Part 2. Biotechnology and genetics

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DEVELOPMENT OF A MULTIPLEX PCR TEST-SYSTEM FOR DETECTION OF BHV-1, BVDV, *CHLAMYDIA* SPP. AND *MYCOPLASMA* SPP.

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Summary. This study describes development of a multiplex PCR assay for detection of BHV-1, BVDV, *Chlamydia* spp. and *Mycoplasma* spp. infections in bovines. The assay was developed using genomic DNA and RNA and four sets of PCR primers targeting 16S rRNA genes of *Chlamydia* spp., *Mycoplasma* spp., 5'-UTR of Bovine viral diarrhea virus, gE of Bovine herpesvirus-1, respectively. A total of 100 tissue samples were collected from cattle suspected to be infected with the viral and bacterial pathogens (BVDV, BHV-1, *Chlamydia* spp. and *Mycoplasma* spp.) from different regions of Ukraine. A part of sample was stored at –50°C for isolation of genomic DNA and RNA. The multiplex PCR assay was optimized in the study. The specific primers designed and used in the study were found sensitive and specific in amplifying target genes viz. 16S rRNA, gE, 5'-UTR of *Chlamydia* spp., *Mycoplasma* spp., BHV-1 and BVDV, respectively. The PCR primers used in the optimization of multiplex PCR assay for detection of Bovine viral diarrhea virus, Bovine herpesvirus-1, *Chlamydia* spp., *Mycolasma* spp. could amplify 221 bp, 111 bp, 386 bp, 279 bp products, respectively. Non specific amplification was not observed

Keywords: Bovine herpesvirus-1, Bovine viral diarrhoea virus, *Chlamydia*, *Mycoplasma*, multiplex PCR, bovines

Introduction. In veal production, bovine respiratory disease (BRD) is the most common and economically important disease. BRD is a multifactorial disease, involving multiple potentially pathogenic microorganisms that causes economic losses due to morbidity, mortality, medication costs, increased time on feeding and associated labor costs (Wisselink et al., 2017; Griffin, 2014; Snowder et al., 2006).

Bovine respiratory disease appears to be precipitated by an imbalance in the triad of interaction among one or more infectious agents, host defenses, and environmental stressors. Viruses isolated from cattle with BRD include Infectious bovine rhinotracheitis virus ((IBRV), Bovine herpesvirus-1 (BHV-1)), Bovine viral diarrhea virus (BVDV), Bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3V), BHV-4 and others. Bacterial pathogens associated with BRD include *Pasteurella haemolytica* Al (now *Mannheimia haemolytica*), *Pasteurella multocida* A:3, *Haemophilus somnus*, *Actinomyces pyogenes*, *Mycoplasma bovis*, *Mycoplasma dispar*, *Mycoplasma hyorhinis*, *Ureaplasma diversum, Chlamydia* spp., etc. (Bowland and Shewen, 2000). Viral and bacterial pathogens together with mycoplasma and environmental risk factors are the most common cause of diseases, ranging from common colds to life-threatening pneumonia. A large number of both RNA and DNA viruses uses the respiratory tract to initiate host infection (Paller et al., 2017).

Infectious abortion is a significant cause of reproductive failure and significant economic losses for the cattle industry. Under optimal laboratory conditions, etiologic diagnosis is achieved in 23.3 to 45.5% of the cases. The variety of infectious agents have also been reported to cause bovine abortion throughout the world, agents such as *Chlamydophila psittaci*, *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Ureaplasma diversum*, Bovine viral diarrhoea virus and Bovine herpesvirus-1 and others (Selim, Elhaig and Gaede, 2014; Maunsell et al., 2011; Miles, 2009; Ellis, 2009).

The purpose of this study was to develop multiplex PCR for detection of four pathogens in a single reaction with high sensitivity and specificity using a DNA template extracted directly from tissue samples.

Materials and methods. ***Collection and screening of clinical materials.*** A total of 100 tissue samples were collected from cattle suspected to be infected with viral and bacterial pathogens (BVDV, BHV-1, *Chlamydia* spp. and *Mycoplasma* spp.) in different regions of Ukraine. A part of each sample was stored at –50°C for isolation of genomic DNA and RNA.

Designing of primers for multiplex PCR. The 16S rRNA, 5'-UTR, gE genes sequences available from public databases GenBank (http://www.ncbi.nlm.nih.gov) were analyzed with BioEdit 7.2.0. PCR primers were derived from these regions with AmplifX primer analysis software (Table).

Table — List of primers used in multiplex PCR

No.	Oligomer name and sequences (5'-3')	Amplicon size
1	*Chl*_F GCGTGTAGGCGGAAAGGAAAGTTA *Chl*_R AAACCACATGCTCCACTGCTTG	386 bp
2	*Myco*_F TGTTTAGCGGGGTTGAGAGACTGA *Myco*_R TTCCGGATAACGCTTGCAACCT	279 bp
3	BHV_F ACGCGGCCATTACAAACCAGTACA BHV_R TGCGCAGGTACTCGGCTTT	111 bp
4	BV_F TGGGAGGTGGAACATGGTGTCATA BV_R ACTAGTTCGATGAGCCTGGTCAGA	221 bp

Our primer design was based of the 16S rRNA, 5'-UTR, gE genes sequences with BioEdit software (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) using CLUSTAL W. The specificity of the primer pairs was also assessed against the NCBI nucleotide sequence database (ftp://ftp.ncbi.nih.gov/blast/db/FASTA). All the primers were checked for their properties like Tm value, length, presence of self and cross dimer using oligonucleotide analyzer software and were also checked for their specificity in BLAST tool of NCBI before custom synthesized.

Optimization of the multiplex PCR assay. PCR conditions were optimized as initial denaturation (95°C for 2 min), for 5 min followed by cycles of denaturation (95°C for 1 min), primer annealing (60°C for 1 min) and extension (72°C for 1 min) along with a final extension of 72°C for 4 min. Reaction was performed in automatic thermal cycler ('Bio-Rad', USA) and total 10 µl PCR product of each sample was electrophorosed in 1.5% agarose gel containing 10 µl/ml ethidium bromide. PCR amplification was visualized under UV light.

Results. ***Optimization of multiplex PCR.*** The PCR primers used in the optimization of multiplex PCR assay for detection of Bovine viral diarrhoea virus, Bovine herpesvirus-1, *Chlamydia* spp., and *Mycoplasma* spp. could amplify 221, 111, 386, and 279 bp products, respectively. Non specific amplification was not observed (Fig.).

Validation of mPCR assay using field samples. DNA and RNA samples (40 known positive and 70 negative samples by using simple PCR), subjected to mPCR revealed comparatively high infection rate for single (35%) as well as for mixed infection (5%). Occurrence of single species infection detected by mPCR was 20% for *Mycoplasma* spp. (20/100), 7% for BVDV (7/100), 5% for *Chlamydia* spp. (5/100), and 3% for BHV-1 (3/100). However, mPCR revealed higher number of mixed infections of BHV-1, BVDV (n = 2), BHV-1, BVDV, *Mycoplasma* spp. (n = 3). Further, all samples tested positive by simple PCR, were also found positive in mPCR with hundred percent agreements.

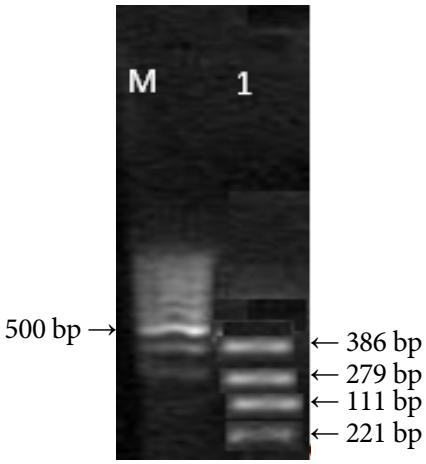

Figure. Standardization of mPCR using positive DNA and RNA of Bovine herpesvirus-1, Bovine viral diarrhoea virus, *Chlamydia* spp., *Mycoplasma* spp. (Lane M: 100 bp DNA ladder, Lane 1: amplicons of 386 bp of *Chlamydia* spp., 279 bp of *Mycoplasma* spp., 221 bp of Bovine viral diarrhoea virus, 111 bp of Bovine herpesvirus-1)

Discussion. In fact, there are many reports available on the development of PCR assays for detection of single pathogens (bacterial or virus) infecting ruminants (Herilovych, Stehnii and Kucheriavenko, 2007; Stehnii, Kovalenko and Kovalenko, 2004).

Some researchers have also developed duplex PCR assays for detection of co-infection of two pathogens infection of bovines (Stehnii et al., 2014).

Detection of multiple pathogens in a single tube will be more convenient for field diagnosis, than the single or duplex PCR assays. Limited reports are available for the development of multiplex PCR. The multiplex PCR assay was optimized in the study.

The specific primers designed and used in the study were found sensitive and specific in amplifying the target genes viz. 16S rRNA, gE, 5'-UTR of *Chlamydia* spp., *Mycoplasma* spp., BHV-1, and BVDV, respectively. Moreover, the size of amplicons ranged between 111 and 386 bp, and the difference between any two fragments ranged between 50 and 100 bp, facilitating easy differentiation of bands on gel electrophoresis.

Conclusion. These results revealed that mPCR developed in the study was capable of specific and sensitive detection of single as well as multiple species infection in cattle.

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