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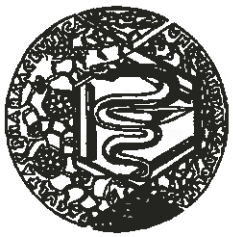

Control positive and negative in construction of microarrays of *Brucella* DNA

Diploma thesis

Supervisors: Prof. Ignacio López-Goñi
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Pamplona 2005/2006

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Pozitivní a negativní kontrola v konstrukci microarray DNA bakterie *Brucella*

Diplomová práce

Vedoucí práce: Prof. Ignacio López-Goñi
Prof. RNDr. Eva Kvasničková, CSc.

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I would like to thank to Prof. Ignacio López-Goñi for professional supervising. Many thanks to Cristina Viadas Martínez for extensive support and many help. My thanks go to Departmento de Microbiologia for nice working environment. I also want to thank to Prof. RNDr. Eva Kvasničková, CSc. for guiding discussions and providing huge assistance.

Content

Brucellosis is one of fifth most common zoonosis in the world. It is virulent for man. Contemporary vaccines are not effective for eradication. Mechanisms of *Brucellas* virulence are subject of study.

Crucial role of *Brucellas* virulence could play two-component system BvrR/BvrS. BvrR/BvrS regulates gene expression of outer membrane proteins on transcriptional level. This system controls an adaptive answer of bacteria in variable conditions, where bacteria occur during infection. Characterization of structural and regulator genes participated in pathogenic features is a basic task for control of disease. Recognition of these genes can help to develop new effective vaccines.

Molecular genetic method, DNA microarrays, allows to study expression of all genes of organism in variable conditions of grow. Brucellas genes coding proteins (ORFs – open reading frame) are printed on a suitable support (array). Arrays are hybridized with cDNA from classic and mutant culture. cDNA reflects gene expression and it is labelled with dye Cyb 3 and Cyb 5. Bounded cDNA generates signal.

Essential role of microarray plays controls. Microarrays include two types of control – control positive and control negative. Control positive is PCR fragment, which represents a gene of *Brucella.* It should be always expressed in variable conditions. Signal generated by control positive confirms that experiment passed well. Control negative is PCR fragment from non-*Brucella* organism. It's gene of plant Arabidopsis thaliana. It should never generate signal in *Brucella* DNA arrays.

This work was part of the research project Molecular Characterization of the Pathogenicity of Brucella using Microarray Technology, which is part of research funding by national and international agencies on Departmento de Microbiologia, Universidad de Navarra. This diploma thesis is focused on control positive and negative of *Brucella* DNA microarrays. My aim was to confirm specificity and effectiveness of microarrays control using principally similar method - Southern blot.

1. Introduction

1.1 Brucella and Brucellosis

1.1.1 Organism

All *brucellae* are obligate intracellular parasites. *Brucellas* are gram-negative bacteria causing one of the fifth most frequent bacterial zoonosis in the world, called brucellosis. *Bruccellae* were first isolated in 1887 from spleen of British soldiers on the island of Malta (Malta fever). The second organism of the group was isolated in Denmark by Bang in 1897 from cattle suffering from infection abortion (Bang's disease) and the third was cultured in the U.S. in 1914 from the foetus of prematurely delivered sow (Brock and Madigan, 2000).

Brucellae are small, gram-negative coccobacilli. They form small, convex, smooth, moist – appearing, nonhemolytic, and transculent colonies. (Brock and Madigan, 2000) The genus *Brucella* consists of six species, designated based on differences in pathogenicity and host preference as *Brucella melitensis* (goats and sheep), *B. abortus* (cattle and bison), *B. suis* (infecting primarily swine, but also hares, rodents and reindeer), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (wood rats). The discovery of *Brucella* bacteria in marine mammals has led to the proposal of two additional species: *B. cetaceae*, infecting cetaceans, and *B. pinnipediae*, infecting pinnipeds. The phenotypic difference and host preference can be attributed to various proteomes, as exemplified by specific outer-membrane protein markers (Manterola et al., 2005). The complete sequencing of the B. melitensis genome was achieved in 2002. The complete sequencing of *B. abortus* and *B. suis* has recently been accomplished as well (Pappas et al., 2005).

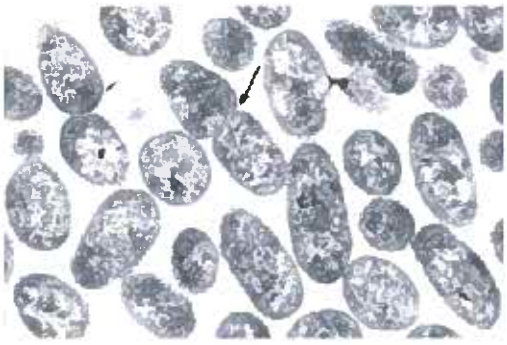

Fig. 1: Brucella melitensis (www.conspiracyarchive.com/images/b/.)

1.1.2 Virulence

Intracellular bacterial infection requires significant interaction between host and pathogen and comprises four steps: adherence, invasion, establishment, and

dissemination. To survive and multiply in host cells, bacteria sense the environment, activate various genes, and secrete proteins that facilitate bacterial adaptation (Eskra et al., 2001). *Brucella* is unusual in several ways: the bacterium does not bear classic virulence factors, such as exotoxins or endotoxins. It exhibits a tendency to invade and persist in the human host through inhibition of programmed cell death (Pappas et al., 2005). Because of its absence of classical virulence factors, *Brucella* has been called "furtive nasty bug". From classical virulence factors (such as envelope molecules, secretion system, flagella, regulation, metal acquisition, stress....) are presented:

O-lipopolysacharide: O-lipopolysacharide (O-LPS) was shown to be involved in inhibition of phagocytosis, protection against bacterial killing inside the phagolysosome and inhibition of the host cell apoptosis.

Type IV of secretion: Type IV of secretion (T4SS) of *Brucella* encoded by the virB operon is a major virulence factor. Type IV secretion system plays a key role in the "vacuolar-jacking" (Dricot et al., 2004). T4SS delivers macromolecules between bacteria and eukaryotic cells. T4SS differs among *Brucella* species and in response to environmental stress (Haine et al., 2005)

Regulation: Bacteria coordinate the expression of an intricate network of factors as an adaptive response mediated by two-component regulatory systems (TCSs) and by the transcriptional regulators. One of these systems is BvrR/BvrS system. BvrS/BvrR system plays a role in the homeostasis of the bacterial surface as well as in setting up the structures required for parasitism. Function of this regulatory system is incompletely understood. It is known that *B. abortus* BvrS/BvrR system regulates transcription of at least two major outer membrane proteins (Omps), which has been implicated in virulence. Dysfunction of the BvrR/BvrS system alters the outer membrane permeability, the expression of several groups of three outer membrane proteins and the pattern of lipid A acylation. It causes LPS structural changes. It has an important effect on HeLa cell pathogen interactions. At the step of penetration, this TCS is involved in the requirement of small GTPases that are required for actin-dependent cell penetration (Haine et al., 2005).

Virulence factors are not yet exactly known. They are still subject of study.

1.1.3 Pathogenesis and clinical features

The organism gain entrance via the broken skin, the conjunctivae, the alimentary tract, or possibly the aerosol routine. In the skin or mucous membranes, the organisms are ingested by polymorphonuclear cells. After ingestion, the majority of brucellae are rapidly eliminated by phagolysosome fusion. They internalize into non-professional phagocytes. Internalization requires the expenditure of energy, and inhibitors of energy metabolism and receptor-mediated endocytosis can suppress this response. BvrS/BvrR system codes for a histidine kinas sensor and controls the expression of molecular determinants necessary for cell invasion. Bacterium replicates in the endoplasmic reticulum without affecting host – cell integrity. Intracellular movement is mediated by induction of virB operon through type IV. secretion system. After replication, brucellae are released with the help of haemolysins and induced cell necrosis (Pappas et al., 2005). Ability of *Brucella* to evade immune detection and adaptation to intracellular survival inside both phagocytic and nonphagocytic cells is related to its chronicity. Chronicity depends on the organism ability to escape host defences. Successful strategies for intracellular survival include the ability to survive in acidified membrane-bound vesicles, inhibition of macrophage apoptosis, prevention of phagosome-lysosome fusion and detoxification and repair mechanisms (Eskra et al., 2001)

Four species of *Brucella* can cause human disease: *B. mellitensis, B. abortus, B. suis, and B. canis*. The transmission of brucellosis to humans occurs through the consumption of infected, unpasterized animal-milk products, through direct contact with infected animal's parts and through inhalation of infected aerosolized particles. The incubation period in human brucellosis is long, often several weeks or months. After entering the human body and local tissue lymphocytes, *Brucella* multiply within them, and are carried via the lymphatic system to regional lymph nodes. There the bacteria enter and multiply within mononuclear cell activation and proliferation. The outcome of this confrontation determines whether the inactive infection is contained. If not, PMNs and mononuclear cells carrying the bacteria reach the blood and soon accumulate in the sinusoids of the liver. These focal aggregations of Kupffer cells containing large number of organisms develop and after another few days form typical small granulomas cell necrosis, similar lesions appears in the in spleen, bone, marrow, and kidney. In certain mammals (cattle, swine, sheep, goats…) *Brucella* also accumulate in mammary

glands, in the genital organs and in the pregnant uterus (Brock and Madigan, 2000). The host response in humans reflects unique features of *Brucella*. Smooth lipopolysacharide does not activate the alternative complement pathway. *Brucella* is resistant to damage from polymorphonuclear cells. An increase of CD4 and CD8 lymphocytes, as the importance of a T-cell receptor, is characteristic for brucellosis. Interferon-γ has a central role in the pathogenesis of brucellosis by activating macrophages, producing reactive oxygen species and nitrogen intermediates, by inducing apoptosis, enhancing cells differentiation and cytokine production, by converting imunoglobin G to imunoglobin G2a, and by increasing the expression of antigen presenting molecules (Pappas et al., 2005).

The onset of symptoms is usually insidious, with malaise, chills, fever, sweats, weakness, myalgia, and headache. Fever may be remittent, particularly with *B. melitensis* (undulant fever). Vague GIT and nervous symptoms are common. Acute illness may be associated with enlarged lymph nodes, spleen and liver (Brock and Madigan, 2000). Osteoarticular disease is universally the most common complication of brucellosis, and three distinct forms exist – peripheral arthritis, sacroliitis and spondylitis. The reproductive system is the second most common site of focal brucellosis. Brucellosis in pregnancy poses a substantial risk of spontaneous abortion. Hepatitis is common, usually manifesting as mild transaminemia. The central nervous system is involved in 5 to 7 percent of cases in most studies. Bacteraemia is present in more than 20% of cases. Meningoencephalitis, osteomyelitis, endocarditis and intestinal nephritis with focal glomerular lesions sometimes occur in the course of the acute disease. The blood count is often characterized by mild leucopoenia and relative lymphocytosis. Circulating Abs are detectable from the time the first sings and symptoms of the disease develop. These Abs do not prevent bacteraemia or reinfection. However, seropositive abattoir workers are less likely to develop clinical brucellosis. The chronicity depends on the marked capacity for multiplication in phagocytic cells. Relapses, at a rate of about 10 percent, usually occur in the first year after infection. Childhood brucellosis generally exhibits a more benign course in terms of the rate and severity of complications and the response to treatment (Pappas et al., 2005).

1.1.4 Treatment and vaccines

Treatment of human brucellosis should involve antibiotics that can penetrate macrophages and can act in the acidic intracellular environment. In 1986, the World Health Organization issued guidelines for the treatment of human brucellosis. The guideline discusses two regiments, both using doxycycline for period of six weeks, in combination with either streptomycin for two to three weeks or rifampin for six weeks. Alternative drug combinations have been used, including other amino glycosides (e.g., gentamicin and netilmicin). Trimethoprim – sulfomethoxazole is a popular compound in many areas, usually used in triple regimens. Quinolones are an alternative. The protracted administration of triple regimens is used for neurobrucellosis. Rifampin is mainstay of treatment in cases of brucellosis during pregnancy, in various combinations that are based on rifampin and trimethoprim – sulfamthoxazole and with aminoglycosides. A human vaccine has not been developed for brucellosis. Numerous vaccines have been used in past, but none have gained wide acceptance (Pappas et al., 2005).

Rev.1 vaccine is an attenuated, live *B. melitensis* strain originated from a virulent *B. melitensis* isolate, which became dependent on streptomycin for its growth but lost this characteristic upon further culture. It gives protection to sheep and goats against infection with *B. melitensis* and to rams against infection with *B. ovis*. This vaccine is attenuated, but it retains some virulence. Depending on the dose administered during pregnancy, abortions will occur frequently; apparently in rams the vaccine is avirulent or of low virulence. The use of Rev. 1 in cattle has been investigated and results indicate that Rev.1 protects well. Although live attenuated vaccines played a crucial role in successful eradication programs (*B. Abortus* strain 19 and *B. Mellitensis* Rev1), these vaccines remain virulent for man. Several other live vaccines have been used in the past although they have not been adopted widely or their use has been discontinued for a variety of reasons (Schuring, 2005).

Available vaccines alone are not sufficient for elimination of brucellosis in any host's species. New brucellosis vaccines with high efficacy and safety are needed. Development of safer and more efficacious vaccines alone, or combined with enhancements or increased emphasis on other regulatory program components, could have tremendous impact on reducing the worldwide prevalence of brucellosis and the

associated zoonotic infections. In general, antibodies are considered to play a minor role in protecting cattle against *B. Abortus*, whereas long-term protection is believed to be associated with stimulation of cellular immunity. Vaccines field of efficacy could be influenced by other factors, such as nutrition, environmental stress or concurrent infection with other microorganisms. Abortion and infection rates are directly related to the exposure dose of virulent *B. Abortus* strain, and high-exposure dosages can overwhelm vaccine-induced protection. Vaccination alone is effective in reducing disease prevalence but is unlikely to eliminate brucellosis from an infected herd (Olsen and Stoffregen, 2005). An understanding of the expression of bacterial genes, both in vivo and in vitro, will provide valuable information for vaccine development (Eskra et al., 2001).

Brucella is also considered as a potential agent of biological warfare or terrorist threat, largely because of its chronic debilitating clinical course, problematic treatment, and lack of suitable vaccines (Dricot et al., 2004).

Eradication of brucellosis depends largely on socioeconomic and political circumstances. Progress in understanding the molecular pathogenesis of disease, vaccine engineering, and postgenomic approaches may lead to new preventive interventions.

1.2 Molecular – genetic methods

1.2.1 Chemical isolation of DNA

Chemical isolation of DNA is a method to obtain nucleic acid available for detection (PCR, blotting...). Optimal extraction protocol should release DNA from an array of target organisms with equal efficiencies and wash out inhibitory factors from various sample types without introducing bacterial DNA contamination to the amplification reaction. There are several methods for bacterial DNA extraction from different material (bacterial colonies, blood, tissues...). They are based on chemical/mechanical lysis of the cell structure to release and separate nucleic acid from bacterial cell. Physical cell wall disintegration methods are for example bead beating and sonication. Bacteria are first incubated with the appropriate enzymes to ensure efficient cell lysis and DNA release from the cells. Then they are lysed in a chaotropic salt-containing solution. Anionic detergent breaks down fatty acids and lipids associated with cell membrane. Heat can help to denature proteins and aid cell lysis. Vortexing

creates the combined chemical/ mechanical lysis conditions required to release desired nucleic acids from microbial cells. Many cell types will not lyse without this chemically enhanced bead beating process. DNA is bound to the silica-based membrane and the remaining lysate is removed by centrifugation. After washing to remove contaminants, the DNA is eluted with buffer into a collection tube (Turner et al., 2000).

1.2.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method used to amplify a sequence of DNA. Amplification is using a pair of oligonucleotide primers, each complementary to the end of the DNA target sequence. There are three major steps in a PCR, which are repeated in cycles. The starting material for polymerase chain reaction is a sample of chromosomal DNA, also called genomic DNA. In addition to the target DNA, a PCR reaction contains several other components:

- DNA template, or cDNA which contains the region of the DNA fragment to be amplified
- two primers which determine the beginning and end of the region to be amplified
- *Taq* polymerase which copies the region to be amplified
- deoxynucleotides-triphosphate, from which the DNA-Polymerase builds the new DNA
- buffer which provides a suitable chemical environment for the DNA-Polymerase

The primers need to be about 18-20 nucleotides long and have similar G + C content, so they anneal to their complementary sequences at similar temperatures. Primers are designed to anneal on opposite strands of the target sequence, so that; they will be extended towards each other by addition of nucleotides to their 3'ends. DNA *Taq* polymerase is derived from thermostable bacterium *Thermus aquaticus*. Operates at 72° C and is reasonably stable above 90° C. The PCR reaction is carried out in a thermal cycler. This machine 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 (typically volumes between 15-100µl per tube), 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.

A PCR reaction lasts several hours and typically consists of 20 to 35 repeating cycles.

Each cycle consists of three steps:

- denaturation
- annealing
- extension

A cycle begins by heating the reaction mixture to 95° C. The heat denatures the DNA, breaking the hydrogen bonds that hold the strands together. 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 they are now single-strand only. Time: 1-2 minutes up to 5 minutes. In addition, certain polymerase are activated at this step Hot-start PCR. After denaturing the DNA, the temperature is reduced, so that the primers can anneal with their complementary sequences in the target DNA. The temperature of this stage depends on the primers and is usually 5°C below their melting temperature (45-60°C). 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. In the next phase, the temperature is raised to 72°C. Taq polymerase functions optimally at this temperature and begins polymerization, adding nucleotides to the 3'end of each primer attached to a DNA strand. After one complete cycle, there are two double-stranded copies of the target DNA. The PCR reaction mixture contains many copies of the primers and an abundant supply of nucleotides to perform many additions cycles. A final extension step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied. This differs from all other extension steps only in that it is longer, typically 10-15 minutes. In the first polymerization step, the target is copied from the primer sites for various distances on each target molecule until the beginning of cycle 2, when the reaction is heated to 95°C again, which denatures the newly synthesized molecules. If PCR were 100% efficient, one target molecule would become 2^n after n cycles. The PCR product can be identified by its size using agarose gel electrophoresis.

There exists many modifications of PCR (multiplex PCR, nested PCR, inverse PCR, RT-PCR, asymmetric PCR, quantitative PCR, quantitative real time PCR, touchdown PCR, colony PCR). Multiplex PCR is one of those modifications using more than one pair of primers. PCR can be used for a broad variety of experiments and analyses. For example genetic fingerprinting, paternity testing, detection of hereditary

diseases, cloning genes, mutagenesis, analysis of ancient DNA, genotyping of specific mutations, comparison of gene expression…(Turner et al., 2000).

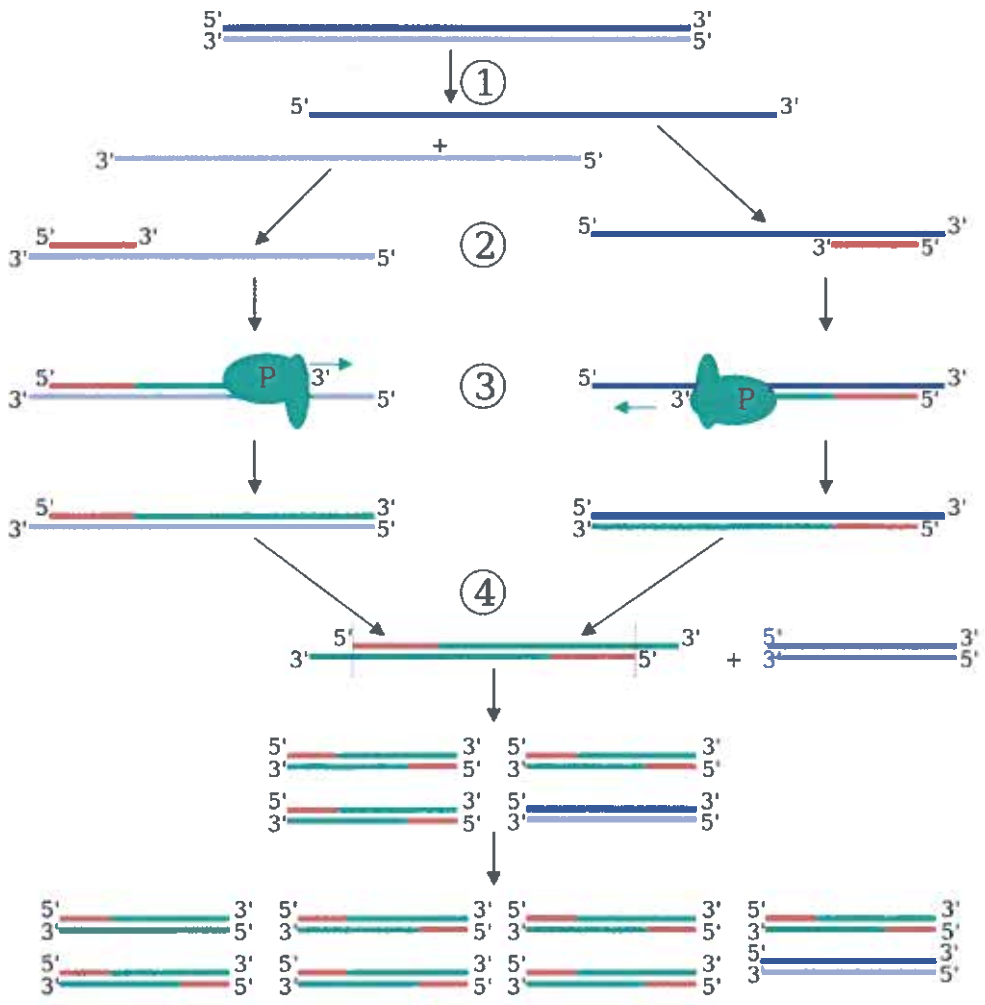

Fig. 1: Mechanism of PCR reaction (http://en.wikipedia.org/wiki/PCR)

1.2.3 Electrophoresis

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely used techniques in biochemistry and molecular biology. When charged molecules are placed in an electric field, they migrate toward either the positive or the negative pole, according to their charge. In contrast to proteins, which can have a net either positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Proteins and nucleic acids are electrophoresed within a matrix or "gel". Most

commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. 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 agarose concentration strengthens the gel. Agarose gels have an extremely easy preparation: mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic. To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, and then heated in a microwave oven until completely melted. Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the $\log_{10}$ of either their molecular weights or number of base pairs, a roughly straight line will appear. DNA fragments are visualized by staining with ethidium bromide. To visualize DNA or RNA, the gel is placed on an ultraviolet transilluminator. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels (Turner et al., 2000).

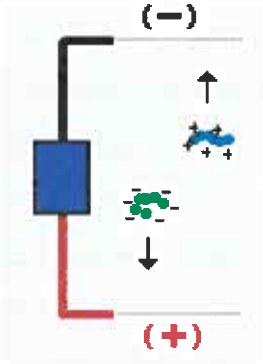

Fig.2: Principal of electrophoresis
(http://www.vivo.colostate.edu/hbooks/genetics/biotech/gels/principles.html)

1.2.4 Southern blotting

Southern blotting (named after its inventor, E.M. Southern) was developed as a method to separate and analyze digested genomic DNA in gels. DNA is digested with specific restriction enzyme. Digested fragments are separated on agarose or

polyacrylamide gel dependently on molecular mass and charges (electrophoresis). After electrophoresis and denaturing the DNA in alkali solution, it is transferred to a membrane surface where DNA fragments of interest are identified by hybridization with labelled, complementary, gene-specific DNA probes. Transfer the fragments on nitrocellulose or nylon membrane can be performed by electrotransfer, capillary action or using vacuum. Vacuum blotting is fast and has excellent recoveries. DNA is fixed into the membrane by baking in the high temperature (nitrocellulose) or cross-linking through the exposure to UV light (nylon).

Hybridization is an incubation of the DNA on the stable matrix (membrane or filter) with a DNA probe. Hybridization efficiency and specificity in Southern analyses depends on temperature, ionic strength, destabilizing agents, mismatched base pairs, duplex length, viscosity and base composition. Temperature of hybridization influences particularity of bound probes. The probe carries DNA sequence from the region of DNA that is of interest. Double stranded probes have to be denatured first. Generation of signal is ensured by labelling of probes. Labelling can be radioactive, fluorescent and chemiluminiscent. Traditionally, the DNA probe (cDNA clone, oligonucleotide, genomic fragment) in Southern blotting procedures is labelled with radioactive phosphorus (e.g., ^{32}P) or sulphur (e.g., ^{35}S) by incorporation of radiolabelled nucleotides during its synthesis. In recent years, alternative, non-isotopic methods have largely replaced radioactive systems. Chemiluminiscent detection is based on specific enzymatic reaction (horseradish peroxidase, alkaline phosphatase) what generates light within seconds that continues at least 6 to 24 hours. It is easy to use, and there is no problem with waste.

There are two popular membrane materials used in nucleic acid blotting. These materials are nitrocellulose (including supported nitrocellulose), and nylon (charged and uncharged). Nitrocellulose is the traditional support medium, and it is still employed by many researchers. In a high salt aqueous environment, nitrocellulose will bind to nucleic acids tightly but non-covalently. After transfer, nucleic acid binding is stabilized by baking the filter for 1 hour. The baked filter can be stripped and reprobed, but because the bound nucleic acid is not covalently linked to the membrane, significant signal loss can occur. A major drawback to nitrocellulose membranes is that they are quite fragile, requiring very careful handling to preserve the blot intact.

Nylon filters have gained great popularity since their introduction, their most obvious advantage over nitrocellulose being their durability. Nylon filters can be

handled roughly with no physical damage. The material withstands prolonged exposure to alkali, which allows use of the convenient alkaline transfer technique. Nylon is blocked by SDS, which simplifies the pre-hybridization steps. Nucleic acids can be covalently linked to nylon membranes through controlled exposure to UV light. The resulting blot may be stripped and reprobed many times with minimal loss of signal. Nylon membranes are available in uncharged form or with immobilized positive charges on their surfaces. Positively charged nylon has a higher affinity for nucleic acids, which are anions, than neutral nylon. Positively charged nylon will bind nucleic acid semi – permanently after alkaline blotting without UV cross-linking. However, in some cases, charged nylon may give higher backgrounds than uncharged membranes.

After labelling, membrane is washed to remove non-specifically bound probe and is then exposed to X-ray film. Although digital image capture is growing in popularity, X-ray film techniques are still widely practiced, efficient and cost-effective methods for chemiluminescent or isotopic imaging. In order to achieve maximum sensitivity with film methods, it is very important to use fresh chemicals and good processing techniques. Obtaining the optimal exposure is often accomplished by trial and error with different exposure times. Southern blots are used in gene discovery and mapping, evolution and development studies, diagnostics and forensics (Turner et al., 2000).

1.2.5 Gene array

Gene arrays are solid support upon which a collection of gene specific nucleic acid have been placed by spotting or direct synthesis, for example glass slide. "Target" is a nucleic acid attached to arrays. The labelled nucleic acid comprising the sample is called "probe". Microarray technology evolved from Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known gene or fragment. Information about sequence of nucleic acid depends on the amount of probe hybridized to each target. Gene arrays can provide thousands of information in single experiment.

Solid supports are glass slides, silicon and nylon or nitrocellulose membranes. Probes are labelled fluorescently or radio labelled. Fluorescently labelled probes are typically used with glass arrays, while radio labelled probes are used with membranes. The array nucleic acids are composed of oligonucleotides, PCR products, cDNA vectors, purified inserts. Many pre-made arrays are available from commercial

manufactures. Many terms exist for naming gene arrays: biochip, DNA chip, Gene Chip, DNA array, micro array and macro array. Micro arrays and macro arrays may be used to differentiate between spot size and the number of spots on the support. Gene arrays can be used for sequence identification or differential expression analysis of two or more RNA samples.

Typical steps of array experiment for detection of gene expression are:

1. Isolating DNA from samples to be compared
2. Converting the RNA samples to labelled cDNA via reverse transcription, this step may be combined with RNA amplification
3. Hybridizing the labelled cDNA to identical membrane or glass slide arrays
4. Removing the unhybridized cDNA
5. Detecting the quantitative data from the various samples

Nylon membranes are typically hybridized with ^{33}P-dNTP labelled probes and they analyzed by phosphor imager along with the appropriate software.

The steps are similar to expression analysis. First RNA is isolated from two tissue or cell samples. Random-sequence primers create two labelled cDNA populations. These two cDNAs are hybridized to two identical arrays in two samples. Then washing and detection of hybridized signal. The genes expressed at different levels in two samples generate different amount of labelled cDNA and this results in the spots on array with different amounts of signal.

Glass slide arrays analysis involves the same steps but probes are labelled with two distinct fluorescently labelled nucleotides and both probes are hybridized to the same array. Typically one is labelled with Cyanine 3-dNTP and the other with Cyanine 5-dNTP. Each dye produces different colour fluorescence. The two-labelled RNA populations are hybridized to one glass slide and are scanned using a fluorescent imager.

Affymetrix's gene chips are glass slide manufactured. Oligonuclotide spots are synthesized directly onto the array substrate. Analysis procedure involves converted RNA samples to biotin labelled cDNA and each sample is hybridized to a separate gene chip.

The hybridized cDNA is stained with a streptavidin-phycoerythrin conjugate and it visualized with an array scanner.

Data are demonstrated by computer. There are some image analysis software including BioDiscovery (ImaGene) and Imaging Research (ArrayVision). For membrane array analysis, a file of the data is generated by phosphoimaging and they are analyzed by software. The software will correlate spots to genes and can compare spot intensities for differential expression studies. Glass array – the image fluorescence is scanned.

Differences in expression of specific sequences are often validated by RT-PCR, northern analysis or nuclease protection assays. These methods can be used for a relative or absolute quantification of specific messages of interest identified by array analysis. Essential for validation of microarray analysis is positive and negative control. Positive control is gene of known sequence, which should be always detectable. Control negative should be a gene, which is not contained in analysing genome (Lockhart and Winzeler, 2000).

One of the most important applications for arrays is monitoring of gene expression (mRNA abundance). Collection of genes that are expressed or transcribed from genomic DNA, sometimes referred to as the expression profile or the 'transcriptome', the major determinant of cellular phenotype and function. The transcription of genomic DNA to produce mRNA is the first step in the process of protein synthesis and differences in gene expression are responsible for both morphological and phenotypic differences as well as indicative of cellular responses to environmental stimuli and perturbations. Unlike the genome, the transcriptome is highly dynamic and changes rapidly and dramatically in response to perturbations or during normal cellular evens such as DNA replication and cell division. Understanding the function of the functions of genes, knowing when, where and to what extent a gene is expressed is central to understanding the activity and biological roles of its encoded protein. Changes in the multi-gene patterns of expression can provide clues about regulatory mechanisms and broader cellular functions and biochemical pathways.

Now the nucleic acid arrays have been constructed for many different organisms and they have been used successfully to measure transcript abundance in a host of different experiments. Expected result act effectively as internal controls that provide a certain amount of validation, while new information is obtained by a systematic search of a larger part of 'gene space'. Arrays often contain probes for genes of unknown function (and often with only partial sequence information); any outcome for these could be considered.

There exist other ways to measure mRNA abundance, gene expression and changes in gene expression, genomic or protein based methods. Importance of protein-based methods is that they measure the final expression product rather than an intermediate and some of them are able to detect post-translational protein modifications and protein complexes. Protein based approaches are generally more difficult, less sensitive and have a lower throughput than RNA – based ones. Messenger RNA is only an intermediate on the way to production of the functional protein products, but mRNA levels are immensely informative about cell state and the activity of genes, and for most genes, changes in mRNA abundance are related to changes in protein abundance.

Most array-based expression measurements are done using RNA from million or more cells. Efficient and reproducible amplification methods are required. There are two primary approaches: PCR-based, that has been used to make single-cell cDNA libraries (but that relative abundance of cDNA products is not well correlated with the original mRNA levels) and the second uses multiple rounds of linear amplification based on cDNA synthesis and template-directed *in vitro* transcription (IVT) reaction. These amplification methods produce sufficient quantities of labelled material starting with as little as 1-50 ηg total RNA is highly reproducible.

Nucleic acid arrays are often equated with gene expression analysis; they may be used to collect much of data that are obtained presently by Southern or northern blot hybridization techniques, but in a parallel fashion. Their utility in polymorphism detection and genotyping is described elsewhere. For example, genomic DNA samples can be manipulated experimentally to select for particular regions before hybridization to obtain specific types of information. As probes for more intergenic regions are synthesized on arrays, it becomes possible to identify protein-binding sites: fragmented chromatin can be crosslinked to a protein and then immunoprecipitated with an antibody to that protein. The DNA fraction of the immunoprecipitate can be labelled and hybridized with to identify the approximate location of the binding site. In addition, full genome arrays can be used in the analysis of plasmid libraries in genetic selection such as two-hybrid screens or, in principal, for any other type of experiment in which the information is contained in the form of RNA or DNA. Arrays also have application in biophysical chemistry and biochemistry (Lockhart and Winzeler, 2000).

2. Objectives

This work is focused to control positive and negative of microarrays.

Objectives of this work were:

1. Obtain purificated probes of control positive (constitutive gene of *Brucella*) and control negative (gene for photosynthesis of *Arabidopsis thaliana*) with PCR method.
2. Hybridize the probe of control positive and than the probe of control negative with genomes of *Brucella* and *Arabidopsis thaliana* by the Southern blotting method.
3. Verification of both steps and availability of controls for microarrays.

3. Material and methods

3. 1 Biological material

For experiments were used genomic DNA of *Arabidopsis Thaliana* (supplied by Departmento de Fisiología vegetal de la Universidad de Navarra) and lysed cells of *Brucella* cultures. Individual cultures of *Brucella (B. mellitensis, B. suis, and B. abortus)* were cultivated on department in conditions that cannot be defined.

3.2 Apparatus

Microcentrifuge, Hermile Labortechnik, Gosheimerstr 5678564 Wehingen, (1000 - 14000 rpm)

Vortex, Heidolph Reax top

Hot plate stirrer, Metrohm AG Herisau

Magnetic stirrer, Velp scientifica

UV crosslinker, Amersham Life science

Gene Amp PCR System 2700 (Applied biosystems)

Developer machine, Agfa gevaertN-V 9462/106

Allgra X-12R Centrifuge (Beckman Coulter)

Water bath, Comercial Assens-Llofriu, S.A

Bio-Rad model 785 vacuum blotter

Analytical balance, CH-Dietkon 300/9206L

Digital pH meter, Crison HI 1311

Electronic balance, Sartorius 1216MP Pacisa

Hybridization oven, Amersham Life science

Shaker, Stuart scientific

Ultraviolet scanner, Gelprinter plus

Specktralphotometer PM2K

Nanodrop (ND-1000)

Power Pac 300 (Bio-Rad)

3.3 Chemicals

DNA extraction: solutions for extraction are provided in UltraClean ™ Microbial DNA Isolation Kit;

PCR: primers 100μM, Sigma-Genosys (primers are specific for each PCR reaction, so they are noted in the method); ddH_2O; other components of PCR mixture are supplied with the kit IMUNOLASE™ DNA Polymerase BIOLINE (ImmoBuffer 10X Perkin-Elmer, 25mM $MgCl_2$ Solution, Taq polymerase, 100 mM dNTPs);

purification: solutions are provided in QIAquick Gel purification Kit (QIAGEN), ethanol (96–100%), Pancreac; isopropanol (100%), Pancreac;

restriction: 10X buffer (TACARA Bio Inc.); ECO R1 (TACARA Bio. Inc.);

electrophoresis: 1 Kb Plus DNA Ladder; tris base, Pancreac; acidum boricum 61.84 Pancreac; glycerina 92.10 Pancreac; orange G 452.4 Sigma-Aldrich; dH_2O; bromphenol blue 1% (MO BIO, Laboratories, Inc.); Ficoll, Pancreac; 0,5M EDTA 372.24 Pancreac; agarosa D-1 Medium Eeo Pronadosa Cat 8021;

Southern blotting: Amersham ECL Direct™ Nucleic Acid Labelling and Detection System Kit; Hybond-N+ Amersham pharmacia biotech; ethanol (96 %), Pancreac; hydrochloric acid 12M, Pancreac; agarosa D-1 Medium Eeo Pronadosa Cat 8021; sodium dodecyl sulphate (SDS), Pancreac; Whatman paper; sodium chloride, Pancreac 58.44; sodium hydroxide 40M, Pancreac; trizma base FW 121,14 Sigma; glycerina 92.10 Pancreac; 0,5M EDTA 372.24 Pancreac (Ethylenediaminetetraacetic acid); ethidium bromide destaining tea bags (MO BIO, Laboratories, Inc.) sodium hydrogen phosphate monohydrate, Pancreac 141.96; sodium citrate Pancreac 294.10; urea, Serva 60.1; Orange G, 452.4 Sigma-Aldrich; redistilled water, Hyperfilm ECL Amersham biosciences.

Solutions for electrophoresis:

Buffer TBE 5X:

135 g of trizma base, 68.75 g of acidum boricum, 9.3 g of 0,5M EDTA are dissolved and filled up 2.5 litters with distilled water.

Buffer TBE 1X:

1 litter of TBE 5X (see above) concentrated dilute in 5 litres of distilled water.

Loading buffer II.

2.5ml of 1% bromphenol blue, 2.5g of Ficoll, 1ml 0.5M EDTA (pH = 8) were mixed and the mixture was filled up to 10ml with ddH2O.

Loading buffer I.

Mix 300µl glycerol with 700µl of distilled water and add a little bit of orange G powder until the colour of solution changes to orange.

Marker solution:

100µl Buffer TBE 1X, 200µl of Loading buffer I., 100µl of 1Kb Plus DNA Ladder) were dissolved and agitated.

Ethidium bromide solution

Add 200ul of ethidium bromide (c= 10mg/ml) to 2.5 litter of distilled water

Stock solutions for Southern blotting:

The solutions are prepared according to the protocols and autoclaved when necessary. All used chemicals are noted above.

SSPE 10X (pH = 7.4)

17.80g $Na_2HPO_4.H_2O$, 105.12g NaCl, 3.7g of 0,5M EDTA are mixed and dissolved in 750ml of dH_2O on magnetic stirrer (Velp scientifica) for at least ½ an hour. Measure pH; adjust with HCl 5M). Fill up 1 litre with distilled water. Autoclave if you do not use the same day.

HCl (0.25M)

Volume for one experiment was 500ml. 10.42ml HCl (M = 12) was added to 489.58ml dH_2O.

NaOH (0.4M)

Volume for one experiment was 1000ml. 16g NaOH (M = 40) was dissolved in distilled water to a total volume of 1 l and mixed on a magnetic stirrer (Velp scientifica) for at least 15 min.

SSC 20X

175.3g of 3M NaCl and 88.2g of 0.3M $Na_3Citrate.2H_2O$ were dissolved in 750ml of distilled water and filled up to 1l with dH_2O.

Primary wash buffer

Dissolve 360g of 6M Urea and 4g of SDS in 25ml SSC 20X. Mix on magnetic stirrer (Velp scientifica) for at least ½ an hour.

Hybridization solution

Dissolve 1.17g NaCl in 2.0ml of Gold Hybridization Buffer (provided in Amersham ECL Direct™ Nucleic Acid Labelling and Detection System Kit). Add 2g of blocking reagent (c = 5%) (Provided in Amersham ECL Direct™ Nucleic Acid Labelling and Detection System Kit) gradually, avoid the clots, keep stirring on magnetic stirrer (Velp scientifica). Keep stirring during one hour at room temperature. Let at 42°C during 30 – 60min with an occasional agitation.

3. 4 Methods

All methods followed steps of laboratory protocol or protocol of kits. Successfully modifications and optimalizations are noted in results.

3.4.1 Extraction of samples

Genomic DNAs of *Brucella* were extracted using UltraClean™ Microbial DNA Isolation Kit. Procedure follows protocol provided by extraction kit. All tubes and chemicals are provided. In each experiment was done control negative of extraction. Control negative of extraction passed through all steps of extraction but does not contain any DNA. All centrifugation steps were carried out at > 12,000 x g in a conventional table-top microfuge (Hermile Labortechnik, Gosheimerstr 5678564 Wehingen). Successful modifications and optimalizations are noted in each experiment.

3.4.2 PCR

Preparation of PCR mixture (composition see below) is very sensitive to contamination. It has to be done in a digestor, using gloves, immediately before use All instruments are sterilized in digestor under an ultraviolet lamp during 30 minutes. Components of mixture are prepared and kept on ice. Using sterilized instrumnets, components are mixed in order (see below). Ratio of components is different for specific experiments using variable primer. The mixture is properly vortexed and

aliquoted into individual PCR tubes. Finally required amount of DNA sample is added. The mixture has to be completely on the bottom of the tube. Avoid drops on the top. Tubes are covered and inserted into thermo cycler machine Gene Amp PCR System 2700 (Applied biosystems). Specific program of PCR reaction (see below) is started. In each experiment a control negative of PCR has been done. Control negative does not contain DNA and confirms that samples were not contaminated.

Chemicals of mixture are provided in IMUNOLASE^{TM} DNA Polymerase BIOLINE kit (see Chemicals). dNTPs has to be diluted. Final concentration of each nucleotide must be 25µl in total volume 200µl.

Three types of PCR reaction have been done: multiplex PCR, PCR with primers of control negative, PCR with primers of control positive.

3.4.2.1 Multiplex PCR

In multiplex PCR, eight pairs of primers to differentiate species of *Brucella* were used. These primers have been invented on department and cannot be defined. They are signed with numbers 1 – 16.

PCR mixture: ddH_2O, ImmoBuffer 10X Perkin-Elmer, 200µM dNTPs, primer 1 – 16, 25mM $MgCl_2$ solution, Taq polymerase, DNA samples in ratio 6.7 : 2.5 : 5 : 0.5 (16x) : 1.5 : 0.3 : 0.2 (v/v/v/v/v/v) in µl.

Program of thermo cycler:

7′.....95°C

25 cycles: 3′.....95°C
45′′.....64°C
180′′.....72°C

6′.....72°C

3.4.2.2 PCR of control positive

In PCR of control positive one pair of primers IF-1 (100µM, Sigma-Genosys) was used. PCR mixture: ddH_2O, ImmoBuffer 10X Perkin-Elmer, 200µM dNTPs, pair of primers, 25mM $MgCl_2$ solution, Taq polymerase, DNA sample in ratio 14.8 : 2.5 : 2.5 : 2 (2x) : 0.5 : 0.2 :(v/v/v/v/v/v) in µl.

Program of thermo cycler:

5′.....95° C

30 cycles: 35′.....95° C

45′′.....64° C

45′′.....72° C

7′.....72° C

3.4.2.3 PCR of control negative

In PCR of control negative a pair of primers porB (100μM, Sigma-Genosys) was used.

PCR mixture: ddH_2O, ImmoBuffer 10X Perkin-Elmer, 200μM dNTPs, pair of primers, 25mM $MgCl_2$ Solution, Taq polymerase, DNA sample in ratio 14.8 : 2.5 : 2.5 : 2 (2x) : 0.5 : 0.2 :(v/v/v/v/v/v) in μl.

Program of thermo cycler:

5′.....95° C

30 cycles: 35′.....95° C

45′′.....64° C

1‘.....72° C

7′.....72° C

3.4.3 Electrophoresis

1. Make a 1%, 1.2% or 2% (defined in each experiment) agarose solution in 1X TBE buffer.

2. Cool to 70°C and pour into the gel former. Insert gel comb to produce wells of up to 5mm in width, removing any air bubbles.

3. Place gel in electrophoresis tank. Fill tank with running buffer TBE 1X, sufficient to cover surface of the gel.

4. Add sufficient amount of loading buffer (10: 3) to the samples and apply DNA samples to wells in gel.

5. Apply 4µl of a marker solution into neighbouring wells.

6. Apply current 120V with Power Pac 300 (Bio-Rad) to gel and allow to run until bromphenol blue indicates that samples has run for sufficient distance, for example two thirds of the way down the gel.

7. After electrophoresis, DNA fragments are visualized by staining with ethidium bromide. Slide the gel from gel-former into a suitable plastic box with dilute solution of ethidium bromide and soak for at least 15 min.

8. Scan the gel under UV light.

3.4.4 Purification

Products of PCR were purificated using JETQUICK Gel Extraction Spin Kit. Procedure followed protocol provided by kit. Before use, absolute ethanol (96-100%) to H2 solution (see Chemicals) was added. All centrifugation steps were carried out at > 12,000 x g in a conventional table-top microfuge (Hermile Labortechnik, Gosheimerstr 5678564 Wehingen). In the last step, samples were diluted in ddH_2O.

3.4.5 Southern blotting

3.4.5.1 Cut of samples

ECO R1 TACARA, 10X H Buffer TACARA, ddH_2O are mixed in ratio (see Table I.) and incubated in 37°C over night.

Table I: x – genomic DNA sample, n – total volume of restriction mixture

Components of restriction mixture	µl
Sample (genomic DNA)	x
ECO R1 TACARA	1
10X H Buffer TACARA	10% of n
ddH_2O	n – (x + 1 + buffer)
Total volume	n

3.4.5.2 Electrophoresis

Make 1.2% agarose gel (see protocol n° 3.4.3), mix total volume of restriction mixture with loading buffer I. (see chemicals) and apply to the gel. If the samples are completely cut and divided, we continue with blotting procedure.

3.4.5.3 Blotting

1. Load the gel into the suitable plastic box with 0.25M HCl (depurinization) and begin agitation on orbital shaker (Amersham Life science), keep agitation for 10 minutes ensuring that the gel moves freely.
2. Discard solution, rinse gel with distilled water.
3. Cover gel with 0,4M NaOH (denaturation) and begin agitation on orbital shaker (Amersham Life science), continue agitation for 20 minutes, realize 2X.
4. Wash gel with distilled water and transfer.
5. Cut Whatmen paper and nylon membrane to the same size of the gel. Do not touch the surface of the membrane, wear gloves. Handle the nylon with forceps.
6. Wet paper and nylon membrane in deionised water, put the wet Whatman paper in the middle of porous membrane of the vacuum blotter (Bio-Rad model 785), collocate wet membrane directly on Whatman paper, avoid air bubbles.
7. Place the plastic foil window of size smaller than gel onto the membrane, place the gel directly on the plastic window and avoid moving it.
8. Apply the vacuum 5mm Hg, holding the gel cover gel with buffer SSPE 10X approximately 1cm upon the edge of gel. Keep transfer for the next 90-120 minutes.
9. Mark membranes wells on the membrane with a ballpoint.
10. Dry membrane at 80°C for 10 minutes and fix DNA with UV. Place gel for 15 minutes into ethidium bromide solution and check effective transfer with Ultraviolet scanner (Gelprinter plus).
11. Preheat the hybridization buffer and SSC 5X to 42°C.
12. Loosely roll the blot, place inside the tube, ensuring no air bubbles between the membrane and the tube, and don't allow the blot to overlap itself.

13. Add 40ml SSC 5X and hybridize in 42°C in hybridization oven (Amersham Life science) for 1 minute.

14. Pour off SSC 5X and add 18ml of hybridization buffer, let hybridize in 42°C for 30-60 minutes (in this time prepare the probe).

3.4.5.4 Preparation of probe

1. Denature sample with required concentration of DNA by heating for 5 minutes in a boiling water bath (Comercial Assens-Llofriu, S.A).

2. Immediately cool the DNA on ice for 5 minutes.

3. Add equivalent volume of DNA labelling reagent (Amersham ECL Direct™ Nucleic Acid Labelling and Detection System Kit) to the cooled DNA, mix gently, but thoroughly with a pipette.

4. Add glutaraldehyde (Amersham ECL Direct™ Nucleic Acid Labelling and Detection System Kit), use volume equivalent to the volume of the labelling reagent, and mix gently but thoroughly with a pipette.

5. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.

6. Incubate for 15-30 minutes at 37°C in a water bath (Comercial Assens-Llofriu, S.A) and use immediately.

3.4.5.5 Hybridization

1. Add the labelled probe (preparation see above) into the tube, but avoid placing directly onto the membrane.

2. Hybridize overnight in a rotisserie hybridization oven (Amersham Life science) at 42°C.

3. Prewarm 100ml of primary wash buffer to 42°C.

4. Discard the hybridization buffer, add 50ml of primary wash buffer to the tube and replace in the rotisserie hybridization oven for 20 minutes, repeat this step a second time.

5. Discard the primary wash buffer.

6. Place membrane to the suitable container and add 200ml of SSC 2X, agitate on orbital shaker (Amersham Life science) for 5 minutes in the room temperature, repeat this wash a second time.

3.4.5.6 Signal generation and detection

1. Mix an equal volume of detection reagent 1 with detection reagent 2 (Amersham ECL Direct™ Nucleic Acid Labelling and Detection System Kit), to cover the blot is necessary approximately 15ml of each reagent and incubate one minute at room temperature.

2. Drain the SSC 2X (see chemicals) from the blot and place it in fresh container DNA side uppermost.

3. Add the mixture of detection reagents directly onto the blot on the side carrying DNA; do not leave the blot to dry out.

4. Incubate for 1 minute at room temperature.

5. Drain the excess of detection reagents mixture and place the blots DNA side up in the film cassette.

6. Cover the film (Hyperfilm ECL Amersham biosciences) with transparent plastic sheet to avoid wetting the film, lever out air bubbles with glass tube, work as quickly as possible to minimize the delay between incubating the blots in substrate and exposing them to the film.

7. Work in the dark room – place on a sheet of autoradiography film on the top of the blots, close the cassette and expose for the wanting time.

8. Remove the film and develop in Agfa gevaertN-V 9462/106. If required, expose a second film for an appropriate length of time.

4. Results

4.1 Obtaining of probes - control positive and control negative

All experiments generally followed the steps of laboratory protocol. Successfully modifications and optimalizations are noted in each experiment.

4.1.1 Control positive

Control positive is a sequence of DNA *Brucella mellitensis*. This sequence is constitutive gene of *Brucella mellitensis* coding bacterial Protein Translation Initiation Factor 1 (IF-1). Locus of gene - BMEI1671. Length of gene - 303bp. Primers for multiplication of this sequence has been designed using Internet tool web page (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

Sequence of gene BMEI1671:

ATGCCGCCTAATCATATAGGTTCCGCCCAAATTAAGGGCCGAAGCTTTTATTCGGCG
GCTTTTATCAAAGAAAGAAACAGGTATCGCATGGCGAAAGAAGAAGTCCTCGAATTTCCGG
GTGTTGTTACGGAACTGCTGCCCAATGCAATGTTCCGCGTAAAGCTCGAAAACGAACATGAA
ATCATTGCCCATACGGCAGGCCGCATGCGCAAGAACCGTATCCGTGTTCTGGCCGGTGACAA
GGTTCTGGTCGAAATGACCCCTTATGACCTGACCAAGGGCCGCATCACCTACCGCTTCAAG

Primers multiply sequence of 164bp. Localization of primers annealing are expressed with red colour.

Pair of primers:

IF-1.F 5'-ATGGCGAAAGAAGAAGTCCT-3'

IF-1.R 5'-ACCAGAACCTTGTCACCGGC-3'

The probe of control positive was prepared and verified by Southern blotting.

To obtain efficious amount of specific sequence of gene IF-1 was used PCR method. For PCR reaction were used genomic DNA samples prepared by the protocol 3.4.1 and diluted 1:50.

Samples: *B. melli 1455* (1:50), *B. melli 16M* (1:50) diluted in ddH_2O.

Samples were multiplied with PCR (see 3.4.2.2) using specific primers (IF-1.F, IF-1.R, 100μM, Sigma Genosys). 7μl of PCR product was divided on 2% agarose gel (protocol see 3.4.3).

Table 1: B. melli 1455: Position of samples in the gel

0	1	2	3
Marker solution	B. melli 1455	Control negative of PCR	Marker solution

Table 2: B. melli 16M: Position of samples in the gel

0	1	2	3	4
Marker solution	B. melli 16M	____	Control negative of PCR	Marker solution

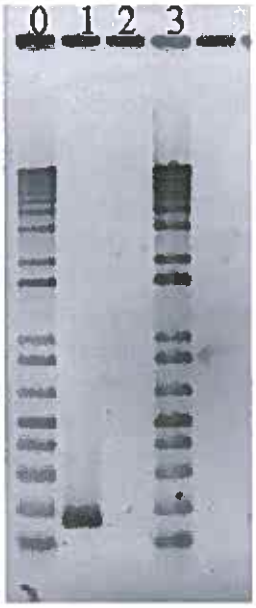

Fig.4: Electrophoresis of B. melli 1455 PCR product

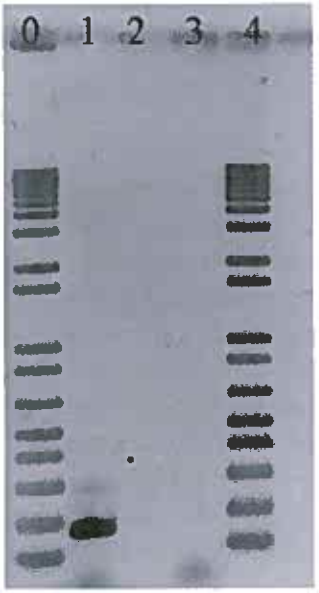

Fig.5: Electrophoresis of B. melli 16M PCR product

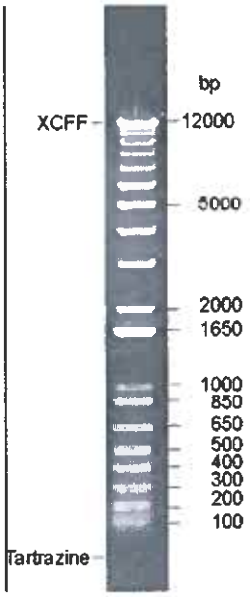

Fig. 6: 1kb DNA ladder

On the electrophoresis, we can see a good division of the samples and purity of controls. We compared the position of a band with 1kb DNA ladder (see fig. 6). Sequence size is 164pb; the band is in the position between last two bands (200pb and 100pb) of 1kb DNA ladder. Multiplication was successfull, bands are in right positions and they are significant. Well multiplied PCR products were purificated following protocol number 3.4.4. Purification purify PCR product from primers dimmers, rest of PCR mixture components and not multiplied fragments. Concentration of DNA in purificated samples was measured on spectrophotometer against blank (ddH_2O) (See Table 3).

Table 3: Concentration of purified samples

Samples	c [µg/ml]
B. melli 1455	10.0
B. melli 16M	18.9

Concentration was sufficient. This product is ready to use such as probes in Southern blotting experiments (see 4.2.1, 4.2.2).

4.1.2 Control negative

Control negative is a sequence of plants DNA *Arabidopsis thaliana*. This sequence is a nuclear gene encoding chloroplast protein (light-dependent NADPH: protochlorophyllide oxidoreductase B (PorB)) of length 1206bp.

Sequence of gene *porB*:

ATGGCCCTTCAAGCTGCTTCTTTGGTCTCCTCTGCTTTCTCTGTTCGCAAAGATGCGAAGTTGAATGCTTCTTCATCATCT
TTCAAGGACTCGAGTCTTTTTGGTGCCTCCATTACCGACCAAATCAAATCCGAACATGGATCTTCCTCGTTAAGATTCAAGAGAGAAC
AGAGCTTAAGGAATCTAGCAATTCGAGCCCAAACCGCTGCGACTTCAAGCCCTACAGTTACAAAATCCGTGGACGGCAAGAAAACGTT
GAGGAAAGGAAATGTGGTGGTCACTGGAGCCTCGTCTGGGTTAGGTCTAGCCACGGCTAAAGCTCTAGCTGAGACAGGGAAATGGAAC
GTGATAATGGCGTGCAGAGACTTCCTTAAAGCCGAGAGAGCTGCTAAATCCGTAGGGATGCCTAAAGACAGCTACACAGTGATGCATT
TAGACTTAGCCTCGTTGGACAGCGTGAGACAGTTTGTTGATAATTTCAGGAGAACAGAGACGCCTCTCGATGTTTTGGTCTGCAATGC
TGCGGTTTATTTCCCGACAGCTAAAGAGCCTACTTACAGTGCTGAAGGGTTTGAGCTTAGTGTTGCGACGAACCATTTGGGACATTTT
CTTCTCGCAAGGTTGTTGCTTGATGACTTGAAGAAATCTGATTACCCTTCAAAGCGTCTCATCATCGTCGGGTCCATTACCGGGAACA
CGAATACATTGGCGGGTAATGTACCACCGAAGGCGAATCTCGGTGATTTGAGGGGTTTAGCCGGCGGATTAAACGGTTTAAACAGCTC
AGCTATGATTGATGGAGGAGATTTCGACGGTGCAAAGGCTTACAAAGACAGTAAAGTCTGCAATATGTTGACAATGCAAGAGTTTCAC
AGGCGTTTCCATGAAGAAACTGGAGTCACTTTCGCTTCGCTTTACCCCGGTTGCATCGCCTCCACAGGTTTATTCCGAGAGCACATTC
CTCTCTTCCGTGCCCTCTTCCCTCCCTTTCAGAAGTACATCACTAAAGGATATGTCTCCGAAACAGAGTCAGGCAAAAGACTTGCTCA
GGTGGTGAGTGATCCAAGCTTGACGAAATCAGGGGTTTATTGGAGCTGGAACAATGCTTCGGCTTCTTTTGAGAACCAGTTATCAGAA
GAAGCAAGTGACGTTGAGAAGGCTCGTAAAGTGTGGGAGATCAGTGAGAAGCTCGTGGGCTTGGCCTAA

Primers for multiplication of this sequence have been designed using Internet tool web page (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

Primers multiply sequence of 300bp. Localization of primers annealing are marked with red colour.

A pair of primers:

LEFT PRIMER 5'-CTCGGTGATTTGAGGGGTTT-3'

RIGHT PRIMER 5'-GCCTGACTCTGTTTCGGAGA-3'

The probe of control negative was prepared and verified by Southern blotting method.

For PCR reaction was used genomic DNA samples commercially supplied. Sample was diluted with ddH_2O 1:50.

Samples: Genomic DNA of *Arabidopsis thaliana* (1:50).

Samples were multiplied with PCR (see 3.4.2.3), using specific pair of primers (PorB, 100μM, Sigma Genosys). 7μl of PCR product was divided on 2% agarose gel (protocol see 3.4.3).

Table 4: Arabidopsis thaliana: Position of samples in the gel

0	1	2	3
Marker solution	Arabidopsis thaliana	Control negative of PCR	Marker solution

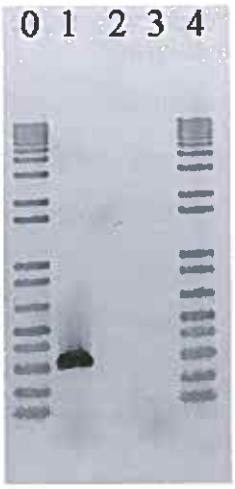

Fig. 7: Electrophoresis of Arabidopsis thaliana PCR product

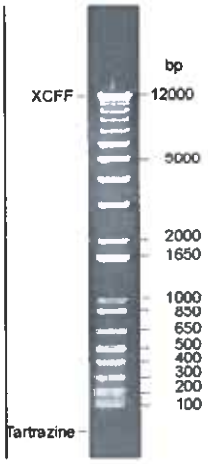

Fig. 8: 1kb DNA ladder

Multiplication was successfull the band is significant. We can see good division of sample and purity of controls on the electrophoresis. We have compared the position of band with 1kb DNA ladder (see fig.8). Sequence size is 300pb; the band is in position of band with size 300pb of 1kb DNA ladder. Multiplication was successful; band is in right positions and is significant. Well multiplied PCR product was purificated following protocol number 3.4.4. Concentration of DNA in purificated sample was measured on spectrophotometer against blank (ddH_2O) (See Table 5).

Table 5: Concentration of purified sample

Sample	c [μg/ml]
Arabidopsis thaliana	29.0

Concentration was sufficient. This product is ready to be used such as probe of control negative in Southern blotting experiments (see 4.2.3, 4.2.4). We have verified

methods to obtain probes, we could continue with proved methods for preparation of probes for microarrays.

4.1.3 Obtaining of probes for microarrays

For microarrays are used same probes such as for Southern blotting, but it's necessary to obtain concentration at least 100μg/ml.

Samples: *B.melli 1507* (1:50), *B. melli 115* (1:50), *B. melli Rev1* (1:50), *Arabidopsis thaliana*

Samples were multiplied with PCR following protocols (for *Brucella* see 3.4.2.2, for *Arabidopsis* 3.4.2.3). We used specific primers for control positive and control negative, they are described above. Multiplied samples of *B. melli* were mixed into one ependorf after purification

Table 6: Position of PCR product on the gel

0	1	2	3	4	5
Marker solution	Mixture of B. melli	Control negative of PCR	Arabidopsis thaliana	Control negative of PCR	Marker solution

Fig.9: Electrophoresis of purified samples

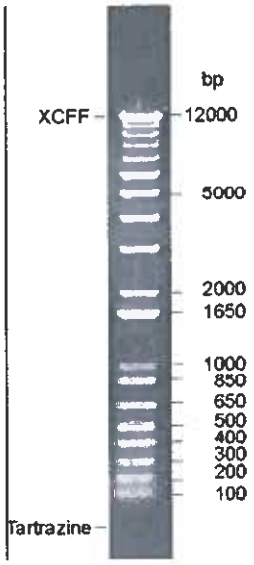

Fig. 10: 1kb DNA ladder

Multiplication was successfull bands are significant. We can watch a good division on the gel and a purity of controls. We compared the positions of bands with 1kb DNA ladder, bands are in the right positions corresponding to their size and multiplication was successful. Multiplied PCR products were purificated following

protocol number 3.4.4. Concentration of DNA in purificated samples was measured on spectrophotometer against blank (ddH_2O) (See Table 7).

Table 7: Concentration of purified samples

samples	c [µg/ml]	A (260/280)
B. melli mixture	40.5	0.89
Arabidopsis thaliana	44.5	0.91

We obtained probe of control positive with c = 40.5µg/ml and probe of control negative with c = 44.5µg/ml. Because these concentrations are lower than concentration required for microarrays, samples were completely evaporated and diluted for required concentration. We obtained 100µl of control positive probe with c = 120µg/ml and 96µl of control negative probe with c = 118µg/ml. These probes were available for microarray use.

4.2 Southern blotting

All experiments generally followed steps of laboratory protocols. Successfully modifications and optimalizations are noted in each experiment.

4.2.1 Phase I.

To obtain material for next experiments, it was necessary to isolate DNA from lyophilized cell culture of *Brucellae*. An extraction was done (following the protocol number 3.4.1, using UltraClean ™ Microbial DNA Isolation Kit Protocol). DNA of *Arabidopsis thaliana* is commercially supplied.

Samples: *B. mellitensis 1553* (3x), *B. mellitensis 1455*, *B. abortus 1 A 280*, *B. abortus 2 A22*, *Arabidopsis thaliana.*

Extracted samples were multiplied with PCR (see 3.4.2.1) and divided by electrophoresis (see 3.4.3). Results of multiplex PCR notified us of achievement of extraction. It was applied 7ul of sample and 3µl of buffer was added to each sample.

Table 8: Positions of samples on the agarose gel.

0	1	2	3	4	5	6	7	8	9
Marker solution	B. melli 1455	B. melli 1553	B. melli 1553	B. melli 1553	B. a 1 A 280	B. a 2 A 22	Control negative of extraction	Control negative of PCR	Marker solution

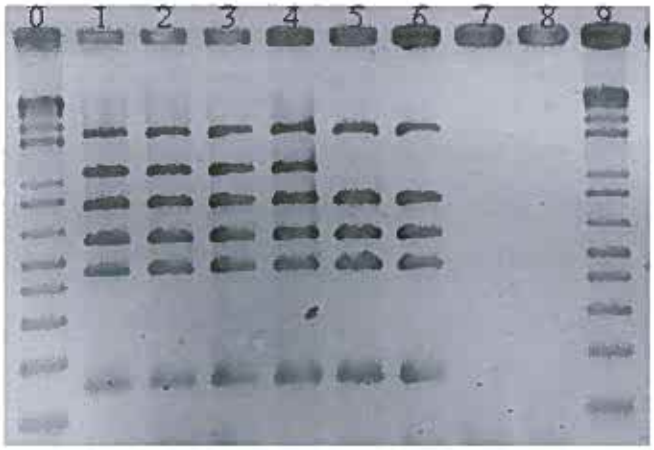

Fig. 11: Electrophoresis after multiplex PCR.

Extraction was right and bands were well separated. In 50µl of sample, concentration of extracted DNA was measured by UV/VIS spectrophotometer. Absorption maximum for DNA is in the wave of 260nm. In table, ratio of absorbencies

in 260 and 280nm is noted. The ratio of these absorbencies of double-stranded DNA sample can be used to access its purity. The value is 1.8. Higher values suggest RNA contamination. Concentration was measured against blank (MD 5 solution – elution buffer) (see Chemicals).

Table 9: Concentration of isolated DNA in samples (c) and ratio of absorbencies (A (260/208))

Samples	c[µg /ml]	A (260/280)
B. melli 1553 (a)	8.7	1.81
B. melli 1553 (b)	29.1	1.77
B. melli 1553 (c)	31.7	1.64
B. melli 1455	17.7	1.93
B. a 1 A 280	24.7	1.90
B. a 2 A 22	11.8	1.51
Arabidopsis thaliana	302.0	1.90

For following steps it is necessary work to with concentration of 1.5µg/µl of sample. We calculated suitable volumes.

Table 10: Optimal concentration of samples

Samples	B. melli 1553	B. melli 1553	B. melli 1455	B. a 1 280	Arabidopsis thaliana
Volumes	52	47	85	61	5

Volumes of extracted samples were lower than calculated volumes, so we used maximum of extracted samples. They were cut with restriction enzyme ECO R1 (TACARA BIO INC.) (see 3.4.5.1). Restriction enzyme recognizes specific fragments of DNA and cut.

5‘– G$^{\downarrow}$AATTC – 3‘

3‘– CTTAA$^{\downarrow}$G – 5‘

The composition of restriction mixture is noted in Table 11.

Table 11: Volumes of components for cut mixture

Symplex	B. melli 1553 (b)	B. melli 1553 (c)	B. melli 1455	B. a 1 280	Arabidopsis thaliana (x)
Sample (µl)	43	43	43	42	5
Eco R1 (µl)	1	1	1	1	1
Buffer (µl)	5	5	5	5	1
H2O (µl)	1	1	1	1	3
Total volume (µl)	50	30	50	50	10

Next steps followed protocol 3.4.5. Gel was assessed under UV light after 15 staining in ethidium bromide. The signal of ethidium bromide was very weak. Amounts of samples weren't sufficient. Separation of restriction fragments wasn't efficient. Based on these results, we worked with optimal or higher concentrations in next experiment.

4.2.2 Phase II.

Isolation was done following the protocol number 3.4.3.

Samples: *B. mellitensis 1849*, *B. mellitensis 1461*, *B. mellitensis 1455*, *B. abortus 330*, *B. suis 13*, *B. suis 1 1330*, *Arabidopsis thaliana*.

In this experiment we used already extracted samples with higher yields. It allowed us to work with optimal concentration. Concentration of samples is noted in Table 12.

Table 12: DNA concentration and ratio of absorbencies of samples

Samples	c [µg/ml]	A (260/280)
B. melli 1461	14.1	1.60
B. melli 1455	17.2	1.91
B. melli 1849	70.5	1.74
B. a 2 A330	37.9	1.82
B. s 1 1330	85.4	1.86
B. s 13	80.4	1.91
Arabidopsis thaliana	302.0	1.90

We calculated volumes of samples with optimal concentration for cut and transfer as described above.

Table 13: Optimal volumes of the samples

Symplex	B. melli 1849	B. s 13	B. melli 1461	B. melli 1455	B. a 2 A330	B. s 1 1330	Arabidopsis thaliana
Volumes (µl)	21	19	106	87	40	18	5

We use optimal or higher volumes to visualize any signal. We used accessible volumes of lower concentrated samples.

Table 14: Volumes of components for cut mixture

Samples	B. melli 1461	B. melli 1455	B. melli 1849	B. a 2 A330	B. s 1 1330	B. s 13	Arabidopsis thaliana
Sample (µl)	41	41	25.5	42	42	25.5	
Eco R1 (µl)	1	1	1	1	1	1	1
Buffer (µl)	5	5	3	5	5	3	2
H2O (µl)	3	3	0.5	2	2	0.5	
Total volume (µl)	50	50	30	50	50	30	20

Next steps followed protocol 3.4.5. Gel was assessed under UV light after 15 staining in ethidium bromide (fig. 12). We watched that samples were visible and well separated. Wells 2, 3, 6 and 7, which contained optimal or higher concentration, afforded significant signal. We continued with Southern blot transfer.

Samples divided on the agarose gel were transferred to nylon following protocols 3.4.5.2 and 3.4.5.3. After transfer, the gel was stained again 15 minutes in ethidium bromide (fig. 13). We could watch an efficiency of transfer. We can compare fig. 12 and fig. 13. We can watch less intensity of ethidium bromide in transferred gel. We continued with hybridization. Gain of probes for hybridization is described in chapter 3.1.

Table 15: Positions of samples in the gel.

0	1	2	3	4	5	6	7	8	9
Marker solution	B. melli 1849	B. s 13	B. melli 1461	B. melli 1455	B. a 2 A330	B. s l 1330	blank	Arabidopsis	Marker solution

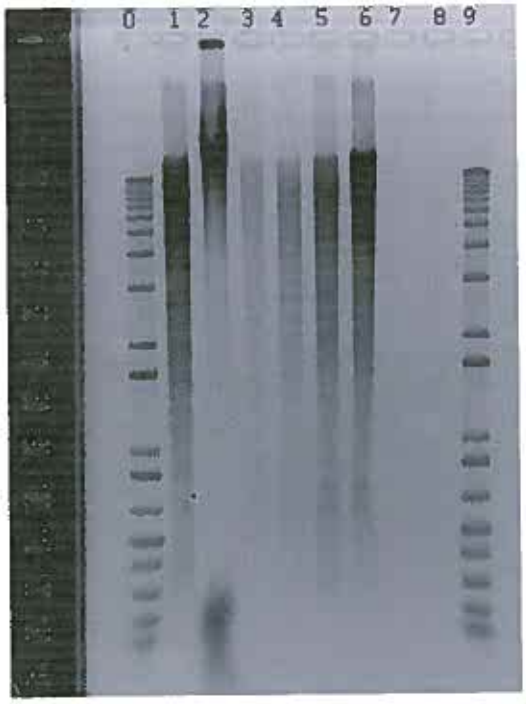

Fig. 12: Agarose gel before transfer

Fig.13: Agarose gel after transfer

Because on the gel the band in the position of *Arabidopsis thaliana* wasn't visible, the membrane was hybridized only with control positive following protocol 3.4.5.5 and 3.4.5.6. Membrane was hybridized with 10µl of control positive - *B. mellitensis 1455* (c = 10µg/ml, A (260/280) = 1.87). We developed 3 films. The first was developed after 1'exposure. No signal was detectable. The second film was exposed for 5 minutes, also without any detectable signal. The third was exposed for 20 minutes and there wasn't signal. In addition, the last one was exposed 65 hours and neither there was any detectable signal.

During the work, some mistakes occurred that could influence the results. The temperature of hybridization with probe was at the beginning exceeded up to 43°C and

then decreased to 39°C over night. Because the temperature of incubation with probe influences the specifity of hybridization, the higher temperature could avoid less specific probes to hybridize. Next availability can be low concentration of probe.

Hybridization on nylon membrane can be repeatable. Next hybridization was done. We hybridized membrane with 10µl of probe – *B. mellitensis 1455* (c = 20µg/ml). We used probe of higher concentration to ensure generating of any signal. We developed 2 films. The first was developed after 5 hours of exposure (fig. 14). Intensity of luminescence depends on time of exposure, so we chose a longer exposure.

Table 16: Positions of samples in the gel wells

0	1	2	3	4	5	6	7	8	9
Marker solution	B. melli 1849	B. s 13	B. melli 1461	B. melli 1455	B. a 2 A330	B. s 1 1330	blank	Arabidopsis	Marker solution

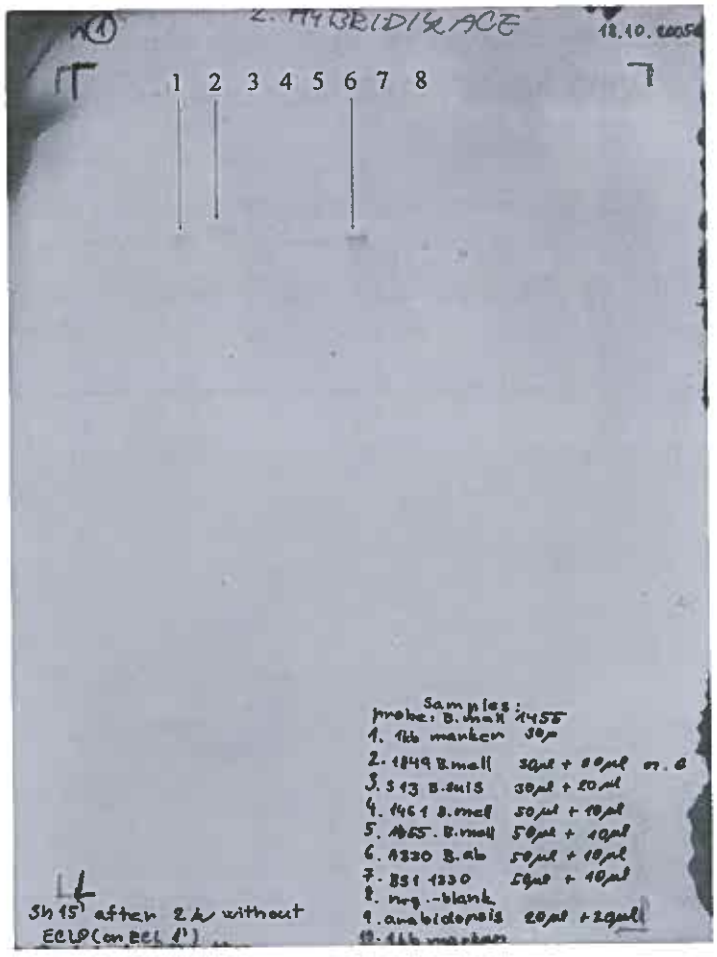

Fig. 14: First film after second hybridization

We could watch four bands in the positions 1, 2, 6. In these positions are samples with higher concentration than 1.5. We can estimate that higher concentration than 1.5μg/ul of DNA is required for transfer of sufficient amount of DNA from gel to membrane. In addition, concentration of probe could play a role in generating visible signal.

The second film was exposed for 16 hours. It wasn't possible to watch any signal. It means that chemiluminiscent substrate generates signal detectable until 5 hours after contact of membrane with ECL solution.

4.2.3 Phase III.

Isolation was done following the protocol number 3.4.1.

Samples: *B. mellitensis 1549*, B. *mellitensis 1534, B. mellitensis 3 ether, B. suis 1 vacuated china, B. suis 13, B. suis 4 APCC 33447, Arabidopsis thaliana*

Multiplex PCR was done following the protocol 3.4.2.1. and separated with electrophoresis (see 3.4.3). We used already extracted samples of *Brucella suis* with high concentration. These samples weren't applied in multiplex PCR.

Table 17: Application of samples into the gel wells

0	1	2	3	4	5	6	7	8
Marker solution	negative	negative	B. melli 1549	B. melli 1534	B. melli 3 Ether	Control negative of extraction	Control negative of PCR	Marker solution

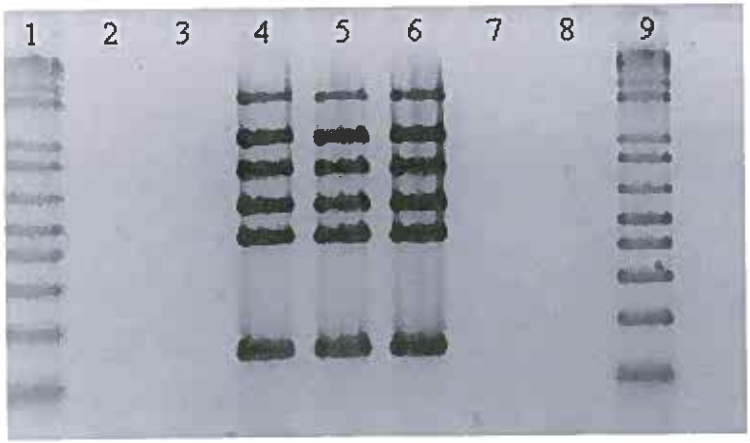

Fig. 15: Electrophoresis after multiplex PCR

Samples were well extracted and separated on electrophoresis. In these samples were measured concentrations of DNA, described above.

Table 18: DNA concentration and ratio of absorbencies of samples

Samples	c [μg / ml]	A (260/280)
B. melli 1549	68.7	1.71
B. melli 1534	68.6	1.72
B. melli 3 ether	25.2	1.59
B. s 1 v.ch.	79.2	1.56
B. s 4 APCC 33447	79.5	1.86
Arabidopsis thaliana	23.1	1.90

Concentration of samples was higher than in previous experiment. According to experiences from previous experiment, we decided to use samples with higher concentration. Efficious concentration could be 1.6 μg/μl. We calculated optimal volumes, which contain 1.6μg of DNA for next steps.

Table 19: Optimal volumes of samples

Samples	B. melli 1549	B. melli 1534	B. melli 3 ether	B. s 1 v.ch.	B. s 4 APCC 33447	Arabidopsis thaliana
volumes	23,14	23,17	63,09	20	21,5	4

Optimal volume of *B. melli3 ether* was higher than volume of extracted samples. In these cases, we used a maximal volume we had. We used higher volume of *B. s1 v.ch*, than necessary to see if it will product higher signal.

Table 13: Volumes of components for cut mixture

Samples	B.melli 1549	B.melli 1534	B. melli 3 ether	B.s 1 v.ch.	B. s 4 APCC 33447	Arabidopsis thaliana
Sample (µl)	23.20	23.20	45	40	21.5	4
Eco R1 (µl)	1	1	1	1	1	1
Buffer (µl)	5	5	3	5	3	2
H2O (µl)	2.8	2.8	4	4	4.5	4
Total volume (µl)	30	30	50	50	30	10

Mixture was separated with electrophoresis. After assessing the gel under UV light, we could watch significant signal only in wells number 9, 10 (fig. 16). The other samples seemed to be low concentrated.

Samples divided on the agarose gel were transferred to nylon membrane (see protocol n° 3.4.5.2 and n° 3.4.5.3). After transfer, the gel was stained again 15 minutes in ethidium bromide (fig. 17). We could watch an efficiency of transfer. We can compare fig. 16 and fig. 17. We can watch less intensity of ethidium bromide in transferred gel. We continued with hybridization. Gain of probes for hybridization is described in chapter 4.1.

Table 20: Application of samples into the gel wells

0	1	2	3	4	5	6	7	8	9	10	11	12	13
1kb marker	Arabidopsis thaliana	—	B. melli 1534	B. melli 1534 (rest)	B. melli 1549	B. melli 1549 (rest)	B. melli ether	B. melli ether (rest)	B. s v.ch.	B. s v.ch. (rest)	B. s 4 APCC 33447	B. s 4 APCC 33447	1kb marker

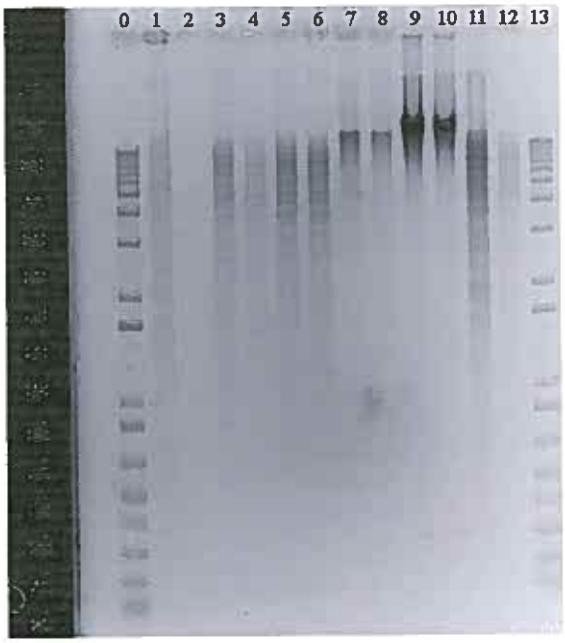

Fig. 16: Agarose gel before transfer

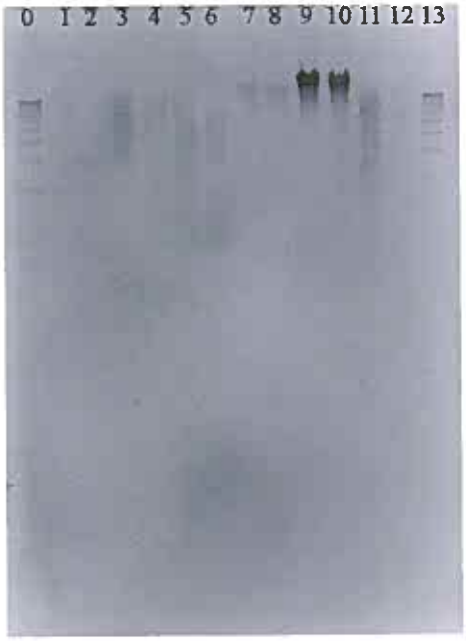

Fig. 17: Agarose gel after transfer

Following protocols 3.4.5.5 and 3.4.5.6 membrane was hybridized with 95µl of control negative – *Arabidopsis thaliana* (c = 5.8µg/ml, A (260/280) = 1.92). The first film was exposed 5 hours in 37°C and no signal was generated. The film was very dirty. The second film was exposed 16 hours in 37° C and also without any signal. The second film was clean.

We did second hybridization with 20µl of control positive – *B. mellitensis 16M* (c = 18.9µg/ml, A (260/280) = 1.82). Three films were developed. The first film was exposed for 30 minutes in 37°C. The film was dirty and no signal was notable. The second film was exposed for 5 minutes. The film was clean, but without any detectable signal. The third film was exposed for 3 hours and 35 minutes and we could notify detectable signal in positions 9 and 10 (see fig. 18).

Table 21: Positions of samples in the gel wells

0	1	2	3	4	5	6	7	8	9	10	11	12	13
1kb marker	Arabidopsis thaliana	—	B. melli 1534	B. melli 1534 (rest)	B. melli 1549	B. melli 1549 (rest)	B. melli ether	B. melli ether (rest)	B. s v.ch.	B. s v.ch. (rest)	B. s 4 APCC 33447	B. s 4 APCC 33447	1kb marker

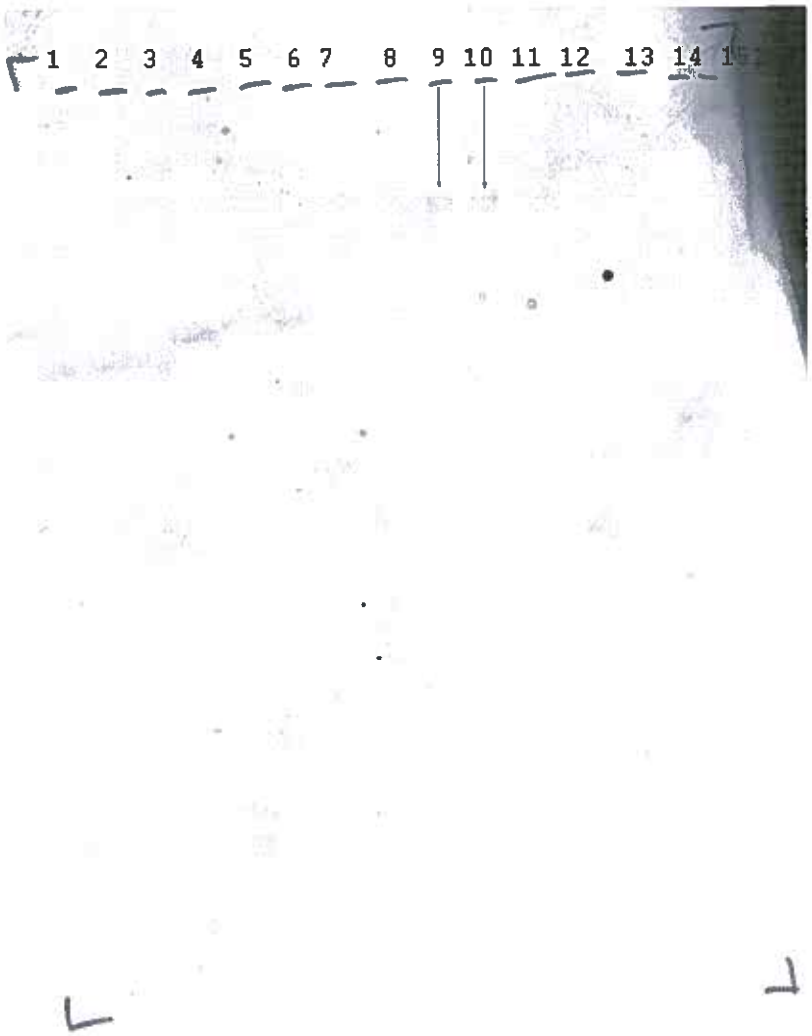

Fig. 18: The third film after second hybridization with C+

4.2.4 Phase IV.

Isolation was done following the protocol number 3.4.1. Every species of *Brucella* were extracted three times to obtain more DNA.

Samples: *B. mellitensis 63/9* (3x), *B. mellitensis 1507* (3x), *B. suis Thomson* (3x), *Arabidopsis Thaliana.*

Multiplex PCR was done following the protocol 3.4.2.1. Samples applied in gel after multiplex are diluted 1:50.

Table 22: Application of samples into the gel wells

0	1	2	3	4	5	6	7	8	9	10	11
Marker solution	B. melli 2 63/9	B. melli 2 63/9	B. melli 1 1507	B. melli 1507	B. melli 1 1507	B. suis 2 Thomson	B. suis 2 Thomson	B. suis 2 Thomson	Control negative of extraction	Control negative of PCR	Marker solution

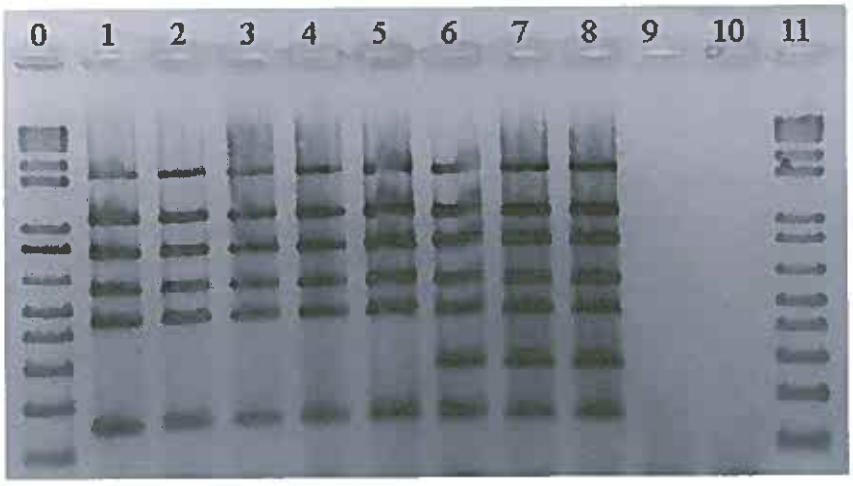

Fig. 19: Electrophoresis after multiplex PCR

We could watch efficious extraction. In samples, concentrations of DNA were measured.

Table 23: DNA concentration and ratio of samples absorbencies

Samples	c [µg/ml]	A (260/280)
B. melli 2 63/9	18.6	1.91
B. melli 1 1507	41.6	1.92
B.suis 2 Thomson	50.9	1.91
Arabidopsis thaliana	849.1	1.91

To obtain higher concentration, volumes of samples were completely evaporated with vacuum evaporation and diluted in 16.5 µl H_2O. From previous experiences, we notified that minimal concentration of sample for transfer of sufficient amount of DNA into the membrane was 1.75µg/µl. Concentrations of other samples were increasing.

Table 24: Concentration and ratio of absorbencies of samples after evaporation

Samples	Concentration [µg/ml]	A (260/280)
B. melli 2 63/9	116.7	1.88
B. melli 1 1507	198.3	1.92
B.suis 2 Thomson	308.6	1.93
Arabidopsis thaliana	849.1	1.91

We used identical volumes of samples to see efficious concentration. Concentration of *Arabidopsis thaliana* is excessive, because until now it didn't generate any signal.

Table 25: Volumes of components for cut mixture

Samples	B. melli 2 63/9	B. melli 1 1507	B. suis 2 Thomson	Arabidopsis thaliana
Sample [µl]	15	15	15	15
Eco R1 [µl]	1	1	1	1
Buffer [µl]	2	2	2	2
H2O [µl]	2	2	2	2
Total volume [µl]	20	20	20	20

Gel was assessed under UV light after 15 staining in ethidium bromide (fig. 20). Samples were visible and well separated, we continued with Southern blot transfer.

Following the protocol we transferred separated samples into the membrane. A swing from the protocol is in the protocol 3.4.5.3 step 8. Vacuum transfer took 1 hour and 45 minutes. After transfer, gel was stained again 15 minutes in ethidium bromide (fig. 21). Transfer in this experiment is significantly efficious. Contrary to previous

experiments, we could watch very intensive signal in position of *Arabidopsis thaliana.* We continued with hybridization. Gain of probes for hybridization is described in chapter 4.1.

Table 26: Application of samples into the gel wells

0	1	2	3	4	5	6
Marker solution	B. melli 2 63/9	B. melli 1 1507	B.suis 2 Thomson	____	Arabidopsis thaliana	Marker solution

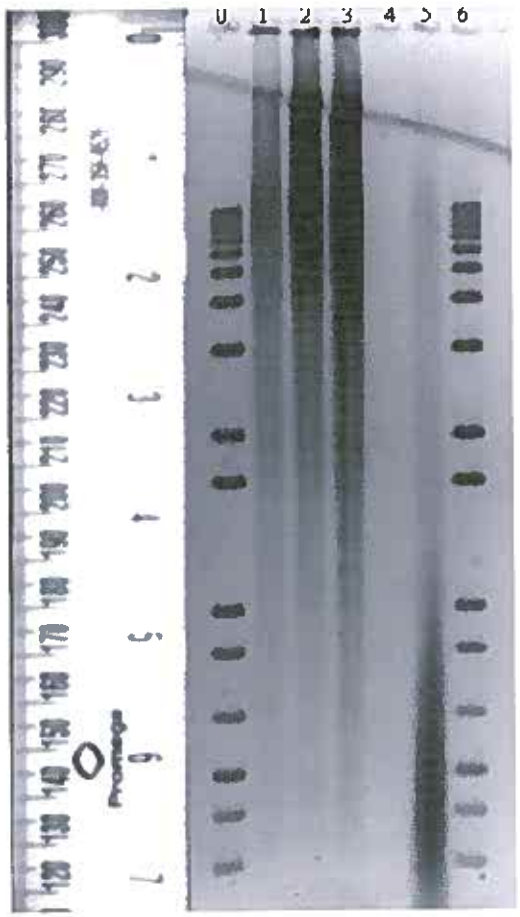

Fig. 20: Agarose gel before transfer

Fig. 21: Agarose gel after transfer

Membrane was hybridized (see 3.4.5.5 and 3.4.5.6) with 30µl control positive (c = 40.5 µg/ml, A) 260/280) = 0.89). We used higher concentration of probe to obtain better signal. The first film was exposed for 35 minutes in 37°C. We could watch signal, but it wasn't very clear and the film was dirty. Next film was exposed only 5 minutes to avoid expressive background. Five minutes were very short and we didn't notify any

signal. The third film was exposed for 5 hours and 35 minutes. The signal was quite weak but distinct.

Table 27: Application of samples into the gel wells

0	1	2	3	4	5	6
Marker solution	B. melli 2 63/9	B. melli 1 1507	B.suis 2 Thomson	_____	Arabidopsis thaliana	Marker solution

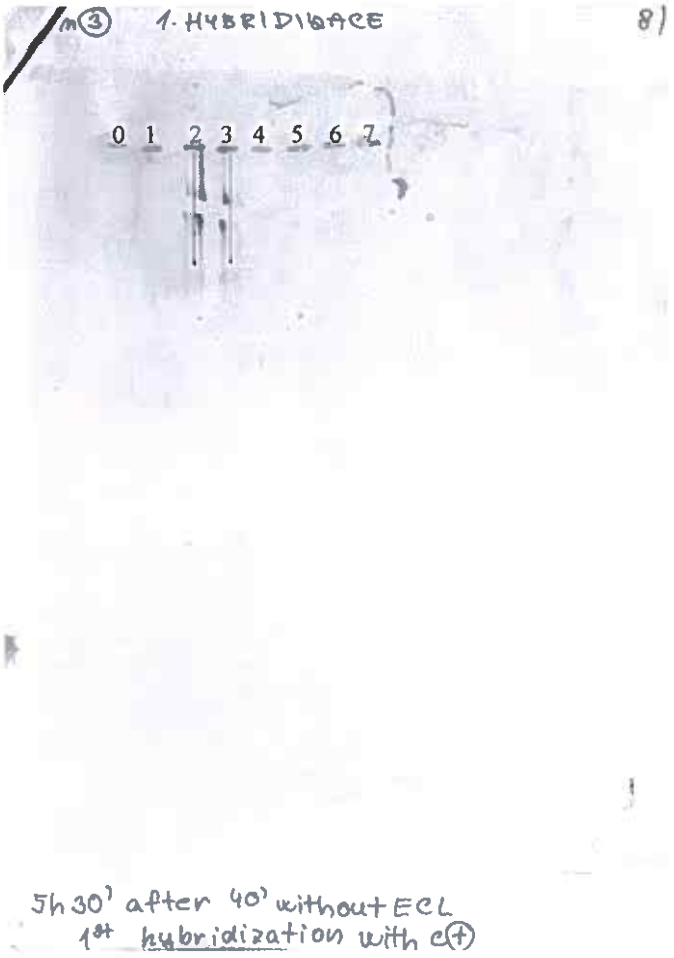

Fig. 22: The third film after the first hybridization with C+

Before the second hybridization, membrane was wetted in 10X SSPE. Membrane was hybridized with 30µl control negative (c = 44.5µg/ml, A (260/280) = 0.91). Two films were developed. The first was exposed for 35 minutes in 37°C. We could watch significant signal in position of *Arabidopsis thaliana*.

Table 28: Application of samples into the gel wells

0	1	2	3	4	5	6
Marker solution	B. melli 2 63/9	B. melli 1 1507	B.suis 2 Thomson	____	Arabidopsis thaliana	Marker solution

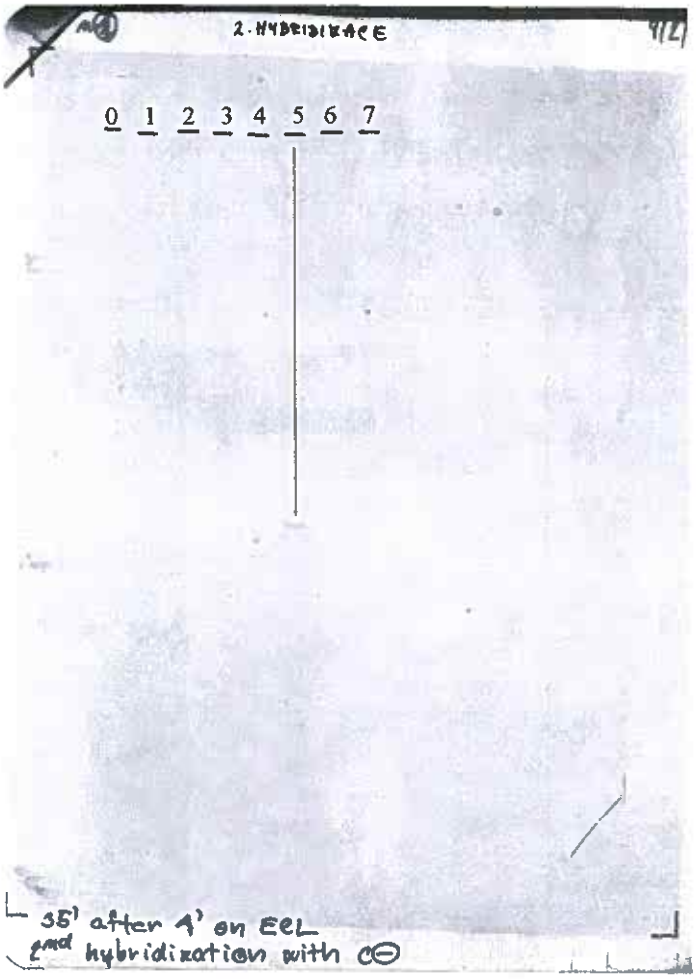

Fig. 23: Second film after second hybridization with control negative

The next film was exposed for 2 hours in 37°C and we also notified signal, but weaker one.

4.3 Confirmation

4.3.1 Confirmation of control positive

Restriction fragment of *brucellas*, which contains sequence of gene IF-1, has size of 12190pb. Sequence of gene IF-1 is complementary to probe of control positive. We measured distance of controls positive band on film (fig. 22) with ruler. Distance was 1.6 – 1.7 units of ruler. We compared distance of band on film with position of band with size of 12000pb (fig. 24 and 25). Distance of band (12000pb) is about 1.6 units on ruler. The distance of band on film is corresponding to position of restriction fragment with gene IF-1. Bounded probes of control positive generated signal in position of restriction fragment containing gene IF-1.

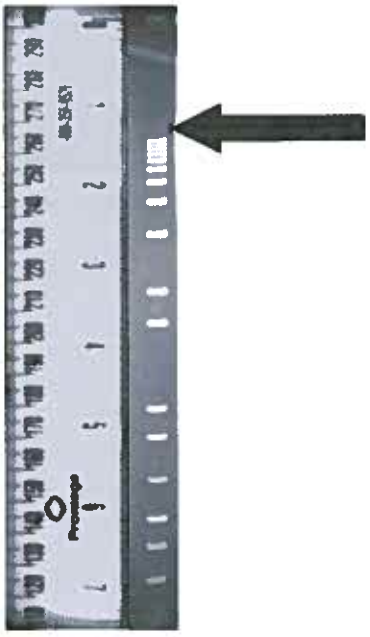

Fig.24: Position of rulers units to 1kb DNA ladder

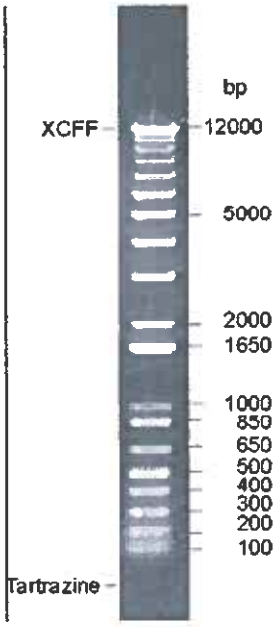

Fig.25: 1kb DNA ladder

4.3.2 Confirmation of control negative

Restriction fragment of *Arabidopsis thaliana*, which contains sequence of gene *porB*, has a size of 3069pb. Sequence of gene *porB* is complementary to probe of control negative. We measured distance of controls negative band on film (fig. 23) with ruler. Distance was 3.13 units of ruler. We compared distance of band on film with position of band with size of 3000pb (fig. 26 and 27). Distance of band (3000pb) is about 3 units on ruler. The distance of band on film is corresponding to position of

restriction fragment with gene porB. Bounded probes of control negative generated signal in position of restriction fragment containing gene porB.

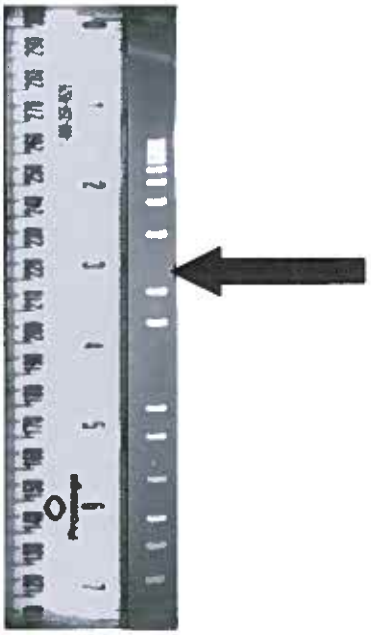

Fig.24: Position of rulers units to 1kb DNA ladder

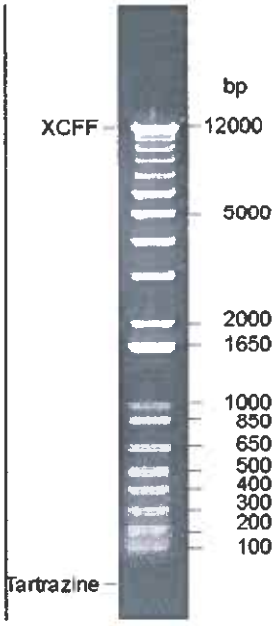

Fig.25: 1kb DNA ladder

5. Discussion

Confirmation of controls specifity using Southern blotting method acquired modification of procedure. Southern blotting method had to be optimalized. Concentration 1.5μg/μl of DNA in sample wasn't convenient for effective transfer and significant signal generation. Generation of signal is related to concentration of transferred DNA, concentration of hybridized probe and time of exposure. Signal of control positive was detectable by the work in defined conditions (concentration of probe was 0.2μg/μl, concentration of transferred DNA 1.7 – 4.6μg/μl and time of exposure 5 hours). It signified that the lowest concentration of transferred DNA had to be 1.7μg/μl, using probe of c = 0.2μg/μl. Higher concentrations of transferred DNA influenced the power of generated signal. Increase of probe concentration above 0.2μg/μl didn't influence the power of generated signal. Exposing time influenced power of signal up to 5 hours, after contact of membrane with ECL solution. After 5 hours chemiluminiscent substrate didn't produce any signal. Generation of signal of control negative required higher concentration of transferred DNA and higher concentration of probe. Only detectable signal was noted by work in defined conditions (concentration of transferred DNA was 12.7 μg/μl, concentration of probe 1.3μg/μl and time of exposure 35 minutes).

Using optimalized method, we acquired signals. Signals as bands on films produced by bounded probes of controls to complementary sequences. Identical complementary sequence to control positive contains *B. mellitensis, B. abortus. B. suis* sequence complementarities differ in one nucleotide. Membrane with genome of *Brucellas* and genome of *Arabidopsis thaliana* was hybridized with probe of control positive (preparation see 4.1.1, procedure see 4.2.4). We watched two bands-signals on the film (see Fig. 22). Bands were in wells positions of *B. melli 1 1507* and *B.suis 2 Thomson.* Both bands were in the same level and their positions were according to positions of restriction fragments with complementary sequence (gene IF-1), (see Results 4.3.1). Because any other signal wasn't detectable, this fact confirmed specifity of control positive. Probe was bounded only with complementary sequence of *Brucellas* gene IF-1; it didn't generate any unspecific signal. Control positive is specific and can be used in microarrays.

The same membrane was hybridized with probe of control negative (preparation see 4.1.2 and procedure see 4.2.4). We watched one band-signal on the film (see Fig. 23). The band was in the well of *Arabidopsis thaliana.* The poosition of band was according to a position of restriction fragment with complementary sequence (gene

porB), (see Results 4.3.2). Because any other signal wasn't detectable, this fact confirmed specifity of control negative probe. Probe was bounded only with complementary sequence of *Arabidopsis thaliana* gene *porB*; it didn't generate any unspecific signal. Control negative is specific and can be used in microarrays.

Controls are important data components of microarrays. Microarrays contain spots of controls (positive and negative). Control act as an information of right done experiment. Specific bounded control positive should always generate signal and confirm accuracy of microarray experiment. Microarrays contain control negative as a gene *PorB.* Microarrays method studies *Brucella* gene expression by hybridizing RNA of *Brucella* with arrays containing genome of *Brucella.* Control negative fragment should never generate signal, because genome of *brucella* doesn't contain any complementary sequence to gene PorB.

6. Conclusion

We obtained probes of controls for microarrays. We prepared 96µl of control negative probe with concentration c = 118µg/ml and 100µl of control positive with c = 120µg/ml. This concentration was efficious. Probes can be used in construction of microarrays of *Brucella* DNA.

Southern blotting method was optimalized. Using optimalized Southern blotting method was confirmed specifity of controls. Controls were bounded only to their complementary sequences. Complementary sequence of control positive was unique for *Brucella* and complementary sequence of control negative was unique for *Arabidopsis thaliana*.

7. Závěr

Získali jsme sondy kontrol pro užití v microarrays. Připravili jsme 96µl sondy pro negativní kontrolu o koncentraci 118µg/ml a 100µl sondy pro pozitivní kontrolu o koncentraci 120µg/ml. Získaná koncentrace byla dostatečná. Sondy mohou být použity v konstrukci microarray DNA bakterie *Brucella*.

Metoda Southern blotting byla optimalizována. Za použití takto optimalizované metody jsme potvrdili specifičnost kontrol. Sondy kontrol se navázaly pouze na jejich komplementární sekvence. Potvrdili jsme, že sekvence pozitivní kontroly je pro genom *Brucella* jedinečná a sekvence negativní kontroly je jedinečná pro *Arabidopsis thaliana.*

8. Abbreviations

A	adenine
Abs	antibodies
B. a	*Brucella abortus*
B. m	*Brucella melitensis*
B. s	*Brucella suis*
c	concentration
C	cytosine
C+	control positive
cDNA	complementary DNA
ddH_2O	redistilled water
DNA	deoxyribonucleicnucleic acid
dNTP	deoxynucleotides
EDTA	ethylendiamintetraacetic acid
G	guanine
GIT	gastrointestinal tract
GTP	guanosin trihosphate
IF-1	initiation factor
Kb	kilobase
LPS	lipopolysacharide
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
ORFs	ORFeomes
pb	pair of bases
PCR	polymerase chain reaction
PMNs	polymorphonuclear cells
porB	protochlorophyllide oxidoreductase B
RNA	ribonucleic acid
Rpm	rotates per minute
RT-PCR	real time PCR
SDS	sodium dodecyl sulphate
T	thymine
Taq	*thermus aquaticus*
Tm	melting temperature
UV	ultraviolet

References

Brock T. D., Madigan M. T.,: Biology of microorganism, 9th edition, Prentice Hall Int. Editions 2000, chapter 3, pages 79 – 77, chap. 5 p. 140 - 141, chap. 14 p. 508, chap. 15 – p. 543

Celis J. E., Kruhöffer M., Gromova I., Frederiksen C., Östergaard M., Thykjaer T., Gromov P., Yu J., Pálsodóttir H., Magnusson N., Örntoft T. F. (2000) Gene expression profiling: monitoring transcription and translation products using DNA microarrays and proteomics. *FEBS Letters.* 480: 2-16

Delrue R. M., Lestrate P., Tibor A., Letesson J. J., De Bolle X. (2004) Brucella pathogenesis, genes identified from random large-scale screens. *FEMS Microbiology letters.* 231: 1-12

Dricot A., Rual J. F, Lamesch P., Bertin N., Dupuy D., Hao T., Lambert C., Hallez R., Delroisse J. M., Vandenhaute J., Goñi I. L., Moriyon I., Garcia-Lobo J. M., Sangari F. J., MacMillan A. P., Cutler S. J., Whatmore A. M., Bozak S., Sequerra R., Doucette-Stamm L., Vidal M., Hill D. E., Letesson J. J., De Bolle X. (2004) Generation of the *Brucella melitensis* ORFeome Version 1.1. *Genome research.* 14: 2201-2206

Eskra L., Canavessi A., Carey M., Splitter G. (2001) *Brucella abortus* Genes Identified following Constitutive Grow and Macrophage Infection. *Infection and Immunity.* 7736-7742

Lockhart D. J., Winzeler E. A. (2000) Genomic, gene expression and DNA arrays. *J. Nature.* 405

López-Goñi I., Guzmán-Verri C., Manterola L., Sola-Landa A., Moriyón I., Moreno E. (2002) Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. *J. Veterinary Microbiology.* 90 (1-4): 329-339

Manterola L., Moriyón I., Moreno E., Sola-Landa A., Weiss D.S., Koch M. H. J., Howe J., Brandenburg K., López-Goñi I. (2005) The Lipopolysaccharide of Brucella abortus BvrS/BvrR Mutants Contains Lipid A Modification and Has Higher Affinity for Bactericidal Cationic Peptides. *JB Journal of Bacteriology.* 187 (16): 5631-5639

Maughan N. J., Lewis F. A., Smith V. (2001) An introdution to arrays. *Journal of Pathology.* 195: 3-6

Olsen S. C., Stoffregen W. S. (2005) Essential role of vaccines in brucellosis control and eradication programs for livestock. *J. Future Drugs.* 4 (6): 915

Pappas G., Akritidis N., Bosilkovski M., Tsianos E. (2005) Medical progress Brucellosis. *The New England Journal of Medicine.* 352: 22

Schena M., Shalon D., Heller R., Chai A., Brown P. O., Davis R. W. (1996) Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes. *Biochemistry.* 93: 10614-10619

Turner et al.,: Molecular Biology, 2nd edition, University of Liverpool, UK, School of biological sciences 2001, section C3, sec. G, sec. J 3

Yoshida S., Ito M., Nishida I., Watanabe A. (2001) Isolation and RNA Gel Blot Analysis of Genes that Could Serve as Potential Molecular Markers for Leaf Senescence in *Arabidopsis Thaliana. Plant and Cell Physiology.* 42(2): 170-178

http://www.conspiracyarchive.com/images/b/.)

http://en.wikipedia.org/wiki/PCR

http://www.vivo.colostate.edu/hbooks/genetics/biotech/gels/principles.html