

Week 7. Multiplex-PCR



ANKARA UNIVERSITY



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

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MULTIPLEX PCR

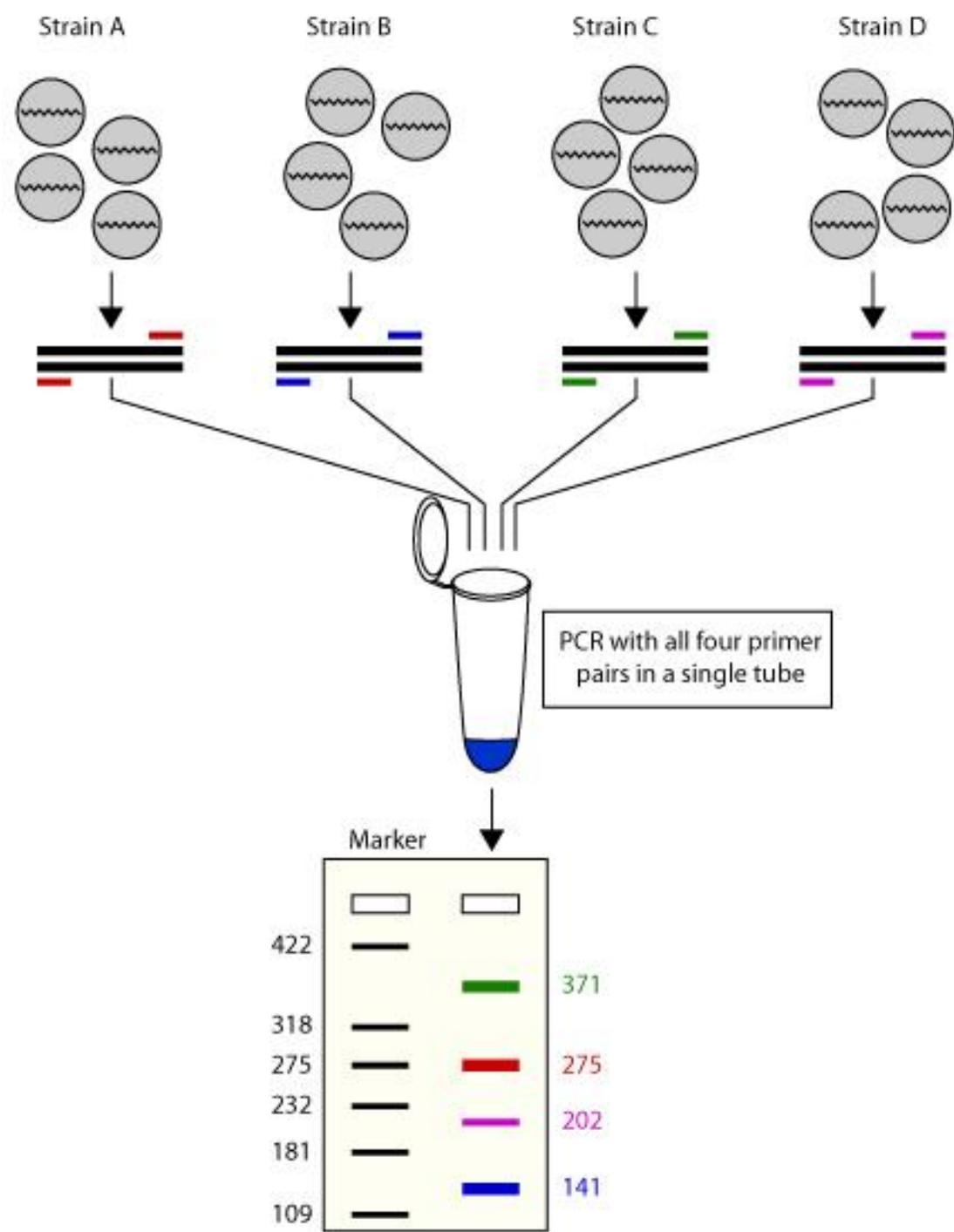
- Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment.
- In a multiplexing assay, more than one target sequence can be amplified by using multiple primer pairs in a reaction mixture.
- As an extension to the practical use of PCR, 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.



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 T_m , preferably between 55°C-60°C are used. For sequences with high GC content, primers with a higher T_m (preferably 75°C-80°C) are recommended. A T_m 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 impracticable
- Since they belong to diverse phylogenetic 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 equipped 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 searches, 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

<i>Brucella</i> spp. / <i>Campylobacter</i> spp. / <i>C. abortus</i> 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		
<i>Brucella</i> spp. / <i>Campylobacter</i> spp. / <i>C. abortus</i> Multiplex PCR-II				
Bru1F	AGGGCAAGGTGGAAGATTTG	20	54°C	337 bp
Bru1R	ATCGGAACGAGCGAAATACC	20		
Campgen3F	TGCCCTACACAAGAGGACAAC	21	54°C	154 bp
Campgen3R	AAGCGTCATAGCCTTGGT	20		
Cabort1F	TCCAATGTAGGCATCACTC	20	54°C	213 bp
Cabort1R	CCCTTGATCCTCTAGGCTTGT	22		
<i>Brucella</i> spp. / <i>Campylobacter</i> spp. Multiplex PCR				
Bru2F	CATGACACCCAACTTAGCC	20	54°C	135 bp
Bru2R	TTACTGCTCTACCTTCTGTGGATT	24		
CampgenF	AGCGCAACCCACGTATTTAG	20	54°C	206 bp
CampgenR	GAACAATCCGAACTGGGACA	20		
<i>Coxiella burnetii</i> / <i>Leptospira</i> spp. / <i>Listeria</i> spp. Multiplex PCR				
Lepto1F	GCGATTATGCCTGACCAAAT	20	54°C	249 bp
Lepto1R	TCCTTCACTTCACCTGGTTT	21		
CoxburF	GACGGCCAATTATCAGAACA	20	54°C	180 bp
CoxburR	CGCTTATTACCAATGACGAAC	22		
Listgen2F	TGACACAAGTAACCGAGAATCA	22	54°C	132 bp
Listgen2R	CGTGCGCCCTTCTAACT	18		

Table. Multiplex-PCR Mix

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

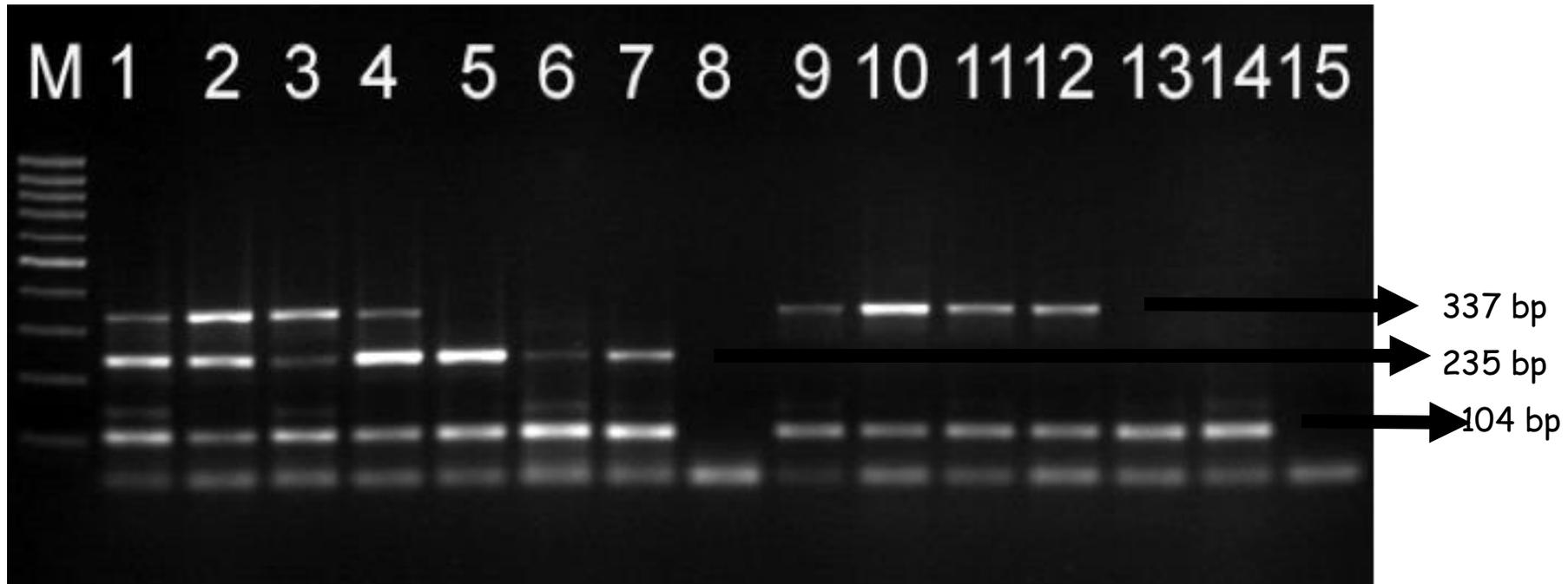
Table. Optimized Multiplex-PCR Amplification Protocol.

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

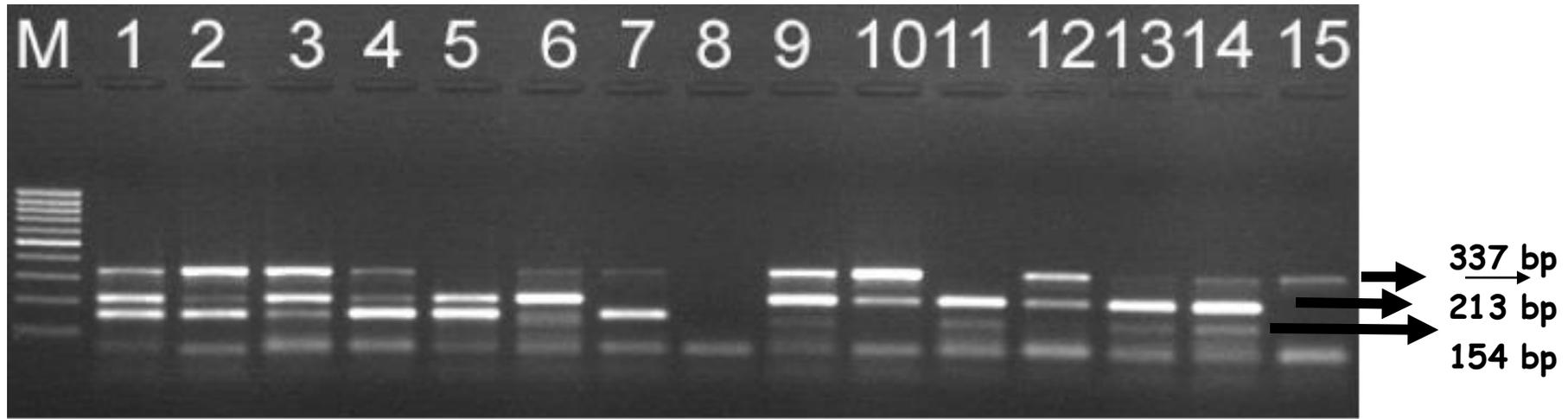
- A total of **59 pairs** of primers were designed in the study, 43 pairs were chosen and tested in vitro and **39 (90.7%)** of these were successful in PCR assays
- Following investigation of bacterial abortifacient agents by singleplex-PCR assays in **112 fetal abomasum contents 32 (28.6%), 24 (21.4%), 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



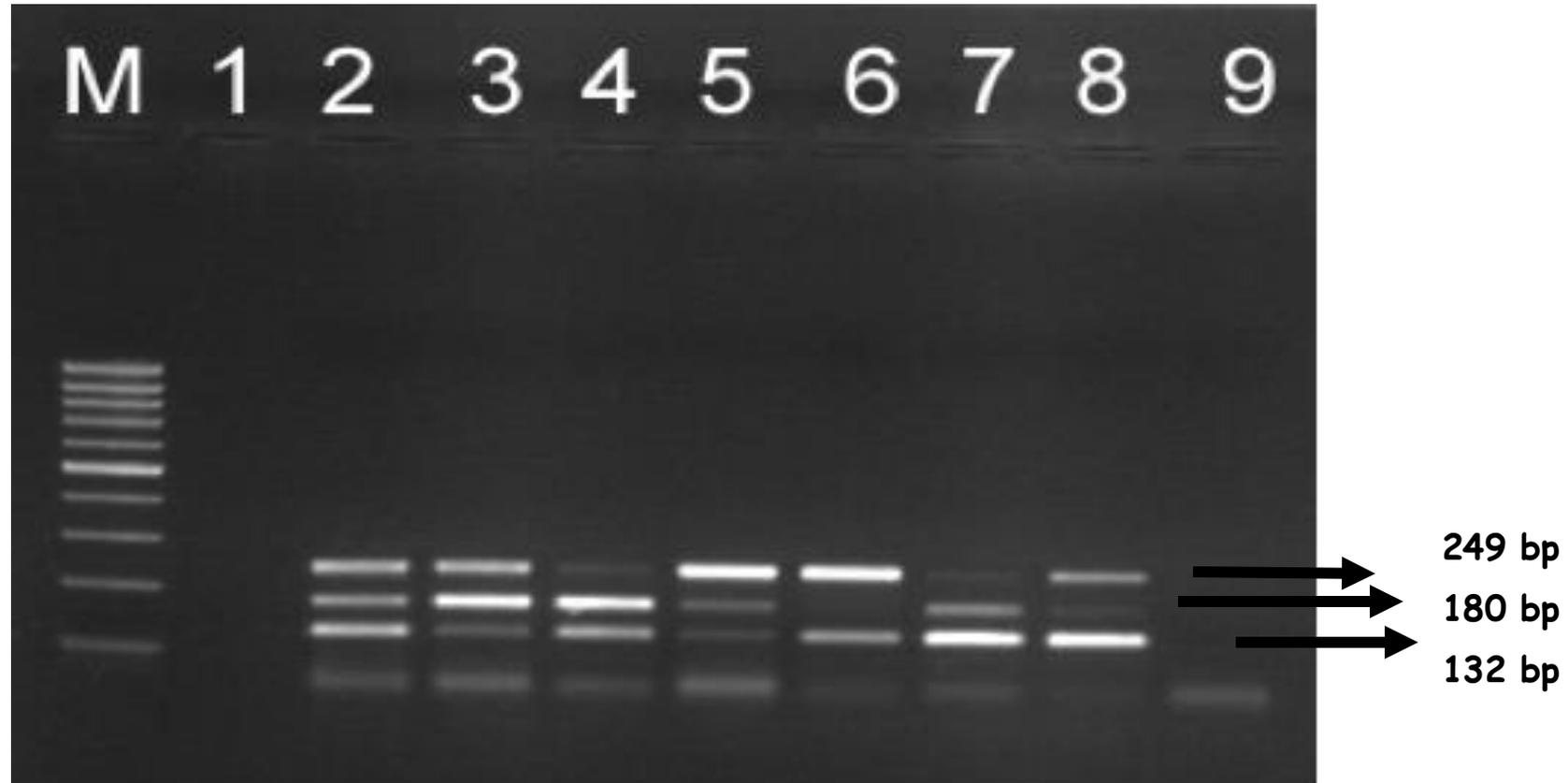
1. Multiplex PCR with template DNAs of 10 ng/ μ l (direct DNA, D) concentrations of bacterial DNA
2. *Brucella* spp. DNA D, *Campylobacter* spp. DNA $D \times 10^{-1}$ (1 ng/ μ l), *C. abortus* DNA $D \times 10^{-2}$ (100 pg/ μ l)
3. *Brucella* spp. DNA D, *Campylobacter* spp. DNA $D \times 10^{-2}$, *C. abortus* DNA $D \times 10^{-1}$
4. *Brucella* spp. DNA $D \times 10^{-1}$, *Campylobacter* spp. DNA D, *C. abortus* DNA $D \times 10^{-2}$
- *Brucella* spp. DNA $D \times 10^{-2}$, *Campylobacter* spp. DNA D, *C. abortus* DNA $D \times 10^{-1}$
1. *Brucella* spp. DNA $D \times 10^{-1}$, *Campylobacter* spp. DNA $D \times 10^{-2}$, *C. abortus* DNA D
2. *Brucella* spp. DNA $D \times 10^{-2}$, *Campylobacter* spp. DNA $D \times 10^{-1}$, *C. abortus* DNA D
3. 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



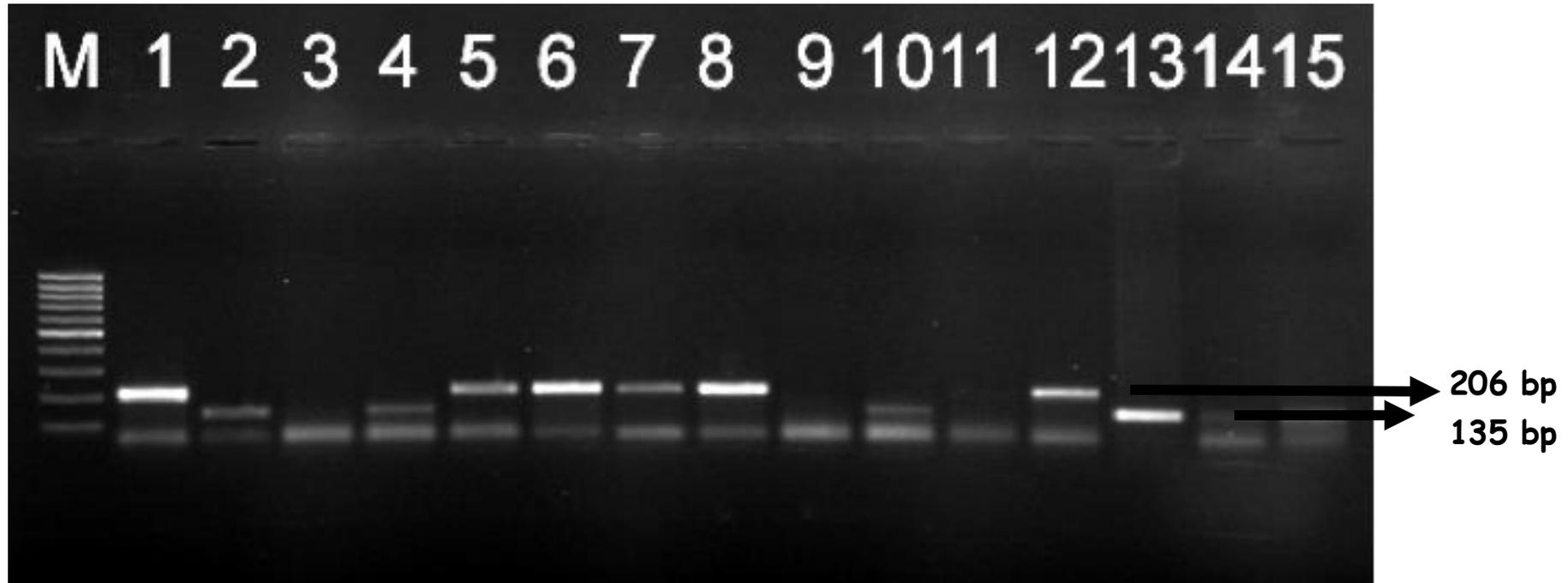
- Multiplex PCR with template DNAs of 10 ng/μl (Direct DNA, D) concentrations of bacterial DNA
- 1. *Brucella* spp. DNA D, *Campylobacter* spp. DNA $D \times 10^{-1}$ (1 ng/μl), *C. abortus* DNA $D \times 10^{-2}$ (100 pg/μl)
- 2. *Brucella* spp. DNA D, *Campylobacter* spp. DNA $D \times 10^{-2}$, *C. abortus* DNA $D \times 10^{-1}$
- 3. *Brucella* spp. DNA $D \times 10^{-1}$, *Campylobacter* spp. DNA D, *C. abortus* DNA $D \times 10^{-2}$
- 4. *Brucella* spp. DNA $D \times 10^{-2}$, *Campylobacter* spp. DNA D, *C. abortus* DNA $D \times 10^{-1}$
- 5. *Brucella* spp. DNA $D \times 10^{-1}$, *Campylobacter* spp. DNA $D \times 10^{-2}$, *C. abortus* DNA D
- 6. *Brucella* spp. DNA $D \times 10^{-2}$, *Campylobacter* spp. DNA $D \times 10^{-1}$, *C. abortus* DNA D
- 7. Negatif kontrol, PCR mix
- 8. *B. melitensis* DNA + *C. jejuni* DNA + *C. abortus* S26/3 DNA
- 9. *B. melitensis* DNA + *C. abortus* S26/3 DNA
- 10. *C. abortus* S26/3 DNA + *C. jejuni* DNA
- 11. *B. abortus* DNA + *C. abortus* S26/3 DNA
- 12. *B. abortus* DNA + *C. fetus subsp. fetus* DNA + *C. abortus* DNA
- 13. *B. ovis* DNA + *C. fetus subsp. fetus* DNA + *C. abortus* DNA
- 14. *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/ μ l (Direct DNA, D) concentrations of bacterial DNA
3. *C. burnetii* DNA D, *Leptospira* spp. DNA Dx10-1 (3 ng/ μ l), *Listeria* spp. DNA Dx10-2 (300 pg/ μ l)
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 μ l)

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

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

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

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

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 μ l)

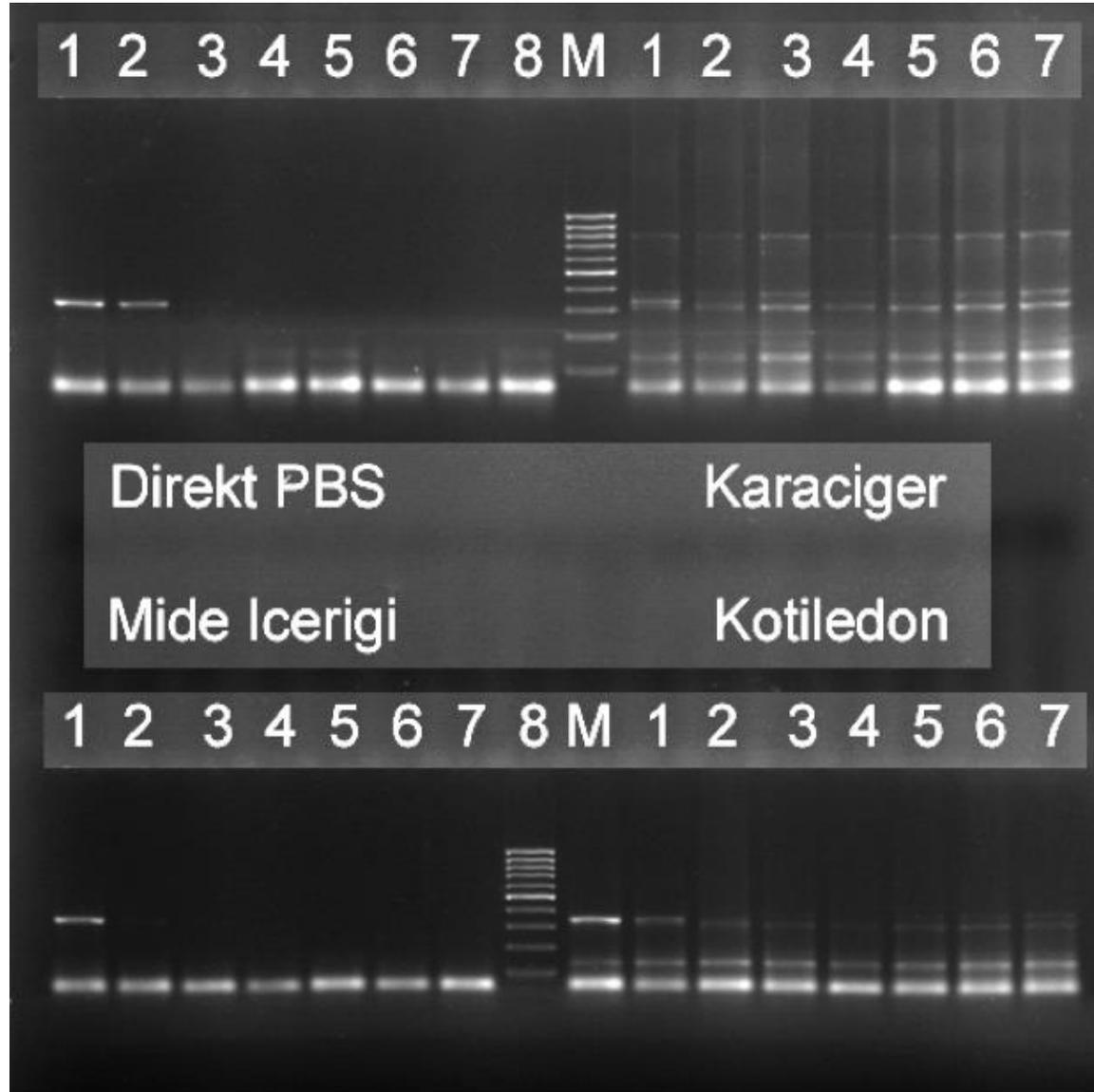
Multiplex-PCR				
	PBS	Liver	Abomasum Content	Cotiledon
B. melitensis	50	5000	500	500
C. jejuni	20	2000	200	200
L. Hardjo	10	100	100	100
L. ivanovii	2	20	2	2
S. Typhimurium	1000	1000	10000	10000

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



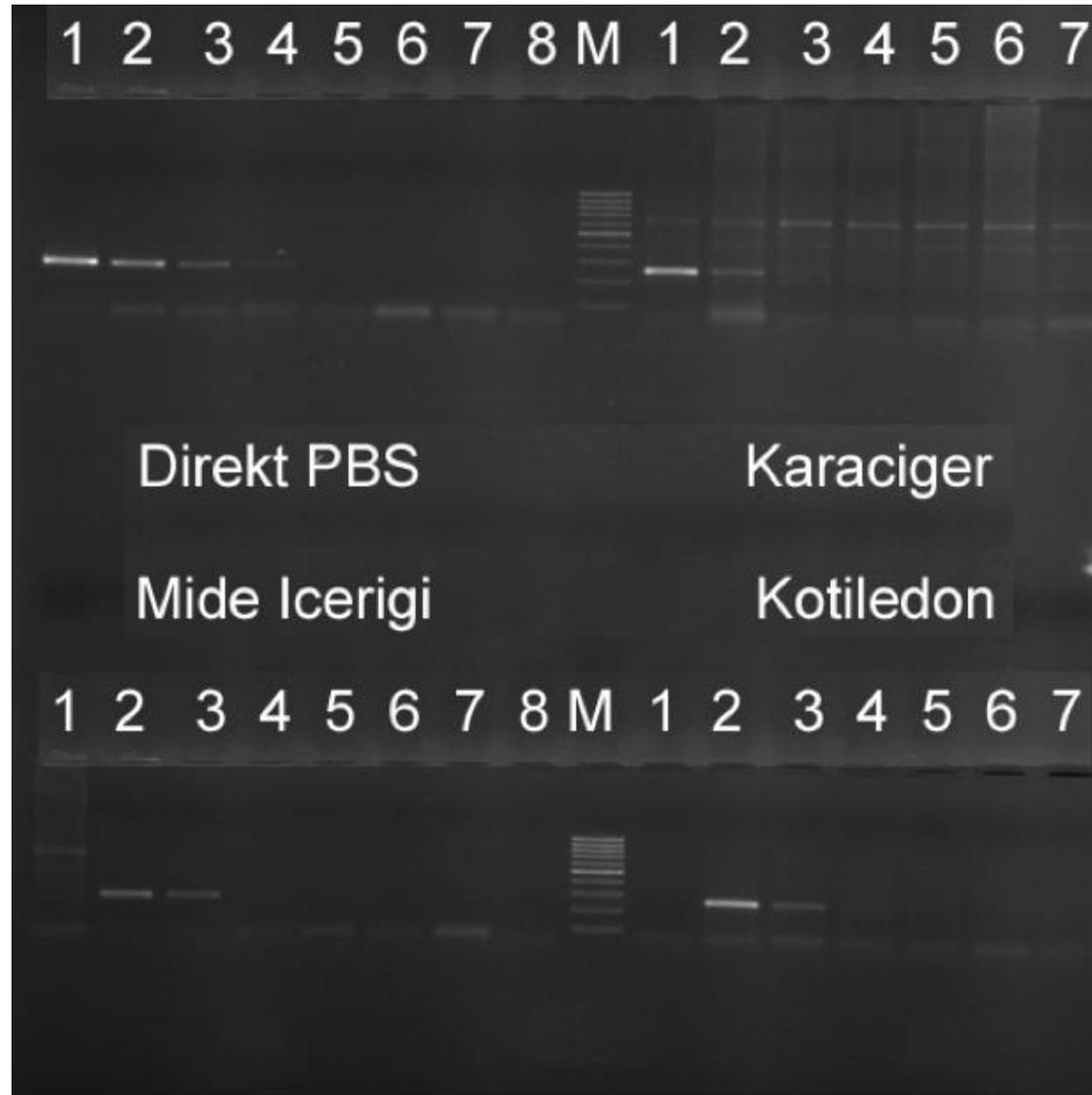
1. 2×10^7 cfu/25 μ l
2. 2000000 cfu/25 μ l
3. 200000 cfu/25 μ l
4. 20000 cfu/25 μ l
5. 2000 cfu/25 μ l
6. 200 cfu/25 μ l
7. 20 cfu/25 μ l
8. negatif kontrol

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



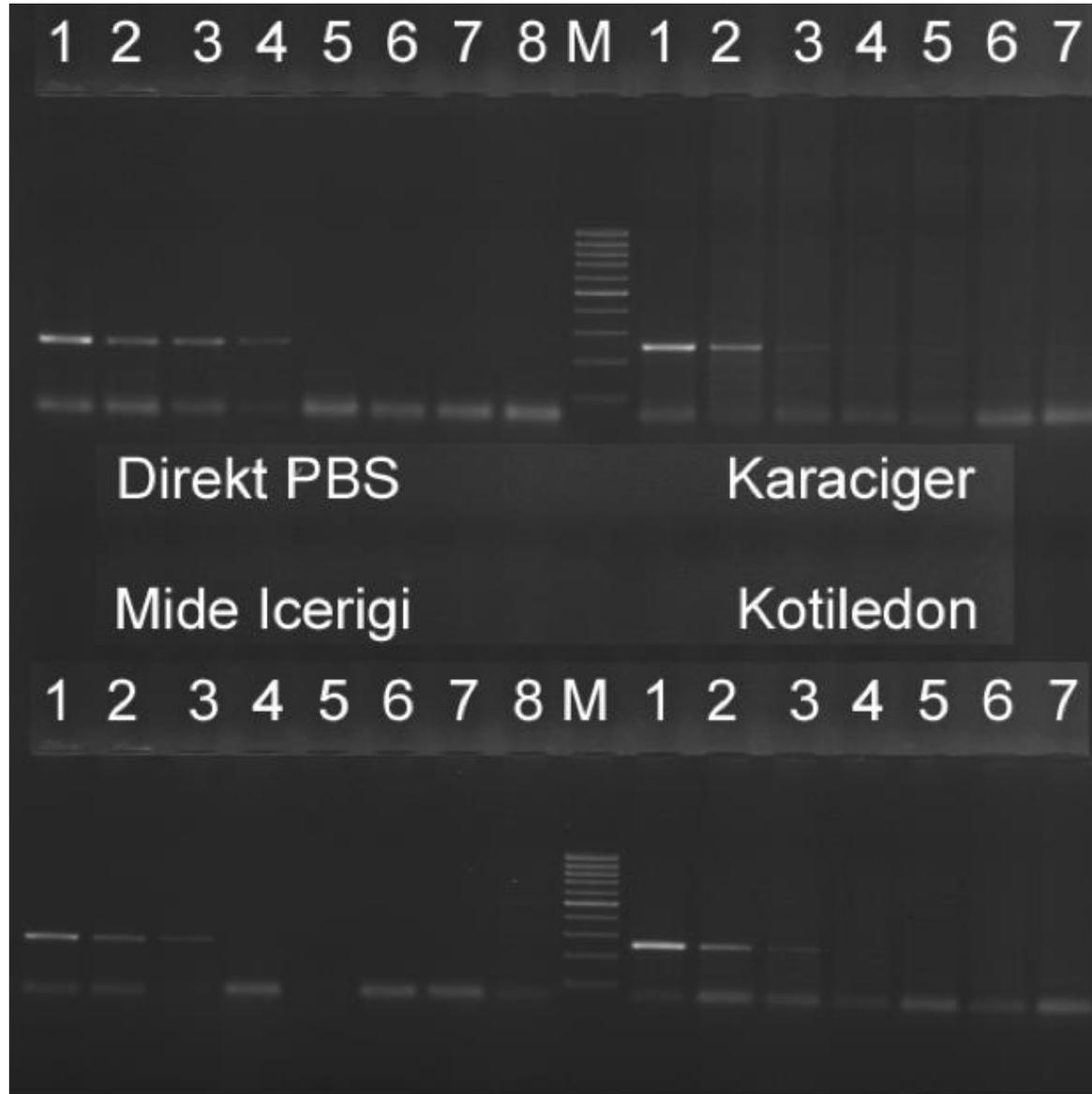
1. 2×10^7 cfu/25 µl
2. 2000000 cfu/25 µl
3. 200000 cfu/25 µl
4. 20000 cfu/25 µl
5. 2000 cfu/25 µl
6. 200 cfu/25 µl
7. 20 cfu/25 µl
8. negatif kontrol

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



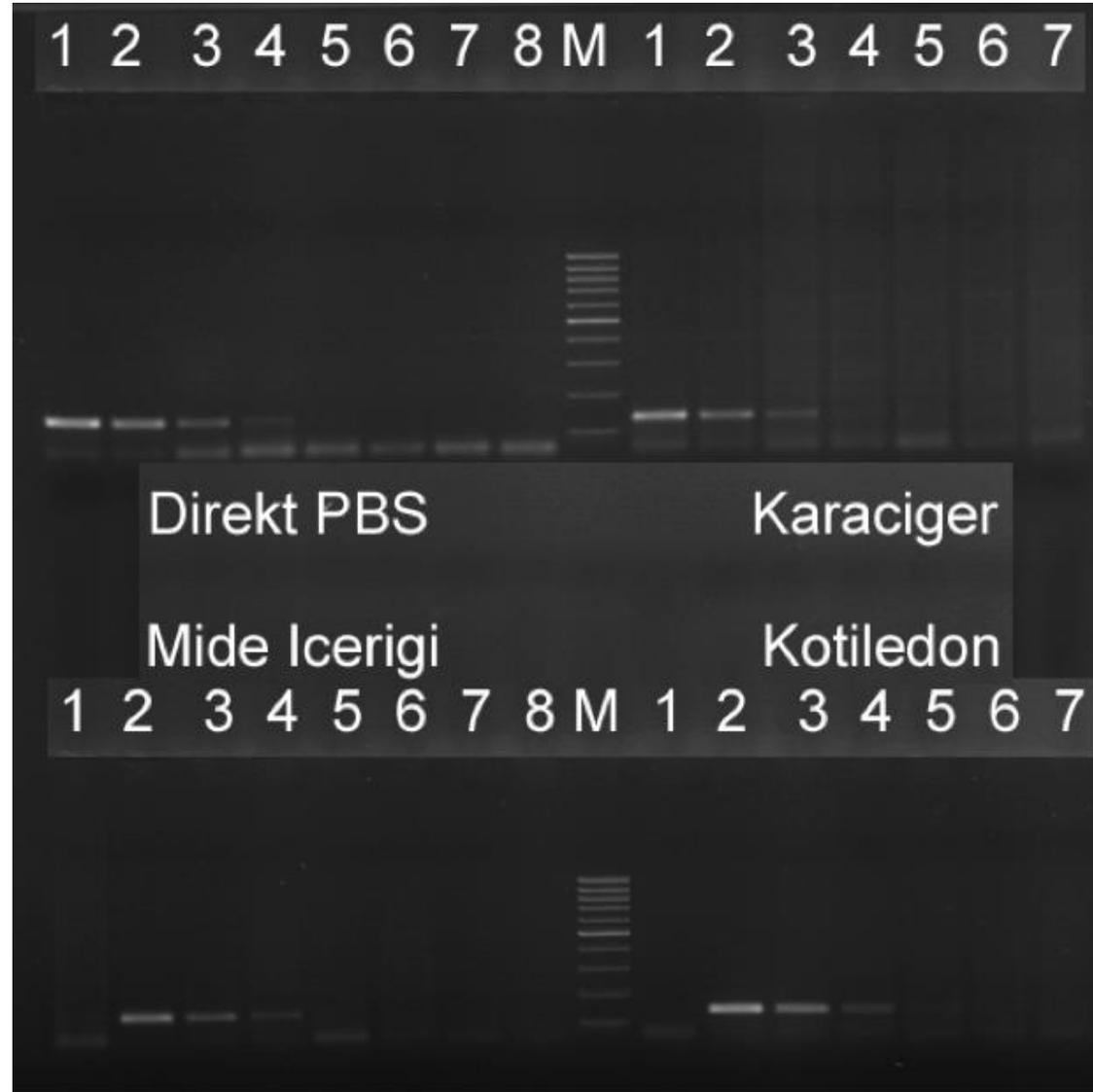
1. 1000000 counts/25 µl
2. 100000 counts/25 µl
3. 10000 counts/25 µl
4. 1000 counts/25 µl
5. 100 counts/25 µl
6. 10 counts/25 µl
7. 1 counts/25 µl
8. negative control

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



1. 200000 cfu/25 μ l 2. 20000 cfu/25 μ l 3. 2000 cfu/25 μ l 4. 200 cfu/25 μ l 5. 20 cfu/25 μ l 6. 2 cfu/25 μ l
7. 0.2-1 cfu/25 μ l 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 μ l)

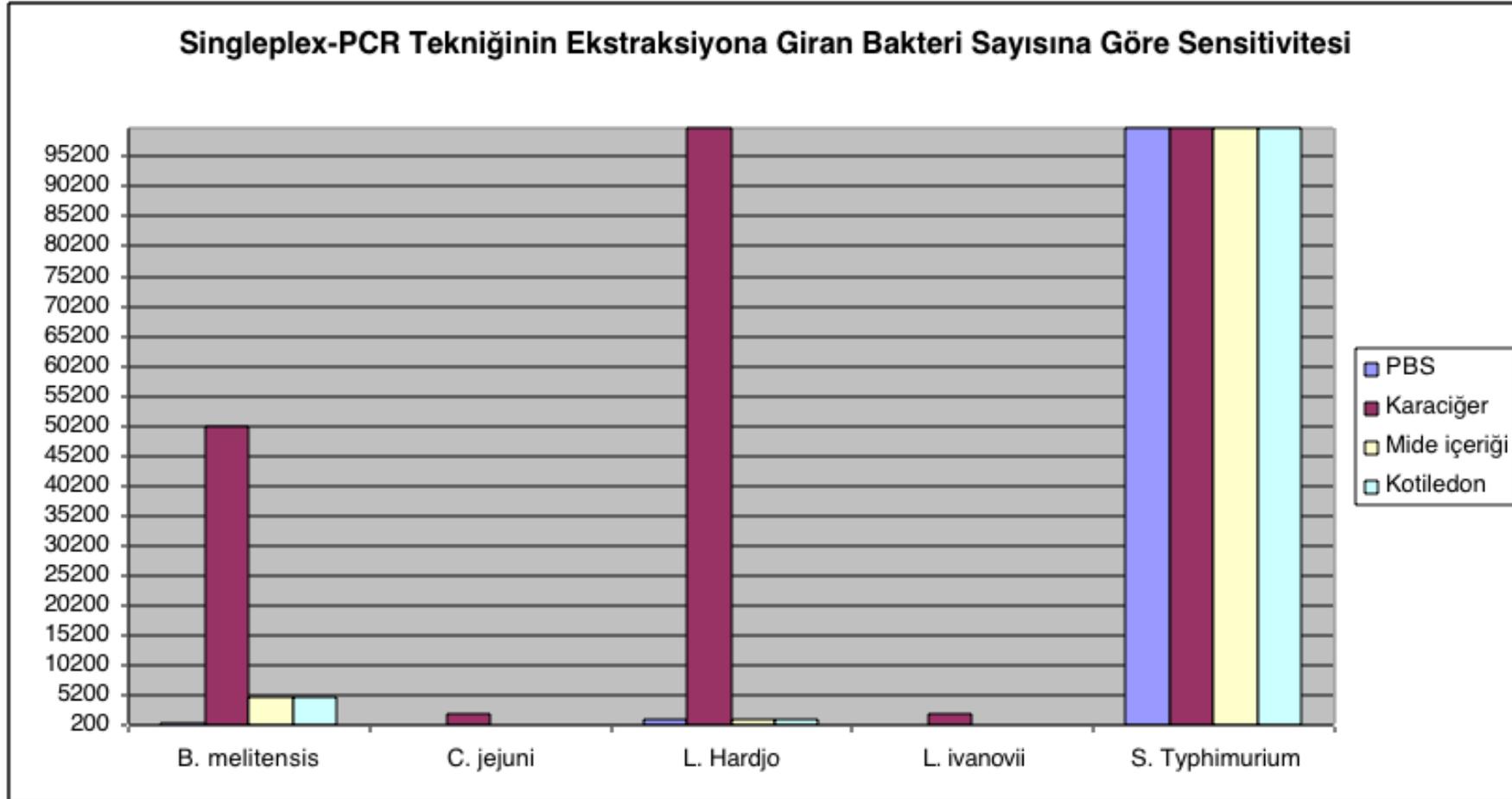


Figure. Sensitivity of Multiplex-PCR Technique Due To Number of Bacteria Extracted (cfu / 25 μ l)

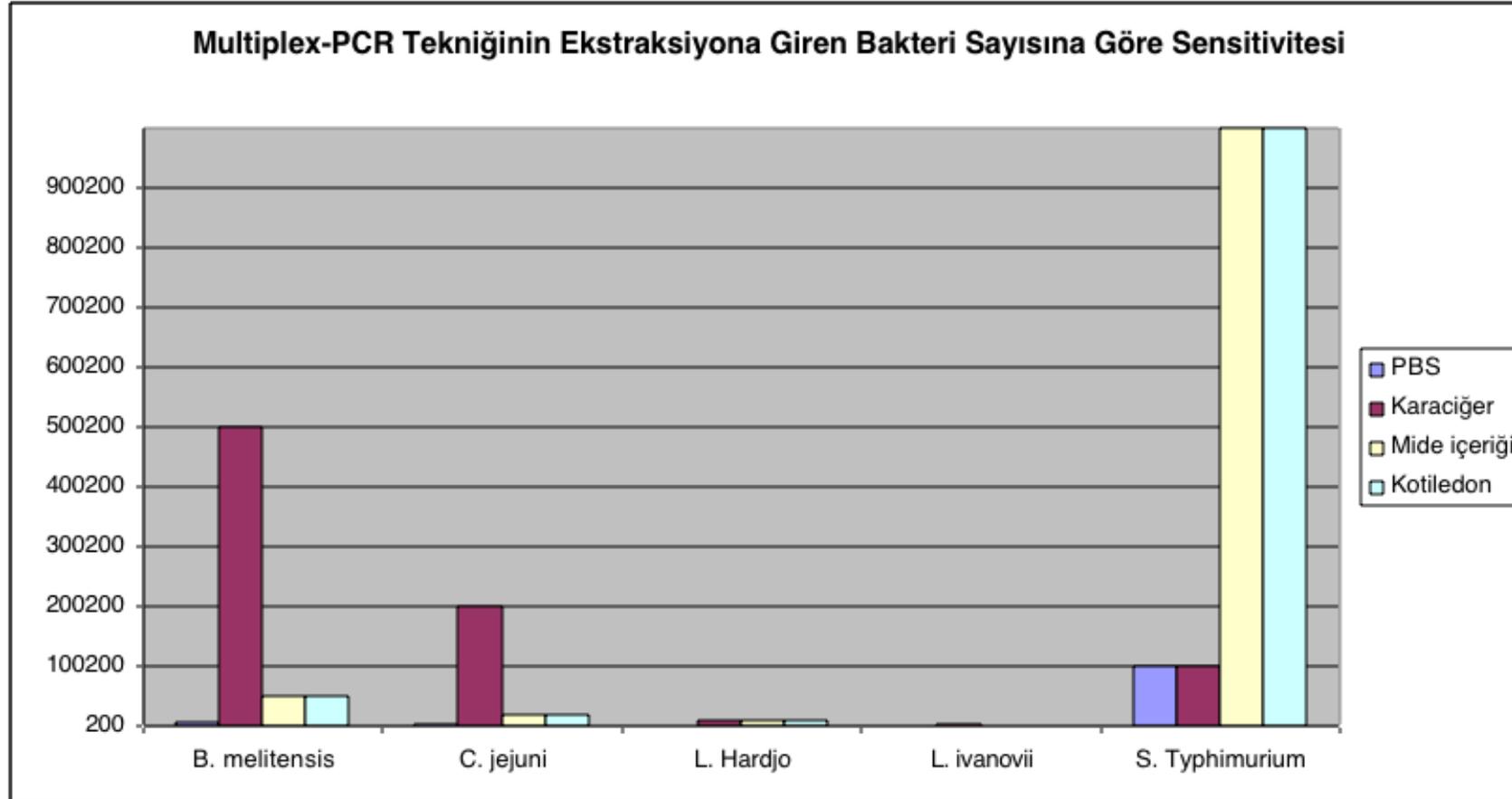


Figure. Sensitivity of Singleplex-PCR Technique Due To Number of Bacteria Per PCR reaction (cfu / 2 μ l)

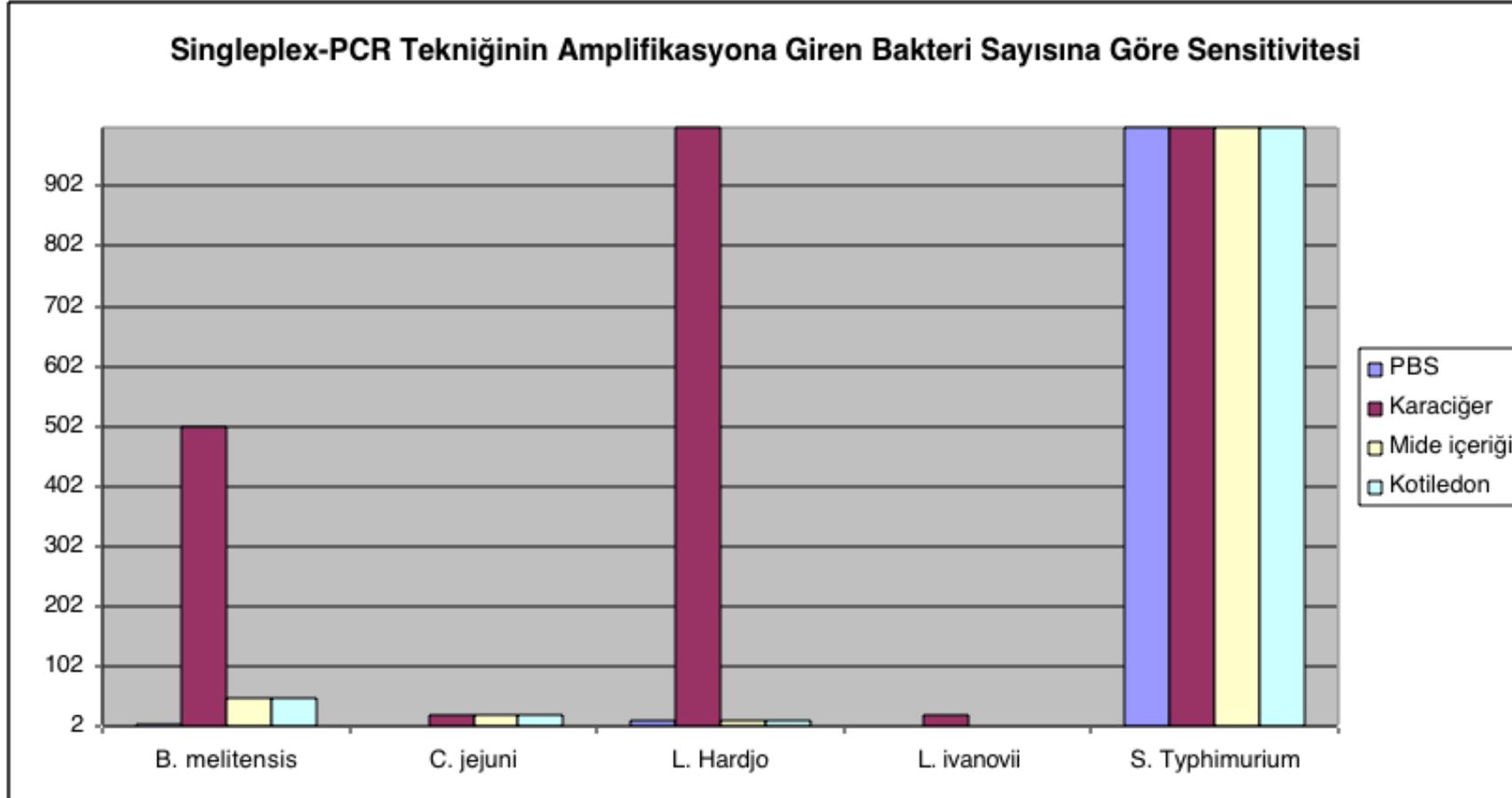
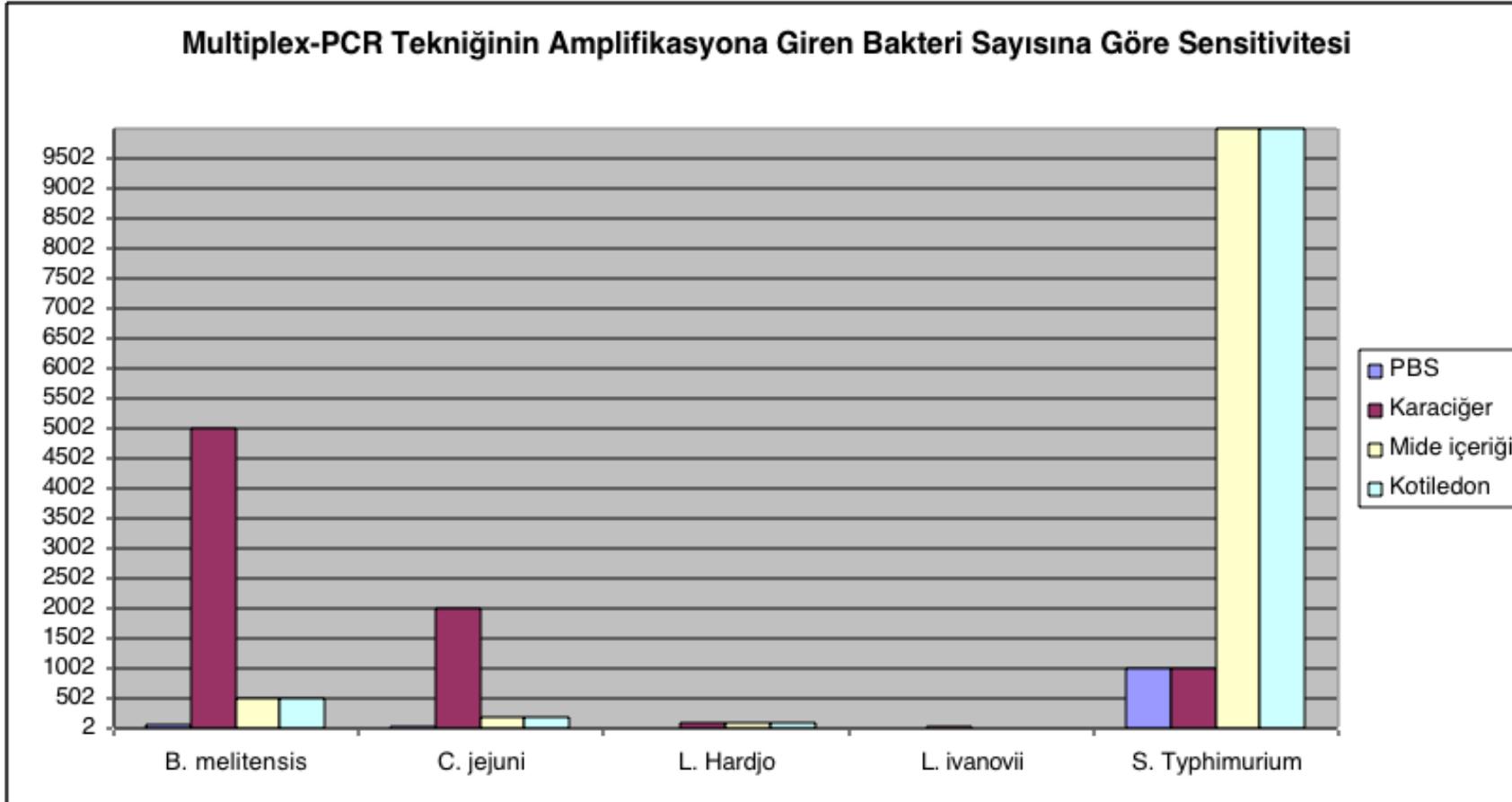


Figure. Sensitivity of Multiplex-PCR Technique Due To Number of Bacteria Per PCR reaction (cfu / 2 μ l)



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 cotyledons have less inhibitors than fetal liver samples
- Placenta 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