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Use of PCR for molecular typing of Brucella strains

DIPLOMA THESIS

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KATEDRA BIOCHEMICKÝCH VĚD

Využití PCR pro molekulární charakterizaci kmenu Brucella

DIPLOMOVÁ PRÁCE

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CONTENS

ABBREVIATIONS

AA	acrylamide
B. melitensis	Brucella melitensis
B. abortus	Brucella abortus
B. ovis	Brucella ovis
B. canis	Brucella canis
B. neo	Brucella neotomae
B.suis	Brucella suis
B. maris	Brucella isolated from marine mammals
Bp	base pairs
DNA	Deoxyribonucleotide acid
DnTp	deoxynucleotide Triphosphates
E.coli	Escherichia coli
Kb	thousand base pairs
MV	molecular weight
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
TBS	tris-buffered saline
T_m	temperature of melting
Tris	Tris(hydroxymethyl)aminomethan

1. INTRODUCTION

Brucella species are responsible for brucellosis, a worldwide zoonotic disease causing abortion in domestic animals and Malta fever in humans.

Six species are recognized within genus Brucella – B.abortus, B. melitensis, B. suis, B. ovis, B.canis, B. neotomae, principally associated with cattle, with goats, sheep, with pigs, dogs, wood rat. They may cause abortion and other reproductive disorder in their hosts, which results in huge economic losses.
The broad spectrum of Brucella isolates has, since the 1990s, been enlarged to include marine mammals. However, their overall characteristics were not assimiable to those of any of the six recognized Brucella species.
With respect to the current classification of Brucella species according to the preferential host two new species names were proposed: Brucella pinnipediae (for pinnipedian isolates) and Brucella cetaceae (for cetacean isolates).
These strains were identified as brucellae by their colonial and cellular morphology, staining characteristic, biochemical activity, agglutination by monospecific antisera, susceptibility to lyses by Brucella specific bacteriophage and metabolic profiles.

Intention of this work was find out specific valid method for identification of Brucellae.
Convenience of method molecular biology is more safety, faster and unfailing to detect this bacterium. Especially the PCR technique is better to detect eventually infection either from animal or from human, in comparing to cultivating method.

The aim of this diploma work is:
1 - To find out suitable condition for new designed pair of primers for PCR reaction to detect individual biovarities in Brucella suis. And take it as background to developed multiplex assay for individual biovarities of Brucella suis.

2 - To confirm the hypothesis that exist more than only 6 species in Brucella genus and tray to find specific pair of primers for those strain. Task of my project was comparing the six known Brucella species to new "Brucella maris" (Brucella strain isolated from marine mammals).

2. THEORETICAL PART

2.1. The Bacterium Brucellae

2.1.1. Typical organism

Brucella is intracellular, facultative anaerobic, bacteria and caused granulomatous infection – Brucellosis. It looks like tuberculosis. Brucella is obligate parasites capable of causing acute / chronic illness or in apparent infection [1].

Brucellaceae a family of Rhizobiales
A family of bacteria containing small, coccoid to rod-shaped, gram-negative cells that are parasites in and pathogens for warm-blooded animals, including humans [1].

2.1.2. History

Brucellosis, a worldwide zoonotic disease, is known to cause reproductive disorders or abortion in domestic animals. It requires combined, protracted antibiotic treatment [1].

Table 1.Taxonomy [1]

Brucellaceae	
Scientific classification	
Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alpha Proteobacteria
Order:	Rhizobiales
Family:	**Brucellaceae**
Genera	
Brucella *Mycoplana* *Ochrobactrum*	

Brucella is named after its researcher Australian doctor Sir David Bruce (Melbourne, May 29, 1855 - November 27, 1931) He was an English pathologist and microbiologist who investigated the Malta-fever and trypanosomes, identifying the cause of sleeping sickness. He won the Leeuwenhoek Medal in 1915. He, in 1886, first isolated the organism from the spleen of a British soldier at *post-mortem* on the island of Malta. From that time a disease known as Malta fever [2].

The source of organism was discovered in 1904, when it was cultured from milk and urine of goats. When the consumption of raw goat's milk was stopped, the incidence of the disease declined sharply [2].

The second organism of the group was isolated in Denmark by Band in 1897 from cattle suffering from infection abortion (Bang's disease). The third was cultured in the U.S. in 1914 from the fetus of a prematurely delivered sow [2].

The genus *Brucella* currently contains six name species: *B. melitensis, B. abortus, B. canis, B. ovis, B. suis and B. neotomae,* which vary in their ability to infect host animals [1].

The disease causes much clinical morbidity as well as a considerable loss of productivity in animal husbandry in the developing world. In this era of international tourism, brucellosis has become a common imported disease in the developed world. Regrettable is existing

possibilities to use Brucella as a biological agents for a germ warfare. Bioterrorism is a real threat in a world of rapid international travel, widespread technical knowledge, and overnight mailings [1].

2.1.3. Morphology and identification

Brucella belongs to the α- 2- subdivision of the proteobacteria, along with ochrobactrum, rhizobium, rhodobacter, agrobacterium, bartonella and rickettsia. The infection agents Brucella are G- negative, nonmotile cocco–bacilli or small rods. Their length varies from 0.6 to 1.5 μm, and width from 0.5 to 0.7 μm. Colonies are small, convex, smooth, moist appearing, nonhemolitic and translucent [3].

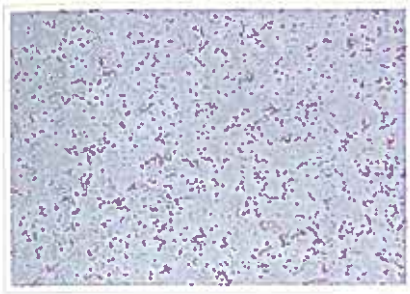


Fig. 1 Heat fixed brucellae stained by the modified Ziehl-Neelsen (Stamp) method. (Courtesy of M. Banai, M. Bernstein and S. Perl, Kimron Veterinary Institute, Bet Dagan, Israel)

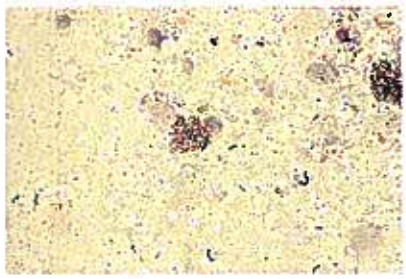


Fig. 2 Heat fixed placenta from aborted material stained by the modified Ziehl-Neelsen (Stamp) method revealing brucellae and non brucella contaminating bacteria. (Courtesy of M. Banai, M. Bernstein and S. Perl, Kimron Veterinary Institute, Bet Dagan, Israel)

2.1.4. Taxonomy

Based on DNA homology, they may represent a single species. Conventionally, however, they are classified into eight species (B. abortus, B. suis, B. melitensis, B. canis, B. ovis, B, neotomae, B. cetaceae, and B. pinnipediae), each comprising several biovars [4].

The traditional classification of Brucella species is largely based on its preferred hosts. There are six classic pathogens, of which four are recognized human zoonoses. The presence of rough or smooth lipopolysaccharide correlates with the virulence of the disease in humans. Two new Brucella species, provisionally called Brucella pinnipediae and B.cetaceae, have been isolated from marine hosts within the past few years [1].

Taxonomic characteristics of Brucella species and biotypes are summarized in table no.2 [1].

Brucella is a monospecific genus that should be termed B. melitensis, and all other species are subtypes, with an interspecies homology above 87 percent. The phenotypic difference and host preference can be attributed to various proteomes, as exemplified by specific outer-membrane protein markers. All Brucella seem to have arisen from a common ancestor to which B. suis biotype 3 shares particular similarity. Although the scientific accuracy of this classification cannot be disputed, its practicality has been under scrutiny [1].

Table 2. Nomenclature and Characteristics of Brucella Species.[1]

Species	Biotype	Animal Hosts	First Described	Human Virulence*	Species Discrimination
B. melitensis	1–3	Goats, sheep, camels	Bruce, 1887	++++	Fuchsin, positive; thionine, positive; safranin inhibition, negative; H_2S production, negative; urease, positive in 24 hr; CO_2 growth, negative; Tiblisi phage lysis, negative; Weybridge phage lysis, negative
B. abortus	1–6, 9	Cows, camels, yaks, buffalo	Bang, 1897	++ to +++	Fuchsin, positive (except biotype 2); thionine, negative (biotypes 1, 2, and 4); safranin inhibition, negative; H_2S production, positive (except biotype 5); urease, positive in 24 hr; CO_2 growth, positive (biotypes 1–4), Tiblisi phage lysis, positive; Weybridge phage lysis, positive
B. suis	1–5	Pigs (biotypes 1–3), wild hares (biotype 2), caribou (biotype 4), reindeer (biotype 4), wild rodents (biotype 5)	Traum, 1914	+	Fuchsin, negative (except biotype 3); thionine, positive; safranin inhibition, positive; H_2S production, positive (biotype 1); urease, positive in 15 min; CO_2 growth, negative; Tiblisi phage lysis, negative; Weybridge phage lysis, positive
B. canis	—	Canines	Carmichael and Bruner, 1968	+	Fuchsin, positive or negative; thionine, positive; safranin inhibition, negative; H_2S production, negative; urease, positive in 15 min; CO_2 growth, negative; Tiblisi phage lysis, negative; Weybridge phage lysis, negative
B. ovis	—	Sheep	Van Drimmelen, 1953	–	Fuchsin, negative for some strains; safranin inhibition, negative; H_2S production, negative; urease, negative; CO_2 growth, positive; Tiblisi phage lysis, negative; Weybridge phage lysis, negative
B. neotomae	—	Rodents	Stoenner and Lackman, 1957	–	Fuchsin, negative; safranin inhibition, negative; H_2S production, positive; urease, positive in 15 min; CO_2 growth, negative; Tiblisi phage lysis, positive or negative; Weybridge phage lysis, positive
B. pinnipediae and *B. cetaceae* (provisional)	—	Minke whales, dolphins, porpoises (pinnipediae), seals (cetaceae)	Ewalt and Ross, 1994	+	Fuchsin, positive; thionine, positive; safranin inhibition, negative; H_2S production, negative; urease, positive; CO_2 growth, negative for pinnipediae and positive for cetaceae; Tiblisi phage lysis, negative; Weybridge phage lysis, positive for pinnipediae and negative for cetaceae

* Virulence is graded on a scale from no virulence (–) to the highest degree of virulence (++++).

2.1.5. Culture (specimens)

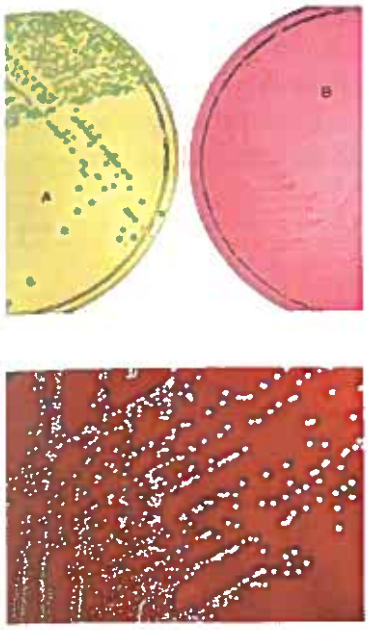


Fig. 3 Typical smooth colony
[University of Navarra]

Smooth, mucoid, and rough variants are recognized by colonial appearance and virulence. The typical virulent organism forms a smooth transparent colony, upon culture; it tends to change to the rough form, which is avirulent [1].

Culture: Blood or tissues are incubated in trypticase – soy broth and on thiokine – tryptosa agar. At intervals of several days, subcultures are made on solid media of similar composition. All cultured are incubated in 10% CO2 and should be observed and subcultured for at the least 3 weeks before being discarded as negative [1].

If organism resembles brucellae are isolated, they are typed by H2S production, dye inhibition, agglutination by absorbed sera. As a rule, brucellae can be cultivated from patients only during the acute phase of the illness or during of recurrence of activity [1].

2.1.6. Growth characteristic

Brucellae are adapted to an intracellular habitat and their nutritional requirements are complex. Brucellae are moderately sensitive to heat and acidity. They are killed in milk by pasteurization [5].

2.1.7. Diagnostic

2.1.7.1. Laboratory tests

Specimens: Blood should be taken for culture, biopsy material also for culture (lymph nodes, bone marrow ...) and serum for serological tests [5].

Inn all cases, at the beginning of determination of sort of G – microbe, have been done for Brucella spp. Within cultures also a quick specific serological battery including the rose Bengal plate agglutination test, al well as Wright's seroagglutination test and the Coombsantibrucella test [5].

The serum of susceptible animals contains a globulin and lipoprotein that suppress growth of nonsmooth, avirulent types and favor the growth of virulent types [5].

2.1.7.2. Antigen structure

Agglutination test is to be reliable, agglutinin tests must be performed with standardized heat killed, phenolized, smooth bacterium. Brucellae antigen available from brucellosis centers and should be incubated at 37°C for 24 hours. Ig G agglutinin titers above 1:8 indicate acute infection [5].

Brucellosis agglutinins are cross – reactive with tularemia agglutinins, and tests for both disease should be done on positive sera, usually, the titer for one disease will be much higher than for the other [6].
Chronic brucellosis is difficult to establish with certainly unless local lesions are present. Brucellae cannot be isolated from the patient at stave with weakness, pains, nervousness, but the agglutinin titer may be high [6].

2.1.7.3. Serology

Ig M antibodies levels rise during the first week of acute illness, peak at 3 month, and may persist during chronic disease [5].

Ig G antibody levels raise about 3 weeks after outset of acute disease, peak at 6 - 8 weeks, and remain high during chronic disease. Ig A levels parallels the Ig G levels [5].

After those procedures cams right moment for typing of Brucella strain. The easy method to differentiate the biovarities between each other is checked it's DNA and the equipment to do it is multiplex PCR.

2.2. Disease

2.2.1. Characterization of brucellosis

Brucellosis, a worldwide zoonotic disease, is known to cause reproductive disorders or abortion in domestic animals [1].

Brucellosis also called *Bangs disease, Gibraltar fever, Malta fever, Mediterranean fever, Rock fever, undulant fever* [3].

Brucellosis is an infections disease caused by the bacteria of the genus Brucella. These bacteria are primarily passed among animals, and they cause disease in many different vertebrates. Various Brucella species affect sheep, goats, cattle, pigs, dogs, and several other animals [7].

2.2.2. Geography

Brucella can be found world wide, it is more common in countries that do not have good standardized and effective public health and domestic animal health programs. Areas currently listed as high risk are the Mediterranean Basin (Portugal, Spain, Southern France, Italy, Greece, Turkey, North Africa), South and Central America, Eastern Europe, Asia, Africa, the Caribbean, and the Middle East [4].

Table 3. **Annual Cases of Human Brucellosis in Various Countries, According to Year.*** [1]

Country	1996	1997	1998	1999	2000	2001	2002	2003
Albania	NA	155	376	458	220	NA	NA	NA
Algeria	4356	3,434	2,232	2,223	NA	3,200	NA	2,766
Argentina	NA	676	NA	353	507	NA	296	325
Australia	38	41	45	52	27	NA	40	17
Azerbaijan	NA	624	494	582	654	660	519	407
Bosnia-Herzegovina	NA	NA	NA	NA	NA	7	NA	48
Colombia	53	42	82	42	NA	27	NA	238
Germany	23	25	18	21	27	25	35	27
Greece	NA	254	435	543	545	405	327	222
Iran	NA	NA	NA	17,168	NA	NA	NA	17,765
Israel	235	151	197	163	131	70	56	56
Italy	1896	1,681	1,461	1,324	1,067	923	813	520
Jordan	957	NA	684	432	288	275	219	159
Kyrgyzstan	NA	NA	NA	973	1,219	1,819	1,771	NA
Lebanon	192	429	136	184	NA	NA	NA	NA
Mexico	3362	3,387	3,550	2,719	2,171	3,013	2,851	3,008
Peru	1691	NA	1,269	NA	1,072	372	991	NA
Portugal	866	1,409	816	683	500	381	206	139
Russia	656	461	NA	352	423	508	595	NA
Saudi Arabia	5997	15,933	5,781	NA	NA	NA	NA	NA
Spain	NA	878	1,520	1,519	1,104	887	886	596
Syria	NA	NA	NA	NA	6,487	4,500	NA	23,297
Tajikistan	257	NA	211	NA	851	752	1,071	1,471
Tunisia	490	291	206	355	NA	321	250	128
Turkey	9480	11,812	11,427	11,462	10,742	15,510	17,553	14,435
Turkmenistan	NA	496	NA	NA	264	246	NA	NA
United Kingdom	15	6	7	76	19	26	38	19
United States	112	98	79	82	87	136	125	93
Uzbekistan	707	459	494	480	NA	NA	408	NA

* Data are from the Office International des Epizooties and various national health ministries. These numbers are believed to be a massive underestimation of the true prevalence of the disease. NA denotes not available.

2.2.3. Occurrence

Brucellosis is not very common for example in the United States, where100 to 200 cases occur each year. But brucellosis can be very common in countries where animal disease control programs have not reduced the amount of disease among animals. In the WHO exist special program for Mediterranean Zoonoses Control Programme (MZCP) of the World Health Organization to eliminate and surveillance among other brucellosis too. The table no.3 [(1)] contains annual cases of Human Brucellosis in various countries, according to year [4].

Brucellosis has been present for millennia and has managed to elude eradication, even in most developed countries. A high prevalence in certain geographic areas is well recognized, although largely underestimated. The relationship between the disease and individual socioeconomic status is exemplified in the United States, where programs to eradicate brucellosis have successfully limited the annual incidence of disease. The disease usually presents in Hispanic populations and is probably related to the illegal importation of

unpasteurized dairy products from neighboring Mexico, where the disease is endemic. Cheeses, sometimes called "village cheeses ", from these areas my represent a particular risk for tourists [1].

60% of the cases of brucellosis occur in workers in the meat-packing industry, less than 10% arise from ingesting raw milk or other unpasteurized dairy products [8].

In their natural animal reservoirs, brucellae show a striking propensity to localize in the pregnant uterus (frequently causing abortion) and in mammary glands; apparently healthy animals may shed brucellae in their milk for years. However, this great variety of wildlife species is bison, elk, feral, swine, wild boars, foxes, hares, African Buffalo, reindeer, and caribou [9].

2.2.4. Brucellosis in animals

2.2.4.1. Host animal

Disease in animals: In infected ruminants, brucellosis commonly induces abortion during the second half of gestation. Calves are often born immature and weak. This is why the disease is also known as Enzootic Abortion [3].
Each Brucella species has a characteristic, albeit not absolute, predilection to infect certain animal species [3].

2.2.4.1.1. Cattle

Bovine Brucellosis is usually caused by B. abortus, less frequently by B. melitensis and rarely by B. suis. B. abortus is differentiated into 7 biovars, with biovars 1 to 4 being predominant in Europe. The disease is usually asymptomatic in non-pregnant females. In pregnant cattle, infected animals develop a placentitis frequently resulting in abortion. Sometimes the leg joints and infrequently the udder are involved in inflammatory processes. Even in the absence of abortion excretion of the infectious organism occurs, for instance via placenta, fetal fluids, vaginal discharge, and milk [3].
Bulls may develop orchitis and epididymitis. Transmission of the organism during serving and artificial insemination is possible. Bovine brucellosis processes enzootic and is a threat to human health. Infections with B. abortus are rare in sheep, goats, and pigs. Mostly they are due to close contact with infected cattle herds [3].
The "Officially brucellosis free" status is currently valid for Germany. Eradication and monitoring of brucellosis is statutorily regulated [3].

2.2.4.1.2. Dog

Dogs can become infected by B. canis. Signs of canine brucellosis are associated principally with the reproductive tract. The ingestion of the organism happens orally. Infections in humans are uncommon and are usually mild [3].

2.2.4.1.3. Pig

Porcine brucellosis is caused by B. suis biovars 1 to 3. Biovars 2 is rarely pathogenic for humans and found in Europe only, whereas biovars 1 and 3 are highly pathogenic and of widespread occurrence. Signs of disease are similar to other animal species. They include abortion in sows and birth of dead or weak piglets. In boars the most prominent sign is orchitis. Inflammatory lesions culminating in abscesses are localized in the reproductive

organs and the bones. The infection is established also in wild boars and in animals other than pigs, in Europe primarily in hares [3].

2.2.4.1.4. Sheep and goat

Ovine and caprine brucellosis is primarily caused by B. melitensis (3 biovars). This disease with its symptoms is very similar to B. abortus infection in cattle. Sheep is considered to be less susceptible. Therefore abortion is less common in this animal species. In Europe, B. melitensis is prevalent in Mediterranean countries. Sporadic infections in bovines, carnivores and other animals are blind ending. Germany is recognized as being officially free of brucellosis (ObmF, Brucella melitensis). Only sheep can be infected by B. ovis. The organism is disseminated worldwide. The disease produces inflammation of the epididymis in rams and the placenta in pregnant ewes. A small proportion of infected ewes may abort. If the kidneys are involved, urinary excretion occurs [3].

2.2.4.1.5. Cetacean, Pinnipediae

The broad spectrum of Brucella isolates has, since the 1990s, been enlarged to include marine mammals, because it showed that they are distinct from terrestrial Brucella species. A number of recent reports have described the isolation and characterization of Brucella strains from wide variety of marine mammals such as bottlenose dolphin, common dolphin, Atlantic whitesided dolphin, striped dolphin, common seal, hooded seal, gray seal, harbor porpoise, otter and minke whale. Brucella was detected in abnormal gonads respectively in granular testes of the whales showing cassation or calcification. One human case, a person infected while working with marine mammal Brucella strains in the laboratory, has been reported, and they thus represent potential zoonotic pathogens [10].

2.2.4.2. Brucellosis in humans

2.2.4.2.1. Way of transmission

Humans became infected by coming in contact with animals or animal products that are contaminated with these bacteria. In humans brucellosis can cause a range of symptoms that are similar to the flu and may include fever, sweats, headaches, back pains, and physical weakness. Severe infections of central nervous systems or lining of the heart may occur. Brucellosis can also cause long-lasting of chronic symptoms that include recurrent fevers, joint pain, and fatigue [4].

Only B. melitensis, B. suis, B. abortus, and B. canis cause disease in man. Infection of humans with B. ovis, B. neotomae and the two marine strains has not been described [3].

Illness occurs, on the one hand, in individuals, such as veterinarians, shepherds, farm workers, slaughterhouse workers and laboratory assistants having occupational exposure to infected animals or infectious materials. Through direct contact with infected animal parts - such as the placenta the birth fluids and the chorion, by inoculation through ruptures of skin and mucous membranes - and through the inhalation of infected aerosolized particles [3].

Most cases now occur in worker in meat – packing plants (90% of cases related to slaughtering of hogs), in livestock raisers [11].

In certain mammals than man brucellae also accumulate in mammary glands (causing infection of milk), in the genital organs, and in the pregnant uterus (often resulting in abortion) [1].

On the other hand, humans acquire the infection by ingestion of unpasteurized dairy products, for example - especially raw milk, soft cheese, butter, and ice cream – is the most

common means of transmission. Hard cheese, yogurt, and sour milk are less hazardous, since both propionic and lactic fermentation takes place. Bacterial load in animal muscle tissues is low, but consumption of undercooked traditional delicacies such as liver and spleen has been implicated in human infection [3].

Examples of human-to-human direct transmission by tissue transplantation or sexual contact are occasionally reported, but extremely rare. Mothers who are breast-feeding may transmit the infection to their infants. Sexual transmission has also been reported. For both sexual and breast-feeding transmission, if the infant or person at risk is treated for brucellosis, their risk of becoming infected will probably be eliminated within 3 days. Although uncommon, transmission may also occur via contaminated tissue transplantation. [4]

2.2.4.2.2. Bioterrorism

Airborne transmission of brucellosis has been studied in the context of using Brucella as a biologic weapon. In fact, B. suis was the first agent contemplated by the U.S. Army as a potential biologic weapon and is still considered in that category. It is known, that in 1955, were Brucella suis filled luster bombs tested. In a hypothetical attack scenario, it was estimated that release of an aerosolized form of brucella under optimal circumstances for dispersion would cause 82,500 cases of brucellosis and 413 fatalities. Cases of laboratory-acquired brucellosis are the perfect examples of airborne spreading of the disease [11].

The use of biological agents such as bacteria and viruses in a terrorist operation (germ warfare) is unfortunately possible. Luckily all US stocks of Brucellae were destroyed in 1969. Later some "lost" munitions were found in the mid- 1990s and were destroyed too. In the principal agents of BW will be bacteria of fungi rather than viruses, since bacteria can readily cultured in large fermentation vats [11]

An effective biological weapon is that the organism be highly infections by the respiratory route, thus permitting effective airborne dispersal, so that each viable unit will be able to initiate an infection. The bacterium Brucellae has all of these abilities [11].

Plague was responsible for decimating the population of Europe at several times in the middle ages. The new plagues are not influenced by the modern variables of political, economic, and military ambition [11].

The most commonly mentioned candidate is Bacillus anthraces, than Smallpox virus .Other bioweapons candidate include Brucella abortus, Yersinia pestis, Francisella tularensis, Salmonella. The problems associated with irresponsible use of our knowledge of microorganisms [11].

Biological diseases and the agents that might be used for terrorism have been listed by the US Centers for Disease Control and Prevention (the CDC). The list includes a sizable number of "select agents" – potential weapons whose transfer in the scientific and medical communities is regulated to keep them out of unfriendly hands. The CDC has classified all of these "select agents" according to the degree of danger. Each agent is felt to pose into one of three categories, within Brucellosis (undulant fever) which fall in category 3.This category 3 means, that organism in it are moderately easy to disseminate and have low mortality rates [11].

2.2.4.2.3. Categorization

The classic categorization of brucellosis as acute subacute, or chronic is subjective and out of limited clinical interest. Four species of brucella can cause human disease: B. melitensis, B abortus, B. suis, and B. canis. Disease from marine species has also emerged. The vast majority of cases worldwide are attributed to B. melitensis. A recent study did not report any clinical differences between cases caused by B. melitensis and those caused by B. abortus. Sufficient data on virulence and clinical presentation of biotypes of B. melitensis are lacking, although separate biotypes that predomination various regions – for example type 2 in northwester Greece, type 3 in Turkey, and type 1 in Spain – may account for variations in clinical presentation [1].

2.2.4.2.4. Symptoms

The incubation period is usually from some days to several weeks. Disease may be abrupt or insidious at the onset and patients usually complain of non-specific symptoms such as fever, sweat, fatigue, shivering fit, anorexia, headache, muscle or joint aches, and sometimes diarrhea. Human brucellosis is traditionally described as a disease of protean manifestations [1].

Table 4. Clinical Presentation of Human Brucellosis.*[1]

Features	Percentage of Cases
Signs and symptoms	
Fever	91
Constitutive symptoms (e.g., malaise, arthralgias)	26
Hepatomegaly	17
Splenomegaly	16
Lymphadenopathy	7
Complications	
Peripheral arthritis	22 (8 in hips, 7 in knees, 4 in elbows, 4 in wrists, 4 in other locations)†
Sacroiliitis	3
Spondylitis	19 (15 lumbar, 3 dorsal, 1 cervical)
Central nervous system disorders	3
Epididymoorchitis	5.7‡
Vomiting and diarrhea	3
Respiratory disorders	6
Rashes	3
Cardiovascular disorders	0
Laboratory findings	
Hematologic	49 (40 relative lymphocytosis, 5 isolated thrombocytopenia, 2 isolated leukopenia, 2 pancytopenia)
Transaminasemia	24
Positive blood cultures	16
Rate of relapse	4

* Data are from the most recent 100 patients who received the diagnosis of brucellosis at the University Hospital of Ioannina and whose cases were followed for at least a year.
† Some of the patients had polyarthritis.
‡ Data are for 70 male patients.

An undulant fever ("Undulant Fever") is observed when patients remain untreated for long periods. Neuropsychiatry symptoms, notably depression, headache, and irritability, occur frequently. In addition, focal infection of bone, joints, or genitourinary tract can cause local pain. Cough, pleurisy chest pain, and dyspepsia may also be noted. Chronically infected patients frequently lose weight. Physical examination is usually normal, although hepatomegaly, splenomegaly, or lymphadenopathy may occur. If inadequately treated, the disease may end fatally in rare cases [3].

The series of host-microbe interactions that takes place in humans differs in many crucial steps from the pathogenetic mechanisms first recognized in animal models. Brucella is unusual in several ways. First, the bacterium does not bear classic virulence factors, such as exotoxins or endotoxins, and its lipopolysaccharide pathogenicity is not typical. Second, it exhibits a tendency to invade and persist in the human host through inhibition of programmed cell death [1].

2.2.4.2.5. Pathogenesis

In naturally acquired brucellosis, the organisms gain entrance via the broken skin, the conjunctiva, the alimentary track, or possibly the aerosol route [9].

At the site of ingested by polymorphonuclear cells, multiply within them, and are carried (mostly in this cells) via lymphatics to the regional lymph nodes. There the bacteria enter and multiply within mononuclear cells, some of these cells die, and the released bacteria cell contents stimulate local mononuclear cell activation and proliferation [9].

The outcome of this confrontation determinates whether the invasive infection is contained. If not, PMN's and mononuclear cells carrying the bacteria reach the blood and soon accumulate in sinusoid of the liver. These focal aggregations of Kupffer cells containing large numbers or organisms develop and after another few days form typical small granulomas. Similar lesions appear in spleen, bone marrow and kidney [9].

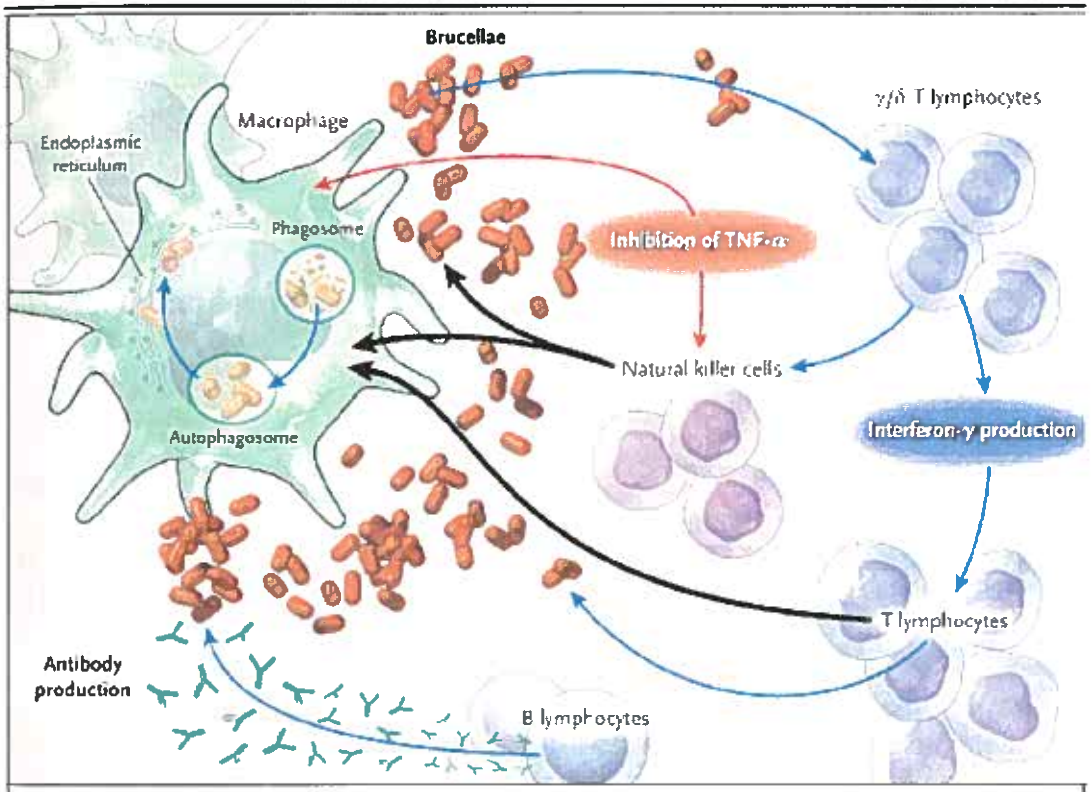


Figure 4 Schematic Representation of Major Events in the Pathogenesis of Brucellosis and the Host Immune Response. Brucellae enter the macrophages, where the minority of the bacteria survive in specialized evolving compartments and multiply in the endoplasmic reticulum. The inhibition of tumor necrosis factor α (TNF-α) by the bacteria disrupts the bactericidal effect of natural killer cells and macrophages. Interferon-γ production induces a bactericidal effect by natural killer cells and T lymphocytes directly and through macrophage induction. Antibody production by B lymphocytes is also induced but plays a minor role in the immune response. T lymphocytes include both helper and suppressor cells, depending on the stage of the disease. Red arrows indicate negative effect, blue arrows positive effect, and black arrows killing effect. [1]

Brucella invades the mucosa, after which phagocytes, internalization requires the expenditure of energy, and inhibitors of energy metabolism and receptor-mediated endocytosis can suppress this response. Brucella has a two-component system called BvrS/BvrR, which codes for histidine kinase sensor and controls the expression of molecular determinants necessary for cell invasion. After ingestion, the majority of brucellae are rapidly eliminated by phagolysosome fusion. Of those bacteria, 15 to 30 percent survive in gradually evolving brucellae – containing compartments, in which rapid acidification takes place. How this

unique environment is formed is incompletely understood, but it is responsible for limiting antibiotic action and explains the discrepancy between in vitro studies and in vivo events [1].
After entering the human's body and being taken up by local tissue lymphocytes, brucellae are transferred through regional lymph nodes into the circulation and are subsequently seeded throughout the body, with tropism for the reticuloendothelial system. The period of inoculation usually ranges from two to four weeks [1].

2.2.4.2.6. Epidemiology and prevention

Through the use of public health measures (testing of cows followed by segregation or slaughter, vaccination of calves with life attenuated vaccine, pasteurization of milk products, inspection of domestic animals for evidence of the disease) in the past – 45 years reduce incidence from 6300 to 130 cases annually. One important epidemiological step in containing brucellosis in the community is the screening of household members of infected persons [9].
The use of goggles and rubber gloves helps protect veterinarians butcher, and slaughter house worker [8].

2.3. Material and methods

2.3.1. PCR reaction

PCR is molecular cloning method. It is a technique for rapid amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase. It can amplify a specific sequence of DNA by as many as one billion times [12].

PCR commonly used in medical and biological research laboratories a variety of tasks, such as the detection of hereditary diseases, the identification of genetic, fingerprints, the diagnosis of infectious disease, bacteria and viruses, study human evolution, clone the DNA of an Egyptian mummy, the cloning of genes and paternity testing etc...Accordingly, PCR has become an essential tool for biologists, DNA forensics labs, and many other laboratories that study genetic material [12].

PCR is a molecular biological technique for amplifying DNA, creating multiple copies of DNA without using a living organism, such as E. coli or yeast [12].
It is the purpose of doing a PCR is the original DNA that one wished to copy need not be pure or abundant. It can be pure but it also can be a minute part of mixture of materials. Thus, each PCR "cycle" involves the following: (1) Heat denaturation of double – stranded target DNA

(2) Cooling to allow annealing of specific primers to target DNA
(3) Primer extension by the action of DNA polymerase.

The beauty of the PCR technique lies in the fact that each cycle literally doubles the content of the original target DNA. In practice, leading to an exponential increase, 20 – 30 cycles are usually run, yielding a 10 to 10^9 fold increase in the target sequence [13].

2.3.2. History

PCR is an ingenious new tool for molecular biology that has had an effect on research similar to that of the discovery of restriction enzymes and the Southern blot. Polymerase chain reaction was introduced to the scientific community at a conference in October 1985 [14].

The concept of PCR was first put forward by H.Ghobind Khrana et al. in 1971, but at the time seemed impractical in a time before gene sequencing or a viable thermo stable DNA polymerase (Sambrook and Russell).It was 15 years later that it was independently conceived by Kary Mullis, given its present name, and put into practice [12]. He was struggling to devise a simple method for determining the identity of specific nucleotides along a stretch of DNA [14].

It seemed that just as he solved one technical problem, another one took its place. Kary Mullis had just conceived of a simple method for producing virtually unlimited copies of a specific DNA sequence in a test tube – the polymerase chain reaction (PCR) [12]. Mullis was awarded the Nobel Prize in Chemistry in October 1993 for this achievement, only seven years after he first published his ideas. The idea was to develop a process by witch DNA could be artificially multiplied though repeated cycles of duplication driven by an enzyme called DNA polymerase [12].

The original PCR technique employed Escherichia coli DNA polymerase, but because of the high temperatures needed to denaturate the double – stranded copies of DNA being made, the polymerase itself also was denaturated and had to be replenished every cycle [7].

This tended to limit the number of cycles that could be run and was very expensive. This problem was solved by employing a thermostable DNA polymerase isolated from the thermophilic bacterium Thermus aquaticus [7].

Patent wars – the PCR technique was patented by Cetus Corporation, where Mullis worked when he invented the technique. The Taq polymerase enzyme is also covered by patents. There have been several high-profile lawsuits related to the technique, including most famously a lawsuit brought by DuPont. The pharmaceutical company Hoffmann-La Roche purchased the rights to the patents in 1992 and currently holds them (K.B.Mullis, U.S:. patent 4, 683, 195, July 1987, U.S. patent 4, 683, 202, July 1987, Saiki et al 1985, Mullis et al 1896, Mullis and Faloona 1987) [12].

2.3.3. Mechanism of PCR similar to in vivo replication

The polymerase chain reaction is a test tube system for DNA replication that allows a "target" DNA sequence to be selectively amplified, or enriched, several million-fold in just a few hours. Within a dividing cell, DNA replication involves a series of enzyme – mediated reactions, whose end result is a faithful copy of the entire genome. Within a test tube, PCR uses just one indispensable enzyme – DNA polymerase – to amplify a specific fraction of the genome [14].

"Target" DNA is almost any source that contains one or more intact template DNA molecule, in theory, be amplify by PCR, providing appropriate primers can be designed [13].

During cellular DNA replication, enzymes first unwind and denature the DNA double helix strands into single strands. Then, RNA polymerase synthesizes a short stretch of RNA complementary to one of the DNA strands at the start site of replication. This DNA/RNA heteroduplex acts as a "priming site" for the attachment of the DNA polymerase, which then are produced the complementary DNA strand. During PCR, high temperature is used to

separate the DNA molecules into single strands, and synthetic sequences of single – stranded DNA (20 – 30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified [14].

The PCR technique requires that the nucleotide sequence of a portion of the desired gene be known. This is necessary because short oligonucleotide – primers – complementary to sequences in the gene or genes of interest must be available for PCR to work [13].

One primer is complementary to one DNA strand at the beginning of the target region; a second primer is complementary to a sequence on the opposite DNA strand at the end of target region [14].

The PCR reaction is carried out in thermal cycler to get similar temperature condition as in vivo. Thermocycler is a machine that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. To prevent evaporation of the reaction mixture, a heated lid is placed on top of the reaction tubes or a layer of oil is put on the surface of the reaction mixture [12].

Some potentially useful observations were made:

- The same PCR program will work slightly different on different thermocycler (temperature and time profiles may be different, depending on the construction), therefore the PCR results using the same primer pair may vary. However, with proper cycling adjustments, the same results can be obtained on most (or any) thermocycler [15].
- Many new PCR machines accommodate the thin-walled 0.2 ml PCR vials (and/or 96 wells microtiter dishes) in their metal block. For this type of vials, the differences in results from vial to vial are usually negligible (contact between the metal and plastic is very good and aided by the downward pressure from the heated lid). Older machine type could accommodate 0.5 or 1.5 ml vials. Because of slight differences in shape and wall thickness among manufacturers, contact between the vials and the metal block of the thermocycler was not always perfect, often resulting in reduced or no amplification. Some manufacturers offer machines controlling the temperature of a small waterbath in which the PCR vials rest during the reaction. In such cases, due to the good thermal change between water and plastic, variations in PCR results are very little (if any) [15].

2.3.3.1. Denaturation

Increased temperature can bring about the denaturation of DNA and RNA. Double – stranded DNA "melts" cooperatively to give single strands at a defined temperature, T_m, which is a function of the G + C content of the DNA. Denaturation may be detected by the change in A_{260} [16].

2.3.3.2. Renaturation

DNA renature on cooling, but will only form fully double-stranded native DNA if the cooling is sufficiently slow to allow the complementary strands to anneal [16].

The process of denaturation can be observed conveniently by the increase in also absorbance as double stranded DNA are converted to single strands [16].

Slow cooling allows time for the wholly complementary DNA strands to find each other, and the sample can became fully double-stranded, with the same absorbance at the original native sample [16].

2.3.4. DNA hybridization

The chemistry of PCR, as with much of molecular biology, depends on the complementarity of the DNA bases. When a molecule of DNA is sufficiently heated, the hydrogen bonds holding together the double helix are disrupted and the molecule unzips or "denatures" into single strands. If the DNA solution is allowed to cool, then complementary base pairs can reform (renature) and original double helix is restored [14].

Experiments performed during the 1960s demonstrated that many DNA sequences were not unique within the genome. Purified DNA solution were denatured by heat and then allowed cool. Using a spectrophotometer it was possible to monitor the rate at which the DNA renatured. Data from these studies revealed that genomes are composed of different classes of DNA sequences that can be distinguished by their repetitive frequency [14].

While useful for studying the broad outline of genome organization, this approach could not be used to investigate the structure of individual genes. This ability came about during the 1970s following the introduction of DNA restriction analysis and nucleic acid hybridization techniques. Hybridization allows a specific DNA sequence to be analyzed against the complex background of a genome. To focus on an individual gene, DNA from the target organism is isolated, fragmented with restriction enzymes, and separated by gel electrophoresis. The DNA fragments are denatured to render them single stranded and exposed to a solution containing a radioactive DNA "probe". The probe consists of single – stranded nucleic acid with a sequence chosen to base pair with the gene of interest [14].

Under appropriate conditions of temperature, salt, and pH, called "stringency", the probe will bind to its corresponding sequence in the target DNA and nowhere else. The presence of a radioactive signal (often by exposure to X-ray film) indicates positions of probe binding [14].

2.3.5. Components of reaction

2.3.5.1. Enzyme

DNA polymerase occurs naturally in living organisms, where it functions to duplicate DNA when cells divide. It works by binding to a single DNA strand and creating the complementary strand. In Mullis's original PCR process, the enzyme was used *in vitro* (in a controlled environment outside an organism from *Escherichia coli*) [12].

The original PCR technique employed *Escherichia coli* DNA polymerase, but because of the high temperatures needed to denature the double-stranded copies of DNA being made, the polymerase itself also was denatured and had to be replenished every cycle [6]. . Mullis's original PCR process was very inefficient since it required a great deal of time, vast amounts of DNA-Polymerase, and continual attention throughout the PCR process. This tended to limit the number of cycles that could be run and was very expensive. [12].

The problem was solved by employing a thermostable DNA polymerase isolated from thermophilic (heat-loving) bacterium *Thermus aquaticus*, which inhabits hot springs geysers at a temperature of over 110°C. This enzyme, called the *Taq polymerase*, remains active despite repeated heating during many cycles of amplification, did not break down when the mixture was heated to separate the DNA strands. Since there was no longer a need to add new DNA-Polymerase for each cycle, the process of copying a given DNA strand could be simplified and automated. This were big step, how to solve problems with automatization

[12]. *Taq polymerase* survives the denaturation step of 95°C for 1- 2 min, having a half – life of more than 2h at this temperature [14].

The use of Taq DNA polymerase also increases the specificity of the PCR reaction because the DNA is copied at 72°C rather than 37°C. At high temperatures, nonspecific hybridization of primers to nontarget DNA rarely occurs, thus making the product of Taq PCR more homogenous than that obtained using the E.coli enzyme [7].

A disadvantage of Taq is that it sometimes makes mistakes when copying DNA. Problem is that the Taq polymerase has no associated 3' to 5' proofreading function (exonuclease activity) and consequently makes more mistake than the *E.coli* enzyme [7].

DNA polymerase from the hyperthermophilic Achaean *Pyrococcus furiousus* (growth temperature optimum, 100°C), called *Pfu polymerase* (or "vent polymerase") is also widely used and even more thermally stable than Taq polymerase. Pfu polymerase has proofreading activity, making it a particularly good enzyme when accuracy is crucial [7].

Polymerases such as *Pfu* or *Pwo,* obtained from *Achaean,* have *proofreading mechanisms* (mechanisms that check for errors) and can significantly reduce the number of mutations that occur in the copied DNA sequence. Combinations of both *Taq* and *Pfu* are available nowadays that provide both high fidelity and accurate amplification of DNA [7].

Because a number of highly repetitive steps are involved in the PCR technique, machines have been developed that can programmed to run through heat time and (cooling cycles automatically [7].

Because each cycle requires only 5 min, the automated procedures allows for large amplifications in only a few hours (in contrast, such amplifications by in vivo cloning methods would taken several days) [7].

To supply the demand for thermostable DNA sequencing markets, the genes for these enzymes have been cloned into E. coli and produced in large quantities. The cost of the PCR method is now just a fraction of what is was when the technique was first introduced [7].

2.3.5.2. Primers

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands-not more than fifty (usually 18-25 bp) oligonucleotide with similar G + C content that exactly match the beginning and end of the DNA fragment to be amplified. They *anneal* (adhere) to the DNA template at these starting and ending points, where the DNA-Polymerase binds and begins the synthesis of the new DNA strand. Primers with some degeneracy can also be used if the target DNA sequence is not completely known [12].

It is important to keep in mind that there are two primers added to a PCR reaction. Both of the oligonucleotide primers should be designed such that they have similar melting temperatures. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer with the higher T_m will miss-prime at lower temperatures and the primer with the lower T_m may not work at higher temperatures [16].

Length of primers

Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR. In general, oligonucleotides between 18 and 24 bases are extremely sequence specific, provided that the annealing temperature is optimal. Primer length is also proportional to annealing efficiency. The primers

should not be too short, however, unless the application specifically calls for it. As discussed below, the goal should be to design a primer with an annealing temperature of at least 50°C [16].

The relationship between annealing temperature and melting temperature is one of the "Black Boxes" of PCR. A general rule-of-thumb is to use an annealing temperature that is 5°C lower than the melting temperature. Thus, when aiming for an annealing temperature of at least 50°C, this corresponds to a primer with a calculated melting temperature(T_m) ~55°C. Often, the annealing temperature determined in this fashion will not be optimal and empirical experiments will have to be performed to determine the optimal temperature. This is most easily accomplished using a gradient thermal cycler [16].

Melting temperature (Tm)

The choice of the length of the primers and their melting temperature (Tm) depends on a number of considerations. The melting temperature of a primer--not to be confused with the melting temperature of the DNA in the first step of the PCR process--is defined as the temperature at which half of the primer binding sites are occupied. The melting temperature increases with the length of the primer. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies [12].

Some simple rules aid in the design of efficient primers. Typical primers are 18 to 28 nucleotides in length having 50 to 60 % G + C composition. The calculated T_ms for a given primer pair should be balanced. For this purpose, one can use the rule-of-thumb calculation of 2°C for A or T and 4°C for C or C [15].

Fortunately, a good working approximation of this value (generally valid for oligos in the 18–24 base range) can be calculated using the formula: $T_m = 2(A+T) + 4(G+C)$.The table below shows calculated values for primers of various lengths using this equation, which is known as the Wallace formula, and assuming a 50% GC content [16].

Table 5 Melting temperature according to the length[12]

Primer Length	**T_m = 2 (A+T) + 4(G+C)**	**Primer Length**	**T_m = 2 (A+T) + 4(G+C)**
4	12°C	22	66°C
6	18°C	24	72°C
8	24°C	26	78°C
10	30°C	28	84°C
12	36°C	30	90°C
14	42°C	32	96°C
16	48°C	34	102°C
18	54°C	36	108°C
20	60°C	38	114°C

Primer concentrations between 0.1 and 0.5 μM are generally optimal. Higher primer concentrations may promote misprinting and accumulation of nonspecific product, and may increase the probability of generating a template-independent artifact termed a primer-dimer. Nonspecific products and primer-dimer artifacts are themselves substrates for RCR and

compete with the desired product for enzyme, dnTps, and primers, resulting in a lower desired product [13].

Depending on the application, T_ms between 55°C and 80°C are desired. One should avoid complementarity at the 3'end pairs as this promotes the formation of primer-dimer artifacts and reduces the yield of the desired product. Also, runs (three or more) of C's or G's at the 3' ends of primers may promote mispriming at G + C- rich sequences and should be avoided, when possible, as should palindromic sequences within primers. If all else fails, it usually helps to try a different primer pair. A less obvious reason for some primers filing to work is the presence of secondary structure in the template DNA. In this case, substitution of 7– deaza – 2'- deoxyGTP for dGTP has been very useful [13].

Above mentioned considerations makes primer design very accurate process, on which depends product yield:

- GC-content should be between 40-60%.
- Calculated Tm for both primers used in reaction should not differ >5°C and Tm of the amplification product should not differ from primers by >10°C.
- Annealing temperature usually is -5°C the calculated lower T_m. However it should be chosen empirically for individual conditions.
- Inner self-complementary hairpins of >4 and of dimers >8 should be avoided.
- 3' terminus is extremely case sensitive - it must not be complementary to any region of the other primer used in the reaction and must provide correct base matching to template.
- The reaction is so specific that, with primers of 15 nucleotides and high annealing temperatures, there will be extremely low levels of " false priming " and therefore the product formed should be relatively homogeneous [12].

Primer Annealing

The temperature and length of time required for primer annealing depend upon the base composition, length, and concentration of the amplification primers. An applicable annealing temperature is 5°C below the true T_m of the amplification primers. Because Taq DNA polymerase is active over a broad range of temperatures primer extension will occur at low temperatures, including the annealing step. The range of enzyme activity varies by two orders of magnitude between 20 and 85°C. Annealing temperature in the range of 55 to 72°C generally yield the best results. A typical primer concentrations [0, 2 μM], annealing will require only few seconds [13].

Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at the 3end of primers. Therefore, stringent annealing temperatures, especially during the first several cycles will help to increase specificity. For maximum specificity in the initial cycle, Taq DNA polymerase can be added after the first denaturation step during primer annealing. Low extension temperature together with high dnTp concentrations favors misextens of primers and extension of misincorporated nucleotides [13].

Primer extension

Extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extension are traditionally performed at 72°C because this temperature

was near optimal for extending primers on an M13-based model template. Estimates for the rate of nucleotide incorporation at 72°C vary from 35 to 100 nucleotides second^{-1} depending upon the buffer, pH, salt concentration, and the nature of the DNA template. An extension time of one minute at 72°C is considered sufficient for products up to 2 kb in length. However, longer extension times may be helpful in early cycles if the substrate concentration is very low, and at late cycles when product concentration exceeds enzyme concentration (approximately 1nM) [13].

Denaturation Time and Temperature

The most likely cause for failure of a PCR is incomplete denaturation of the target template and /or the PCR product. Typical denaturation conditions are 95°C for 30 seconds to denature DNA at its strand-separation temperature (T_{ss}), however, there may be lag time involved in reaching T_{ss} inside the reaction tube. It is a good idea to monitor the temperature inside one reaction tube with a low mass thermocouple probe. Incomplete denaturation allows the DNA strands to "snap back" and, thus, reduces product yield. In contrast, denaturation steps that are too high and /or too long lead to unnecessary loss of enzyme activity. The half - life of Taq DNA polymerase activity is less than 2 hours, 40 minutes, and 5 minutes at 92, 5°C, respectively 97, 5 [13].

2.3.5.3. Magnesium chloride

It is beneficial to optimize the magnesium ion concentration. The magnesium concentration may affect all of the following: primer annealing, strand dissociation temperatures of template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity. Taq DNA polymerase requires free magnesium on top of that bound by template DNA, primers, and dnTps. Accordingly, PCRs should contain 0.5 to 2.5 mM magnesium over the total dnTp concentration. Note that the presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent magnesium optimum [13].

2.3.5.4. Buffer

A recommended buffer for PCR is 10 to 50 mM Tris – HCl (between pH 8, 3 and 8, 8) when measured at 20°C, however, an extensive survey of other buffers has not been performed. Tris is a dipolar ionic buffer having a pK_a of 8, 3 at 20°C a ▲ pK_a of – 0.021/°C. Thus, the true pH of 20 mM Tris (pH 8,3) at 20°C varies between 7,8 and 6,8 during typical thermal cycling conditions [13].

Up to 50 mM KCl can be included in the reaction mixture to facilitate primer annealing. NaCl at 50mM, or KCl above 50 mM, inhibits Taq DNA polymerase activity. While DMSO is useful in PCRs performed with the Klenow fragment of *E. coli* DNA polymerase I, 10% DMSO inhibits the activity of Taq DNA polymerase by 50% and its use is not recommended for most applications [13].

Gelatin or bovine serum albumin (100μg/ml) and nonionic detergents are included to help stabilize the enzyme, although many protocols work well without added protein [13].

2.3.5.5. DnTp

Stock deoxynucleotide Triphosphates solutions should be neutralized to pH 7, 0 and their concentration should be determined spectrophotometrically. Primary stocks are diluted to 10 mM, aliquoted, and stored at – 20 °C [13].

A working stock containing 1mM each dnTp is recommended. The stability of the dnTp during repeated cycles of PCR is such that approximately 50% remains as dnTp after D0 cycles. Deoxynucleotide concentration between 20 and 200 nM each result in the optimal balance among yield, specificity, and fidelity. The four dnTp should be used at equivalent concentration to minimize misicoporation error [13].

Low dnTp concentrations minimize mispriming at nontarget sites and reduce the likelihood of extending misincorporated nucleotides. One should decide on the lowest dnTp concentration appropriate for the length and composition of the target sequence [13].

2.3.6. Process of reaction

How is PCR done?

The PCR process consists of a series of twenty to thirty cycles. Each cycle consists of three steps. As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty (forty) such cycles, a theoretical amplification factor of one billion is attained. The cycles are done on an automated cycler, which rapidly heats and cools the test tubes containing the reaction mixture. Each step -- denaturizing (alteration of structure), annealing (joining), and extension -- takes place at a different temperature [12].

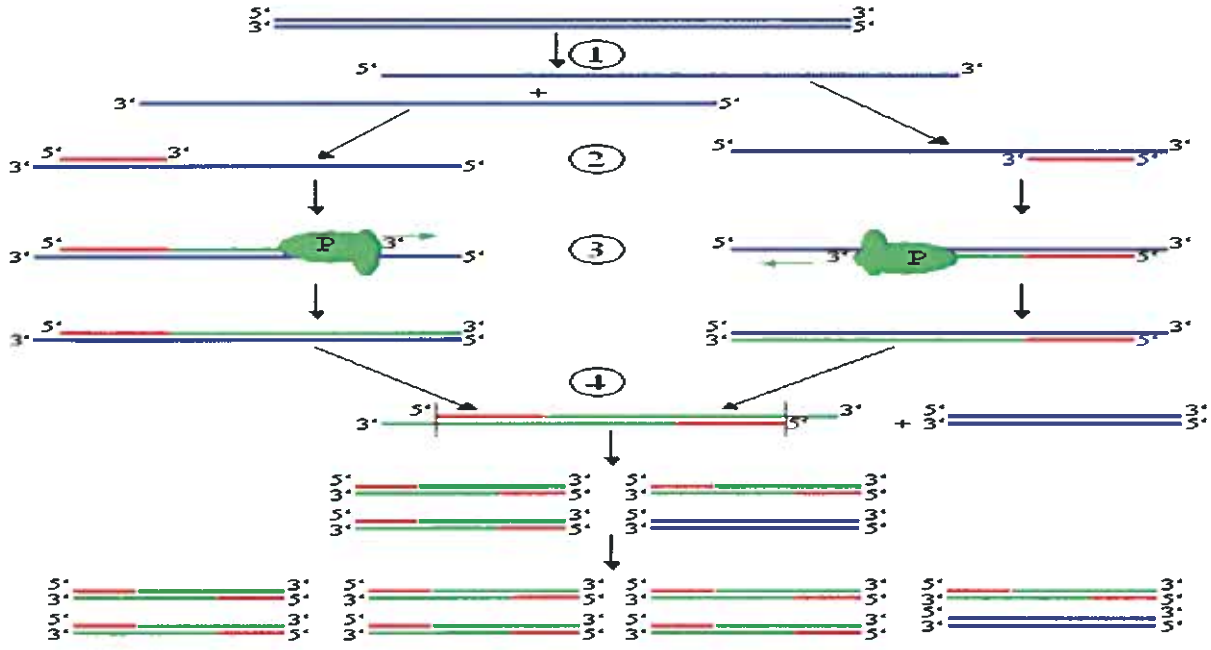


Fig. 5: Schematic describing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at (eg) 68°C. (3) Extension at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle. [17].

2.3.6.1. Denaturation

The double-stranded DNA has to be heated to 94-96°C in order to separate the strands. This step is called *denaturing;* it breaks apart the hydrogen bonds that connect the two DNA strands, during which the double-stranded DNA melts and opens into single-stranded DNA [14]. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that

both the template DNA and the primers have completely separated and is now single-strand only. Time: 1-2 minutes [12].

2.3.6.2. Annealing

After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called *annealing*. The temperature of this stage depends on the primers and is usually 5°C below their melting temperature (45-60°C). During this range the primers hybridize or "anneal "(by way of hydrogen bonds) to their complementary sequences on either side of the target sequence. Hydrogen bonds form and break between the single-stranded "primer" and the single-stranded "template." (The template provides the pattern to be copied.) The more stable bonds last longer and on that little length of double-stranded DNA (the joined primer and template), the polymerase attaches and starts copying the template [14]. A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random. Time: 1-2 minutes [12].

2.3.6.3. Extension

Finally, the DNA-Polymerase has to fill in the missing strands. It starts at the annealed primer and works its way along the DNA strand. This step is called *elongation.* The elongation temperature depends on the DNA-Polymerase. It is usually at 72°C, during which the polymerase binds and extends a complementary DNA strand from each primer. The polymerase works best. As a result, the attraction, created by the hydrogen bonds, of the primers to the template is stronger than the forces breaking these attractions. The upshot is that bases complementary to the template are coupled to the primer [14].
The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified. (as a rule-of-thumb, 1 minute per 1000bp) [12].

2.3.7. PCR optimalization

Since PCR is very sensitive, adequate measures to avoid contamination from other DNA present in lab environment (bacteria, viruses, own DNA etc.) should be taken. Thus DNA sample preparation, reaction mixture assemblage and the PCR process, in addition to the subsequent reaction product analysis, should be performed in separate areas. For the preparation of reaction mixture, a laminar flow cabinet with UV lamp is recommended. Fresh gloves should be used for each PCR step as well as displacement pipettes with aerosol filters. The reagents for PCR should be prepared separately and used solely for this purpose. Aliquots should be stored separately from other DNA samples. A control reaction (inner control), omitting template DNA, should always be performed, to confirm the absence of contamination [15].

2.3.7.1. "Plateau" effect

The term "plateau effect" is used to describe the attenuation in the exponential rate of product accumulation that occurs during late PCR cycles concomitantly with the accumulation of 0.3 to 1 pmol of the intended product. Depending on reaction conditions and thermal cycling, one or more of the following may influence plateau: (1) utilization of substrates (dnTps or primers, (2) stability of reactants (dnTps or enzyme, (3) end –product inhibition (pyrophosphate, duplex DNA), (4) competition for reactants by nonspecific

products or primer – dimer, (5) reannealing of specific product at concentrations above 10-8 M (may decrease the extension rate or processivity of Taq displacement of primers), and (6) incomplete denaturation/strand separation of product at high product concentration [13].

An important consequence of reaching plateau is that an initially low concentration of nonspecific products resulting from mispriming events may continue to amplify preferentially. Optimizing the number of PCR cycles is the best way to avoid amplifying background products [13].

2.3.8. Multiplex PCR

Multiplex PCR is a variant of PCR which enabling simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers [14].

Since its first description in 1988 by Chamberlain at al, this method has been applied in many areas of DNA testing, including analyses of deletions alike in this diploma [14].

Advantages

The multiple sets of primers are often used as a quick test to detect the presence of microorganisms that may be contaminating food or water, or be infecting tissue [18].

The diagnosis of focal forms of brucellosis is sometimes difficult, at the yield conventional cultures. Brucella spp. are slowly growing pathogens, the cultures requires prolonged incubation, which can at time lead to excessive delays in diagnosis [18].

Next to high degree of sensitivity and specificity of the PCR assay, the technique almost completely obviates the necessity for direct handling of the pathogen, thus drastically reducing the risk of infection of laboratory personal [18].

Disadvantages

Multiplex assays can be tedious and time-consuming to establish, requiring lengthy optimalization procedures [18].

2.3.9. Component of reaction

Like another PCR, multiplex reactions should be done at a stringent enough temperature, allowing amplification of all loci of interest without "background" products. Perhaps the most critical parameter for successful PCR is the design of Primers [19].

2.3.9.1. Primer

First step in design a multiplex PCR is choosing the primer pairs which can be combined. One important requirement is to find a PCR program allowing optimal amplification of all loci when taken individually. This is achieved by adjusting the annealing and extension time and temperature [15]. The primer sequence determines several things such as length of the product, its melting temperature and ultimately the yield [16].

Combining is the desired primer pairs in multiplex mixture(s), using equimolar amounts of each primer. PCR amplification of the multiplex mixtures can be performed, first using exactly the same PCR program as with individual primer pairs. Result in preferential amplification of some loci. Unspecific products can be seen in single-locus PCR; these unspecific products usually become invisible when the multiplex reaction is performed. Due

to the concurrent amplification of many specific loci, this overwhelms the unspecific products. This is probably the reason why small increases in the dnTp concentrations can rapidly inhibit the PCR reaction (Mg gets "trapped") whereas increases in magnesium concentration often have positive effects [15].

Basic Principles is to amplify a specific locus without any unspecific by-products. Annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA-DNA matches to occur in the reaction. For any given primer pair, the PCR program can be selected based on the composition (GC content) of the primers and the length of the expected PCR product. In the majority of the cases, products expected to be amplified are relatively small [15].

Each primer pair should be tested for primer-primer interactions. Primer sequences should be aligned with all DNA sequences entered in the databases (using BLAST programs) and checked for similarities with repetitive sequences or with other loci, where in the genome. If two loci are very similar (for example across species) it is useful to design the primers so that at least 1-2 bases at the 3' end are specific for the locus to be amplified [15].

The amount of DNA primer(s) available during the PCR reaction influences the results. Primer concentration taken in a common PCR reaction (for example when amplifying a single locus is about 100-500 nM each primer). Although equimolar primer mixtures did not usually provide optimal amplification of all loci too high and too low primer amounts may need to be avoided. Too high primer concentrations may inhibit the multiplex reaction whereas too low amounts may not be sufficient [15].

Design new pair of primers

Genus Brucella is α -2- subdivision of the Proteobacteria. It is composed of 2 circular chromosomes. On chromosome I are essential genes, on Ch. II, smaller one is plasmid like. For significant pair of primers is find some sequence, which delete in other Brucella strains, gen specific only for the individual biovarities to easy distinguish on gel their different amplifying sequence of nucleotides after PCR [1].

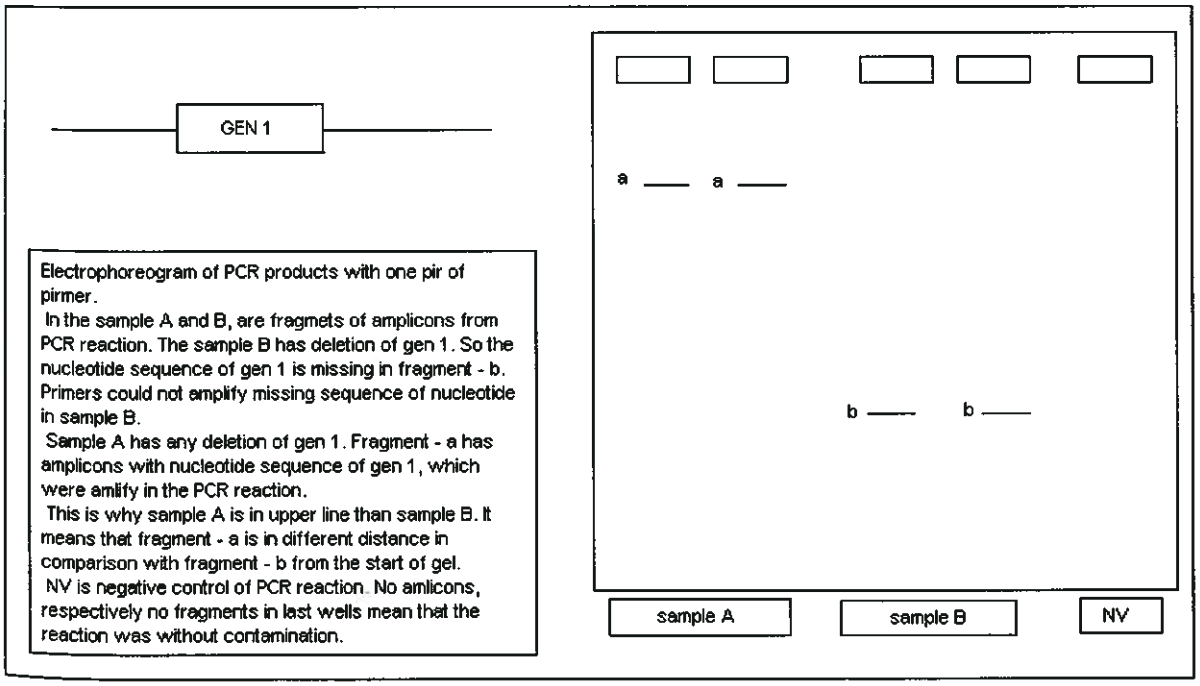


Fig. 6: Electrophoreogram of PCR products

2.3.9.2. $MgCl_2$ and dnTp

Common dnTp use in PCR and multiplex PCR the best results were achieved at 200 and 400 μm dnTp; reaction was rapidly inhibited after these values. Lower than usual dnTp concentrations still allowed PCR amplification, but with somewhat less efficiency [15].

DnTp concentrations of about 200μM each are usually recommended for the Taq polymerase, at 1.5mM $MgCl_2$. In a 25 μl reaction volume, theoretically these nucleotides should allow synthesis of about 6-6.5 μg of DNA. This amount should be sufficient for multiplex reactions in which 5 to 8 or more primer pairs are used at the same time [15].

DnTp stocks are very sensitive to cycles of thawing/freezing. During long-term freezing, small amounts of water evaporate on the walls of the vial changing the concentration of the dnTp solution. Before using, it is essential to centrifuge these vials at high speed in a microfuge. This low stability of the dnTp is not so obvious when single loci are amplified. Another important observation is that, anytime nucleotides are diluted in water, the solution should be buffered (for example with 10mM Tris pH 7.7-8.0, final concentration). An acid pH will promote hydrolysis of dnTp into dnDp. [15].

Two of other important ingredients influencing the results of a PCR reaction are the buffer (especially salt) and the magnesium concentrations [15].

At 1x salt concentration and 200 mM each dnTp, reaction worked best at about 1.5 mM magnesium. At higher magnesium concentrations unspecific products appeared, but they gradually decreased in intensity towards 21.6 mM (probably because $MgCl_2$ is a salt, decreasing the stringency of the buffer - same way KCl does) [15].

At 3x salt concentration and 200 mM each dnTp, reaction worked best between 1.5 and 3.5 mM magnesium. As the stringency of the buffer was already lower than usual (due to the high KCl concentration), further increase in $MgCl_2$ the "combined" stringency of the reaction even more. Thus, fewer long unspecific products were obtained and the reaction was almost completely inhibited towards 21.6 mM magnesium [15].

At 10.8 mM $MgCl_2$ and 200 mM each dnTp, reaction worked best around 2x salt (KCl) concentration (mostly specific products amplified). However, it is obvious that overall amount of PCR product is reduced compared to the reactions taking place at 1.5 mM magnesium. In this respect, high magnesium concentrations seem to inhibit the reaction more than high KCl (3x) concentrations. Therefore, it is likely that this magnesium inhibition is more than just a reduction in stringency of the reaction mixture. The overall amplification became gradually more "specific" (unspecific bands disappeared) and the products acquired comparable intensities (at 10.8mM) [15].

However, higher concentrations of $MgCl_2$ appeared to inhibit the polymerase activity, decreasing the amount of all products. Taking into consideration the amount of PCR products, the best magnesium concentration should be between 1.8 and 3.6 mM [15].

An increase in salt concentration makes longer DNA denature slower than shorter DNA, so shorter molecules will be amplified preferentially. Some primers, however, worked well over a wide range of buffer/salt concentrations [15].

2.3.9.3. Buffer

A commonly used PCR buffer includes only KCl, Tris and $MgCl_2$ (for example, Perkin Elmer Cetus). For the successful PCR or multiplex PCR amplification of many loci (especially products between 100-1000 bp) raising the buffer concentration to 1.4x-2x (or only the KCl concentration to about 70-100mM) dramatically improves the efficiency of the

reaction. In fact the effect of the KCl concentration was more important than any of the adjuvant tested (DMSO, glycerol or BSA). Generally, many primer pairs producing longer amplification products worked better at lower salt concentrations [15].

Adjuvant in PCR reactions could be DMSO and glycerol to improve amplification efficiency (higher amount of product) and specificity (no unspecific products) of PCR, when used in concentrations varying between 5-10% (v/v). In the multiplex reaction, however, this adjuvant gave conflicting results. The usefulness of these adjutants needs to be tested in each case [15].

BSA, in concentrations of up to 0.8μg/μl (higher than previously described) appeared to increase the efficiency of the PCR reaction much more than either DMSO or glycerol. It should be noted that BSA did not have an inhibitory effect on any of the loci amplified [15].

2.3.9.4. Enzyme

The activity of the Taq polymerase is abut 2000 nucleotides/ minute at optimal temperature (72 - 78 ° C) and the extension time in the reaction can be calculated accordingly [17].

As the activity of the enzyme may not be always optimal during the reaction, an easy rule applied successfully by the author was to consider an extension time (in minutes) equal to the number of kb of the product to be amplified (1 min for a 1 kb product, 2 min for a two kb product etc.). Later on, after the product(s) become "known", extension time may be further reduced [15].

The most efficient enzyme concentration seemed to be around 0.4μl or 2 Units/25μl reaction volumes. Too much enzyme, possibly because of the high glycerol concentration in the stock solution, resulted in an unbalanced amplification of various loci and a slight increase in the background. Too little enzyme resulted in the lack of some of the amplification products [15]

2.3.10. Condition of reaction

2.3.10.1. Temperature

Annealing time increase in annealing time up o 2-3 minutes did not appreciably influence the outcome of the PCR reactions, one of the most important parameters that need adjustment in the PCR reaction. Moreover, the flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients (especially template DNA). However, if the same reaction is performed in the presence of a higher amount of DNA template, the low annealing temperature results in the appearance of many unspecific secondary products. Thus, it appears that by decreasing the amount of DNA template, the number of potentially unspecific sites is also decreased, making possible the drop in annealing temperature [15].

Influence of annealing temperature and number of loci amplified: Like any other PCR, multiplex reactions should be done at a stringent enough temperature, allowing amplification of all loci of interest without "background" by-products. Although many individual loci can be specifically amplified at an annealing temperature of 56°-60° C, experiments showed that lowering the annealing temperature by 4-6° C was required for the same loci to be co-amplified in multiplex mixtures. In PCR, due to differences in base composition, length of

product or secondary structure some loci are more efficiently amplified than others when many loci are simultaneously amplified (multiplexed), the more efficiently amplified loci will negatively influence the yield of product from the less efficient loci. This phenomenon is due in part to the limited supply of enzyme and nucleotides in the PCR reaction. Therefore, in the multiplex procedure the more efficiently amplified loci compete better and take over the less efficiently amplified products, thus rendering them less visible or invisible [15].

The annealing time can be chosen based on the melting temperature of the primers (which can be calculated using other many applications, freely available for molecular biologists). This may work, but sometimes the results may not match the expectations. Therefore, a simple procedure used many times by the author was to use an initial annealing temperature of 54 ° C (usually good for most primers with a length around 20 bp or more). If unspecific products result, this temperature should be increased. If the reaction is specific (only the expected product is synthesized) the temperature can be used as is [15].

For the seventy or so primers used during this work, a denaturing time of 30-60 seconds was sufficient to achieve good PCR products. To long a denaturing time, will increase the time the Taq polymerase is subjected at high temperatures, and increases the percentage of polymerase molecules that lose their activity [15].

Extension time: In multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products [15].

Extension temperature: There is a higher yield of PCR products for A(72°C), B and D when program A was used. This shows that the 72° C extension temperature, negatively influenced amplification of some loci, while also making some unspecific products visible. The higher annealing temperature is probably detrimental to the stability of the DNA helix, so less strands of DNA have the chance to become "copied" by the polymerase after annealing [15].

2.3.10.2. Number of cycles

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized. A common mistake is to execute too many cycles. To quote Kary Mullis,: "If you have to go more than 40 cycles to amplify a single-copy gene, there is something seriously wrong with your PCR". Too many cycles can increase the amount and complexity of nonspecific background products. Of course, too few cycles give lox product yield [15].

In general, 30 cycles should be sufficient for a usual PCR reaction. An increased number of cycles will not dramatically change the amount of product. Cycling conditions and buffer concentrations should be adjusted for each primer pair, so that amplification of the desired locus is specific; with no secondary products (see other pages). If this is not possible, the sequences of the primers should be either elongated with 4-5 bases or simply, changed entirely [15].

First PCR program: For any given primer pair, the PCR program can be selected based on the composition (GC content) of the primers and the length of the expected PCR product. In the majority of the cases, products expected to be amplified are relatively small (from 0.1 to 2-3 kb). As the activity of the enzyme may not be always optimal during the reaction, an easy rule applied successfully by the author was to consider an extension time (in minutes) equal to the number of kb of the product to be amplified (1 min for a 1 kb product, 2 min for a

two kb product etc.). Later on, after the product(s) become "known", extension time may be further reduced [15].

2.3.10.3. Reaction volume

If using older model thermocyclers (without a heated lid), to run small volume PCR reactions, mineral oil is necessary to cover the reaction mixture [15].

It is important to mention, that small volume PCRs may be very beneficial when using small amounts of DNA template. In general, at a constant amount of template DNA, the yield of PCR product per micro liter reaction is higher when the reaction volume is 5 μL compared to 100 μL. This may allow visualization of the PCR products; sometimes invisible when larger reaction volumes are used [15].

Important in finding and documenting polymorphisms is slight mismatches, (even 1 base-pair mutations) in one of sequences bound by the two primers used to amplify a DNA locus, can be detected by slight variations in annealing temperature and/or by multiplex PCR [15].

2.3.10.4. Pipetting and DNA template

It is best to start pipetting water first, followed by the other ingredients. There was no difference in results when various components of the reaction were pipetted in different orders [15].

To minimize the chance of primer binding to the DNA template and to prevent the polymerase from working (even theoretically) prior to the first denaturing step, it is useful to keep the vials on ice while pipetting the ingredients of the reaction [15].

Depending on the profile of the laboratory (i.e. current DNA probes in use), pipetting can be done under a laminar flow of sterile air (when plasmids are commonly used in the lab) or at the bench (when the template DNA is genomic DNA or when a larger amount of DNA is used) [15].

Another problem when pipetting small volumes (1-2 μL) of a complex DNA sample (like genomic DNA) is the likelihood of differences in the amount of DNA actually taken in each PCR vial [15].

2.3.11. Characterization of amplycons

2.3.11.1. Electrophoresis

Electrophoresis is separating method by which charged molecules are allowed to migrate in an electric field the rate of migration being determined by the size of the molecules and their electrical charge [19].

The PCR product (amplicons) can be identified by its size using (agarose) gel electrophoresis. Gel electrophoresis is a technique that consists of injecting DNA into gel and then applying an electric current to the gel.

The gel is a complex network of fibrils and the pore size on the gel can be controlled by the way in which the gel is prepared. The molecules migrate trough the pores a matrix of "gel" at rates depending upon their molecular weight and molecular shape. The DNA samples are placed in wells in the gel surface, the power supply is switched on and the DNA is allowed to migrate through the gel in separate lanes or tracks. As a result, the smaller DNA strands (amplycons, fragments) or compact molecules move more rapidly than the larger strands or loose molecules, through the gel toward the positive current. The size of the PCR product can be determined by comparing it with a *DNA ladder*, which contains DNA fragments of known size, also within the gel. The gel is located into an electrophoresis chamber and dived by the comb in line for one sample (mixture of amplycons) [14].

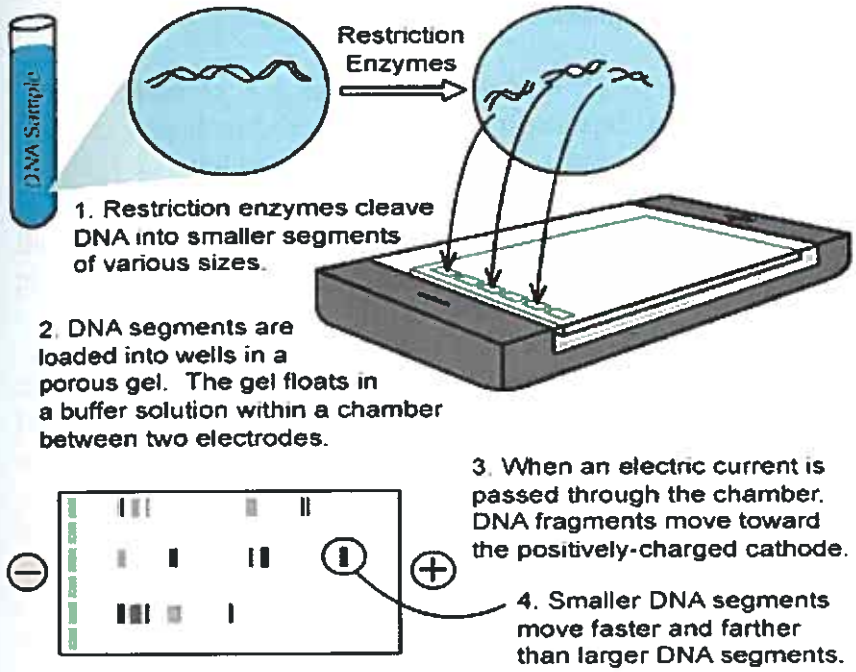


Fig. 7: Schematic describing of gel electrophoresis [6]

The horizontal frame, made of Lucite plastic, holds the gel. The ends of the gel are in immersed in buffer which makes an electrical connection to the power supply. The chamber has two electrodes on opposite ends, which can be attached to a DC power supply. Gel chambers are available with single (J8-21-3668) and double (J8- 21 – 3654) casting trays. The double casting tray enables you to run two gels at one time in the same gel box [7].

An electrophoresis buffer fills the chamber and conducts the electricity between two electrodes. When current is applied, the negatively charged DNA migrates toward the positive electrode. The agarose gel acts as a sieve allowing the smaller-sized fragments to migrate faster than the larger fragments, thus separating fragments by size [19].

The speed of electrophoresis is dependent on the size of the gel and the amount of voltage applied to the gel box by the power supply. The higher voltage does the faster of the migration of the fragments. Each gel box has a maximum optimal voltage range, and exceeding this range results in smearing of the DNA bands. Lower voltages generally give cleaner separation of bands [19].

In each lane, a mixture DNA fragments had been applied. A computerized scanner can be used to locate the positions of the DNA bands. After a defined period of time of migration, the locations of the DNA molecules in the gel are assessed by making the DNA molecules fluorescent and observing the gel with UV [19].

2.3.11.1.1. Type of gels

The gel itself is composed of either agarose or polyacrylamide, each of which has attributes suitable to particular tasks:

Agarose is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose is concentration the "stiffer" the gel. Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic [7].

Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoresis techniques [19].

Polyacrylamide is a cross-linked readily polymer of acrylamide (AA), C_3H_5NO, derived from acryl acid and used in synthetic fibers and sewage treatment. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders) [14].

Acrylamide is a potent neurotoxin and should be handled with care! Wear disposable gloves when handling solutions of acrylamide, and a mask when weighing out powder. Polyacrylamide is considered to be non-toxic, but polyacrylamide gels should also be handled with gloves due to the possible presence of free acrylamide [7].

Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing is length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins [7].

Although the gels had the same thickness, results also indicate that the "special" NuSieve agarose is more transparent than the regular agarose. Although NuSieve agarose is much more expensive, it provides some cost reduction by requiring less amount of agarose for the same separation power and by requiring less amount of separation time. These particular advantages can make such "specialized" agarose useful for particular applications. It is worth mentioning that other agarose (from different manufacturers) used, perform similarly. Agarose gels can be run at various voltages, depending on the separation desired and the available time. It was noticed that, at least for PCR products smaller than 600 bp, separation is better and bands are sharper if gels are run very fast (3-4 hours for a 15-20 cm long 2-3% agarose gel). When the same gel runs at a low voltage overnight (14-16 hours) the products become less separable or "puffy" due to the diffusion in the gel [7].

2.3.11.1.2. Type of labeling

After electrophoresis, view the DNA by staining with ethidium bromide, methylene blue, or Carolina Blue (TM) DNA stain [14].

Ethidium bromide is the stain most commonly used by researchers because of its sensitivity to DNA and the speed of staining. The DNA shows up as an orange band on illumination by UV light, sizes can be estimated by comparison with the marker tracks. Drawbacks include the cost involved for its visualization (it requires a UV light source), and its suspected

carcinogenicity. If you wear rubber gloves and work in a sink, the low concentrations required for staining can be used safely [14].

Methylene blue stain is less sensitive than ethidium bromide, but this can be compensated for by using higher concentrations of DNA. It is also a visible – light stain, which means UV light sources are not required. Many instructors purchase inexpensive white light boxes, or utilize an overhead projector protected by a sheet of acetate. Another method is to place a clears piece of acetate over the gel and trace the DNA band patterns. A disadvantage of methylene blue is the time required for staining and distaining the gel. An alternative to these two stains is the Carolina BLU(TM) staining system [14].

2.3.11.2. Spectroscopic properties

2.3.11.2.1. UV absorption

The aromatic bases of nucleic acids absorb light with a λ_{max} of 260 nm. The absorption properties of DNA can be used for detection quantization and assessment of purity. Although the λ_{max} for DNA in constant, the extinction coefficient depends on the environment of the bases. The absorbance at 260 nm (A_{260}) is greatest for isolated nucleotides, intermediate for ssDNA least for dsDNA. This effect is caused by the fixing of the bases in a hydrophobic environment by stacking. The classic term for this change in absorbance is hypochromicity (From the Greek "less colored") [19].

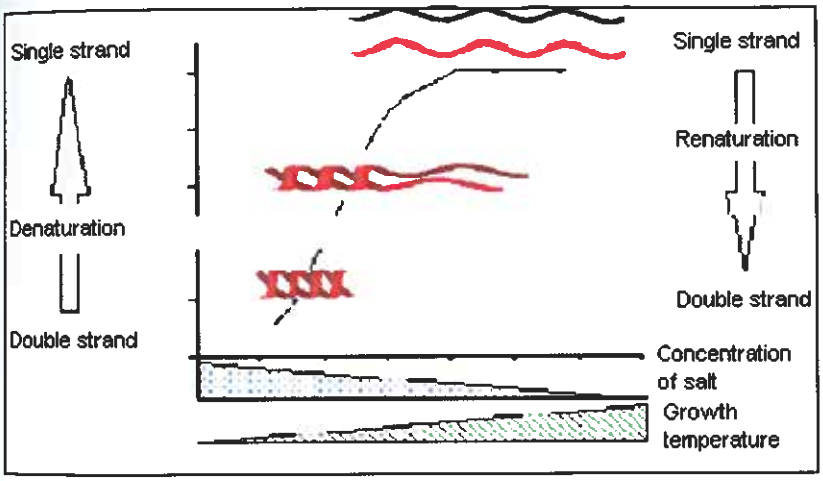


Fig. 8: General UV - VIS spectroscopic properties of DNA in dependence on temperature [16]

2.3.11.2.2. Quantities of amplycons

The absorbance at 260 nm is used to determine the concentration of DNA. At a concentration of 1 mg ml^{-1} and 1 cm path length, double-stranded DNA has $A_{260} = 20$.

It is not convenient to speak of the molar extinction coefficient (ε) of DNA, since its value will depend on the length of the molecule in question, instead,, extinction coefficients are usually quoted in terms of concentration in mg ml^{-1} ds DNA has an $A_{260} = 20$. The absorbance values also depend on the amount of secondary structure (double-stranded regions) in a given molecule, due to hypochromicity [19].

2.3.11.2.3. Purity of DNA

The A_{260}/A_{280} ratio of a double-stranded DNA sample can be used to assess its purity. For pure DNA, the value is 1, 8. Values above 1, 8 suggest RNA contamination and those below 1,8 suggest protein contamination. The approximate purity of dsDNA preparations may be estimated by determination of the ratio of absorbance at 260 and 280 (A_{260}/A_{280}). The shape of absorption spectrum as well as the extinction coefficient varies with environment of the bases such that pure dsDNA has an A_{260}/A_{280} of 1, 8 [19].

Hence, if a DNA sample has an A_{260}/A_{280} greater than 1, 8 this suggest RNA contamination, whereas one less than 1, 8 suggest protein in the sample [19].

3. EXPERIMENTAL PART

3.1. Material and instruments

3.1.1. Cell line

Brucella melitensis, Brucella abortus, Brucella ovis, Brucella canis, Brucella neotomae, Brucella suis, Brucella cetaceae, Brucella pinnipediae comes from Zaragosa Institute- gen bank

3.1.2. Machines

Freezer -80 ° C, Ardo, Zanussi, Spain
Fridge -4/8 ° C, Ardo, Zanussi, Spain
Apparatus for the preparation of the deionized water milli Q, Millipore, USA
Vortex, Heidolph Reax top
Microcentrífuga: Hermile Labortechnik, Gosheimerstr 5678564 Wehingen, (1000-14000 rpm)
Microwave, Phillips
Analytical balance: CH-Dietkon 300/9206L, Spain
Sterile laminar box, Herasafe HS/HSP, Kendro, Germany
Shaking water bath, Comercial Assens-Llofriu, S.A
UV – VIS spectrophotometer, Hitachi 1100, UK
Automatic Ice maker, AUCMA, China
PCR thermocyclers :GeneAmp PCR System 2700, Perkin-Elmer
Machine for capturing images: Gelprinter Plus, TDI, Madrid
Electrophoresis power supply, Bio Rad, USA
Source of the electric current Power Pac 200, Power Pac 300, Bio Rad, USA

3.1.3. Instruments

Tank for horizontal electrophoresis, combs, glass plates, HE 99X Max, Hoefer, Germany
Pipettes, Pipette tips Eppendorf, Germany
Tubes, Microtubes MicroAmp Reaction (Perkin-Elmer)
Beakers, Erlenmeyer flasks, volumetric flasks
Forceps, syringe
Funnel, spoon, filtrated paper
Latex Glows
Bin for ethidium bromide,
Eppendorf UVette, Eppendorf AG, Germany

3.1.4. Chemicals

Tris (hydroxymethyl) aminomethane, Trisma Base, Sigma, Germany
Acidum boricum, Panreac
Deionizated water, Sigma, Germany
Boric acid, Sigma, Germany

Hydrochlorid acid, Sigma, Germany
Sodium dodecyl sulfate, Sigma, Germany
EDTA, EDTA : Panreac
Ethanol, Sigma, Germany
Tryptosa, Sigma, Germany
Soya, Sigma, Germany
Agarose: medium EEO (D-1 Medium Eeo Pronadosa Cat 8021), Pronadisa, Spain
Ethidium Bromide (2 ug/mL), Sigma, Germany
dNTP Mix, Promega Biotech Iberica
Mg^{2+}, BIOLINE, UK
Buffer, 10x InmoBuffer, BIOLINE, UK
Orange G, Sigma-Aldrich, Germany
Enzyme Taq polymerase (0.5 U) ,Imolase DNA polymerase, BIOLINE, UK
Marker ladder: 1 Kb plus DNA ladder (Invitrogen)
Ultraclean Microbial DNA isolation Kit, MoBio,Carlsbad
Primers: Sigma-Genosys, Germany

3.2. Laboratory procedure

3.2.1. Preparation of samples

Samples were received either from farm, where domestic animal had suspicion of brucellosis, or from Zaragosae institute.

Lot of them came in lyophilized form like ready DNA for further research, but some of samples were need to cultivate and than prepare DNA yourself.

It was necessary controlled in each case, if the microbes weren't contaminated in transfer for example or through the way of cultivating. I had to be sure, that yield of extraction of DNA from Brucellae is without contamination.

It is done, because for molecular methods are wanted to have clear strains without negative influence of other substances in results (protein which left next to DNA from Brucella genus after extraction from cell).

3.2.1.1. Growing condition

Lyophilized strains were resuspended in saline water like physiological solution (9g NaCl/ 1 l H_2O).

Clean living colony was transferred, after growing colony in normal solid medium Mc Conkey, in liquid medium. Living really free cells had better condition in liquid medium to grow and obtain more quantity of DNA. Liquid medium contained tryptosa, soya and agar, which supply enough substrates for multiplying of colony of Brucellae.

Inoculated liquid medium were situated in thermostat at 37 °C with agitating and with / without CO_2 (belongs to which strains want what) for 24 hours.

Milkiness, enough growing mass of living Brucellae were heated in 100 – 80 °C for 30 minutes to kill them. Dead cells were aliquoted and it is one of form for storing at - 80°C.Like this, cells are prepared for further steps, respectively for extraction.

3.2.1.2. Extraction of DNA

I used extraction kit from MoBio, names Ultraclean™ Microbial DNA Isolation Kit. This kit contains all the regents and tubes, which are necessary. Only centrifuge, tips 1000ml, 200ml and their pipettes are tools from laboratory. All of that equipment we let before uses in UV for 15 min to decontaminated and destroy possible exogenous DNA.

The UltraClean™ Microbial DNA Kit is designed to isolate high-quality genomic DNA from microorganisms. A variety of microorganisms, including bacterial and fungal spores, have been tested successfully with this kit.

Microbial cells, resuspended in bead solution are added to a bead beating tube containing beads, followed by lyses solution. The principal is to lyse the microorganisms by a combination of heat, detergent, and mechanical force against specialized beads. The cellular components are lysed by mechanical action using a specially designed MO BIO Vortex Adapter on a standard vortex. From the lysed cells, the released DNA is bound to silica Spin Filter. The filter is washed, and the DNA is recovered in certified DNA-free Tris buffer.

Steps of extracting protocol (kit is for research purposes only, not for diagnostic use)

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Decant the supernatant and spin the tubes at 10,000 x *g* for 30 seconds at room temperature and completely remove the media supernatant with a pipet tip. NOTE: Based on the type of microbial culture, it may be necessary to centrifuge longer than 30 seconds.

This step concentrates and pellets the microbial cells. In some cases it may take longer to completely pellet the cells. It is important to pellet the cells completely and remove all the culture media in this step.

2. Resuspended the cell pellet in 300 µl of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to MicroBead Tube with particles.

The MicroBead Solution contains salts and a buffer which stabilizes and homogeneously disperses the microbial cells prior to lysis.

3. Add 50 µl of Solution MD1 to the MicroBead Tube.

Solution MD1 contains SDS and other disruption agents required for cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will precipitate. Heating at 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. In addition, Solution MD1 can be used while it is still warm.

MD_1 is amphiphiles compound, so this hydrophobic – hydrophilic solution easy go between double layers of bacterial membrane (from phospholipids) and after mixing in the tube with particles, this deals to make pores in membrane.

4. Optional: To increase yields, heat at 65°C for 10 minutes.

This optional step can be beneficial for hard to lyse organisms. Heat can help denature proteins and aid in cell lysis.

In part of results is statistic measuring with this step, but is not definition like that is helpful in each case.

5. Secure bead tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. (See "Alternative lyses method" for less DNA shearing).

This step creates the combined chemical/ mechanical lyses conditions required to release desired nucleic acids from microbial cells. Many cell types will not lyse without this chemically enhanced bead beating process. The vortex action is typically all that is required, however, more robust bead beaters may also be used. In most cases the times may be shorter with other devices but you may run the risk of increased DNA shearing. This process is compatible with fast prep machines.

6. Make sure the 2 ml MicroBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x *g* for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x *g* or tubes may break.

The cell debris is sent to the bottom of the tube while DNA is remains in the supernatant.

7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

The volume to expect will vary depending on the size of the original cell pellet from step 1.

8. NOTE: Expect 300 to 350 µl of supernatant.

9. Add 100 µl of Solution MD2, to the supernatant. Vortex 5 seconds. Then incubate at 4°C for 5 minutes.

10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

Solution MD2 contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications

11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Expect approximately 450 µl in volume.

The pellet at this point contains non-DNA organic and inorganic materials, including cell debris and proteins. For the best DNA quality and yield, avoid transferring any of the pellet.

12. Add 900 µl of Solution MD3 to the supernatant and vortex 5 seconds.

Solution MD3 is a highly concentrated salt solution. It sets up the high salt condition necessary to bind DNA to the Spin Filter membrane in the following step.

13. Load about 700 µl into the Spin Filter and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x *g* for 30 seconds at room temperature. NOTE: A total of 2 to 3 loads for each sample processed are required. Discard all flow through liquid.

DNA is selectively bound to the silica membrane in the Spin Filter device. Contaminants pass through the filter membrane, leaving only the DNA bound to the membrane.

14. Add 300 µl of Solution MD4 and centrifuge at room temperature for 30 seconds at 10,000 x *g*. For example small peptides go out through the membrane, so it is not interference in further investigating procedures.

Solution MD4 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residues of salt, and other contaminants while allowing the DNA to stay bound to the silica membrane.

15. Discard the flow through.

This flow through is waste containing ethanol wash solution and contaminants that did not bind to the silica Spin Filter membrane.

16. Centrifuge at room temperature for 1 minute at 10,000 x *g*.

This step removes residual Solution MD4 (ethanol wash solution). It is critical to remove all traces of wash solution because it can interfere with down stream DNA applications.

17. Being careful not to splash liquid on the spin filter basket, place Spin Filter in a new 2 ml Collection Tube (provided).

It is important to avoid any traces of the ethanol based wash solution.

18. Add 50 µl of Solution MD5 to the center of the white filter membrane.

Placing the Solution MD5 (elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in more efficient release of bound DNA

19. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
As the Solution MD5 (elution buffer) passes through the silica membrane, DNA is released, and it flows through the membrane, and into the Collection Tube. The DNA is released because it can only bind to the silica Spin Filter membrane in the presence of salt. Solution MD5 is 10mM Tris pH 8 and does not contain salt.
20. Discard Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required and it is recommend storing DNA frozen (-20°C). Solution MD5 contains no EDTA.

Additional Information
Alternative lysis methods
For less DNA shearing, skip step # 4 in the protocol and heat MicroBead Tubes at 65°C for 10 minutes. Vortex for a few seconds, every 3 minutes, throughout the 10 minute incubation. This procedure will reduce DNA shearing and at the same time reduce the yield of total DNA.

If cells are difficult to lyse, 10 minute incubation at 70°C, after adding Solution MD1, can be performed. Follow by continuing with protocol step 4.

Concentrating the DNA
The final volume of eluted DNA will be 50µl. The DNA may be concentrated by adding 5 µl of 5M NaCl and inverting 3-5 times to mix. Next, add 100 µl of 100% cold ethanol and invert 3-5 times to mix. Incubate at -20°C for 30 minutes and Centrifuge at 10,000 x g for 15 minutes at room temperature. Decant all liquid. Remove residual ethanol in a speed vac or desiccators or air dry. Precipitated DNA resuspended in sterile water or Solution MD5 (10 mM Tris).
DNA floats out of well when loaded on a gel
This usually occurs because residual Solution MD4 remains in the final sample. Prevent this by being careful in step 14 not to transfer liquid onto the bottom of the spin filter basket. Ethanol precipitation (described in "Concentrating the DNA") is the best way to remove residual Solution MD4.
Storing DNA
DNA is eluted in Solution MD5 (10mM Tris) and must be stored at -20°C to -80°C to prevent degradation. For long term storage, we recommend aliquoting DNA into appropriate volumes and store at -80°C. DNA can be eluted in TE without loss, but the EDTA may inhibit downstream reactions such as PCR and automated sequencing. DNA may also be eluted with sterile DNA-Free PCR Grade Water (MO BIO Catalog No. 17000-10).
Extracted DNA from dead cells by kit is other possible form of storing. Also this is perfectly prepared state for further research.

3.2.1.3. Control of quality
Concentration and purity of DNA were measured by using spectrophotometer.
Before the measurement, the spectrophotometer was calibrated by placing the cuvette filled with 50 µl of MD_5 solution in the spectrophotometer (as blind sample), and canceling any possible absorbance at 260 nm and 280 nm automatically. After this step, the sample absorbance was measured and automatically transferred into valuation of amount of DNA

accordingly to Lambert – Behr law. After the measurement, the cuvette was thrown away. Each sample had own new clean cuvette. Purity of DNA was obtained by using the following formulas:

Purity of DNA = A_{260} / A_{280}

Purity is expressed as the ratio of measurement at 260 nm and 280 nm. This ratio provides an estimate of the purity of DNA with respect to contaminants such as proteins. Factor of purity 1, 8is equivalent to clean DNA without any contamination.(Rates are in the tables in part 4).

The extracted DNA by kit from Mobio were checked with itself running electrophoresis without any interference, for be sure with effectually using of PCR mixture in each individual PCR reaction. It means that control of quality was done by running samples of DNA in electrophoresis. Condition of electrophoresis was 120 V, for 60 min, in 1 % gel (1% TBE and agarose). TBE consist of 54g Tris, 27,5g Acid Boric, 3, 72g EDTA (boric acid 89 mM, EDTA 2 mM, Tris-HCl 89 mM [pH 8,0]).
Separated samples in gels were labeling with ethidium bromide bin. Ethidium solution were prepared from stock bottle and the final concentration of labeling solution were 0,5 %.
Record of results was developed by camera with UV, which visualized staining bands of DNA in separating gel.

3.2.2. Multiplex PCR

Extracted strain was controlled, next to electrophoresis separation of DNA, also in well-tried multiplex PCR reaction with perfect consistence of 8 pairs of primers.

3.2.2.1. Condition of PCR reaction

I checked Control of purity of strains with multiplex PCR (8 pairs of primers) for all types of Brucellae. Negative control were added next to the samples for calculating, if the reaction is without contamination (instead samples was H_20). Volume for each sample was 25 µl.

Table 6 Parameters for multiplex PCR reaction

Components of reaction			**Condition of reaction**	
solution	**volume (µl)**		**Temperature (° C)**	**Time (second)**
2,5 µl 10x Buffer	2, 50		95	420
200 uM DnTp mix	5, 00		95	30
25 uM Primer 1 – 16	0, 50		72	45
1 mM $MgCl_2$	1, 50		64	180
0.5 U Taq	0, 30		72	360
DNA	1, 00			
H_2O	16,05		**Number of cycle**: 25	

3.2.2.2. Agarose detection of amplicons

The agarose gel of 2 % was prepared for visualization of amplicons; the volume of gel was 90 ml. Agarose of 0, 9g was resuspended in 90 ml in 1 % TBE. TBE consist of boric acid 89 mM, EDTA 2 mM, Tris-HCl 89 mM [pH 8, 0]. Suspension was 2 minute let in microwave.

This solution was filled in module for electrophoresis with comb. Gel was transferred, after congelation, into tank for electrophoresis and the DNA samples were placed in wells in the gel surface. The power supply was switched on and the DNA was allowed to migrate through the gel in separate lanes or tracks. The size of the PCR product was determined by comparing it with a *DNA ladder* (1 Kb plus DNA ladder (Invitrogen), which contains DNA fragments of known size. The DNA ladder was also placed in same gel.

Here is for the simplification representative description of one of photo. Picture showed gel with PCR products, respectively amplicons. Amplicons were photographed in agarose gel for archiving. Extracted DNA was multiply in PCR reaction, than separated in agarose gel by electrophoresis and developed in machine for capturing images.
These lines are written, because each extracted samples were developed in same way. It is useless in each figure to repeat it.

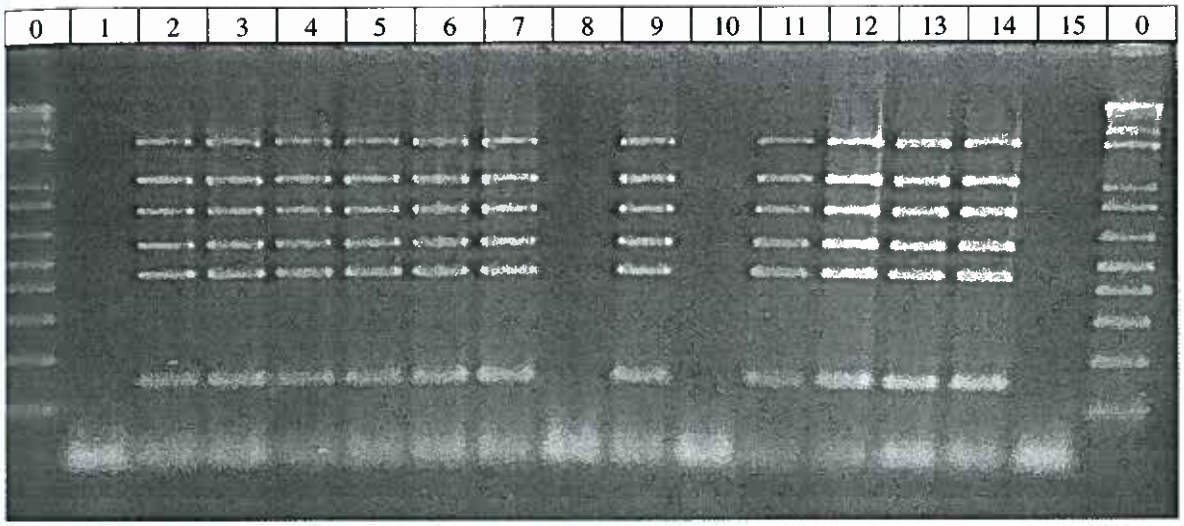


Fig. 9 Representative picture of agarose gel of PCR products made with 8 pair of primers using for detection of Brucella strains. NG is negative control and it is placed next to the DNA ladder. MW is standard molecular weight of 1 Kb DNA leader (it was apply in right and left corner in each gel all the times).Number of samples reply for the number of samples in tables 7.

Table 7 Survey of samples in fig. 9

No	**Species**	**biovarities**	**strain**	concentration (µg/ml)	factor of purity
0	MW	-	-	-	-
1	NV	x	x	x	x
2	Brucella melitensis	3	1553	15,0	1,51
3		3	1553	24,1	1362
4		3	1553	28,3	1358
5		3	1559	15,5	1,70
6		3	1559	25,3	1,78
7		3	1559	14,2	1,72
8	NV of extraction	x	x	X	x
9	Brucella melitensis	2	1461	16,4	1,57
10		2	1461	Bad amplify in PCR reaction	
11		**2**	**1461**	20,1	1,66
12	Brucella melitensis	2	1455	54,9	1,74
13		2	1455	32,3	1,81
14		2	1455	27,7	1,78
15	NV of extraction	x	x	x	x

3.2.3. Experiment 1

I participate with my teacher to develop a new multiplex PCR for Brucella suis. First was controlled extracted DNA, which I preparing for research (chapter 3.2.1).

This experiment should distinguish individual biovarities between each other after developing best condition for all the pairs of primers in single PCR.

We try new access, because in researches field, it is not be done. Different biovarities has variety virulence, level of danger, and it is good in treatment know, which infection agent induce the disease.

It was design new pairs of primers and planed expected result according to dates from articles [21 – 25] (it stand on the basis describing in chapter 2.3.9.1)

DNA ladder	number of bp	**Brucella suis 1**	**Brucella suis 2**	**Brucella suis 3**	**Brucella suis 4**	**Brucella suis 5**
-	1600					
-						
-						
-	1000					
-						
-	800					
-						
-	600					
-	500					
-	400					
-	300					
-	200					
-	100					

Fig. 10 Expected view of electrophoreogram of new multiplex PCR reaction

It was calculated new pair of primers in design steps with similarities in T_m and other parameters belong to theoretical part (chapter 2.3.5). Each primer should be specific for different sequence region of gene Brucella suis. Primers amplified, their distinguish sequence of nucleotides, in PCR reaction different part of DNA samples. Different part of DNA PCR products was possible to make separation in electrophoresis. Amplicons could be seen in final electrophoreogram in different distance from start of gel (fig. 10). Respectively, it is eventually to say, each individual biovarities has own different level of amplicons. Knowledge about number of bands specific for each biovars of Brucella suis finally may detect, which infect agent induce the disease.

3.2.4. Experiment 2

In second part of my project was to confirm or to exclude the hypothesis, if there exist exactly 2 new strains special for sea mammals.

Some articles [10, 20] show from recent studies, that Brucella strains are able to infect mammals in sea too. These strains were identified as brucellae by them colonial and cellular morphology, staining characteristics, biochemical activity (see chapter 2.2.4.1.5.).

I try support this theory in single PCR with 7 pairs of primers. You can see that only in one photo of gel is distinguish in amplifying DNA of different biovarities of "Brucella maris". Each PCR products had variant condition of PCR reaction and it is describing together to results. The molar concentration is same as for multiplex PCR (see chapter 3.2.2.1.). The visualization was done in same steps like for Brucella suis. DNA was multiply in PCR reaction, than separated in agarose gel by electrophoresis and developed in machine for capturing images The agarose gel of 2 % was prepared for visualization of amplicons; the volume of gel was 90 ml. Agarose of 0, 9g was resuspended in 90 ml in 1 % TBE. TBE consist of boric acid 89 mM, EDTA 2 mM, Tris-HCl 89 mM [pH 8, 0](chapter 3.2.1.2.2.).

4. RESULTS and DISCUSSION

4.1. Verification of extracted DNA

Selection only really correct DNA fragment from Brucella genus was important to obtain, for further investigating part of my diploma project, how I describe in upper lines.

It were proven some of the extracted DNA, which was ground in next experiments, with validated system of 8 pair of primers in multiplex PCR assay(condition in chapter 3.2.1.2)

*Table 8.*Survey of used samples in this diploma project

Table 8 a) species: **Brucella melitensis**

No	biovars	strain	host / source	geographic origin	No	biovars	strain	host / source	geographic origin
1	1	1507	goat	Spain	24	2	63/9	goat	India/Turkey
2	1	1507	goat	Spain	25	2	1449	goat	Spain
3	1	1507	goat	Spain	26	2	1449	goat	Spain
4	1	1534	human	Spain	27	2	1449	goat	Spain
5	1	1534	human	Spain	28	2	1455	human	Spain
6	1	1534	human	Spain	29	2	1455	human	Spain
7	1	1534	human	Spain	30	2	1455	human	Spain
8	1	1534	human	Spain	31	2	1461	goat	Spain
9	1	1534	human	Spain	32	2	1461	goat	Spain
10	1	H 38	goat	Mexico	33	2	1461	goat	Spain
11	1	H 38	goat	Mexico	34	3	1549	human	Spain
12	1	H 38	goat	Mexico	35	3	1549	human	Spain
13	1	Rev 1	goat	USA	36	3	1549	human	Spain
14	1	Rev 1	goat	USA	37	3	1553	bovine	Spain
15	1	Rev 1	goat	USA	38	3	1553	bovine	Spain
16	1	16 M	goat	USA	39	3	1553	bovine	Spain
17	1	16 M	goat	USA	40	3	1559	goat	Spain
18	1	16 M	goat	USA	41	3	1559	goat	Spain
19		115			42	3	1559	goat	Spain
20		115			43	3	Ether	human/goat	Italy
21		115			44	3	Ether	human/goat	Italy
22	2	63/9	goat	India/Turkey	45	3	Ether	human/goat	Italy
23	2	63/9	goat	India/Turkey					

Table 8 b) species: **Brucella abortus**

No	biovars	strain	host / source	geographic origin	No	biovars	strain	host / source	geographic origin
1	1	45/20			7	1	1280		
2	1	45/20			8	1	1280		
3	1	45/20			9	1	1280		
4	1	544	cattle	England	10	1	2308	bovine	USA
5	1	544	cattle	England	11	1	2308	bovine	USA
6	1	544	cattle	England	12	1	2308	bovine	USA

Table 8 b) species: **Brucella abortus** (continued)

No	biovars	strain	host / source	geographic origin	No	biovars	strain	host / source	geographic origin
13	2	91/75			30	3	GI/2		
14	2	91/75			31	4	292	cattle	England
15	2	91/75			32	4	292	cattle	England
16	2	A - 300			33	4	292	cattle	England
17	2	A - 300			34	5	-	bovine	UK
18	2	A - 300			35	5	-	bovine	UK
19	3	15.III			36	5	-	bovine	UK
20	3	15.III			37	R	51	cattle	USA
21	3	15.III			38	R	51	cattle	USA
22	3	12420			39	R	51	cattle	USA
23	3	12420			40	6	870	cattle	Africa
24	3	12420			41	6	870	cattle	Africa
25	3	Tulya	human	Uganda	42	6	870	cattle	Africa
26	3	Tulya	human	Uganda	43	9	-	bovine	UK
27	3	Tulya	human	Uganda	44	9	-	bovine	UK
28	3	GI/2			45	9	-	bovine	UK
29	3	GI/2							

Table 8 c) species: **Brucella suis**

No	biovars	strain	host / source	geographic origin	No	biovars	strain	host / source	geographic origin
1	1	vacual China			12	3	686	human/swine	USA
2	1	vacual China			13		19 USA		
3	1	vacual China			14		19 USA		
4	2	Thomsen 1	porcine/swine	Denmark	15		19 USA		
5	2	Thomsen 1	porcine/swine	Denmark	16		19 INDIA		
6	2	Thomsen 1	porcine/swine	Denmark	17		19 INDIA		
7	2	S - 65			18		19 INDIA		
8	2	S - 65			19	4	40	reindeer	Russia
9	2	S - 65			20	4	40	reindeer	Russia
10	3	686	human/swine	USA	21	4	40	reindeer	Russia
11	3	686	human/swine	USA					

Table 9 Identification number of used samples in this diploma

TAXA IN GENUS Brucella, marked by American type of culture collection (ATCC)				
No	**species**	**biovars**	**strain**	**ATCC**
1	Brucella melitensis	1	16 M	23456
2		2	63/9	23457
3		3	Ether	23458
4	Brucella suis	2	Thomsen 1	23445
5		3	686	23446

Table 9 Identification number of used samples in this diploma (continued)

TAXA IN GENUS Brucella, marked by American type of culture collection (ATCC)				
No	**species**	**biovars**	**strain**	**ATCC**
6	Brucella suis	4	40	23447
7	Brucella abortus	1	544	23448
8		3	Tulya	23450
9		4	292	23451
10		5	-	23450
11		6	870	23453
12		9	-	23455

Control extracted samples that they are verify (condition of multiplex PCR in table 4)

- FIRST Multiplex PCR

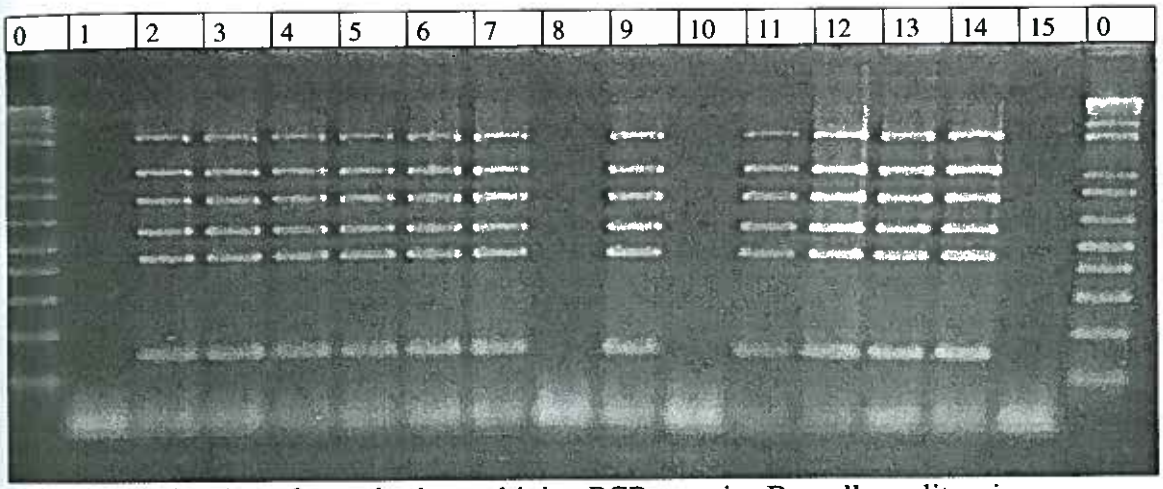


Fig.11 Verification of samples by multiplex PCR, species Brucella melitensis (Condition of multiplex PCR in table 6, order of samples in table 10)

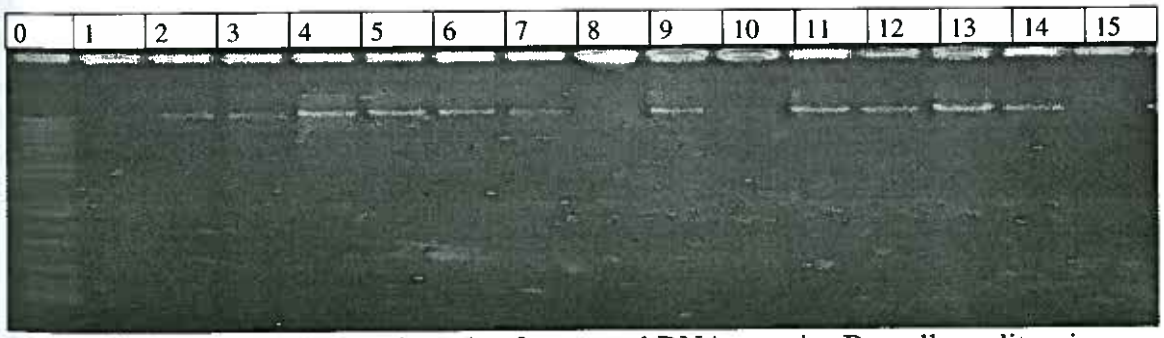


Fig. 12 Agarose gel electrophoresis of extracted DNA, species Brucella melitensis (Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 10)

Table 10 Data of samples illustrated in Fig 11, 12

No	species	biovarities	strain	concentration (μg/ml)	factor of purity
0	MW	DNA ladder			
1	Negative control	x	x	x	x
2	Brucella melitensis	3	1553	15,0	1,51
3		3	1553	24,1	1362

Table 10 Data of samples illustrated in Fig.11, 12(continued)

No	species	biovarities	strain	concentration (µg/ml)	factor of purity
4	Brucella melitensis	3	1553	28,3	1358
5		3	1559	15,5	1,70
6		3	1559	25,3	1,78
7		3	1559	14,2	1,72
8	Negative control of extraction	x	x	X	x
9	Brucella melitensis	2	1461	16,4	1,57
10		2	1461	Bad amplify in PCR reaction	
11		2	1461	20,1	1,66
12		2	1455	54,9	1,74
13		2	1455	32,3	1,81
14		2	1455	27,7	1,78
15	Negative control of extraction	x	x	x	x

- SECOND Multiplex PCR

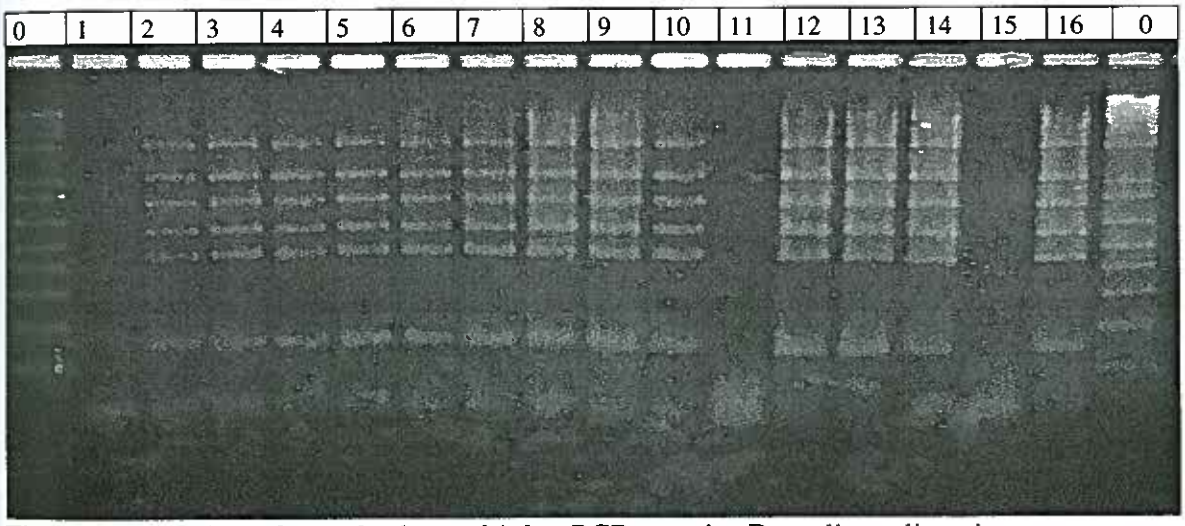


Fig.13 Verification of samples by multiplex PCR, species Brucella melitensis
(Condition of multiplex PCR in table 6, order of samples in table 11)

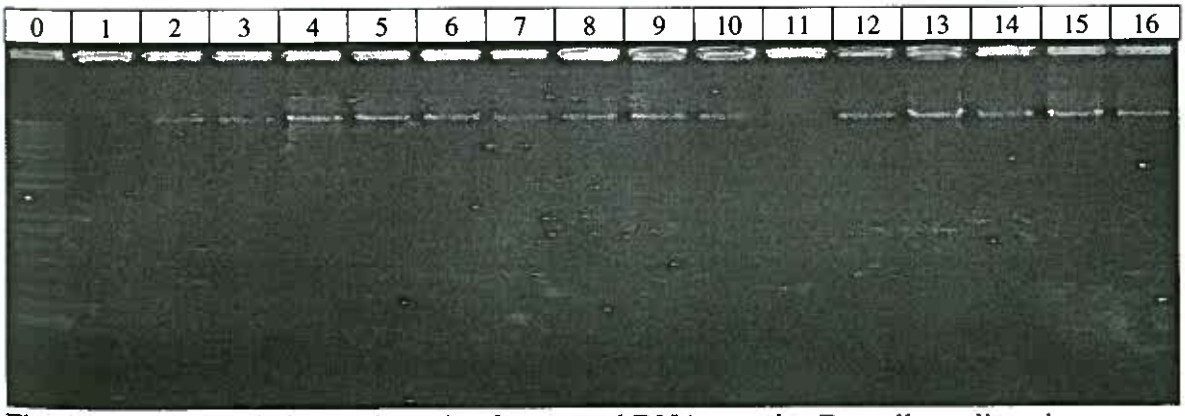


Fig. 14: Agarose gel electrophoresis of extracted DNA, species Brucella melitensis
(Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 11)

Table 11 Data of samples illustrated in Fig.13, 14

No	species	biovarities	strain	concentration (µg/ml)	factor of purity
1	Negative control	x	x	x	x
2	Brucella melitensis	3	1549	42,9	1,77
3		3	1549	20,2	1,59
4		3	1549	68,7	1,33
5		1	H 38	21,7	1,70
6		1	H 38	19,3	1,64
7		1	H 38	26,7	1,71
8		1	1534	14,5	1,70
9		1	1534	30,6	1,65
10		1	1534	68,6	1,22
11	Neg.control of extraction	x	x	x	x
12	Brucella melitensis	1	1507	81,1	1,44
13		1	1507	47,1	1,63
14		1	1507	38,7	1,77
15	Neg.control of extraction	x	x	x	x
16	Brucella melitensis	2	1461	18,3	1,62

- THIRD Multiplex PCR

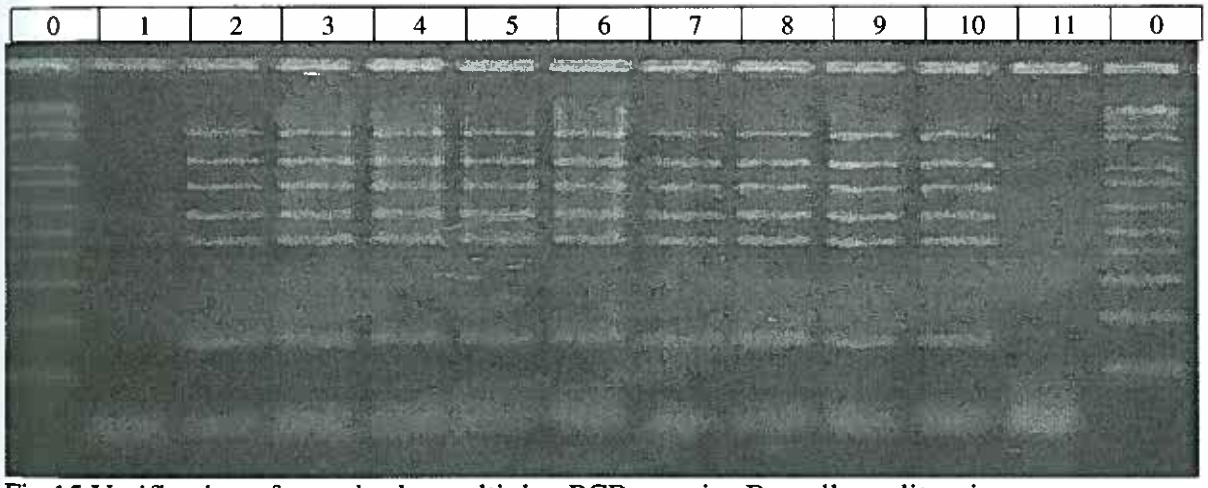


Fig.15 Verification of samples by multiplex PCR, species Brucella melitensis
(Condition of multiplex PCR in table 6, order of samples in table 12)

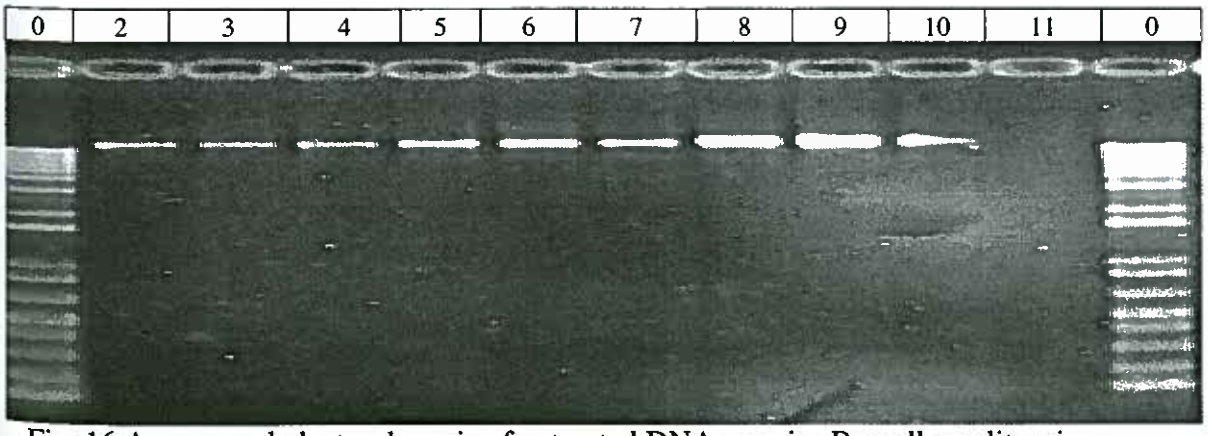


Fig. 16 Agarose gel electrophoresis of extracted DNA, species Brucella melitensis
(Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 12)

Table 12 Data of samples illustrated in Fig.15, 16

No	species	biovarities	strain	concentration (µg/ml)	factor of purity
1	Negative control	x	x	x	x
2	Brucella melitensis	3	Ether	32,2	1,63
3		3	Ether	23,4	1,70
4		3	Ether	29,9	2,19
5		1	Rev 1	22,1	1,87
6		1	Rev 1	30,3	1,95
7		1	Rev 1	33,9	1,56
8			115	38,9	1,86
9			115	43,9	1,86
10			115	26,6	1,87
11	Negative control of extraction	x	x	x	x

- FOURTH Multiplex PCR

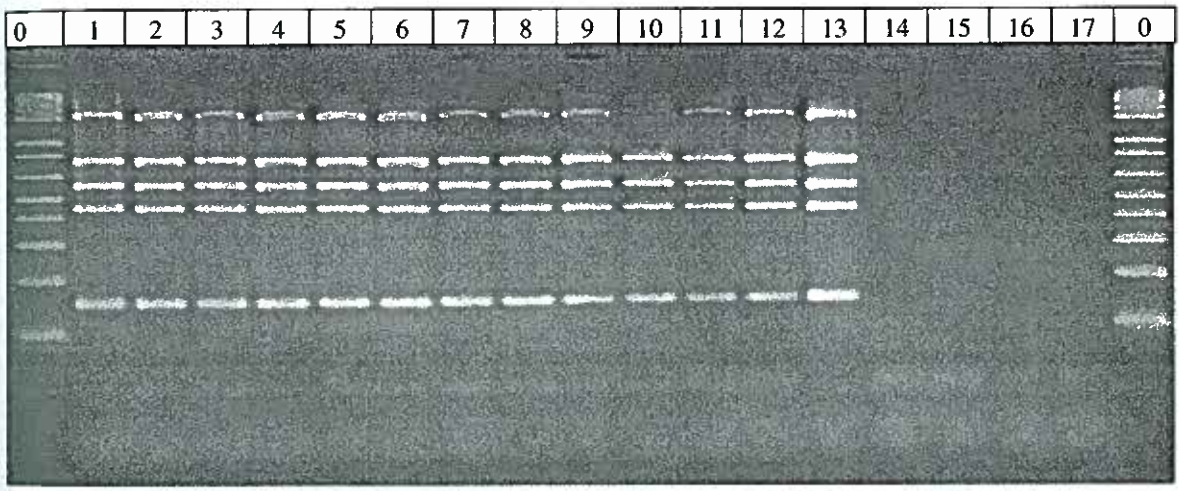


Fig.17 Verification of samples by multiplex PCR, species Brucella abortus
(Condition of multiplex PCR in table 6, order of samples in table 13)

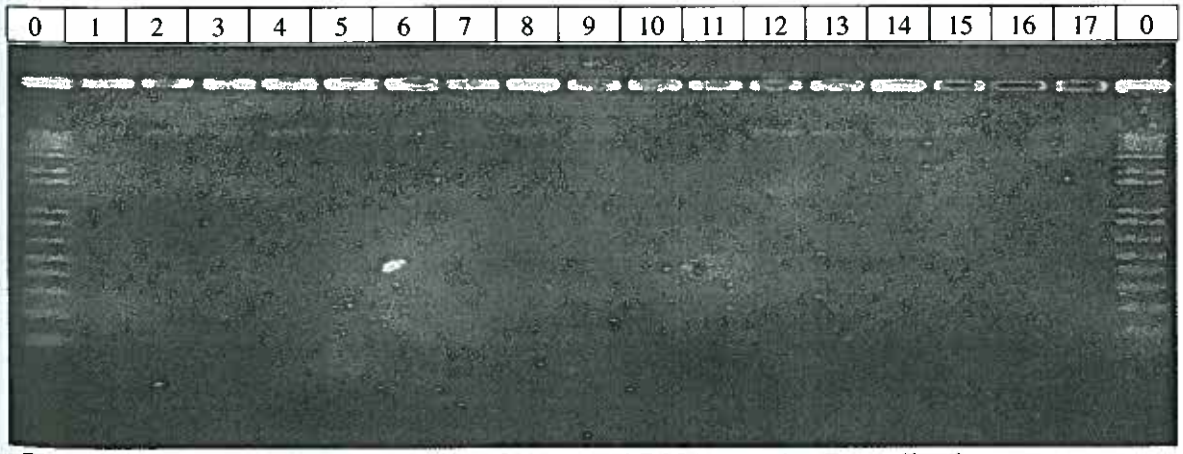


Fig. 18 Agarose gel electrophoresis of extracted DNA, species Brucella abortus
(Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 13)

Table 13 Data of samples illustrated in Fig.17, 18

No	species	biovarities	strain	concentration (µg/ml)	factor of purity
1	Brucella abortus	5		12,20	1,56
2		5		13,00	1,69
3		6	870	20,10	1,57
4		3	Thulya	18,40	1,63
5		3	Thulya	18,30	1,69
6		3	Thulya	17,50	1,68
7			12420	8,20	1,56
8			12420	9,90	1,6
9			12420	9,30	1,46
10			Indi	41,10	1,52
11			544	7,00	1,77
12			544	7,70	1,64
13			544	20,40	1,61
14	Bruc.melitensis		1449	104,80	1,81
15			1449	74,20	1,83
16			1449	81,80	1,86
17	Negative control	x	x	x	x

- FIFTH Multiplex PCR

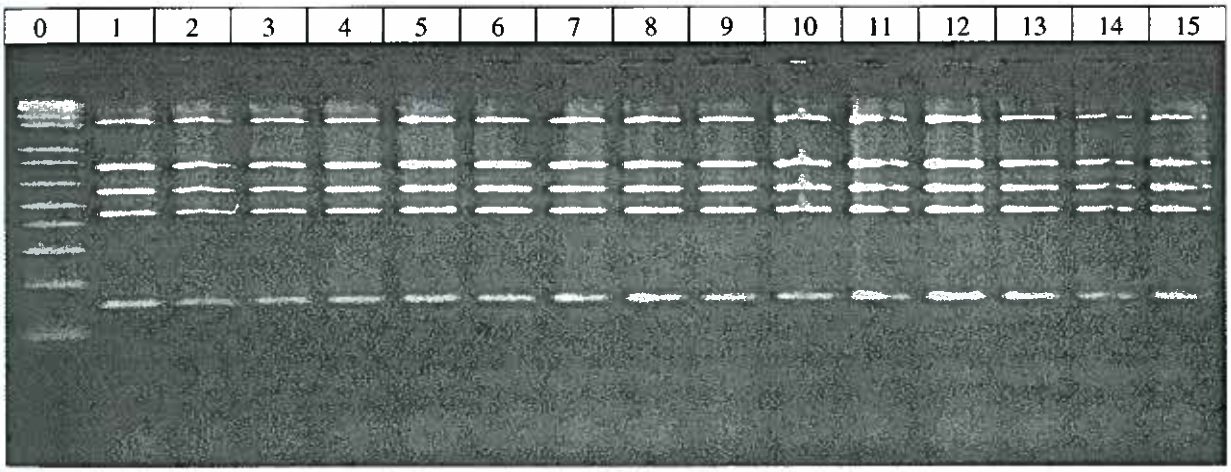


Fig. 19 Verification of samples by multiplex PCR, species Brucella abortus
(Condition of multiplex PCR in table 6, order of samples in table 14)

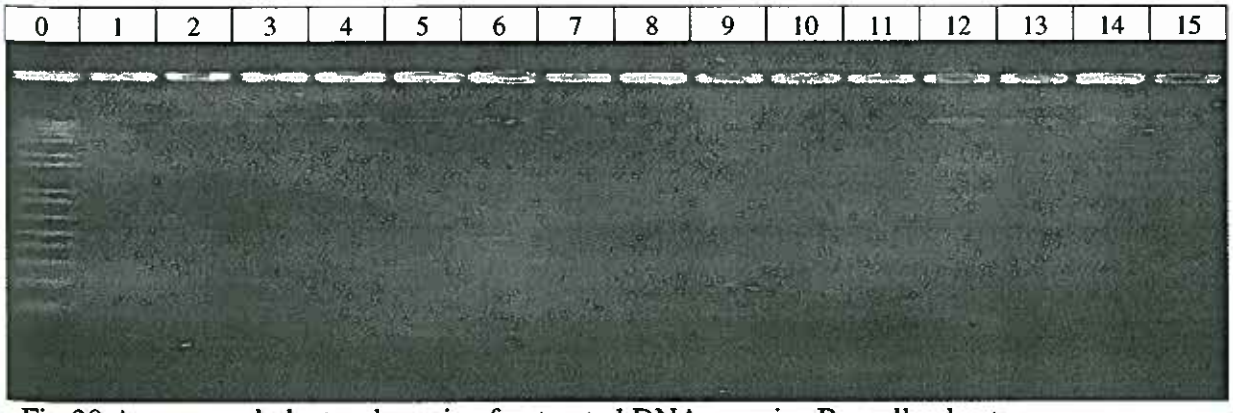


Fig.20 Agarose gel electrophoresis of extracted DNA, species Brucella abortus
(Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 14)

Table 14 Data of samples illustrated in Fig.19, 20

No	species	biovarities	strain	concentration (µg/ml)	factor of purity
1	Brucella abortus	1	45/20	19,7	1,72
2		1	45/20	24,0	1,60
3		1	45/20	22,8	1,65
4		1	1280	23,1	1,74
5		1	1280	20,1	1,76
6		1	1280	20,9	1,65
7		1	2308	16,3	1,76
8		1	2308	20,3	1,56
9		1	2308	18,3	1,68
10		2	91/75	8,5	1,45
11		2	91/75	7,4	1,43
12		2	91/75	14,5	1,73
13		9	-	12,8	1,66
14		9	-	11,8	1,66
15		9	-	14,1	1,66

- SIXTH Multiplex PCR

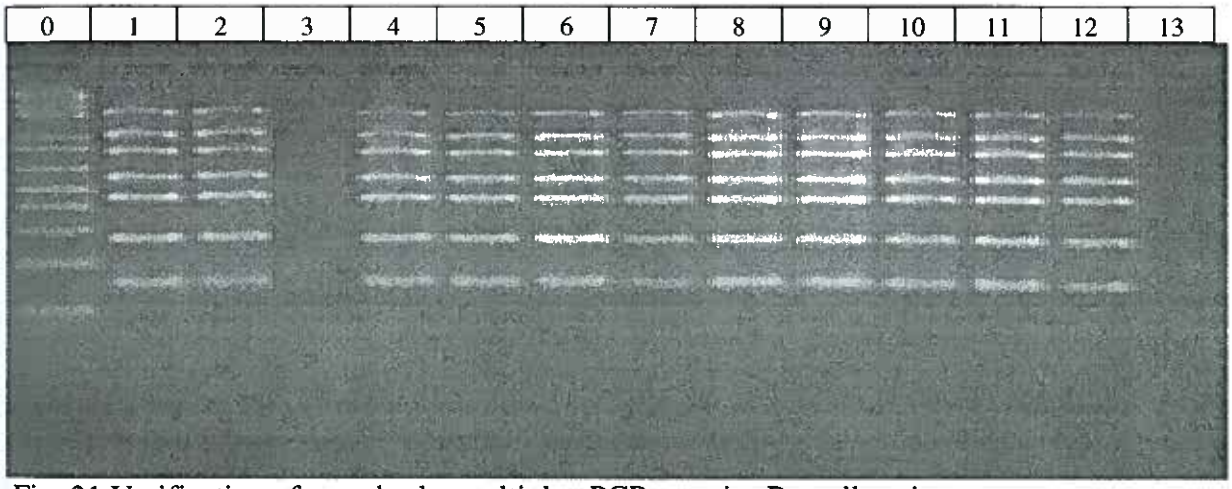


Fig. 21 Verification of samples by multiplex PCR, species Brucella suis
(Condition of multiplex PCR in table 6, order of samples in table 15)

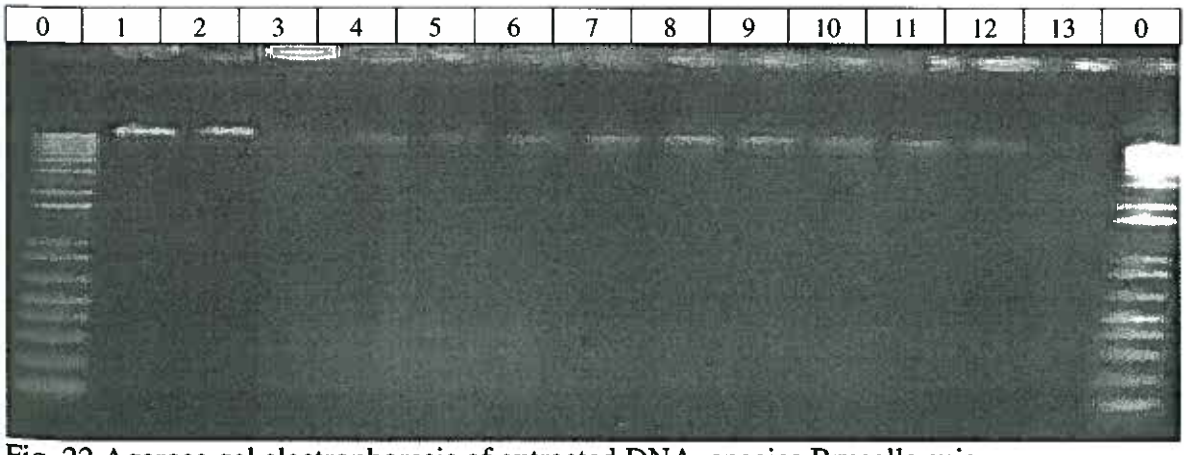


Fig. 22 Agarose gel electrophoresis of extracted DNA, species Brucella suis
(Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 15)

Table 15 Data of samples illustrated in Fig. 21, 22

No	species	biovarities	strain
1	Brucella suis	1	vacual China
2		1	vacual China
3	Negative control	x	x
4	Brucella suis	3	686
5		3	686
6		3	686
7		2	Thomsen 1
8		2	Thomsen 1
9		2	Thomsen 1
10		4	40
11		4	40
12		4	40
13	Negative control of extraction	x	x

- SEVENTH Multiplex PCR

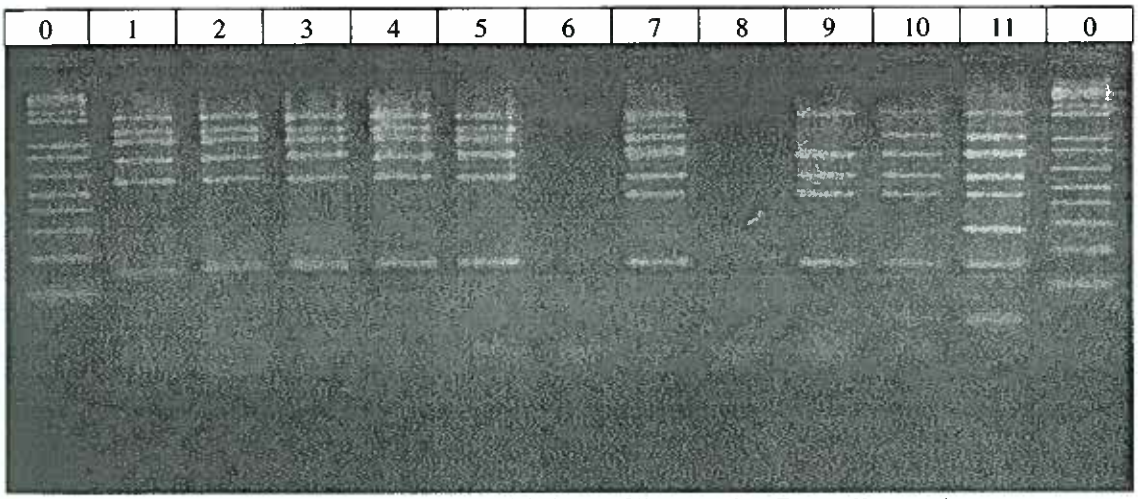


Fig. 23 Verification of samples by multiplex PCR, species Brucella maris
(Condition of multiplex PCR in table 6, order of samples in table 16)

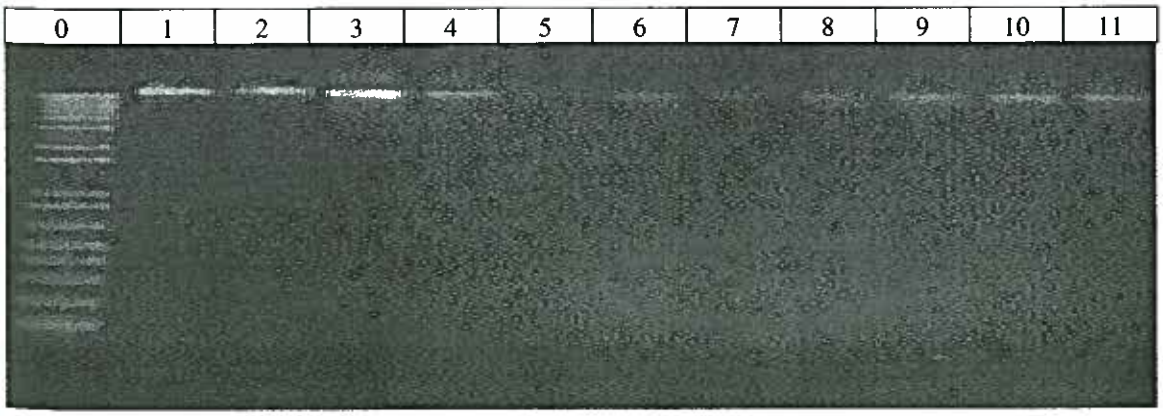


Fig. 24 Agarose gel electrophoresis of extracted DNA, species Brucella maris
(Condition of gel electrophoresis see chapter 3.2.1.3, order of samples in table 16)

Table 16 Data of samples illustrated in Fig. 23, 24

No	sample	concentration (µg/ml)	factor of purity
0	DNA ladder	x	x
1	B.M3 05 - 684	17,00	x
2	B.M s 7-77-63	17,00	x
3	B.dolphin	17,00	x
4	B.porpoise	17,00	x
5	B.seal	17,00	x
6	B.MCITA	17,00	x
7	B.16 M	17,00	x
8	Negative control	17,00	x
9	B. a 5	17,00	x
10	B.1534	17,00	x
11	B. s 2 S65	17,00	x

It was extracted 110 samples according to table 8. I did in such huge number of extraction the statistic classification of effect of optional steps by extracting kit from Mobio (for example increase of yields Mobio Company recommends heat at 70 ° C 10 minutes or for higher concentration of product of extraction at the end of extraction to repeated last step (see chapter 3.2.1.2).

We supposed that to be interesting effectually influence the amount of extracted DNA. But the statistic classification showed that the optional steps are only recommendation. Statistic classification did not make any significant parameters, which deserved mention.

Verification of extracted DNA

Samples were found be ready for analyzing in the experiment 1, 2, according to described protocol (see chapter 3.2.1.2).

All the parts of control of quality were confirmed. The task of this part was to learn how to proceed in accordance with good laboratory practice.

4.2. New primer designed for multiplex PCR for Brucella suis

Experiment 1

Goal of this part was to differentiate biovarities in Brucella suis in detail and find appropriate primers for multiplex PCR.

New pair of primers - specific for Brucella suis biovars 1, 2, 3, 4, 5 - was used in single PCR. The figure below showed how results seem after electrophoresis of amplicons.

It were projected the parameters and final face of the expected final electrophoreogram, after choosing right pair of primers in programs (in web pages) for design new primers (see chapter 3.2.2.).

Contents of this work were reserved the optimalization of the choosing primers. The work describes what is necessary to change in parameters of PCR reaction in order to acquire perfect multiplex PCR assay.

"In representative part of results", there are photos, which allowed in project to come up to higher steps, respectively that were possible mixed single pair of primers together.

"In non representative part of results", there are photos, which said that the condition for detecting searched deletion is not enough stringent. It means that was in number of time repeating same condition for relevancy PCR reaction to detect, if the result is only falls in pipetting, falls in dosage of components of reaction, or possible of contamination, or if the primers is not enough specific for targets DNA. Those photos showed us, in which modification could we suppose mixing single PCR together or not. The conditions were still changing until the pairs of primers did enough distinguishable products of PCR.

TABLE 17 Survey of results acquired by using new primers

		strain										
number of primers	**length (bp)**	**B. melitensis**	**B. abortus**	**B. ovis**	**B.canis**	**B. neotomae**	**B. suis 1-104**	**B.suis 2 24**	**B. suis 2-Thomsen 1**	**B.suis 3 686**	**B.suis 4 40**	**B.suis 5 elf**
1	376	P1	P1	P1	P2	P1	P2	P1	P1	P2	P2	P1
2	1871	P1	P1	P1	P2	P	P2	P1	P1	P2	P2	P1
3		P1	P1	P2	P2	P1	P3	P2	P1	P3	P3	P3
4	2683	P1	P1	P1	P2	P1	P2	P1	P1	P2	P2	P1
5	1491	P1	P2	P1	P2	P1	P2	P3	P3	P3	P1	P4
6	2343	P2	P2	P2	P1	P1	P1	P1	P1	P1	P1	P1
7	2701	P2	P2	P2	P2	P2	P1	P2	P2	P2	P2	P2
8	1639	P1	P2	P1	P3	P1	P1	P2	P2	P1	P1	P1

ABBREVIATIONS

use short cut in table means, in wchich position is the band

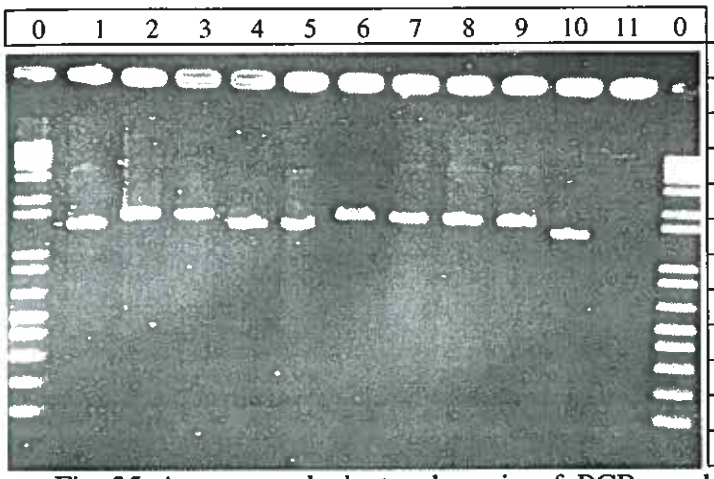
P1	Pattern 1 : level 1 from the start of gel in electrophoresis to detect amplicons
P2	Pattern 2 : level 2 from the start of gel in electrophoresis to detect amplicons
P3	Pattern 3 : level 3 from the start of gel in electrophoresis to detect amplicons
P4	Pattern 4 : level 4 from the start of gel in electrophoresis to detect amplicons

New primer designed for multiplex PCR for Brucella suis

- Single PCR – strain: **Brucella suis**

Table 18 Condition of PCR reaction for primers no. 5

Components of reaction			Condition of reaction	
solution	volume (µl)		Temperature (° C)	Time (second)
Buffer	2,50		95	420
DnTp	2,50		95	35
Primer 1 – 2	2,00		63	45
$MgCl_2$	0,75		72	105
Taq	0,20		72	360
DNA	1, 0		Number of cycles : 30	
H2O	14,05			



No	species	biovarities	strain
1	B. melitensis	1	16M
2	B. abortus	1	45/20
3	B. suis	1	104
4	B.suis	2	24
5	B.suis	2	Thomsen
6	B.suis	3	686
7	B.suis	3	686
8	B.suis	4	40
9	B.suis	4	40
10	B.suis	5	ELF
11	NG	x	x

Fig 25 Agarose gel electrophoresis of PCR products with primers no. 5 (condition of reaction in table 18)

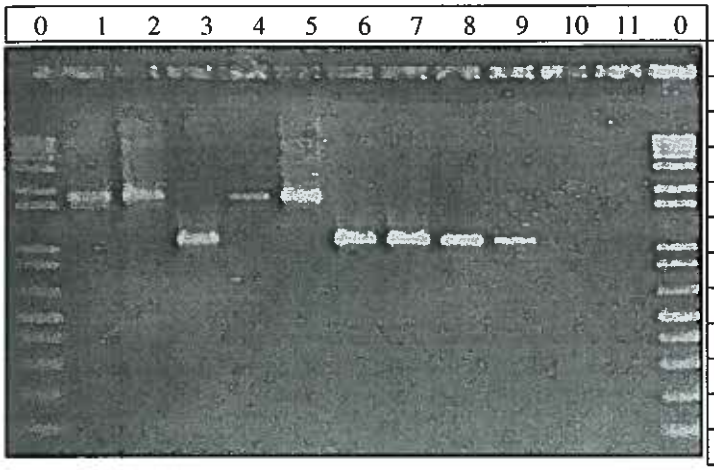
The pair of primer no.5 used for detection of all biovars of Brucella suis, NG is negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard molecular weight of 1 Kb (it was apply in right and left corner in each gel). Numbers of samples agree with those the samples in tables next to the fig. 25.

Fig 25 All of the samples were amplify with pair of primer no 5 in single PCR reaction. It has between each other different level (distinguish distance from start of gel in running electrophoresis). These results are noted in table 17 together with other results. Primers no. 5 is good for further incorporation in multiplex PCR. The condition could not to much change in response of amplification for obtaining representative bands in electrophoreogram.

- Single PCR – strain: **Brucella suis**

Table 19 Condition of PCR reaction for primers s no. 2

Components of reaction			Condition of reaction	
solution	volume (µl)		Temperature (° C)	Time (second)
Buffer	2,50		95	420
DnTp	2,50		95	35
Primer 1 – 2	2,00		63	45
$MgCl_2$	0,75		72	120
Taq	0,20		72	360
DNA	1, 0		Number of cycles : 30	
H2O	14,05			



No	species	biovarities	strain
1	B. melitensis	1	16M
2	B. abortus	1	45/20
3	B. suis	1	104
4	B.suis	2	24
5	B.suis	2	Thomsen
6	B.suis	3	686
7	B.suis	3	686
8	B.suis	4	40
9	B.suis	4	40
10	B.suis	5	ELF
11	NV	x	x

Fig 26 Agarose gel electrophoresis of PCR products with primers no. 2 (condition of reaction in table 19)

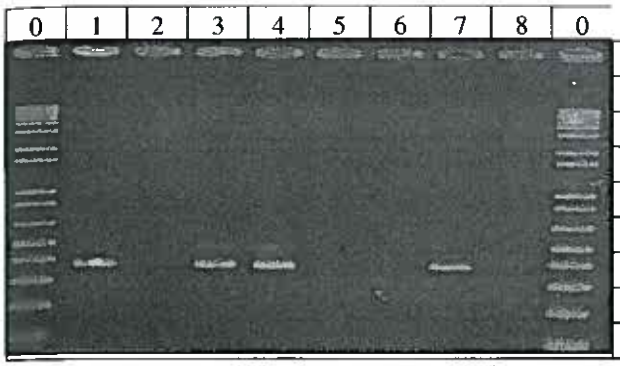
The pair of primer no.2 used for detection of all biovars of Brucella suis, NG is negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard molecular weight of 1 Kb (it was apply in right and left corner in each gel). Numbers of samples agree with those the samples in tables next to the fig. 26.

This condition for pair of primer no. 2 is little bit different against to condition for pair of primers no. 5, but it is fain in single PCR. We calculated with this pair of primers also for further using in designed multiplex PCR, because the amplicons analyzed also as different level or band in strain in running electrophoresis. These results are noted in table 17 together with other results.

- Single PCR – strain: **Brucella suis**

Table 20 Condition of PCR reaction for primers s no. 4

Components of reaction			Condition of reaction	
solution	volume (µl)		Temperature (° C)	Time (second)
Buffer	2,50		95	420
DnTp	2,50		95	30
Primer 1 – 2	2,00		63	45
$MgCl_2$	0,75		72	45
Taq	0,20		72	360
DNA	1, 0		Number of cycles : 25	
H2O	14,05			



No	species	biovarities	strain
1	B.ovis		
2	B.canis		
3	B. neotomae		
4	B.suis	2	Thomsen
5	B.suis	3	686
6	B.suis	4	40
7	B.suis	5	ELF
8	NV	x	x

Fig 27 Agarose gel electrophoresis of PCR products with primers no. 4 (condition of reaction in table 20)

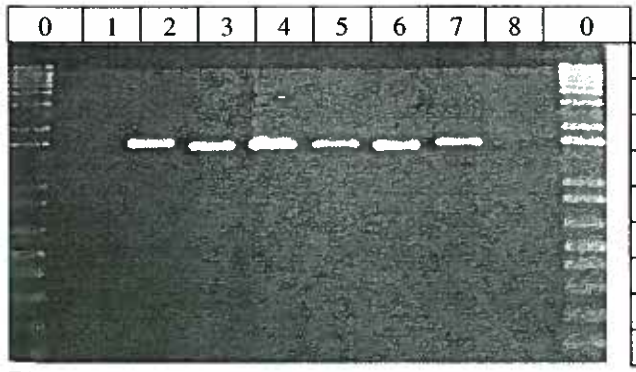
The pair of primer no.4 used for detection of all biovars of Brucella suis, NG is negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard molecular weight of 1 Kb (it was apply in right and left corner in each gel). Numbers of samples agree with those the samples in tables next to the fig. 27.

The pair of primer no. 4 is supposed perspective for multiplex PCR too. The bands look, how we expected. The amplicons were appeared in right strips of comb, probable deletion were detected correctly in genes in individual biovarities of Brucella suis.

- Single PCR – strain: **Brucella suis**

Table 21 Condition of PCR reaction for primers s no.8

Components of reaction			Condition of reaction	
solution	volume (µl)		Temperature (° C)	Time (second)
Buffer	2,5		95	420
DnTp	2,5		95	35
Primer 1 – 2	2		62	45
$MgCl_2$	0,75		64	60
Taq	0,2		72	360
DNA	1, 0		**Number of cycles** : 30	
H2O	14,05			



No	species	biovarities	strain
1	B.suis	5	ELF
2	B.suis	2	Thomsen
3	B. suis	3	686
4	B.suis	4	ATTC
5	B.suis	4	40
6	B.suis	1	104
7	B. suis	5	24
8	NV	x	x

Fig. 28 Agarose gel electrophoresis of PCR products with primers no. 8 (condition of reaction in table 21)

The pair of primer no. 8 used for detection of all biovars of Brucella suis, NG is negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard

molecular weight of 1 Kb (it was apply in right and left corner in each gel). Numbers of samples agree with those the samples in tables next to the fig. 28.

This photo is like the other with using only one pair of primers. The reaction goes well, and it is good point to combination different pair of primers with different sequence of nucleotide together. Further figure represent, if it arise unspecific amplicons or if the reaction is still well specific to multiply right targets DNA.

- Multiplex PCR – strain: **Brucella suis**

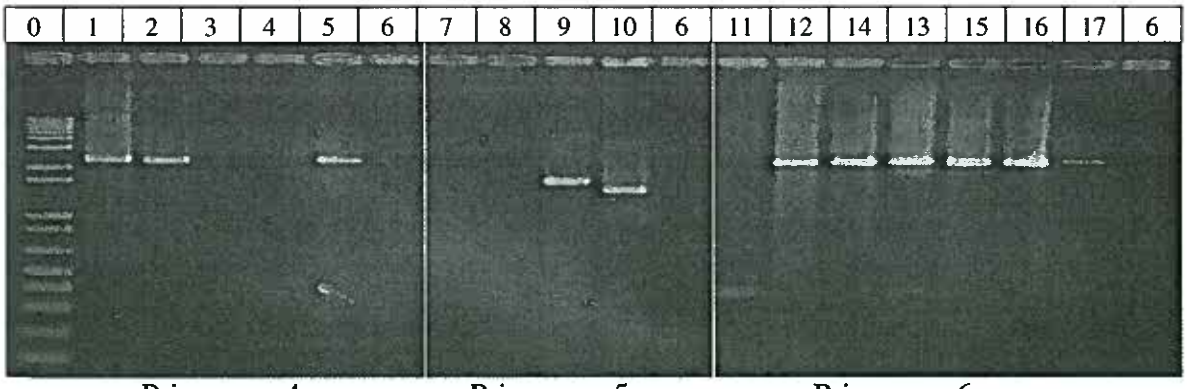
Table 22 summarized 3 pair of primers together. PCR products are in fig. 29, and bordered bands in photos showed, that conditions were suitable for all 3 primers. It is possible in this condition continue for other pair of primers as basis developing new multiplex PCR reaction.

Table 22 Condition of PCR reactions for primers no. 4, 5, 6 together

Components of reaction			**Condition of reaction**	
solution	**volume (µl)**		**Temperature (° C)**	**Time (second)**
Buffer	2,5		95	420
DnTp	2,5		95	35
Primer 1 – 2	2		63	45
$MgCl_2$	0,75		72	180
Taq	0,2		72	360
DNA	1, 0		**Number of cycles** : 30	
H2O	14,05			

Table 23 Survey of samples in fig.29

No	species	biovarities	strain	No	species	biovarities	strain
1	B.melitensis	1	16 M	10	B.suis	5	ELF
2	B.suis	2	Thomsen	6	NV	x	x
3	B.suis	3	686	11	B. ovis		
4	B.suis	4	40	12	B.canis		
5	B.suis	5	ELF	13	B. neotomae		
6	NV	x	x	14	B.suis	2	Thomsen
7	B.suis	2	Thomsen	15	B.suis	3	686
8	B.suis	3	686	16	B.suis	4	40
9	B.suis	4	40	17	B.suis	5	ELF



Primer no. 4 Primer no. 5 Primer no. 6

Fig 29 Agarose gel electrophoresis of PCR products with primers no. 4, 5, 6 (condition of reaction in table 22, order of samples in table 23)

Fig. 29 the pair of primer no. 4, 5, and 6 used for detection of all biovars of Brucella suis, NG is negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard molecular weight of 1 Kb.

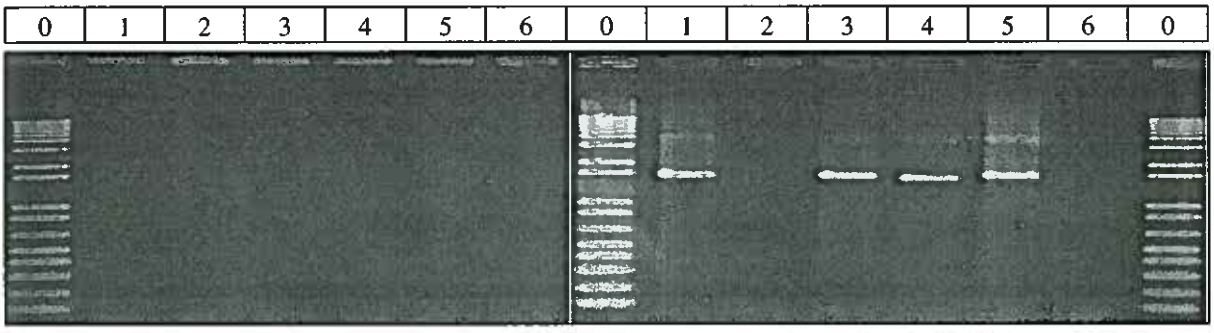
- Multiplex PCR – strain: **Brucella suis**

Table 24 Condition of PCR reactions for primers no. 7, 8 together

Components of reaction		**Condition of reaction**	
solution	volume (µl)	Temperature (° C)	Time (second)
Buffer	2,50	95	420
DnTp	2,50	95	35
Primer 1 – 2	2,00	62	45
$MgCl_2$	0,75	72	60
Taq	0,20	72	360
DNA	1, 0	**Number of cycles** : 30	
H2O	14,05		

Table 25 Survey of samples in fig.29

No	species	biovarities	strain
1	B.melitensis	1	16 M
2	B. abortus		
3	B.ovis		
4	B. canis		
5	B. neotomae		
6	NV	x	x



Primer no. 7 Primer no. 8

Fig 30 Agarose gel electrophoresis of PCR products with primers no. 7, 8 (condition of reaction in table 24)

The pair of primer no. 7, 8 used for detection of all biovars of Brucella suis, NG is negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard molecular weight of 1 Kb (it was apply in right and left corner in each gel). Numbers of samples agree with those the samples in tables 25.

Table 24 summarized 2 pair of primers together. It is other case, which allowed mixing primers. PCR products have bordered bands in fig. 30. Those conditions gave stringent amplicons. The primer no. 7 (fig. 30) has no amplicons, because it is specific for Brucella suis biovars 1, and this strain is not in order of samples to make band in the PCR reaction . The fig is well as we expected. Primers no.7 did not amplicons, and primer no. 8 do amplicons agree with those nucleotide sequence. They are suitable as basis for developing new multiplex PCR reaction.

Table 17 summarized which pair of primers is allowed to mix to give distinguish amplicons in PCR reaction. It is agree with results fig. 29 – 30. They are suitable as basis developing new multiplex PCR reaction.

- Single PCR – strain: **Brucella suis**

Following photos are "not representative". It means that the picture is developing well, but the condition is not all right to give bordering bands. It is necessary to amplify one more time for checking to find, where falls is. Mistakes showed us direction of the way of investigating. It belongs also to result; it showed investigators that it is necessary switch over some parameters of PCR reaction.

Pair of primer no 7 should be specific for Brucella suis 1, but in bad temperature condition couldn't be see. It was necessary to change Tm to obtain representative result, how is written in the articles (see in photo in upper part).

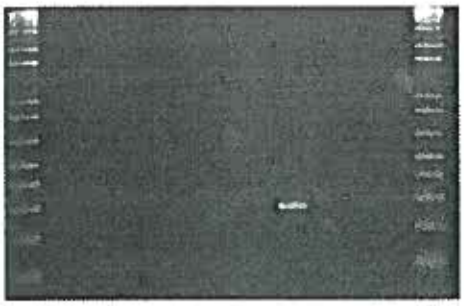


Fig.31 Agarose gel electrophoresis of PCR products with primers no. 7, species Brucella suis, (condition of reaction in table 26)

Table 26 Condition of PCR reactions for primers no. 7, 8 together

Components of reaction		**Condition of reaction**	
solution	Volume (µl)	Temperature (° C)	Time (second)
Buffer	2,50	95	420
DnTp	2,50	95	35
Primer - 1	2,00	62	45
Primer - 2		72	60
$MgCl_2$	0,75	72	360
Taq	0,20		
DNA	1, 0	**Number of cycles** : 30	
H2O	14,05		

This result is not right according to articles [21]. The band is badly amplified in position 7. We expected band in different place in electrophoreogram. That is why we repeated it one more time for convincing of falls, where mistake is.

Similar cases like with pair of primers no.7.

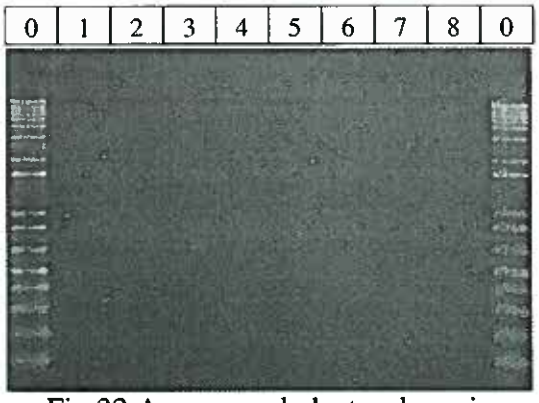


Fig.32 Agarose gel electrophoresis of PCR products primer no.3

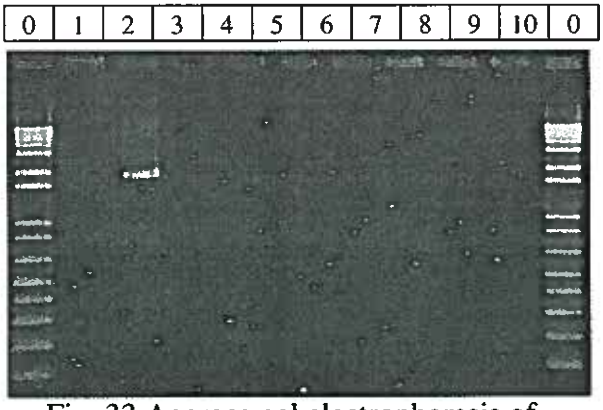


Fig. 33 Agarose gel electrophoresis of PCR products primer no.7

Several PCR assays have been reported and in laboratory of microbiology department, University of Navarra developed to identify the genus Brucella and discriminate among species.

The identification of Brucella phenotypes by conventional methods is dependent on the isolation of pure strains of Brucella. Contamination by other bacteria can occur in the tissues used to isolate Brucella [20]. An extra step to remove these other bacteria is necessary, based on the observation that the genetic element IS 711 occurs at several species-specific or biovars-specific chromosomal loci. So the effect of foreign DNA on the assay to identify stands like other methods at the start with problems of good laboratory practice.

Individual biovars of Brucella strain within a species are not differentiated, and task of experiment 1 were to solve this problem. Swine brucellosis is caused by the biovars 1, 2 and 3 of Brucella suis. The identification up to now relies on microbiological tests lacking, but it has not adequate specificity together with time consuming and expensive. Molecular procedures as PCR can helps to be more safety, faster and unfailing to detect this bacterium.

The assay exploits the polymorphism arising from species-specific localization of the genetic element IS711, and omp2b gene in the Brucella chromosome. Identity is determined by the size(s) of the product(s) amplified from primers hybridizing at various distances from the element. . The assay exploits the single nucleotide polymorphisms found in omp2b gene of B. suis reference biovars which are conserved in B. suis field isolates from different geographic origins and hosts. However, some B. suis field isolates identified as biovars 2 or 3 according AMOS-PCR, PCR-RFLP of omp31 and omp2 genes and classical bacteriological methods, resulted also in S1 patterns, limiting the typing usefulness of the method. [21]

We try to describe a multiplex PCR where should be comprises 7 oligonucleotide primers, which can identify individual biovars of Brucella suis. Until the steps where were mix 3 pair of primers shows that is good background for the further investigating in multiplex assay and the purpose were realized.

4.3. Expression of Brucella pinnipediae, Brucella cetacean

In second part of my project was to confirm or to exclude the hypothesis, if there exist exactly 2 new strains special for sea mammals.

Some articles [10, 19] showed in recent studies, that Brucella strains are able to infect mammals in sea too. These strains were identified as brucellae by them colonial and cellular morphology, staining characteristics, biochemical activity....

I try with some pairs of primers support this theory. In single PCR with 7 pairs of primers in following results, you can see that only in one photo of gel is distinguish in amplifying DNA of different biovarities of "Brucella maris".

Table 27 Order of samples responding to fig. 34 - 38, species: Brucella cetaceae, Brucella pinnipediae

No	species	strain
1	Brucella cetaceae	MCITA
2	Brucella pinnipediae	seal
3	Brucella cetaceae	porpoise
4	Brucella cetaceae	dolphin
5	Brucella maris	MS 05-684
6	Brucella maris	M3 97-7763
7	Brucella melitensis	16 M
8	Brucella abortus	45/20
9	Brucella suis	686
10	NV	x

- Single PCR – strain: Brucella maris

Table 28 Condition of PCR reactions for primers no. 1, 2

Components of reaction		Condition of reaction	
solution	volume (µl)	Temperature (° C)	Time (second)
Buffer	2,50	95	420
DnTp	2,50	95	45
Primer 1 – 2	2,00	62	45
$MgCl_2$	0,75	72	120
Taq	0,20	72	360
DNA	1, 0		
H2O	14,05	Number of cycles : 30	

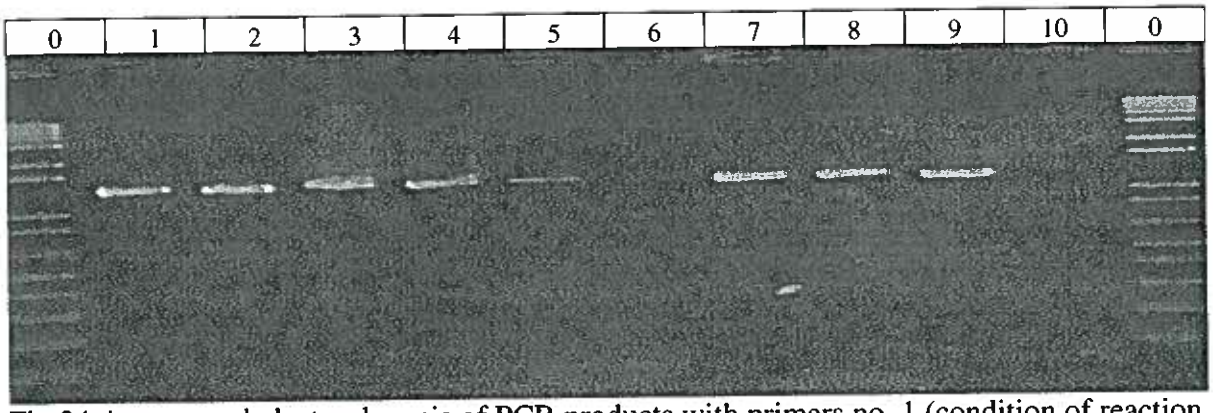
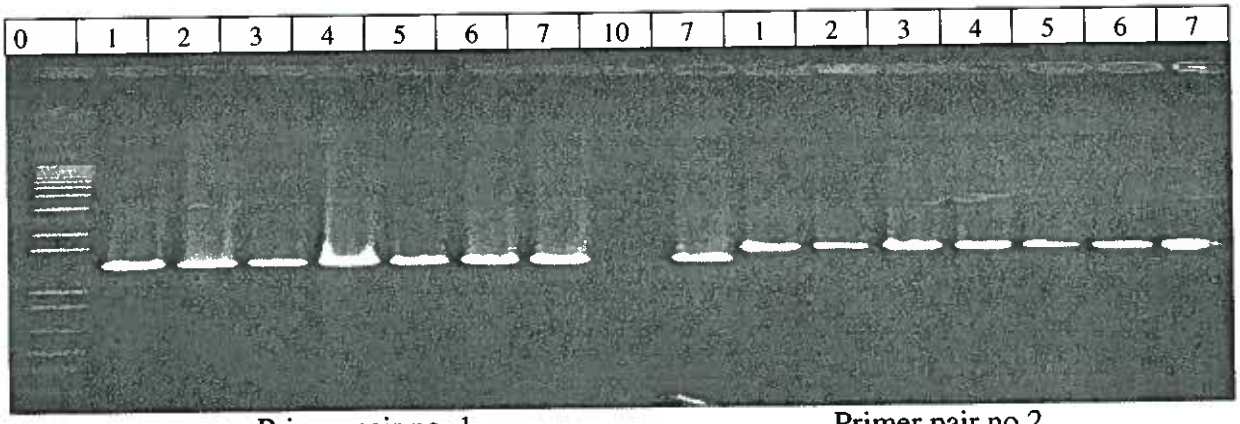


Fig.34 Agarose gel electrophoresis of PCR products with primers no. 1 (condition of reaction in table 28)

Fig.34 this pairs of primer 1 is not such significant for eventually projecting PCR reaction, because strains of Brucella maris are seem as other species of Brucella. This pair of primer has no sense for detect eventually deletion in Brucella maris, primers are not suitable differentiate strain between each other (fig.30).

The pattern were not enough stringent, we decide to increase the volume of primer, in this case to obtain better result. It means that was used instead of 1 µl 2 µl of primers.



Primer pair no. 1 Primer pair no.2

Fig.35 Agarose gel electrophoresis of PCR products with primers no. 1. 2 together (condition of reaction in table 28)

Fig.35 It was used same primer as for fig. 34 and added one more primer pair with same condition for reaction. The primers were combined in one thermocycler, but with two mixtures separated. Primer 1, 2 were used for detection of biovars of Brucella suis, NG is

negative control of reaction (instead sample is H_20). Position no. 0 is DNA leader with standard molecular weight of 1 Kb. Numbers of samples agree with those the samples in tables 27.

- Single PCR – strain: Brucella maris

Table 29 Condition of PCR reactions for primers no. 2

Components of reaction			**Condition of reaction**	
solution	volume (µl)		Temperature (° C)	Time (second)
Buffer	2,50		95	420
DnTp	2,50		95	45
Primer 1 – 2	2,00		62	45
$MgCl_2$	0,75		72	45
Taq	0,20		72	360
DNA	1, 0		**Number of cycles** : 30	
H2O	14,05			

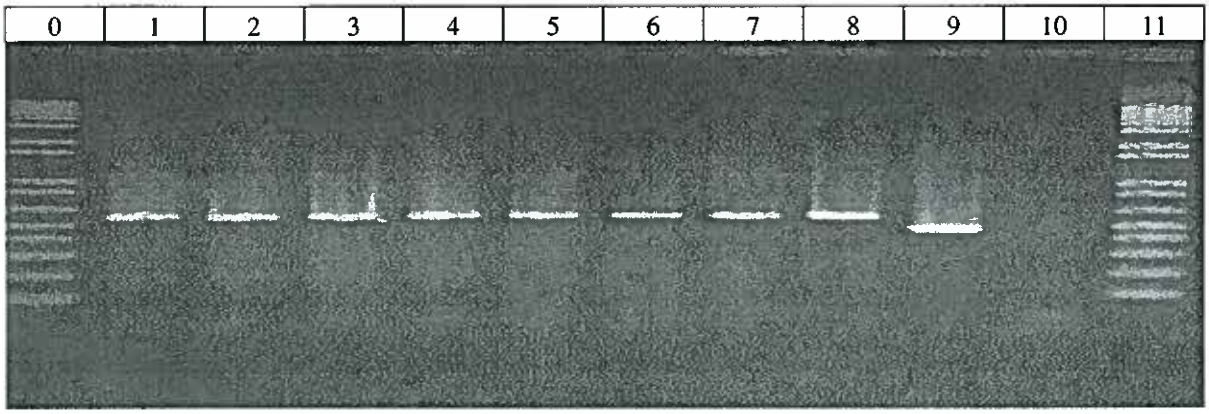


Fig.36 Agarose gel electrophoresis of PCR products with primers no. 2 (condition of reaction in table 29)

Fig. 36 showed that Brucella suis has different sequence in targets part of DNA, although in this part of experiment is not important, because sample in 9 position is Brucella suis. We need different distance from start of gel in position 1-6. The primers number 2 could not help to clear up the hypotheses of this experiment.

- Single PCR – strain: Brucella maris

Table 30 Condition of PCR reactions for primers no. 3

components of reaction			**Condition of reaction**	
solution	volume (µl)		Temperature (° C)	Time (second)
Buffer	2,50		95	420
DnTp	2,50		95	45
Primer 1 – 2	2,00		62	45
$MgCl_2$	0,75		72	45
Taq	0,20		72	360
DNA	1, 0		**Number of cycles** : 30	
H2O	14,05			

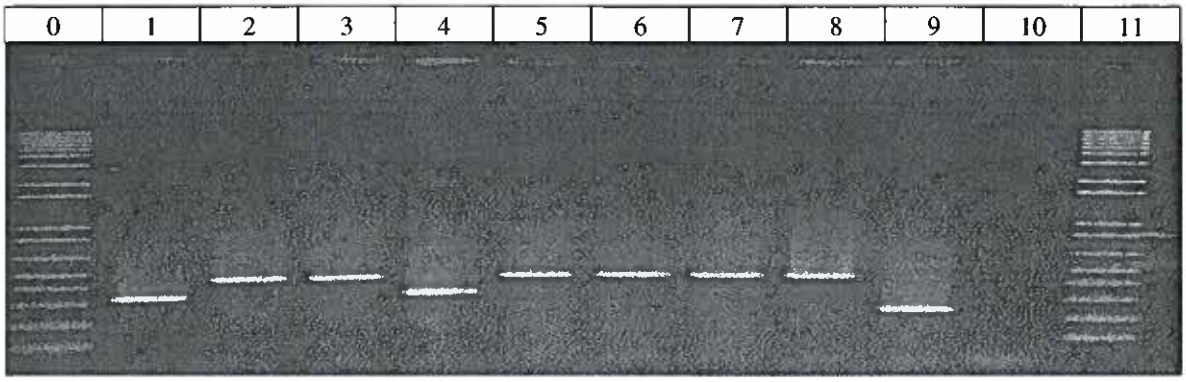


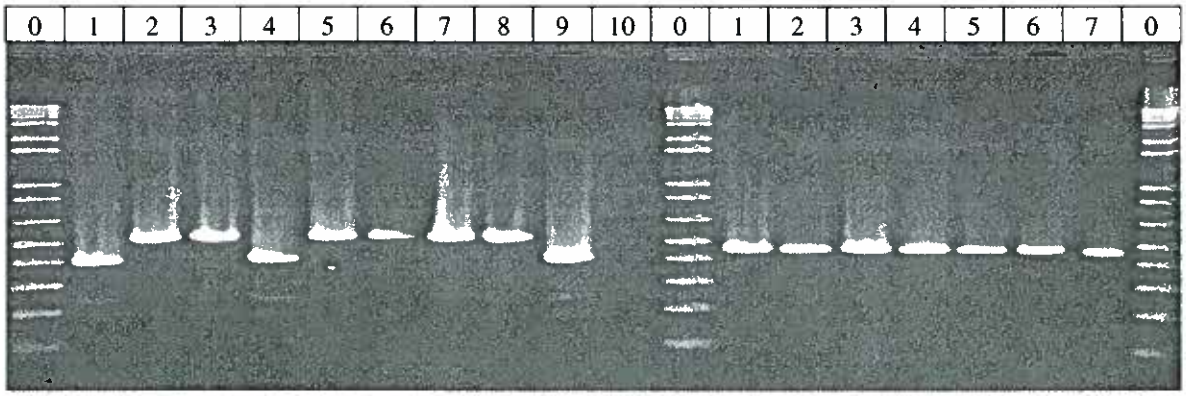
Fig. 37 Agarose gel electrophoresis of PCR products with primers no. 3 (condition of reaction in table 30)

Pair of primers number 3 was the most interesting in this experiment. It shows that idea about enlargement of taxonomy of family Brucellae is seems to be correct. The samples in position no. 1, 4 are in different level – respectively in different distance from start of gel. Sample in position no. 9 is representative of "old" group of Brucella species. Sample 9 is in comparison in different level from amplicons of sample 1, 4. It means that they have distinguished sequence nucleotide and primer no. 3 is able to recognize it.
The fact could be basis for future designed new multiplex PCR assay.

- Single PCR – strain: Brucella maris

Table 31 Condition of PCR reactions for primers no. 4, 5 together

Components of reaction			**Condition of reaction**	
Solution	Volume (µl)		Temperature (° C)	Time (second)
Buffer	2,50		95	420
DnTp	2,50		95	45
Primer 1 – 2	2,00		62	45
$MgCl_2$	0,75		72	45
Taq	0,20		72	360
DNA	1, 0			
H2O	14,05		**Number of cycles** : 30	



Primer pair no. 4 Primer pair no. 5

Fig.38 Agarose gel electrophoresis of PCR products with primers no. 4, 5 together (condition of reaction in table 31)

Fig.38 shows "double PCR", where two mixtures were used with same condition but with different pair of primers in one PCR cycler. Primer no.4 amplifies fragments similar to result in fig. 37 by primers no. 3, but the bands have distinguished molecular weight. The pair of primer no. 5 (part behind the strips of the marker in the middle) is not shows, on the other hands, any of interesting distinguished between individual of strains Brucella.

The existence of two new strains of Brucella cetaceae and Brucella pinnipediae was not clear. It were investigated Brucella maris with same pair of primer like for experiment 1. The goal of this experiment was certified the new primers also if it is able to detect any deletion of nucleotides specific for the Brucella maris. Primers should finally recognized Brucella suis and Brucella maris between each other. [20].

Studies mainly by Cloeckaert, the significant proof was presented that Brucella genus should be extending with B. cetacean and B. pinnipediae. It was confirmed according to articles, that Brucella maris has a specific marker of marine mammal Brucella isolates, consisting of an IS711 element downstream of the bp26 gene. [10]

The results represent, only in small amount of PCR reaction that Brucella cetaceae and Brucella pinnipediae has different deletion in comparison to six acknowledged representative of Brucellae family.

Experiment 2 valued only as the really start, because the unknown sequence of new no defined genome of Brucella maris is not describe enough by only this limited pair of primers.

5. SUMMARY

Conditions for isolation of DNA and its use in new design of primers were tested. The sequence of primers and optimal condition should serve as the basis for future multiplex PCR assay of Brucella genus.

The new designed pair of primers was proved on the cell line of Brucella genus. These cell lines were verified before using in experimental part. DNA for PCR reaction is prohibited to contain contaminants. This fact were controlled by validated multiplex PCR and alone electrophoresis.

Conclusion from the experiment

Experiment 1

The newly designed pair of primers gave a good basis for the further multiplex PCR of Brucella suis.

Experiment 2

Single PCR showed that, the individual amplicons are at different levels, which mean that, they are not identical.

The results support the hypothesis the existence of two new species of Brucella strains.

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