Validation of the multiplex PCR for identification of *Brucella* spp.

Validação da técnica de PCR multiplex para identificação de *Brucella* spp.

**Lívia de Lima Orzil[I] Ingred Sales Preis[I] Iassudara Garcia de Almeida[I]
Patrícia Gomes de Souza[I] Paulo Martins Soares Filho[I]
Fabrício Barcelos Jacinto[II] Antônio Augusto Fonseca Júnior[I*]**

ABSTRACT

A multiplex PCR technique for detection of ***Brucella*** spp. in samples of bacterial suspension was validated as a complementary tool in the diagnosis of the disease. This technique allows the characterization of the agent without performing biochemical tests, which greatly reduces the time for a final diagnosis, and provides more security for the analyst by reducing the time of exposure to microorganisms. The validation was performed in accordance with the Manual of Diagnostic Tests from OIE (2008) and following the requirements present in the ABNT NBR ISO/IEC 17025:2005. The mPCR validated in this study identified the different species of ***Brucella (Brucella abortus, B. suis, B. ovis e B. melitensis)*** of bacterial suspension obtained from the slaughterhouse samples, as well as distinguished the biovars (1, 2 e 4; 3b, 5, 6 e 9) of ***B. abortus*** in grouped form and differentiated the field strains from vaccine strains, as a quick, useful and less expensive technique in diagnosis of brucellosis in Brazil.

Key words: *Brucellosis, molecular identification, zoonosis, Brucella, multiplex PCR.*

RESUMO

Validou-se neste trabalho uma técnica de PCR Multiplex (mPCR) para detecção de ***Brucella*** spp. em amostras de suspensão bacteriana, como ferramenta complementar no diagnóstico da doença. Esta técnica possibilita a caracterização do agente sem que seja necessária a realização de testes bioquímicos, o que diminui consideravelmente o tempo para o diagnóstico final, além de oferecer mais segurança ao analista ao diminuir o tempo de exposição ao agente infecioso. A validação foi realizada de acordo com o Manual de Testes de Diagnósticos da OIE (2008), seguindo as exigências presentes na norma de qualidade da ABNT NBR ISO/IEC 17025:2005. A mPCR validada neste trabalho identificou as diferentes espécies de ***Brucella (Brucella abortus, B. suis, B. ovis e B. melitensis)*** em suspensão bacteriana, obtidas a partir de amostras de frigorífico. Além disso, discriminou os biovares (1, 2 e 4; 3b, 5, 6 e 9) de ***B. abortus,*** de forma agrupada, e diferenciou cepa vacinal de cepa de campo, sendo esta uma técnica rápida, útil e de menor custo para o auxílio no diagnóstico de brucelose no Brasil.

Palavras-chave: *Brucelose, identificação molecular, zoonose, **Brucella**, PCR multiplex.*

INTRODUCTION

Brucellosis is an infectious zoonotic disease of chronic evolution and global distribution listed as notifiable by the World Organization for Animal Health (OIE, 2008). The species of greatest economic interest are ***Brucella abortus***, ***Brucella melitensis***, ***Brucella suis***, ***Brucella canis*** and ***Brucella ovis***, the latter being the only one of those species that do not cause disease in humans (NIELSEN & DUNCAN, 2000; GALIŃSKA & ZAGÓRSKI, 2013). In infected animals, the main clinical signs are linked to reproductive problems such as retained placenta, abortion and birth of weak calves which lead to major economic losses, especially in cattle herds (LAGE et al., 2009).

Brazilian law provides guidelines for indirect tests (serologic tests) using the Buffered Acidified Antigen (BAA) test as a screening test and 2-Mercaptoethanol (2-ME) as a confirmatory test, but only in live animals (BRASIL, 2006). Thus, to

[I]Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Pedro Leopoldo, MG, Brasil. E-mail: antonio.a.fonsecajr@gmail.com.
*Corresponding author.
[II]Serviço de Inspeção Federal, Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Pedro Leopoldo, MG, Brasil.

reach the final characterization of the agent, there is a long and laborious process of bacterial isolation that involves the manipulation of highly pathogenic bacterial colonies, which puts the analyst involved at risk (ALTON et al., 1975). Polymerase chain reaction (PCR) is an important aid in the diagnosis of brucellosis, making it especially useful in the process of bacterial identification due to factors such as high sensitivity and specificity, as well as to increase the biosecurity as it reduces time of exposure of the analyst to the infectious agent. The exposure time is less due to less manipulation of the agent, as biochemical tests are no longer necessary. This is important because, of all reported cases of human brucellosis, 2% infection are associated with laboratory work (COLLINS, 1983), with an attack rate of 30 to 100% (ERGÖNÜL et al., 2004), including a severe case recorded in Brazil (RODRIGUES et al., 2013).

Thus, it becomes important the validation of a safest alternative to the diagnosis of brucellosis. Molecular results are obtained in a short period of time, they are also relatively simple, involve lower costs when compared to bacterial isolation and the infectious agent can be inactivated (YU & NIELSEN, 2010). This work aimed to validate a multiplex PCR (mPCR) for identification of different species of *Brucella* spp. in samples of bacterial suspension in accordance with the Manual of Diagnostic Tests (OIE, 2008). This technique was introduced into the routine of the Laboratório Nacional Agropecuário de Minas Gerais (Lanagro/MG) as an aid in the official diagnosis of brucellosis in the country.

According to the OIE (2008), validation is a process that determines the ability of a given test developed properly, optimized and standardized for a purpose. Thus, validation must guarantee, through experimental studies, that the method meets the requirements of the analytical applications, ensuring the reliability of the results. This is important for proper accreditation of the laboratory in ISO/IEC 17025:2005 for quality insurance applicable to calibration and testing laboratories. The objective of this study is to validate a technique for diagnosis of brucellosis faster, safer and less costly to be used in routine LANAGRO/MG.

MATERIAL AND METHODS

The mPCR used was based on previous studies by BRICKER & HALLING (1994), with modifications and inclusion of primers designed by LÓPEZ-GOÑI et al. (2008). This method, called AMOS (a short for abortus, melitensis, ovis and suis)

PCR, has as principle the amplification of *is711* region by PCR, which enables to differentiate the species of *Brucella*, since the number of repetitive copies of the genetic element is unique for each species or biovar with different positioning in the genome. Different primers, specific for the various *Brucella* species (*B. abortus*, *B. abortus* S19, *B. melitensis*, *B. suis* and *B. ovis*), generate PCR products of different sizes, approximately 498bp for *B. abortus* (biovars 1, 2 and 4), 537bp for *B. abortus* S19, 590bp for *B. abortus* field strain, 731bp for *B. melitensis*, 976bp for *B. ovis,* and 285bp for *B. suis* (Figure 1). The primers sequences are shown in table 1 and the profile according to the primers used are shown in table 2.

We used a pair of primers as internal for the mPCR which amplifies the region responsible for the expression of the 16S bacterial ribosomal RNA resulting in a 800bp amplified product (BRICKER et al., 2003). Samples of *B. abortus*, *B. abortus* S19, *B. abortus* biovar 1, *B. abortus* biovar 3, *B. melitensis* and *B. suis*, provided by Lanagro/MG, were used during the validation process of the mPCR as positive controls. Samples suspended in saline were inactivated at 60°C for 1 hour. The inactivated bacterial suspension was used for PCR without special methods or kits for DNA extraction.

Different species of bacteria were used as negative control. Samples of *Trueperella pyogenes* were collected from the primary isolation in the Laboratory of Diagnostics of Bacterial Diseases in Lanagro/MG. Because of the similarity between the morphological characteristics of this
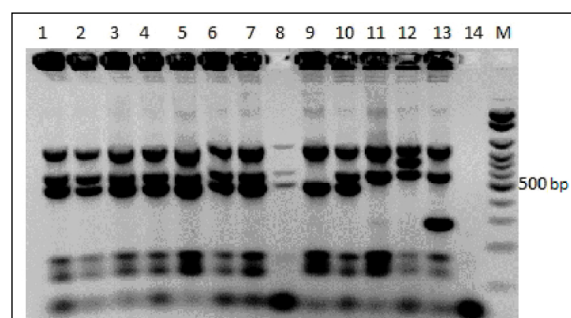

Figure 1 - 1.5% agarose gel electrophoresis showing the different patterns for each species and biovars of *B. abortus* using the *GoTaq* enzyme. Markers at intervals of 100 base pairs. 1 to 7: *B. abortus*, samples used in repeatability test. 8: *B. abortus* biovar 1. This same profile is observed to *B. abortus* biovars 2 and 4; 9: *B. abortus* S19. 10: *B. abortus* biovar 1; 11: *B. abortus* biovar 3b. This same profile is observed to *B. abortus* biovars 5, 6 and 9; 12: *B. melitensis* and 13: *B. suis*, 14: blank control (No DNA template). 15: MM (Molecular Marker).

Table 1 - DNA sequence of the primers used in the validation of the mPCR, gene target and reference.

Primer	DNA sequence (5' – 3')	Gene target	Reference
Bru-AMOS-Ab	GAC GAA CGG AAT TTT TCC AAT CCC	alpha-ketoglutarate-dependent dioxygenase	BRICKER & HALLING, 1994
Bru-AMOS-Me	AAA TCG CGT CCT TGC TGG TCT GA	hypothetical protein	BRICKER & HALLING, 1994
Bru-AMOS-Ov	CGG GTT CTG GCA CCA TCG TCG	TRAP transporter solute receptor, TAXI family protein"	BRICKER & HALLING, 1994
Bru-AMOS-Su	GCG CGG TTT TCT GAA GGT TCA GG	indole-3-glycerol phosphate synthase	BRICKER & HALLING, 1994
Bru-AMOS-IS711:	TGC CGA TCA CTT AAG GGC CTT CAT	is711	BRICKER & HALLING, 1994
16s.Universal.800F:	GTG CCA GCA GCC GCC GTA ATA C	16S	BRICKER et al.,2003
16s.Universal.800R	TGG TGT GAC GGG CGG TGT GTA CAA G	16S	BRICKER et al.,2003
Bru-BMEii0428F	GCC GCT ATT ATG TGG ACT GG	eriD	LÓPEZ-GOÑI et al., 2008
Bru-BMEii0428R	AAT GAC TTC ACG GTC GTT CG	eriD333	LÓPEZ-GOÑI et al., 2008

bacterium with *Brucella,* the use of *T. pyogenes* was important during the isolation process from suspicious material submitted to the diagnosis of brucellosis. Other samples were also used such as *Rhodococcus equi*, *Salmonella enterica*, *Staphylococcus aureus* and *Escherichia coli*.

Bacterial suspensions isolated from 164 samples of bovine tissues collected from slaughterhouses were used for validation. The bacterial strains were isolated using tryptose agar with 5% bovine serum and incubation performed at 37+-2°C with 5-10% CO_2. Typical colonies of *Brucella* spp. were selected and subjected to biochemical tests (catalase, oxidase, urease, citrate, and nitrate reduction). Confirmation of species and biovars of *Brucella* spp. were determined by growth tests in an atmosphere of CO_2 and O_2, acriflavine agglutination test, H_2S production, growth in the presence of thionine (20mg mL^{-1} and 40mg mL^{-1}) and basic fuchsin (20mg mL^{-1}) and forward assemblage anti-A serum and anti-F (ALTON et al., 1988). The colonies diluted in saline from the primary isolation

were inactivated in a water bath at 65°C for an hour and sent for realization of mPCR.

All isolates from the primary isolation were submitted to biochemical tests. One hundred and twelve isolates had a certificate of origin according to ISO 17025 (data such as biovars and the Brazilian state of the sample origin are listed in table 3). The remainder was collected previous to accreditation and had no certificate of origin but was submitted to biochemical tests so it could be certified as *Brucella*.

The determination of limit of detection (LOD) was performed on ten-point decimal dilutions of a sample of *B. abortus*. The dilutions were tested in triplicate in the mPCR. The LOD was determined by the lowest concentration at which the agent was detected in all replicates. For the repeatability tests, it was used seven *Brucella* spp. and seven bacteria belonging to others genera previously cited as negative controls. These tests were carried out in three days by two analysts. Regarding the reproducibility, 20 samples were used in the mPCR with three high performance enzymes from different manufacturers:

Table 2 - Electrophoresis profiles of *Brucella* spp. observed according to the primers used on mPCR.

			-----Species/Biovar-----			
Primers	*B. abortus* S19	*B. abortus* 1, 2 e 4	*B. abortus* 3, 5, 6 e 9	*B. melitensis*	*B. suis*	*B. ovis*
Bru-AMOS-Ab is711	+	+	-	-	-	-
Bru-AMOS-Me is711	-	-	-	+	-	-
Bru-AMOS-Ov is711	-	-	-	-	-	+
Bru-AMOS-Su is711	-	-	-	-	+	-
16s.Universal	+	+	+	+	+	+
Bru-BMEii	-	+	+	+	+	+

Table 3 - List of biovars of **Brucella abortus** identified in bacterial isolation and their Brazilian State.

Brazilian State	*B. abortus* 1	*B. abortus* 2	*B. abortus* 3	*B. abortus* 4
Pará	18	45	10	1
Mato Grosso	10	2	2	0
Mato Grosso do Sul	1	1	0	0
Rondônia	2	1	0	0
Tocantins	2	1	2	0
Rio Grande do Sul	13	1	0	0
Total	46	51	14	1

GoTaq (Promega, USA) Platinum Taq (Invitrogen, USA) and Jump Start (Sigma Aldrich, USA).

RESULTS AND DISCUSSION

Standardization

The best concentrations for AMOS PCR were defined by trial and error as 0.20pmol uL^{-1} of each primer, 0.05U uL^{-1} GoTaq (Promega, USA), 1.2mmol L^{-1} MgCl$_2$, 0.25mmol L^{-1} dNTP, and 1X Buffer Green (Promega, USA) to a final volume of 25μL with 2.5μL of this DNA at 10 to 100ng μL^{-1}. The reaction went through a period of denaturation at 95°C for 3min, followed by 35 cycles at 95°C for 75s, 80s and 55.5ºC by 72°C for 80s and a final extension cycle at 72°C for 5min. In order to confirm the PCR results, products were visualized on 1.5% agarose gel electrophoresis with 60 minutes running time at 100V.

All 164 samples submitted to bacterial biochemical assays were concordant in the mPCR results. In EWALT & BRICKER (2000), the number of samples used in the validation of a simplified AMOS PCR was 231, which is a close number to the present research.

Limit of detection

The LOD found was 0.35ng μL^{-1}. This concentration is considered sufficient for this particular test, given the high number of bacteria in each colony obtained during the isolation process. The amount of DNA is so high that some samples may need to be diluted for a better visualization of each PCR band in gel electrophoresis (DATA NOT SHOWN). The main concern is to obtain a better test specificity, so the LOD is important only to clarify the minimum amount of DNA recommended to be used in the PCR.

Repeatability

The result of the repeatability of tests conducted by two analysts was considered satisfactory as all samples had the same molecular profiles defined during test standardization. Repeatability is the ability of a method to produce the same response with different operators in the same laboratory. It is therefore important to understand the factors that influence the outcome of a particular technique in order to ensure its reliability.

Reproducibility

The variables used in the reproducibility test did not show satisfactory results since it was not observed the pattern of bands expected using those enzymes even though various concentrations of enzymes or different MgCl$_2$ concentrations have been tested. Therefore, enzymes Taq Platinum and Jump Start were excluded as an option in molecular test to identify **Brucella** spp. by this mPCR (Figures 2 and 3).
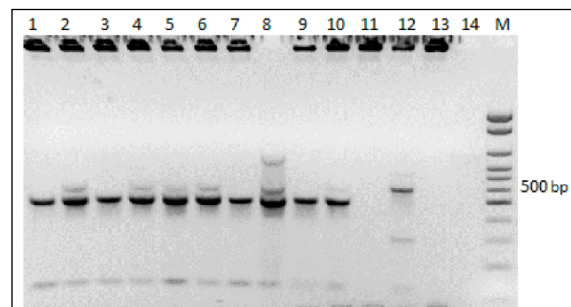

Figure 2 - 1.5% agarose gel electrophoresis demonstrating the results after the mPCR was performed with the *Jump Start* enzyme (final concentration 0.1U uL^{-1}). Markers at intervals of 100 base pairs. In this robustness test, we did not observe the pattern of bands expected using this enzyme, and is therefore unsuitable for this reaction. Samples: 1 - DB 012/12 23 T1; 2 - DB 012/12 16 T1; 3 - DB 012/12 1 T1; 4 - DB 012/12 2 T1; 5 - DB 012/12 25 TI; 6 - DB 012/12 41 T1; 7 - DB 012/12 26 T1; 8 - *B. abortus* biovar 1. This same profile is observed to *B. abortus* biovars 2 and 4; 9 - *B. abortus* S19; 10 - *B. abortus* biovar 1; 11 - *B. abortus* biovar 3b. This same profile is observed to *B. abortus* biovars 5, 6 and 9; 12 - *B. melitensis*; 13 - *B. suis*; 14 - Blank (No DNA template); 15 - MM (Molecular Marker).
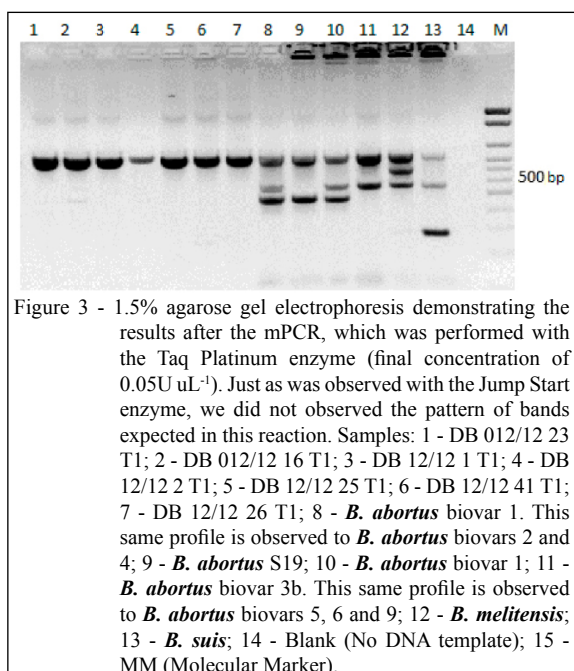
Figure 3 - 1.5% agarose gel electrophoresis demonstrating the results after the mPCR, which was performed with the Taq Platinum enzyme (final concentration of $0.05U\ uL^{-1}$). Just as was observed with the Jump Start enzyme, we did not observed the pattern of bands expected in this reaction. Samples: 1 - DB 012/12 23 T1; 2 - DB 012/12 16 T1; 3 - DB 12/12 1 T1; 4 - DB 12/12 2 T1; 5 - DB 12/12 25 T1; 6 - DB 12/12 41 T1; 7 - DB 12/12 26 T1; 8 - *B. abortus* biovar 1. This same profile is observed to *B. abortus* biovars 2 and 4; 9 - *B. abortus* S19; 10 - *B. abortus* biovar 1; 11 - *B. abortus* biovar 3b. This same profile is observed to *B. abortus* biovars 5, 6 and 9; 12 - *B. melitensis*; 13 - *B. suis*; 14 - Blank (No DNA template); 15 - MM (Molecular Marker).

Not even the differing changes in the concentrations of these enzymes were enough to increase efficiency in the reaction. This type of result is common since different enzymes have different activities depending on the conditions in which they are used (TERPE, 2013). Thus, in this mPCR, the most efficient enzyme was GoTaq (Promega, USA).

The mPCR validated in the present research has identified different *Brucella* species and was able to discriminate the vaccine strain S19, field strain and different groups of biovars found in Brazil (MINHARRO et al., 2013). Originally described AMOS PCR can not detect biovars 3, 5, 6 and 9 and does not any internal control for DNA extraction and PCR inhibition, except when working with differentiation of vaccine samples (BRICKER & HALLING, 1995). Discriminating biovars is an important step as it contributes significantly to future epidemiological studies. The presence of an extraction control to verify the possibility of PCR inhibition is also fundamental to the process of quality assurance and to determine if a negative result occurs due to the absence of a specific *Brucella* DNA or to the presence of inhibitors or lack of DNA (RUSSO & MALNATI, 2014).

The mPCR is a low-risk diagnostic technique because there is only the manipulation of genetic material from the agent in the laboratory. This is important in the case of a highly pathogenic microorganism to men. Brucellosis is one of the most commonly acquired diseases in laboratories due to the manipulation of the agent in culture media (FIORI et al., 2000). The bacterial isolation for diagnosis of brucellosis requires a well structured laboratory with biosafety level 3 and highly qualified staff for handling these microorganisms. We estimated that the time taken for bacterial characterization by molecular method proposed in this research is five times lower when compared to the bacterial isolation method. In order to overcome the disadvantages mentioned, the introduction of molecular diagnostic technique substantially reduces handling of the colonies, which reduces the risk of contamination and the cost for determining the final diagnosis (YU & NIELSEN, 2010).

The method validated according to international guidelines in this research represents an important advance to biosafety and speed in the diagnostic of *Brucella* spp. It has also advantages compared to the original molecular methods described in literature as it is able to distinguish the biovars of *B. abortus* in grouped form.

ACKNOWLEDGEMENTS

REFERENCES

ALTON, G.G. et al. **Laboratory techniques in brucellosis**. Geneva, Switzerland: World Health Organization Monograph Series, 1975. N.55, p.11-63.

ALTON, G.G. et al. **Techniques for the brucellosis laboratory**. Paris: Institut National de la Recherche Agronomique, 1988. 190p.

BRASIL - PROGRAMA NACIONAL DE CONTROLE E ERRADICAÇÃO DA BRUCELOSE E DA TUBERCULOSE ANIMAL (PNCEBT). **Manual técnico – Ministério da Agricultura, Pecuária e Abastecimento**. Brasília, 2006. 188p.

BRICKER, B.J.; HALLING, S.M. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. **Journal of Clinical Microbiology**, v.32, n.11, p.2660-2666, 1994. Available from: <http://jcm.asm.org/content/32/11/2660.full.pdf>. Accessed: Jan. 20, 2014.

BRICKER, B.J.; HALLING, S.M. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. **Journal of Clinical Microbiology**, v.33, n.6, p.1640-1642, 1995. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC228233/pdf/331640.pdf>. Accessed: Jan. 15, 2014.

BRICKER, B.J. et al. Evaluation of the *Brucella abortus* species–specific polymerase chain reaction assay, an

improved version of the **Brucella** AMOS polymerase chain reaction assay for cattle. **Journal of Veterinary Diagnostic Investigation**, v.15, n.4, p.374-378, 2003. Available from: <http://vdi.sagepub.com/content/15/4/374. long>. Accessed: Jan. 20, 2014.

COLLINS, CH. **Laboratory-acquired infections**. London: Butterworths; 1983.

ERGÖNÜL, Ö. et al. Analysis of risk factors for laboratory-acquired **Brucella** infections. **Journal of Hospital Infection**, v.56, n.3, p.223-227, 2004.

EWALT, D.R.; BRICKER, B.J. Validation of the abbreviated **Brucella** AMOS PCR as a rapid screening method for differentiation of **Brucella abortus** field strain isolates and the vaccine strains, 19 and RB51. **Journal of Clinical Microbiology**, v.38, n.8, p.3085-3086, 2000. Available from: <http://jcm.asm. org/content/38/8/3085.long>. Accessed: Mar. 10, 2014.

FIORI, P.L. et al. **Brucella abortus** infection acquired in microbiology laboratories. **Journal of Clinical Microbiology**, v.38, n.5, p.2005-2006, 2000. Available from: <http://jcm.asm. org/content/38/5/2005.long>. Accessed: Mar. 10, 2014.

GALIŃSKA, E.M.; ZAGÓRSKI, J. Brucellosis in humans – etiology, diagnostics, clinical forms. **Annals of Agricultural and Environmental Medicine**, v.20, n.2, p.233-238, 2013. Available from: <http://aaem.pl/fulltxt.php?ICID=1052322>. Accessed: Jan. 15, 2014.

LAGE, A.P. et al. Brucelose bovina: uma atualização. **Revista Brasileira de Reprodução Animal**, v.32, n.3, p.202-212, 2009. Available from: <http://www.cbra.org.br/pages/publicacoes/ rbra/download/RB206%20Lage%20vr2%20pag202-212.pdf>. Accessed: Mar. 10, 2014.

LÓPEZ-GOÑI, I. et al. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all **Brucella** species, including the vaccine strains. **Journal of Clinical Microbiology**,

v.46, n.10, p.3484-3487, 2008. Available from: <http://jcm.asm. org/content/46/10/3484.long>. Accessed: Jan. 20, 2014.

MINHARRO, S. et al. Biotyping and Genotyping (MLVA16) of **Brucella abortus** isolated from cattle in Brazil, 1977 to 2008. **PLoS ONE**, v.8, n.12, p.1-7, 2013. Available from: <http://journals.plos.org/plosone/article?id=10.1371/journal. pone.0081152>. Accessed: Mar. 10, 2014.

NIELSEN, K.; DUNCAN, J.R. Animal brucellosis. In: BLASCO, J.M. **Brucella ovis**. Florida: CRC, 2000.

OIE (WORLD ORGANIZATION FOR ANIMAL HEALTH). **Manual of diagnostic tests and vaccines for terrestrial animal**. 6.ed. Paris, 2008. V.1.

RODRIGUES, A.L. et al. Outbreak of laboratory-acquired **Brucella abortus** in Brazil: a case report. **Revista da Sociedade Brasileira de Medicina Tropical**, v.46, n.6, p.791-794, 2013. Available from: <http://www.scielo.br/scielo.php?script=sci_ arttext&pid=S0037-86822013000600791&lng=en&nrm=iso&tl ng=en>. Accessed: Mar. 10, 2014.

RUSSO, D.; MALNATI, M.S. Absolute quantification of viral DNA: the quest for perfection. **Methods in Molecular Biology**, v.1160, p.75-86, 2014. Available from: <http://link. springer.com/protocol/10.1007%2F978-1-4939-0733-5_7>. Accessed: Mar. 10, 2014.

TERPE, K. Overview of thermostable DNA polymerases for classical PCR applications: from molecular and biochemical fundamentals to commercial systems. **Applied Microbiology and Biotechnology**, v.97, p.10243-10254, 2013. Available from: <http://link.springer.com/article/10.1007%2 Fs00253-013-5290-2>. Accessed: Jan. 15, 2014.

YU, W.L; NIELSEN, K. Review of detection of **Brucella** sp. by polymerase chain reaction. **Croatian Medical Journal**, v.4, n.51, p.306-313, 2010. Available from: <http://www.ncbi.nlm. nih.gov/pmc/articles/PMC2931435/>. Accessed: Jan. 20, 2014.