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Multiplex SYBR green real time PCR for the simultaneous detection and differentiation of four important reproductive infectious pathogens

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ABSTRACT

Brucellosis, Leptospirosis, Mycoplasmosis and Listeriosis are important zoonosis and represent an important cause of reproductive losses in animals worldwide, especially in Mediterranean countries and Egypt. Aiming at improvement in the diagnostic scheme, a quick method for the simultaneous detection of these microorganisms in different clinical samples using a SYBR green multiplex real-time polymerase chain reaction (m RT-PCR) has been developed. The m 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. This study was applied to 161 different clinical samples (milk, blood, fetal fluids, semen, tissue and vaginal discharges). Real time PCR assay revealed a specific dissociation peak at Tm=79.0°C, 80.0°C, 85.2°C and 88.0°C (± 0.5) for Listeria, mycoplasma, brucella and Leptospira respectively. The real time assay neither revealed interferences between primers nor nonspecific florescence. In conclusion the high output, time saving and cost effective SYBR green m RT- PCR method established, could offer an effective tool for simultaneous, quick and reliable identification of these microbial agents in different clinical samples.

Key word: *Brucella, Leptospira, Listeria, Mycoplasma, mRT-PCR*

INTRODUCTION

Abortion due to infectious agents represents a significant cause of reproductive failure and economic losses in animals (Da silva *et al.,* 2009). Brucellosis, Leptospirosis, Mycoplasmosis and Listeriosis are accepted as important diseases of domestic animals, and they are commonly spread in Egypt and Mediterranean countries (Hamdy, 1992; Sangari *et al.,* 1994; Eissa *et al.,* 2012; Wareth *et al.,* 2014 and Gwida *et al.,* 2016). The traditional bacteriological isolation of these microorganisms is rarely performed in routine diagnosis, as most of them are fastidious bacteria, difficult to culture and slow growing (McAuliffe *et al.,* 2004 and Boonslip *et al.,* 2011). Moreover, culture has serious disadvantages, as it is time consuming, complex, laborious, need highly skilled personal, as well as the zoonotic nature of most species and its biological hazards, the results are not always conclusive (Bricker, 2002; Medarla *et al.,* 2003 and Awan *et al.,* 2009). Serological tests could be too sensitive yielding false positive results, or too specific yielding false negative results, beside misdiagnosis due to cross reactivity of other bacteria (Shakuntala *et al.,* 2006)

Early and accurate diagnosis is serious issue in public health policy (Rijpens and Herman, 2002), it is the corners stone in any control and eradication programs it helping in early treatment and prevention protocols. Recently, The PCR appear as a promising alternative, offering several advantages over the classical methods, such as shorter time of analysis, low detection limits and potential for automation (Germini et al ., 2009). Also it proved to be much faster, highly sensitive and specific for accurate diagnosis (Abo-Elnaga *et al.*, 2012).The described system relies on SYBR green as the reporter dye, thus, demanding subsequent melting curve analysis to differentiate each pathogen.

Permitting a rapid DNA extraction procedure, Results could be obtained in less than 1working day, which could be performed on a large variety of samples. Detecting Multipathogens on a single-assay platform reduces the cost for testing and also provides data on the presence of pathogens in a single experiment.

The aim of the study is to diagnose some of important reproductive diseases such as Brucellosis, Leptospirosis, Mycoplasmosis and Listeriosis as abortifacient infectious agents using multiplex real time PCR trying to reach rapid diagnosis of these infectious bacterial agents by effective, sensitive and safe methods which will fasten the process of treatment, and control effectiveness.

MATERIALS AND METHODS

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 (Brucella, Leptospira, Mycoplasma and Listeria organisms) are classified as biohazard (OIE, 2002); the appropriate precautions and biosafety measures were followed during the study.

Clinical samples: A total of 161 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 used in this study.

DNA extraction methods: DNA extraction 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.

Primers: specific primers for detection of 31kDa omp gene in Brucella (Traumata *et al.*, 2011, Baily *et al.*, 1992), lig gene in Leptospira (Palaniappan *et al.*, 2005), hlyA gene in Listeria (Jami *et al.*, 2010) and 16S rDNA in Mycoplasma spp. (Marios *et al.*, 2000) using m-PCR were used in this study. The sequences of the primers, their expected products sizes and species specificity are listed in (Table 1).

Qualitative SYBR®GreenqPCR assay (Multiplex RT-PCR detection method) All qPCR techniques were applied in accordance with the general requirements (Anonymous, 2011) those specific for the SYBR®Green. All PCR assays were done on Rotor Gene 6000 Real time detection system (Corbett Research, Australia).

The RT-PCR was done in a final volume of 50 uL reaction volume; with the following constituents: 25uL of 2x absolute Master Mix (with SYBR Green Mix, Thermoscientific, ABgene, UK), 0.2 uM of each primer (listed in table1), 2.5 uL of genomic DNA (which previously measured with biophtometer for conc. and purity) were used as a template.

All the previous components were used with the following thermal profile: a single cycle of 3 min hold at 95 °C for the initial denaturation, followed by 40 amplification cycles; each cycle consisted of a first denaturation step of 10 sec. at 95 °C; second annealing step at 53 °C for 15 sec. and third extension step at 72 °C for 20 sec. (acquiring to cycling A green). Subsequently,

melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 60 to 95 °C in 20 min (± 0.6 °C/20s) i.e. temperature change rates were 20°C/s, except in the final step was 0.1°C/s. The melt peak generated represents the specific amplified product. The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and the threshold limit setting was performed in automatic mode, consistent with the detection Software version (Applied Biosystems), the "No template" controls (NTC) using DNase and Rnase free water were placed in each reaction to assess primer dimer formation or non-specific amplification.

For understanding a SYBR Green q PCR assay, two important characters should be taken in consideration: firstly; the quantification cycle (Cq) value and secondly; the melting temperature of the amplicon (Tm). The Cq value denotes the fractional cycle at which PCR amplification reaches the threshold level for their action (Bustin, 2000). As it is a screening assay, a qualitative response is needed. To be regarded as positive, a signal created in SYBR Green q PCR analysis should exhibit an exponential amplification above the threshold level, showing a single peak on melting analysis giving a specific Tm value. A signal was reported negative when no Cq value or a nonspecific Tm was obtained. Each run must contain a positive (contain the target DNA) and negative control (NTC) samples.

Table 1: Primer sets used in this study

Primer Code	Sequence (5` to 3`)	Species Specificity
B4	TGGCTCGGTTGCCAATATCAA	All *Brucella* species
B5	CGCGCTTGCCTTTCAGGTCTG	
Lig1	GACGAACGGAATTTTTCCAATCCC	Pathogenic *leptospira*
Lig2	TCGCGATCACTTAAGGGCCTTCAT	
L.mono1	AAATCGCGTCCTTGCTGGTCTGA	*Listeria monocytogenes*
L.mono2	TCGCGATCACTTAAGGGCCTTCAT	
Myco1	CCCCGGAAGATATGCTTCGATCC	*Mycoplasma spp.*
Myco2	TCGCGATCACTTAAGGGCCTTCAT	

RESULTS

Specific simultaneous multiplex detection of each pathogen was achieved with its corresponding primers with the conditions described before and no interferences were detected between them; also Specificity was checked against a number of non-target bacterial strains and no specific fluorescence was detected. In the real time PCR, each spp. give a unique melt peak at specific Tm (-/+0.5); Leptospira Tm= 88.0°C; Brucella Tm= 85.2°C; Mycoplasma Tm= 80.0°C; Listeria Tm= 79.00C. The results were demonstrated in (fig.1).

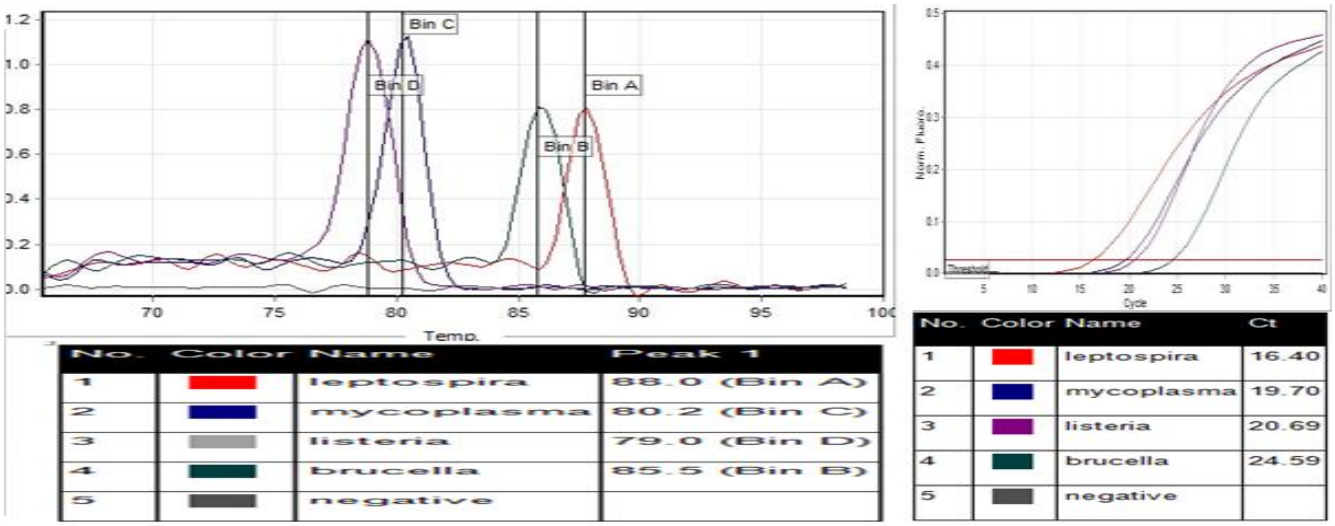

Fig.1: Detection of brucella, Mycoplasma, Leptospira and Listeria using the primer sets (B4, B5, myco1, myco2, L.mono1, L. mono2, lig1 and lig2); Detection of brucella, leptospira, Mycoplasma and Listeria spp. by real time PCR. (Green Melt curve analysis: The X axis indicates the melting temp., while the Y axis indicates the fluoresce intensities).

Application on clinical samples:

Table (2): Positive results by real time PCR in different clinical samples

Sample type	Total No.	+Ve Real time PCR				
		Brucella	Mycoplasma	Listeria	Leptospira	Mixed
Milk	115	24	9	-	-	4 (brucella + mycoplasma
Blood	15	2	-	-	1	
Semen	10	-	2	-	2	
Fetal fluid	10	4	2	-	-	1 (brucella + Leptospira)
Tissue	6	-	1	-	2	
Vaginal discharge	5	2	-	-	-	
Total	161					

Results of clinical samples using m-RT PCR: (Green Melt curve analysis: The X axis indicates the melting temp., while the Y axis indicates the fluoresce intensities).it shows the 4 melting peaks of 4 positive control strains, the positive samples give the specific Tm, under the specific melting peak. Melting curves of amplified fragments generated by m-RT PCR, specific signals for +ve brucella had melting temp. of 85.2±0.5℃

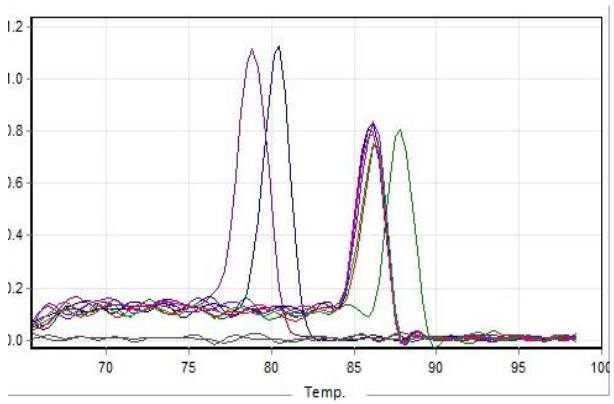

Fig.2: diagnosis of some milk samples

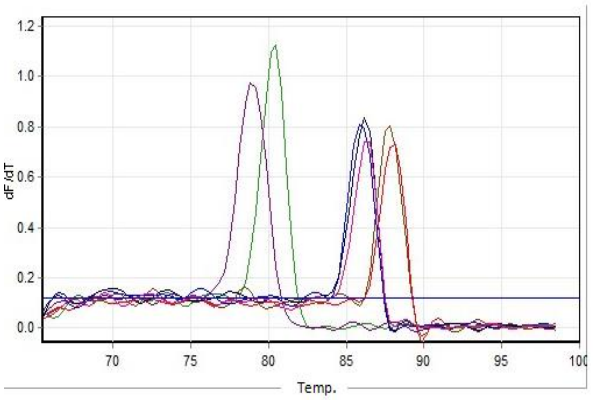

Fig.3: diagnosis of blood samples

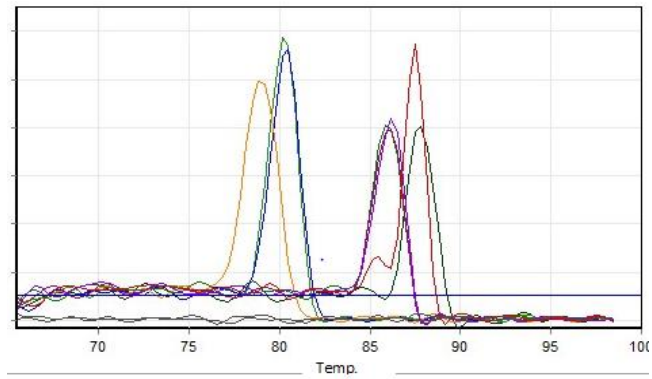

Fig.4: diagnosis of fetal fluid samples

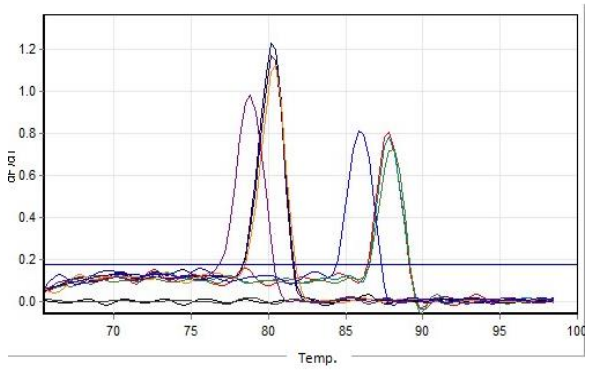

Fig.5 : diagnosis of semen samples

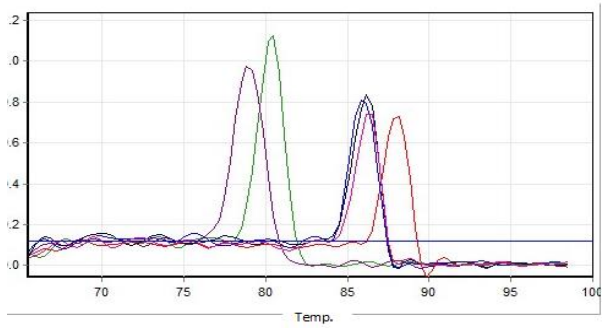

Fig.6: diagnosis of tissue samples

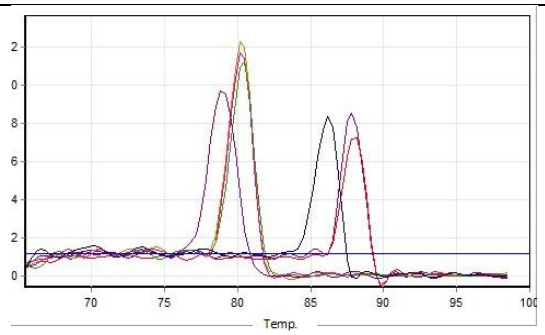

Fig.7: diagnosis of vaginal discharge samples

DISCUSSION

The arrival of the real-time PCR has offered an opportunity to meet the requirements for rapid diagnosis (Saini *et al.,* 2017)

PCR offers a better method for swift and sensitive detection of microorganism. On the downside conventional PCR usually consists of 3 phases: DNA isolation, PCR amplification, and post-PCR analysis, which includes gel electrophoresis, hybridization and/or sequencing. Real –time PCR is a desirable technique as the method does not require post-PCR analysis and has an outstanding detection limit (Khanna *et al.,* 2005 and Mouozumi, *et al.,* 2006).

In this study, different pre-optimized PCR based detection systems were combined to acquire a tool enabling the synchronized analysis of Brucella spp., Mycoplasma spp.; Listeria spp. and Leptospira spp. in a sample. The grouping of the previous individual reaction set up permitted the co-amplification of each target

when either equal or variable amounts of DNA were mixed without meddling in efficiency or specificity. Hence, amplification of multiple targets did not compromise the act of the individual assays in terms of selectivity or amplification result.

Results of clinical samples (that are present in table 1), it was found that the most detectable microorganism was Brucella and/or mycoplasma, this may be due to the type of samples used (i.e. mostly milk sample and aborted foeti), or due to the high incidence of these microorganisms as abortifacient agents compared with other microorganisms; especially in those examined field samples; in addition to high sensitivity of these primers (B4, B5, Myco1, Myco2) comparing to other primers. All the results have been compared with conventional PCR and a primarily serological identification and a primer sensitivity test to each primer.

The Listeria was negative in all sample, it may be due to the type of sample, or due to low incidence of Listeria comparing to other microorganisms.

The bacterial strains that were kindly provided by Animal reproduction research institute (ARRI) were used as positive controls; we compared all results with positive and negative controls used in each run; firstly, we used them to optimize the technique either in a single or in a multiplex format. We mixed the bacterial strains together in one Eppendorf tube (make a mixture of colonies from all these bacteria) and after DNA extraction, we applied previously described assays either in single or in a multiplex format.

The real time PCR technology offers a significant improvement for the detection of infections; in addition to, the absence of post-amplification manipulation of PCR products, significantly reduces the risk of contamination which results from amplicon carryover.

Many reasons explain the use of a simple SYBR Green I dye format to deliver the fluorescent signal. First, the selected targets amplified are highly specific to the selected genus and is previously experimented and validated. Second, melt-curve analysis of the augmented DNA allows clear discrepancy of the specific products from artifacts, such as primer -dimers. Third, as this technique used as a qualitative, screening test; SYBR Green dye seems the most suitable for this. Fourth, this approach is much simpler and cost efficient, since many fluorescent labels, rather than a single molecule, are combined into the amplified fragment (Newby *et al.,* 2005 and Shu *et al.,* 2003).

The melt-curve analysis improved the specificity of the assay by checking that a positive fluorescence signal gained during the real-time PCR was related with an amplified product with a characteristic Tm. The existence of some false-negative results was astonishing. Considering that some of these false-negatives may result from the presence of inhibitors in the sample (Zerva *et al.,* 2001)

The small reaction volume used in the RT PCR poses a possible drawback compared to conventional PCR, since only a minor volume of template could be added to the master mix. The use of very small volume samples with low concentrations of circulating pathogens could result in an absence of target DNA in the sample tested.

The assay was considered to have good specificity. The high sensitivity of PCR-based methods allows the amplification of probable fragments of circulating DNA in asymptomatic subjects, although these have no clinical significance (Kami *et al.,* 2013). Overall, the results of this study are in accordance with those described previously (Queipo-Ortuno *et al.,* 1997; Mortata *et al.,* 2003; Zerva *et al.,* 2001and Vironi *et al.,* 2004)

Finally, the multiplex format of the assay will reduce reagent fee, reduce risk of contamination and staff time. Moreover, it achieves the ultimate goal of using molecular biology technique for the early detection of pathogens.

In conclusion, a quick and sensitive multiplex assay was established for simulations detection of four important pathogens; this assay appears to be a promising means for high -through put screening of large number of samples that require either single or multiple pathogen detection.

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