African Journal of Biotechnology Vol. 10(75), pp. 17291-17296, 28 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2016
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Improvement of specific polymerase chain reaction (PCR) for the identification of *Mycoplasma capricolum* subsp. *capripneumoniae*

He Ying, Zhang Nian-zhang, Zhao Ping, Chu Yue-feng, Gao Peng-cheng, Zhang Jian-jun and Lu Zhong-xin*

State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Epizootic Diseases of Grazing Animal of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China.

Accepted 7 October, 2011

A polymerase chain reaction (PCR) test for the identification of *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) was optimized using the H2 gene sequences of M1601. The test was evaluated on 20 strains including six representative strains of the *Mycoplasma mycoides* cluster as well as 13 field isolates from China, *Pasteurella multocida and Mannheimia haemolytica*. To obtain a test that was specific for Mccp, the PCR produced an amplicon of approximately 680 bp only with the Mccp strains. The specificity of the present PCR was found to be 100% specific for Mccp. The sensitivity of the PCR showed that it could detect a minimum of 0.75 ng of purified DNA. For further evaluation, clinical samples tested by PCR included lung and liquor pleurae from 14 goats artificially infected with Mccp. The PCR was positive in all the clinical samples both in the undiluted and 50-fold dilutions of extracted DNA except for a sample of liquor pleurae. However, the latter showed weak bands. An epidemiological survey was also performed using the constructed PCR methods and results indicated that 36 of 61 of these samples including tissue and percolate from Western China were considered positive. The coincidence rate was 82% compared with the performance of a PCR test earlier published. The present PCR represents a rapid and reliable method for genetically based identification of Mccp. The specificity of the test makes it suitable for detection of Mccp in clinical samples.

Key words: *Mycoplasma capricolum* subsp. *capripneumoniae,* PCR, identification.

INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe infectious disease of goats caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (MCCP), which occurs in many countries of Africa and Asia (Woubit et al., 2004) and is a disease of major economic relevance characterized by high morbidity and mortality. The mortality rate often approaches 100% in susceptible herds (Rurangirwa et al., 1987). A mycoplasma strain designated F-38 was isolated in Kenya (MacOwan and Minette, 1976) and is a member of the *Mycoplasma mycoides* cluster, which includes *M. mycoides* subsp. *mycoides* SC (MmmSC), *M. mycoides* subsp. *mycoides* LC (MmmLC), *M. mycoides* subsp. *capri* (mmc), *M. capricolum* subsp. *capricolum* (Mcca) and *Mycoplasma* sp. bovine group7 (bg7).

Definite diagnosis is made by culture of the causative agent from lung samples or pleuritic fluid taken post mortem. Isolates may be identified by biochemical, immunological and molecular tests. At present, serological tests for the detection of specific antibodies have relied on the complement fixation, a test prescribed by the Office International des Epizooties (OIE) for international trade. A latex agglutination test is also available and is used routinely in some parts of Africa, but thus far,

*Corresponding author. E-mail: luzhongxin@hotmail.com. Tel: 86 931 8342676.

Abbreviation: Mccp, *Mycoplasma capricolum* subsp. *Capripneumoniae;* **PCR,** polymerase chain reaction.

Table 1. Collection of strains used to test the sensitivity and specificity of the *Mycoplasma capricolum subsp. capripneumoniae* PCR test.

Species	N	Description	Origin	PCR
M. capricolum subsp. capripneumoniae	6	F38, M1601, SD,8711,8713,116	F38 CIRAD M1601, SD,8711,8713,116 China	+
M. ovipneumoniae	5	Y98, MoGH3-3,TX,GS-17, Y116-1	Y98 NTCC MoGH3-3,TX,GS-17, Y116-1 China	-
M. mycoides subsp. Capri	1	PG3	China	-
M. mycoides subsp. mycoides Large colony	1	Y- goat	CIRAD	-
M. capricolum subsp. capricolum	1	C. Kid	CIRAD	-
M. agalactiae,	1	GS. 12	China	-
M. arginini,	1	PG 1	China	-
M. bovis	2	M.B1 ;M.B2	China	-
Pasteurella multocida	1	*Pasteurella multocida*	China	-
Mannheimia haemolytica	1	*Mannheimia haemolytica*	China	-

there is no way to detect this pathogen rapidly and specifically. Both assays are based on an initial *M. mycoides* cluster-specific PCR amplification of a fragment of the 16SrRNA (Bascunana et al., 1994) or the CAP21 loci (Hotzel et al., 1996). The specificity for Mccp identification is obtained by *Pst*I cleavage, which detects a point mutation in one of the two rRNA operons coding for Mccp (Bascunana et al., 1994), or by a nested PCR using a second set of primers (Hotzel et al., 1996). In 2004, a new PCR assay was developed that would not have the aforementioned drawbacks and would allow a one-step specific detection of Mccp strains (Woubit et al., 2004), but the result of this PCR allows strain YG (MmmLC) to yield a faint amplification of a smaller fragment (Woubit et al., 2004). The objective of this study was to develop an improved specific PCR test for the detection and identification of Mccp strains.

MATERIALS AND METHODS

Strains and cultivation

The origin of the 20 strains of the *M. mycoides* cluster and field isolated in China are listed in Table 1. The *Mycoplasma* strains were cultivated in modified KM_2 (Hank's solution with 1.7% lactalbumin hydrolysate, 1% MEM, 20% decomplemented horse serum, 5% fresh yeast extract, 1% thallium acetate and 0.4% sodium pyruvate) in a high security laboratory. The DNA of *Pasteurella multocida* and *Mannheimia haemolytica* was maintained in the State Key Laboratory of Veterinary Etiological Biology.

Clinical samples

28 samples from 14 artificially infected animals with *M. capricolum* subsp. *capripneumoniae* were used in this study. The clinical samples were collected when the goats exhibited primary clinical signs, including coughing, anorexia, labored breathing with painful grunting and a rise in temperature up to 41°C. Because the gross pathological lesions were localized exclusively in the lungs, hepatized tissue samples were collected for experimentation (Table 2). 61 clinical samples, including tissue and percolate were collected from GANSU province, QINHAI province and NIXIA province of Western China from 2009 to 2011 and used for the epidemiological survey. These samples were kept at -80°C until analyzed.

Preparation of samples

Aliquots (1 ml) of culture were centrifuged at 13,000 rpm for 20 min at 4°C. The cell debris was pelleted, then washed in PBS and re-suspended in 50 µL ddH_2O, vortexed, lysed by boiling for 10 min, centrifuged and diluted to concentrations of 1:50. For the clinical samples, DNA extraction was performed using the Invitrogen kit (Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted DNA was used for PCR in both an undiluted form and in a 1:50 dilution.

PCR conditions

Based on the conserved sequence of the H2 gene (GenBank accession number: AF162991.1) of Mccp, suitable primers were designed using the primer 5.0 software (Primer Premier) (F 5′-AAA AGT CCC TGA AAC ATT AC-3′, bp 319 to 338 and R 5′-GGT GTA CCC ACT GCT AAA GA 3′, bp 1032 to 1013). These primers were synthesized by TaKaRa (Dalian, China). The 50 µL reaction mixture contained 3 µL $MgCl_2$ (1.5 mM), 0.5 µL dNTP (150 µM for dCTP and dGTP, 300 µM for dATP and dTTP), 5 µL 10 × *Taq* Buffer (TaKaRa), 1 µL of each primer, 0.5 µL *Taq* polymerase (1 unit, TaKaRa), 5 µL DNA sample and complement with 34 µL ddH_2O. PCR conditions consisted of an initial denaturation step of 5 min at 94°C followed by 35 cycles of 45 s at 94°C, 30 s at 50°C and 45 s at 72°C and a final extension step of 10 min at 72°C. Samples of PCR amplification products (5 µL) were subjected to electrophoresis in a 1% agarose gel in tris/borate buffer according to standard protocols. DNA was visualized by UV-fluorescence after staining with ethidium bromide.

Table 2. Isolation of *Mycoplasma capricolum subsp. capripneumoniae* and PCR-detection from artificial infected animals with Mccp.

Goat number	Macroscopic finding	Sample type	Microbiology result	PCR	
				Undiluted	1:50
1	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
2	slight hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
3	slight hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
4	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
5	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
6	slight hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
7	slight hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
8	slight hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
9	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
10		lung	-	+	+
		liquor pleurae	-	+	-
11	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
12	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
13	serious hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+
14	serious hepatized hepatized	lung	Mccp	+	+
		liquor pleurae	Mccp	+	+

Investigation of sensitivity

The sensitivity of the PCR was evaluated using a ten-fold DNA dilution of F1601 (starting with 75 ng/ml), where each dilution was used as a template for PCR in triplicate. Each PCR was repeated three times.

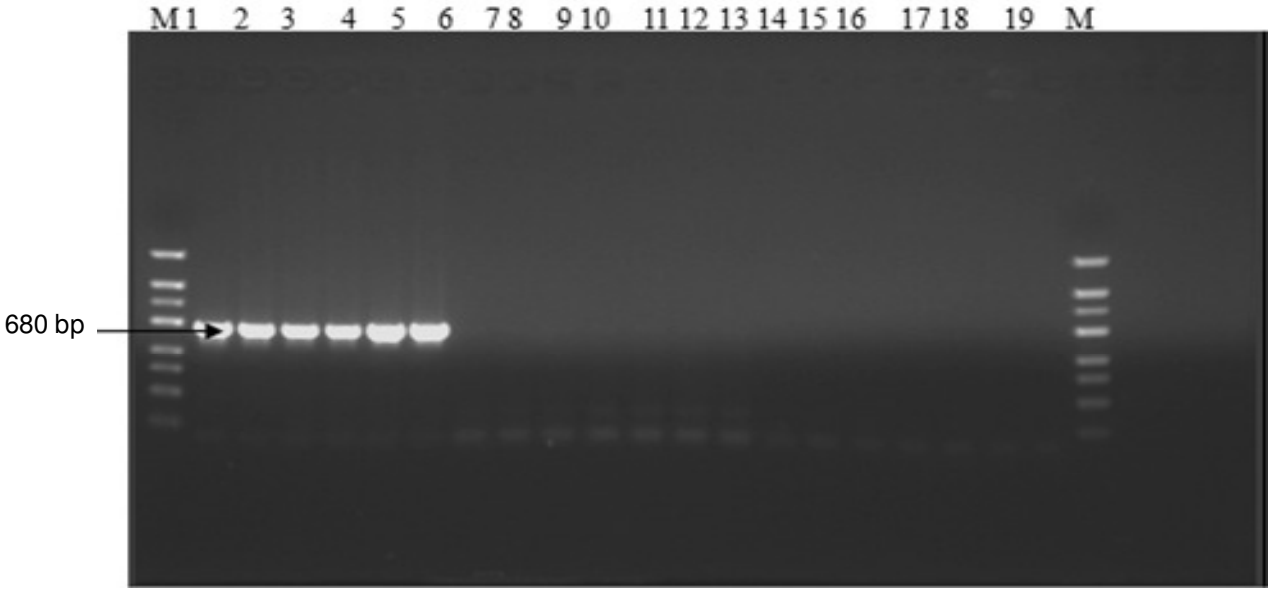

Figure 1. Gel electrophoresis of PCR products showing a specific amplicon of 680 bp for *M. capricolum* subsp. *Capripneumoniae* (Mccp) strains. Lane M, Molecular weight marker; Mccp strains in lane 1, M1601; lane 2, F38; lane 3, SD; lane 4, 8711; lane 5, 8713; lane 6, 116; lanes 7 – 11, *M. ovipneumoniae* strains; Y98; MoGH3 – 3; TX; GS-17; Y116-1; lane 12, *M. mycoides subsp. Capri* PG3; lane 13, *M. mycoides subsp. Mycoides large colony* Y-goat; lane 14, *M. capricolum subsp. Capricolum C.* Kid; lane 15, *M. agalactiae* GS. 12; lane 16, *M. arginine* PG 1; lanes 17 and 18, *M. bovis* strains M.B1 AND M.B2; lane 19, *Pasteurella multocida*; lane 20, *Mannheimia haemolytica.*

RESULTS

Validation of PCR method

The PCR produced an amplicon of approximately 680 bp with all six *M. capricolum* subsp. *capripneumoniae* samples. However, the other field isolates and representative strains of the *M. mycoides* cluster as well as *P. multocida* and *M. haemolytica* listed in Table 1 were negative (Figure 1).

Test sensitivity

The sensitivity of the tests was evaluated on boiling culture. The PCR test of Woubit et al. (2004) could not show the sensitivity, while the present PCR test was positive from the fourth PCR sample when tested using ten-fold diluted DNA, but not in the fifth PCR sample (Figure 2). The least detectable concentration was 750 pg. Identical results were observed in all replicates of the tests. Addition of 1.0×10^5 pg of extracted DNA from *P. multocida* and *M. haemolytica* to each PCR tube did not alter the sensitivity of the tests (data not shown).

Clinical samples

28 clinical samples originating from 14 animals showing serious clinical symptoms were included in the analysis. 28 samples using undiluted templates and 28 samples using 1:50 diluted templates were positive (Figures 3 and 4). In addition, two samples from one animal were positive in the PCR assay, but cultivations were negative. To further evaluate the present PCR, the comparison to the PCR assay of Woubit et al. (2004) was performed through an epidemiological survey using the samples from western China.

The results are shown in Table 3. Overall, the present PCR from 61 clinical samples gave a total of seven more positive results than the earlier published PCR test (Woubit et al., 2004). Furthermore, the positive results performed by the PCR test of Woubit et al. (2004) were in agreement with the present PCR tests, except for two lung samples (Table 3).

DISCUSSION

The present PCR can recognize all strains of *M. capricolum* subsp. *capripneumoniae*, both from the sample strains and collections. Further, it can distinguish Mccp and Y-goat strains, although, *M. capricolum* strain *California kid* can be amplified in a smaller fragment of nearly 250 bp. The present PCR could also distinguish Mccp from *P. multocida* and *M. haemolytica,* which are also common pathogens of the goat respiratory tract. The present PCR consequently represents a clear

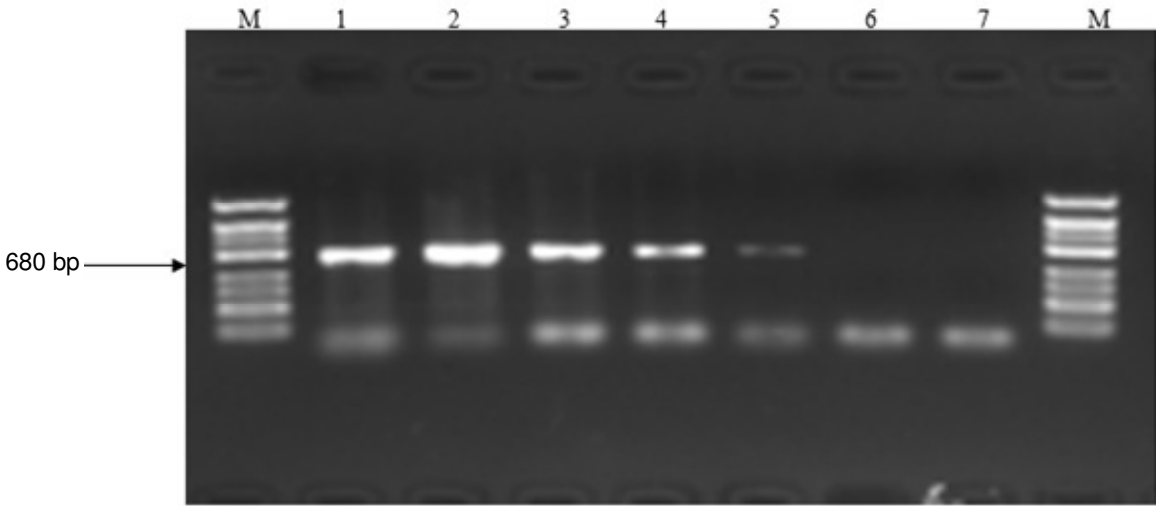

Figure 2. The result of the sensitivity of PCR. Lane M, Molecular weight marker; lanes 1 to 5, 7.5 µg; 750 ng; 75 ng; 7.5 ng; 750 pg respectively. The last concentration by detection was 750 pg.

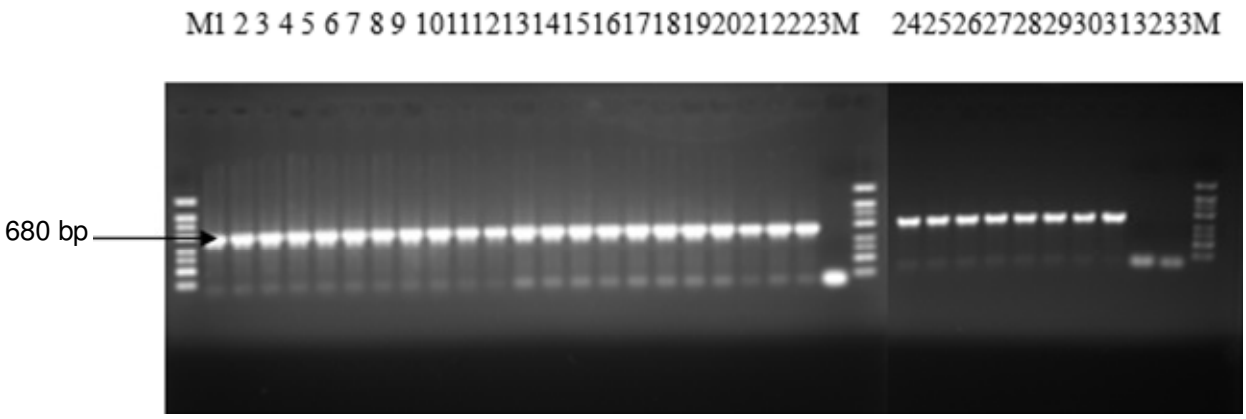

Figure 3. The result of PCR detection from artificially infected animals with Mccp in undiluted samples. Lane M, Molecular weight marker; lanes 1 to 21 and 24 to 30, clinical samples; lanes 22 and 31, positive control; lanes 23 and 32, negative control; lane 33, normal lung tissue of goat.

complement compared to the earlier published PCR of Woubit et al. (2004). Lack of PCR specificity makes conclusions difficult; consequently, the interpretation of the earlier published PCR test is uncertain when it is used for the detection of *M. capricolum* subsp. *capripneumoniae* in clinical lung samples. There was a high sensitivity when tested on purified 10-fold diluted DNA.

The lowest detectable concentration was 0.75 ng. For better evaluation, the PCR tests on clinical samples were dependent on whether the samples were used undiluted or diluted 1:50 before use as templates (Table 2). In the undiluted samples, high DNA concentrations might give positive results. The performance of the tests might be improved using high concentrations of DNA or a more optimal DNA concentration. However, if the DNA concentration in the starting material is too high, the PCR using diluted samples will give the best test results. The best results can be expected when both undiluted and diluted templates are used for the PCR test.

The results of the PCR of the clinical samples, performed with both the established Woubit method and the present protocol, showed coherence. 27 positive results out of 61 clinical samples were found by both PCR tests. The present PCR test detected 36 positive results in total, while only two positive results out of 29 clinical samples were found using the earlier published PCR methods. The results therefore showed that the two PCR methods may be used for different purposes. Moreover, the present PCR has been shown to represent a rapid and reliable method for genetically based identification of Mccp. The high specificity and sensitivity of the test makes it suitable for detection of Mccp in clinical samples, regardless of the presence of affiliated species

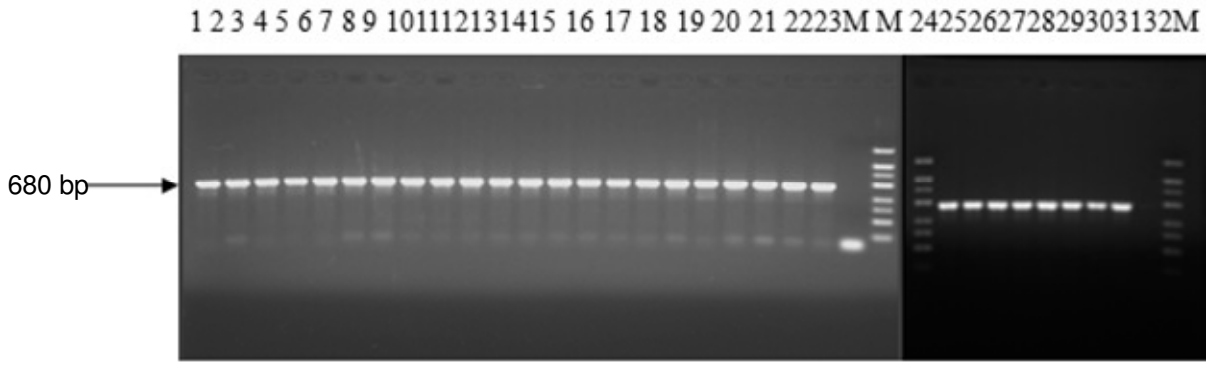

Figure 4. The result of PCR detection from artificially infected animals with Mccp samples diluted as 1:50. Lane M, Molecular weight marker; lanes 1 to 21 and 24 to 30, clinical samples; lanes 22 and 31, positive control; lane 23, negative control, lane 32, normal lung tissue of goat.

Table 3. Isolation of *Mycoplasma capricolum subsp. capripneumoniae* and PCR-detection from clinical samples obtained from animals suspected for being infected with Mccp.

Sample type	Number of positive results (%)	
	PCR 1 (this work)	PCR2[a]
Lung tissue	26 (42)	21 (42)
liquor pleurae	10 (19)	8 (19)
Total	36	29

PCR2[a] Source, Woubit et al. (2004).

and contaminating flora. Hence, we believe that the present PCR test will be more suited for the diagnosis of Mccp, especially for laboratories that are not experienced with the culture of Mccp or in situations where culture is not possible.

ACKNOWLEDGEMENTS

We are grateful to Dr. Thiaucourt at CIRAD, who provided us with strains F-38, Y-goat and C. Kid. We are also grateful for the funding from the State Key Laboratory of Veterinary Etiological Biology, The Technology R&D Program of Gansu Province (1011NKCA054), Gansu Province Agricultural Biotechnology Research and Application Development Project (GNSW-2010-09), and The National Scientific-Basic Special Fund (2008FY210200), and The Natural Science Foundation of Gansu Province (1107RJZA107)

REFERENCES

Bascun˜ana CR, Mattsson JG, Bolske G, Johansson KE（1994）.Characterization of the 16S rRNA genes from Mycoplasma sp.strain F38 and development of an identification system based on PCR. J. Bacteriol. 176: 2577–2586.

Hotzel H, Sachse K, Pfu¨tzner H (1996). A PCR scheme for differentiation of organisms belonging to the Mycoplasma mycoides cluster. J. Vet. Microbiol. 49(1-2): 31–43.

MacOwan KJ, Minette JE (1976). A mycoplasma from acute contagious caprine pleuropneumonia in Kenya. Trop. Anim. Health Prod. 23(8): 652-661.

Rurangirwa FR, McGuire TC, Musoke AJ, Kibor A (1987). Differentiation of F38 mycoplasmas causing contagious caprine pleuropneumonia with a growth-inhibiting monoclonal antibody. J. Infect. Immun. 55(12): p. 3219.

Woubit S, Lorenzon S, Peyraud A, Manso-Silva´n L, Thiaucourt F, (2004). A specific PCR for the identification of *Mycoplasma capricolum* subsp. capripneumoniae, the causative agent of contagious caprine pleuropneumonia (CCPP). J. Vet. Microbiol. 104(1-2): 125-132.