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## EXPERIENCES WITH THE ELISA FOR DETECTION OF THE *E. COLI* K99 ANTIGEN IN CALF FAECES

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### Abstract

This paper summarizes experiences with the use of an enzyme-linked immunosorbent assay (ELISA) for the detection of K99<sup>+</sup> *E. coli* in calf faeces. ELISA results were compared with those of conventional bacteriological examination (BE) using a total of 1668 faecal samples of calves in the field, collected during aetiological and epidemiological studies. In addition, faecal samples obtained from four experimentally inoculated calves were examined with ELISA, BE and quantitative BE i.e. determination of the number of viable K99<sup>+</sup> ETEC organisms per gram of faeces. The ELISA proved to be very useful, though slightly less sensitive than BE, when dealing with large numbers of faecal samples of calves of less than 6 days of age. Clinical ETEC-induced diarrhoea cases appeared to be diagnosed particularly reliably. In calves of over five days of age, however, positive BE-results were usually not confirmed by ELISA, most likely due to the presence of specific copro-antibodies. This last conclusion was suggested by the observation that in experimentally inoculated calves, ELISA results became negative abruptly after day 6 post-infection. The quantitative BE showed no significant decrease of the number of ETEC organisms at that time. In some faecal samples after day 6 post-infection an excess of K99 antibodies was demonstrated. It is concluded that ELISA is of no value in epidemiological studies on the spread and persistence of K99<sup>+</sup> ETEC organisms in older calves and cows. However, ELISA is a very reliable diagnostic tool for use in large-scale field surveys on the aetiology of neonatal calf diarrhoea.

During the last decade, evidence has accumulated that enterotoxin producing strains of *E. coli* (ETEC) of bovine origin usually carry the K99 antigen. The reverse is nearly always true (Moon *et al.*, 1976; Contrepois *et al.*, 1979; Guinée *et al.*, 1979; Larivière *et al.*, 1979; WHO, 1980). Consequently, the diagnosis of ETEC induced diarrhoea in calves may be based on the detection of the K99 antigen in faecal samples or intestinal contents. As a rule this is done by a combination of bacteriological cultural methods and slide agglutination tests.

When dealing with large numbers of samples

this procedure is rather laborious. In 1979, an enzyme-linked immunosorbent assay (ELISA) for the detection of the K99 antigen was developed at our institute (Ellens *et al.*, 1979). This assay together with ELISA's for the detection of enteric viruses, has facilitated large-scale field studies on neonatal diarrhoea of which preliminary results have been published elsewhere (Moerman *et al.*, 1982). In the course of these studies, data were compiled on the sensitivity and the usefulness of the K99-ELISA as compared to conventional bacteriological methods.

The present report deals with the experiences of

the use of the K99-ELISA during these field studies together with the experiences obtained in experimentally inoculated calves.

## Materials and Methods

### Experimental infections

The experimental infections in colostrum-deprived Dutch-Friesian calves which were delivered by caesarean section, were described previously (Zijderfeld *et al.*, 1982). The ETEC strain used for this purpose was of the OK type O9: K35: K99 (ST<sup>+</sup>) and was isolated from a field case. The chloramphenicol-resistant strain was also made resistant to nalidixic acid.

Doses of  $70$ ,  $7 \times 10^3$ ,  $7 \times 10^5$ ,  $7 \times 10^7$  and  $7 \times 10^9$  ETEC organisms per calf were employed. Faecal samples for bacteriological and virological examination were collected twice a day for the first three days, then once a day and from the 12th day onwards every 2-3 days.

In this paper only the results of the faeces examination of the four surviving calves are presented. Three of these calves were orally inoculated with  $70$  ETEC organisms and one with  $7 \times 10^7$  ETEC organisms within 3-8 h after birth.

### Faecal samples of calves in the field

One thousand-six hundred-eighty-eight samples from about 900 calves, collected during a large field survey on neonatal calf diarrhoea from April 1980 to May 1982 (Moerman *et al.*, 1982) were examined with ELISA and with conventional bacteriological methods. Most of these samples came from 0-3 week old calves, but occasionally samples were obtained from older animals (up to 66 days). The calves originated from more than 30 herds. Often two or more samples from the same calf, taken at different days were examined.

Faeces consistency was noted as normal, semi-liquid or liquid. Part of the faecal material was homogenized in four volumes of PBS containing 0.05% Tween 80. This suspension was tested by ELISA at once or after storage at  $-20^\circ\text{C}$ .

### Laboratory methods

Conventional bacteriological examination (BE) of faecal samples for the presence of K99<sup>+</sup>*E. coli* was performed as described previously (Zijderfeld, 1981) using modified brilliant green agar and Minca-Isovitalex agar.

Suspected colonies were examined by slide agglutination with a specific K99 antiserum, prepared as described by Guinée *et al.* (1976).

In addition to this faecal samples of experimental calves were plated out in tenfold dilutions on 5% sheep blood agar and modified brilliant green

agar plates with and without the addition of  $20\ \mu\text{g}$  nalidixic acid and chloramphenicol per ml of medium.

Subcultures of colonies from these plates were made on Minca-Isovitalex agar and checked for the K35 and K99 antigens by slide agglutination.

The ELISA for the detection of the K99 antigen was the double antibody sandwich technique as described by Ellens *et al.* (1979) with minor modifications. Specific K99-antisera were prepared in goats and rabbits with purified K99 antigen, isolated by preparative electrophoresis from *E. coli* strain H416 (O101: K?: K99) according to the method of Guinée *et al.* (1976).

Specificity of the sera was confirmed by double immunodiffusion tests and immunoelectrophoresis with the use of ultrasonic extracts of different K99<sup>+</sup> and K99<sup>-</sup> *E. coli* strains, grown at 18 and  $37^\circ\text{C}$ .

The IgG fraction of the goat serum, prepared by ammonium sulphate precipitation and subsequent DEAE-cellulose chromatography was used at its optimal dilution for coating of polystyrene microtiter plates. The IgG fraction of rabbit antiserum was conjugated with horseradish peroxidase as described by Wilson and Nakane (1978).

Faecal suspensions were incubated in precoated microtiterplates for 16-18 h at  $4^\circ\text{C}$ .

Two hours after the addition of the substrate solution, plates were read with a Titertek<sup>®</sup> Multiskan at 450 nm.

Each sample which showed an absorbance value  $\geq 2/10$  of the absorbance of a standard K99 antigen preparation and which was blocked in the confirmation test, was scored positive.

Positive samples had absorbance values  $\geq 0.2 - 0.24$ .

For the detection of K99-specific antibody in faecal homogenates or in serum, the ELISA was performed as a blocking test (Ellens *et al.*, 1979), using a standard K99 preparation.

ELISA titers are expressed as the reciprocal of the sample dilution giving an absorbance value at 450 nm, equal to 50% of the absorbance value of the standard antigen preparation.

## Results

The lower detection level of ELISA was determined previously for 15 different K99<sup>+</sup> ETEC strains, grown *in vitro* in Minca-Isovitalex broth. This detection level proved to be strain-dependent and varied between  $5 \times 10^5$  and  $5 \times 10^7$  ETEC organisms per gram of medium. After previous ultrasonic treatment of the cultures the detection level was reduced to  $5 \times 10^4 - 1 \times 10^6$  organisms per gram.

### Experimental infections

The excretion patterns of three calves, each orally inoculated with 70 O9:K35:K99 ETEC organisms are shown in figure 1.

Two calves did not show clinical signs of ETEC infection; the other calf (calf number 1) had severe diarrhoea at day 2 and mild diarrhoea at day 3 after infection. Faecal samples, taken at the second or third day after inoculation reacted positively in the ELISA. During these days the numbers of ETEC organisms per gram of faeces usually exceeded  $10^5$ . The ELISA was not able to detect the K99 antigen in faecal samples obtained after day 5 or 6. At that time the numbers of ETEC organisms had decreased to less than  $10^5$  per gram. The ETEC strain was demonstrated from day 1 or 2 up to day 6 or 7 by conventional BE.

The calf that survived inoculation with the  $7 \times 10^7$  dose (fig. 2) excreted ETEC organisms at a high level for well over four weeks.

The animal showed severe diarrhoea up to day 6 after infection. With ELISA K99 antigen shedding by this calf could not be demonstrated after day 6. BE was found positive on every occasion for more than 20 days after infection. The numbers of ETEC organisms remained above  $10^5$  per gram of faeces up to 28 days after infection.

Specific K99 antibodies were detected in faecal extracts of this calf from day 20 to day 46 after infection.

### Faecal samples obtained from calves in the field

One thousand-six-hundred eighty-eight faecal samples of calves in the field were examined both by BE and ELISA (table 1). One thousand five hundred sixty four samples were negative and 48 positive by both methods, an agreement of 95 %.

In 73 samples K99<sup>+</sup> ETEC was only detected by BE, whereas in three samples only ELISA scored positive. One of these three samples showed no bacterial growth on the culture media, probably because of bad storage conditions. Of the total of 124 K99 positive samples, 59 were obtained from calves of less than 6 days of age. Of these 72.8 % was found to be positive by both methods, 23.7 % by BE only and 3.3 % by ELISA only.

Table 2 shows that of the 73 samples found positive by BE only, 8 (10.9 %) were obtained from calves with diarrhoea. One of these 8 samples came from a calf older than five days.

Of the 48 samples found positive by BE and by ELISA, 39 (81.3 %) were obtained from calves suffering from diarrhoea.

### Discussion

Experimental inoculation of three calves with

70 ETEC organisms per calf resulted in a mild clinical disease in one calf and subclinical infections in the others.

K99 was not detected in the faeces by ELISA in the first and later stages of the infection, when the number of ETEC organisms per gram was less than the detection level of approximately  $10^5$ . Some of the ELISA negative samples obtained during these stages were positive by BE, indicating that BE is more sensitive. In theory BE is positive if the K99<sup>+</sup> ETEC in the faeces are present in a sufficient quantity in proportion to the numbers of K99<sup>-</sup> *E. coli* organisms thus facilitating the selection of individual K99<sup>+</sup> colonies.

In our experience, K99<sup>+</sup> *E. coli* should constitute at least 1-2 % of the total coliform flora of the

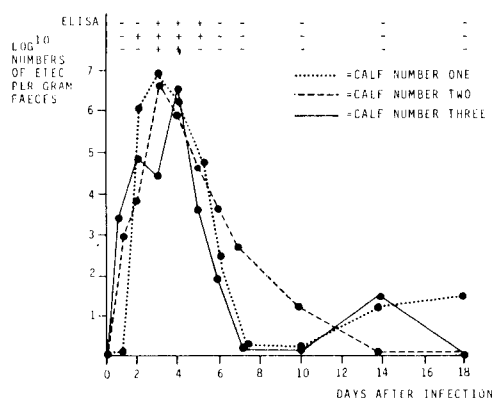


Fig. 1. — The excretion pattern of the challenge strain by three calves after an experimental infection with 70 ETEC O9:K35:K99 organisms/calf.

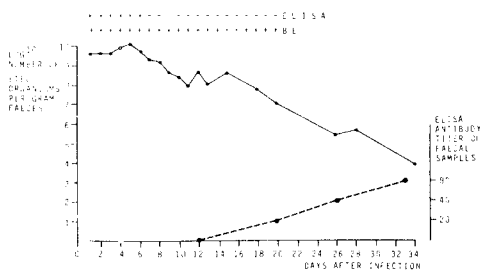


Fig. 2. — ETEC excretion by one calf, experimentally inoculated with  $7 \times 10^7$  O9:K35:K99 organisms and the detection of the K99 antigen or K99<sup>+</sup> ETEC by ELISA or conventional bacteriological examination in its faeces.

Table 1. — Comparison of the results of the ELISA and those of conventional bacteriological examination (BE) for detection of K99-positive *E. coli* in 1688 faecal samples of calves (0-66 days of age).

	ELISA	
	+	-
Bacteriological examination		
positive	48 (43) <sup>a</sup>	73 (14) <sup>a</sup>
negative	3 (2) <sup>a</sup>	1564

a: number of samples in this category, obtained from calves < 5 days of age.

sample and the numbers of K99<sup>+</sup> ETEC organisms per gram of faeces should amount to at least  $10^2$  —  $10^3$ .

The calf challenged with  $7 \times 10^7$  organisms showed severe clinical disease. From six days after infection, ELISA was not able to detect the K99 antigen in its faeces, whereas the numbers of ETEC organisms per gram were not reduced significantly (fig. 2). BE proved to be more sensitive.

Several possibilities may be put forward to explain this observation. Firstly actively formed coproantibodies against the K99 antigen may appear in the faeces after day 5 post-infection, cover the antigenic sites and thus prevent a positive result of an immunological test like ELISA. Coproantibodies however, were first detected on day 20, while a sample taken on day 12 did not react.

This unexpectedly late detection may be the result of an «insensitive» technique, rather than the absence of K99-specific antibodies. In this respect, it should be remembered that an antibody excess in comparison to the antigen in the faeces is necessary to allow its detection. A second explanation, which cannot be excluded, is the possibility that the K99 antigen is not fully expressed *in vivo* during the later stages of an infection.

Table 2. — Distribution of faecal samples, found positive for the K99 antigen by ELISA and/or BE, according to diarrhoea score.

		Total number of samples	Number with diarrhoea score <sup>a</sup>		
			--	+	++
BE <sup>+</sup>	ELISA <sup>+</sup>	48	9	14	25
BE <sup>+</sup>	ELISA <sup>-</sup>	73	65	4	4
BE <sup>-</sup>	ELISA <sup>+</sup>	3	1	2	—

a: —: normal faeces; +: semi-liquid faeces; ++: liquid faeces.

At first sight the ELISA for the detection of K99 positive ETEC in faeces of field calves of varying age appears considerably less sensitive than BE (table 1).

Most of the 73 BE<sup>+</sup>, ELISA<sup>-</sup> samples were obtained from non-diarrheic calves of more than five days of age. K99 positive ETEC infections usually occur in calves of less than six days of age (Moerman *et al.*, 1982). The different results of the two techniques in the older age category may be explained by the same mechanism responsible for the observations made in experimentally infected calves, as discussed above.

It was also striking that most of these 73 samples were obtained from three herds. This observation indicates that differences in the expression of the K99 antigen by the various strains occurring in the herds may contribute to the divergent results, i.e. BE<sup>+</sup>, ELISA<sup>-</sup>. The detection level of the ELISA for *in vitro* cultured K99<sup>+</sup> ETEC strains proved to be strain-dependent.

In calves of less than six days of age, the results of the K99-ELISA were closer to those of BE, especially when dealing with samples of diarrheic animals (tables 1, 2). Clinical ETEC infections in calves are confined to animals of this age-category.

A few samples (only three) were scored positive by ELISA and not by BE. In general, such a difference could be caused by samples in which the K99 negative *E. coli* flora predominated the K99 positive flora. In one of these samples bacteria were dead because of bad storage conditions before examination. Furthermore, absence of K99 expression *in vitro* or a false positive reaction in the ELISA may be involved.

Based on the results discussed above, we feel that the K99 ELISA is a reliable method to detect K99<sup>+</sup> ETEC in the faeces of calves if less than one week of age. Combined with the usual advantages of ELISA this makes the test particularly suitable for use in large-scale field surveys on the aetiology of neonatal calf diarrhoea. The usefulness of the assay is maximal in the case of longitudinal studies, i.e. surveys in which samples of the same animal, obtained at different days, are examined.

Our results also confirm previous observations (Zijderveld *et al.*, 1982) that ELISA is of no value in epidemiological studies in herds on the spread and persistence of K99<sup>+</sup> ETEC organisms in older calves and cows. To a large extent this is also true for conventional bacteriological examination.

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## Question

From Dr Larvor to Dr Van Zijderveld

In your slides you presented two experiments of infection by K99 *E. coli*. In the first the ELISA was able to detect levels of  $10^5$  or more bacteria K99<sup>+</sup> and in the second ELISA could only detect an amount of bacteria  $\geq 10^9$ . Could you comment on this?

## Answer

That's exactly the main point I tried to explain to you. The calves shown in the first figure, infected, with the lower dose, in general, had a subclinical infection. The detection level of the ELISA was approximately  $10^5$  org/gram.

The calf which survived the higher dose was shown in fig. 2. In the first stage of the infection, the numbers of K99<sup>+</sup> ETEC exceeded the detection level of  $10^5$  very rapidly. After day 6 ELISA was negative, despite the fact that the numbers of ETEC organisms were much higher than  $10^5$ . Probably there is an equilibrium between day 6 and approximately day 17-20 with respect to the amount of antigen and coproantibody. This causes a negative result with respect to antigen detection by any immunological technique ELISA, after day 17-20 there is an excess of antibody compared to the antigen resulting in its detection in the serological variant of the ELISA.

## Question

From Dr Scherrer to Dr Zijderveld

1. Even in the absence of coproantibodies, does your talk mean that you expected your ELISA to be as sensitive or even more sensitive than a biological test?

2. I also understood that you love a lot of subclinical infection in your studies. Have you any idea of the frequency of such an infection?

## Answer

1. In the absence of coproantibodies, the conventional bacteriological examination also proved to be more (slightly) sensitive than ELISA.

2. Of course there are many subclinical infections in older animals but we also observed subclinical ETEC infections in the very young calf: subclinical ETEC infections do occur quite regularly.