研究用

www.biox.com

コスモ・バイオ株式会社

Cryptosporidium ELISA KIT

BIO K 346/2

Diagnosis test of *Cryptosporidium* for all species.

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 *E. coli* K99 (F5), or by protozoa such as *Cryptosporidium*. The diagnosis of the aetiological agent of diarrhoea can be performed in the laboratory only, because clinical signs do not enable one to differentiate between the different microorganisms. It is possible to identify *Cryptosporidium* by means of flotation or staining techniques (modified Ziehl Neelsen). However, these techniques are labour-intensive and unpractical. These conventional techniques can be replaced by ELISA because of its simplicity, and the limited laboratory equipment requirements. The sensitivity and specificity of the ELISA technique for the detection of *Cryptosporidium* are at least as good as those of the more conventional techniques ; the results are very similar. The ELISA technique is rapid and reliable and particularly suited to the analysis of large numbers of samples.

Reliable Results

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

Ease-of-Use

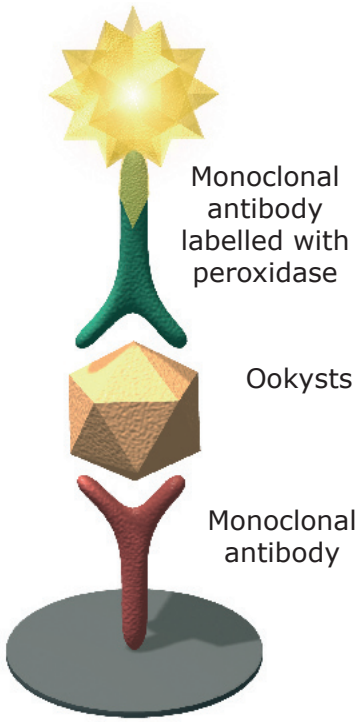
Minimal hands-on-time
Room temperature incubation
Results available in 140 minutes. All reagents are ready to use.

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 chromogen (TMB)
Wait 10 minutes.
Add stop solution.
Read at 450 nm



Bio-X Diagnostics
Site du complexe des Postes 49, rue Joseph Wauters BE-5580 Jemelle BELGIUM (Europe)
Tel: +32 (0)84.32.23.77 Fax: +32 (0)84.31.52.63 E-mail: info@biox.com http://www.biox.com

Example of results

Flotation

ELISA BIO K 346

	+	-	
+	33	6	39
-	1	60	61
	34	66	100

Specificity: 90.9 %
Sensitivity: 97.1 %

Days after birth

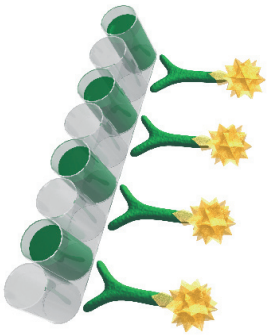
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Calf 1	Crypto						+	+	+	+	+	+	+	+	+			
	Rota																	
	Diarrhoea						+	+	+	+	+	+	+					
Calf 2	Crypto							+	+	+	+	+	+	+	+	+	+	+
	Rota					+	+											
	Diarrhoea					+	+	+	+	+	+	+	+	+	+		+	

Bio-X Diagnostics
Site du complexe des Postes 49, rue Joseph Wauters BE-5580 Jemelle BELGIUM (Europe)
Tel: +32 (0)84.32.23.77 Fax: +32 (0)84.31.52.63 E-mail: info@biox.com http://www.biox.com

Composition of the kit

BIO-X *CRYPTOSPORIDIUM* ELISA KIT : BIO K 346/2

	BIO K 346/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 25 ml (1 X)
Control antigen	1 X 4 ml (1 X)
Single component TMB	1 X 25 ml (1 X)
Stopping solution	1 X 15 ml (1 X)



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

Bibliography

Evaluation of a Bovine Concentrated Lactoserum for Preventing Neonatal Diarrhoea in Belgian Blue Calves.
S. Vandeputte, J. Detilleux, S. Carel, B. Bradfer, H. Guyot and F. Rollin
The Open Veterinary Science Journal, 2010, 4, 36-40

Bio-X Diagnostics
Site du complexe des Postes 49, rue Joseph Wauters BE-5580 Jemelle BELGIUM (Europe)
Tel: +32 (0)84.32.23.77 Fax: +32 (0)84.31.52.63 E-mail: info@biox.com http://www.biox.com

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

C. van Maanen[1], M.H. Mars[1], A.M. van der Meulen[1], H. v.d. Sande, H.A. Blok[2] and C.B.E.M. Reusken[2]

[1] Dutch Animal Health Services (GD), Deventer, the Netherlands

[2] Center for Infectious Disease Control, Institute for Public Health and the Environment (rivm), Bilthoven, the Netherlands

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

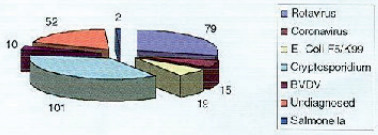
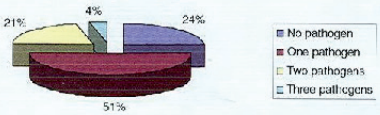


Fig. 2 Simultaneous detection of enteropatnogens in faecal samples of young calves with diarrhoea



4

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

1. Kapil,S., Trent,A.M. and Goyal,S.M., 1990. Excretion and persistence o bovine coronavirus in neonatal calves. Arch.Virol., 115 (1-2): 127-132
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 *CRYPTOSPORIDIUM*

Sandwich ELISA test

Direct test for faeces

Diagnosis test of *Cryptosporidium* for all species

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 *E. coli* F5, or by protozoan microorganisms such as *Cryptosporidium.*
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. It is possible to identify *Cryptosporidium.* by means of floating or staining techniques (Ziehl Neelsen modified). However, these techniques are labour intensive and unpractical.
These classical techniques can be replaced by the ELISA technology because of its simplicity, and the limited requirements in laboratory equipment.
The sensitivity and specificity of the ELISA technique for the detection of *Cryptosporidium.* is at least as good as that of the more classical techniques ; results are very similar. The ELISA technique is rapid and reliable and is particularly suited to the analysis of important numbers of samples.

II - PRINCIPLE OF THE TEST

The test uses 96-well microtitration plates sensitised by specific antibodies for *Cryptosporidium* ookysts. 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. A large number of false positives are eliminated.
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, then the conjugate, a peroxidase labelled anti-*Cryptosporidium* specific monoclonal antibody, is added to the wells. Plate is incubated for 1 hour at 21°C +/- 3°C.
After this second incubation step, 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 *Cryptosporidium* 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 *Cryptosporidium* 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**: Two 96-well microtitration plates. Rows A, C, E, G are sensitised by anti-*Cryptosporidium* 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 25-ml bottle of anti-*Cryptosporidium* peroxidase conjugate (horseradish peroxidase-labelled anti-*Cryptosporidium* monoclonal antibody). This solution is ready to use.
- **Positive Control:** 1 vial of 4 ml. The reagent is ready to use.
- **Single component TMB**: One 25-ml bottle of the chromogen tetramethylbenzidine. Store between +2°C and +8°C. protected from light. This solution is ready to use.
- **Stopping solution**: One 15-ml bottle of the 1 M phosphoric acid stop solution.

	BIO K 346/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 25 ml (1 X)
Control antigen	1 X 4 ml (1 X)
Single component TMB	1 X 25 ml (1 X)
Stopping solution	1 X 15 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.
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 step 2, as follows: empty the microplate of its contents by flipping it over sharply over a sink. Tap the microplate upside down against a piece of clean absorbent paper to remove all the liquid. Fill all the used wells with the washing solution using a spray bottle or by plunging the plate in a vessel of the right dimensions, then empty the wells once more by turning the plate over above a sink. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times proceed 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 above
9- Add 100µl 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 for 10 minutes at 21°C +/- 3°C and away from the light. Do not cover. This time 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 per microwell.
11- Read the optical densities in the microwells using a plate reader and a 450 nm filter. Results must be read fairly soon after the stopping solution has been added since the chromogen may cristallize in wells with strong signals and distort the results accordingly.

VII – INTERPRETING THE RESULTS

Calculate the net optical density for 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 antigens.
The test is validated only if the positive control antigens yield a difference in optical density at 10 minutes that is greater than the values given on the QC data sheet.

Divide each resulting value by the corresponding value obtained for the corresponding positive control and multiply this result by 100 to express it as a percentage.

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

Using the first table in the quality control procedure, determine each sample's status (positive, negative).

VIII – ORDERING INFORMATION

BIO-X *CRYPTOSPORIDIUM* ELISA KIT: 2 X 48 tests BIO K 346/2