

## Comparison of immunohistochemistry, electron microscopy, and direct fluorescent antibody test for the detection of bovine coronavirus

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**Abstract.** Bovine coronavirus (BCV) is 1 of the major causes of calf diarrhea and has also been implicated in respiratory infections of young calves and winter dysentery of adult cattle. Currently, transmission electron microscopy (TEM), direct fluorescent antibody (DFA), and enzyme-linked immunosorbent assay (ELISA) techniques are considered standard methods for the diagnosis of BCV infection. However, these techniques are not useful if fresh tissues and intestinal contents are not available for examination. The detection of viral antigens in formalin-fixed, paraffin-embedded tissues using immunohistochemistry (IHC) is a suitable alternative. In the present study, 166 tissue specimens were tested by IHC for the presence of BCV. These tissues were from animals whose feces were positive for rotavirus and/or coronavirus by TEM. Some of these samples were also tested by DFA. Thus, TEM, DFA, and IHC were compared for the detection of BCV. There was 56% agreement among the 3 methods (overall kappa = 0.368). When IHC was compared with TEM, 78% agreement was observed (kappa = 0.475). Similarly, IHC and DFA had 64% agreement (kappa = 0.277). These kappa values indicate a moderate degree of agreement between IHC and TEM; agreement between IHC and DFA was fair. The results of this study indicate that IHC may be a suitable adjunct for the detection of BCV because of its simplicity, ease of use, and relatively close correlation with TEM results.

Calf scours is a major cause of economic losses to the cattle industry resulting in annual losses of up to \$1.7 billion around the world,<sup>28</sup> increasing in recent years due to the adoption of intensive beef and dairy practices.<sup>30</sup> Bovine coronavirus (BCV) is one of the leading causes of calf scours,<sup>32</sup> with coronaviruses and rotaviruses estimated to account for 20–26% and 27–36% of all cases of calf diarrhea, respectively.<sup>3</sup> More severe diarrhea and higher mortality is caused by BCV than by rotaviruses because it involves both the large and the small intestines.<sup>38</sup> BCV has also been implicated in winter dysentery of adult cattle<sup>31</sup> and in respiratory tract infection of 2–16-week-old calves.<sup>20</sup> BCV has also been implicated in pneumonia in adult dairy cattle<sup>9</sup> and in cattle after shipping.<sup>35</sup>

BCV can be detected by transmission electron microscopic examination of fecal samples, direct fluorescent antibody assay (DFA) on frozen sections of spiral colon,<sup>23</sup> and enzyme-linked immunosorbent assay (ELISA)<sup>10</sup> and occasionally through virus isolation *in vitro*.<sup>21</sup> Currently, transmission electron microscopy (TEM) and DFA are the most widely used methods for BCV diagnosis.<sup>27</sup> Results from TEM can be obtained in a few hours, but the technique has the limi-

tation of detecting nonviral particles that may look like coronavirus and detecting virus only when the number of virions is  $>10^6$ /g of feces.<sup>12</sup> After the onset of diarrhea, the amount of BCV in feces may fall rapidly below the detection limits of TEM.<sup>20</sup> Another difficulty is that a relatively small number of samples can be examined by TEM in any given day.<sup>37</sup>

Many different types of ELISA systems have been used for the detection of BCV antigen in feces. ELISA is often preferred over TEM because it is rapid and a large number of samples can be tested in a relatively short period of time. However, these ELISA systems lack sensitivity when compared with similar assays for other enteric pathogens.<sup>33,34</sup>

Although virus isolation is a sensitive method for the detection of animal viruses, no cell culture system is currently available in which all field strains of BCV can grow consistently to a significantly high titer during primary isolation.<sup>21</sup> In addition, virus isolation is time consuming and labor intensive.<sup>37</sup>

Immunofluorescence staining of frozen tissue sections is rapid and simple as compared with virus isolation and TEM<sup>29</sup> and is commonly used for the detection of many viruses of veterinary interest.<sup>5,17</sup> However, successful application of this technique requires rapid transportation of fresh samples to the diagnostic laboratory and careful attention to packaging and shipping of refrigerated specimens. Other problems associated with this technique are nonspecific fluorescence, fading of fluorescence on exposure to ordinary and

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Received for publication April 21, 1997.

ultraviolet light, and intolerance of sections to a permanent mounting system.

Other difficulties encountered in these diagnostic techniques include the need for highly sophisticated facilities and trained personnel. The worldwide incidence of BCV may have been underestimated because most of the laboratories in developing countries do not have facilities required for virus isolation, electron microscopy, and fluorescent microscopy, leading to an inability to identify viral agents in many cases of diarrhea. Also, it is not easy to find infected cells and to correlate the target cell morphology with viral antigen-positive cells with fluorescence microscopy.

Recent advances in the use of enzyme-labeled antibody has resulted in improved detection of viral antigens in fixed, paraffin-embedded tissues prepared for histologic examination through the application of immunohistochemical methods.<sup>1,8,19,24</sup> The objective of this study was to compare immunohistochemistry (IHC) with DFA and TEM as a diagnostic tool for BCV. The use of IHC should permit retrospective epidemiologic studies of BCV on routine necropsy material without requiring specialized equipment and procedures. In addition, this method should be within the scope of most histopathologic and disease investigation laboratories, even in developing countries. In addition, IHC can be used for detailed studies on the pathogenesis of BCV.

### Materials and methods

**Specimens.** A total of 166 paraffin blocks of intestinal tissues collected by the histopathology section of the Minnesota Veterinary Diagnostic Laboratory during 1993–1995 were included in this study. Block selection was based on the TEM results of fecal samples from animals from which these blocks were prepared. Tissue blocks were obtained from animals whose fecal samples gave the following TEM results: 101 calves positive for BCV, 17 pigs positive for porcine coronavirus, 3 dogs positive for canine coronavirus, 4 cats positive for feline coronavirus, 9 calves positive for bovine rotavirus, 6 calves positive for both rota- and coronaviruses, and 26 calves negative for any virus.

**TEM.** Fecal samples were examined using TEM as previously described.<sup>14</sup> A 10% fecal suspension in distilled water was sonicated for 20 sec and then cleared by centrifugation at  $3,000 \times g$  for 10 min. The supernatant was then centrifuged at  $30,000 \times g$  for 1 hr to obtain a pellet. The pellet was resuspended in distilled water and stained with phosphotungstic acid (pH 6.4). The suspension was then nebulized on a 200-mesh collodion-coated grid and viewed under an electron microscope at a magnification of  $180,000\times$ .

**DFA.** Cryostat sections of small intestine and colon were tested for BCV by DFA as previously described.<sup>27</sup> Cryostat sections (4–6  $\mu\text{m}$  thick) of fresh tissues were mounted on glass slides. Sections were fixed with acetone and stained with fluorescein conjugate (DVL 620-BDV)<sup>a</sup> against BCV.

Counterstaining was done with Evan's blue. Sections were examined under a fluorescent microscope at a magnification of  $400\times$ .

**IHC.** Tissue sections (4  $\mu\text{m}$  thick) from selected blocks were placed on microscopic glass slides coated with a bonding reagent.<sup>b</sup> Tissue sections were left to dry overnight at room temperature. The next day, the slides were placed in a hot air oven at 60 C for 25 min followed by deparaffinization for 21 min (3 consecutive changes of deparaffinizing agent at 7-min intervals). The sections were rehydrated in increasing dilutions of ethyl alcohol for 6 min (2 min each in 100%, 95%, and 80% dilution) with a final dip in 50% ethanol.

After rehydration, the sections were treated with 0.05% trypsin–ethylenediaminetetraacetic acid buffer for 10 min and then washed for 5 min with distilled water. To quench endogenous peroxidase activity, tissue sections were treated with 3% hydrogen peroxide solution for 5 min followed by a 5-min wash in distilled water and a 10-min wash in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T; pH 7.2).

Nonspecific protein binding was blocked by treating the sections with a protein blocking agent<sup>c</sup> for 10 min. The sections were then incubated with a 1:300 dilution of primary antibody in PBS-T for 2 hr at 37 C in a moist chamber. The primary antibody used was monoclonal antibody (MAb) Z3A5.<sup>d</sup> This murine MAb recognizes the 86-kD spike protein of BCV (Zhang Z et al.: 1995 Abstr Conf Res Workers Anim Dis no. 240).

After a 10-min wash with PBS-T, the sections were treated with a 1:200 dilution of secondary antibody (biotinylated, equine anti-mouse IgG) for 20 min. After another wash with PBS-T for 10 min, the slides were treated with avidin–biotin peroxidase solution<sup>b</sup> for 30 min at room temperature. Chromogen (3,3-diaminobenzidine-4HCl [DAB]) was applied for 10–15 min, and the development of color was watched under a light microscope. The slides were then placed in distilled water for 5 min before counterstaining with hematoxylin<sup>e</sup> for 30 sec. The slides were then washed for 5 min in running tap water, coverslipped with water-based glycerol–gelatin mounting medium,<sup>e</sup> and examined under a light microscope at magnifications of  $200\times$  and  $400\times$ .

Slides processed without incubation with primary antibody served as negative controls. Twenty-six intestinal tissue samples from animals negative for the presence of any virus by TEM were also used as negative controls. Thirty-three samples positive for porcine, canine, and feline coronaviruses and for bovine rotavirus were also considered negative controls because the Z3A5 MAb recognizes BCV only.

**Statistics.** Overall agreement between different tests was estimated using kappa ( $\kappa$ ) statistics;  $\kappa$  is an appropriate measure of the diagnostic agreement between tests beyond the agreement due to chance.<sup>25</sup>

The results were weighted on the basis of the  $\kappa$  value, which ranges from 1 to  $-1$ , where a value of  $-1$  indicates complete disagreement between tests, 0 indicates agreement by chance only, 0.01–0.20 indicates slight agreement, 0.21–0.40 indicates a fair amount of agreement, 0.41–0.60 indicates moderate agreement, 0.61–0.80 indicates substantial agreement, and 0.81–1 indicates almost perfect agreement.<sup>22</sup> To find out whether variation among 3 tests in detecting a

**Table 1.** Comparison of 3 diagnostic methods for the detection of BCV in 108 samples.

Methods*			No. (%) samples	Observed agreement (%)
TEM	IHC	DFA		
+	+	+	43 (40)	56
-	-	-	18(17)	
+	+	-	24 (22)	79
+	-	+	11 (10)	
+	-	-	8 (7)	64
-	+	-	4 (4)	71
-	-	+	0	

$\kappa = 0.368$

$SE_{\kappa} = 0.05$

$Z = 7.36^*$

$Q_a = 28.63^{**}$  for 2 df at the 0.05 level

\* TEM = transmission electron microscopy, fecal samples; IHC = immunohistochemistry, intestinal tissue from the same animals; DFA = tested by direct fluorescent antibody assay, intestinal tissue from the same animals.

sample as positive or negative significantly differ, Cochran's  $Q$  value ( $Q_a$ ) for 3 tests was determined and compared with critical values at 2 degrees of freedom at the 0.05 level of significance. To evaluate agreement among the tests being beyond chance, a  $Z$  value was calculated for each set of comparisons. The standard errors of estimates of ( $SE_{\kappa}$ ) were also calculated.

## Results

The results in Tables 1–5 were analyzed by considering TEM as the “gold standard.” A total of 166 samples were tested in this study, of which 108 (either feces or intestinal tissue obtained from the same animal) were tested by all 3 techniques. Of these 108 samples, 43 (40%) were positive and 18 (17%) were negative for BCV by all 3 techniques (Table 1), showing an agreement of 56% among the 3 techniques. Eighty-six samples (80%) were positive and 22 (20%) were negative for BCV by TEM. Twenty-four (22%) samples were positive by TEM and IHC but not by DFA, whereas 11 (10%) samples were positive by TEM and DFA but were negative by IHC. In addition, 8 (7%) samples were positive by TEM alone, and four (3%) samples were positive by IHC alone. None of the samples was positive by DFA only. Overall  $\kappa$  value of 0.368 among 3 tests indicates a fair amount of agreement among the tests. The  $Q_a$  for Table 1 is 28.63, which exceeds the critical value, indicating that the tests differ significantly from each other in detection of samples as positive or negative.<sup>11</sup> A  $Z$  value of 7.36, compared with a standard normal distribution, indicates that the amount of agreement is beyond that expected by chance.

To determine the specificity of IHC, 24 nonbovine samples positive for their respective coronaviruses (17

**Table 2.** Test results for 19 bovine fecal/intestinal samples where sections for DFA were not available for detection of BCV.

Methods*			No. samples
TEM	IHC		
+	+		12
-	-		3
+	-		3
-	+		1

\* TEM = transmission electron microscopy; fecal samples; IHC = immunohistochemistry, intestinal tissue from the same animals.

porcine coronavirus, 3 canine coronavirus, 4 feline coronavirus) were tested for BCV using the IHC technique. In addition, 9 bovine fecal samples positive for bovine rotavirus by TEM were also tested by IHC. All 33 of these samples were negative for BCV by the IHC test. The IHC also detected BCV in 4 of 6 bovine tissue samples that were positive for both BCV and bovine rotaviruses by TEM.

Table 2 shows the results of 19 samples that were tested by TEM and IHC but not by DFA. Tables 3–5 show overall agreement between 2 of the 3 techniques, using TEM as the “gold standard.” Table 3 shows an agreement of 78% between TEM and IHC. A  $\kappa$  value of 0.475 shows a fair degree of agreement, with  $SE_{\kappa} = 0.083$  and  $Z = 5.722$ , indicating significant agreement. Of the 101 samples positive by TEM, 79 (78%) were detectable by IHC. Of the 26 samples negative for any virus in TEM, 21 (81%) were negative by IHC. Five samples negative by TEM were positive by IHC. McNemar's test for comparison between TEM and IHC was  $\chi^2 = 12$  for 1 df at the 0.05 level, showing that TEM produced significantly more positive results than did IHC.

Table 4 shows a comparison of DFA and TEM and indicates 70% observed agreement between the two techniques ( $\kappa = 0.407$ ,  $SE_{\kappa} = 0.07$ ,  $Z = 5.29$ , and  $\chi^2$

**Table 3.** Number of samples evaluated by TEM\* and IHC† for the detection of BCV. Observed proportion of agreement between the 2 tests was 78%.

IHC*	TEM		Total
	+	-	
+	79	5	84
-	22	21	43
Total	101	26	127

$\kappa = 0.475$

$SE_{\kappa} = 0.083$

$Z = 5.722^*$

$\chi^2 = 12^*$  for 1 df at the 0.05 level

\* Fecal samples tested by transmission electron microscopy.

† Intestinal tissue from the same animals tested by immunohistochemistry.

**Table 4.** Number of samples evaluated by TEM\* and DFA† for the detection of BCV. Observed proportion of agreement between the 2 tests was 70%.

DFA	TEM		Total
	+	-	
+	54	0	54
-	32	22	54
Total	86	22	108

$\kappa = 0.407$   
 $SE_{\kappa} = 0.083$   
 $Z = 5.288^*$   
 $\chi^2 = 30.03^{**}$  for 1 df at the 0.05 level

\* Fecal samples tested by transmission electron microscopy.

† Intestinal tissue from the same animals tested by direct fluorescent antibody.

**Table 5.** Number of samples evaluated by IHC\* and DFA† for the detection of BCV. Observed proportion of agreement between the 2 tests was 63.8%.

DFA	IHC		Total
	+IHC <sup>a</sup>	-IHC <sup>a</sup>	
+	43	11	54
-	28	26	54
Total	71	37	108

$\kappa = 0.277$   
 $SE_{\kappa} = 0.06$   
 $Z = 4.61^*$   
 $\chi^2 = 8.3^*$  for 1 df at the 0.05 level

\* Intestinal tissue tested by immunohistochemistry.

† Intestinal tissue from the same animals tested by direct fluorescent antibody.

= 30.03 for 1 df at the 0.05 level). The relative sensitivity and specificity of DFA as compared with TEM were 63% (54/86) and 100%, respectively. Table 5 shows a comparison of IHC with DFA, both methods showing 64% agreement ( $\kappa = 0.277$ ,  $SE_{\kappa} = 0.06$ ,  $Z = 4.61$ , and  $\chi^2 = 8.3$  for 1 df at the 0.05 level). The relative sensitivity and specificity of DFA as compared with IHC were 76% and 100%, respectively.

### Discussion

Coronaviruses and rotaviruses are considered to be the major infectious causes of calf scours.<sup>2</sup> Of the several methods used for the detection of enteropathogenic viruses, TEM appears to be an indispensable diagnostic tool with negatively stained fecal samples.<sup>18</sup> TEM has been reported to be a more sensitive and valuable technique than DFA and virus isolation for detecting rotaviruses and coronaviruses.<sup>27</sup> However, the presence of coronavirus-like particles presents difficulty in the diagnosis of BCV by TEM and requires confirmation using other diagnostic methods.<sup>4</sup> A comparison of virus isolation (by shell vial technique) and DFA, as alternatives to TEM and considering TEM as the “gold standard,” has shown relative sensitivities of 98% and 80%, respectively.<sup>37</sup> In our study, comparison of IHC and DFA, considering TEM as the “gold standard,” indicated relative sensitivities of 83% and 63%, respectively, which favors the use of IHC over DFA as an alternative to TEM. Other studies designed to detect different animal viruses have favored the use of IHC in parallel with TEM, e.g., in the investigation of porcine epidemic diarrhea,<sup>36</sup> bovine viral diarrhea virus,<sup>6</sup> and rinderpest virus.<sup>7</sup> In another study, IHC, ELISA, and TEM were compared for the detection of BCV in cases of calf diarrhea.<sup>13</sup> Agreement among the 3 techniques was 93%, which supports our findings of fairly close agreement between IHC and TEM for the detection of BCV.

In the present study, IHC was used for the detection of BCV in formalin-fixed, paraffin-embedded intestinal tissues, and the results were compared with those of TEM and DFA. An overall agreement of only 56% was found among the 3 methods; the overall  $\kappa$  value of 0.368 ( $SE_{\kappa} = 0.05$ ) indicated that the observed agreement was greater than that expected by chance. The results of IHC and TEM showed a 78% association with a  $\kappa$  value of 0.623, indicating a substantial degree of agreement between the 2 methods and thus supporting the suitability of IHC as an alternative to TEM. As shown in Table 3, 22 of 127 fecal samples were positive for BCV by TEM, but their corresponding intestinal tissues were negative by IHC. These results may be attributed to 1) the destruction of antibody-specific viral epitopes during tissue processing,<sup>15</sup> 2) difference in the number of virus particles present in feces as compared with those in the intestinal tissues, and 3) presence of nonviral particles in TEM preparations that are morphologically similar to coronaviruses but are not true virus particles. The recognition of virus-specific antigenic epitopes by antigen-specific antibody decreases the possibility of false virus detection by IHC.<sup>4</sup> The detection of 5 samples that were negative by TEM but positive by IHC (Table 3) may be explained by low numbers of virus particles in feces, which were beyond the detection limits of TEM.<sup>12</sup>

Overall agreement of 70% ( $\kappa = 0.407$ ) and 64% ( $\kappa = 0.277$ ) (Tables 4, 5) between DFA and TEM and between DFA and IHC, respectively, indicated a moderate amount of agreement between DFA and TEM and a relatively lesser degree of agreement between DFA and IHC. These results are consistent with those of previous studies.<sup>27</sup>

Initially, nonspecific staining was observed in IHC, which was later eliminated by adjusting the incubation period of tissue sections with secondary antibodies and

DAB. The use of MAb Z3A5 for IHC allowed BCV antigen to be revealed in paraffin-embedded tissue as much as 4 years of age and hence can be recommended for use as an epidemiologic and diagnostic tool for BCV infections. The sensitivity of the technique can further be improved by testing a range of MAbs against different epitopes,<sup>16</sup> the use of different proteolytic enzymes to improve reagent accessibility to antigen,<sup>1</sup> and the use of chromogen other than DAB because DAB may be problematic in the presence of large amounts of heme pigment in the specimen.<sup>26</sup>

Bovine intestinal specimens received for routine pathologic examination can be used for the detection of BCV without the need for specialized procedures for preservation of specimens or need of extraordinary skill and sophisticated equipments such as cryostat, electron microscope, or fluorescent microscope. IHC seems to be convenient and sensitive enough for widespread use as a diagnostic tool for the detection of BCV.

### Acknowledgements

We thank Kinley Larntz and Will Marsh for help in statistical analysis of data and Kem Pomeroy, Chris Thurn, Jackie Evanson, Donna Gulbranson, and Mary Ito for expert technical assistance.

### Sources and manufacturers

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- c. Immunon, Pittsburgh, PA.
- d. Veterinary Medical Research and Development, Pullman, WA.
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