研究用

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E. Coli F5 (K99) ELISA KIT

BIO K 345/1

Diagnosis test of *E. Coli* F5 attachment factor for bovines, small ruminants and pigs

Diarrhoea is a major cause of mortality in young cattle under six weeks.
Bovine neonatal gastroenteritis is a multifactorial disease. It can be caused by viruses : coronavirus or rotavirus, by bacteria: *Salmonella* or by protozoan microorganisms such as *Cryptosporidium*. In calves younger than three days, K99 (F5) positive enterotoxigenic *E.Coli* are frequently isolated, particularly in colostrum-deprived calves or in calves which received colostrum free of anti - K99 (F5) specific antibodies. The diagnosis of the etiological agent of diarrhoea can only be performed by laboratory technics because clinical signs do not allow to differentiate between the different microorganisms. The *E. coli* bacteria can be isolated from faeces on an appropriate growth medium. The Minca medium is often used because allows optimal expression of the attachment factor F5 (K99). However, the culture may be unsuccessful if the calf has previously undergone antibiotherapy. In this case, the ELISA test can be very useful because it will detect the attachment factor K99 (F5) even on dead bacteria. Moreover, when the ELISA test for the detection of the attachment factor K99 (F5) is performed directly on fecal samples, it will give more reliable results than the isolation of bacteria because it allows the quantification of the attachment factor K99 (F5) in the sample rather than the presence or absence of the factor in a limited number of isolated strains. The identification of *E. coli* bacteria on a growth medium is not sufficient on its own. It must be coupled to a detection test for the attachment factor K99 (F5) or the toxin. The ELISA test can be used to detect the attachment factor K99 (F5) produced by the *E. coli* bacteria in culture. The ELISA method is particularly useful when a large number of samples must be analyzed. The test is fast and reliable, and it can be evaluated at a glance if a spectrophotometer is not available.

Reliable Results

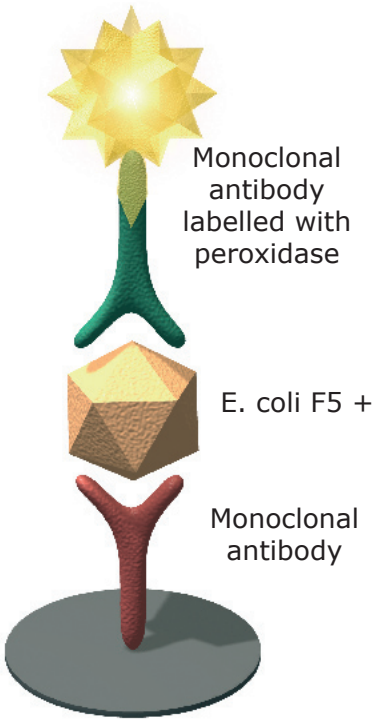
The use of monoclonal antibody as conjugate and capture antibody ensures excellent specificity and very reliable results.

Ease-of-Use

Minimal hands-on-time
Room temperature incubation
Results available in 140 minutes. All reagents are ready to use.

Flexibility

Results can be read visually or spectrophotometrically.



EIA Procedure

1- Microplate coated with monoclonal antibody
2- Add samples and positive control.
Incubate 1 hour at 21°C +/- 3°C
Wash
3- Add conjugate.
Incubate 1 hour at 21°C +/- 3°C .
Wash
4- Add TMB chromogen.
Wait 10 minutes.
Add stop solution.
Read at 450 nm

Bio-X Diagnostics
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Example of results

Isolated strain on Minca	ELISA (BIO K 345) O.D.	Strips (BIO K 154)	PCR (314 bp)
16179	2,1	+	+
7951	1,2	+	+
6785	0,06	-	-
2180	1,55	+	+
785	0,069	-	-
03-029429	1,94	+	+
03-005404/1	0,061	-	-

PCR

ELISA BIO K 345

	+	-	
+	20	0	20
-	2	64	66
	22	64	86

Specificity: 100 %
Sensitivity: 90.9 %

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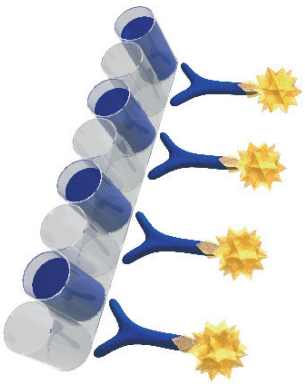
Composition of the kit

BIO-X *E. COLI* F5 ELISA KIT : BIO K 345

	BIO K 345/1
Microplates	1
Washing solution	1 X 100 ml (20 X)
Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 12 ml (1 X)
Control antigen	1 X 2 ml (1 X)
Single component TMB	1 X 12 ml (1 X)
Stopping solution	1 X 6 ml (1 X)



Stability : One year between +2°C and +8°C.

Bibliography

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S. Vandeputte, J. Detilleux, S. Carel, B. Bradfer, H. Guyot and F. Rollin

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DETECTION OF ENTEROPATHOGENS INVOLVED IN CALF NEONATAL DIARRHOEA:
VALIDATION OF ELISAs AND LATERAL FLOW IMMUNOASSAYS AS COMPARED WITH REFERENCE METHODS

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Key words: Calves; neonatal diarrhoea; diagnosis; enteropathogens; ELISA;lateral flow immunochromatography; PCR

1. Introduction and Objectives

Several pathogens play a role in calf neonatal diarrhoea. The major enteropathogens involved are *Escherichia coli* F5⁺/K99 (*E. coli*), *Cryptosporidium parvum*, bovine enteric coronavirus, bovine rotavirus and bovine viral diarrhoea virus. In our laboratory different methods – e.g. selective culture for *E. Coli* F5⁺/K99, microscopic examination of faecal smears for *Cryptosporidium parvum*, a commercially available latex agglutination test for bovine rotavirus, and a commercially available antigen-detection-ELISA for BVDV are routinely used for detection of these agents. For bovine enteric coronavirus no routine diagnostic method was implemented until now.

The objectives of this study were to evaluate two commercially available antigen-detection-ELISA kits and two lateral flow immunochromatography tests (on site tests) for the detection of four of the above-mentioned pathogens.

2. Materials and Methods

2.1 Samples At necropsy rectal contents were sampled from calves between 0 and 6 weeks of age with diarrhoea (n=216). Samples were investigated by routine procedures and then stored at -20 °C to enable batchwise testing.

2.2 ELISAs Samples were tested in two different ELISA kits according to the instructions of the manufacturers. Samples positive for bovine coronavirus in one or both ELISAs were tested by a coronavirus-specific PCR for confirmation.

2.2 Lateral flow immunochromatography tests. A subset of 100 samples with a more or less equal distribution of positive results for the four pathogens of interest, were tested by two lateral flow strip tests (C and D). Tests A and C were produced by the same manufacturer. All samples of this subset were also tested for bovine coronavirus by PCR.

3. Results

Agreement is presented in table 1. For *E. coli* F5⁺/K99, the number of positives in the reference test and other tests was comparable. For rotavirus and cryptosporidium, slightly more samples were positive in ELISAs and slightly less samples were positive in fast tests then in the reference tests. Agreement between ELISA tests was also good, and correlation coefficients between ELISA results were high for the four enteropathogens evaluated.

Table 1. Level of agreement between different tests for four pathogens associated with neonatal diarrhoea in calves, displayed as κ-values (Kappa)

		Reference method			
		E. coli K99	bovine rotavirus	bovine coronavirus	Cryptosporidium parvum
BIO K 348	ELISA kit A	0.93	0.80	0.55	0.81
	ELISA kit B	0.96	0.72	0.54	0.70
BIO K 156	Fast test kit C	0.89	0.91	0.37	0.85
	Fast test kit D	0.91	0.72	0.05	0.73

For coronavirus all positive samples in ELISA kit A wer confirmed by PCR, whereas ELISA kit B scored some fals positives. In the comparative study on a subset of 100 sample PCR scored 26 samples positive for coronavirus, of which 12 an 14 samples scored positive in ELISA kits A and B, respectivel Fast test C was as sensitive as ELISA kit A, but scored a additional 14 samples positive, discrepant, however, from th additional PCR positives. Fast test D only scored 1 sampl positive.

Fig. 1 shows the numbers of samples for each pathogen detected b ELISA kit A (four pathogens) or routine methods for BVDV an Salmonella typhimurium/dublin. Fig. 2 demonstrates detection c more than one pathogen in 25 % of the samples.

Fig. 1 Frequency distribution of defined enteropathogens in faecal samples of young calves with diarrhoea

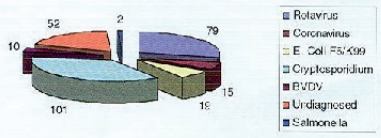
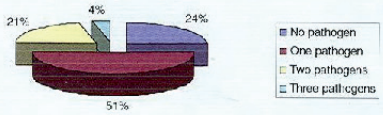


Fig. 2 Simultaneous detection of enteropathogens in faecal samples of young calves with diarrhoea



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Discussion and Conclusions

Hardly any literature is available concerning diagnosti performance of commercially available ELISA kits and lateral flo kits for detection of the major enteropathogens involved in cal neonatal diarrhoea (2, 3). All kits showed satisfactory diagnosti performance for detection of E. coli K99, bovine rotavirus an cryptosporidium parvum, with kits A and C showing the highes kappa-values. For detection of bovine coronavirus, kit D faile almost completely, whereas kappa-values of the other kits wer rather poor. The reference test, however, was PCR. Considering th relative low detection limits of PCRs in general, the clinica significance of these PCR results remain to be seen (1).

Also the significance of – frequently occurring – combinations o enteropathogens in calf neonatal diarrhoea may cause a headach for the veterinary practitioner.

5. References

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2. Khattar,S. and Pandey,R., 1990. A comparison of four methods fo detecting rotavirus in faeces of bovine calves. J.Diarrhoeal Dis.Res., (1-2): 31-33. 3. Trotz-Williams,L.G.,Martin,S.W., Martin,D. Duffield,T., et al., 2005. Multiattribute evaluation of two simple tests fo the detection of *Cryptosporidium parvum* in calf faeces. Vet. Parasitol

Bio-X Diagnostics

ANTIGENIC ELISA KIT FOR DETECTION OF *ESCHERICHIA COLI F5* (K99)

Sandwich ELISA test

Direct test for faeces and bacterial culture

Diagnosis test of *E. Coli* attachment factor for bovines, small ruminants and pigs

I - INTRODUCTION

Diarrhoea is a major cause of mortality in young cattle under one month.
Bovine neonatal gastroenteritis is a multifactorial disease. It can be caused by viruses : coronavirus or rotavirus, by bacteria : *Salmonella* or by protozoan microorganisms such as *Cryptosporidium parvum*. In calves younger than three days, F5 positive enterotoxigenic *E.Coli* are frequently isolated, particularly in colostrum-deprived calves or in calves which received colostrum free of anti – *E.coli* F5 specific antibodies.
The diagnosis of the etiological agent of diarrhoea can only be performed in the laboratory because clinical signs do not allow to differentiate between the different microorganisms.
The *E. coli* bacteria can be isolated from faeces on an appropriate growth medium. The Minca medium is often used because allows optimal expression of the attachment factor F5. However, the culture may be unsuccessful if the calf has previously undergone antibiotherapy. In this case, the ELISA test can be very useful because it will detect the attachment factor F5 even on dead bacteria. Moreover, when the ELISA test for the detection of the attachment factor F5 is performed directly on faecal samples, it will give more reliable results than the isolation of bacteria because it allows the quantification of the attachment factor F5 in the sample rather than the presence or absence of the factor in a limited number of isolated strains. The identification of *E. coli* bacteria on a growth medium is not sufficient on its own. It must be coupled to a detection test for the attachment factor F5 or the toxin. The ELISA test can be used to detect the attachment factor F5 produced by the *E. coli* bacteria in culture. The ELISA method is particularly useful when a large number of samples must be analyzed. The test is fast and reliable, and it can be evaluated at a glance if a spectrophotometer is not available.

II - PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by specific for an antigenic determinant of *E. coli* F5. These antibodies allow a specific capture of the corresponding pathogens which are present in the faeces samples. Rows A, C, E, G have been sensitized with these antibodies and rows B, D, F, H are containing non specific antibodies. These control rows allow the differenciation between specific immunological reaction and non specific bindings so as to eliminate false positives.
Faeces are diluted in dilution buffer and incubated on the microplate for 1 hour at 21°C +/- 3°C.
After this first incubation step, the plate is washed and incubated for 1 hour with the conjugate, a peroxidase labelled anti-*E. coli* F5 specific monoclonal antibody. After this second incubation, the plate is washed again and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.
If F5 positive *E. coli* is present in the tested faeces, the conjugate remains bound to the corresponding microwells and the enzyme catalyses the transformation of the colourless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titre of *E. coli* F5 in the sample. Enzymatic reaction can be stopped by acidification and resulting optical density at 450 nm can be recorded using a photometer. The signals recorded for the negative control microwells are substracted from the corresponding positive microwells. Positive control is provided with the kit so as to validate the test results.

III - COMPOSITION OF THE KIT

- **Microplates**: One 96-well microtitration plate. Rows A, C, E, G are sensitised by anti-*E. coli* F5 specific antibodies, while rows B, D, F, H are sensitized by the non specific antibodies.
- **Washing solution**: One 100- ml bottle of 20x concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water.
- **Dilution buffer**: One 50-ml bottle of 5x concentrated buffer for diluting samples. Dilute this concentrated dilution buffer 1:5 with distilled or demineralised water. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.
- **Conjugate**: One 12 ml vial of coloured conjugate. **This solution is ready to use.**
- **Positive Control:** 1 vial of 2 ml. The reagent is ready to use.
- **Single component TMB** One 12-ml bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C protected from light. **This solution is ready to use.**
- **Stopping solution**: One 6-ml bottle of the 1 *M* phosphoric acid stop solution.

	BIO K 345/1
Microplates	1
Washing solution	1 X 100 ml (20 X)
Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 12 ml (1 X)
Control antigen	1 X 2 ml (1 X)
Single component TMB	1 X 12 ml (1 X)
Stopping solution	1 X 6 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, plastic tubes, tube rack, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for "in vitro" diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- The concentrated wash solution and dilution buffer may be stored at room temperature. Once diluted, these solutions remain stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope's seal airtight. If these precautions are taken, the strips' activity can be conserved up to the kit's shelf-life date.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.
- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

1- Bring all the reagents at 21°C +/- 3°C before use.
2- Dilute the concentrated washing solution 20 fold in distilled water. Be sure that all crystals have disappeared before dilution.
   Dilute the concentrated dilution buffer 5 fold in distilled water.
   Keep these solutions between +2°C and +8°C when not used.
3- Dilute faecal samples volume per volume into dilution buffer prepared as instructed in step 2.
   This is a qualitative dilution only, which must allow the pipetting of faecal suspensions. Discard any gruds by natural decantation for about 10 minutes. Do not centrifuge the suspensions.
   If one wishes to test an isolated strain on culture medium, it is better to make it grow one night at 37°C on liquid Minca medium (see composition attached). The culture is used without dilution.
4- Add 100-µl aliquots of the diluted samples to the wells as follows: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1, etc. Proceed in the same manner for the positive reference (ex.: G1 and H1).
5- Incubate the plate at 21°C +/- 3°C for 1 hour. Cover the plate with a lid.
6- Rinse the plate with the washing solution prepared as instructed in the 'Composition of the Kit' section. To do this, empty the microplate of its contents by flipping it over suddenly over a sink. Tap the upside-down microplate against a sheet of clean absorbent paper so as to remove all liquid. Fill the used wells with a washing solution either by means of a spray bottle or by plunging the microplate in a suitably-dimensioned container, then empty the plate once again by flipping it over a sink. Repeat the entire operation twice, taking special care to avoid the formation of bubbles in the wells. Upon completing these three washes, go on to the next step.
7- Distribute the conjugate solution at the rate of 100 µl per well. Incubate at 21°C +/- 3°C for 1 hour. Cover the plate with a lid.
8- Wash the plate as described in Step 6.
9- Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated. Incubate at 21°C +/- 3°C and away from light for 10 minutes. Do not cover. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
10- Add 50 µl of stop solution to each well.
11- Read the optical densities by means of a microplate spectrophotometer with a 450 nm filter. The results must be read as quickly as possible after the stop solution has been applied, for in the case of a strong signal the chromogen can crystallise and lead to incorrect measurements.

Minca medium composition

Minca base: for 950 ml distilled water.
KH2PO4 : 1.36 g.
Na2HPO4.2H2O : 10.1 g.
Caseine Hydrolysate (ACID) : 1g.

Minca saline: for 100 ml distilled water.
MgSO4.7H2O : 1 g.
MnC12.4H2O : 0.1 g.
FeC13.6H2O : 0.135 g.
CaC12.2H2O : 0.04 g.

Minca Glucose : for 100 ml distilled water.
Glusose : 2.5g

Autoclave the three solutions in 20 minutes at 121°C. Let it cool.

Mix 950 ml de Minca Base + 10 ml de Minca saline + 40 ml de Minca glucose.

Check sterility by incubating 5 ml of medium overnight at 37 °C and store between +2°C and +8°C.

VII – INTERPRETING THE RESULTS

Calculate the net optical density of each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control.
Proceed in the same way for the positive control antigen.
The test is validated only if the positive control antigen yields a difference in the optical density at 10 minutes that is greater than the value given on the QC data sheet.

Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage. Using the first table in the quality control procedure, determine each sample's status (positive, negative).

$$Val = \frac{\text{Delta DO spl} * 100}{\text{Delta DO pos}}$$

VIII – ORDERING INFORMATION

BIO-X *E. COLI* F5 ELISA KIT: 1x48 tests BIO K 345/1