonstrating different levels of anti-K88 IgG in unvaccinated and vaccinated sows, and for the study of vaccine potency, further field studies under strictly controlled management conditions are necessary to clarify the influence of these additional parameters in the protection due to vaccines. It seems that the number of doses administered does not enhance the IgG levels against K88 antigen, although re-inoculation could be necessary to maintain protective IgG levels against K88 antigen.

This study was supported by grants from the Xunta de Galicia (XUGA 26104B92, 26104B94) from DGICYT (Plan PETRI PTR 91-0050 y CICYT PM 142/90). F.V. and J.I.G. acknowledge Xunta de Galicia and Ministerio de Educación y Ciencia for their research grants, respectively. S.V. acknowledges the Instituto de Cooperación Iberoamericana to stay in Spain during this study. We thank I. Ferreiro, M.K. López, M.J. Ares and C. Fernández for skilful technical assistance. Collaboration maintained with Zeltia-Veterinaria S.A. laboratories for field studies on porcine immunisation is gratefully acknowledged.

References

30. de Graaf PK, Roorda I. Production, purification, and characterization of the fimbrial adhesive antigen F41 isolated from the calf enteropathogenic Escherichia coli strain B41M. Infect Immun 1982; 36: 751-758.