Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 61 (2): 52-57. April 2019
DOI: 10.5455/ajvs.42471

Simultaneous Detection of Brucella, Leptospira, Mycoplasma and Listeria Species by Multiplex-PCR

Omnia M. El-Sayed[1], Alaa E.H. Moustafa[2], Eman Abdeen[2], Adel S. Amin[1]
1 Biotechnology Research Unit, Animal Reproduction Research Institute, Giza, Egypt
[2] Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt

Key words:
Brucella, Leptospira, Mycoplasma, Listeria, Multiplex-PCR Conventional PCR, Abortion

***Corresponding to:**
dr_omnia_ms@hotmail.com

Article History
Received Oct 30 2018
Revised Dec 15 2018
Accepted Jan 01 2019

ABSTRACT

Reproductive disorders in farm animals represent a great importance due to economic losses for breeder and country economy. The main cause of reproductive failure in cattle is infectious abortion. Brucella, Leptospira, Mycoplasma and Listeria are important zoonosis and leading to sever economic losses in animals around the world, especially in Mediterranean countries and Egypt. Since early detection is crucial in control and treatment, molecular techniques proved to be a more rapid, sensitive and specific diagnostic tool. The present study is aimed to detect these four important pathogens using multiplex- PCR. The multiplex PCR has been standardized by using 4-pairs of primers to amplify 31kDa gene encoding protein in Brucella spp., lig gene in pathogenic Leptospira, hlyA gene in Listeria monocytogenes and 16S rDNA in Mycoplasma spp. A total of 161 different clinical samples were used, an expected band at 223bp, 468p, 370 bp and 270bp were obtained from Brucella spp., pathogenic Leptospira, Listeria monocytogenes and Mycoplasma spp., respectively. In conclusion, the results of this conventional m-PCR assay revealed that it can be a valuable tool for simultaneous, rapid, cost saving and reliable detection of these microbial agents causing bovine abortion.

1. INTRODUCTION

High economic lose was resulting due to the reproductive disorders in cattle and buffaloes such as abortions, repeat breeding and infertility (Yoo et al., 2010). Bovine abortion may be due to many causes, but infectious causes are the most important one (Silva et al., 2009). Worldwide, Brucellosis, Leptospirosis, Mycoplasmosis and Listeriosis are considered serious zoonosis and represent an important cause of reproductive losses in animals, especially in Mediterranean countries and Egypt (Gwida et al., 2016). These diseases are characterized by fast spreading, difficulty of control and prevention, time consuming and the cost of treatment (Selim et al., 2014).

As a replacement of time consuming traditional methods, PCR has been used as an important diagnostic tool of abortion in cattle (Anderson, 2007). Several single PCR assays have been used for detection of abortion microbial agents, including Brucella species, pathogenic Leptospira species, Mycoplasma species and Listeria monocytogenes (Sharifzadeh et al., 2008; Poltronieri et al., 2009; Bhure et al., 2012; Chiebao et al., 2015; Mahajan et al., 2017 and Mori et al., 2017). The aim of this work was to develop a rapid, sensitive and specific single-reaction m-PCR for detection of these four abortificient bacterial agents.

2. MATERIAL AND METHODS

2.1. Bacterial strains:

Brucella strains (*B. melitensis* biovar-3), Leptospira strains (*Leptospira icterohaemorrhagiae*), Listeria strains (*Listeria monocytogenes*) and Mycoplasma strains (*Mycoplasma bovis*) were kindly provided form Animal Reproduction Research Institute (ARRI) and Faculty of Veterinary Medicine, Cairo University, and used as positive control in the PCR reaction. All infectious agents used are classified as biohazard (OIE, 2002); the appropriate precautions and biosafety measures were followed during the study.

2.2. Clinical samples:
A total of 161 samples (115 milk samples, 10 semen samples, 6 tissue samples and 15 blood samples 6 tissue samples, 15 blood samples, 5 vaginal swabs and discharges and15 fetal fluids and fetal organs from aborted foeti) were used in this study.

2.3. DNA extraction: Samples were prepared in Tris-EDTA buffer (TE buffer): Tris Hcl (pH 7.8) 10 mM, EDTA (pH 8.0) 1 mM.
DNA extraction was done using DNA preparation kits: 1-Animal and fungi DNA preparation kit (Jena Bioscience), Germany: for genomic DNA purification from animal tissue. 2-DNA Mini kit, (Bio Basic Inc.), Canada: Genomic DNA kit for Extraction of DNA from blood samples.

2.4. Primers: specific primers for detection of 31kDa omp gene in all Brucella spp.: 5'-TGG CTC GGT TGC CAA TAT CAA- 3'(r) and 5'-CGC GCT TGC CTT TCA GGT CTG-3'(f) B4,B5 for all Brucella spp. (Tramuta et al., 2011; Baily et al., 1992); 5'-TCA ATC AAA ACA AGG GGC T-3'(r) ; lig gene in pathogenic Leptospira spp. only 5'- ACT TGC ATT GGA AAT TGA GAG-3'(f) Lig1, Lig2 for pathogenic Leptospira (Palaniappan et al., 2005); hlyA gene in Listeria 5'AAA TCG CGT CCT TGC TGG TCT GA3'(r) and 5'TCG CGA TCA CTT AAG GGC CTT CAT -3'(f) L.mono1,L.mono2 for detection of Listeria monocytogenes (Jami et al., 2010); and 16S rDNA in Mycoplasma spp. 5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3'(f) and 5'- GGG AGC AAA CAG GAT TAG ATA CCC T-3'(r) for all Mycoplasma spp. (Marios et al., 2000).

2.5. m-PCR conditions: In each phase of standardization of multiplex PCR, concentration of primers in the cocktail, annealing temperatures, concentration of template DNA were optimized. The m-PCR was carried out in a 200ul PCR tube with a final volume of 50ul reaction volume; with the following components: 25ul of 2x absolute Master Mix (Thermo scientific, ABgene, UK); 0.2ul of each primer; 3ul of extracted genomic DNA were used as a template and Milli-Q water up to the final volume. The PCR reaction was performed on eppendorf master cycler, nexus gradient, Germany; with the following thermal profile: a single cycle of 5 min at 94°C for the initial denaturation, followed by 30 amplification cycles; of a denaturation step of 40 sec. at 95 °C; then annealing step at 53 °C for 1 min. and finally extension step at 72 °C for 1min. followed by a final extension at 72 °C for 10 min.

2.6. Evaluation of m-PCR: Subsequently, agarose gel electrophoresis of the amplified products was performed, 10 µl of each PCR product were mixed with 2 µl of the orange / Blue 6X loading dye (Promega). Then they were electrophoresed on 1.5 % agarose gel in 0.5 X TBE buffer containing 0.5 µg/ml ethidium bromide for 60 minutes at 70 volts in a minigel electrophoresis unit using 0.5X TBE as electrophoresis buffer (Sambrook et al., 1989). Amplification products were visualized using ultraviolet trans-illuminator and photographed. The sizes of the amplification products were compared with the 100 bp DNA marker that was loaded with the samples. Negative "No template" controls (NTC) using DNase and Rnase free water and positive controls were always run with each PCR assay, to monitor for reagents and laboratory contamination.

2.7. Sensitivity evaluation of multiplex PCR assay: DNA from Brucella, Leptospira, Listeria and Mycoplasma was quantified in spectrophotometer. Serial tenfold dilutions of DNA were carried out to determine the detection limit of multiplex PCR assay.

2.8. Application of multiplex PCR assay on clinical samples: A total of 161clinical samples including: 115 milk samples, 10 semen samples, 5 vaginal swabs and discharges,15 fetal fluids and fetal organs from aborted foeti, 6 tissue samples and 15 blood samples) were collected and used in this study. DNA was extracted directly from the clinical samples and used for m-PCR.

3. RESULTS

3.1. PCR detection limit: The result of the PCR assay sensitivity was determined by amplification of extracted genomic DNA products from the 10-fold serial dilutions from different positive control strains. The reproducible detection limits were $1.7x\ 10^2$ fg/ul, 0.14×10^3fg/ul, 0.16×10^3fg/ul and 0.74×10^4 fg/ul for Brucella, Mycoplasma, Listeria and Leptospira, respectively. Specific simultaneous detection of all pathogens was achieved with its corresponding primers with the conditions described before, and no interferences were detected between them, therefore there is no false negatives were obtained. The results of conventional m-PCR revealed the specific band at 223bp, 270bp, 370bp and at468bp for detection of Brucella, Mycoplasma, Listeria and Leptospira species simultaneously, as shown in Fig.1.

3.2. Application on clinical samples: Previously described m-PCR assay has been applied on different field samples. Results summarized in Table (1) and Plate (1). The results revealed specific PCR products, with minimal primer dimmer and without nonspecific products.

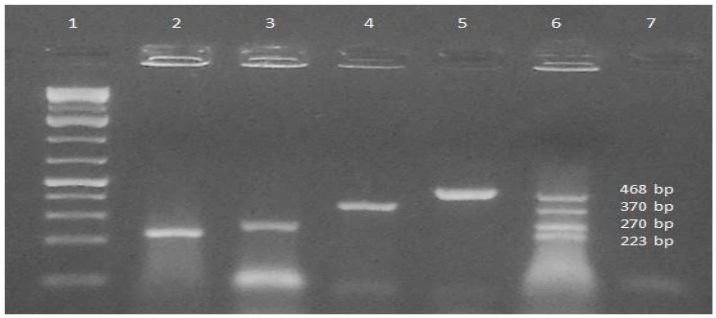

Figure 1: multiplex PCR-detection of Brucella, Mycoplasma, Leptospira and Listeria positive control strains extracted DNA using the primer sets (B4, B5, myco1, myco2, L.mono1, L. mono2, lig1 and lig2), Lane 1: 100 bp DNA marker; lane 2: single Brucella positive strain; lane 3: single Mycoplasma positive strain; lane 4: single Listeria positive strain; lane 5: single Leptospira positive strain; lane 6: Multiplex Brucella, Mycoplasma, Listeria and Leptospira positive strains; lane 7: Negative control

Table (1): Results of m-PCR in different clinical samples

Sample type	Total No.	+ Ve Conventional PCR				
		Brucella	Mycoplasma	Listeria	Leptospira	Mixed
Milk	115	18	8	-	-	4(br+myco
Blood	15	2	-	-	1	-
Semen	10	-	2	-	2	-
Fetal fluid	15	2	-	-	2	2(br+ lepto)
Tissue	6	-	1	-	-	-
Vaginal discharge	5	2	-	-	-	-
Total	161					

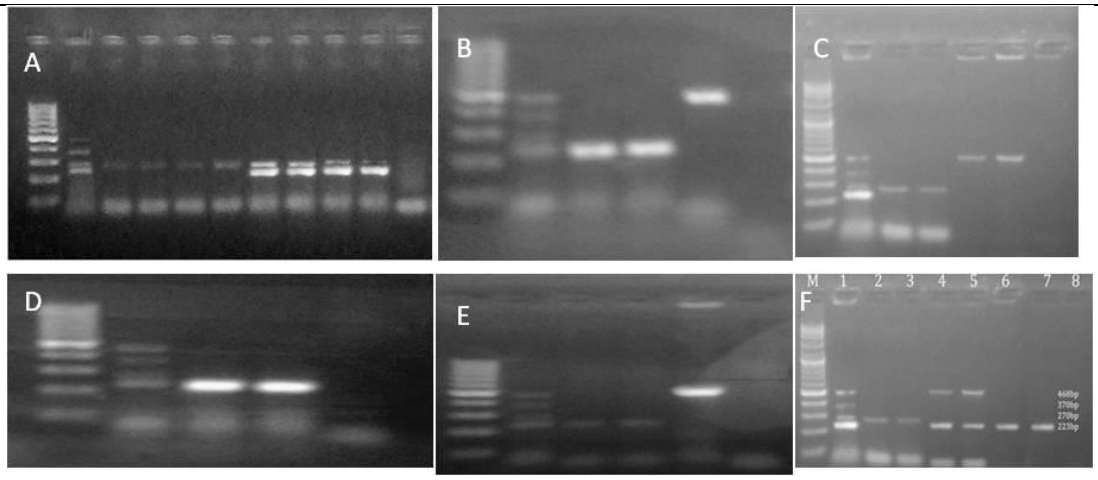

Plate 1: Detection of Brucella, Mycoplasma, Leptospira and Listeria in different clinical samples, **A**: detection in milk samples; Lane 1: 100 bp molecular marker; lane 2: +ve m-PCR sample; lane 3-6: +ve mycoplasma samples; lane9-10: +ve mycoplasma and brucella samples; lane 11: -ve control; **B**: detection in blood samples; Lane 1: 100 bp molecular marker; lane 2: +ve m-PCR sample; lane 3, 4: +ve brucella samples; lane 5: +ve leptospira sample; lane 6: -ve control.; **C:** detection in semen samples. Lane 1: 100 bp molecular marker; lane 2: +ve m-PCR sample; lane 3, 4: +ve mycoplasma samples; lane 5, 6: +ve leptospira samples; lane 7: -ve control; **D:** detection in tissue samples Lane 1: 100 bp molecular weight marker; lane 2: +ve m-PCR sample; lane 3, 4: +ve brucella samples; lane 5: -ve control.; **E**: detection in vaginal discharge samples; Lane 1: 100 bp molecular weight marker; lane 2: +ve m-PCR sample; lane 3, 4: +ve brucella samples; lane 5: +ve leptospira sample; lane 6: -ve control.; **F**: detection in fetal fluids samples; Lane 1: 100 bp molecular weight marker; lane 1: +ve m-PCR sample; lane2, 3:+ve mycoplasma samples; lane 4,5: +ve brucella and leptospira sample; lane 6, 7: +ve brucella samples; lane8: -ve control.

4. DISCUSSION

Abortion in cattle considered a multifactorial disease, usually caused by many different infectious agents (Selim et al. 2014). Brucella, Leptospira, Mycoplasma and Listeria are considered an important causative pathogens for reproductive disorders in cattle and buffaloes and continue to be a serious problem for animals through the world leading to severe economic losses, moreover, they considered to be a threat to community health and a critical issue in public health in developing countries (Yoo et al., 2010, Gunyeli et al., 2011)

Specific and decisive diagnosis of the etiological agent in reproductive diseases is highly demandable since control measures vary according to the causative agent involved (Kaur et al., 2015). In many cases, traditional techniques are not sufficient alone to detect the cause of the abortion, as they are inconclusive, difficult, time consuming, dangerous and require expert personnel (Selim et al. 2014). Therefore the PCR has become a valuable diagnostic tool because of its speed, simplicity, accuracy and applicability to different clinical samples. Such advances represent a promising alternative for hazardous, time consuming, less sensitive traditional diagnostic techniques especially for those important Zoonotic bacterial pathogens (Bhure et al., 2012).

In this study, monoplex PCRs, carried out separately for each bacterium, specific amplicon sizes of 223bp, 270bp, 370bp and 468bp for detection of Brucella, Mycoplasma, Listeria and Leptospira species simultaneously, since diagnosis by monoplex PCR allows the detection of a single bacterium at a time, it is relatively costly and wasting a lot of time. Multiplex PCR assay is a technique for the amplification of multiple target DNA sequences in a single tube PCR experiment. It offers a considerable potential for saving of time and effort without affecting the utility of the test, the multiplex PCR assay has an advantage of the simultaneous detection of different pathogens and proved to be more sensitive, specific and cost effective. It can also be helpful in diagnosis, screening and surveillance of the animal flocks (Henegariu et al., 1997).

Standardization of multiplex PCR requires careful optimization of all the ingredients along with the PCR conditions. Standardization of the primer cocktail is important since the inclusion of many primer pairs can lead to primer dimerization and mis-priming. Each primer set has amplified a DNA fragment specific for the corresponding gene and of the predicted size. The sharpness and intensity of the amplified DNA fragments in monoplex PCR assay was more as compared to that obtained in multiplex PCR. This could be because of the reason that in monoplex PCR where only single gene is targeted, we can use the optimal conc. of $MgCl_2$, Taq, annealing temperature according to the specific primer, but, in case of multiplex PCR, the ingredients as well as PCR conditions should suit all primer pairs and target genes and so may not be optimal for each individual pair of primers, Similar observations have been reported (Selim et al. 2014; Moustacas et al., 2015).

The primers used in this multiplex assay has been carefully chosen to be specific for those 4 bacterial pathogens according to literatures (Jami et al., 2010), the B4 & B5 primer set which used for brucella species, amplifying a 223 bp product which encoding for a (BCSP31) 31-KDa immunogenic brucella cell surface protein, conserved among all Brucella species. The major outer membrane lipo-protein gene (Lig), which amplifies a 468 bp product, is an important virulence factor and is confined to pathogenic leptospira species and not found in non-pathogenic Leptospira and a 370-bp product for hly gene which encodes for listeriolysin o (a pore forming cytolysine) that is one of virulence factors in Listeria monocytogenes.

The sensitivity of selected PCR assays have been assessed, When applied on 10- fold serially diluted DNA samples of positive control strains of the 4 pathogens, the Brucella PCR assay could detect as low as $1.7x10^2$fg/ul which agree with (Bhure et al., 2012; Arasoglu et al., 2013); While, the mycoplasma assay could detect down to 0.14 x 103fg/ul and that agree with (Higuchi et al., 2011). In case of L. monocytogene the detection limit was 0.16 x 103fg/ul as with (Kumar, 2009); and for Leptospira the results was 0.74 x 104 fg which close to (Richtzenhain et al., 2002; Bhure et al., 2012 and Lam et al.,2012).

This assay has been applied for the direct detection and identification of these four organisms in different clinical samples; in the present study, one sample of fetal stomach content was positive for

Brucella and Leptospira by m-PCR. Similarly, infection by both Brucella and Leptospira spp. has earlier been observed aborted fetuses of bovine by multiplex PCR (Kaur et al., 2015 and Dehkordi et al., 2012). When two or more target sequences present in different amount in the same foetal tissue it may result in a false negative results specially when concerning the lower prevalent agent. This situation cans potentially affecting other multiplexes targeting including in molecular assays (Selim et al. 2014). However, in this work, the results obtained by using multiplex PCR assays were in accordance with those obtained by the same PCR assay run in simplex, thus showing the high reliability and sensitivity of the proposed panel.

Only one sample is positive in tissue samples may be due to many causes; as the inhibition of PCR may be due to unknown inhibitors which can be released from tissue in DNA extraction step. (Lester et al., 2003)

All tested field samples (unknown samples) were negative for Listeria monocytogenes, as we found Brucella, Mycoplasma, Leptospira and did not find Listeria, it may be due to low incidence of listeria comparing to other microorganisms or the type of sample itself.

In conclusion the multiplex PCR assay proved to be a valuable method for the diagnosis of co-infection for its accuracy, specificity and low cost in comparison to traditional techniques. The assay could be used for routine screening and molecular epidemiology of Brucella, Mycoplasma, Listeria and Leptospira infection; the performance of this assay can minimize the number of testes required with rapid delivery of the results.

REFERENCES

Anderson, M.L.2007. Infectious causes of bovine abortion during mid- to- late gestation. Therio. 68: 474-486.

Arasoglu, T., Gulluce, M., Özkan, H., Adiguzel, A. Şahin, F. 2013. PCR detection of Brucella abortus in cow milk samples collected from Erzurum, Turkey. Turk. J. Med. Sci. 43: 501-508

Baily G.G, Krahn J.B, Drasar B.S, Stoker N.G. 1992. Detection of Brucella melitensis and Brucella abortus by DNA amplification. J. Trop. Med. Hyg. 95: 271–275.

Bhure, S.K., Chandan, S., Amachawadi, R.G., Patil, S.S., Shome, R. Prabhudas, K. 2012. Development of a novel multiplex PCR for detection of Brucella, Leptospira and Bovine herpsvirus-1. Ind. J. An. Sci. 82(11): 1285-1289.

Chiebao, D.P., Valadas, S.Y.: Minervino, A.H., Castro,V., Romaldini, A.H., Gennari,S.M., Keid, L.B., Soares,R.M. 2015. Variables associated with infections of cattle by brucella abortus, Leptospira spp. and Neospora spp. in Amazon region in Brazil. Transboundary and Emerging diseases, 62, 30-36

Dehkordi, F. S., Taghizadeh, F. 2012. Prevalence and some risk factors associated with brucellosis and leptospirosis in aborted fetuses of ruminant species. Res. Op. Ani. Vet. Sci. 2: 275–281

Gunyeli, I., Abike, F., Dunder, I., Tapisizi, O., Temizkan,O. 2011. Chlamydia, Mycoplasma and Uraeplasma infections in infertile couples and effects of these infections on fertility. Arch. gyne. Obst. 283: 379-385

Gwida, M., E-Ashker, M., El-Diasty, M., Neubauer, H. 2016. Use of serology and real time PCR to control an outbreak of bovine brucellosis at dairy cattle farm in the Nile Delta region, Egypt. IRL. Vet. J. 69: 3-10

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H., Vogt, P.H. 1997. Multiplex PCR: Critical parameters and step by step protocol. Biotechniques. 23, 504-511.

Higuchi, H., Iwano, H., Kawai, K., Ohta, T., Hirose, K., Ito, N., Tamura, Y., Nagahata, H. A. 2011. Simplified PCR assay for fast and easy Mycoplasma mastitis screening in dairy cattle. J. Vet. Sci. 12(2): 191-193.

Jami, S., Jamshidi, A., Khanzadi, S. 2010. The presence of Listeria monocytogenes in raw milk samples in Mashhad, Iran. Ira. J. Vet. Res., Shiraz University. Vol.11, No.4, Ser.No.33

Kaur, P., Sharma, N. S., Arora, A .K. 2015. Development of a multiplex PCR assay for detection of different bacterial pathogens associated with reproductive disorders in cattle and buffaloes. Ind. J. An. Sci. 85 (12): 1306–1310.

Kumar, A., Tuteja, U., Sing, D., Kumar, A., Kumar, O. 2009. Rapid multiplex PCR assay for the simultenous detection of the Brucella genus. J. Micro. Biotech. 21(1): 89-92.

Lam, T.Q, Yoon, B., Hahn, T. 2012. Development of a multiplex PCR to identify Salmonella, Leptospira and Brucella spp. in tissue samples. Korean J. Vet. Res. 52 (2): 75-82.

Lester, J.W., LeFebvre, R.B. 2003. Detection of Leptospira interrogans. Methods Mol Biol. 216, 193-200.

Mahajan, V., Banga, H. S., Filia, G., Gupta, M. P., Gupta, K. 2017. Comparison of diagnostic tests for the detection of bovine brucellosis in the natural cases of abortion. Ira. J. Vet. Res., Shiraz University. IJVR. Vol. 18, No. 3, Ser. No. 60, 183-189

Marios, C., Dufour,G.F. and Kemp,F.I. 2000. Detection of Mycoplasma synoviae in poultry samples by culture and PCR. Vet. Microb. 73:311-318.

Mori, M., Bakinahe, R., Vannoorenberghe, P., Maris J., De Jong, E., Tignon, M., Marin, M., Desqueper, D., Fretin, D., Behaeghel, I. 2017. Reproductive Disorders and Leptospirosis: A Case study in a Mixed-Species Farm (Cattle and Swine). Vet. Sci. 4, 64

Moustacas, V. S., Silva, T. M., Costa, L. F., Xavier, M. N., Carvalho, C. A., Costa E .A., Paixao, T. A., Santos, R. L. 2013. Species specific multiplex PCR for the diagnosis of Brucella ovis, Actinobacillus seminis and Histophilus somni infection in rams. BMC Vet. Res. 9: 51–53.

OIE (World organization for animal health) OIE manual of diagnostic tests and vaccines for terrestrial animals 5th ed. 2002

Palaniappan, R.U., Chang, Y.F., Chang, F.C., Pan, M.J., Dubovin, E., Divers, T., Roe, B. 2005. Evaluation of lig-based conventional and real time PCR for detection of pathogenic leptospira. Molecular and cellular probes 19, 111-117.

Poltronieri, P., de Blasi, M.D., Durso, O.F. 2009. Detection of Listeria monocytogenes through real time PCR and biosensor methods. Plant soil environo. 55(9):363-369.

Richtzenhain, L.J., Cortez, A., Heinemann, M.B., Soares, R. M., Sakamoto, S.M., Vascoooncellos, S.A., Morais, Z. M., Scarrcelli, E., Genovez, M.E. 2002. A multiplex PCR for the detection of Brucella spp. and Leptospira spp. DNA from aborted bovine fetuses. Vet. Micro. 87: 139-147.

Sambrook, J., Fritsch, E., Maniatis, T. 1989. Molecular cloning: A laboratory manual, second edition. Cold Spring Harbor Laboratory Press

Selim, A., Elhaig, M., Gaede, W. 2014. Development of multiplex real-time PCR assay for the detection of Brucella spp., Leptospira spp. and Campylobacter foetus. Veterinaria Italiana 50 (4), 269-275.

Sharifzadeh, A., Doosti, A., Khaksar, K. 2008. Simultaneous detection of Brucella species and Salmonella abortus ovis by multiplex PCR. Res. J. Bio. Sci. 3: 109– 111.

Silva, T. M. A., Oliviera, R. G., Mol, J. P. S., Xavier, M. N., Paixao, T. A., Cortez, A., Heinemann, M., Richtzenhain, L. J., Lage, A. P., Santos, R. L. 2009. Etiological diagnosis of bovine infectious abortion by PCR. Ciência Rural. Santa Maria 39 (9): 2563– 2570

Tramuta, C., Daniela, L., Simona, Z., Mariella, G., Alessandro, D., Ezio, F., Patrizia, N., Sergio, R. 2011. Development of a set of multiplex standard polymerase chain reaction assays for the identification of infectious agents from aborted bovine clinical samples. J. Vet. Diag. Inves. 23: 657–664.

Yoo, H. S. Infectious causes of reproductive disorders in cattle. 2010. J. Rep. Dev. 56: 53-60.