Asian-Aust. J. Anim. Sci.
Vol. 21, No. 1 : 124 - 130
January 2008

www.ajas.info

Development of a Rapid PCR Test for Identification of *Streptococcus agalactiae* in Milk Samples Collected on Filter Paper Disks

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ABSTRACT : *Streptococcus* (*Strep.*) *agalactiae* is one of the major pathogens of bovine mastitis and is the main cause of subclinical infection. This study attempted to develop a rapid PCR diagnosis procedure using milk samples collected on filter paper disks. Chromatographic filter paper was employed as the preservation media and kept at room temperature for one to four weeks. The revival rate of *Strep. agalactia*e kept on dried filter paper disks was affected by the pretreatment preservation time. The revival test suggested that not all the bacteria in artificially contaminated milk samples on the filter paper disks could be recovered. After that, a PCR based on the 16-23S intergenic spacer region of *Strep agalactiae* was performed. The results distinguished the *strep. agalactiae* from major pathogens of bovine mastitis at a 2×10^2 colony forming units (CFU)/ml level, which showed similar sensitivity to the results from liquid milk samples. The results also showed that milk samples collected on filter paper disks could be kept at room temperature for one to four weeks with little negative effect on sensitivity and specificity. The field test showed that the diagnostic sensitivity and specificity was 96.15% and 98.60%, respectively. In conclusion, the protocol will provide a rapid and economic procedure for the detection of bovine mastitis. (**Key Words :** Filter Paper Disk, Bovine Mastitis, *Streptococcus agalactiae*)

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland. It is a major worldwide problem in dairy industry. The mammary gland infection referred to as infectious mastitis causes dairy producers to lose billions of dollars every year worldwidely. Economic losses of bovine mastitis caused by *Strep. agalactiae* were contributed to production losses, milk quality penalties and decreases in milk quality (Keefe, 1997; Kalorey et al., 2001). The most common method being used to identify potential chronic infections is the California Mastitis Test (CMT) in field conditions and the automated method in the diagnosis laboratory (Middleton et al., 2004; Mungube et al., 2004). The major pathogens of bovine mastitis can be identified by *in vitro* culture, which regarded as the "gold standard" for other protocols (Pyorala, 2003). However, this technique is a labor-intensive and time-consuming procedure. The whole procedure takes about one week. Those procedures based on the change of milk components (such as ion, lactose, protein etc.) emphasize whether the mastitis happened or did not, at the same time, their sensitivity and specificity are also influenced by many factors (Pyorala, 2003). A rapid, simple and accurate diagnosis in the early stage of mastitis could rise the cure rate and control the spread of contagious pathogens.

Strep. agalactiae are mainly responsible for the mastitis situation in many farms (Merl et al., 2003). It is one of the major pathogens caused subclinical mastitis which can be diagnosed only in the laboratory (Meiri et al., 2002). It is important to identify *Strep. agalactiae* as early as possible for dairy producer to prevent serous infection in the herd. Current methods for identifying *Strep. agalactiae* are hemolytic pattern and biochemical tests based on bacterial culture on blood agar plates. Although they have high sensitivity and specificity, they also need an *in vitro* culturing period and time-consuming. For its rapidity, sensitivity and precision, several PCR techniques (Jayarao et al., 1992; Forsman et al., 1997; Bohnsack et al., 2004; Zhou et al., 2006) has been widely used to amplify DNA fragment of *Strep. agalactiae* (Estuningsih et al., 2002; Dmitriev et al., 2003; Zschock et al., 2005) in liquid milk samples.

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Received February 4, 2007; Accepted July 28, 2007

There are several methods for the collection of liquid biological sample, e.g. filter paper disks (Abdulrazzaq and Ibrahim, 2001; Wollants et al., 2004) and the conventional tube-like container samples. The filter paper disks and strips are inexpensive, bio-safe, reliable and applicable method in field tests with limited technical facilities to collect, transport and store samples for different study aims. This procedure has been in use for several years to collect many clinical samples in diagnosing limited diseases (Yamamoto et al., 2001; Wollants et al., 2004; Minzi et al., 2005). The RNA of virus samples stored on this way could still be detected for up to 6 months (Vilcek et al., 2001; Yamamoto et al., 2001). Filter paper pretreated with EDTA will help to keep the nucleic acid of the organism for PCR (Eckerbom and Berggvist, 1989; Wollants et al., 2004). Therefore, sampling can be performed by the farmers or the veterinarians in small or remote farms and the samples then can be mailed to the laboratory within several weeks. Based on these reports, the bacterial information of milk samples collected on filter paper disks could be detected using a PCR technique. In order to develop a portable, rapid and cheap diagnosis procedure for identify the *Strep. agalactiae* with suitable sensitivity and specificity for small-scale dairy producer all over the world, a set of primers based on *Strep. agalactiae* 16S-23S rRNA intergenic spacer region (ISR) were applied to identify *Strep. agalactiae* in milk samples collected on filter paper disks.

MATERIALS AND METHODS

Filter paper disks preparation

Xinhua grade 3 chr paper (Xinhua paper inc. Hangzhou, China) was used. It is 0.92 mm thick and highly absorbent pure cellulose chromatography paper with a flow rate of 190 mm/30 min. Filter paper disks for analysis were produced by a standard punch device, which punched out 0.50 cm disks, and handled using disposable gloves. The filter paper disks were divided into five groups. The first to 5th groups were treated with (A) double distilled water, (B) Tris-HCl buffer (20 mM, pH 8.0), (C) Tris-HCl buffer (20 mM, pH 8.0) with 5 mM EDTA, (D) Tris-HCl buffer (20 mM, pH 8.0) with 10 mM EDTA, (E) Tris-HCl buffer (20 mM, pH 8.0) with 20 mM EDTA overnight respectively. Then, the paper disks were sterilized at 121°C for 15 minutes and allowed to dry at 60°C overnight. One hundred blank filter paper disks were used to analyze the volume of milk saturating the disks by an analytical balance.

Sample collection and field test

826 CMT-positive quarter milk samples were collected from 620 Holstein cows in the 2nd, 3rd, 4th lactations during the middle stage of the lactation cycle. In order to increase its reliability, a stratified random sampling was carried to balance between the lactation times, days postpartum, age and milk production. The samples were collected while the cows were restrained in a standing position. Udder and teats were washed with water, and 70% ethyl alcohol was applied after drying. The first several streams were discarded and a CMT was conducted before 5 ml of milk were collected in sterile tubes. Meanwhile each milk sample was dropped on filter paper disks mounted on pen-like samplers (our laboratory invented, a container which could accelerate the dry and protect the sample) and threw away the redundant milk immediately. Milk samples were transported to the laboratory on ice. Filter paper disks were stored at room temperature for two weeks. After that, the filter paper disks were used to extract DNA for field tests. The liquid milk samples were cultured in blood agar within 6 h to isolate the *Strep. Agalactiae*. The isolated organisms were identified by API strep systems (BioMerieux Inc, France).

Bacterial strains and growth conditions

The organisms used in this study include *Staphylococcus aureus Nu305* and two clinical isolates from cases of bovine mastitis, *Strep. agalactiae* 2002-007, and *Strep. dysgalactiae* 2002-004, which were identified biochemically by the API Strep systems. All organisms were cultured in trypticase soy broth (TSB, supplied by Tianhe Company, China) at 37°C with a horizontal rotator for about 18 h before DNA extraction. Cell numbers were determined by the preparation of serial dilutions of an overnight culture in phosphate-buffered saline (PBS) and plating on blood agar (Tianhe Company, China).

The recovery of *Strep. agalactiae* artificially contaminated on filter paper disks

The culture of *Strep. agalactiae* in TSB (2.01×10^7 CFU/ml) was diluted to 2×10^4 CFU/ml. Twenty μl of the diluted solution was used to artificially contaminate pretreated filter paper disks and dried by chemical desiccant on sealed containers. After that they were kept on 25°C and 37°C for one to four weeks in the original container. The filter paper disks were put into a 1.5 ml sterilized eppendorf tube, one hundred μl of incubation buffer (20 mM Tris-HCl, pH 8.0, sterilized by filtration) was added and mixed in a vortex mixer for 20 min. The mixtures were cultured at TSB for 48 h. The change of TSB was recorded.

Preparation of bacterial DNA for PCR

About 1.5 ml of the bacterial culture or 1 ml of liquid milk sample was transferred into an eppendorf tube and centrifuged at 5,000 rpm for 10 min. The pellets were resuspended in 500 μl of enzyme incubation buffer (20 mM Tris-HCl, pH 8.0, 1.2% Triton x-100, 20 mg/ml of lysozyme, 2 mM EDTA) followed 30 min, incubated for

Table 1. The recovery rate of Strep. agalactiae collected on filter paper disks by artificial contamination

Preserved condition		Number of filter paper disks	Growth percentage
Temperature	25°C	333	84.08
(x^2 = 3.23)	37°C	340	88.82
Time	Liquid milk	140	100.00
(x^2 = 85.36**)	One week	181	97.24
	Two weeks	176	81.25
	Four weeks	176	69.89
Media	distilled water	128	75.78
(x^2 = 21.61**)	Tris-HCl	136	88.24
	Tris-HCl with 5 mM EDTA	137	86.86
	Tris-HCl with 10 mM EDTA	136	86.76
	Tris-HCl with 20 mM EDTA	136	86.03

** Means having significant effect (p<0.01) on the revived ratio of the *Strep. agalactiae* on filter paper disks, i.e. time and pretreated media significantly affect on the recovery rate.

37°C, followed by the addition of 25 μl of proteinase K (20 mg/ml) and incubation at 56°C for 2 h. After that, the DNA was extracted with phenol-chloroform-isoamylol (25:24:1) and precipitated by ethanol. The DNA was dissolved in 500 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration and purity was determined by the absorbance at 260/280 nm with a UV 2,000 colorimeter (Amersham Pharmacia), then stored at 4°C for later usage.

Preparation of bacterial DNA from filter paper disk

The filter paper disks were put into a 1.5 ml eppendorf tube, Five hundred μl of incubation buffer (20 mM Tris-HCl, pH 8.0, 1.2% Triton x-100, 2 mM EDTA, sterilization by filtration) was added; mix in a vortex mixer for 20 min. Then add 1 mg of lysozyme and followed by an incubation period of 30 min at 37°C, followed by the addition of 25 μl of proteinase K and incubation at 56°C for 2 h. After that, the DNA was extracted with phenol-chloroform-isoamylol (25:24:1) and precipitated by ethanol. The DNA was dissolved by 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then stored at 4°C for later usage.

Preparation of bacterial DNA for the sensitivity and specificity study

The cultures of *Strep. agalactiae* in TSB (2.01×10^7 CFU/ml) were transferred into tubes and centrifuged at 5,000 rpm for 15 min. The pellets were submitted to serial dilutions with sterile bovine milk (121°C for 15 min, whole milk, SCC 102,000 cells/ml) to reach the following concentrations 2×10^5, 2×10^4, 2×10^3, 2×10^2, 20, 2 CFU/ml. The bacterial concentration of *Staphylococcus aureus*, *Strep. dysagalactiae*, *Strep. uberis* and *Escherichia coli* were determined by limit dilution and plating on blood agar and diluted to 2×10^4 CFU/ml. The filter paper disks were artificially contaminated with the diluted bacterial solutions and dried at 60°C overnight. After that the filter paper disks were put into a 1.5 ml eppendorf tube and treated with the protocol described in Preparation of Bacterial DNA from Filter Paper Disk.

Amplification and detection of *Strep. agalactiae* by PCR

The oligonucleotide primers were selected from the DNA coding for the 16S to 23S rRNA ISR based on previously published paper (Phuektes et al., 2001). The sense primer (AAGGAAACCTGCCATTTG) and antisense primer (TTAACCTAGTTTCTTTAAAACTAGAA) were used to amplify a 270 bp DNA sequence. Reaction mixtures (50 μl each) were prepared by using 2.5 U of Taq DNA polymerase (Biobasic Inc, USA). 1× Taq polymerase buffer minus Mg (Sangon, China), 6 μl of 25 mM $MgCl_2$, and 0.2 mM of each dNTPs (supplied by Sangon, China), 0.5 μM each primer, and 5 μl template DNA. A pre-PCR step at 94°C for 3 min was applied. The reaction mixture was cycled 40 times in a GeneAmp PCR System 2400 (Perkin Elmer): denaturation at 94°C for 30 s, annealing at 58°C for 45 s , and extending at 72°C for 1 min. After the final cycle, the reaction mixture was kept at 72°C for 7 min to complete the reaction. The resulting products were resolved by electrophoresis on 1.5% agarose (1× Tris-borate-EDTA). Gels were visualized under UV illumination (Jeda company, Nanjing, China) and photographed.

Statistics analysis

The specificity and sensitivity and its 95% confidence intervals of the PCR method was calculated in comparison with *in vitro* culture method. A Kappa test was performed to test the fitness of PCR to bacterial culture. A Chi-square test was carried to calculate the impact of preservation time and temperature, and pretreatment of media on the reviving rate of bacterium artificially contaminated on filter paper disks.

RESULTS

The revived rate of *Strep. agalactiae* kept on filter paper disks

The volume of milk saturating the filter paper disk is

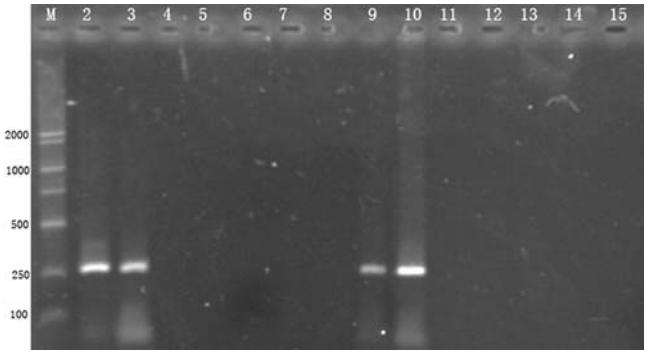

Figure 1. Agarose gel showing the amplification products of milk samples with *Strep. agalactiae* and other major pathogens of bovine mastitis collected in tubes (lane 2 to 8) and filter paper disks (lane 9 to 15). Lane 1, DNA marker; lane 2, 3, 9 and 10, *Strep. agalactiae*; lane 4 and 11, *Strep. dysgalactiae*; lane 5 and 12, *Strep. uberis*; lane 6 and 13, *Staphylococcus aureus*; lane 7 and 14, *Escherichia coli*; lane 8 and lane 15, negative control.

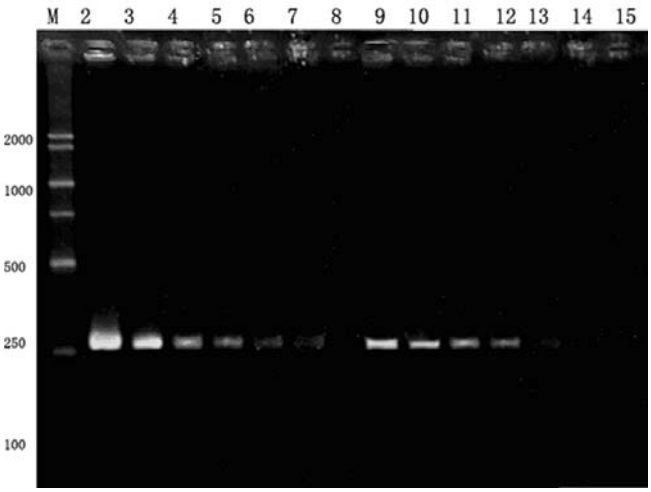

Figure 2. Agarose gel electrophoresis illustrating the sensitivity of PCR test using the primers list above for *Strep. agalactiae* samples kept in tubes (lane 2 to 8) and filter paper disks (lane 9 to 15). Lane 1, DNA marker; lane 2 and 9, samples artificially contaminated with 2×10^5 CFU/ml of *Strep. agalactiae*; lane 3 and 10, artificially contaminated with 2×10^4 CFU/ml of *Strep. agalactiae*; lane 4 and 11, artificially contaminated with 2×10^3 CFU/ml of *Strep. agalactiae*; lane 5 and 12, artificially contaminated with 2×10^2 CFU/ml of *Strep. agalactiae*; lane 6 and 13, artificially contaminated with 20 CFU/ml of *Strep. agalactiae*; lane 7 and 14, artificially contaminated with 2 CFU/ml of *Strep. agalactiae*; lane 8 and 15, negative control.

24.99±3.05 μl liquid milk. The revived test presented that most of the *Strep. agalactiae* kept on filter paper disks at different temperature for one to four weeks could revive on TSB (Table 1). The pretreatment method of filter paper disks and preservation time significantly influence on the reviving rate of *Strep. agalactiae* ($p<0.01$), but the preserved temperature shows little effect on the results ($p>0.05$). The Chi square analysis also demonstrated that the preservation time and pretreatment method have reciprocity with the reviving ability of *Strep. agalactiae* ($p<0.01$). In conclusion, pretreatment of the filter paper disks with 10 mM EDTA, and prompt use after pretreatment as soon as possible will give a high recovery rate of *Strep. Agalactiae* under certain preserved temperature and preserved time.

Specificity of primers

A pair of primers for these tests was derived from DNA coding for the 16S to 23S rRNA intergenic spacer region based on previously published papers. The primers were aligned and examined by a blast tool provided by GenBank. An e-PCR software (a shareware presented by NCBI: ftp://ftp.ncbi.nlm.nih.gov/pub/schuler/e-PCR/) was used to predict the product. The results showed that the primers can only amplify the 16S-23S rRNA intergenic spacer region of *Strep. agalactiae* and distinguished it from other major pathogens of bovine mastitis. The blast result also demonstrated the amplification region were 99-100% homology to 14 strains of *Strep. agalactiae* whose genomes was found in GenBank (NEM316, A909, ATCC27956, ATCC13813, 18RS21, 090, H36B, 2603V/R, SS1214, SS3139, CJB111, JM19, 7271, M781 etc.). Agarose gel electrophoresis revealed that both the liquid samples and dried samples collected on filter paper disks can give unique 270 bp bands from the amplification products (Figure 1). The samples contaminated with *staphylococcus aureus*, *Strep. dysgalactiae*, *Strep. uberis* and *Escherichia coli* could not be amplified by the given primers.

Sensitivity of PCR assay on filter paper disk collected sample

Agarose gel electrophoresis demonstrated that all PCR results of 2×10^5 CFU/ml to 2×10^2 CFU/ml dilution have unique 270 bp amplification product. With the dilution increasing, the lightness of electrophoresis bands decreased and completely disappeared at 2 CFU/ml dilution. The band pattern also presented the lightness of amplification products of samples collected on filter paper disks was somewhat weaker than the products gained on artificially contaminated liquid milk samples. With the protocol described in methods, sensitivity tests presented that limit of detect-ability of the liquid milk is 2×10^2 CFU/ml, which is the same as the sensitivity gained in dried milk samples collected in filter paper disks (Figure 2).

Identification of *Strep. agalactiae* artificially contaminated filter paper disks stored at room temperatures for different periods

Artificially contaminated milk samples collected on filter paper disks pretreated with double distilled water or Tris-HCl buffer (20 mM, PH8.0) or Tris-HCl buffer with 5 mM EDTA, 10 mM EDTA or 20 mM EDTA can preserve

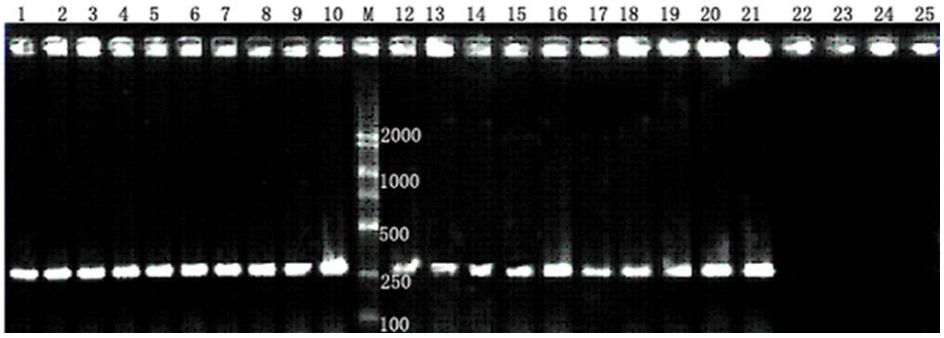

Figure 3. Agarose gel electrophoresis demonstrating the PCR products of milk samples artificially contaminated with 2×10^4 CFU/ml of *Strep. agalactiae.* The treatments of samples collected on filter paper disks are as follows: (A) no preservation (lane1 to 5), (B) preserved for one week (lane 6 to 10), (C) two weeks (lane 12 to 16) and (D) four weeks (lane 17 to 21). Lane 11, DNA marker; lane 1, 6, 12 and 17, filter paper disks pretreated with distilled water; lane 2, 7, 13 and 18, filter paper disks pretreated with 20 mM Tris-HCl buffer (pH 8.0); lane 3, 8, 14 and 19, filter paper disks pretreated with 20 mM Tris-HCl buffer (pH 8.0) with 5 mM EDTA; lane 4, 9, 15 and 20, filter paper disks pretreated with 20 mM Tris-HCl buffer (pH 8.0) with 10 mM EDTA; lane 5 ,10, 16 and 21, filter paper disks pretreated with 20 mM Tris-HCl buffer (pH 8.0) with 20 mM EDTA; lane 22 to 25, negative control.

Table 2. Comparison of bacterial culturing and PCR results in field tests

	Number of *Strep. agalactiae* culture positive	Number of *Strep. agalactiae* culture negative	Total
Number of PCR positive	175	9	184
Number of PCR negative	7	635	642
Total	182	644	826

for 1 to 4 weeks at room temperature for PCR. The stability tests demonstrated that all of these pretreatment media show good PCR results. The milk samples collected on filter paper disks give the same PCR results up to four weeks and appear little effect on the PCR sensitivity and specificity. The filter paper disks pretreated by Tris-HCl buffer with 20 mM EDTA make the best band pattern (Figure 3).

Field test

Strep. agalactiae was isolated from 182 samples from the 826 milk samples of field tests. There are 184 samples showing positive in a PCR test among the 826 milk samples preserved on filter paper disks (Table 2). 175 PCR positive samples show the same results as *Strep. agalactiae in vitro* culture. Other 9 PCR positive samples show no *Strep. agalactiae* growth. Seven S*trep. agalactiae* culture positive samples presented negative in PCR tests. The diagnosis sensitivity is 96.15% (95% confidence intervals is 91.92-98.30%) and the specificity is 98.60% (95% confidence intervals is 97.27-99.32%). The false positive rate is 1.09%, and the false negative rate is 0.85% respectively. A *kappa* tests was performed to compare the fitness of the results from PCR with the results of bacterial culture. The results revealed that the PCR diagnosis of *Strep. agalactiae* presented high consistency in comparison with *in vitro* culture (*kappa* = 0.9438, U = 67.89, $p<0.01$) i.e. PCR method has the same discriminating ability to identify the milks with *Strep. agalactiae* from healthy milk and mastitis milks caused by other pathogens as the method of bacterial culturing *in vitro* can do.

DISCUSSION

PCR based on milk samples collected on filter paper disks is less labor-intensive, more rapid and portable methods than conventional bacterial identification. It is well known that bovine mastitis is a worldwide problem that causes serious economical loss in dairy industry. Up to now there are not efficient vaccines against bovine mastitis. To control efficiently against bovine mastitis requires sensitive, rapid and specific tests to detect and identify the major pathogens (Cremonesi et al., 2006). *In vitro* culture can give the "gold standard" of other method, but it is labor-intensive and time-consuming protocol. PCR as a diagnosis method can be suitable for this. There are many farms located in remote region with a limited technical facilities. It is important that a rapid, portable and economical protocol is being developed in detecting *Strep. Agalactiae* (Merl et al., 2003). The traditional diagnosis strategy included bacterial culturing, isolation and identification procedure. It was well documented that the traditional diagnosis strategies almost need one week to give the diagnosed results. We present a rapid diagnostic method for pathogen (*Strept. agalactiae*) detection from milk samples collected on filter paper disks without cultivation which provides cost and time reduction. The present PCR protocol based on milk samples collected

on dried filter paper disks can get the results within 6 hours. The current payment of PCR is also very cheaper than the direct bacterial diagnosis process. About 2 dollars (USD) could be saved per sample. It is very important for a diagnosis procedure to be accepted by the farmers in relative poverty regions and undeveloped countries. Because of its portability and easiness to preserve, we attempted to collect liquid milk samples on sterile filter paper disks to replace the traditional liquid milk samples. But, a filter paper disk can only keep about 20μL liquid milk, thus it needs a relative sensitive and specific PCR protocol to detect the bacteria. The reviving test also suggested the bacterial collected can not completely revive by a direct culture method. This also a reason why a PCR procedure was adapted to discover the bacterial information collected on the filter paper disks.

PCR based on milk samples collected on filter paper disks presented no cross-reaction with other major pathogens. During the last decades, it was reported that many PCR protocols based on 16S-23S rRNA intergenic spacer region (ISR) have been developed to a efficient way for the diagnosis of *Strep. agalactiae* from major pathogens of bovine mastitis (Phuektes et al., 2001; Estuningsih et al., 2002; Hassan et al., 2002). In present paper, 16S-23S rRNA ISR was employed to diagnose *Strep. agalactiae* with high specificity. The results revealed that the given primers could only amplify the 16S-23S rRNA ISR of *Strep. agalactiae*, which can discriminate it from other major pathogens of bovine mastitis.

PCR based on milk samples collected on filter paper disks is sensitive. It enables to detect one bacterium in 1 ml of raw milk and can be easily incorporated as a part of routine screening protocol in dairy industry (Meiri et al., 2002). The results also presented that the least limit of bacterial number detected by PCR in liquid milk was 2×10^2 CFU/ml, while the result reported by the earlier (Chotar et al., 2006) was 6,000 CFU/ml. The sensitivity of PCR for the detection of bacteria in liquid milk was also affected by protein and calcium ion when the crude DNA extracted from bovine mastitis milk to be used as a templates. A high concentration of casein and calcium ion can interfere the activity of Taq in PCR. The sensitivity of the milk samples collected on filter paper disks showed a little worse than that of liquid milk. There are several factors that can cause the sensitivity of PCR to decrease. The physical and chemical features of the filter paper can cause several problem. Firstly, the DNA molecule can adhere to the fiber matrix of paper which may decrease the DNA concentration of extract (PCR template). Secondly, the chemical substance in the filter paper can also change the pH value and DNA configuration, thus it causes the sensitivity to reduce.

The preservation test demonstrated that the sensitivity and specificity of PCR for the milk collected on filter paper disk could keep stable at room temperature for up to four weeks. It stands to reason that in a dry state, the activity of intrinsic nuclease of milk and bacteria could be inhibited and reduced, thus the decomposing speed of DNA slowed. Meanwhile, the EDTA on the filter paper disks combined with magnesium ion, which can inhibit the activity of nuclease. Based on these reasons, it is possible that all preservation treatments may not affect the PCR results.

The suitability of a protocol for routine diagnosis depends on several factors such as specificity, sensitivity, time required and its applicability to large numbers of samples. CMT technique is commonly used in the screening test of bovine mastitis for the liquid milk samples. It is a sensitive and rapid diagnosis protocol. For its relative low specificity, the CMT can not be adapted as "gold standard" in the analysis of bovine mastitis. The common diagnosis ("gold standard") of *Strep. agalactiae* mastitis is based on identification of pathogens by *in vitro* culture (Shet and Ferrieri 2004). The present study suggested that the sensitivity and specificity of PCR based on the milk samples collected on filter paper disks were quite good in comparison with bacterial culturing.

By *in vitro* culture, 25 μl of milk has to be used, and there is only about 2.5 μl of milk is used for a PCR reaction. And the latter is also affected by the DNA extracting and diluting procedure. The sensitivity is still likely increased by modifying extracting protocol and reducing the dilution volume. The specificity of the PCR reaction is possibly interfered with the milk components and the amount of DNA extracted from somatic cells which greatly increase in mastitis.

However, the PCR methods based on 16S-23S rRNA ISR sequences were successfully used to identify *Strep. Agalactiae* and could be simply implemented into veterinary practice. It is hoped that the PCR diagnosis protocol for the milk samples kept on filter paper disks become a common tool used to identify *Strep. Agalactiae* in bovine mastitis.

ACKNOWLEDGMENT

This research was supported by project of the Department of Sciences and Technique of Zhejiang, China (NO. 2005C12009-02) and the project of Department of Education of Zhejiang, China (G20060313).

REFERENCES

Abdulrazzaq, Y. M. and A. Ibrahim. 2001. Determination of amino acids by ion-exchange chromatography on filter paper spotted

blood samples stored at different temperatures and for different periods: comparison with capillary and venous blood. Clin. Biochem. 34:399-406.

Bohnsack, J. F., A. A.Whiting, G. Martinez, N. Jones, E. E. Adderson, S. Detrick, A. J. Blaschke-Bonkowsky, N. Bisharat and M. Gottschalk. 2004. Serotype III *Strep. agalactiae* from bovine milk and human neonatal infections. Emerg. Infect. Dis. 10:1412-1419.

Cremonesi, P., B. Castiglioni, G. Malferrari, I. Biunno, C. Vimercati, P. Moroni, S. Morandi and M. Luzzana. 2006. Technical note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. J. Dairy Sci. 89:163-169.

Chotar, M., B. Vidova and A. Godany. 2006. Development of specific and rapid detection of bacterial pathogens in dairy products by PCR. Folia Microbiol. (Praha). 51:639-46.

Dmitriev, A., M. Yang, E. Shakleina, L. Tkacikova, A. Suvorov, I. Mikula and Y. H. Yang. 2003. The presence of insertion elements IS861 and IS1548 in group B streptococci. Folia Microbiol. (Praha). 48:105-110.

Eckerbom, S. and Y. Bergqvist. 1989. Improved sample collection technique for capillary blood on filter paper for determination of glycated haemoglobin. Ann. Clin. Biochem. 26:148-150.

Estuningsih, S., I. Soedarmanto, K. Fink, C. Lammler and I. W. Wibawan. 2002. Studies on *Strep. agalactiae* isolated from bovine mastitis in Indonesia. J. Vet. Med. Ser. B Infect. Dis. Vet. Public Health. 49:185-187.

Forsman, P., A. Tilsala-Timisjarvi and T. Alatossava. 1997. Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. Microbiol. 143:3491-3500.

Hassan, A. A., O. Akineden, C. Lammler and R. Huber-Schlenstedt. 2002. Molecular characterization of phenotypically CAMP-negative *Strep. agalactiae* isolated from bovine mastitis. J. Vet. Med. Ser. B Infect. Dis. Vet. Public Health. 49:257-259.

Jayarao, B. M., J. J. Dore, Jr. and S. P. Oliver. 1992. Restriction fragment length polymorphism analysis of 16S ribosomal DNA of Sp. and Enterococcus species of bovine origin. J. Clin. Microbiol. 30:2235-2240.

Kalorey, D. R., N. V. Kurkure, N. K. Nigot, M. P. Patil and V. P. Pathak. 2001. Effect of subclinical mastitis on milk of cross bred Sahiwas×Jersey cows: a biochemical study. Asian-Aust. J. Anim. Sci. 14:382-383.

Keefe, G. P. 1997. *Strep. agalactiae* mastitis: a review. Can. Vet. J. 38:429-437.

Meiri, B., I. E. Lipkin, A. Friedmann, G. Leitner, A. Saran, S. Friedman and Y. Kashi. 2002. A PCR-based method for the detection of *Strep. agalactiae* in milk. J. Dairy Sci. 85:1717-1723.

Merl, K., A. Abdulmawjood, C. Lammler and M. Zschock. 2003. Determination of epidemiological relationships of *Strep. agalactiae* isolated from bovine mastitis. FEMS Microbiol. Lett. 226:87-92.

Middleton, J. R., D. Hardin, B. Steevens, R. Randle and J. W. Tyler. 2004. Use of somatic cell counts and California mastitis test results from individual quarter milk samples to detect subclinical intramammary infection in dairy cattle from a herd with a high bulk tank somatic cell count. J. Am. Vet. Med. Assoc. 224:419-423.

Minzi, O. M., A. Y. Massele, L. L. Gustafsson and O. Ericsson. 2005. Simple and cost-effective liquid chromatographic method for determination of pyrimethamine in whole blood samples dried on filter paper. J. Chromatogr. Ser. B Analyt. Technol. Biomed. Life Sci. 814:179-183.

Mungube, E. O., B. A. Tenhagen, T. Kassa, F. Regassa, M. N. Kyule, M. Greiner and M. P. Baumann. 2004. Risk factors for dairy cow mastitis in the central highlands of Ethiopia. Trop. Anim. Health Prod. 36:463-472.

Phuektes, P., P. D. Mansell and G. F. Browning. 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. J. Dairy Sci. 84:1140-1148.

Pyorala, S. 2003. Indicators of inflammation in the diagnosis of mastitis. Vet. Res. 34:565-578.

Shet, A. and P. Ferrieri. 2004. Neonatal and maternal group B streptococcal infections: a comprehensive review. Indian J. Med. Res. 120:141-150.

Vilcek, S., L. Strojny, B. Durkovic, W. Rossmanith and D. Paton. 2001. Storage of bovine viral diarrhoea virus samples on filter paper and detection of viral RNA by a RT-PCR method. J. Virol. Methods. 92:19-22.

Wollants, E., P. Maes, I. Thoelen, F. Vanneste, M. Rahman and M. Van Ranst. 2004. Evaluation of a norovirus sampling method using sodium dodecyl sulfate/EDTA-pretreated chromatography paper strips. J. Virol. Methods. 122:45-48.

Yamamoto, A. Y., M. M. Mussi-Pinhata, P. C. Pinto, L. T. Figueiredo and S. M. Jorge. 2001. Usefulness of blood and urine samples collected on filter paper in detecting cytomegalovirus by the polymerase chain reaction technique. J. Virol. Methods. 97:159-164.

Zhou, L., Y. Yang, Z. Li, L. Kong, G. Xing, H. Di and G. Wang. 2006. Detection and characterization of PCR-SSCP markers of the bovine lactoferrin gene for clinical mastitis. Asian-Aust. J. Anim. Sci. 19:1399-1403.

Zschock, M., S. Manhold-Maurer, A. Wescher, K. Merl, I. Khan and C. Lammler. 2005. Evaluation of tRNA intergenic spacer length polymorphism analysis as a molecular method for species identification of streptococcal isolates from bovine mastitis. J. Dairy Res. 72:333-337.