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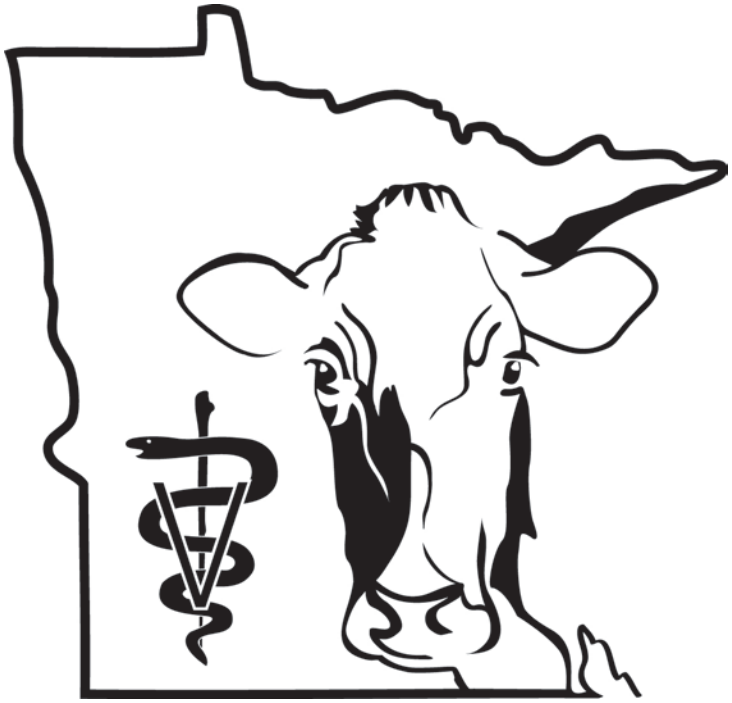

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New Laboratory Techniques for Diagnosis of Dairy Cattle Diseases

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Introduction

Of all the techniques available for the analysis of DNA and RNA, none has had the potential value of the polymerase chain reaction (PCR). PCR can selectively amplify a single molecule of DNA or RNA several million fold in a few hours so highly sensitive analyses can be performed on extremely small sample volumes. Contemporary dairy practitioners should be well informed about the potential applications and limitations of PCR testing because PCR-based diagnosis is becoming widely applied in veterinary diagnostic laboratories.

The Polymerase Chain Reaction Method

Enzymatic amplification of a DNA or RNA fragment is the basis of PCR. Short pieces of DNA consisting of a small number of nucleotides, (oligonucleotide primers) specifically bind (hybridize) to the opposite strands of the DNA fragment and "prime" synthesis of the complementary DNA sequence. Repeated cycles of heat denaturation (separates the double strands of DNA), primer hybridization, and DNA synthesis by the enzyme DNA polymerase result in exponential amplification (2,4,8,16,32,...copies) of the target DNA sequence. With the use of automated PCR machines (thermal cycler) a round of amplification takes only minutes. In only a few hours, millions of sequence copies can be produced; after 30 rounds of amplification, over a billion copies of sequence are created! With a few additional steps, PCR can be applied to RNA analysis thereby enabling RNA as well as DNA detection.

PCR Applications for Dairy Cattle Disease Diagnosis

PCR is rapidly becoming a standard method for analysis of DNA and RNA samples in veterinary research laboratories. PCR tests have been developed for many infectious agents. Nevertheless, few of the assays are routinely used for diagnosis because of concerns about expense, sensitivity (too sensitive), and specificity (contamination). Fortunately, new PCR technologies that are faster, less expensive, more sensitive, and less prone to contamination are overcoming the limitations of earlier procedures.

PCR testing offers several advantages over conventional biologic assays including speed, improved sensitivity, and ability to detect agents that are difficult to culture. The ability to detect infectious agents regardless of their viability is another advantage of PCR. For example, abortigenic agents can be detected by PCR even though fetuses are partially decomposed and the infectious agent is no longer viable.

The BVD TaqMan™ test

We developed a TaqMan™ test to automate the detection of BVD virus in tissue and serum (1ml required). The test detects as little as 10 $TCID_{50}$ BVD virus types I and II and Border Disease viral RNA (Table 1). The test is highly specific and does not cross react with Bovine Respiratory Syncytial, Bovine Rhinotracheitis or $Parainfluenza_3$ viruses. Stability studies indicate that BVD virus RNA is stable for years at –70C, 6 months at –20C, 5 days at 4C, 3 days at 25C and 3 days at 37C (Table 2). Thus, submission of refrigerated serum and/or tissue provides satisfactory samples.

The BVD virus TaqMan™ PCR Test is reliable. Fifty tissue homogenates found to be positive by virus isolation were tested using TaqMan™ PCR. Samples from dairy cattle in Minnesota, Wisconsin and Iowa were collected from 1990 through 1997. Forty-five of the 50 samples were found to be positive by PCR. Five samples that tested negative by PCR but positive by virus isolation may have had age-related degradation of BVD genetic material because some specimens were > 10 years old. There were no false positives in the study. To date, the PCR test results correlate favorably with other tests used to detect BVD virus. Preliminary results indicate that TaqMan™ PCR is more sensitive than virus isolation and immunohistochemistry for detection of fetal BVD virus infection.

The TaqMan™ BVD test has the capacity for full-scale automation enabling us to process many samples at lower cost and provide results rapidly (48-72 hours for serum). All BVD positive samples are confirmed by repeating the extraction and the PCR.

Mycobacterium paratuberculosis TaqMan™ PCR Test

The University of Minnesota has developed a TaqMan™ PCR test for *Mycobacterium paratuberculosis* in milk (5 ml required) and in culture. The test detects the genetic material (DNA) of the bacterium *M. paratuberculosis*. The *M. paratuberculosis* TaqMan™ PCR Test will detect bovine *M. paratuberculosis* and elk *M. paratuberculosis*. We have not tested strains of *M. paratuberculosis* from sheep and goats but anticipate that the test will detect those strains as well. The test is highly specific (Table 3). There is no cross reaction with *M. avium, M. phlei, M. terrae, M. fortuitum, M. scrofulaceum, M. intracellullare, and M. smegmatis*. The *M. paratuberculosis* TaqMan™ PCR Test detects about 100 organisms per ml (Table 4). The genetic material in these samples is stable for years at –70C and –20C, and weeks at 4C. Stability at 22C and 39C is unknown. Application of the TaqMan™ PCR test will enable more rapid identification of *M. paratuberculosis* from culture and more efficient screening to detect Johne's Disease herds.

Summary

The advantages offered by TaqMan™ PCR and other molecular technologies will ensure widespread application of these technique in laboratories throughout the world. It is plausible that veterinary practitioners in the near future will submit specimens for "PCR panels" to detect respiratory, enteric, or reproductive pathogens. The availability of relatively new and improved PCR procedures will greatly assist pathologists and veterinarians in their quest to achieve accurate and timely diagnoses.

References

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Table 1. BVD Taqman™ PCR sensitivity.

Concentration (TCID50 per ml)	Rn Values	Positive or Negative
1,000,000	26.73	Positive
100,000	23.36	Positive
10,000	18.86	Positive
1,000	14.23	Positive
100	9.18	Positive
10	3.95	Positive
1	2.23	Negative
0.01	2.21	Negative
Negative	2.63	Negative

Table 2. Stability of BVD RNA.

Extraction #	Condition	Result	Rn Value
8	Day 0	positive	18.81
9	24 hrs, 37 ^{0}	positive	18.03
10	24 hrs, 25 ^{0}	positive	18.02
11	24 hrs, 4 ^{0}	positive	17.85
12	48 hrs, 37 ^{0}	positive	18.47
13	48 hrs, 25 ^{0}	positive	18.29
14	48 hrs, 4 ^{0}	positive	18.50
15	72 hrs, 37 ^{0}	positive	18.33
16	72 hrs, 25 ^{0}	positive	19.26
17	72 hrs, 4 ^{0}	positive	18.04
18	96 hrs, 37 ^{0}	positive	16.99
19	96 hrs, 25 ^{0}	positive	17.11
20	96 hrs, 4 ^{0}	positive	18.82
21	114 hrs, 37 ^{0}	positive	16.35
22	114 hrs, 25 ^{0}	positive	16.86
23	114 hrs, 4 ^{0}	positive	18.66

	Temperature		
Time (hrs)	37 ^{0}C	25 ^{0}C	4 ^{0}C
0 hrs	18.81	18.81	18.81
24 hrs	18.03	18.02	17.85
48 hrs	18.47	18.29	18.5
72 hrs	18.33	19.26	18.04
96 hrs	16.99	17.11	18.82
114 hrs	16.35	16.86	18.66

Table 3. Specificity of the TaqMan™ PCR test for *Mycobacterium paratuberculosis.*

Sample	Sample Type	# Organisms per ml	Ave Rn Value	Pos / Neg
7	M. paratb ATCC	1,000,000	16.99	Positive
10	M. paratb ATCC	100,000	18.02	Positive
13	M. paratb ATCC	10,000	15.35	Positive
16	M. paratb ATCC	1,000	7.32	Positive
19	M. paratb ATCC	100	1.39	Neg
22	M. paratb ATCC	10	1.55	Neg
25	M. paratb DT 100	1,000,000	18.94	Positive
28	M. paratb DT 100	100,000	18.18	Positive
31	M. paratb DT 100	10,000	16.69	Positive
34	M. paratb DT 100	1,000	16.59	Positive
37	M. paratb DT 100	100	7.69	Positive
40	M. paratb DT 100	10	7.33	Positive
70	M. avium JTC	1000	1.46	Neg
73	M. phlei ATCC	1000	1.55	Neg
76	M. terrae JTC	1000	2	Neg
79	M. fortuitum JTC	1000	2.11	Neg
82	M. avium complex	1000	1.8	Neg
85	M. scrofulaceum	1000	1.85	Neg
94	M. intracelullare	1000	1.64	Neg
97	M smegmatis	1000	1.47	Neg

Table 4. Sensitivity of the TaqMan™ PCR test for *Mycobacterium paratuberculosis.*

Sample #	Dilution (organisms per ml)	Ave Rn Value	Pos/ Neg
1	6,000,000	17.04	Positive
2	600,000	16.77	Positive
3	60,000	16.65	Positive
4	6,000	12.55	Positive
5	600	8.24	Positive
6	60	3.71	Positive
7	6	1.93	Neg
8	0	1.67	Neg
9	6,000,000	17.65	Positive
10	600,000	17.04	Positive
11	60,000	15.77	Positive
12	6,000	10.07	Positive
13	600	6.22	Positive
14	60	2.57	Neg
15	6	1.84	Neg
16	0	1.94	Neg