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 E.coli F5 (K99), or by protozoa such as Cryptosporidium.
Coronavirus and rotavirus are often associated with episodes of neonatal diarrhoea.
The diagnosis of the aetiological agent of diarrhoea can be performed by laboratory technics only, because the clinical signs do not enable one to differentiate between the different microorganisms. It is possible to identify these agents by means of different techniques including culture, staining, electron microscopy and floating techniques. However, these techniques are labor-intensive, unpractical and time consuming. These conventional techniques can be replaced by ELISA because of its simplicity, and limited laboratory equipment requirements.
The sensitivity and specificity of the ELISA technique for the detection of these pathogens 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 is particularly suited to the analysis of important numbers of samples.

**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.

**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.
Example of results

**Electron microscopy**

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>-</td>
<td>4</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>39</td>
</tr>
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</table>

Specificity: 90 %
Sensitivity: 95 %

**RT-PCR**

<table>
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<tr>
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<th>-</th>
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</thead>
<tbody>
<tr>
<td>+</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>4</td>
<td>77</td>
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<tr>
<td>Total</td>
<td>18</td>
<td>81</td>
</tr>
</tbody>
</table>

Specificity: 98.7 %
Sensitivity: 77.8 %

**Days after birth**

<table>
<thead>
<tr>
<th>Days</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
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<th>16</th>
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</thead>
<tbody>
<tr>
<td>Corona</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rota</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crypto</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Detectability**

The kit gives a positive signal with a minimum of 100,000 TCID\(_{50}/\text{ml}\)
## Composition of the kit

**BIO-X ELISA CORONAVIRUS KIT ANTIGENIC : BIO K 344/2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Qty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microplates</td>
<td>2</td>
</tr>
<tr>
<td>Washing solution</td>
<td>1 X 100 ml (20 X)</td>
</tr>
<tr>
<td>Dilution buffer</td>
<td>1 X 50 ml (5 X)</td>
</tr>
<tr>
<td>Conjugate</td>
<td>1 X 25 ml (1 X)</td>
</tr>
<tr>
<td>Control antigen</td>
<td>1 X 4 ml (1 X)</td>
</tr>
<tr>
<td>Single component TMB</td>
<td>1 X 25 ml (1 X)</td>
</tr>
<tr>
<td>Stopping solution</td>
<td>1 X 15 ml (1 X)</td>
</tr>
</tbody>
</table>

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


1. Introduction and Objectives

Several pathogens play a role in calf neonatal diarrhea. The major enteropathogens involved are Escherichia coli F57/K99 (E. coli), Cryptosporidium parvum, bovine rotavirus, bovine coronavirus, bovine rotavirus and bovine viral diarrhea virus. In our laboratory different methods – e.g. selective culture for E. coli F57/K99, microscopic examination of fecal 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: A necropsy material was sampled from calves between 0 and 6 weeks of age with diarrhea (n=210). 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 set of 100 samples with a true positive and true negative 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 F57/K99, the number of positives in the reference test and other tests was comparable. For rotavirus and bovine coronavirus, slightly more samples were positive in ELISAs and slightly less samples were positive in the reference tests. Agreement between ELISAs 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 enteropathogens associated with neonatal diarrhea in calves, displayed as x-ratio (Kappa)

<table>
<thead>
<tr>
<th>Reference method</th>
<th>E. coli F57/K99</th>
<th>bovine rotavirus</th>
<th>bovine coronavirus</th>
<th>Cryptosporidium parvum</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA kit A:</td>
<td>0.93</td>
<td>0.80</td>
<td>0.55</td>
<td>0.81</td>
</tr>
<tr>
<td>ELISA kit B:</td>
<td>0.96</td>
<td>0.72</td>
<td>0.84</td>
<td>0.70</td>
</tr>
<tr>
<td>E. coli test kit C:</td>
<td>0.89</td>
<td>0.91</td>
<td>0.37</td>
<td>0.85</td>
</tr>
<tr>
<td>E. coli test kit D:</td>
<td>0.91</td>
<td>0.72</td>
<td>0.86</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Discussion and Conclusions

Hardly any literature is available concerning diagnostic performance of commercially available ELISA kits and lateral flow kits for detection of the major enteropathogens involved in calf neonatal diarrhea (2, 3). All kits showed satisfactory diagnostic performance for detection of E. coli K99, bovine rotavirus and Cryptosporidium parvum, with kits A and C showing the highest kappa-values. For detection of bovine coronavirus, Kit D failed almost completely, whereas kappa-values of the other kits were rather poor. The reference test, however, was PCR. Considering the relative low detection limits of PCR in general, the clinical significance of these PCR results remain to be seen (1).

Also the significance of – frequently occurring – combinations of enteropathogens in calf neonatal diarrhea may cause a headache for the veterinary practitioner.

5. References

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 parvum*. Coronavirus and rotavirus are often associated with episodes of neonatal diarrhoea. The diagnosis of the etiological agent of diarrhoea can only be performed in the laboratory because clinical signs do not allow to differentiate between these different microorganisms. It is possible to identify these agents by means of different techniques including culture, staining, electron microscopy and floating techniques. However, these techniques are labor intensive, unpracticle and time consuming. These classical techniques have rapidly been 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 these pathogens 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 the Coronavirus. 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 differentiation 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-coronavirus 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 coronavirus 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 coronavirus 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-coronavirus specific antibodies, while rows B, D, F, H are sensitised 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 vial of coloured conjugate. **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 (TMB). 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.

<table>
<thead>
<tr>
<th>Bio X 344/2</th>
<th>2</th>
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</thead>
<tbody>
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</tr>
</tbody>
</table>

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 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.

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).

\[ \text{Val} = \frac{\text{Delta DO spl} \times 100}{\text{Delta DO pos}} \]
VIII - ORDERING INFORMATION

BIO-X ELISA CORONAVIRUS KIT ANTIGENIC: 2 X 48 tests

BIO K 344/2