

## DETECTION OF SPECIFIC ANTIBODIES AGAINST STRAINS OF ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) IN THE RABBIT BY AN ELISA USING 94-KILODALTON PROTEINS.

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**ABSTRACT :** A protein band of 94 kilodalton specific for EPEC strains, was detected and partially purified by elution electrophoresis. The protein band was used in an ELISA at 1 µg per well. Sera were taken every fortnight after experimental infections with six enteropathogenic *Escherichia coli* (EPEC) strains belonging to the pathotypes 1+/O109, 2+/O128, 2+/O132, 3-/O15, 4+/O26 and 8+/O103, and with three non pathogenic *E. coli* strains.

Furthermore, sera were collected from slaughter rabbits of commercial rabbitries with known EPEC status. The two groups of sera were tested at a dilution of 1:625 with the ELISA based on the 94-kilodalton proteins. The serological results were compared with the EPEC status of fecal samples of the corresponding animals. The test offers a specificity of 73 % and a sensitivity of 96 % ( $P < 0,05$ ).

**RESUME :** Détection des anticorps spécifiques des *Escherichia coli* enteropathogènes (EPEC) chez le lapin par un test ELISA utilisant une bande de protéines de 94 kilodaltons.

Une bande de protéines de 94 kilodaltons spécifique des souches EPEC, a été détectée et purifiée partiellement par élution. Elle a été utilisée dans un test ELISA à raison de 1 µg de protéine par puits. Lors d'infections expérimentales, d'une part avec six souches d'*Escherichia coli* entéropathogènes correspondant aux pathotypes 1+/O109, 2+/O128,

2+/O132, 3-/O15, 4+/O26 et 8+/O103, et d'autre part avec trois souches d'*E. coli* non pathogènes, des sérums ont été récoltés toutes les deux semaines. Des sérums de lapins provenant d'élevages commerciaux, au statut EPEC dûment identifié, ont été collectés lors de l'abattage. Les deux groupes de sérums ont été testés à une dilution de 1:625 par ELISA à base des protéines de 94 kilodaltons. Les résultats sérologiques ont été comparés avec le statut EPEC fécal d'animaux homologues. Le test donne une spécificité de 73 % et une sensibilité de 96 % ( $P < 0,05$ ).

### INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) cause important economic losses through digestive disorders in the rabbit. They can be divided into three groups. A first group is formed by strains belonging to bio/serotype 1+/O109, highly pathogenic for young rabbits before weaning. A second group consists of strains with variable pathogenicity (bio/serotypes 2+/O128 and 2+/O132) for rabbits before and after weaning, and a third group contains highly pathogenic strains affecting mostly weaned rabbits (bio/serotypes 3-/O15, 4+/O26 and 8+/O103). Colibacillosis causes losses through mortality, growth delay and poor feed conversion. At this moment it is extremely difficult to eradicate this bacterium from a rabbitry. There is no vaccine available which is both efficient and safe. Antibiotic resistance is increasing and sooner or later the breeder is forced to empty his rabbitry and repopulate it with new animals. There is no guarantee that the newly purchased rabbits are not carriers of EPEC strains, except if a bacteriological test is performed which is time consuming and expensive. A serological test which is not influenced by the use of antibiotics would in a relatively cheap way permit a swift detection of contact with EPEC strains.

### MATERIAL AND METHODS

#### Strains.

Thirty-six EPEC strains were isolated from farms suffering from colibacillosis, while 14 *E. coli* strains

were isolated from healthy farms (no isolation of EPEC strains in the previous 12 months). The choice of these 50 strains was based on previously published results (PEETERS *et al.*, 1988).

The isolation, the biotyping and the serotyping were performed as described by PEETERS *et al.* (1988). The strains were stored by lyophilisation. Thirty-nine strains originated from the VAR collection and 11 strains were provided by Prof. A. Milon of the *Ecole Nationale Vétérinaire* of Toulouse (France). The strains were cultured according to the method of PEETERS *et al.* (1988). They were used in experimental infections of Medium Disease Level (MDL) rabbits. During this experiment the virulence of the strains was analyzed by clinical observation, histopathology, bacteriology, feed intake and weight gain (CHOW *et al.*, 1994). Based on these results, six pathogenic strains, belonging to six different bio/serotypes, and three non pathogenic strains were selected as reference strains and used in later experiments.

#### Animals.

Four-week-old "Cunistar MDL" rabbits were purchased from the N.V. Verlabreed (Nevele, Belgium). Their feed contained no coccidiostatics nor antibiotics. Water was put at their disposition in individual bottles. Before the experimental infection was carried out, the animals were checked for the presence of EPEC, *Clostridium spiroforme* and *Eimeria* as described by PEETERS *et al.* (1986). A check for rotavirus was carried out according to the procedure of VANOPDENBOSCH (1980).

**Table 1 : Choice of number of CFU/ml used in the experimental infections with different EPEC strains**

Strain	Biotype/ serotype	Dilution	Number of CFU	Mortality %	Dilution chosen for infection experiment	Corresponding number of CFU				
82/123	1+/O109	1/10	$2.7 \times 10^{11}$	0	1/10	$2.7 \times 10^{11}$				
		1/100	$2.7 \times 10^{10}$	0						
		1/1000	$2.7 \times 10^9$	25						
82/183	2+/O128	1/10	$2.8 \times 10^8$	0	1/10 <sup>2</sup>	$2.8 \times 10^7$				
		1/100	$2.8 \times 10^7$	25						
		1/1000	$2.8 \times 10^6$	0						
82/90	2+/O132	1/10	$3.9 \times 10^9$	75	1/10 <sup>3</sup>	$3.9 \times 10^7$				
		1/100	$3.9 \times 10^8$	50						
		1/1000	$3.9 \times 10^7$	50						
83/39	3-/O15	<i>1<sup>st</sup> series</i>			1/10 <sup>3</sup>	$2.5 \times 10^9$				
		1/10	$2.5 \times 10^{11}$	100						
		1/100	$2.5 \times 10^{10}$	100						
		1/1000	$2.5 \times 10^9$	50						
		<i>2<sup>nd</sup> series</i>								
		1/100	$4.2 \times 10^7$	not tested						
		1/10.000	$4.2 \times 10^6$	25						
		1/1.000.000	$4.2 \times 10^5$	0						
		452.11b	4+/O26	1/10			$2.0 \times 10^{10}$	50	1/10 <sup>3</sup>	$2.0 \times 10^8$
				1/100			$2.0 \times 10^9$	50		
1/1000	$2.0 \times 10^8$			50						
85/91.2	8+/O103	<i>1<sup>st</sup> series</i>			1/10 <sup>6</sup>	$1.6 \times 10^5$				
		1/10	$7.3 \times 10^8$	50						
		1/100	$7.3 \times 10^7$	50						
		1/1000	$7.3 \times 10^6$	50						
		<i>2<sup>nd</sup> series</i>								
		1/100	$1.6 \times 10^9$	not tested						
		1/10.000	$1.6 \times 10^7$	50						
		1/1.000.000	$1.6 \times 10^5$	0						

**Experimental infection.**

The pathogenicity of the strains has been described by PEETERS *et al.* (1988), POHL *et al.* (1993) and CHOW *et al.* (1994). Each of the six EPEC reference strains belonged to a bio/serotype important in Belgium : the bio/serotypes 1+/O109, 2+/O128, 2+/O132, 3-/O15, 4+/O26 and 8+/O103. The three non pathogenic strains belonged to the bio/serotypes 2+/O103, 3+ and 18+.

For the experiment, eight rabbits were infected per strain. For every group of infected animals, there were four non-infected control animals, which were kept in a separate room.

The number of Colony Forming Units (CFU) per ml of inoculum is shown in table 1. To determine the size of the inoculum that had to be ingested per rabbit, an experimental infection was performed beforehand for the six reference EPEC strains, infecting three groups of four animals per strain with ten-fold dilutions of a

culture of six hours. Dilutions of 1:10, 1:100 and 1:1000 were used. For the 3-/O15 and 8+/O103 strains, a second series of dilutions (1:100, 1:10.000 and 1:1.000.000) was tested because of the high mortality even at a dilution of 1:1000 (see table 1). Counts of the bacterial suspensions were done using Coliform Count Plates (Petrifilm, 3M). For 17 days the feed intake and weight gain were registered. The animals were screened for fecal *E. coli* excretion on day seven and day 15. The strains found were biotyped and serotyped. The proportion of mortality per dilution and numbers of CFU chosen for the final experimental infections are shown in table 1.

Starting on the day of infection, fecal material was collected once a week. The evening before collection the trays underneath the cages were covered with a clean sheet of paper, and 24 hours later a sample of the fecal material was taken. The fecal material was homogenised

**Table 2 : Numbers of animals surviving and excreting EPEC after experimental infection, and results of the ELISA for the surviving animals**

Strains	Animals surviving >28 d.		Animals excreting EPEC		Positivity of the ELISA in the surviving animals	
	I	NI	I	NI	I	NI
<b>EPEC</b>						
1+/O109	6/8	1/4	6/6	0/1	6/6	0/1
2+/O128	7/8	3/4	7/7	0/3	7/7	0/3
2+/O132	3/8	3/4	3/3	0/3	3/3	1/3
3-/O15	5/8	3/4	5/5	0/3	5/5	0/3
4+/O26	4/8	3/4	4/4	0/3	4/4	0/3
8+/O103	5/8	4/4	0/5	0/4	0/5	2/4
TOTAL	30/48	17/24	25/30	0/17	25/30	3/17
<b>non path.</b>						
2+/O103	5/8	3/4	0/5	0/3	2/5	1/3
3+	8/8	2/4	1/8	0/2	4/8	0/2
18+	8/8	1/4	0/8	0/1	3/8	0/1
TOTAL	21/24	6/12	1/21	0/6	9/21	1/6

I : infected animals, NI : non-infected animals.

with Phosphate Buffered Saline (PBS) in a dilution of 1:5 (w/v) and pelleted. The supernatant was stocked at -20°C until use. Blood was taken every fortnight, and the sera were stocked at -20°C. During the first week, rectal swabs were taken three times, to detect excretion of the strain used for infection. If this strain was not detected, the animals were infected a second time nine days after the first infection. Eight weeks after the experiment had begun, the surviving animals were euthanised.

#### SDS-PAGE and immunoblot.

The 50 *E. coli* strains were cultured and precipitated by centrifugation (17,000 g), washed and resuspended in Tris buffer (10 mM, pH 6.8). The bacterial suspension (0.3 mg of protein per ml) was prepared and analyzed by SDS-PAGE and western blotting as described by PEETERS *et al.* (1992). For the immunostaining we used the supernatant of the fecal material (IgA) and sera (IgG) taken from 12-week-old rabbits which had been experimentally infected with one of the reference strains at 4 weeks of age, or from the control animals. The fecal supernatant was used without further dilution, while sera were used at a dilution of 1:100. Before staining, excess binding sites on the blotting membranes were blocked by incubation for 15 minutes with gentle rocking in wash buffer (50 mM Tris HCl, 140 mM NaCl, 5 mM EDTA [pH 7.4]) containing 0,25 % gelatine and 0,1 % Bovine Serum Albumin. After rinsing three times with wash buffer the membranes were probed for 18 hours (4°C) with IgA present in the fecal supernatant or with IgG present in

the serum dilution, and rinsed again. For detection of the IgA the membranes were treated with goat-anti-rabbit IgA (ICN) followed by biotinylated rabbit-anti-goat IgG (Amersham). For detection of serum IgG the membranes were treated with biotinylated goat-anti-rabbit IgG (Amersham). Hereafter both membranes were treated in the same way. They were incubated with alkaline phosphatase-conjugated streptavidine (Boehringer, Mannheim, Germany). All steps were performed at an ambient temperature for one hour with gentle rocking, and between each step the membranes were rinsed three times with wash buffer. After the last step the membranes were washed with a buffer containing 100 mM Tris HCl (pH 9,5), 100 mM NaCl and 50 mM MgCl<sub>2</sub>, after which they were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Immunoselect ; Life Technologies). The reaction was stopped with a buffer containing 10 mM Tris HCl (pH 8,0) and 1 mM EDTA. The molecular weights of the antigens were estimated by comparing them with biotinylated molecular weight standards (Bio-Rad) which had been transferred to the same nitrocellulose membranes. For this purpose the membranes were scanned with a Model GS-670 Imaging Densitometer (Bio-Rad), and analyzed first with the software "Molecular Analyst™/PC 1.0" (Bio-Rad), followed by the software "GelCompar 3.0" (Applied Maths, Kortrijk, Belgium).

#### Purification by Prep Cell.

The 94-kilodalton (kDa) proteins specific for EPEC that were detected after SDS-PAGE and immunoblot

were partially purified by continuous elution electrophoresis using the Model 491 PrepCell of Bio-Rad (37 mm internal diameter) according to the firm's recommendations. The running gel was cast at a concentration of 7% (50 ml), the stacking gel at a concentration of 4% (12 ml). A culture of the reference strain 82/123 (bio/serotype 1+/O109) was precipitated by centrifugation (17,000 g), washed and resuspended in Tris buffer, then 25 % (v/v) of SDS buffer (4% SDS, 1.25 M Tris {pH 6.8}, 20% glycerol, 10% 2-mercaptoethanol, 0.0004% bromofenolblue) was added. The mixture was heated to 100°C for five minutes, cooled on ice, and centrifuged at 2,000 g. Subsequently 10 % (v/v) of a saturated solution of trichloroacetic acid was added to the supernatant to precipitate the proteins present in the supernatant. The resulting pellet was washed three times with ethanol absolute, then dissolved in a mixture of electrophoresis buffer (Tris 0,025 M ; glycine 0,192M ; SDS 0,1%) and SDS-buffer (4:1). The solution was heated to 100°C for five minutes, cooled on ice and centrifuged at 2,000 g. The supernatant was charged onto the Prep Cell column. The elution buffer and the electrophoresis buffer were identical. The resulting fractions were tested by SDS-PAGE and immunoblot for the presence of the 94-kDa protein band and then concentrated with centricons 30 (Amicon). A protein analysis was then performed on the concentrated fractions.

#### Production of monospecific antiserum.

A 4-week-old MDL-rabbit was immunized with three series of subcutaneous and intramuscular injections of respectively 220 µg, 130 µg and 80 µg of the partially purified 94-kDa band emulsified in the Ribi Adjuvant System (Sigma), at three-week intervals. The animal was bled three weeks after the third immunization. Every ml of serum was absorbed with an equal volume of a culture of the non pathogenic strain with bio/serotype 2+/O103, which does not show an immunogenic 94-kDa protein band on immunoblot. The mixture was shaken constantly during six hours at 4°C, then centrifuged at 9000 g for 10 minutes. The supernatant was recovered, and subsequently the absorption protocol was repeated.

#### Cross reactions.

The absorbed serum was tested to determine if there were any cross reactions between the antigens present in the 94-kDa protein band and those of *Salmonella typhimurium*, *Salmonella enteritidis*, *Clostridium spiriforme*, *Citroba ter freundii*, *Enteroba ter loa ea*, *Serratia mar es ens*, *Klebsiella pneumoniae*, *Eimeria spp.*, *Cryptosporidium parvum*, and rotavirus. The bacteria were tested by SDS-PAGE and immunoblot, the rotavirus by immunodiffusion, and

the *Eimeria* and *Cryptosporidia* by an ELISA. The *Eimeria* and *Cryptosporidia* oocysts were purified from respectively fecal material of rabbits and calves. The oocysts were prepared and used to coat microtiter plates as described by DE GRAAF and PEETERS (1997). A direct ELISA was then performed using 100 µl per well of the monospecific serum in dilutions of 1: 25 to 1:819,000, in two-fold dilution steps with dilution buffer (PBS pH 7,2 ; 0,5 % bovine serum albumine ; 0,05 % tween 20), followed by 100 µl per well of goat-anti-rabbit IgG-peroxidase conjugate diluted to 1:2,000, also with dilution buffer. For both steps, the microtiter plates were incubated at 37°C during one hour. After each of the steps mentioned above, the wells were washed four times with Phosphate Buffered Saline (PBS) containing 0,05 % Tween-20 (PBS-Tween). The substrates used were 3,3',5,5'-tetramethylbenzidine or TMB (Kirkegaard & Perry Laboratories Inc.) and H<sub>2</sub>O<sub>2</sub>. These were mixed immediately before use with PBS in the following respective proportions : 7:10:3. Per well, 100 µl of substrate was added. The microtiter plates were incubated in the dark for 10 minutes at 25°C. Subsequently the reaction was stopped by adding 100 µl 1 M *o*-phosphoric acid per well. OD values were read at 450 nm (counter filter 690 nm).

A positive control serum of an animal infected with and excretor of the reference strain 1+/O109, and a negative control serum of a 4-week-old non-infected MDL-rabbit were also tested on each ELISA. The *Cryptosporidium* antigen was supplementary tested by SDS-PAGE and immunoblot (serum dilution : 1:10,000).

#### ELISA.

The microtiter plates (Nunc, Maxisorp) were coated overnight (4°C) with 10 µg ml<sup>-1</sup> of partially purified 94-kDa protein in carbonate buffer (pH 9.6), at a rate of 100 µl per well. Before use, the microtiter plates were incubated with 250 µl coating buffer (carbonate buffer, pH 9,6 ; 0,5 % bovine serum albumine) at 37°C for one hour. After both steps, the wells were washed four times with PBS-Tween. A direct ELISA was then performed using the rabbit sera diluted to 1:625, following the protocol as described above. The sera obtained after the experimental infections and the sera collected during the slaughter of commercial rabbits were double-tested. Generally 40 sera were tested per commercial rabbitry.

## RESULTS

#### Animals.

None of the MDL-rabbits excreted EPEC strains or rotavirus at the beginning of the experiment. In 2 % of the animals some *Eimeria spp.* were found. *Clostridium spiriforme* was detected only sporadically and in small

numbers. Only one animal (<1 %) showed lesions of enterotoxemia.

### Experimental infection.

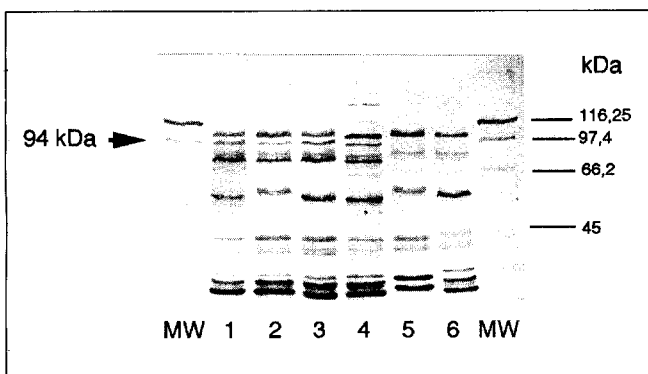
The proportions of infected and non-infected animals, surviving for at least 28 days after infection, and the proportions of animals that became excretors of EPEC, are presented in table 2.

### SDS-PAGE and immunoblot.

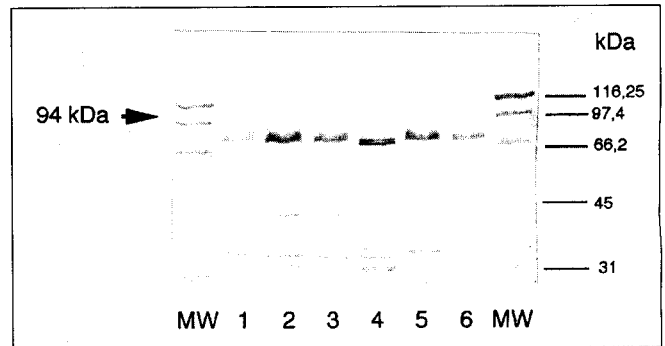
The IgG of the serum and the IgA of the fecal material collected from animals infected with the bio/serotypes 1+/O109, 2+/O128, 2+/O132, 3-/O15 and 4+/O26 react with a protein band with a molecular weight of about 94-kDa, which is present in all EPEC strains tested but cannot be found in the non pathogenic *E. coli* strains tested in this experiment (see figure 1). The serum and fecal antibodies of animals infected with non pathogenic *E. coli*, with the reference strain for bio/serotype 8+/O103, and of non-infected animals did not recognize this 94-kDa protein band (see figure 2). However, with the antibodies of the animals infected with EPEC-strains other than the 8+/O103 bio/serotype, the 94-kDa protein band was proved to be present in the 8+/O103 EPEC strains (see figure 1).

### Purification of the 94-kDa protein band.

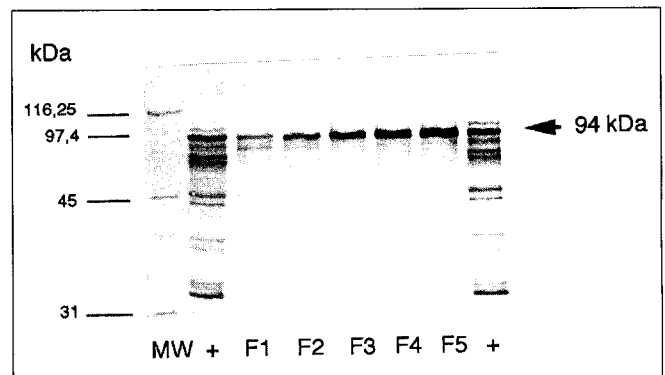
A partial purification of the 94-kDa protein band was obtained using the Prep Cell. Weak contaminating protein bands, with a molecular weight close to 94-kDa, remained present (see figure 3).



**Figure 1 :** SDS-PAGE followed by immunoblot of a molecular weight standard (MW), EPEC strains 82/123 (1), 83/39 (2), 452.11b (3) and 85.91.2 (4), and commensal strains Deprez (5) and Vande Velde (6). After immunostaining with 1+/O109-antiserum the 94-kDa protein band is clearly detected in the EPEC strains, but absent in the commensal *E. coli* strains.



**Figure 2 :** SDS-PAGE followed by immunoblot of a molecular weight standard (MW), EPEC strains 82/123 (1), 83/39 (2), 452.11b (3) and 85.91.2 (4), and commensal strains Deprez (5) and Vande Velde (6). After immunostaining with Deprez-antiserum no 94-kDa protein band is detected, neither in the EPEC strains nor the commensal *E. coli* strains.



**Figure 3 :** SDS-PAGE followed by immunoblot of a molecular weight standard (MW), the crude protein extract, which was used as a positive control (+), and fractions collected after continuous elution electrophoresis (F1 - F5) using a PrepCell (Bio-Rad). After immunostaining with 1+/O109-antiserum the 94-kDa band and weak contaminating bands, with a molecular weight close to 94 kDa, are detected.

### Cross reactions.

No antigen tested was recognized by the monospecific antiserum, except for the native *Cryptosporidium* antigen in the ELISA, which showed a positive reaction up to a dilution of 1:12,800. The denaturated *Cryptosporidium* antigen tested by SDS-PAGE and immunoblot with the same monospecific antiserum at a dilution of 1:10,000, gave a negative reaction.

ELISA with sera obtained after experimental infection.

Based on the average of the Optical Densities (OD) and the Standard Deviation (SD) obtained with the ELISA's negative control sera, a cut-off value has been

**Table 3 : Results of the ELISA performed on sera obtained from rabbits of commercial rabbitries**

Pathotype isolated in the rabbitry	N	Proportion of the sera with OD > CO	% of sera with OD > CO	Distribution of the proportions
1+/O109	3	55/120	46 %	7/40 - 27/40
2+/O132	5	49/178	28 %	3/40 - 20/40
3-/O15	7	99/290	34 %	2/40 - 26/40
8+/O103	2	7/48	15 %	4/40 - 3/8
2+/O132	2	30/80	38 %	11/40 - 19/40
3-/O15				
1+/O109	1	10/40	25 %	10/40
2+/O132				
3-/O15				
1+/O109	1	10/40	25 %	10/40
2+/O128				
2+/O132				
3-/O15				
NONE, AB +	1	10/40	25 %	10/40
NONE, AB +	1	4/50	8 %	4/50
NONE, AB +	1	1/40	2 %	1/40
NONE, AB -	1	2/50	4 %	2/50
NONE, AB -	1	2/40	8 %	2/40
NONE, AB -	1	11/48	23 %	11/48

N : number of rabbitries ; OD : optical density ; CO : cut-off level ; AB + : antibiotics positive ; AB - : antibiotics negative

calculated in the following way : average + 1.96 x SD. Statistically, this cut-off value is only exceeded by a maximum of 5 % of the negative samples. Of the animals used in the experimental infections and the respective negative control animals, 74 have survived sufficiently long to permit a complete bacteriological and serological evaluation. The results are presented in table 2.

#### ELISA with sera obtained from animals in commercial rabbitries.

As shown by table 3, the ELISA based on the 94-kDa protein, gives similar numbers of positive results, no matter what pathogenic bio/serotype or combination of bio/serotypes is found in the rabbitry. The use of antibiotics does not seem to have an influence on the serological results (data not shown). Six rabbitries were bacteriologically negative for EPEC. Of these six, three had been using antibiotics. Two of these three show positive results for 4/50 and 10/40 animals. In the third rabbitry, only one serum gives a weak positive result. In the three remaining rabbitries, which hadn't been using antibiotics, two had sera reacting weakly positive (2/50 and 2/40). The third rabbitry had a proportion of 11/48 positive sera.

#### Sensitivity and specificity.

For these calculations (ALTMAN, 1991), the data obtained during the experimental infections were used. The sensitivity of the ELISA has been calculated according to the formula (true positive x 100 %) / (true positive + false negative), giving a result of 96 % (25/26). The specificity of the ELISA was calculated as (true negative x 100 %) / (true negative + false positive), resulting in 73 % (35/48).

#### DISCUSSION

The choice of the six EPEC and three non pathogenic *E. coli* reference strains which were used for the experimental infections was based on the data available from previous experiments (PEETERS *et al.*, 1988 ; POHL *et al.*, 1993), and the results of more recent infection experiments (CHOW *et al.*, 1994). An important conclusion from the latter experiments was that strains belonging to the same bio/serotype showed clear differences in pathogenicity. Selection criteria for the EPEC strains were presence of clinical disease without excessive mortality and histological evidence of adhesion in the gut mucosa. Each of the six selected EPEC strains was representative for its respective bio/serotype. The selected non pathogenic *E. coli* strains showed low to no virulence and no adhesion to the gut mucosa. Their selection was also partly based on the presence of a biotype or serotype similar to a pathogenic bio/serotype (strain C127 : bio/serotype 2+/O103).

During the infection experiments with these reference strains, a number of the control animals succumbed to iota-enterotoxemia (*Clostridium spiroforme* infection). This was due to stress as a result of a malfunctioning automatic lighting system.

SDS-PAGE and immunoblot revealed an immunogenic 94-kDa protein band specific for the EPEC strains tested. The fact that it was not recognised by the antibodies of animals infected with the reference strain for the bio/serotype 8+/O103, may be due to a weak antigenic stimulation as a result of a low infection dose.

When observing the 94-kDa protein band in the different reference strains, there is no noticeable difference between the bio/serotypes. Initial purification experiments with strains 82/123 (1+/O109) and 85/91.2 (8+/O103) yielded better results with strain 82/123, therefore it was decided to continue the purification tests with this strain.

In a recent study (data not shown) the partially purified 94-kDa protein band was submitted to two-dimensional electrophoresis, which revealed that it was composed of a large number of proteins with varying pI. The identity of the various protein spots should be proved by further experiments. POHL *et al.* (1993) have described a rabbit *eae* gene, which is EPEC-specific. This gene encodes intimin, an outer membrane protein (OMP) of 94 - 97-kDa (JERSE and KAPER, 1991). The *eae* gene is a part of the Locus for Enterocyte Effacement (LEE) (MCDANIEL *et al.*, 1995), which is responsible for the attachment and effacement (a/e) lesions of enterocytes caused by EPEC. The a/e lesions are initialised by a loose attachment, which is followed by an intimate attachment mediated by intimin. This intimate attachment is accompanied by the secretion of a number of proteins which induce a rearrangement of the enterocyte's cytoskeleton and a disruption of the microvilli, which is described as "effacement". It would be logical to suppose that the 94-kDa protein band, which was found specific for EPEC strains, contains a protein homologous to the EPEC-specific intimin.

As for cross reactions with the 94-kDa protein band, there were none with the antigens tested except for one, which was observed with the native antigen of *Cryptosporidium*. This reaction could not be confirmed by immunoblot : the denaturated antigen was not recognized by the absorbed serum.

The false positive results of the ELISA, obtained from animals experimentally infected with non pathogenic strains and from non-infected control animals, may be explained by the presence of contaminating proteins in the purified fraction. The only false negative result was found with a strain belonging to bio/serotype 2+/O132, whose pathogenicity is quite variable. The sera of the commercial rabbitries, where the presence of EPEC strains was established, generally reacted in a very strong way, even if antibiotics had been used, or if bacteriologically only a few animals had been found positive for EPEC. One of the three rabbitries that were bacteriologically negative and had been using antibiotics, produced only one positive serum, and its OD was low. In this rabbitry the problems caused by EPEC had started only a couple of weeks before the blood sampling. The experimental infections (data not shown) proved that the production of a detectable level of antibodies after EPEC infection takes two to four weeks. In this case the blood may have been sampled too soon after the infection to permit positive serological results. This

shows that it is important to repeat the ELISA in a rabbitry frequently, e.g. every three months. Among the EPEC negative rabbitries that had not been using antibiotics, one gave a proportion of positive sera of 11/48 (23 %). The bacteriological results in this case may have been falsely negative. This is possible because after infection, an animal excretes the pathogenic strain continuously only during the first few weeks. Afterward the excretion becomes intermittent. The OD values of the sera of the two other rabbitries were, if positive, close to the cut-off level. When the cut-off level is increased by 50 %, the percentages of positive sera in the EPEC positive rabbitries go from an average of 30 % to an average of 23 %, and the rabbitry suspected of being falsely bacteriologically negative for EPEC, still shows a proportion of positive sera of 6/48 (12 %). At the same time, in the two remaining rabbitries, which were bacteriologically EPEC negative and antibiotics negative, these percentages go from an average of 4 % to 0 %.

We may conclude that the results of the ELISA based on the 94-kDa proteins are promising. A further purification of these proteins and, if present, intimin, will be necessary to improve the test. This improvement may permit an even clearer distinction between EPEC positive and EPEC negative rabbitries.

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