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Molecular genetic techniques and oligonucleotides for mycoplasma identification – a review

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Abstract

Control of distribution of mycoplasmal infections in cattle herds is essential in the majority of countries world-wide. Various PCR procedures are available to detect mycoplasmas in cell cultures and bovine mycoplasma in different types of samples. We reviewed some common PCR techniques and specific primers targeted to different bacterial genetic regions of mycoplasma. Several researchers used the same PCR approach and *Mycoplasma* spp. as a target but their results could not be compared because different primer pairs were used. These methods and primers were first developed to identify mycoplasma species that contaminate animal cell cultures, and then were used by other researchers to differentiate mycoplasmas as a cow infecting agent. Our analysis of the specificity of these primer pairs to nucleotide sequences of five *Mycoplasma* spp. showed that oligonucleotides have less specificity to them. Numerous commercially available PCR kits are applicable to find mycoplasma contamination in cell cultures and fewer of them can be used in veterinary diagnostics. Although serological and culture techniques are still used, it is necessary to develop a new multiplex PCR technique with a more specific primer set especially in agrarian countries.

PCR, species-specific primers, sequencing, nested PCR, real-time PCR, cow disease

It is known that in industrial cattle breeding, the use of a basic method of reproduction such as artificial insemination does not always guarantee a high fertility rate of cows due to the possibility of semen contamination. Breeding efficiency depends largely on timely prevention and treatment of the diseases of reproductive organs that cause infertility, lowering the number of calves born. Poor uterine conditions due to obstetrical and genital diseases (such as retention of placenta, endometritis, subinvolution of the uterus etc.) cause alterations in the fertilization process and can also lead to cattle infertility (Marusich 2017).

In recent years, farms in Kazakhstan have suffered major economic losses due to the wide distribution of non-contagious animal diseases. Among the opportunistic microorganisms associated with non-contagious diseases, mycoplasmas are of great importance, and in synergy with the viral and bacterial microbiota, they can increase the severity of various diseases in the cattle, including respiratory diseases, diseases of the reproductive organs, urogenital tract, arthritis, otitis, and others.

Inflammation of the cattle mammary gland is often caused by various microorganisms, including streptococci and staphylococci (Zhumanov et al. 2015). However, in 1961, mycoplasmas were isolated in cows with severe mastitis in the USA. It has been identified that *Mycoplasma bovis* is the most important mycoplasmal pathogen in farm animals world-wide. This species of mycoplasma is the causative agent of mastitis and arthritis in adult cows and is also reportedly associated with cases of abortions (Hotzel et al. 1993). The second most common mycoplasma in cattle is *M. californicum*, associated with mastitis in adults, as well as arthritis and pneumonia in calves. The species of *M. bovigenitalium* is isolated from the cow's reproductive tract and is associated with vulvovaginitis and

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infertility, as well as postnatal diseases such as dystocia and endometritis (Parker et al. 2017).

At present, more than 100 mycoplasma species have been described, many of which are pathogenic to humans and animals (cows, sheep, pigs, birds) whereas other mycoplasmas infect plants and insects. Studies have shown that not all species of mycoplasma can cause disease, animals are often carriers of the pathogen and the infection process can be asymptomatic for a long time. The percentage of cows affected by mastitis is known to be 25% annually, while the disease can be progressing in a latent form in more than 50% of animals (Adilbekova et al. 2015).

The traditional method of mycoplasma detection is their cultivation on highly enriched isolation media such as Spiroplasma medium SP-4, Difco pleuropneumonia like organism broth (PPLO), Friis medium, Hayflick's agar medium containing 10–20% of horse serum (Friis 1975; Nakagawa et al. 1992; Tang et al. 2000). However, the microbiological method of cultivation has a number of limitations, including their slow growth within 7–30 days, as well as difficulties in differentiating *Mycoplasma* species and its discrimination from non-pathogenic *Acholeplasma* bacteria with similar colonial morphology (Hotzel et al. 1993). Because of the highly contagious nature of the pathogen and the effects that bovine mycoplasma may have on the entire herd, it is important to rapidly obtain the final results to minimize the spread of infection. Methods of serological analysis can be used to screen *M. bovis* antibodies or antigens in clinically diseased animals, so early detection of infection is not possible in this case (Sachse et al. 1993). Diagnosis of genital infections associated with mycoplasmas through PCR analysis is a much quicker, sensitive and specific method, and has several advantages, especially if some strains of mycoplasma are non-culturable. In this study, we investigated the main molecular genetic techniques and primer pairs used to detect and identify mycoplasmal pathogens, including infectious agents that can be involved in the decrease of cattle fertility.

PCR techniques for mycoplasma detection

Developed in the 1990s, the traditional PCR method used for mycoplasma identification is based on the amplification of conserved bacterial 16S rRNA gene. The use of available NCBI GenBank and EMBL nucleotide databases with alignment of 16S rRNA sequences made it possible to select genus-specific MGSO-GPO-1 primers to detect species of the *Mycoplasma* genus, as well as other mycoplasmas such as *Ureaplasma* spp., *Spiroplasma* spp. and *Acholeplasma* spp. (Van Kuppeveld et al. 1992). Although PCR with universal pH-pA pair primers and further partial 16S rRNA sequencing related to the gold standard method are often used for identification of mycoplasma species. To identify 60 strains from 27 different mycoplasma species and subspecies, Stakenborg (2005) and co-authors used the ARDRA method, based on the digestion of the amplified fragments of the conserved 16S rRNA locus. However, this method requires a large number of restriction endonucleases, including *Alu*I (AGˆCT), *Bfa*I (CˆTAG), *Hpy*FI0VI (GCNNNNNˆNNGC) (Stakenborg et al. 2005).

Like most prokaryotic organisms, mycoplasmas have 1–2 operons containing ribosomal RNA (rRNA) genes. It has been established that in all mycoplasmas, the DNA sequence of the spacer region located between the 16S rRNA and 23S rRNA genes is rich in A and T nucleotides, and also does not contain genes encoding transport RNA (tRNA). In a study of 16S-23S rRNA spacer region in *Acholeplasma laylawii* and using primers developed for the identification of lactobacilli, it was found that unlike mycoplasma, acholeplasma contains two loci of tRNA (Nakagawa et al. 1992). Comparative study of the sequences of 16S-23S rRNA spacer region was carried out for phylogenic analysis of mycoplasma species belonging to cluster *M. mycoides* (Harasawa et al. 2000). In that work, PCR

Table 1. List of primers used to identify *Mycoplasma* spp. by the sequencing method.

Name of primers	Sequence of Forward primer 5' -3'	Sequence of Reverse primer 5' -3'	Size of the PCR product	Genetic locus	References
pA - pH	5'-AGA GTT TGA	5'-AAG GAG GTG	1500 bp	Bacterial 16S rRNA for sequencing	Stakenborg et al. 2005
	TCC TGG CTC AG-3'	ATC CAG CCG CA-3'			Boonyayatra et al. 2012
MGSO-GPO-1	TGC ACC ATCTGT	ACT CCT ACG GGA	715 bp	Mycoplasma genus specific 16S rRNA	Van Kuppeveld et al. 1992
	CAC TCT GTT AAC CTC	GGC AGC AGTA			
F-R	CCC GTC ACA CCA	TCG GCT CCA TTT	226-228 bp	16S-23S rRNA spacer region for sequencing	Harasawa et al. 2000
	TGA GAG TT	TCC AAG GC			
F1-R1	ACA CCA TGG GAG (Y)TG GTA AT	CTT C(W)T CGA CTT (Y)CA GAC CCA AGG CAT	250 bp	16S-23S rRNA spacer region for sequencing	Harasawa et al. 2002 Harasawa 1999

W - A or T, Y - C or T

was carried out with F-R primers, and then products of DNA amplification were sequenced. However, in other studies, as well as in studying the genetic relationships of genetically related mycoplasmas and the species of *M. haemomuris*, which was previously identified as *Haemobartonella muris*, researchers already provided sequences of other F1-R1 primers (Harasawa 1999; Harasawa et al. 2002). Table 1 shows the nucleotide sequences of primers used in genus-specific PCR and in the sequencing genetic loci of mycoplasmas.

The PCR amplification with primers specific to the conserved region of the 16S rRNA gene must be accompanied by using primers peculiar to each bacterial species. For the detection and discrimination between different mycoplasma species as contaminants in cell cultures, Harasawa et al. (1993) have developed a nested PCR method with two pairs of primers necessary to amplify a heterogeneous 16S-23S rRNA spacer region. This work is based on PCR with flanking MCGpF11-R23-R1 and internal R16-2-MCGpR21 primers, which were derived from the known nucleotide sequence of the conserved region of rRNA operon of mycoplasma (Nakagawa et al. 1992). As a result of PCR, the primer set generated one specific DNA fragment of 236-365 base pairs (bp) with each of the 8 mycoplasma species of cell culture contaminants, while *A. laylawii* gave a specific pattern with two discrete bands of 430 and 223 bp in length. Due to the fact that some closely related species of mycoplasma give a cross-reaction during serological analysis, as well as for the differentiation of strains within a single species, the RFLP analysis of PCR products is often used. Thus, the identification of 9 species and strains of mycoplasma was done by digestion of DNA fragments using *Vsp*I, *Hind*III, *Hinc*II, *Cla*I, *Pvu*II, *Hae*III enzymes (Harasawa et al. 1993). The sequences of oligonucleotides used in these studies are shown in Table 2.

The sensitivity for the PCR detecting *A. laidlawii* was increased to the level of 20–180 CFU/ml by incorporating three additional primers specifically selected from 16S and 23S rRNA region of the species (Tang et al. 2000). In the process of mycoplasma detection, Tang et al. (2000) used the same primer set of MCGpF11-R23-R1 and R16-2-MCGpR21 indicated as F1-R1 and F2-R2, but in this work with reference to Nakagawa (1992), we can see another reverse primer R1 with sequence of 5'-CTT C(A/T)T CGA CTT (C/T/)CA GAC CCA AGG CAT-3' that is recommended by Harasawa (1996) and Harasawa

Table 2. List of primers used in nested PCR to detect the *Mycoplasma* spp.

Name of primer pair	Sequence of Forward primer 5'-3'**	Sequence of Reverse primer 5'-3'**	Size of the PCR product	Target species. of Mycoplasma spp	References
Flank MCGpF11 -R23-R1	ACA CCA TGG GAG (C/T)TG GTA AT	CTC CTA GTG CCA AG(C/G) CAT (C/T)C	-	-	Harasawa et al. 1993
FlankF1-R1	ACA CCA TGG GAG (C/T)TG GTA AT	CTT C(A/T)T CGA CTT (C/T)CA GAC CCA AGG CAT	370-500 bp	9 mycoplasmal species	Tang et al. 2000; Harasawa et al. 2002; Sung et al. 2006;
FlankF1-R1	ACA CCA TGG GAG (C)TG GTA AT	CTT C(A)T CGA CTT (T)CA GAC CCA AGG CAT	-	-	Baird et al. 1999
FlankF1 / R1	ACA CCA TGG GAG (C)TG GTA AT	GCA TCC ACC A(A/T) A (A/T)AC (T)CT	340-660 bp	11 species of *Mycoplasma* spp.	Uemori et al. 1992
Nested R16-2 -MCGpR21 (Nested F2-R2**)	GTG (C/G)GG (A/C) TG GAT CAC CTC CT	GCA TCC ACC A(A/T) A (A/T)AC (C/T)CT T***	236-365 bp	*M. pirum, M. fermentans, M. orale, M. arginini, M. hominis, M. genitalium, M. hyorhinis, M. pneumoniae, M. salivarium*	Harasawa et al. 1993; Tang et al. 2000**
Nested FN2-R2	ACC TCC TTT CTA CGG AGT ACA A	GCA TCC ACC A(A/T) A (A/T)AC (C/T)CT T***	223-353 bp	11 mycoplasma species	Sung et al. 2006
Nested FN3-RN3	TAT TTG CTA TTC AGT TTT CAA AGA AC	GGG GTG AAG TCG TAA CAA GGT AT	165-233 bp	*M. hyorhinis, M. neurolyticum*	Sung et al. 2006
Nested F2 / R2	GTT CTT TGA AAC TGA AT (F2 / R1**)	GCA TCC ACC A(A)A (A)AC (T)CT	210-215 bp	*M. bovis*, other *M. californicum* and species of *Mycoplasma* spp.	Baird et al. 1999; Uemori et al. 1992**11
One-step PCR F2-R2	GTG (C/G)GG (A/C) TG GAT CAC CTC CT	GCA TCC ACC A(A/T) A (A/T)AC (C/T)CT T***	370 bp	*M. bovis*, *M. bovigenitalium*, *M. californicum*	Boonyayatra et al. 2012

* - some authors showed primers with degenerate nucleotide base, shown in squares and in bold; ** - researchers showed the renamed primer pair; *** - primer sequences have an additional nucleotide base, shown as underlined

et al. (2002). Except for research by Tang et al. (2000), these universal F1-R1 primers are found in studies to identify 14 serovars of *Ureaplasma urealyticum* (Harasawa and Kanamoto 1999). For the first time, these oligonucleotides F1 and R2, homologous to the corresponding genes of 16S and 23S rRNA, were developed and used in the first round of PCR and denoted as F1 and R1 (Uemori et al. 1992). In the nested PCR with 8 different species of mycoplasma contaminants in the cell cultures, the results of Tang et al. (2000) showed generating a single DNA fragment in the range of 236–365 base pairs (bp), while *A. laylawii* produced a characteristic two-banded pattern with amplicons of 426 and 219 bp. The researcher carried out further species identification of mycoplasmas using endonucleases of *Vsp*I, *Cla*I and/or *Hind*III to cleave the obtained PCR products. However, some of the PCR results of Tang et al. (2000) and 80 et al. (1993) do not coincide while using the same restriction endonucleases and studying the same *Mycoplasma* spp. It is clear that these discrepancies are related to the use of different pairs of oligonucleotides in PCR.

For the purpose of PCR discrimination of different *Mycoplasma* spp., primers are being designed from sequences not only conserved regions of the 16S *r*RNA gene and variable 16S-23S rRNA spacer region, but researchers have also utilized primers specific to other targets (Harasawa et al. 2004). Although many researchers used a two-stage PCR method developed by Harasawa et al. (1993) with the above-mentioned primer pairs and various modifications to detect the *Mycoplasma*. Initially, Harasawa et al. (1993) and Tang et al. (2000) optimized the PCR method to differentiate 9 mycoplasma species of *M. pirum, M. fermentans, M. orale, M. arginini, M. hominis, M. genitalium, M. hyorhinis, M. pneumoniae, M. salivarium,* and *A. laylawii* as the most common contaminants of cell cultures. Sung et al. (2006) used the nested-PCR with F1-R1 primers for the first round to detect 13 species of mycoplasmal contaminants, including the above mentioned species, and also *M. arthritidis, M. bovis, M. neurolyticum, M. pulmonis*. However, after the first stage of PCR, DNA fragments were not found for all species of *Mycoplasma*. Therefore, the researchers had to use R2 and designed FN2 primers in the second round of PCR for 11 species of *Mycoplasma*, and another internal pair FN3-RN3 for the species of *M. hyorhinis* and *M. neurolyticum*. Accurate species specific PCR identification of mycoplasmas may require up to three or more pairs of oligonucleotides for each species (Kong et al. 2001).

Boonyayatra et al. (2012) developed new real-time PCR assays for detecting three mycoplasma species causing cattle mastitis, and the obtained results were compared using 16S rRNA partial sequencing. These researchers also used a single-stage PCR described earlier by Tang et al. (2000) with the previously mentioned F2-R2 primers, the published sequences of which coincide, as can be seen in Table 2 (Nakagawa et al. 1992). As a result, PCR products for type strains of *Mycoplasma* spp. were 370 bp, that were processed by enzyme *Ase*1 having the same restriction site of AT^TAAT as well as for *Vsp*I. After the restriction, digested fragments 140, 125 and 115 bp were produced for *M. bovis*, 265 and 115 bp are for *M. bovigenitalium*, while the DNA amplicon of *M. californicum* could not be cut by this enzyme. Species identified by 16S rRNA sequence analysis were confirmed in 100% agreement by PCR-RFLP and real time PCR methods for all investigated 228 of *M. bovis* and 22 of *M. californicum* field isolates. However, none of 5 field isolates of *M. bovigenitalium* were correctly identified, since the restriction profile of two strains was similar to those of *M. californicum*, and the other three isolates had quite different DNA pattern (Boonyayatra et al. 2012).

In a nested PCR reaction consisting of two rounds of amplification, Baird et al. (1999) with reference to Uemori et al. (1992) used the same commercially available outer F1 and R1 primers as well as internal F2 and R2 primers. It is indicated that flanking primers F1 and R1 are designed to amplify the 16S-23S rRNA spacer region, while internal F2 and R2 are annealed at the intergenic 16S-23S rRNA sequence and at the 3'-end of the 23S rRNA gene, respectively. From Table 2 it can be seen that the nucleotide sequence of

forward F2 primer differs from the sequences presented in the published researches of Tang et al. (2000) and Boonyayatra et al. (2012). Baird et al. (1999) used F1, F2, and R2 primers in PCR, the sequences of which are presented in the study by Uemori et al. (1992) but were labelled as F1, F2, and R1, respectively. Due to DNA amplification in the first and second stages of nested PCR, fragments with different lengths were produced for mycoplasmas *M. bovis, M. alkalescens, M. californicum, M. bovigenitalium* and *M. canadense*. Two-stage PCR using these primers made it possible to differentiate the two species of *M. bovis* and *M. californicum*, which had unique amplicons of 210 and 215 bp, respectively. The standard PCR was used by other researchers to quickly and easily screen mycoplasma mastitis in dairy cattle, and to develop a simplified version of PCR detection procedure that excludes the DNA isolation stage (Higuchi et al. 2011).

Oligonucleotides specificity for PCR detection

Above is described the use of the PCR technique for the detection of bovine mycoplasmas along with the primer pairs originally designed to identify the mycoplasmal species contaminating animal cell cultures. The international nucleotide sequence databases for microorganisms are being updated, which makes it possible to check the specificity of previously designed primers in relation to different mycoplasma genomes (https: //www.ncbi.nlm.nih.gov/nuccore/). We used the commonly known PrimerBlast program to check the Flank F1-R1 primers specificity for five selected organisms of *Mycoplasma* spp. Genome sequences of *M. bovis* PG45 (NC_014760.1), *M. californicum* strain ST-6 (NZ_CP007521.1), *M. bovigenitalium* strain NCTC10122 (NZ_LR214970.1), *M. canadense* strain HAZ360 (NZ_AP014631.1) and *M. bovirhinis* strain NCTC10118 (NZ_LR214972.1) are available in GenBank database.

In comparison to *M. canadense* strain HAZ360 and *M. bovirhinis* strain NCTC10118, the sequences of two loci in *M. bovis* PG45 and *M. bovigenitalium* strain NCTC10122 do not have perfect matches to the F1-oligonucleotide, due to the difference in one nucleotide (Fig. 1, underlined nucleotide A). Whereas one of the two locus sequences of *M. californicum* ST-6 have two mismatches (underlined GA) to this primer on the 5'-end. The targeted sequences of 5 mycoplasma species have near perfect matches to the reverse R1-primer because of differences concerning the degenerate bases already represented in this oligonucleotide sequence (Figs 1 and 2). In PCR with the F1-R1 primers, it is assumed that the sizes of amplicons for these selected targets were ranging between 478 and 490 bp, except for *M. canadense* HAZ360 (367 bp).

Flank F1 5'->3'		**A C A C C A T G G G A G C/T T G G T A A T**
NZ_LR214972.1 *Mycoplasma bovirhinis* strain NCTC10118	822815-822834	. . . . C . . . .
//-//	694584-694603	. . . . C . . . .
//-//	611534-611553	. . . . C . . . .
NZ_AP014631.1 *Mycoplasma canadense* strain HAZ360_1	605663-605682	. . . . C . . . .
//-//	331959-331978	. . . . C . . . .
NZ_LR214970.1 *Mycoplasma bovigenitalium* strain	256316-256335	. A . . . . C . . . .
//-//	567991-567972	. A . . . . C . . . .
NZ_CP007521.1 *Mycoplasma californicum* strain ST-6	595800-595781	. A . . . . C . . . .
//-//	555814-555795	G A . . . . C . . . .
NC_014760.1 *Mycoplasma bovis* PG45	322481-322500	. A . . . . C . . . .
//-//	317487-317506	. A . . . . C . . . .

Fig 1. Flank F1 primer specificity to selected *Mycoplasma* spp. sequences

Flank R1 5'->3'		C	T	T	C	A/T	T	C	G	A	C	T	T	C/T	C	A	G	A	C	C	C	A	A	G	G	C	A	T
NZ_LR214972.1 *Mycoplasma bovirhinis* strain NCTC10118	823292-823266	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	695061-695035	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	612011-611985	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NZ_AP014631.1 *Mycoplasma canadense* strain HAZ360_1	606029-606003	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	332325-332299	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NZ_LR214970.1 *Mycoplasma bovigenitalium* strain	256801-256775	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	567505-567531	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NZ_CP007521.1 *Mycoplasma californicum* strain ST-6	595315-595341	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	555329-555355	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
NC_014760.1 *Mycoplasma bovis* PG45	322968-322942	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	317976-317950	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Fig 2. Flank R1 primer specificity to selected *Mycoplasma* spp. sequences

Nested F2 5'->3'		G	T	G	C/G	G	G	A/C	T	G	G	A	T	C	A	C	C	T	C	C	T
NZ_AP014631.1 *Mycoplasma canadense* strain HAZ360_1	605774-605793	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
//-//	332070-332089	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
NZ_LR214970.1 *Mycoplasma bovigenitalium* strain	256419-256438	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
//-//	567888-567869	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
NZ_CP007521.1 *Mycoplasma californicum* strain ST-6	555711-555692	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
//-//	595698-595679	.	.	.	G	.	.	A	A	.	.	.	.	T	.	.	.	.	.	.	.
NC_014760.1 *Mycoplasma bovis* PG45	322584-322603	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
//-//	317590-317609	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
NZ_LR214972.1 *Mycoplasma bovirhinis* strain NCTC10118	822927-822946	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
//-//	694696-694715	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.
//-//	611646-611665	.	.	.	G	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.

Fig 3. Nested F2 primer specificity to selected *Mycoplasma* spp. sequences

Nested R2 5'->3'		G	C	A	T	C	C	A	C	C	A	A/T	A	A/T	A	C	C/T	C	T	T
NZ_AP014631.1 *Mycoplasma canadense* strain HAZ360_1	606006-605988	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	T	.	.	.
//-//	332302-332284	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	T	.	.	.
NZ_LR214970.1 *Mycoplasma bovigenitalium* strain	256778-256760	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.	.	.
//-//	567528-567546	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.	.	.
NZ_CP007521.1 *Mycoplasma californicum* strain ST-6	555352-555370	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.	.	.
//-//	595338-595356	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.	.	.
NC_014760.1 *Mycoplasma bovis* PG45	322945-322927	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.	.	.
//-//	317953-317935	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	T	.	.	.
NZ_LR214972.1 *Mycoplasma bovirhinis* strain NCTC10118	823269-823251	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.	T	.	.	.
//-//	695038-695020	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.	T	.	.	.
//-//	611988-611970	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.	T	.	.	.

Fig 4. Nested R2 primer specificity to selected sequences of *Mycoplasma* spp.

Analyzing the nested F2-R2 primer specificity, it was found that the sequences of the investigated mycoplasma genomes have one mismatch to the F2-primer sequence excluding the almost fully matched sequence of *M. canadense* HAZ360. As shown in Fig. 3, one of the two studied loci of *M. californicum* ST-6 has 2 nucleotides mismatched to the F2-primer. The reverse R2 primer is not fully specific to the sequences of 2 loci in *M. bovis* PG45, *M. bovigenitalium* NCTC10122, and *M. californicum* ST-6, having one mismatch. It is interesting that sequences located in three genetic loci of *M. bovirhinis* NCTC10118 have significant number of mismatches (3 nucleotides) to the R2 sequence (Fig. 4).

It is predicted that nested PCR with F2-R2 and targets of *M. bovirhinis* NCTC10118, *M. bovigenitalium* NCTC10122, *M. californicum* ST-6 and *M. bovis* PG45 can produce fragments with 343–364 bp in size, and 233 bp amplicon is for *M. canadense* HAZ360. In general, the PrimerBlast allowed to check the flank F1-R1 and nested F2-R2 primer pairs specificity to 83 and 82 of RefSeq representative genomes. The specificity of F2/R2 primers using by Baird S.C. in second-stage of nested PCR was also checked but against to 170 genome sequences of *Mycoplasma* spp. It was found that these primers have 2–5 mismatches to different loci of many genome sequences available through the GenBank database.

Mycoplasma species specific primers

The computer alignment of the known 16S rRNA gene sequences for 36 species and mycoplasma strains, revealed some consistent differences in the V2 and V3 regions of the gene. Based on these differences in 16S rRNA nucleotide sequences, species-specific primers have been selected for 8 species of *Mycoplasma* spp. and also for *U. urealyticum* isolated from human biomaterials and rats (Van Kuppeveld et al. 1992). Three species-specific pairs of primers have been developed using the pMB2, pMB900, pMB920, pJ5 plasmid library containing inserts of the *M. bovis* genome (Hotzel et al. 1993). It was shown that PCR with *M. bovis*-specific PpMB2 primers do not cross-react with DNA samples of 27 other mycoplasmal species. However, the PpMB920 F/R primers along with the previously designed oligonucleotides for *M. alkalescens, M. bovigenitalium*, and *M. bovirhinis* associated with mastitis and mycoplasma pneumonia in cattle were used by Hirose et al. (2001) to detect the *M. bovis* species. By using species-specific PCR, *M. bovirhinis* was detected in 70% of milk samples from cows with clinical mastitis symptoms whereas *M. bovis* found only in 7% of them.

For mycoplasma detection, Brazilian researchers used previously developed primers species-specific to the 16S rRNA gene of *M. bovigenitalium* and *U. urealyticum* (Masedo et al. 2018). These oligonucleotides were used in a multiplex PCR detection of *M. bovis*, *M. bovigenitalium*, and *U. urealyticum* species in the same biological sample (Tramuta et al. 2011). Three pairs of primers were used to detect the three mycoplasmal species and as can be seen in Table 3, the sequences of the forward primer in these pairs were identical.

Real time PCR for mycoplasma detection

Among the molecular typing techniques, the multilocus variable number of tandem repeats analysis (MLVA) method is new and used to characterise and differentiate the field and vaccine strains of *M. synoviae*, as an important poultry pathogen (Kreizinger et al. 2018). The same approach with evaluated multilocus sequence typing (MLST) showed the highest resolution power to differentiate 40 strains of 44 genotypes of *M. hyopneumoniae*, a common swine pathogen (Feldea et al. 2018). However, the multilocus sequencing analysis based on identifying up to 302 nuclear genes of the *M. synoviae* genome, is very

Table 3. List of species-specific primers used in PCR for mycoplasma detection.

Name	Sequence of forward primer 5' -3	Sequence of reverse primer 5' -3''	Size of PCR produc	*Mycoplasma* spp. targets t	References
PpMB2 F/R	ATA TTG AAA AAG TTA TAT	TAA ACT CTC AGA ATC TA	254 bp	*M. bovis* specific	Hotzel et al. 1993
PpMB920 F/R	GGC TCT CAT TAA GAA TGT C	TTT TAG CTC TTT TTG AAC AAA T	2 kbp	*M. bovis* specific	Hotzel et al. 1993
Pp15 F/R	CTA AAG AAG AAA GTG GC	CAC ATT GAG AGA TGT TC	120 bp	*M. bovis* specific	Hotzel et al. 1993
MboF - MboR	GGC TCT CAT TAA GAA TGT C	TTT TAG CTC TTT TTG AAC AAA T	-	*M. bovis* specific	Hirose et al. 2001
MakF - MakR	GCT GTT ATA GGG AAA GAA AAC T	AGA GTC CTC GAC ATG ACT CG	-	*M. alkalescens* specific	Hirose et al. 2001
MbgF - MbgR	CGT AGA TGC CGC ATG GCA TTT ACG G	CAT TCA ATA TAG TGG CAT TTC CTA C	-	*M. bovigenitalium* specific	Hirose et al. 2001
MbrF - MbrR	GCT GAT AGA GAG GTC TAT CG	ATT ACT CGG GCA GTC TCC	-	*M. bovirhinis* specific	Hirose et al. 2001
F/R	GTT TGA TCC TGG CTC AGG AT	CAA ACG CTT CCT TTT ATA TTA C	198 bp	*M. bovis* specific	Tramuta et al. 2011
F/R	GTT TGA TCC TGG CTC AGG AT	AAG GTA CAT TCA ATA TAG TGG	476 bp	*M. bovigenitalium* specific	Tramuta et al. 2011
F/R	GTT TGA TCC TGG CTC AGG AT	CTC ATA AGC GAG CCG ACA TT	831 bp	*U. diversum* specific	Tramuta et al. 2011

U. diversum - Ureaplasma diversum

expensive and therefore not used in routine diagnostics for mycoplasma detection (Ghanem and El-Gazzar 2018).

In over than the last decade, the real-time PCR method based on using hybridization probes has been introduced into clinical microbiology. This technique allows not only to detect the presence of mycoplasmas in mastitis milk samples and animal tissues in real time within a few hours, but also to differentiate between the closely related mycoplasma species (Cai et al. 2005). To detect *M. bovis* in the milk and lung tissue of cattle, Cai et al. (2005) developed the real-time PCR method with MbsF and MbsR primers and a pair of FRET probes, the sequences of which are presented in Table 4. Selection of MbsF and MbsR oligonucleotides was based on its specificity to the 16S rRNA genes of infectious mastitis pathogens including *M. bovis*, *M. agalactiae*, *Staphylococcus aureus* and *Streptococcus agalactiae*.

The DNA probe set contained at least 2 nucleotides mismatches to the sequence of the *M. agalactiae 16S* rRNA gene and was therefore used to detect differences in melting temperatures of hybridized probe and the PCR fragment of *M. bovis* or *M. agalactiae*. Of all the tested bovine mycoplasma and other bacteria, only *M. bovis* strains were positive in the real time PCR, with a melting peak of 66.6 °C for the DNA fragments, and a positive result was obtained with *M. agalactiae* strain PG2 having a lower melting peak of 63.1 °C.

The protocol utilizing cyanine SYBR-green dye was found to be a less expensive version for the real time PCR technique, which does not require labelled DNA-probes. Harasawa et al. (2005) used a real-time-SYBR-PCR with F1-R1 primers developed previously for conventional PCR to detect 5 mycoplasma species contaminating the cell lines. This analysis showed that mycoplasma

Table 4. List of specific primers used in real-time PCR for mycoplasma detection.

Primers and probes	Sequence of Forward primer 5'-3'	Sequence of Reverse primer 5'-3'	Size of PCR product	Locus	Target species	References
MbsF- MbsR	GAG GCA GCA GTA GGG AAT	GTA TTA CCG CGG CTG C	190 bp	*16S rRNA*	*M. bovis, M. agalactiae, S. aureus, S. agalactiae*	Cai et al. 2005
MbsFL	GAT GAA GGC CCT ATG GGT TGT AAA CTG- FL	-	-	//	-	//
MbsLC	LCRed640 -GTG GTT AGG GAA GAA AAA GTA GCA TAG GAA ATG AT-phosphate	-	-	//	-	//
MbF - MbR	TAA TGC ACG CAA ACT CTC GTA GT	TGT CAC CAG TTG TTG TGC CTT	-	*fusA*	*M. bovis*	Boonyayatra et al. 2012
MbP	6-FAM -ACC AAC AGC AGC AAC AAT ATC ACC TGC-BHQ1	-	-	//	//	//
MbovF - MbovR	TCT AAT TTT TTC ATC ATC GCT AAT GC	TCA GGC CTT TGC TAC AAT GAA C	112 bp	*uvrC*	//	Clothier et al, 2010, Parker et al. 2017
MbovP	FAM -AAC TGC ATC ATA TCA CAT ACT-BHQ1	-	-	//	//	Clothier et al. 2010, Parker et al. 2017
McF - McR	GCA CTT AGA CGA AAG AGG GAT T	GGA TTA TCA TCA CCT TTG GGA CT	-	*rpoB*	*M. californicum*	Boonyayatra et al. 2012
McP	6-FAM -CGT GTT GGT TCG GAA GTG GTT CCA G-BHQ1	-	-	//	//	//
McalP	CAL FluorOrange560 -CGT GTT GGT TCG GAA GTG GTT CCA G-BHQ1	-	-	//	//	Parker et al. 2017
MbvgF -MbvgR	CTT TCT ACG GAG TAC AAA GCT AAT	GAG AGA ATT GTT CYC TCA AAA CTA	-	*16S-23S rRNA* spacer region	*M. bovigenitalium*	Boonyayatra et al. 2012
MbvgP	6-FAM -TAT CGT CAT GGC TTG GTT AGG TCC CA-BHQ1	-	-	//	//	//
MbvgP	CAL FluorRed610-TAT CGT CAT GGC TTG GTT AGG TCC CA-BHQ2	-	-	//	//	Parker et al. 2017

M. bovis - Mycoplasma bovis, M. agalactiae - Mycoplasma agalactiae, S. aureus - Staphylococcus aureus, S. agalactiae - Streptococcus agalactiae, M. californicum - Mycoplasma californicum, M. bovigenitalium - Mycoplasma bovigenitalium

strains have different melting temperatures (*T*m) of double-stranded DNA in respect with *Mycoplasma* species. The values of *T*m were 89.14–89.54 °C for *M. orale*, 89.02–89.42 °C for *M. fermentans*, 88.54–88.94 °C for *M. hyorhinis*, 87.68–87.98 °C for *M. arginini*, and 87.25–87.65 °C for *M. salivarium*.

By Justice-Allen et al. (2011), the SYBR-PCR method was used to detect the presence of and to identify several mycoplasma species other than *M. bovis*. The researchers reported using primers that were previously developed and specific to the conserved locus of rRNA intergenic region of the *Mycoplasma* genus. In addition to this technique, PCR amplicon sequencing was used to confirm species identity in cases where the melting temperature of the DNA fragment did not correspond to the typical *T*m value of 73.2 °C for the *M. bovis* species. The results of this study showed that milk samples from a bulk tank with a negative result of microbiological assessment had a positive test for the presence of *M. bovigenitalium* in the SYBR-PCR assays. So this culture negative sample was identified as *M. bovigenitalium* positive and in PCR analysis produced the amplicon with size of 222 bp and *T*m 73.18 °C. The PCR *M. bovis*-positive samples had an amplified product of 207 bp and *T*m = 73.39 °C, compared to mycoplasmas other that *M. bovis* tended to have lower *T*m and plus a different PCR product in length (approximately 156 bp). In comparison to the traditional microbiological method, the SYBR-PCR analysis did not show any significant sensitivity when detecting different species of *Mycoplasma* spp. in bulk tank milk.

In order to avoid cross-amplification and to precisely identify the closely related mycoplasma species including *M. bovis* and *M. agalactiae*, the researchers recommend to use designed primers specific to different genetic loci of genome DNA isolating from complex biomaterials. Thus Boonyayatra et al. (2012) developed 3 assays of real-time PCR with primer pairs and FAM-labelled DNA probes specific to different loci of the three mastitis agents *M. bovis*, *M. californicum*, and *M. bovigenitalium*.

Table 4 presents sequences of the primer pairs Mb-F and Mb-R, McF and McR, Mbvg-F and Mbvg-R that are specific to the *fusA* gene (encoding for elongation factor G) of *M. bovis*, the gene *rpo*B (encoding for RNA polymerase β subunit) of *M. californicum*, and the 16S-23S rRNA spacer region of *M. bovigenitalium*, respectively. The results of the mycoplasma detection were fully consistent with the 16S rRNA gene sequencing analysis, except for 4 samples identified by real time PCR as *M. bovis* and *M. californicum*. It is known that the method of 16S rRNA partial sequencing allows detecting a dominant species of microorganisms, therefore the sequencing of all the produced amplicons can confirm the presence of two mycoplasma species in samples which was done by the authors for these cases.

Moreover, a multiplex real-time PCR with different species-specific DNA probes labelled with FAM, CAL Fluor Orange 560 and CAL Fluor Red 610 has been developed in order to detect these three mycoplasma species in bovine milk, semen and swab samples (Parker et al. 2017). In their research for rt-PCR identification of *M. bovigenitalium* and *M. californicum*, Parker et al. (2017) applied DNA probes and primers MbvgF and MbvgR, McalF and McalR described by Boonyayatra et al. (2012) and also MbovP, MbovF and MbovR oligonucleotides specific to *M. bovis uvr*C gene and developed by Clothier et al. (2010).

By using the mentioned DNA extraction methods, the multiplex rt-PCR detection limits for mycoplasma *M. bovis* and *M. californicum* in milk samples were 1.3×10^2 CFU/ml and 6.0×10^2 CFU/ml respectively. According to Clothier et al. (2010), the detection level of *M. bovis* in all spikes of milk, lung tissue and suspension culture samples was 2.4×10^2 CFU/ml whereas 10–30 CFU/ml were reported by Boonyayatra et al. (2012) in the single rt-PCR assay. Although the newly developed multiplex real-time PCR had a poorer limit for *M. bovigenitalium* detection in contaminated milk (5.0×10^5 CFU/ml) compared to other mycoplasma species (Parker et al. 2017).

Table 5. List of commercial kits used in PCR for mycoplasma detection.

Kit	PCR technique	Gene target	Samples	PCR amplicon	Mycoplasma detection level	Targets	Manufacturer
LookOut Mycoplasma PCR Detection Kit	16S rRNA PCR	cell suspension, coding region	260 +/-6 bp DNA samples	> 10 genomes/	19 species ul of sample	Sigma-Aldrich, of *Mycoplasma*	MP0035
MycoReport	PCR	16S rRNA	cell suspension, DNA samples	516-519 bp	25-50 copies of DNA	*M. fermentans, M. orale, M. arginini, M. hominis, M. hyorhinis, A. laidlawii*	Evrogen, cat.# MR001
Microsart AMP Mycoplasma	TagMan-real -time PCR	16S rRNA	cell cultures, medium compounds, pharmaceutical products	-	10 cfu/ml *Spiroplasma*,	70 species of *Mycoplasma. Acholeplasma*	Sartorius
PCR Mycoplasma test kit	PCR	16S rRNA coding region	cell cultures	270 bp	110-240 cfu/mL	*M. fermentans, M. hyorhinis, M. penetrans, M. capricolum*	Applichem. A3744, 0020
PCR Mycoplasma test kit II	PCR	16S rRNA coding region	biomaterials	265-278 bp		*Mycoplasma* spp.	Applichem. cat.# A8994
Test system Mik-Dif	real-time PCR	-	biomaterials	-	10^3 genomes/ ml of sample	*M. hyopneumoniae, M. hyorhinis*	LLC InterLabService Vet-60-FRT(RG,iQ)-K2 mycoplasmosis of pigs
Test system Mik-KOM	PCR	-	biomaterials	509 bp	-	*Mycoplasma* spp.	Vet-4-RO, 2-K mycoplasmosis pathogens
Bactotype Mastitis PCR kit	real-time PCR	-	milk	-	-	*S. agalactiae* + *M. bovis, S. aureus, S. dysagalactiae* + *S. uberis,* IC	Indical Bioscience, screening cat.# BT280005
Bactotype Mastitis HP2+ PCR kit	real-time PCR	-	milk	-	-	*S. agalactiae, M. bovis,* pathogenic Mycoplasma	Indical Bioscience, cat.# BT280025

Table 5. List of commercial kits used in PCR for mycoplasma detection.

Kit	PCR technique	Gene target	Samples	PCR amplicon	Mycoplasma detection level	Targets	Manufacturer
VetMax *M. bovis* PCR ki	real-time PCR	-	biomaterial, bovine milk	-	-	*M. bovis*	AppliedBiosystem. Real-time cat.# MPBO50
VetMax Masti Type Myco8 kit	2 step real-time PCR	-	bovine milk	-	-	*Mycoplasma* spp., *M. alkalescens*, *M. bovis*, M. *bovigenitalium*, *M. canadense*, *M. californicum*, *S. aureus*, *S. agalactiae*	AppliedBiosystem, cat. # A39236

A. laidlawii - Acholeplasma laidlawii, S. agalactiae - Streptococcus agalactiae, S. aureus - Staphylococcus aureus, S. dysagalactiae - Streptococcus dysagalactiae, S. uberis - Streptococcus uberis

PCR detection kits for *Mycoplasma* spp.

Most Russian companies offer PCR kits to detect the mycoplasmas belonging to human pathogens, and also mycoplasma contaminants in cell line cultures (Table 5). The MycoReport and Myco real-time PCR assay kits for detecting mycoplasma contaminants in cell cultures are commercially available from the Eurogen company. The PCR test systems developed by the Federal Budget Institution of Science "The Central Research Institute of Epidemiology" (FBIS CRIE) are offered through LLC InterLabService and used to detect the swine pathogens of *M. hyopneumoniae*, *M. hyorhinis*, the poultry pathogens *M. gallisepticum*, *M. synoviae*, and include the Mik-Kom test system for detecting various mycoplasma species.

Numerous foreign manufacturers such as Norgen Biotek Corp. (Canada), PromoCell and Sartorius (Germany) also offer mainly PCR detection systems for mycoplasma in cell culture and for use in research only and not for diagnostics. The MycoSEQ Mycoplasma real-time PCR system based on SYBR green technology for detecting 90 different mycoplasma species, acholeplasma and spiroplasma as contaminants of cell cultures and nutrient media, was developed for use in research only by Applied Biosystems. The AB company developed the VetMax *M. bovis* real-time PCR kit to *M. bovis* diagnosis in various samples, including milk, as well as the VetMax MastiType Multi Kit, MastiType Myco8 Kit, MastiType Micro4 Kit for the detection of mastitis pathogens. Sigma-Aldrich (USA) offers the LookOut Mycoplasma PCR Detection Kit, which can be used for research and in diagnostic procedures to detect mycoplasmal contamination of cell cultures. This kit contains 8-strip microtubes that are pre-coated with dNTPs and mycoplasma-specific primers and also DNA-positive tubes as an internal control (Dobrovolny and Bess 2011). For veterinary use only, the assays with qPCR detection of *Streptococcus agalactiae, M. bovis*, *Staphylococcus aureus*, *Streptococcus dysagalactiae*, and *Streptococcus uberis* are available from the Indical Bioscience (Germany), formerly Qiagen Leipzig.

Conclusions

Among more than 100 species of *Mycoplasma*, many species are recognized as common contaminants in cell cultures while others are of interest in cattle. *Mycoplasma* species are associated with a variety of clinical diseases such as mastitis, arthritis, pneumonia, vulvovaginitis, endometritis and cause the abortion and infertility in infected herds. Outbreaks of the most important mycoplasmas can lead to serious economic losses in farm animals worldwide.

Microbiological culture is an available standard method for routine testing of mycoplasmas and should be followed by serological analysis. For the definitive species identification, one of these techniques is laborious and requires up to a month for mycoplasma growth on several appropriate culture media; the second test can be used with a species-specific antibody and only on clinical animals. At the present time, using PCR technique for mycoplasma detection is becoming more common because it allows to obtain diagnostic results quickly so that the cows shedding pathogens can be effectively removed from the herd at an early stage of infection.

Different PCR protocols for mycoplasma detection have been developed over the last three decades. This process is ongoing with collecting new data on the disease pathogenesis and strains of mycoplasma agents (Gondaira et al. 2017). Compared to methods of gene sequencing, multilocus typing and real-time PCR, traditional and multiplex PCR methods are the simplest and less expensive (Higuchi et al. 2011). It is known that the efficiency of PCR detection of microorganisms largely depends on the level of contamination of the biological sample and the type of material under study. Due to the low initial level of the infection agent, particularly in subclinical animals, many researchers have to optimize the PCR protocol to directly detect mycoplasmas in various materials, including milk, lung tissue, excretion, swabs (Rossetti et al. 2010; Justice-Allen et al. 2011; Parker et al. 2017).

In order to avoid cross-amplification and to precisely identify the closely related mycoplasma species, the researchers recommend to use designed primers specific to different genetic loci of the mycoplasmal genome. So, according to peer-reviewed reports, we can see the use of the PCR technique for the detection of bovine mycoplasmas along with the primer pairs originally designed to identify the mycoplasma species contaminating animal cell cultures. Therefore, it was interesting to verify the specificity of some of these primers to available genome sequences of five *Mycoplasma* spp., as clinically important agents in cattle especially in the international nucleotide sequences databases for microorganisms are always being updated. We have to notice an interesting finding given that the F1-R1 and F2-R2 primer pairs recommended by the researchers for the nested-PCR were specific to *M. bovirhinis* NCTC10118 sequences located in three genetic loci (Baird et al. 1999; Tang et al. 2000; Harasawa et al. 2002; Sung et al. 2006). This case may be explained by a low primer specificity for the target because it is known that mycoplasmas have 1 or 2 *r*RNA operons, although the genome of *M. bovirhinis* species is still not very much established (Nakagawa et al. 1992).

The commercially available PCR mycoplasma detection kits have been developed based on the approaches revised in this study. However, it is important to develop a new multiplex PCR assay for the purpose of identifying *Mycoplasma* spp. other than *M. bovis* on animal farms with mastitis outbreaks as reported by some researchers. Along with this, a new multiplex PCR protocol should be adapted to accurately identify all species without depending on a concentration of them in complex type samples (Parker et al. 2017).

Government programs are aimed to support the development of livestock industry in Kazakhstan but breeders have problems with decreased cow fertility and abortion along with the breeding of highly valuable and productive animals. As showed in the literature, the said problems are widely encountered on large farms using artificial insemination and having

animals transported from foreign countries. Antimicrobial therapy for mycoplasmosis is not effective because of mycoplasma mutations responsible for resistance to a wide range of drugs (Sulyok et al. 2018).

In Kazakhstan, no studies have been conducted on the most prevalent and clinically important mycoplasma species in cattle and no data on the circulating mycoplasma strains are available. In general, the practical importance of PCR methods for mycoplasma detection lies in early diagnosis of the pathogens and providing effective veterinary control, which is especially important in countries with a predominance of livestock industry.

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