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Research Article

Comparison of PCR and Culture Methods for Diagnosis of Subclinical Mastitis in Dairy Cattle [1]

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Abstract

Bovine mastitis is the greatest source of economic loss in the dairy industry. Rapid and definitive detection of causative agent is very important for treatment and control of the disease. The aim of this study was to compare culture and polymerase chain reaction techniques for diagnosis of agents in subclinical bovine mastitis. For this purpose, after conducting the California Mastitis Test on 540 cows, 79 milk samples were analysed by the classical culture method and simplex polymerase chain reaction. Forty-three of samples were found positive by both methods, differences were found only seven samples. While coagulase negative staphylococci these seven samples were determined by culture method, coagulase negative staphylococci and *S. dysgalactiae* were determined together by polymerase chain reaction. The results of this study indicate that the polymerase chain reaction is more sensitive than culture method and could detect pathogens at the species level within a few hours from directly milk samples. Rapid and relieable molecular techniques can be useful method in farm level detection for fast decision about the culling or treatment.

Keywords: *Bovine mastitis, Culture, Polymerase Chain Reaction*

Süt Sığırlarında Sub-klinik Mastitisin Tanısında Kültür ve PCR Yöntemlerinin Karşılaştırılması

Özet

Sığır mastitisleri süt sığırcılığı endüstrisinde ekonomik kayıpların en önemli nedenidir. Mastitiste etkeninin hızlı ve kesin tanısı hastalığın tedavisi ve kontrolü açısından çok önemlidir. Bu çalışmanın amacı subklinik sığır mastitislerinde etkenin tanısı için kültür ve polimeraz zincir reaksiyonu tekniklerinin karşılaştırılmasıdır. Bu amaçla, 540 ineğin California Mastitis Test ile muayenesinden sonra, pozitif bulunan 79 adet süt örneği, klasik kültür metodu ve direkt sütten yapılan polimeraz zincir reaksiyonu ile analiz edildi. Kırk üç örnekte her iki yöntemde de mikroorganizma belirlenirken, sadece 7 örnekte iki teknik arasından fark görüldü. Bu 7 örnekte kültür yöntemiyle sadece Koagülaz Negatif Stafilok belirlenirken, polimeraz zincir reaksiyonu ile Koagülaz Negatif Stafilok dışında *S. dysgalactiae* yönünden de pozitif bulundu. Bu çalışmanın sonuçları polimeraz zincir reaksiyonunun kültür yönteminden daha duyarlı olduğunu ve direkt olarak süt numunesinden hedef etkenin bir kaç saat içinde saptanabileceğini gösterdi. Hızlı ve güvenilir moleküler tekniklerin kullanımı mastitiste sürüden çıkarma veya tedavi etme konusunda hızlı karar verilebilmesinde yardımcı olabilir.

Anahtar sözcükler: *Sığır mastitisi, Kültür, Polimeraz Zincir Reaksiyonu*

INTRODUCTION

Mastitis is one of the most common causes of economic loss in dairy cattle farming. It has been reported that the economic loss in a mastitis case can range from 107 to 344 (U.S. Dolars) per cow in some countries [1,2].

More than 130 different microorganisms have been identified in cases of bovine mastitis [3]. According to primary sources, the bacteria responsible for bovine mastitis can be divided into environmental *(Escherichia coli, Streptococcus dysgalactiae*, *Streptococcus parauberis* and *Streptococcus uberis)* and contagious *(Staphylococcus*

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aureus, *Streptococcus agalactiae, Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*, *Actinomyces pyogenes*, *Corynebacterium pyogenes)* and *Mycoplasma* spp.) categories [4]. In recent years, the increasing importance of Coagulase-Negative *Staphylococci* (CNS) has been emphasised [5,6]. In addition; yeast such as *Candida* spp. has been reported to be more isolated in areas with higher moisture than in other regions [7].

Mastitis is classified as clinical or subclinical according to clinical appearance. The diagnosis of subclinical mastitis cannot be made from symptoms; diagnosis requires special tests. Due to latent infection in subclinical mastitis, it may spread throughout the farm and causes a high level of economic loss [4,8]. Early diagnosis of subclinical mastitis is important in order to treat and prevent the spread of the disease by applying the appropriate security measures [9,10].

Although the milk culture is considered the gold standard test for diagnosing mastitis, there are several disadvantages associated with this technique. Growing microorganisms *in vitro* can be suppressed by factors such as antibiotic residue, inflammatory cells and mediators in milk, and infectious agents can be very low in number in subclinical mastitis [11]. The prevalence of false negative results using bacterial culture methods has increased the importance of Polymerase Chain Reaction (PCR) analysis; its usage in diagnosing mastitis has been proposed for the rapid and reliable detection of agents with high sensitivity. And a few of microorganisms in the samples can be detected by PCR [11-15]. In addition, the use of molecular techniques has been suggested for detecting fastidious microorganisms such as *Mycoplasma* spp.[16-18] and for discriminating of strictly related microorganisms such as *S. parauberis* and *S. uberis* [19]. For the prevention and control of the disease, routine and periodical control and detection of mastitis agens is very important point [20].

The aim of this study was to compare the reliability of classical culture and PCR as means of detecting bacterial or yeast agents in bovine subclinical mastitis.

MATERIAL and METHODS

Sampling

Prior to sampling, ten well-managed farms with an average fifty cattle per farm were selected from around Hatay, Turkey. Somatic Cell Counts in the bulk milk sampled farm were in the range between 125.000 and 380.000/ml. The average clinical mastitis in the sampled herd rate was below 2%, the rate of subclinical mastitis was 14.6%. Totally 2160 milk samples from 540 cows were tested with California Mastitis Test (CMT). CMT was performed according to Schalm et al.[21]. According to visible reactions the results were classified into 5 scores: (0) = negative, (±) = trace, (+1) = weak positive, (+2) = distinct positive, and (+3) = strong positive. In the study, the milk samples were only taken from subclinical infected udder halves, but clinical infected milk (positive with strip-cup test) was not taken into consideration for this study. Totally CMT positive 79 subclinical milk samples were collected aseptically according to a standard procedure [22] and transferred to the laboratory within 1-3 h in a 4-8°C cooler for the microbiological analyses (The clinical samples were taken with permission with MKÜ Local Ethics Commitee. Meeting Date 17.06.2010: Meeting No: 2010/02: Decision No: 30).

Bacteriological Culture

The milk samples were mixed and 100 µl of milk were streaked onto Blood Agar and (supplemented with 7% defibrinated sheep blood) and Mac Conkey's Lactose Agar and Sabouraud Dextrose Agar. Bacteriological and mycological isolation and identification were performed by the classical culture method and standard biochemical tests according to accepted standards [23-25]. For the isolation of yeast, after incubation 5 days at 30°C, colonies growth in the Sabouraud Dextrose Agar were stained Gram method and they were identified according to their macroscopic and microscopic properties. For the bacterial identification, after incubation for 24 and 48 h at 37°C, colonies in Blood Agar Plates were examined for colony characteristics, morphology and haemolysis properties. Mixed colonies in the plates were sub-cultured by transferring into new agar plates for the obtain pure culture. Sub-cultured pure colonies were evaluated macroscopically and stained with Gram method. Then, catalase and oxidase test put in the implement for all of isolates.

Catalase positive and oxidase negative, coccus-shaped isolates were included member of *Staphylococcus* spp. After tube coagulase test with rabbit plasma, *Staphylococcal* isolates were classified as coagulase positive and coagulase negative. Further characterisation of coagulase positive isolates were made with thermostable nuclease test and mannitol fermentation.

Gram positive, coccus-shaped, catalase negative and oxidase negative isolates were included member of *Streptococcus* spp. For the further characterization of these isolates, the Christie-Atkins-Munch-Petersen (CAMP) reaction, esculin hydrolysis on Edwards Medium (Oxoid, Basel, Switzerland), sodium hippurate hydrolysis.

Gram positive, small curved rod-shaped, catalase negative and oxidase negative isolates were confirmed as *T. pyogenes* (formerly *Arcanobacterium pyogenes)*. And other routine biochemical tests, nitrate reduction, gelatin hydrolyzation, urease production, Oxidation-Fermentation, were carried out to identify the isolates.

For the *Mycoplasma* spp. isolation, 1 ml of milk sample was transferred to 9 ml of PPLO broth medium (supplemented with horse sera, thallium acetate, and penicillin) and incubated at 37°C for two weeks under

microaerophilic conditions. After the incubation, 100 µl aliquots were transferred from the PPLO broth medium to PPLO agar (supplemented with horse sera, thallium acetate, and penicillin) and incubated at 37°C for two weeks under microaerophilic conditions according to Carter [24] and Quinn et al.[25].

Molecular Diagnosis

For the PCR analyses, *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228) from department collection, and *Mycoplasma bovis* ATCC 25025 DNA (Dr. Jessie Trujillo, IOWA State University of Science and Technology, College of Veterinary Medicine, Department of Veterinary Microbiology and Preventive Medicine) were used as positive control DNA. One ml of milk sample was transferred into a sterile plastic tube and centrifuged at 5.000 x g for 5 min, after which the pellets were resuspended with 1 ml of sterile PBS (Phosphate-Buffered Saline, pH 7.4). This washing was performed three times to remove calcium ions and other inhibitors [15]. The pellets were then resuspended in 300 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and a nucleic acid extraction was implemented according to the method of Sambrook and Russell [26]. Extracted DNA pellet was dissolved in 100 µl of TE buffer and stored –20°C until used in the PCR analyses. The properties of the primers are shown in *Table 1*. The simplex PCR protocols and procedures were carried out according to their references. After amplification, ten microliters of each amplification reaction mixture was analysed by electrophoresis performed with a 1.5% (wt/vol) agarose gel stained with ethidium bromide (0.7 µg/ml). After migration with 160 volts for 30 min, amplification products were visualized under ultraviolet light.

Cost Analyses

The total costs for culture and PCR analyses were determined for only reagents and plastic consumables used in the analyses, not labor costs and laboratory equipment. PCR reagents were calculated for 79 samples and ten simplex PCR analyses because of the all samples were used PCR analyses for each studied primer sets. In the culture, cost for the first isolation were calculated for 79 samles, but costs for biochemical test were calculated for 43 isolates after first isolation. Price quotations were obtained two commercial companies, and in the calculation the most lower prieces were used.

Statistical Analysis

Kappa analysis was used the determination of agreement in the results in the both methods used in this study. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows Statistical Package Version 14.0.

Table 1. *Properties of primers used in the study*
Tablo 1. *Çalışmada kullanilan primerlerin özellikleri*

Agent	Target Gene	Primer Name	Primer Sequences	Annealing Temp (°C)	Reference
Staphylococcus spp.	*16s rRNA*	Staph294-318	5′-GCCGGTGGAGTAACCTTTTAGGAGC-3′	55	[27]
		Staph 1522-1540	5′-AGGAGGTGATCCAACCGCA-3′		
E. coli	23S r RNA	Eco 2083	5′-GCTTGACACTGAACATTGAG-3′	64	[19]
		Eco 2745	5′-GCACTTATCTCTTCCGCATT-3′		
S. aureus	23S rRNA	Sau 327	5′-GGACGACATTAGACGAATCA-3′	64	[19]
		Sau 1645	5′-CGGGCACCTATTTTCTATCT-3′		
Strep. agalactiae	16S rRNA gene	Sag 40	5′-CGCTGAGGTTTGGTGTTTACA-3′	60	[19]
		Sag 445	5′-CACTCCTACCAACGTTCTTC-3′		
Strep. dysgalactiae	16S rRNA gene	Sdy 105	5′-AAAGGTGCAACTGCATCACTA-3′	57	[19]
		Sdy 386	5′-GTCACATGGTGGATTTTCCA-3′		
Strep. parauberis	23S rRNA gene	Spa 301	5′-GCGACGTGGGATCAAATACT-3′	57	[19]
		Spa 1219	5′-TACCATTACCTCTAAAGGTA-3′		
Strep. uberis	23S rRNA gene	Sub 302	5′-CGAAGTGGGACATAAAGTTA-3′	56	[19]
		Sub 396	5′-CTGCTAGGGCTAAAGTCAAT-3′		
M. bovis	membrane lipoprotein P81gene	Mb 1113–1133	5′-TATTGGATCAACTGCTGGAT-3′	55	[28]
		Mb 1542–1560	5′-AGATGCTCCACTTATCTTAG-3′		
T. pyogenes	Plo gene	Plo1	5′-GGCCCGAATGTCACCGC-3′	55	[29]
		Plo2	5′-AACTCCGCCTCTAGCGC-3′		
Candida spp.	rRNA gene	Cab1	5′-TATTAAAGTTGTTGCAG-3′	52	[30]
		Cab2	5′-CCTGCTTTGAACACTCTAATTT-3′		

RESULTS

In the result of the analyses, 43 (54.43%) of 79 CMT positive samples were found positive by both culture method and simplex PCR. Of 36 (45.57%) samples were not any isolation by culture method and they were also found negative by PCR. Totally eight different results profiles were determined both PCR and culture methods. The types of result profiles were shown in *Table 2*. Forty-three of samples were found positive by both methods, differences were found only seven samples. While coagulase negative staphylococci these seven samples were determined by culture method, coagulase negative staphylococci and *S. dysgalactiae* were determined together by polymerase chain reaction. Comparison of results for each samples were shown in the *Table 3*.

In the statistical analysis of eight results profiles, results for CNS and *S. dysgalactiae* in PCR (26 samples positive) and culture (19 samples positive) were found as substantial agreement (κ 0.785) and other results were found as almost perfect agreement (κ 1.000).

In the result of cost analyses, total cost for culture method were calculated to be 465.28 ₺ (Turkish Liras aproximately 206.42 U.S. Dollars), and for PCR analyses were determined 1076.52 ₺ (1076.52 Turkish Liras aproximately 477.60 U.S. Dolars). Of the costs in the PCR were consisted of 210.62 ₺ (93.44 U.S. Dollars) and 865.90 ₺ (384.16 U.S. Dollars) PCR tests.

Table 2. *Comparison results of culture method and PCR from milk samples*
Tablo 2. *Süt örneklerinden kültür ve PCR sonuçlarının karşılaştırılması*

Results	PCR Method	Culture Method
1 *CNS + S. dysgalactiae*	26 samples	19 samples
2 CNS	4 samples	4 samples
3 *S. dysgalactiae*	2 samples	2 samples
4 *S. aureus + S. dysgalactiae*	2 samples	2 samples
5 *CNS + S. uberis*	5 samples	5 samples
6 *CNS + S. agalactiae*	1 samples	1 samples
7 *CNS + S. dysgalactiae + T. pyogenes*	1 samples	1 samples
8 *Candida* spp.	2 samples	2 samples

DISCUSSION

The early and accurate diagnosis is important for treatment and control of mastitis that can effects animal and human health. The PCR method is widely used for the diagnosis of mastitis [11-19]. In this study, it was compared the results of diagnosis of subclinical mastitis using the bacterial culture and PCR analysis methods. Although seven samples positive for only CNS by culture method, these seven samples in the PCR analyses were found positive CNS and *S. dysgalactiae*. Other results for each samples in the culture method were the same with results of PCR analyses. In the result of this study, PCR was found more sensitive than culture method on the analysing of milk samples from subclinical bovine mastitis. Similarly our study, Phuektes et al.[11] found that multiplex PCR was more sensitive than culture for *S. aureus* and *S. uberis*, but not significantly different for *S. agalactiae* and *S. dysgalactiae* in bovine milk samples. Amin et al.[31] reported that simplex and multiplex PCR were more sensitive than culture in detecting *S. aureus*, *E. coli*, and *S. agalactiae* in milk. And, they suggested that PCR could be used as a rapid and sensitive method for detecting those microorganisms. Karahan et al.[32] compared the culture and multiplex PCR methods for diagnosing bovine mastitis and reported that multiplex PCR was more successful than culture for detecting *S. aureus* and *S. agalactiae*. And, Gillespie and Oliver [33] reported that, the real-time PCR technique correctly identified 91.7% of *S. aureus*, 98.2% of *S. agalactiae*, and 100% of *S. uberis*. They noted that multiplex real-time PCR has the potential for simultaneous identification of these agents with 95.5% sensitivity and 99.6% specificity. Above all Koskinen et al.[34] reported that PCR was more sensitive than culture methods especially multiple species in the milk samples. And their results supported that in this study,altough seven samples were positive for CNS in culture method, but in the PCR analyses these seven samples were positive for CNS and *S. dysgalactiae*. All of these studies about comparing PCR and culture in milk samples from bovine mastitis suggested that PCR had more useful than conventional culture in for speed, interpretation of results, and sensitivity.

Nelson et al.[35] compared that phenol-chloroform extraction method with two different commercial DNA extraction kits in the bacterial DNA extraction from human fecal specimens for analyses by Real Time PCR. They reported that phenol-chloroform extraction method was te cheapest (0.25 Australian Dollars per samples) extraction methods. Turenne et al.[36] compared costs of culture method and bacterial 16S rRNA gene targeted a fluorescence-based PCR–single-strand conformation polymorphism (SSCP) protocol for the identification of bacteria from blood samles. They calculated all costs for reagents and labor costs in their study, and time for each analyses. Researchers reported that the average cost for conventional identification per blood culture isolate was ranged from $39 to $45 (U.S. currency) and in the molecular analyses wiht SSCP was to be $21 (U.S. currency). Also, they reported the identifaciton time was 24 h SSCP, but in the culture was changed 1 to 8 days. In this study, PCR costs (477.60 U.S. Dollars) found nearly 2,3 times higher than culture costs (206.42 U.S. Dollars). These differences may be caused from high prices of PCR reagents in our country or didn't calculation of labor cost in this study.

In conclusion, PCR might has the potential for the rapid and reliable diagnosis of a large number of milk samples. And also, it would be beneficial for use as an

Table 3. *Comparison of culture method and PCR results for the each sample*
Tablo 3. *Her bir örnek için kültür ve PCR sonuçlarının karşılaştırılması*

No	SC	Staph		Sau		Tp		Sag		Sdy		Sub		Can	
		CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR	CCM	PCR
1	2	+	+	-	-	-	-	-	-	-	-	+	+	-	-
2	9	+	+	-	-	-	-	-	-	-	-	+	+	-	-
3	10	+	+	-	-	-	-	-	-	-	-	+	+	-	-
4	11	+	+	-	-	-	-	-	-	-	-	-	-	-	-
5	12	+	+	-	-	-	-	-	-	-	-	+	+	-	-
6	14	+	+	-	-	-	-	-	-	-	-	+	+	-	-
7	16	+	+	-	-	-	-	-	-	-	+	-	-	-	-
8	20	+	+	-	-	-	-	-	-	+	+	-	-	-	-
9	21	+	+	-	-	-	-	-	-	+	+	-	-	-	-
10	22	+	+	-	-	-	-	-	-	+	+	-	-	-	-
11	24	+	+	-	-	-	-	-	-	-	-	-	-	-	-
12	25	+	+	-	-	-	-	-	-	+	+	-	-	-	-
13	28	+	+	-	-	-	-	-	-	+	+	-	-	-	-
14	29	+	+	-	-	-	-	-	-	+	+	-	-	-	-
15	32	+	+	-	-	-	-	-	-	+	+	-	-	-	-
16	35	+	+	-	-	-	-	-	-	+	+	-	-	-	-
17	36	+	+	-	-	-	-	-	-	-	-	-	-	-	-
18	38	+	+	-	-	-	-	-	-	+	+	-	-	-	-
19	42	+	+	-	-	-	-	-	-	-	-	-	-	-	-
20	43	+	+	+	+	-	-	-	-	+	+	-	-	-	-
21	46	+	+	+	+	-	-	-	-	+	+	-	-	-	-
22	47	+	+	-	-	-	-	-	-	+	+	-	-	-	-
23	48	+	+	-	-	-	-	-	-	+	+	-	-	-	-
24	51	+	+	-	-	-	-	-	-	+	+	-	-	-	-
25	52	+	+	-	-	-	-	-	-	-	+	-	-	-	-
26	53	+	+	-	-	-	-	-	-	-	+	-	-	-	-
27	54	+	+	-	-	-	-	-	-	-	+	-	-	-	-
28	55	+	+	-	-	-	-	-	-	-	+	-	-	-	-
29	56	-	-	-	-	-	-	-	-	+	+	-	-	-	-
30	57	+	+	-	-	-	-	-	-	-	+	-	-	-	-
31	58	+	+	-	-	-	-	+	+	-	-	-	-	-	-
32	60	+	+	-	-	-	-	-	-	-	+	-	-	-	-
33	62	-	-	-	-	-	-	-	-	+	+	-	-	-	-
34	63	+	+	-	-	-	-	-	-	+	+	-	-	-	-
35	64	+	+	-	-	-	-	-	-	+	+	-	-	-	-
36	66	+	+	-	-	-	-	-	-	+	+	-	-	-	-
37	67	+	+	-	-	+	+	-	-	+	+	-	-	-	-
38	68	+	+	-	-	-	-	-	-	+	+	-	-	-	-
39	71	+	+	-	-	-	-	-	-	+	+	-	-	-	-
40	72	-	-	-	-	-	-	-	-	-	-	-	-	+	+
41	73	-	-	-	-	-	-	-	-	-	-	-	-	+	+
42	77	+	+	-	-	-	-	-	-	+	+	-	-	-	-
43	79	+	+	-	-	-	-	-	-	+	+	-	-	-	-
Total	PR	39	39	2	2	1	1	1	1	24	31	5	5	2	2
	NR	4	4	41	41	42	42	42	42	19	12	38	38	41	41

SC: *Sample Code,* ***Staph:*** *Staphylococcus spp.,* ***Sau:*** *Staphylococcus aureus,* ***Tp:*** *Trueperella pyogenes,* ***Sag:*** *Streptococcus agalactiae,* ***Sdy:*** *Streptococcus dysgalactiae,* ***Sub:*** *Streptococcus uberis,* ***Can:*** *Candida spp.* ***CCM:*** *Conventional Culture Method,* ***PCR:*** *Polymerase Chain Reaction,* ***+*** *positive,* ***-*** *negative,* ***PR:*** *Positive results,* ***NR:*** *Negative results*

auxiliary diagnosis technique to culture. Further studies on developing advanced molecular tecniques based PCR analyses for contagious or major mastitis agents can be useful tool for carrying out the checks at farm level.

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