Multiplex PCR

Week 8

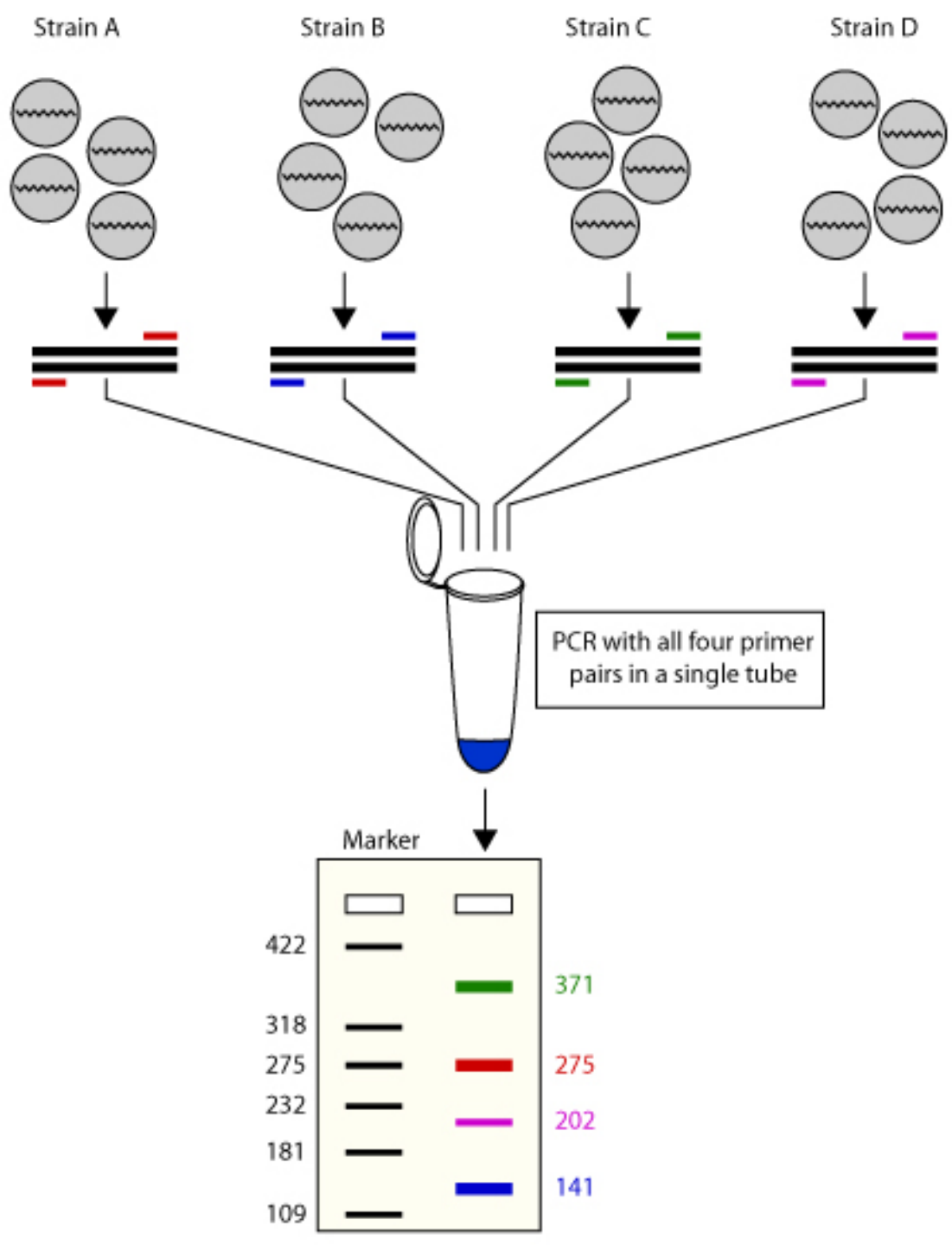
DEVELOPMENT OF MULTIPLEX-PCR TECHNIQUES FOR DETECTION OF ABORTIVE BACTERIAL INFECTIONS OF SHEEP

MULTIPLEX PCR

- Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment.
- In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture.
- As an extension to the practical use of PCR, this technique has the potential to produce considerable savings in time and effort within the laboratory without compromising on the utility of the experiment.

TYPES OF MULTIPLEX PCR

- Multiplexing reactions can be broadly divided in two categories:
  1. Single Template PCR Reaction
  This technique uses a single template which can be a genomic DNA along with several pairs of forward and reverse primers to amplify specific regions within a template.
- **2. Multiple Template PCR Reaction**
  It uses multiple templates and several primer sets in the same reaction tube. Presence of multiple primers may lead to cross hybridization with each other and the possibility of mis-priming with other templates.


Strain A
Strain B
Strain C
Strain D
PCR with all four primer pairs in a single tube
Marker
422
318
275
232
181
109
371
275
202
141

Primer Design Parameters for Multiplex PCR

- Design of specific primer sets is essential for a successful multiplex reaction. The important primer design considerations described below are a key to specific amplification with high yield.
- **1. Primer Length**
  Multiplex PCR assays involve designing of large number of primers, hence it is required that the designed primer should be of appropriate length. Usually, primers of short length, in the range of 18-22 bases are used.
- **2. Melting Temperature**
  Primers with similar Tm, preferably between 55° C-60° C are used. For sequences with high GC content, primers with a higher Tm (preferably 75° C-80° C) are recommended. A Tm variation of between 3° -5° C is acceptable for primers used in a pool.

Primer Design Parameters for Multiplex PCR

- **3. Specificity**
  It is important to consider the specificity of designed primers to the target sequences, while preparing a multiplex assay, especially since competition exists when multiple target sequences are in a single reaction vessel.
- **4. Avoid Primer Dimer Formation**
  The designed primers should be checked for formation of primer dimers, with all the primers present in the reaction mixture. Dimerization leads to unspecific amplification.
- All other parameters are similar to standard PCR primer design guidelines.

Advantages of Multiplex PCR

- **1. Internal Controls**
  Potential problems in a simple PCR include false negatives due to reaction failure or false positives due to contamination. False negatives are often revealed in multiplex assays because each amplicon provides an internal control for the other amplified fragments.
- **2. Efficiency**
  The expense of reagents and preparation time is less in multiplex PCR than in systems where several tubes of uniplex PCRs are used. A multiplex reaction is ideal for conserving costly polymerase and templates in short supply.
- **3. Indication of Template Quality**
  The quality of the template may be determined more effectively in multiplex than in a simple PCR reaction.
- **4. Indication of Template Quantity**
  The exponential amplification and internal standards of multiplex PCR can be used to assess the amount of a particular template in a sample. To quantitate templates accurately by multiplex PCR, the amount of reference template, the number of reaction cycles, and the minimum inhibition of the theoretical doubling of product for each cycle must be accounted.

Applications of Multiplex PCR

- Pathogen Identification
- High Throughput SNP Genotyping
- Mutation Analysis
- Gene Deletion Analysis
- Template Quantitation
- Linkage Analysis
- RNA Detection
- Forensic Studies

INTRODUCTION

One of the most important problems of sheep breeding in Turkey is bacterial abortion

Amongst the bacterial infectious diseases causing ovine abortions in Turkey are **brucellosis**, **campylobacteriosis**, **chlamydiosis**, **listeriosis** and **salmonellosis**

More than **9 genus** of bacteria are known to be regularly encountered in ovine abortion cases

Most of these are **zoonotic agents** threatening human health

Bacterial agents causing abortions in sheep

Brucella abortus
Brucella melitensis
Campylobacter fetus subsp. *fetus*
Campylobacter jejuni
Chlamydophila abortus
Coxiella burnetii
Salmonella Abortusovis
Listeria monocytogenes
Listeria ivanovii
Actinobacillus seminis
Histophilus (ovis) somni
Leptospira spp.

- Rapid and reliable detection of abortifacient bacteria is the first step in the prevention of economical losses due to abortions
- Most of the abortifacient bacteria are fastidious agents requiring specific growth conditions
- In vitro culture of some of them is almost inpractible
- Since they belong to diverse philogenetic groups each require special and specific culture
- Isolation and identification of abortifacient agents by conventional methods is a time taking, labor-intensive, sometimes an expensive process often requiring a equiped lab and good lab practice
- An alternative to conventional culture may be the serological diagnosis, however it has some drawbacks like serological examination could only be applied to the aborted animal (mother), and only after an appropriate period, results are affected by many factors, high ratio of false results, etc.

- Considering the disadvantages of bacteriological and serological identification methods, molecular techniques seem to be the promising alternatives
- They provide rapid results, are sensitive, economic, and labor-saving
- Many PCR techniques were developed for the identification of infectious diseases in both human and veterinary medicine
- However, when the literature are searched intensively, very few studies were encountered in molecular identification of sheep infectious abortions.
- Since different bacteria cause ovine abortions, diverse PCR applications should be performed for the molecular detection of each of these. Problems with conventional identification methods like time, labor and cost can be solved by multiplex-PCR applications.

OBJECTIVE OF THE STUDY

Development of multiplex-PCR techniques which provides rapid, reliable, and simultaneous detection of bacterial agents causing sheep abortions and zoonotic infections in Turkey

MATERIALS AND METHODS

Control strains and DNAs

- Research institutes, universities, research groups, culture collections, researchers, colleagues

Primer Design

- determination of target sequences by searching sequence databases (Entrez PubMed, Nucleotide, GeneBank) and DNA sequence analysis of isolates
- web based services and primer design softwares
- in silico and in vitro analysis, alignments, BLAST searchs, etc.)
- Testing primers for possible interactions

DNA Extraction

- Phenol chloroform extraction
- Extraction with a commercial kit

Development and Optimization of PCR and multiplex-PCR (mPCR) assays

Validation of mPCR assays

- Testing efficacy of primers with different DNA concentrations
- Testing specificity and sensitivity of mPCR assays with control strains/DNAs and bacterial dilutions in PBS (phosphate buffered saline), fetal liver, cotiledon tissues, and abomasum contents

***Brucella* spp. / *Campylobacter* spp. / *C. abortus* Multiplex PCR-I**				
Name of Primer	Primer sequence	Length	Ta*	Target Size
Bru1F	AGGGCAAGGTGGAAGATTTG	20	54° C	337 bp
Bru1R	ATCGGAACGAGCGAAATACC	20		
Campgen2F	AGCGCAACCCACGTATTTAG	20	54° C	235 bp
Campgen2R	ATTCCGGCTTCATGCTCTC	19		
Cabort5F	CCCATCACATTATCAGCAGGA	21	54° C	104 bp
Cabort5R	CCTAGATCCATGACAACGGTAGA	23		
***Brucella* spp. / *Campylobacter* spp. / *C. abortus* Multiplex PCR-II**				
Bru1F	AGGGCAAGGTGGAAGATTTG	20	54° C	337 bp
Bru1R	ATCGGAACGAGCGAAATACC	20		
Campgen3F	TGCCCTACACAAGAGGACAAC	21	54° C	154 bp
Campgen3R	AAGCGTCATAGCCTTGGT	20		
Cabort1F	TCCCAATGTAGGCATCACTC	20	54° C	213 bp
Cabort1F	CCCTTGTATCCTCTAGGCTTGT	22		
***Brucella* spp. / *Campylobacter* spp. Multiplex PCR**				
Bru2F	CATGACACCCAAACTTAGCC	20	54° C	135 bp
Bru2R	TTACTGCTCTACCTTCTGTGGATT	24		
CampgenF	AGCGCAACCCACGTATTTAG	20	54° C	206 bp
CampgenR	GAACAATCCGAACTGGGACA	20		
***Coxiella burnetii* / *Leptospira* spp. / *Listeria* spp. Multiplex PCR**				
Lepto1F	GCGATTATGCCTGACCAAAT	20	54° C	249 bp
Lepto1R	TCCTTTCACTTCACCTGGTTT	21		
CoxburF	GACGGCCAATTATCAGAACA	20	54° C	180 bp
CoxburR	CGCTTTATTACCAATGACGAAC	22		
Listgen2F	TGACACAAGTAACCGAGAATCA	22	54° C	132 bp
Listgen2R	CGTGCGCCCTTTCTAACT	18		

Table. Multiplex-PCR Mix

PCR Content	Concentration	Final Conc.
10 x PCR Buffer	1 x PCR Buffer	2.5 µl
$MgCl_2$	25 mM	3 µl
dNTP mix	10 mM	1 µl
Forward primer 1	100 µM	0.1 µl
Reverse primer 1	100 µM	0.1 µl
Forward primer 2	100 µM	0.1 µl
Reverse primer 2	100 µM	0.1 µl
Forward primer 3	100 µM	0.1 µl
Reverse primer 3	100 µM	0.1 µl
Taq polimeraz	5 U / µl	0.4 µl
DEPC-water	-	15.5 µl
Extracted DNA	>10 ng	2 µl

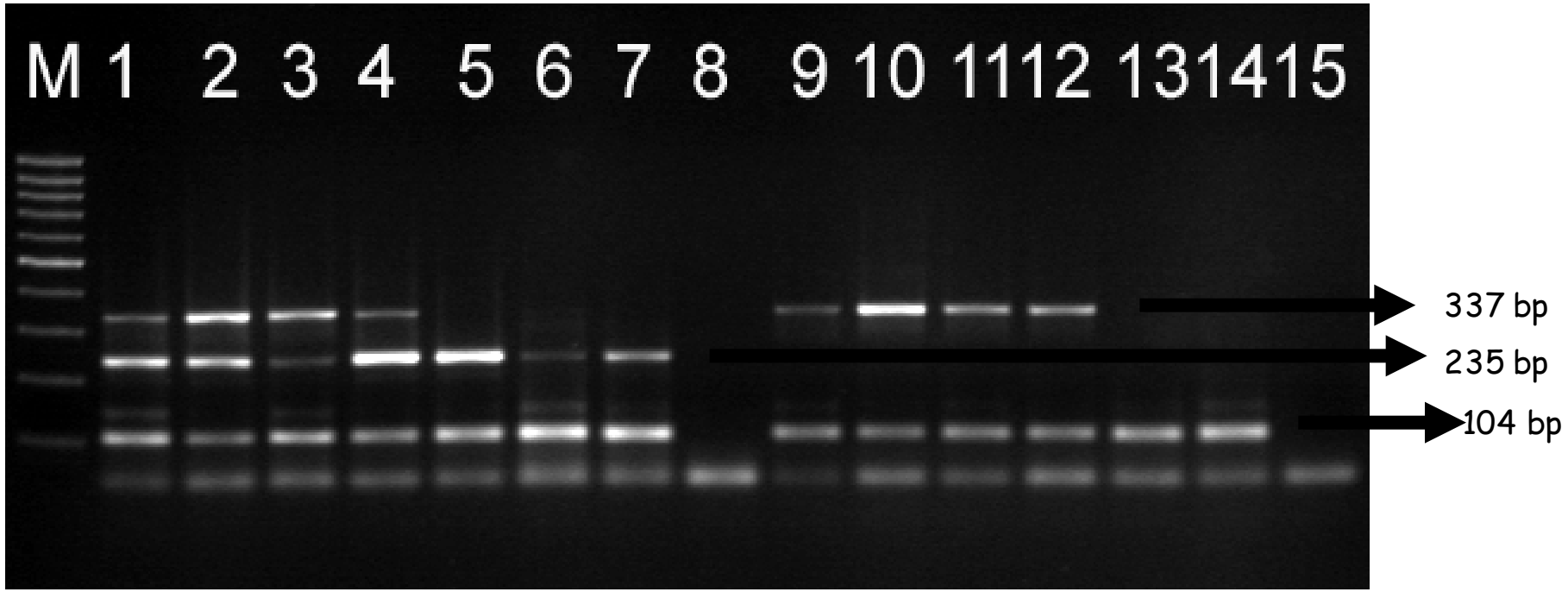
Table. Optimized Multiplex-PCR Amplification Protocol.

PCR Step	**Cycling Conditions**	**Cycles**
Initial Denaturation	94° C 4 min.	1 cycle
Denaturation	94° C 30 s.	30 cycles
Primer annealing	54° C 30 s.	
Extension	65° C 1 min.	
Final extension	65° C 3 min.	1 cycle

RESULTS

- A total of **59 pairs** of primers were designed in the study, 43 pairs were choosen and tested in vitro and **39 (90.7%)** of these were successful in PCR assays
- Following investigation of bacterial abortifacient agents by singleplex-PCR assays in **112 fetal abomasum contents 32 (28.6%)**, **24 (21.4%)**, **4 (3.6%)**, and **4 (3.6%)** samples were ***Brucella* spp.**, ***Campylobacter* spp.**, ***Listeria* spp.**, ***C. burnetii*** positive, respectively. (**Poster # 773**)
- Following evaluation of primers (*in silico* and *in vitro* analysis) **10 pairs of primers** were selected for development of multiplex-PCR (mPCR) techniques
- **4 different mPCR techniques** were developed (2 mPCR for the detection of *Brucella* spp., *Campylobacter* spp.and *C. abortus*, one duplex-PCR for the detection of *Brucella* spp. and *Campylobacter* spp., and 1 mPCR for the detection of *Listeria* spp., *Leptospira* spp. and *C. burnetii*)

Figure 13. Testing efficacy of primers in *Brucella* spp., *Campylobacter* spp., *C. abortus* Multiplex PCR-I with different DNA concentrations



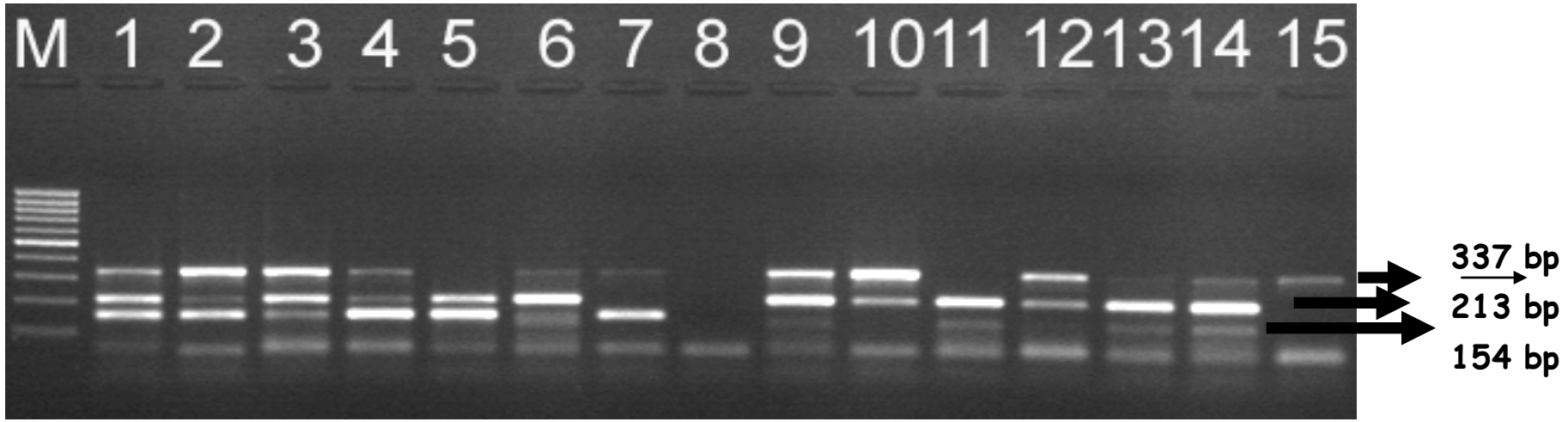
1. Multiplex PCR with template DNAs of 10 ng/µl (direct DNA, D) concentrations of bacterial DNA
2. *Brucella* spp. DNA D, *Campylobacter* spp. DNA Dx10^{-1} (1 ng/µl), *C.* abortus DNA Dx10^{-2} (100 pg/µl)
3. *Brucella* spp. DNA D, *Campylobacter* spp. DNA Dx10^{-2}, *C.* abortus DNA Dx10^{-1}
4. *Brucella* spp. DNA Dx10^{-1}, *Campylobacter* spp. DNA D, *C.* abortus DNA Dx10^{-2}
5. *Brucella* spp. DNA Dx10^{-2}, *Campylobacter* spp. DNA D, *C.* abortus DNA Dx10^{-1}
6. *Brucella* spp. DNA Dx10^{-1}, *Campylobacter* spp. DNA Dx10^{-2}, *C.* abortus DNA D
7. *Brucella* spp. DNA Dx10^{-2}, *Campylobacter* spp. DNA Dx10^{-1}, *C.* abortus DNA D
8. Negative control, PCR mix only

9-12. *Brucella* spp. + *Campylobacter* spp. DNA

13-14. Only *C. abortus* DNA

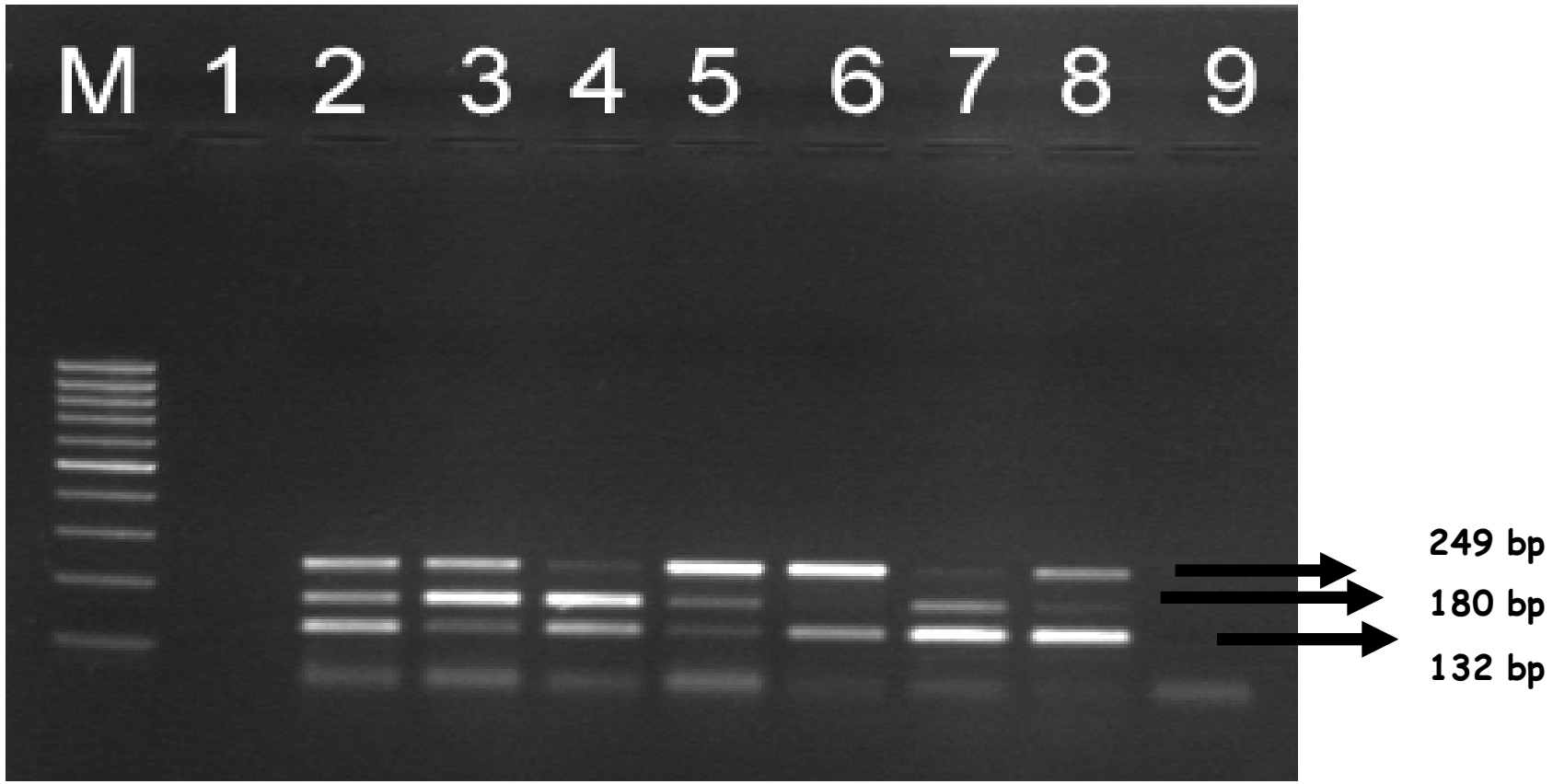
15. Negative control, *Leptospira* spp. DNA

Figure 13. Testing efficacy of primers in *Brucella* spp., *Campylobacter* spp., *C. abortus* Multiplex PCR-II with different DNA concentrations



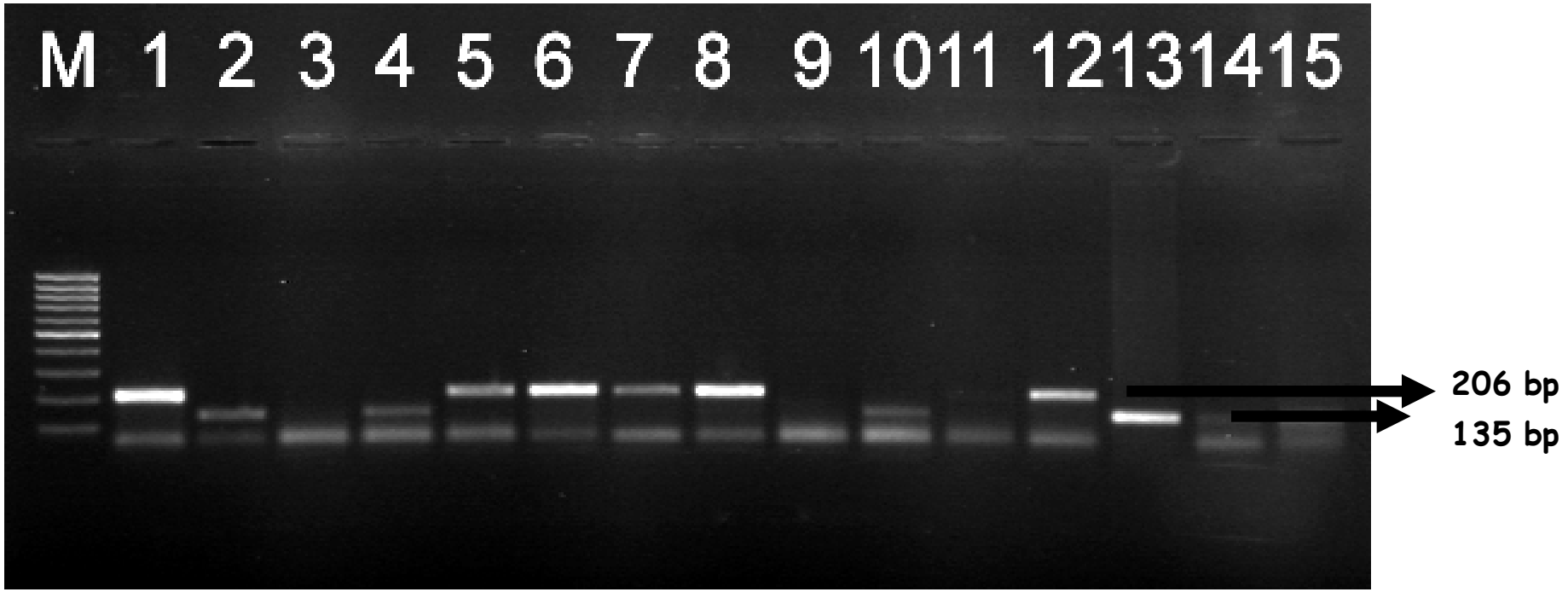
- Multiplex PCR with template DNAs of 10 ng/µl (Direct DNA, D) concentrations of bacterial DNA
- *Brucella* spp. DNA D, *Campylobacter* spp. DNA $Dx10^{-1}$ (1 ng/µl), *C.* abortus DNA $Dx10^{-2}$ (100 pg/µl)
- *Brucella* spp. DNA D, *Campylobacter* spp. DNA $Dx10^{-2}$, *C.* abortus DNA $Dx10^{-1}$
- *Brucella* spp. DNA $Dx10^{-1}$, *Campylobacter* spp. DNA D, *C.* abortus DNA $Dx10^{-2}$
- *Brucella* spp. DNA $Dx10^{-2}$, *Campylobacter* spp. DNA D, *C.* abortus DNA $Dx10^{-1}$
- *Brucella* spp. DNA $Dx10^{-1}$, *Campylobacter* spp. DNA $Dx10^{-2}$, *C.* abortus DNA D
- *Brucella* spp. DNA $Dx10^{-2}$, *Campylobacter* spp. DNA $Dx10^{-1}$, *C.* abortus DNA D
- Negatif kontrol, PCR mix
- *B. melitensis* DNA + *C. jejuni* DNA+ *C. abortus* S26/3 DNA
- *B. melitensis* DNA + *C. abortus* S26/3 DNA
- *C. abortus* S26/3 DNA + *C. jejuni* DNA
- *B. abortus* DNA + *C. abortus* S26/3 DNA
- *B. abortus* DNA + *C. fetus* subsp. *fetus* DNA + *C. abortus* DNA
- *B. ovis* DNA + *C. fetus* subsp. *fetus* DNA + *C. abortus* DNA
- *B. ovis* DNA

Figure 14. Testing efficacy of primers in *Leptospira* spp., *Listeria* spp., *C. burnetii* Multiplex PCR with different DNA concentrations



1. Negative control, PCR mix without DNA
2. Multiplex PCR with template DNAs of 30 ng/µl (Direct DNA, D) concentrations of bacterial DNA
3. *C. burnetii* DNA D, *Leptospira* spp. DNA Dx10-1 (3 ng/µl), *Listeria spp.* DNA Dx10-2 (300 pg/µl)
4. *C. burnetii* spp. DNA D, *Leptospira* spp. DNA Dx10-2, *Listeria spp.* DNA Dx10-1
5. *C. burnetii* DNA Dx10-1, *Leptospira* spp. DNA D, *Listeria spp.* DNA Dx10-2
6. *C. burnetii* DNA Dx10-2, *Leptospira* spp. DNA D, *Listeria spp.* DNA Dx10-1
7. *C. burnetii* DNA Dx10-1, *Leptospira* spp. DNA Dx10-2, *Listeria spp.* DNA D
8. *C. burnetii* DNA Dx10-2, *Leptospira* spp. DNA Dx10-1, *Listeria spp.* DNA D
9. Negatif kontrol, *Brucella* spp. DNA

Figure 16. Results of *Brucella* spp. and *Campylobacter* spp. duplex-PCR assay with extracted DNA from the fetal abomasum contents obtained from Eastern Anatolia in Turkey.



M. 100 bp DNA Ladder, (Fermentas, Lithuania).
1, 5-8, 11, 12. *Campylobacter* spp. 2, 4, 10, 13-15. *Brucella* spp.

Table. Sensitivity of Singleplex-PCR Technique Due To Number of Bacteria Extracted (cfu / 25 µl)

PCR				
	PBS	**Liver**	**Abomasum Content**	**Cotiledon**
B. melitensis	500	50000	5000	5000
C. jejuni	200	2000	200	200
***L.* Hardjo**	1000	100000	1000	1000
L. ivanovii	200	2000	200	200
***S.* Typhimurium**	100000	100000	100000	100000

Table. Sensitivity of Multiplex-PCR Technique Due To Number of Bacteria Extracted (cfu / 25 µl)

Multiplex-PCR				
	PBS	**Liver**	**Abomasum Content**	**Cotiledon**
B. melitensis	5000	500000	50000	50000
C. jejuni	2000	200000	20000	20000
***L.* Hardjo**	1000	10000	10000	10000
L. ivanovii	200	2000	200	200
***S.* Typhimurium**	100000	100000	1000000	1000000

Table. Sensitivity of Singleplex-PCR Technique Due To Number of Bacteria Per PCR reaction (cfu / 2 µl)

PCR				
	PBS	Liver	Abomasum Content	Cotiledon
B. melitensis	5	500	50	50
C. jejuni	2	20	20	20
L. Hardjo	10	1000	10	10
L. ivanovii	2	20	2	2
S. Typhimurium	1000	1000	1000	1000

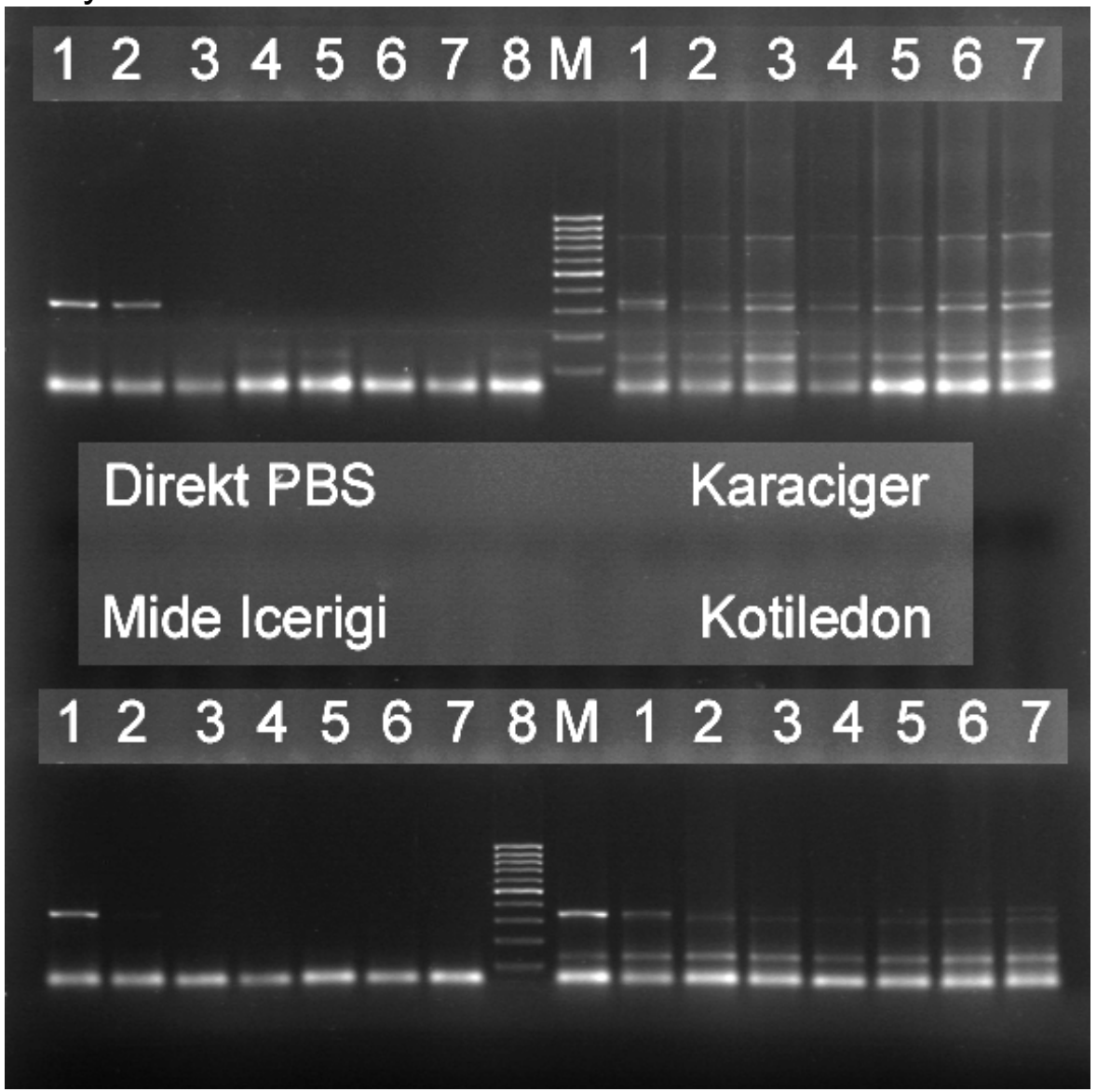
Figure. Sensitivity of Multiplex-PCR Technique Due To Number of Bacteria Per PCR reaction (cfu / 2 µl)

Multiplex-PCR				
	PBS	Liver	Abomasum Content	Cotiledon
B. melitensis	50	5000	500	500
C. jejuni	20	2000	200	200
L. Hardjo	10	100	100	100
L. ivanovii	2	20	2	2
S. Typhimurium	1000	1000	10000	10000

Figure. Sensitivity of Singleplex *Brucella* PCR assay with bacterial dilution in PBS and tissue samples, respectively

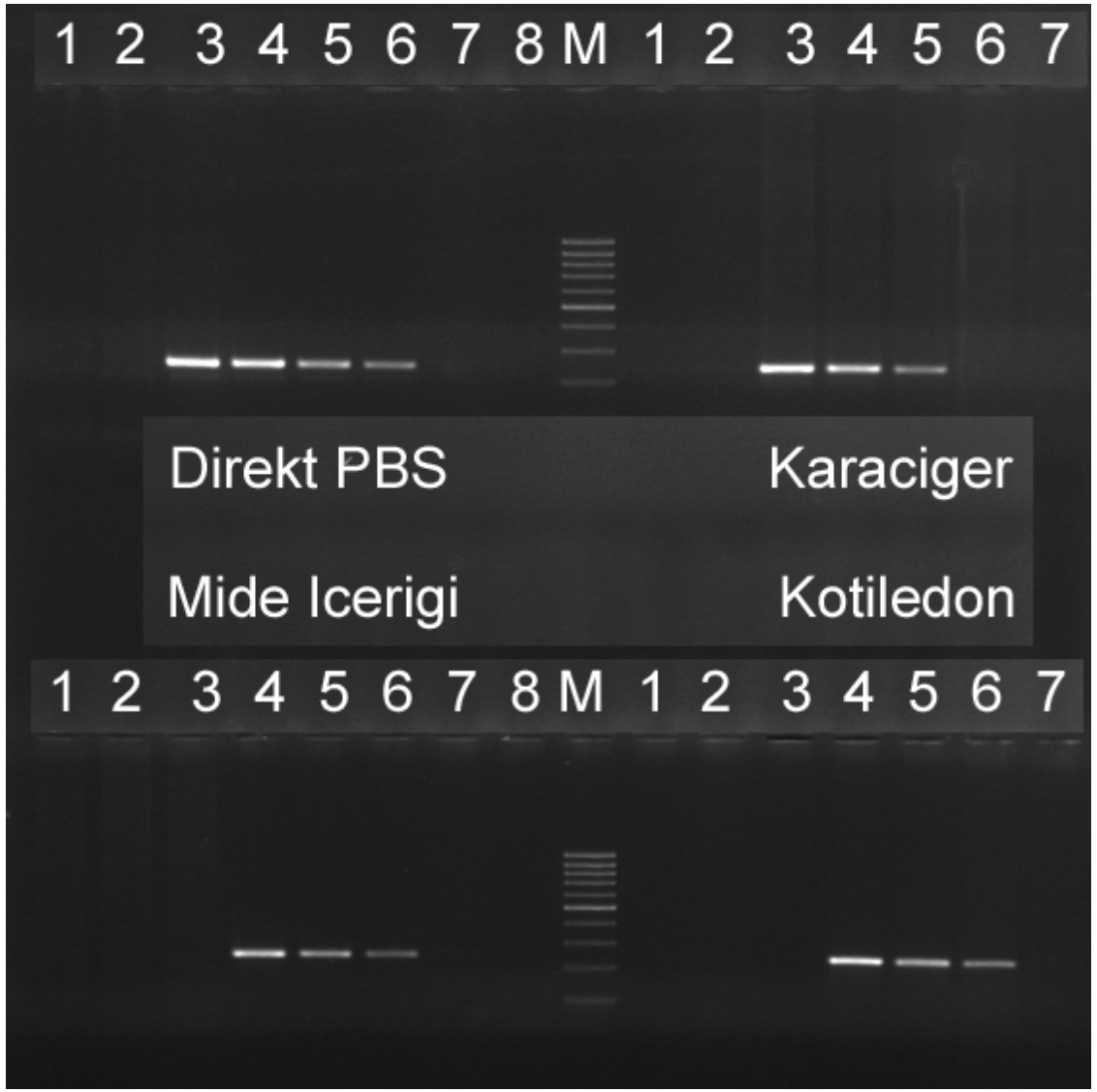
1. 500000 cfu/25 µl 2. 50000 cfu/25 µl 3. 5000 cfu/25 µl 4. 500 cfu/25 µl 5. 50 cfu/25 µl 6. 5 cfu/25 µl
2. 7. 1 cfu/25 µl 8. negative control

Şekil. Sensitivity of Multiplex PCR assay with bacterial (*Brucella* spp.) dilution in PBS and tissue samples, respectively



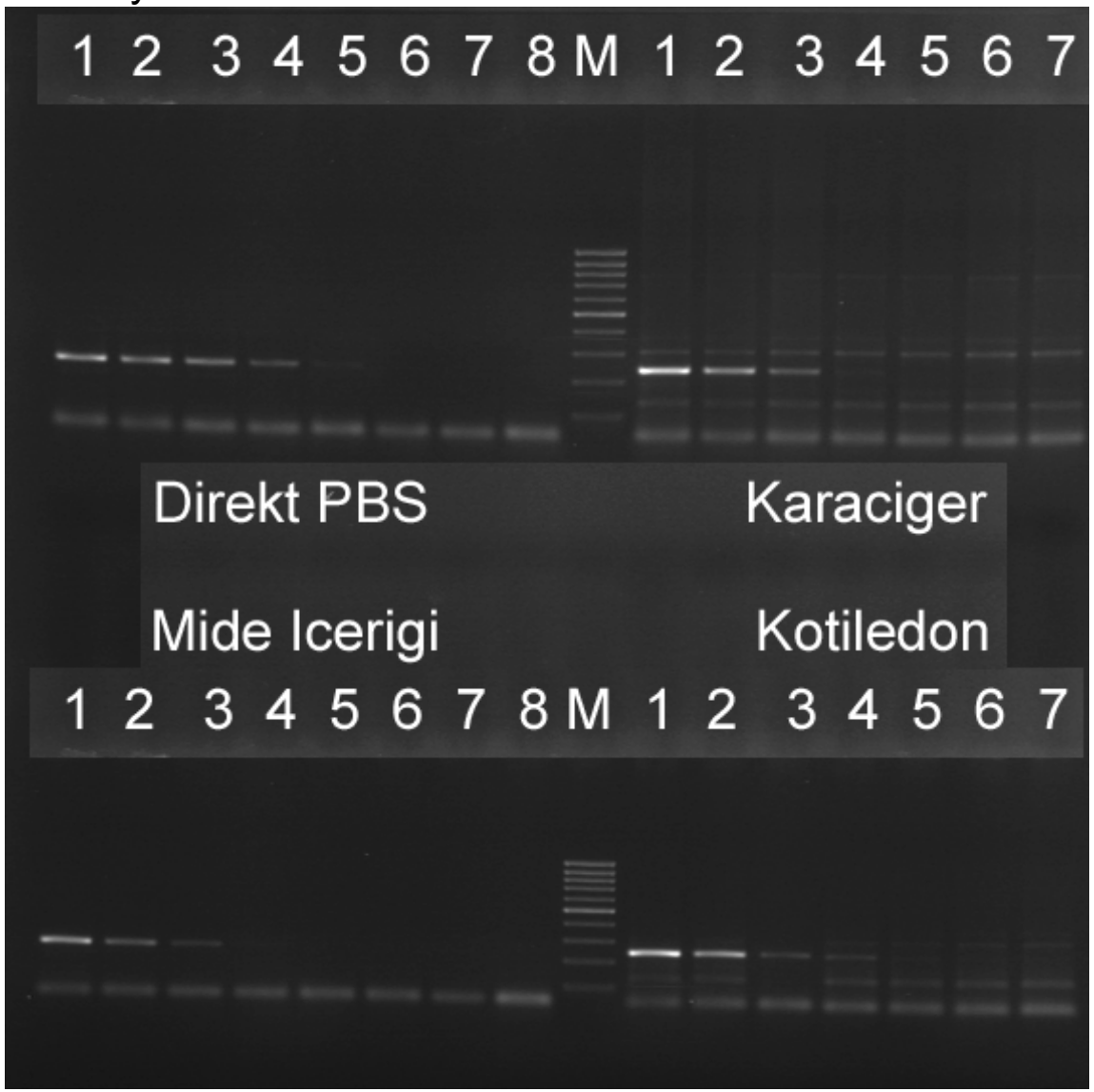
1. 500000 cfu/25 µl 2. 50000 cfu/25 µl 3. 5000 cfu/25 µl 4. 500 cfu/25 µl 5. 50 cfu/25 µl 6. 5 cfu/25 µl
2. 7. 1 cfu/25 µl 8. negatif kontrol

Figure. Sensitivity of Singleplex *Campylobacter* PCR assay with bacterial dilution in PBS and tissue samples, respectively



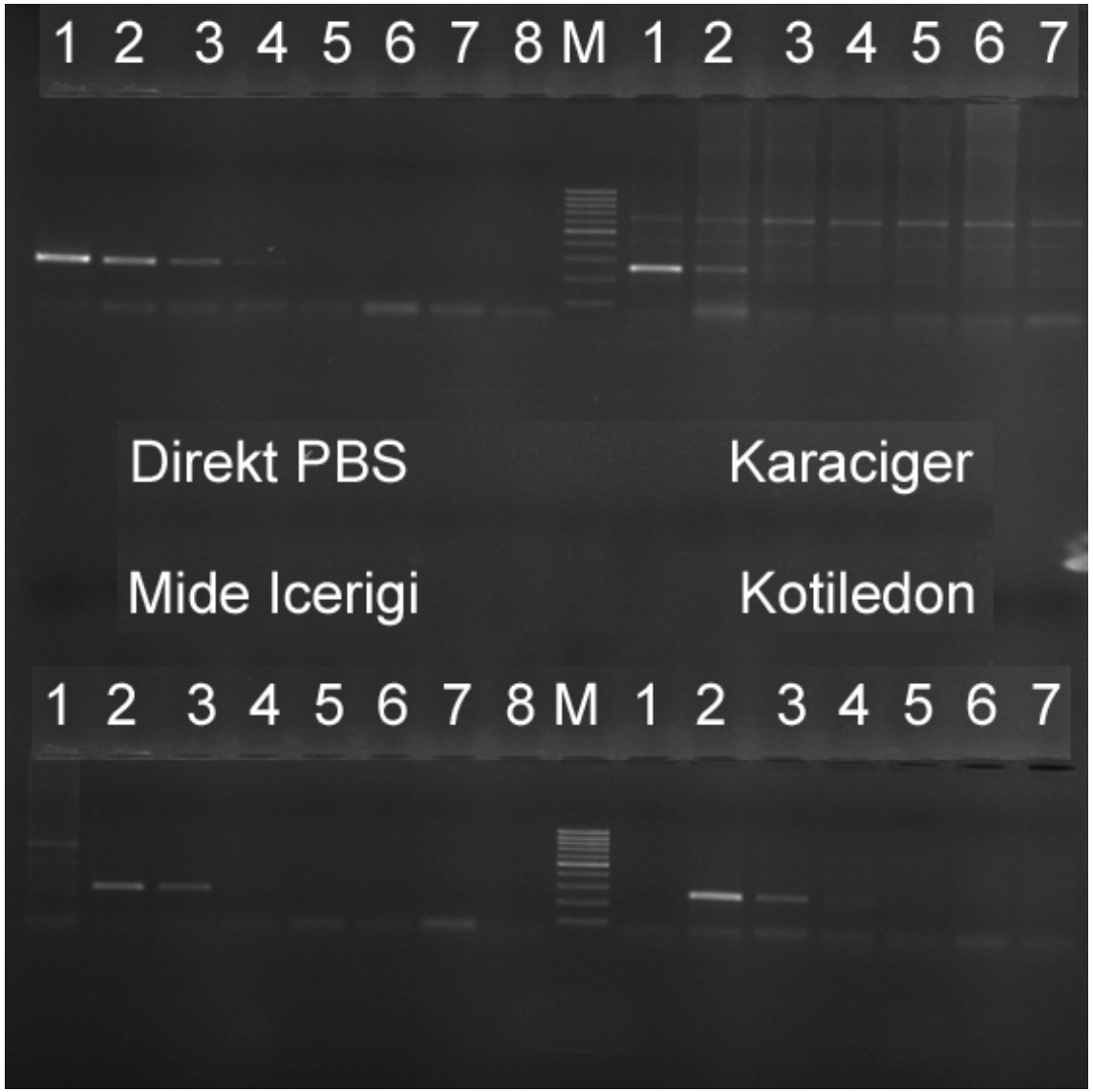
1. 2x107 cfu/25 µl 2. 2000000 cfu/25 µl 3. 200000 cfu/25 µl 4. 20000 cfu/25 µl 5. 2000 cfu/25 µl 6. 200 cfu/25 µl
2. 7. 20 cfu/25 µl 8. negatif kontrol

Şekil. Sensitivity of Multiplex PCR assay with bacterial (*Campylobacter* spp.) dilution in PBS and tissue samples, respectively



1. 2x107 cfu/25 µl 2. 2000000 cfu/25 µl 3. 200000 cfu/25 µl 4. 20000 cfu/25 µl 5. 2000 cfu/25 µl 6. 200 cfu/25 µl 7. 20 cfu/25 µl 8. negatif kontrol

Figure. Sensitivity of Singleplex *Leptospira* PCR assay with bacterial dilution in PBS and tissue samples, respectively

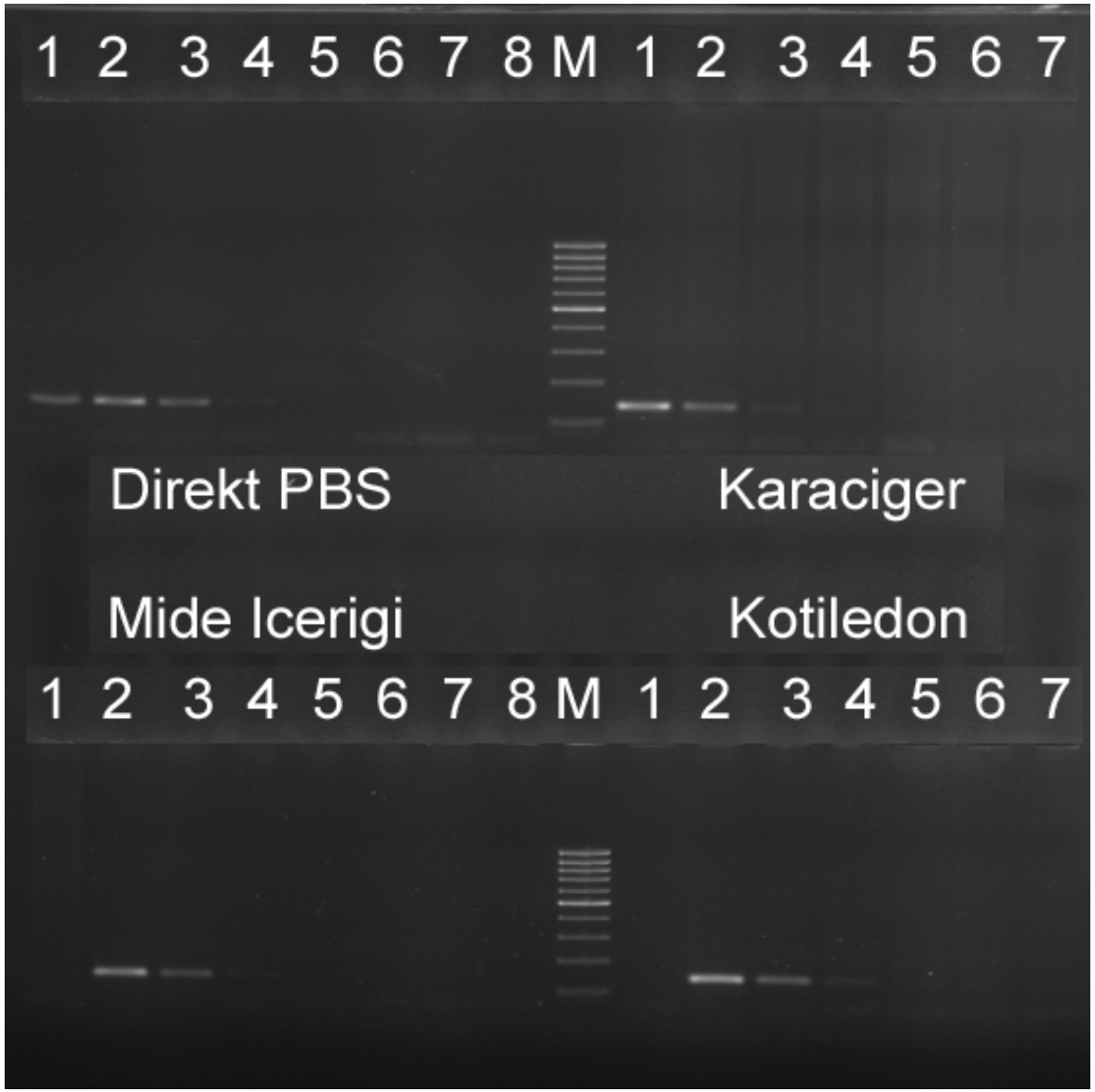


1. 1000000 cfu/25 µl 2. 100000 cfu/25 µl 3. 10000 cfu/25 µl 4. 1000 cfu/25 µl 5. 100 cfu/25 µl 6. 10 cfu/25 µl 7. 1 cfu/25 µl 8. negative kontrol

Şekil. Sensitivity of Multiplex PCR assay with bacterial (*Leptospira* spp.) dilution in PBS and tissue samples, respectively

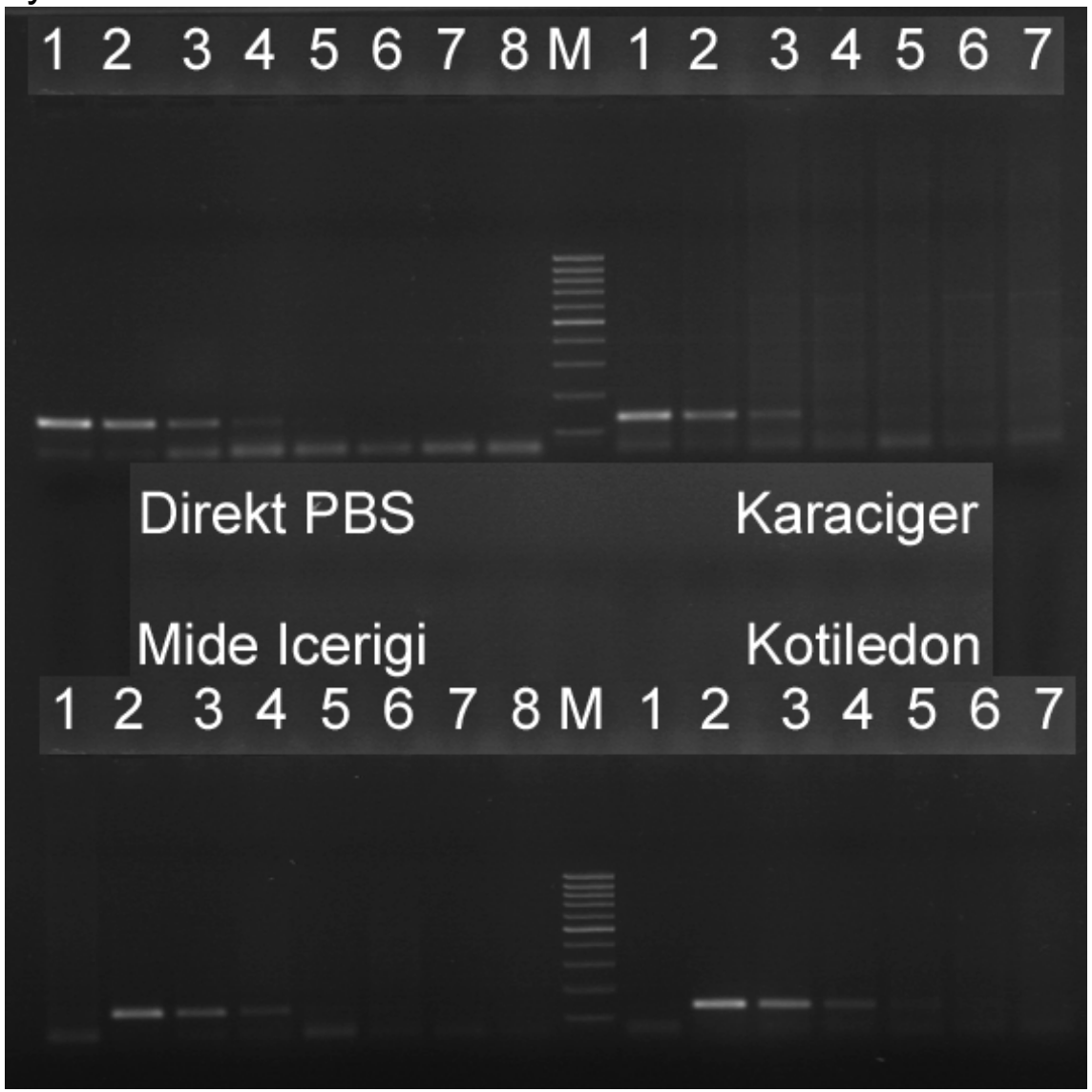
1. 1000000 counts/25 µl 2. 100000 counts/25 µl 3. 10000 counts/25 µl 4. 1000 counts/25 µl 5. 100 counts/25 µl 6. 10 counts/25 µl 7. 1 counts/25 µl 8. negative control

Figure. Sensitivity of Singleplex *Listeria* PCR assay with bacterial dilution in PBS and tissue samples, respectively



1. 200000 cfu/25 µl 2. 20000 cfu/25 µl 3. 2000 cfu/25 µl 4. 200 cfu/25 µl 5. 20 cfu/25 µl 6. 2 cfu/25 µl 7. 0.2-1 cfu/25 µl 8. negatif kontrol

Şekil. Sensitivity of Multiplex PCR assay with bacterial (*Listeria* spp.) dilution in PBS and tissue samples, respectively



1. 200000 cfu/25 µl 2. 20000 cfu/25 µl 3. 2000 cfu/25 µl 4. 200 cfu/25 µl 5. 20 cfu/25 µl 6. 2 cfu/25 µl 7. 0.2-1 cfu/25 µl 8. negatif kontrol

Figure. Sensitivity of Singleplex-PCR Technique Due To Number of Bacteria Extracted (cfu / 25 µl)

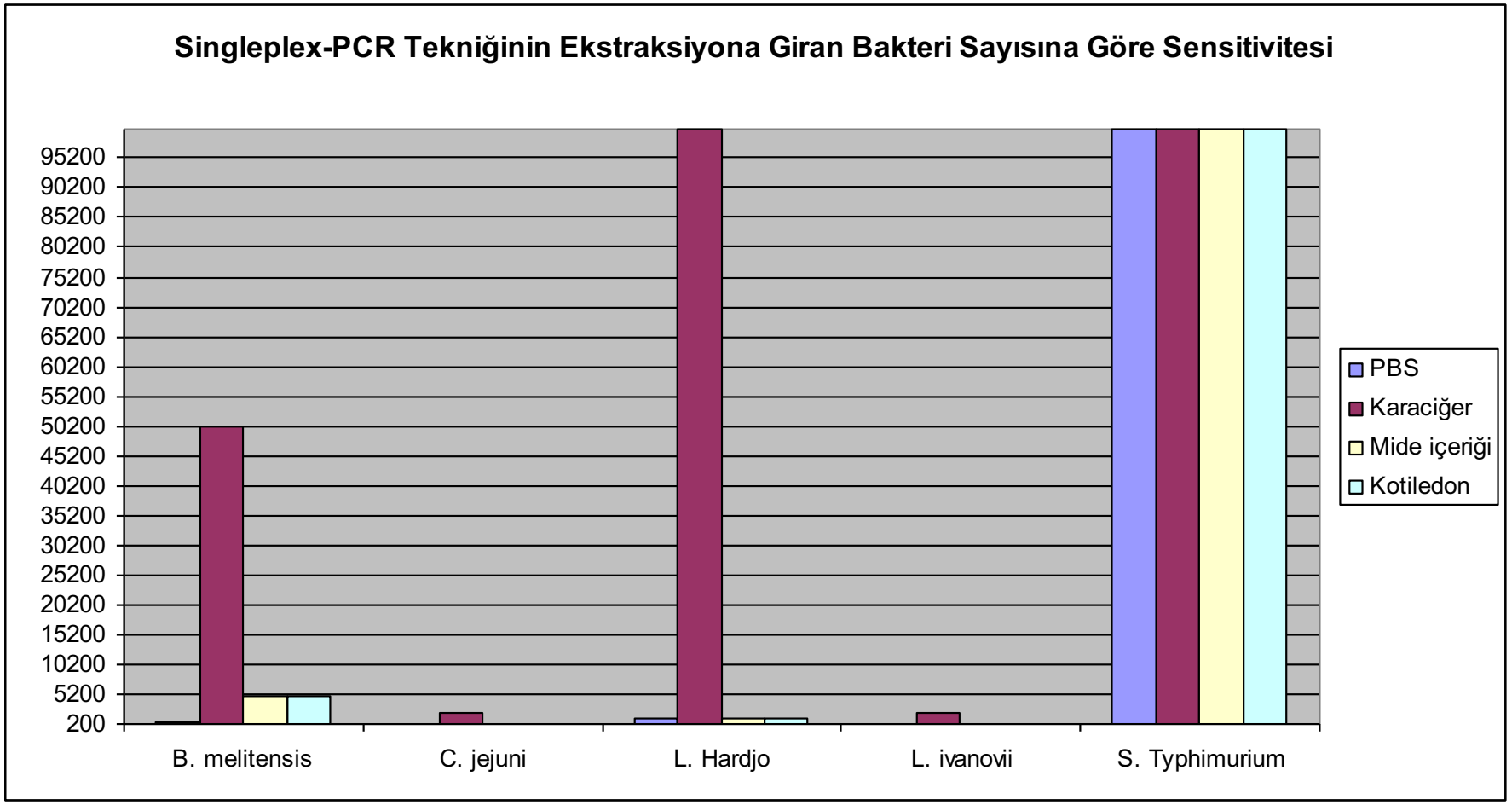


Figure. Sensitivity of Multiplex-PCR Technique Due To Number of Bacteria Extracted (cfu / 25 µl)

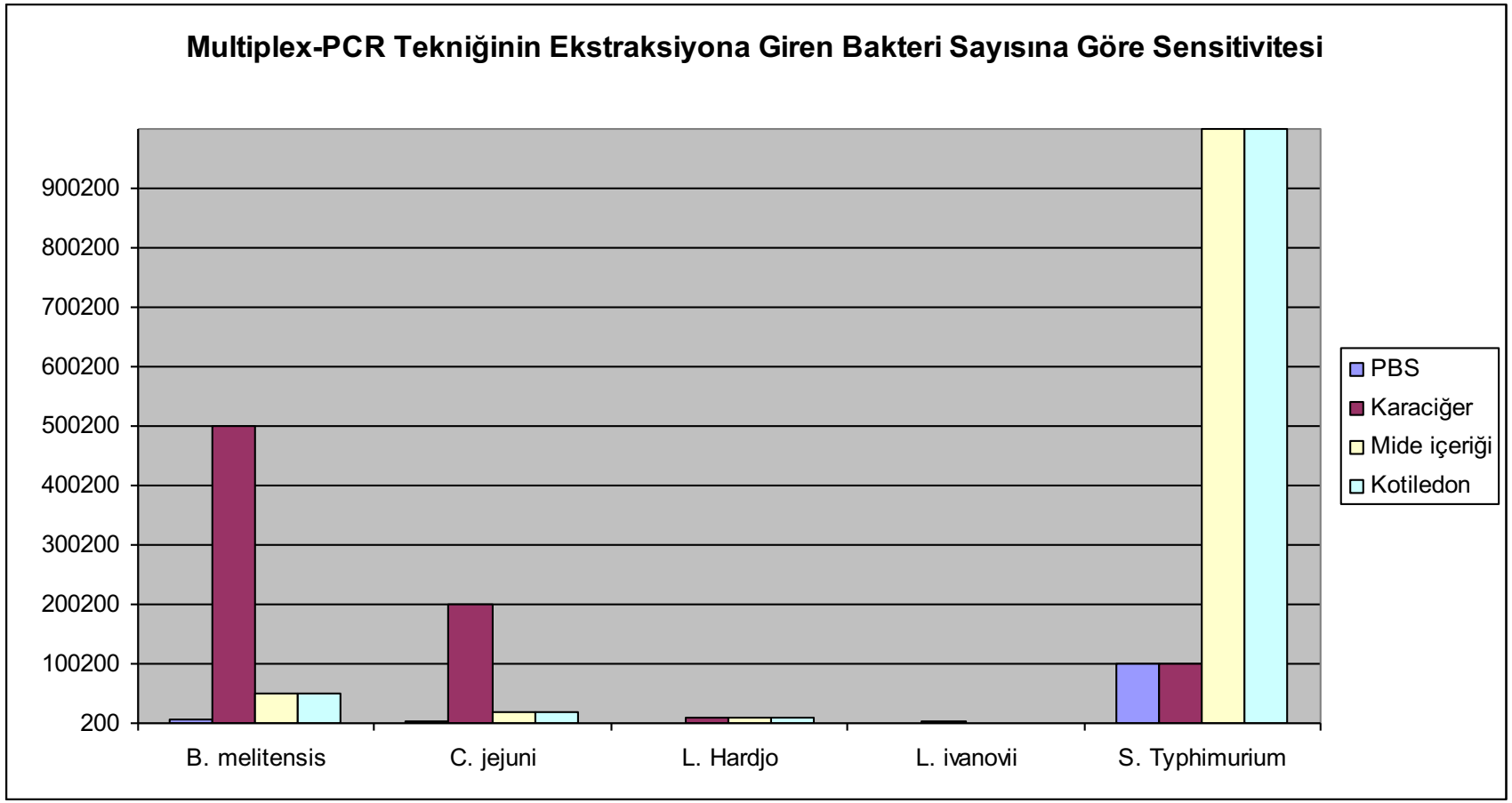


Figure. Sensitivity of Singleplex-PCR Technique Due To Number of Bacteria Per PCR reaction (cfu / 2 µl)

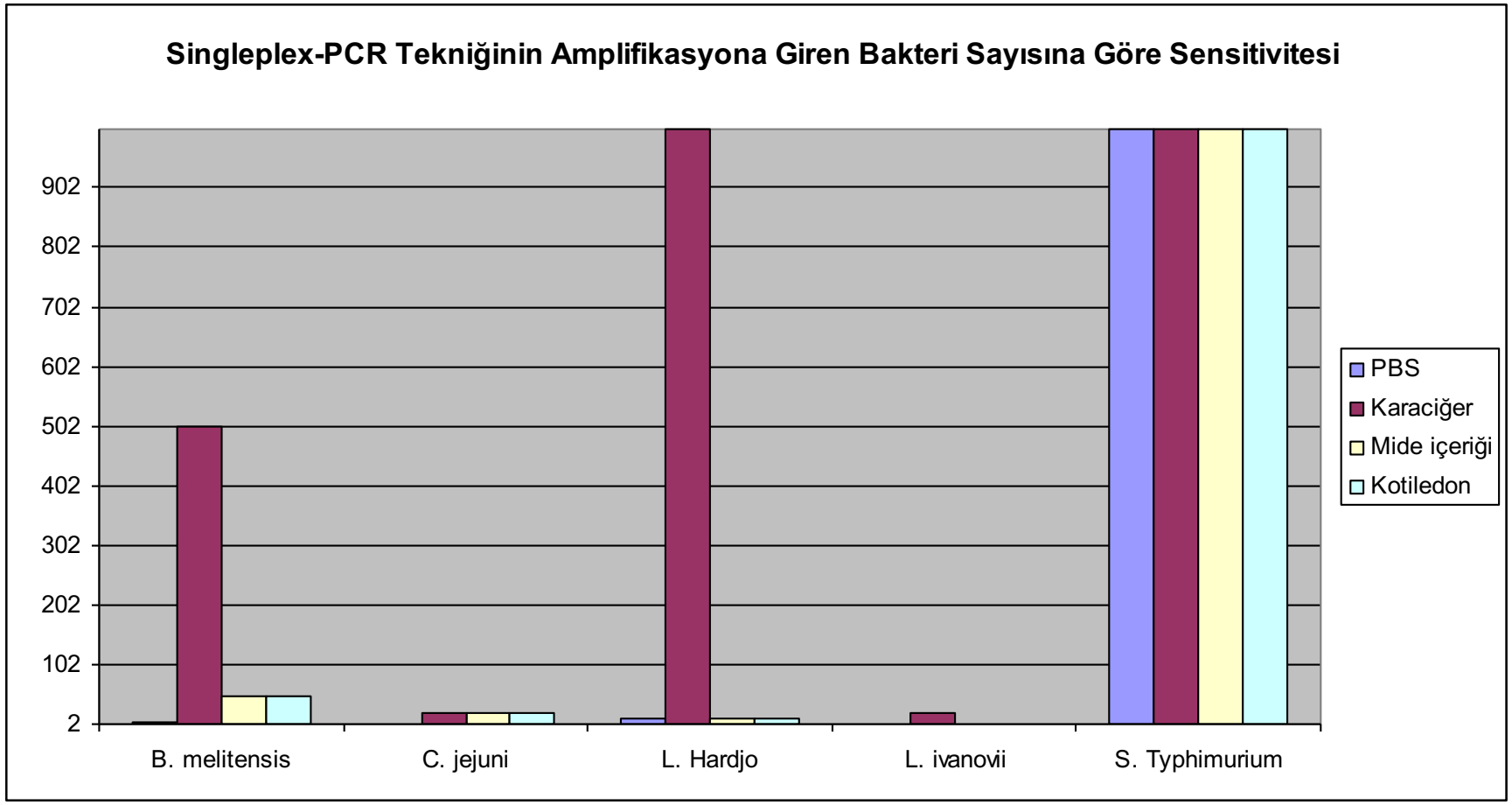
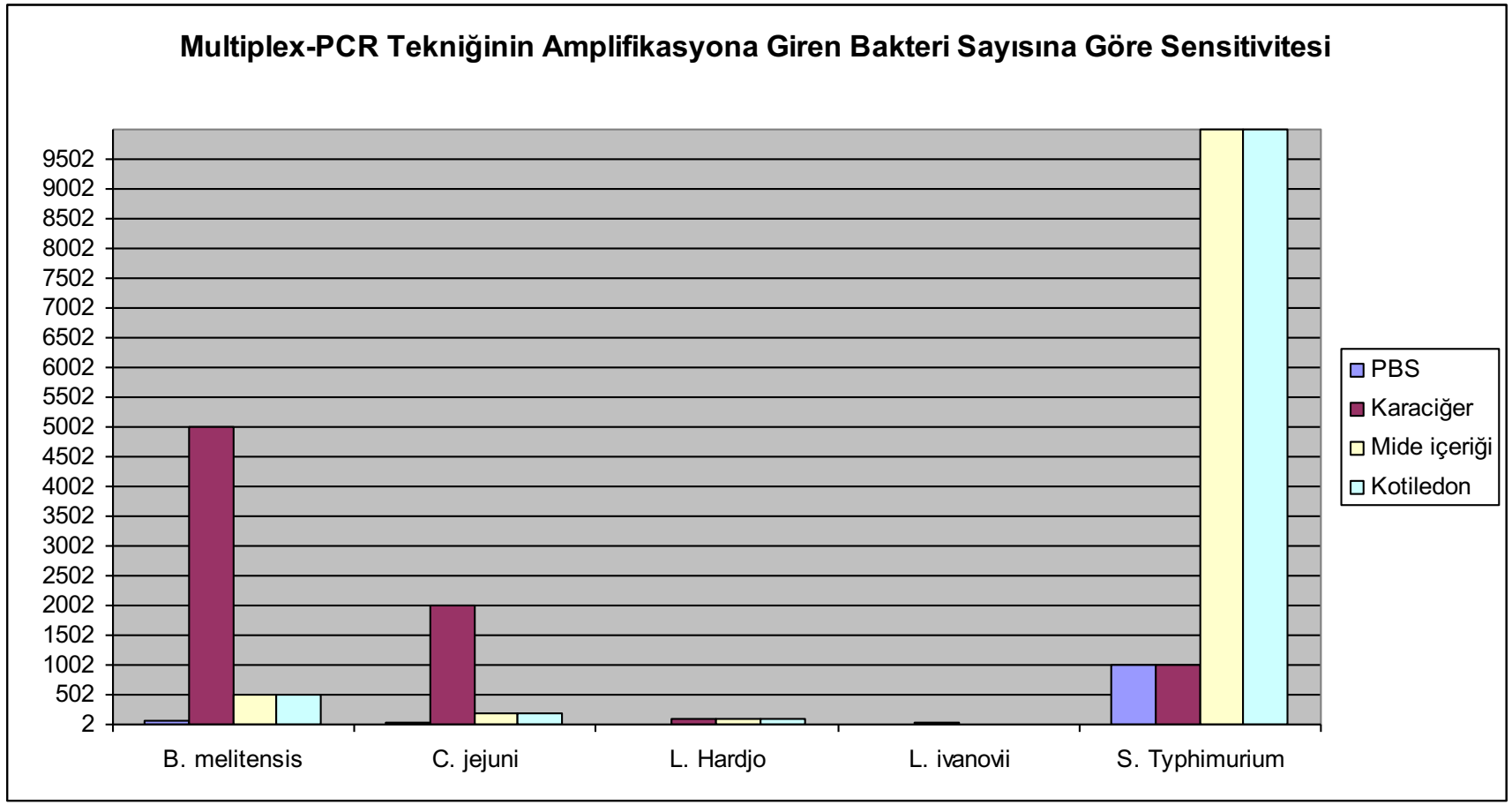


Figure. Sensitivity of Multiplex-PCR Technique Due To Number of Bacteria Per PCR reaction (cfu / 2 µl)



DISCUSSION

- PCR specificity depends on how good you design the primers
- PCR sensitivity depends on how many agents and inhibitors (and their ratio!!!) are there in your clinical sample
- You can increase your test sensitivity by optimizing your DNA extraction
- You can increase the primer sensitivity and test sensitivity by choosing multicopy targets (insertion sequences, 16S rRNA genes, etc.)
- Fetal abomasum contents are the best clinical materials for PCR and/or multiplex-PCR assays, and cotiledons have less inhibitors than fetal liver samples
- Plasenta is the best clinical sample in the identification of Chlamydial abortions

CONCLUSION

- In this study, novel detection techniques were developed which lack the drawbacks of conventional identification methods, and having the advantage of simultaneously investigating several agents
- PCR techniques developed in this project could be used for the molecular microbiological investigation of abortions in different animal groups in Veterinary Medicine and for the identification of zoonotic infections, as well
- The techniques are thought to be original according to literature search