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Identification of Intrauterine Mycoplasma in Endometritis of Dairy Cow Isolates by 16S rDNA Nested PCR-DGGE

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Abstract: Molecular diagnostics methods are used to investigate mycoplasma infection status but this approach to infection analysis of mycoplasma in endometritis of dairy cow has not been reported. In this study, a nest Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (nested PCR-DGGE) assay for detection of 16S ribosomal RNA of mycoplasma was used as reference of 4 isolates which were identified in a previous survey of dairy cow's reproductive system in Jilin province, China. A reference PCR-DGGE profile was generated using DNA extracted and amplified from the strain of these cultures. The effectiveness of the procedure was assessed by application to test the samples from endometritis dairy cow's uterus mucus. The bands from the nested PCR-DGGE were extracted for sequencing.

Key words: Mycoplasma, endometritis, cultures, cow's reproductive system, China

INTRODUCTION

Mycoplasma, an important gynecological infectious pathogens has been implicated in spontaneous abortion, preterm labor, endometritis, chorioamnionitis and premature birth (Quinn *et al*., 1987; Izraeli *et al*., 1991). The main injury to the host of mycoplasma is Intrauterine Growth Retardation (IUGR) (Germain *et al*., 1994). The DNA or RNA, damaged by mycoplasma infection, leads to express of new proteins and change cell behavior (Rui-Ling and Yuan-Zhe, 2008).

Mycoplasma interacted with other bacteria is involved in important tissue damage in dairy cow's reproductive system. Evidence of the damage of mycoplasma is abundant but understanding of the specific species of mycoplasma is very limited. Serological assays are the traditional methods for taxonomic identification and pathogenicity studies. The species recovered from dairy farm are very difficult to identify and require trap cultivation on host cells to produce specific reaction to identify (Rosendal, 1975). However, this approach is not enough to make sense of it because the mycoplasma infection is dependent on the species, host, growth conditions and other environmental factors (Luo *et al*., 2000). Molecular diagnostics methods can be used for identification of mycoplasma from uterus directly, avoid the deficiency of culture, difficulties and could potentially provide information on the active phase of the infection by mycoplasma (Rikihisa *et al*., 1997; Neimark and Kocan, 1997; Neimark *et al*., 2001).

Muyzer was the first one to use PCR-DGGE to profile microbial identification (Muyzer *et al*., 1993). After that this technique was widely used for identifying pathogenic bacterium on dental and oral disorders and many other diseases (Fujimoto *et al*., 2006; De-Oliveira *et al*., 2007; Donskey *et al*., 2003). At present, PCR-DGGE has proven to be a powerful technique for the culture-independent detection and characterization of microorganisms (Kocherginskaya *et al*., 2001; Konstantinov *et al*., 2003; Zoetendal *et al*., 1998). PCR-DGGE was demonstrated to be complimentary to cloning strategies for pathogenic bacterium of bovine mastitis studies by tentatively identifying cloned 16S rDNA fragments (Kuang *et al*., 2009). Vainio and Hantula showed DGGE assay is more accurate than culturing technique for detecting microorganism species from environmental samples (Vainio and Hantula, 2000).

The objective of this study was to develop a nested PCR-DGGE procedure for detecting intrauterine mycoplasma from endometritis of dairy cows from Jilin

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province, China. To prove its efficacy, the technique was applied to test uterine mucus samples from a survey of 10 cows from 5 dairy farms. To the knowledge, this is the first use of nested PCR-DGGE to evaluate the infection by mycoplasma from endometritis of dairy cow.

MATERIALS AND METHODS

Reference mycoplasma species: Four species of mycoplasma were used for the development of the procedures (Table 1) which had been putatively identified in a previous survey of endometritis in dairy cow (Nicholas *et al*., 2000). These isolations were regenerated in pot cultures. All reference cultures were separated prior to use in DNA extraction.

Samples collection from dairy farm: Uterus mucus samples were kindly provided by Jilin, Baishan, Fengman, Yanbian and Xin Xiwang dairy farms in China. All of them were aseptically collected from dairy cows with endometritis of different severity while sample H1 was from a healthy cow. Table 2 shows detailed information about these samples and the corresponding cows. All samples were kept on liquid nitrogen until their transportation to the lab and then they were stored at -20°C until further processing.

Before transporting, the samples culturing was performed to clarify the major and minor pathogens in these samples. Briefly, the uterus mucus was plated onto selective agar including blood agar. To further identification of these organisms, researchers performed Giemsa staining and morphology tests and these samples with positive results were diagnosed as infected with mycoplasma while negative was identified as no mycoplasma infectious in H1.

DNA extraction from uterus mucus samples: Selected uterus mucus were centrifuged at 3000×g for 30 sec and washed twice with 2 mL of stroke-physiological saline solution. Three times of crush/freeze/thaw were performed on the samples using a flame sterilized mortar and liquid N2. Raw lysates were suspended in 2 mL TE buffer. An equal volume of phenol:chloroform:isoamylalcohol (12:10:1) was added to each lysate and centrifuged twice for 45 sec at 6000×g. The tubes were centrifuged for 10 min at 4000×g. Aqueous layers were placed in new tubes in ice bath. To remove phenol an equal volume of chloroform:isoamylalcohol (24:1) was added to the collected aqueous layer and the tube was gently inverted for 5 sec, placed in a new tube with 35% (w/v) polyethylene glycol and incubated at room temperature to precipitate the DNA. Precipitated DNA was spun at 13,000×g for 10 min to pellet. The supernatant was removed and the pellet was washed with 70% (v/v) ethanol. Ethanol was drained and the pellet allowed to air dry for 20 min. Finally, the pellet was suspended in 1 mL autoclaved, distilled and deionized water (ddH_2O).

DNA extraction and nested PCR strategy: DNA extraction from the uterus mucus sample was subjected to the first PCR using primers (Table 3) PM1 and PM2 to amplify the *16S rDNA* gene about 492 kb (Uemori *et al*., 1992). These primers were universal 16S rDNA mycoplasma primers. PCR was performed in 50 μL volume with 2.0 μL template DNA using the Taq PCR Master Mix System (Takata, Dalian, China) with the manufacture's recommended buffer, enzyme and nucleotide conditions. Product was amplified on GStorm Gradient PCR (Dongsheng, Beijing, China) using the following conditions: 95°C for 10 min; 30 cycles (95°C, 45 sec; 64°C, 60 sec; 72°C, 45 sec); 72°C, 10 min. PCR products were analyzed by 1.2% agarose gel electrophoresis.

First stage PCR products were used as templates in second stage PCR using the reaction mixture described above except for primers. The second stage primers (PMS1, PMS2 in Table 3) and GC-Clamp produced a 240 bp fragment (based on sequences matched from GenBank). Thermo cycling used the following condition: 95°C for 5 min; 30 cycles (94°C, 45 sec; 67°C, 60 sec; 72°C, 45 sec), 72°C, 10 min. Nested PCR products were analyzed as the first PCR Method.

Table 1: Reference Mycoplasma species used as controls in this study

No.	Species	Source
MG2009A	*Mycoplasma genitalium* (MG)[a]	University of Jilin,
UU2009B	*Ureaplasma urealyticum* (UU)[a]	College of Animal Science
MB2009C	*Mycoplasma bovis* (MB)[b]	Veterinary Medicine Clinic
MA2009D	*Mycoplasma agalacia* (MA)[b]	Immunology Lab Culture Collection

[a]They were isolated form endometritis cows in Hei Longjiang, China by our lab in 2009; [b]They were isolated form mastitis cows in Nei Menggu China by the lab in 2009

Table 2: Information on cows which proided uterus mucus samples

Cow No.	Source (dairy farm)[a]	Age of cow (year)	Postpartum time (day)
J1, J2	JI LIN	2	20
B1, B2	BAI SHAN	2	20
F1, F2	FENG MAN	2	20
Y1, Y2	YAN BIAN	2	20
X1, X2	XIN XIWANG	2	20

[a]They were discovered endometritis cows according to clinical symptoms

Table 3: Oligonucleotide primers used for nest-PCR

Primers	Sequence (5'-3')	Product size (bp)
PM1	AGAGTTTGATCCTGGCTCAG	492
PM2	CTACGGCTACCTTGTTACGA	
PMS1	GC-clamp[a]-CCTACGGGAGGCAGCAG	240
PMS2	ATTACCGCGGCTGCTGGTGCGGCT	

[a]GC-clamp:CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

Denaturing Gradient Gel Electrophoresis (DGGE) analysis: DGGE was performed with the DcodeTM System (Bio-Red). The nest-PCR products were loaded onto a 6% polyacrylamide gel which was made with a denaturant gradient ranging form 40-60%. The 100% denaturant was 3.5 M urea and 40% deionized form amide. Electrophoresis was carried out in 1× TAE buffer at 120 V for 6 h at 60°C. The gels were stained for 90 min in 0.5× TAE buffer with ethidium bromide and visualized by UV illumination. Images of the gels were obtained by Fluor-STM MultiImager (BioRed, USA) and stored as TIFF files.

Sequence analysis of DGGE bands and partial 16S rDNA sequences: Prominent DGGE bands were excised from the UV illuminated acrylamide gels and DNA eluted from the excised gel by incubation in 100 μL ddH_2O at 28°C overnight. Eluted DNA was used for PCR amplification as described above and analyzed again by DGGE using a narrower gradient (45-55%). PCR products with single bands on the second DGGE were purified for sequence analysis using the PAGE DNA purification kit (AOXING; BEI JING, China) with a final elution volume of 30 μL and be sequenced by Sheng Gong Biotechnological Co., Ltd. in Shanghai, China. The sequencing results were compared with the National Centre for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/). Screening for possible chimeric sequences was done using the Ribosomal Database Project (RDP) online Chimera Check program (http://rdp.cme.msu.edu/html/analyses.html).

Detection limit of optimized procedures: The detection limit of the optimized procedures for the lab culture collection ureaplasma urealyticum was determined. The 1, 10, 10^2 and 10^3 were spiked into each of five 1 mL uterus mucus samples from the H1 dairy cow and subjected to the optimized extraction and PCR-DGGE procedures previously described. The detection limit is expressed as the concentration of spores required for detection by DGGE per milliliter of uterus mucus sample.

RESULTS AND DISCUSSION

DNA extraction and PCR-DGGE results of reference mycoplasma samples: Initially, mycoplasma samples genomic DNA was extraction by a Bacterial Genome Extraction kit (TianGen, Changchun, China). No PCR amplifiable template was produced by this method. The optimized method or requirement is the use of mortar and pestle to consistently extract amplifiable DNA from reference samples. Upon sequential amplification with the PM and PMS primer pairs, mycoplasma PCR products of target (492 and 240 bp, respectively) were applied to all the reference isolates tested. The 16S rDNA amplified products were reclaimed from gel of DGGE bands for the reference samples of *Mycoplasma genitalium* MG2009A, *Ureaplasma urealyticum* UU2009B, *Mycoplasma bovis* MB2009C and *Mycoplasma agalacia* MA2009D. Table 4 shows the sequences recovered (Fig. 1) and their most closely related isolate (s) determined by BLAST search of GenBank. BLAST searches yielded a minimum of 98% sequence similarity between the reference mycoplasma and the GenBank database.

Researchers tested whether the procedure was able to delineate members of a simple mycoplasma community. The analysis of reference mycoplasma by nested PCR-DGGE was performed. The DGGE analysis of the amplificated products within the range of 60 and 40% denaturant (Fig. 1). Isolates and reference mycoplasma samples were distinguishable from each other based upon DGGE mobility.

Detection limit of the developed molecular procedure when applied to reference samples: The DNA extraction method by bacterial genome extraction kit was able to extract amplifiable template from reference mycoplasma samples but the desired mycoplasma 16S rDNA fragments were not produced consistently (Fig. 2). To overcome the inconsistent DNA extraction from samples, a scaled-up Version of the Mortar and Pestle method adapted from the reference samples DNA extraction was used to generate the PCR-DGGE profile of Fig. 3. The optimized

Table 4: Sequences recovered from DGGE bands of reference mycoplasma samples

Accession No.	Species classification (Sequence designation)[b]	Most related isolate (s) from GenBank (Percentage sequence similarity by BLAST)[a]	GenBank Accession No. of most related sequences
MG2009A	*Mycoplasma genitalium* (mg-1)	*Mycoplasma genitalium* (98)	X77334, GI: 459531
UU2009B	*Ureaplasma urealyticum* (uu-3)	*Ureaplasma urealyticum* (99)	M23935, GI: 176284
MB2009C	*Mycoplasma bovis* (mb-4)	*Mycoplasma bovis* (99)	U02968, GI: 409968
MA2009D	*Mycoplasma agalacia* (ma-2)	*Mycoplasma agalacia* (100)	U44763, GI: 1174206

[a]97% sequence similarity is minimum requirement for identity; Stackbrandt and Goebel demonstrated that at sequence similarity values <97%, it is unlikely that two organisms will have >70% DNA-DNA reassociation after complete denaturation (the standard for species identity) and hence, they are related at no more than the species level; [b]Sequence designations are as labeled in Fig. 1

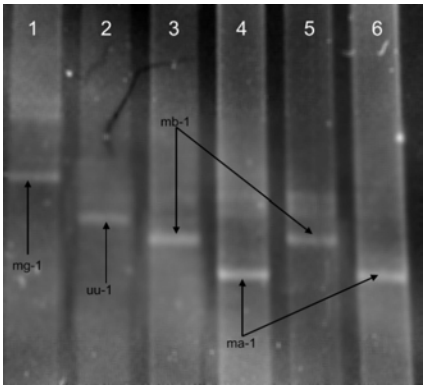

Fig. 1: DGGE profiles of 16S rDNA fragments for reference mycoplasma samples. Lane 1: *Mycoplasma genitalium* MG2009A; Lane 2: *Mycoplasma agalacia* MA2009D; Lane 3, 5: *Ureaplasma urealyticum* UU2009B; Lane 4, 6: *Mycoplasma bovis* MB2009C. Each arrow locates a single band. Each band is labeled with the accession number in Table 4 followed by a sequential designation (e.g., uu-2 denotes the PCR-DGGE band from reference mycoplasma *Ureaplasma urealyticum* UU2009B and it was the second band excised from the gel)

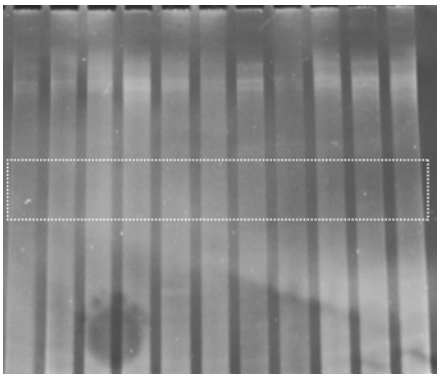

Fig. 2: DGGE profiles of 16S rDNA fragments for reference samples. 16S rDNA DGGE signatures generated from reference mycoplasma *Ureaplasma urealyticum* UU2009B extrcted DNA using the bacterial genome extraction kit; non-mycoplasma bands (as determined by sequencing) are encompassed by white dotted box

methodology produced detectable mycoplasma signatures in all reference samples. However, the detection limit of the procedure for mycoplasma concentration must be considered. The concentration of reference samples was 10^3 mL^{-1} uterus mucus samples.

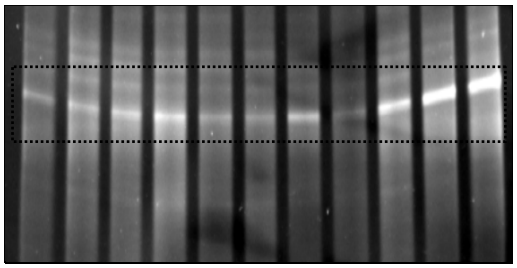

Fig. 3: DGGE profiles of 16S rDNA fragments for reference samples. 16S rDNA DGGE signatures generated from reference mycoplasma extracted DNA using the mortar and pestle method; the black dotted box: 16S rDNA DGGE profiles generated from reference mycoplasma Urealyticum UU2009B

From Fig. 4 an UU2009B signature from uterus mucus extracts was produced when spiked with 10^3 mL^{-1} mucus; therefore the minimum detection limit of the method for UU was 10^3 mL^{-1} uterus mucus samples.

PCR-DGGE detection of mycoplasma from uterus mucus of endometritis dairy cow: The success of the developed assay for detecting mycoplasma was judged by its ability to detect mycoplasma from uterus mucus samples of endometritis dairy cows.

All these samples were done in duplicate and the results were almost the same. Ten treatment samples had prominent DGGE bands of mycoplasma origin (i.e., bands within mobility range of reference bands; Fig. 4). The samples from healthy dairy cow had no detectable mycoplasma band.

The majority of recovered DGGE bands were identified as UU and MB by DGGE mobility and sequencing. The attempts were made to sequence all bands within the mobile range of the reference bands but some bands could not be recovered from DGGE gels or did not produce usable sequences.

The bands that produced usable sequences are shown in Table 5 along with the identity of their closest related sequences from GenBank. The majority of sequenced bands were identified as UU and MB. In addition, MB a previously undetected mycoplasma in Jilin, China was found in this survey (Fig. 5). Bands corresponding to all species were represented by the reference cultures. However, UU and MB were the sequence-confirmed reference species found in these endometritis in dairy cows. The effect of mycoplasma in endometritis in dairy cows is still very poor. Up to now, most of studies were constrained by the limitations of

Table 5: Sequences recovered from DGGE bands of endometritis cattle's uterus mucus

Sequence designation[a]	Most related isolate (s) from GenBank (Percentage sequence similarity by BLAST)[b]	GenBank Accession No. of most related sequences
B1-1	*Ureaplasma urealyticum* (99)	M23935, GI: 176284
F1-2	*Mycoplasma bovis* (99)	U02968, GI: 409968
F2-3	*Mycoplasma bovis* (97)	U02968, GI: 409968

[a]Sequence designations are as labeled in Fig.5; [b]97% sequence similarity is minimum requirement for identity

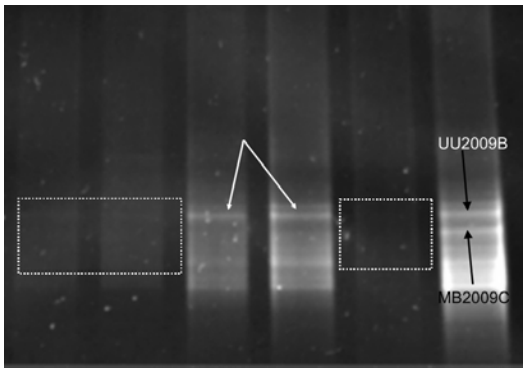

Fig. 4: DGGE profiles of 16S rDNA fragments from reference mycoplasma with different concentrations of *Ureaplasma urealyticum* (UU2009B) added to the uterus mucus sample prior to DNA extraction. Lane 6: 16S rDNA DGGE signature generated from mycoplasma extracted DNA from reference culture UU2009B and MB2009C; Lanes 1-4: 16S rDNA DGGE profiles generated from reference samples extracted DNA from reference culture UU2009B using the optimized method with the concentrations of 1, 10^1, 10^2, 10^3 per mini liter of uterus mucus, respectively, spiked into the mucus prior to DNA extraction. White arrow locates UU2009B bands. Non-UU2009B bands are encompassed by dotted box. The Lane 5 was the uterus mucus sample which we choose prior to adding the reference mycoplasma

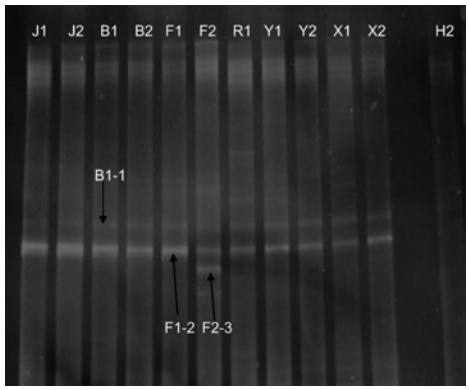

Fig. 5: Sample DGGE profiles of 16S rDNA fragments from uterus mucus of endometritis cattle. Lane R1 is a ladder constructed with reference mycoplasma PCR products of *Mycoplasma genitalium* MG2009A (the top band), *Mycoplasma agalacia* MA2009D (second band), *Ureaplasma urealyticum* UU2009B (third band), *Mycoplasma bovis* MB2009C (the last band). Lane H2 is a contrast constructed with PCR product of DGGE gel eluted DNA from the healthy dairy cow. Other lane designations denote the corresponding uterus mucus sample number (e.g., Lane J1 denotes the uterus mucus sample from the first cow in Jilin dairy farm whereas Lane B2 denotes the uterus mucus sample from the second cow in BaiShan dairy farm). Bands with usable sequences are located by arrows and labeled with the respective lane designation and a sequential number (e.g., Band F1-2 was the second sequenced band and it is from Lane F1)

traditional microbiological techniques. Serological diagnoses are the traditional methods for mycoplasma taxonomic identification and pathogenicity studies (Razin *et al*., 1998). Indeed, some important pathogens may substantially escape from these detection assays, particularly some microbes in low abundance and specificity. In the present study, the mycoplasma was identified at species level by a 16S rDNA-based nested PCR-DGGE approach. Researchers performed an optimized nested PCR-DGGE technique to identify mycoplasma isolates form reference mycoplasma and uterus mucus samples (Fig. 1 and 4) and uterus mucus samples with endometritis (Fig. 5). The primer PMS was only specifically for mycoplasma. From the results and the recent check of GenBank database, this primer specificity for mycoplasmas was stringent to exclude non-mycoplasmas samples during PCR amplification. But the result will be turned to significantly when PCR is conjuncted with community profile techniques such as DGGE. In retrospect, selection or development of specific primers may improve detection of mycoplasmas in those fields (Fig. 4). The primers were like the operon in DGGE. Operon heterogeneity was important in DGGE banding patterns (Sanders *et al*., 1995; Clapp *et al*., 1999; Kuhn *et al*., 2001). Researchers observed only a band pattern for all reference isolates (Fig. 1). The observed *16S rDNA* gene is from one single mycoplasma. This

observation made the interpretation of field DGGE profiles easily because all of mycoplasmas isolates can be represented by one band.

Extraction and concentration efficiency of mycoplasma genomic DNA was relative to other non-purpose DNA must be sufficiently high to overcome the detection limit of PCR procedures (Sung *et al*., 2006; Razin, 1994). Martin-Laurent demonstrated that different extraction protocols may provide conflicting estimates of microbial diversity depending on their efficiency (Martin-Laurent*et al*., 2001). This is illustrated by the Detection Limit test performed on UU (Fig. 4). Above the detection limit, UU DNA was amplified and visualized by DGGE (Lanes 3-5).

The dominant number of observed mycoplasma-like bands was not surprising (Fig. 5 and Table 5). In particular, the large number and wide distribution of observed UU-like and MB-like bands (those with similar mobility to UU and MB bands) agree with the literature's general assessment of UU as a common mycoplasma found in a variety of famine genital system (Bayraktar *et al*., 2010; Govender *et al*., 2009), MB as a common mycoplasma found in cattle (Maunsell and Donovan, 2009; Angen *et al*., 2009; Petit *et al*., 2008). The near absence of members from the MA concurs with the MA was usually found in goat milk (Amores *et al*., 2010a, b). This survey of ten dairy cows with endometritis from five farms by Molecular Diagnostics Methods found >2 species per site (assuming each band represented a different isolate). In the ten assessed dairy cows, all were collected from the cows which showed the most severe endometritis symptoms. Although, the MB and UU were found in most of them, it is quite difficult to establish a relationship between the characteristics and composition of mycoplasma and the severity of mastitis. It is accepted that environment, management and cow factors may affect the occurrence and severity of endometritis (Nagatomo *et al*., 2001). Indeed some reports have indicated that endometritis mainly depends on cow factors as shown by cases where cows were infected by the same species (Nicholas *et al*., 2002).

In the study, most of the endometritis cattle were found MB and UU which wasn't incidental. The effect of MB and UU on endometritis in dairy cow should be further studied. In conclusion, the nested PCR-DGGE Method described here gives an increasingly comprehensive and more precise picture of the mycoplasma populations associated with bovine endometritis. Although, more studies on endometritis need to perform, the results showed that each sample was infection by mycoplasma. The attempts to identify them within uterus mucus also demonstrate that currently used traditional Serological method may not be the best way.

The nested PCR-DGGE technique can improve the diagnosis method of mycoplasma endometritis and identify of different species involved. Thus, it will be a useful diagnostic tool in the dairy industry, especially for endometritis control and prevention. However, the availability of this method for the on-farm investigators deserves more careful studies; besides, limitations concerning nest PCR-DGGE should be addressed. Based on the study, researchers proposed some opinions. First, the error rate of the proprietary Taq used in the Master Mix is unknown but non-proofreading polymerases have reported error rates ranging from 4.0×10^{-2} to 2.2×10^{-4} (Innis and Gelfand, 1999; Meier *et al*., 1996). The potential error is doubled because of the nested PCR strategy used. Second, primer specificity could contribute to the singleness pattern if related but non-mycoplasma templates were present in a sample. Poor specificity at the primer could produce non-specific products (Wirth *et al*., 1994; Tang *et al*., 2000; Eldering *et al*., 2004). Finally, the concentration rang of denaturing agent for DGGE must be accurate so that the DNA molecule here had been spread apart, opening up two single stranded DNA's completely. Kocherginskaya *et al*. (2001) demonstrated that single-stranded and double-stranded molecules from the same template have different mobility during DGGE (Kocherginskaya *et al*., 2001).

Although, the efforts to improve are still needed, the resolution of nested PCR-DGGE technology is quite mature nowadays. The combination of DGGE with other Molecular Diagnostics Methods may lead to more accurate and comprehensive analysis of the mycoplasma communities and infectious agent features.

CONCLUSION

Most of endometritis in dairy cows were infected with mycoplasma. The majority of these sequencing strains were *Ureaplasma urealyticum* (UU) which is a very common pathogenic mycoplasma in dairy cow's reproductive tract. Comparing with the traditional serological methods this approach was fast and convenient in mycoplasma testing.

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