BABEŞ-BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY

Summary of doctoral thesis

CLONING AND EXPRESSION OF SOME GENES INVOLVED IN PROKARYOTIC CELLS DIVISION

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1. Introduction

Keywords: FtsZ, *Escherichia coli*, *Pseudomonas aeruginosa*, antibiotic resistance, murine polyclonal anti-FtsZ antibodies, cross-reactivity, actin, tubulins

The bacterial cells division, named also binary fission, starts with bacterial chromosome replication, followed by chromosome segregation and citokinesis, with division septum formation and separation of daughter cells. After the selection of the division site, usually at the middle of the cell, between newly replicated and segregated chromosomes, citokinetic ring assembly follows, which in prokaryotes invariably involves FtsZ.

FtsZ is a GTPase which binds and hydrolyze GTP, providing energy for cell shape remodeling and septum formation, by possibly counteracting forces due to turgor and existing cellular architecture. FtsZ is a protein whose molecular weight ranges between 37 to 43 kDa, ubiquitous in bacteria. FtsZ is a homologue of eukaryotic tubulins, primary structure comparison of FtsZ and tubulins reveals the sequence homology in the N-terminal domain. It is remarkable that these proteins have a sequence rich in glycine ,,glycine-rich cluster" [GGGTG (S\T)G], which in tubulin is known to be part of structure of the GTP binding site of the N-terminal domain. No other known protein has this sequence, besides tubulins and FtsZ. Moreover, [GGGTG(S\T)G] is one of three highly conserved sequences in tubulins family which counts over 150 proteins. [Mukherjee et al., 1993] FtsZ and tubulins polymerize unidirectionally into linear filaments in GTP-dependent manner and in similar conditions, FtsZ filaments form bundles and sheets, elastic structures which help to maintain the Z ring under the pressure generated by the septal constriction. There is no evidence that FtsZ could form *in vitro* or *in vivo* microtubular structures. [Sun & Margolin, 1998; Gonzalez et al., 2005] *In vivo*, FtsZ is assembled in the middle of the cell in a supramolecular structure called Z ring, which in conventional fluorescent microscopy appears as a homogeneous structure, circular-closed. PALM showed a Z ring with a width of 110nm, with protofilaments that are not tightly aligned, but relaxed arranged, with longitudinal and radial overlaps, forming a heterogeneous structure with dynamic remodeling by exchanges between helical conformation and the ring.[Fu et al.., 2010]

Eukaryotic cells have a complex and dynamic cytoskeleton with essential functions in maintaining cell shape, in cell signaling, in the transport of DNA molecules, organelles and secretion vesicles and in the cell division. Citokinetic ring of eukaryotes is a actomyozin structure. There is a remarkable interdependence between microtubular structures that occur post-anaphase and constriction determined by components of cleavage furrow until the completion of citokinesis. The middle zone of the spindle and the central body contain structural and signaling elements that are assembled after chromosome segregation, and are involved in the location of the division septum and citokinesis.

Interactions between the mitotic spindle, contractile ring and the cell membrane ensure correct positioning of the cleavage furrow between segregated chromosomes, and the addition of new membranes by vesicular transport along microtubules of the specialized areas provide citokinesis completion and separation of daughter cells. [Hales et al., 1999; Straight & Field, 2000; Guertin et al., 2002; Pollard, 2010]

It is likely that FtsZ originated in a common ancestor and was passed to bacteria and archaea . Eukaryotes later evolved an actin-based machine for cytokinesis, and in eukaryotes FtsZ underwent a drastic change in as it evolve into tubulin [Erickson, 2007].

ftsZ gene is an essential gene and therefore conserved in all bacteria cells, and FtsZ protein has a central role in cell division, an event which is critical to the survival of bacteria, so it ca be considered as an alternative target for new generation of therapeutic antibacterial agents, which would have in this way the quality of broad-spectrum agents.

Considering these issues and the increasing resistance of bacteria to current use antibiotics, it was evaluated the immunogenicity of FtsZ from two Gram-negative bacterial species, *Escherichia coli* and *Pseudomonas aeruginosa* in mice, and than the cross-reactivity between FtsZ from *Escherichia coli* and *Pseudomonas aeruginosa*. It has been studied subsequently cross-reactivity between these antibodies and the eukaryotic proteins actin and tubulin.

2. Research premises

Essential and conserved components of the cytoskeleton, involved in bacterial cell division, can be regarded as new target structures for antibacterial agents active against pathogenic bacteria that have developed resistance against to antibiotics frequently used.

FtsZ is a protein with an essential role in prokaryotic cell division, which is although present in all bacterial cells whose *ftsZ* gene has been studied, is nevertheless absent in the mithocondria of eukaryotic cells. Due to divergent evolution of FtsZ and its eukaryotic homologue, tubulin, FtsZ can be considered an attractive target for antibacterial agents with a broad spectrum but also, with a selective toxicity against bacterial pathogens.

3. Research objectives

The main research objective was to express and evaluate the immunogenicity of FtsZ from two Gram-negative bacteria, well-known as opporthunistic human pathogens, *Escherichia coli* and *Pseudomonas aeruginosa*. Cytokinetic ring is considered as a new target, susceptible to antibacterial therapy, and FtsZ as the presumptive ideal molecule for interactions with agents that would block bacterial cells cytokinesis.

Initial objective consisted in isolation and amplification of *ftsZ* genes from *Escherichia coli K12* genome and from *Pseudomonas aeruginosa PAO1,* by PCR, in order to cloning and overexpression these two FtsZ proteins.

Recombinant proteins FtsZ were purified and used to obtain antiftsZ *Escherichia coli* and antiftsZ *Pseudomonas aeruginosa* polyclonal antibodies, by inoculation in mice.

These antibodies were then analyzed in terms of titer, of affinity, specific reactivity and cross-reactivity characteristics. Study of cross-reactivity of these antibodies more precisely targeted the cross-reactivity of FtsZ from the two bacterial species, *Escherichia coli* and *Pseudomonas aeruginosa*, at this stage. Considering the literature data according to which ftsZ gene is an essential gene, conserved in almost all prokaryotes, therapeutic agents

that interact specifically with FtsZ, inhibit cell division and would have a broad-spectrum of action.

Also in agreement with literature data according to which there is large sequence omology between N-terminal domains of FtsZ and tubulins, especially in the GTP binding and hydrolysis site, and actin plays a central role in eukaryotic cytokinesis (the major protein in prokaryotic cytokinesis is FtsZ), next objective was selected: to analyze the cross-reactivity of FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa* with the most important proteins of eukaryotic cytoskeleton: actin and tubulins. Cross-reactivity between this eukaryotic antigens and antiFtsZ polyclonal antibodies was analyzed in order to obtain data concerning the action selectivity of antibodies exclusive against bacterial agents without affecting the eukaryotic cytoskeleton.

4. Matherials and methods

4.1. Amplification of *ftsZ Escherichia coli K12* gene

For gene cloning, a set of primers have been designed by the author. In order to clone the gene into pET21b and pET28b vector, the forward primer: 5'GGCATATGTTTGAACCAATGGAACTTAC3'
and the reverse primer:
5'GTACTCGAGTTAATCAGCTTGCTTACGCAG3' were used, with underlined positions stand for restriction sites of *Nde*I (forward primer) and *Xho*I (reverse primer) enzymes.

Vent polymerase from New England BioLabs (NEB) has been used for *ftsZ E. coli* amplification by PCR.

The conditions used for the PCR reaction were: 95°C for 5 minutes, 30 cycles of 93°C (30 s), 57°C (30 s), 72°C (2 min) and 72°C for 5 minutes. [McPherson şi colab, 1991; Sambrook şi Russell, 2001]

4.2. Cloning *ftsZ Escherichia coli* gene in pGEM-T cloning vector

ftsZ E.coli was amplified by PCR, purified from gel with a kit NucleoSpin® Extract II (Macherey-Nagel)and introduced into pGEM-T vector (Promega). The cloning process was necessary in order to facilitate gene sequencing and to perform a new cloning of this gene into pET 21b and pET 28b expression vectors. pGEM-T vector is a PCR product direct cloning small vector, of 3003 bp. The cloning kit which includes this vector (pGEM®-T Vector Systems) contains the linearized vector which has a free thymine at the 5' end. It can be used to obtain a large amount of plasmidic DNA generating many copies when cloning. The cloning site of this vector is integrated inside *lacZ'* gene allowing selection of recombined molecules by IPTG (Isopropyl β-D-1-thiogalactopyranoside) and X-Gal (bromo-chloro-indolyl-galactopyranoside) which confers ampicilin resistance to transformed cells.

Ligation was performed at 4ºC overnight. Ligation mix was used to transform

Escherichia coli DH5α competent cells by electroporation. *E.coli* DH5α were inoculated on ampicilin containing (100mg/l) solid LB. To select colonies with correctly recombined vectors (pGEM-*ftsZEc*), IPTG (50mM) and X-Gal (10mg/ml) were added.[Promega Technical Manual pGEM®-T and pGEM®-T Easy Vector Systems; Sambrook & Russell, 2001; Ausubel et al., 2003; Clark, 2005]

4.3. Verification of recombined molecules

White colonies appeared after the transformation were separated in small 5ml LB liquid medium cultures, supplemented with ampicilin (100mg/ml), overnight incubated at 37ºC on a rotative shaker. Plasmidic DNA was purified out of these colonies using a GeneJET™ Plasmid Miniprep Kit (Fermentas). Final verification of cloned sequence was performed by sequencing with a ABI Prism 310 Analysor, using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and M13 primers which are specific for pGEM-T vector. [MacBeath et al., 2001; Ausubel et al., 2003]

4.4.Cloning *ftsZ Escherichia coli* gene into pET21b and pET28b expression vectors

Molecules were digested with *Xho*I and *Nde*I restriction enzymes from Fermentas and were inserted into pET21b and pET28b expression vectors. Samples were incubated over night at 37ºC. Digestion products were separated in 1% agarose gel. Digested plasmidic DNA was purified from agarose gel using a NucleoSpin® Extract II Macherey-Nagel kit. Overnight ligation was performed at 4ºC with T4 DNA ligase (NEB). The mix was used to transform *Escherichia coli* DH5α strain which are electrocompetent cells. [Glover şi Hames, 1995] The *E.coli* DH5α cells were then inoculated on solid LB medium with 100 mg/l ampicillin for pET21b and 30mg/l kanamycin for pET28b. .[Sambrook & Russell, 2001; Ausubel et al., 2003; Novagen pET System Manual ediția a 10-a, 2002; http: //www.fermentas.com/templates/files/tiny_mce/media_pdf/ broch_genejet_P19.pdf]

4.5. Verification of pET21b-*ftsZ* and pET28b-*ftsZ* recombined molecules

White colonies appeared after transformation (four colonies were analyzed for each recombined vector) were inoculated in 5ml LB culture medium including 100mg/l ampicillin and 30mg/l kanamycin, respectively, and they were incubated overnight at 37ºC. Plasmidic DNA was purified out of these cultures using a GeneJET™ Plasmid Miniprep Kit from Fermentas. In order to verify the recombination, digestion of recombined molecules was performed using *Nde*I/*Xho*I restriction enzymes set. Digestion products were analyzed in 1% agarose gel.

4.6. FtsZ *Escherichia coli* expression and purification

Recombinated vector pET28b was used to transform *Escherichia coli* BL21(DE3) which are also electrocompetent cells. BL21(DE3) is the host strain selected for expression of recombinant proteins, because target genes were cloned in pET plasmids under control of bacteriophage T7 promoter and expression is induced by providing T7RNA polymerase in BL21(DE3). Transformed BL21(DE3) were inoculated on kanamycin containing (30mg/l) solid LB. Colonies appeared after transformation, were inoculated in 5ml LB culture medium including 30mg/l kanamycin, and they were incubated overnight at 37ºC. 50 ml LB culture medium with kanamyicin (30 mg/l) were inoculated with all transformed bacteria from the solid medium and incubated at 37°C until OD_{600nm} had reached 0,9997. Addition of 40 µl IPTG (1 mM) induced the expression of target protein, at 37 ºC. The induced cells were then harvested by centrifugation. Pellets were suspended in 3ml of lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH8, 10mM imidazol) and lysated by sonication. The bacterial lysate was centrifuged 20 minutes at 21000*g and 4ºC. [Ausubel et al., 2003] Both the supernatant and the lysate were analyzed by SDS–PAGE using a 12,5% polyacrylamide gel in order to verify the Escherichia *coli* FtsZ expression. [Popescu, 1990; Rosenberg, 2005] The supernatant of the cell lysate was passed through the nickel–NTA column (Qiagen). The column was washed with lysis buffer containing 10 mM imidazol. Then the protein passed through the column. Next the.column was eluted with increasing imidazole concentration (250 mM). Purity of the recombinant protein was assessed by SDS–PAGE using a 12,5% polyacrylamide gel, and then dialyzed against PBS. Quantitation of recombinant proteins was carried out using Bradford protein assay. [Mikkelsen & Cortón, 2004]

4.7. Amplification of *ftsZ Pseudomonas aeruginosa PAO1* gene

For *ftsZPseudomonas aeruginosa* gene cloning, the primers have been designed by the author. In order to clone the gene into pET 28a vector, the forward primer:

5’ GGCATATGTTTGAACTGGTCGATAAC 3’ and the reverse primer:

5’GTGAATTCTCAATCGGCCTGACGACG 3’ were used, with the underlined positions stand for restriction sites of *Nde*I (forward primer) and *EcoR*I (reverse primer) enzymes. Amplification by PCR of *ftsZ Pseudomonas aeruginosa* gene was performed with a mix of *Taq* and *Pfu* polymerases.

The conditions used for the PCR reaction were: 95°C for 5 minutes, 35 cycles of 93°C (30 s), 57°C (30 s), 72°C (2 min) and 72°C for 5 minutes. Products of amplification by PCR were verified in 1% agarose gel. [McPherson et al., 1991; Sambrook & Russell, 2001]

4.8. Cloning *ftsZ Pseudomonas aeruginosa* gene in PTZ57R cloning vector

ftsZ Ps.aeruginosa was amplified by PCR, purified from gel with a Nucleospin kit and introduced into pTZ57R vector (Fermentas) . The cloning process was necessary in order to facilitate gene sequencing and perform a new cloning of this gene into pET28a expression

vector.

pTZ57R vector is a PCR product direct cloning small vector, of 2886 bp. The cloning kit which includes this vector (InsTAclone™ PCR Cloning Kit) contains the linearized vector which has a free dideoxythymine at the 5' end. It can be used to obtain a large amount of plasmidic DNA generating many copies when cloning. The cloning site of this vector is integrated inside *lacZ'* gene allowing selection of recombined molecules by IPTG (Isopropyl β-D-1- thiogalactopyranoside) and X-Gal (bromo-chloro-indolyl-galactopyranoside) and β-lactamase gene, which confers ampicilin resistance to transformed cells.

Ligation was performed at 4ºC overnight. Ligation mix was used to transform *Escherichia coli* XL1Blue competent cells. In purpose of inducing competence, bacteria were chemically prepared with TSS. They were inoculated on ampicilin containing (100mg/l) solid LB. To select colonies with correctly recombined molecules, 80µl IPTG (50mM) and X-Gal (10mg/ml) were added. [Sambrook & Russell, 2001; Ausubel et al., 2003; Nair, 2007]

4.9. Verification of recombined molecules

White colonies appeared after the transformation were separated in small 5ml LB liquid medium cultures, supplemented with ampicilin (100mg/ml), overnight incubated at 37ºC on a rotative shaker. Plasmidic DNA was purified out of these colonies using a GeneJET™ Plasmid Miniprep Kit (Fermentas) and verified on 1% agarose gel. Final verification of cloned sequence was performed by sequencing with a Beckman Coulter CEQ8800 using GenomeLab DTCS Quick Start Kit and M13 primers which are specific for pTZ57R vector. [Graham & Hill, 2001; Ausubel et al., 2003]

4.10. Cloning *ftsZ Pseudomonas aeruginosa* gene into pET28a expression vector

Molecules were digested with *EcoRI* and *NdeI* restriction enzymes from Fermentas and were inserted into pET28a expression vector. Samples were incubated over night at 37ºC. Digestion products were separated in 1% agarose gel. Digested plasmidic DNA was purified from agarose gel using a NucleoSpin® Extract II Macherey-Nagel kit. Overnight ligation was performed at 4ºC with T4 DNA ligase (NEB). The mix was used to transform *Escherichia coli* DH5α strain which are competent cells prepared chemically, on solid LB medium with 30mg/l kanamycin for pET28a. [Sambrook & Russell, 2001; Ausubel et al., 2003; Novagen pET System Manual ediția a 10-a, 2002; http://www.fermentas.com /templates/files/tiny_mce/media_pdf/ broch_genejet_P19.pdf]

4.11. Verification of pET28a-*ftsZ Pseudomonas aeruginosa* recombined molecules

White colonies appeared after transformation (four colonies were analyzed for each recombined vector) were inoculated in 5ml LB culture medium including 30mg/l kanamycin, respectively, and they were incubated overnight at 37ºC. Plasmidic DNA was purified out of these cultures using a GeneJET™ Plasmid Miniprep Kit from Fermentas. In order to verify the recombination, digestion of recombined molecules was performed using *Nde*I/*Eco*RI restriction enzymes set. Digestion products were analyzed in 1% agarose gel.

4.12. FtsZ *Pseudomonas aeruginosa* expression and purification

The purified plasmids were further introduced into *E. coli* BL21(DE3) cells. The transformed *E. coli* cells were grown on LB solid medium containing kanamycin (30 μg/ml). An overnight culture was used to inoculate 50 ml LB broth medium supplemented with kanamycin. When the OD_{600} had reached 0,8-1,00, expression was induced by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) 1mM, at 37 °C. The induced cells were then harvested by centrifugation. Pellets were suspended in lysis buffer and lysate by sonication. The supernatant of the cell lysate was passed through the nickel–NTA column (Qiagen). The column was washed with lysis buffer containing 10 mM imidazol. Then the protein passed through the column. Next the column was eluted with increasing imidazole concentration (250 mM). [Ausubel şi colab., 2003] Expression and purity of the recombinant protein was assessed by SDS–PAGE using a 12,5% polyacrylamide gel, and then the protein was dialyzed against PBS [Popescu, 1990; Rosenberg, 2005] Quantitation of recombinant proteins was carried out using Bradford protein assay. [Mikkelsen & Cortón, 2004]

4.13. Generation of FtsZ-specific mouse antibodies

Six- to eight-week-old BALB/c mice with a body weight of approximately 20 g were used. Mice were obtained from the Cantacuzino Institute (Bucharest, Romania).

Four mice were assigned to two different groups and immunized subcutaneously with either 50 μg of *E. coli* (group 1) or *P. aeruginosa* FtsZ (group 2), respectively mixed with Freund's complete adjuvant. The animals were boosted twice with the same protein preparation in incomplete Freund's adjuvant at two weeks intervals. Preimmune sera were collected before the first immunization and immune sera were obtained at regular intervals thereafter and characterized by immunofluorescence immunoblot and ELISA.

4.14. Characterisation of murine FtsZ-specific antibodies

Recombinant FtsZ were fractionated by 12.5% and 5% SDS-PAGE, respectively, transferred to nitrocellulose, and analyzed by immunoblotting. Gels were cast with recombinant FtsZ proteins and some, were stained with Coomasie Blue in order to check the migration pattern whereas duplicate gels were transferred for 2h to 0.2 microM nitrocellulose membranes (Bio-Rad). Membranes were blocked overnight in TBST 1%BSA 3% skim milk and washed 2 x 10 min in TBST. Strips were cut and each was incubated with a different dilution of primary antibodies (mouse serum diluted 100, 250, 500 and 1000x, respectively) for 2h at room temperature under continuous shaking. After washing the strips 2 x 10 min, another incubation with a 1/3000 dilution of secondary goat-anti mouse HRP-labeled antibody was carried out for 1h at room temperature. Color reaction was observed using 3,3' diaminobenzidine (Merck) as a chromogenic substrate. For ELISA analysis, 96 well plates were coated with 500 ng recombinant proteins in 100microL of carbonate/bicarbonate buffer pH 9.6 for 1h at room temperature. After washing the plates five times with PBST, blocking was performed with 1% BSA in PBST for 1h at room temperature. Primary antibodies (i.e.

polyclonal antibodies from mice immunized with recombinant proteins) and the secondary HRP-labeled antibody were diluted as described for the immunoblot. Color reaction was developed using ortho-phenylenediamine (DakoCytomation) as a chromogenic substrate and the reaction was stopped with 0.5M H_2SO_4. Absorbance was read at 490nm using a multiplate reader.

Investigation of the cross-reactivity among the polyclonal anti-FtsZ antibodies and between these antibodies and the eukaryotic proteins actin and tubulin

Gels were cast using either FtsZ or actin from bovine heart (SigmaAldrich) or tubulin beta from bovine brain (tebu-bio) using the protocol described above. The gels were either stained with Coomassie blue or transferred to nitrocellulose. Strips were cut and different combinations of substrate and primary antibody were used to investigate the cross-reactivity potential of our newly produced antibodies. Antibodies from group 1 (mice immunized with *E. coli* FtsZ) were incubated with either *E. coli* FtsZ, *P. aeruginosa* FtsZ, actin or tubulin; antibodies from group 2 were also incubated with all the four mentioned substrates. A monoclonal mouse anti-actin antibody was likewise incubated with all substrate and used as a control for the assay. [Chiriac et al., submitted; Coligan et al.,2005].

5. Results

5.1. PCR amplification of *ftsZ* genes

The amplification products of *ftsZ* gene from *Escherichia coli* were visualized in agarose gel 1% (Fig.1). As it can see in the image gel, amplified fragment is of the desired length, amount of amplified product is sufficiently high. Reaction performed with *Vent* polimerase was specific, without unspecific amplifications.

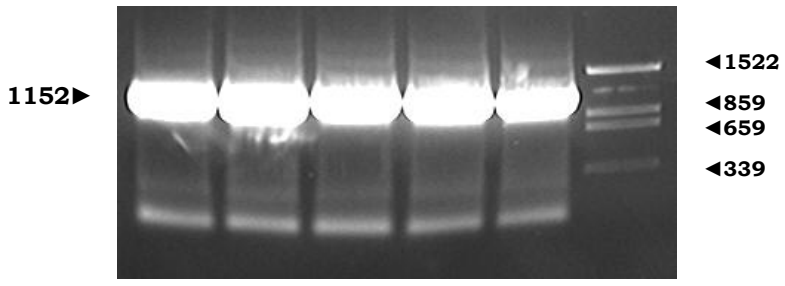

Figure 1. PCR amplification of *ftsZ* gene from *Escherichia coli*
1-5- 50 µl amplification products
6- molecular marker (pQE60 digested with *Dra*I)

The amplification products of *ftsZ* gene from *Pseudomonas aeruginosa* were also visualized in agarose gel 1% (Fig.2). As it can see in the image gel, amount of amplified product is sufficiently high and the reaction, performed with a mix of *Taq* and *Pfu* polimerases, had undergone without unspecific amplifications.

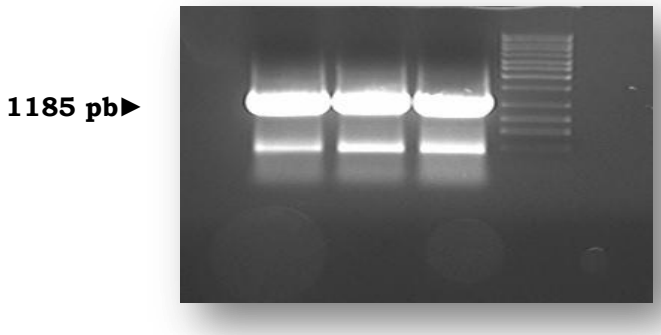

Figure 2. PCR amplification of *ftsZ* gene from *Pseudomonas aeruginosa*
1-3- 50µl PCR products; **4**– O'GeneRuler™ 1kb Ladder (Fermentas)

5.2. Cloning *ftsZ Escherichia coli* gene in pGEM-T cloning vector

The recombinant colony screening was done with white-blue screening (X-Gal 20mg/ml and IPTG 100mM). The plasmid DNA containing *ftsZ* was purified from an overnight culture (5ml) and digested with *Nde*I and *Xho*I restriction enzymes (Fig. 3).

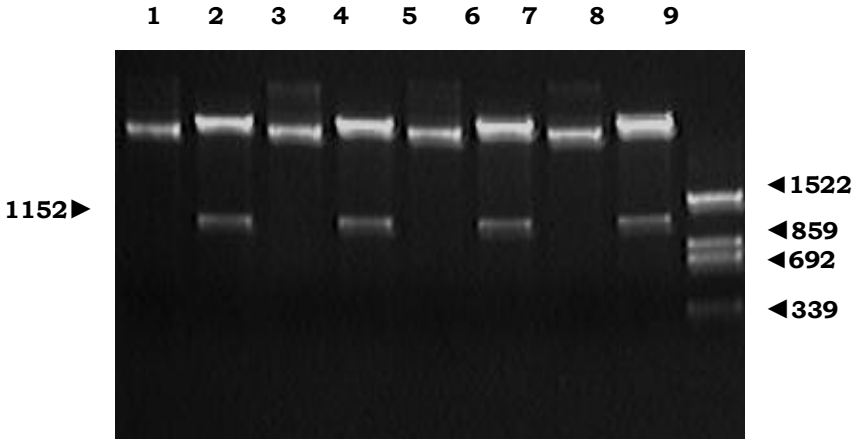

Figure 3. Recombinant plasmid screening.
1,3,5,7- Plasmid DNA
2,4,6,8- Plasmid DNA digested with *Nde*I and *Xho*I restriction enzymes
9- molecular marker (pQE60 digested with *Dra*I)

The recombinant plamids pGEM-TftsZEc were sequenced using specific primers M13/pUC (forward and reverse sequencing primers) The cloned gene was 100% identical with the sequence of *ftsZ Escherichia coli K-12* from databases.

5.3. Cloning *ftsZ Pseudomonas aeruginosa* gene in pTZ57R cloning vector

FtsZ gene amplified by PCR and purified from gel was inserted into pTZ57R cloning vector from Fermentas. Ligation mix was used to transform *Escherichia coli* XL1Blue

competent cells, which had been sown on solid LB medium with ampicillin. For the recombinant colonies selection, X-Gal and IPTG were added to the medium. From the white colonies that are result of transformation, plasmid DNA was purified. Recombinant molecules were screened initially by digestion with *Nde*I and *Eco*RI restriction enzymes and then by sequencing. Products of digestion have been analyzed on agarose gel 1% (Fig4.). The fragments obtained have appropriate length: the lengths of the gene sequence and the length of vector. (Fig4.)

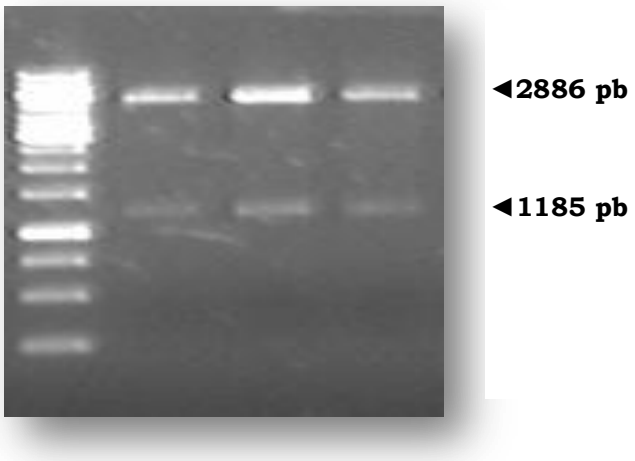

Figure 4.Recombinant plasmid pTZ57R-*ftsZPs* screening

1 –O'GeneRuler™ 1kb Ladder Fermentas;

2, 3, 4 - Plasmid DNA was digested with *Nde*I and *Eco*RI restriction enzymes

The recombinant plamids pTZ57R*ftsZPs* were sequenced using specific primers M13/pUC (forward and reverse sequencing primers), with Beckman Coulter CEQ8800 and a GenomeLab DTCS Quick Start Kit. The cloned gene was 100% identical with the sequence from database (FTSZ_PSEAE P47204).

5.4. Cloning *ftsZ Escherichia coli* gene into pET21b and pET28b expression vectors

The ftsZ gene was excised from pGEM by digestion with restriction enzymes *Nde*I and *Xho*I and ligated into pET21b and pET28b vectors from Novagen. Products of digestion were migrated on1% agarose gel (Fig.5).

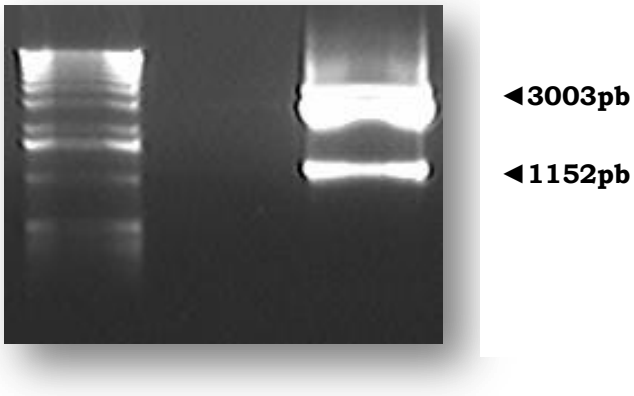

Figure 5. Digestion of recombinant molecules pGEM-T*ftsZE.coli*

1- 1kpb Ladder (Invitrogen)

2- Plasmid DNA pGEM-T digested with *NdeI* and *XhoI* restriction enzymes.

pET21b and pET28b were digested with the same restriction enzymes, *NdeI* and *XhoI*. The digestion products, *ftsZEc* gene, pET28b and pET21b have been verified on 1% agarose gel (Fig. 6).

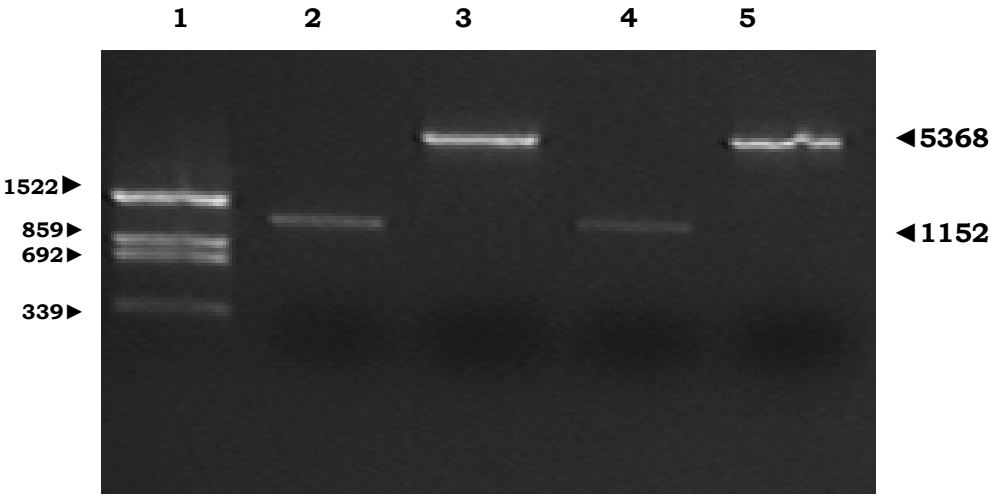

Figure 6. *ftsZ* gene and pET21b, pET28b vectors after digestion with *Nde*I and *Xho*I restriction enzymes

1-molecular weight marker (pQE60 vector digested with *Dra*I);

2,4- *ftsZ* gene

3- pET21b vector; **5**- pET28b vector.

Recombinant molecules obtained after ligation were used for *Escherichia coli* DH5α.cells transformation. From colonies appeared on the medium with antibiotics (ampicillin for pET21b and kanamycin for pET28b), plasmidic DNA was isolated (4 precultures for each vector) and analyzed on1% agarose gel (Fig.7)

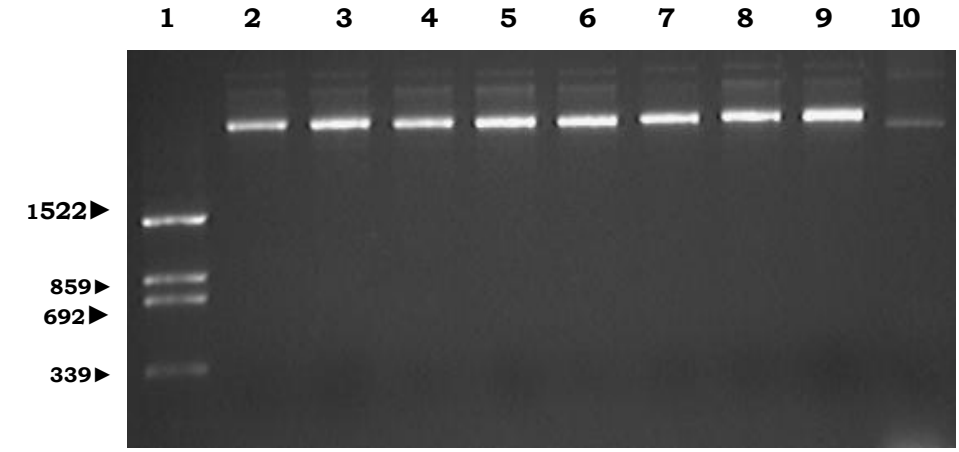

Figure 7. Verification of *ftsZ* gene presence in pET21b and pET28b.

1 - molecular weight marker (pQE60 vector digested with *DraI*)

2-5 - 5µl recombinant pET21b

6-9 – 5µl recombinant pET28b vector

10 – 3µl pET21b vector

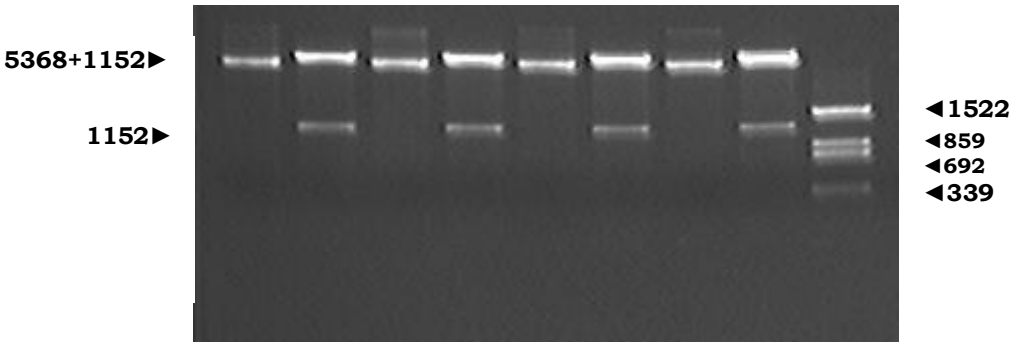

Figure 8. Verification of *ftsZEscherichia coli* gene (1152pb) presence in pET21b after digestion with *XhoI* şi *NdeI*.

1,3,5,7- pET21b*ftsZE.coli*

2,4,6,8- pET21b digested

9- molecular weight marker (pQE60 vector digested with *Dra*I)

5.5. Cloning *ftsZPseudomonas aeruginosa* gene in pET28a vector

Correct recombinant plasmids pTZ57R*ftsZPs* were digested with *NdeI* şi *EcoRI* restriction enzymes. Products of digestion were separated on 1% agarose gel, then excised from gel and purified. Products of purification were further used to clone gene in pET28a vector, which was also digested with the same restriction enzymes and purified then from agarose gel. Ligation mixture was used to transform *Escherichia coli* DH5α cells, wich were sown on solid LB medium with kanamycin. From the colonies appeared after transformation, were performed several precultures suplimented with kanamycin, from which plasmid DNA was purified. Recombinant molecules pTZ57R*ftsZPs* were initially verified by digestion with the restriction enzymes, digestion products were then analyzed on 1% agarose gel (Fig. 9). As

it can see from the gel image, by digestion of plasmids, there are two fragments resulted, one of larger size which correspond to linearized vector (5369pb) and another of smaller size, which correspond *ftsZ* gene from *Pseudomonas aeruginosa* (1185 pb).

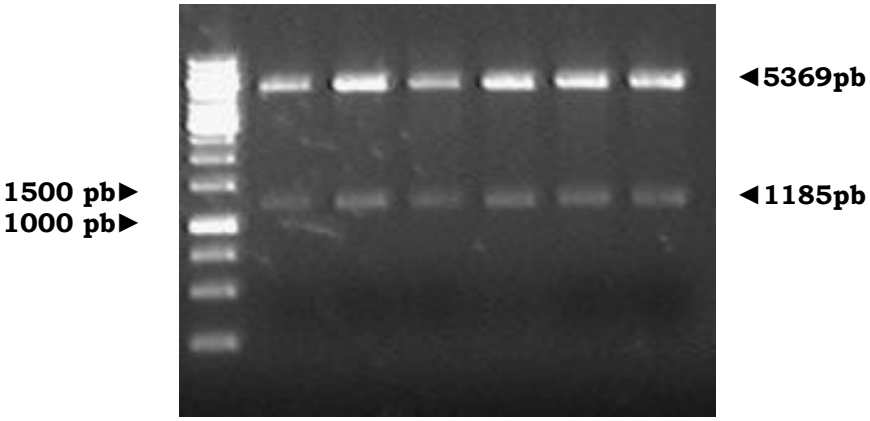

Figure 9. Digestion of recombinant molecules

1 - O'GeneRuler™ 1kb Ladder (Fermentas);

2-7- digestion products of pET28a-*fstZPs* recombinant vectors with *Nde*I and *EcoR*I restriction enzymes.

5.6. FtsZ proteins expression and purification

For protein expression, the recombinant plasmid containing *ftsZ* from *Escherichia coli* and also, recombinant plasmid with *ftsZ* from *Pseudomonas aeruginosa* was introduced by chemical transformation into a *E. coli* cells BL21(DE3) strain. An overnight small culture was used to inoculate 200 ml LB broth medium supplemented with kanamycin (30 mg/L). The expression was induced with 1 mM IPTG when the OD_{600} had reached 0.8-1.00 for 4-5 hours. Bacterial cells were then harvested by centrifugation. Pellets were suspended in lysis buffer containing 10 mM imidazole, 50 mM Tris HCl pH 8.0 and 300 mM NaCl. Cell lysis was done by sonication. The total lysate was clarified by centrifugation at 20 000 g× at 4ºC for 30 min. The supernatant of the cell lysate was passed through the nickel–NTA column (Qiagen) echilibrated with lysis buffer. The column was washed with 10 columns volumes of washing buffer (50 mM imidazole, 50 mM Tris HCl pH 8.0 and 300 mM NaCl). The recombinant protein was eluted with the same buffer containing 250 mM imidazole. Expression and purity of the recombinant protein was analyzed with SDS–PAGE using a 12.5 % polyacrylamide gel. The fraction with protein was pooled and dialyzed against PBS at 4ºC for 24 hours. Protein quantification was carried out using Bradford protein assay.

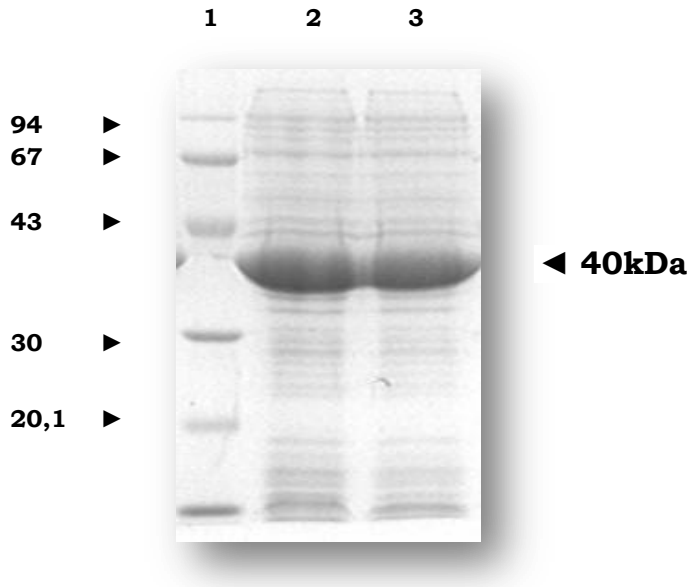

Figure 10. FtsZ *Escherichia coli* expression in *Escherichia coli* BL21(DE3).

1- protein weight marker
2- bacterial lysates
3- supernatant with recombinant protein.

As it can see from the gel image, (Fig.10) FtsZ from *Escherichia coli* expresses soluble in *Escherichia coli* BL21(DE3) cells.

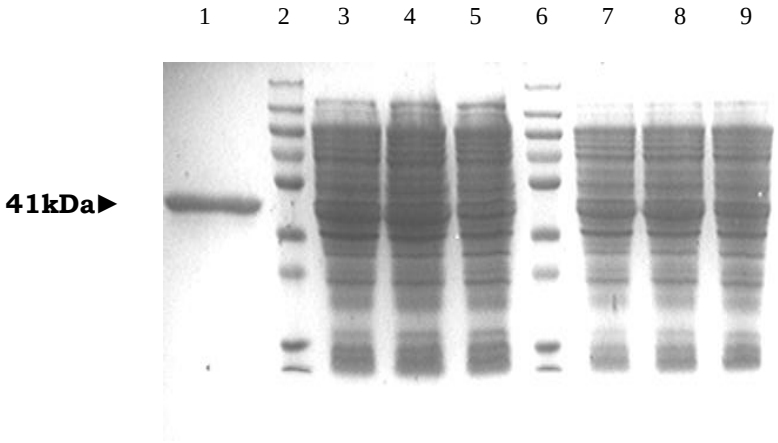

Figure 11. FtsZ *Pseudomonas aeruginosa* expression in *Escherichia coli* BL21(DE3).

1- 20 µl eluted protein
2- 4µl Page Ruler Prestained Protein Ladder Plus (Fermentas SM1181)
3- 5µlbacterial lysate
4- 5µl supernatant
5- 5µl rest (after passing supernatant through column)
6- 4 µl Page Ruler Prestained Protein Ladder Plus (Fermentas SM1181)
7- 3 µl bacterial lysate
8- 3 µl supernatant
9- 3 µl rest (after passing through column)

FtsZ *Pseudomonas aeruginosa* recombinant protein expresses soluble in *Escherichia coli* BL21(DE3).

The final concentration of FtsZ *Pseudomonas aeruginosa* after elution from the column and pooling fractions was 0,8mg/ml. Repeated expression and purification of FtsZ *Pseudomonas aeruginosa* led to a final concentration of 0,56mg/ml in a final volume of 3ml (Fig.12).

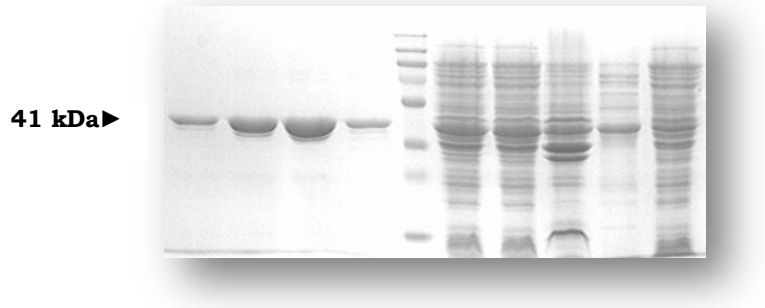

Figure 12. Expression and purification of FtsZ *Pseudomonas aeruginosa*

1– 4 eluted fraction 2, 3 4 and 5 respectively
5 – protein weight marker (Page Ruler Prestained Protein Ladder Plus from Fermentas).
6- bacterial lysate; **7**- supernatant; **8**- pellet; **9**-wash column;
10- rest (after passing supernatant through column)

5.7. Production of murine polyclonal antiFtsZ antibodies

Mice immunized with recombinant proteins FtsZ produce polyclonal antibodies as soon as 8 weeks after the first immunization and by immunoblotting, the sera collected at 8 weeks after the onset of immunization showed reactivity with the recombinant protein used to immunize the animals (Fig.14).

Previously, the recombinant FtsZ proteins were separated on polyacrylamide gel and together with a protein molecular weight marker, and then transferred to a nitrocellulose membrane. Checking the transfer was made by staining the gel with Coomasie Blue.(Fig.13)

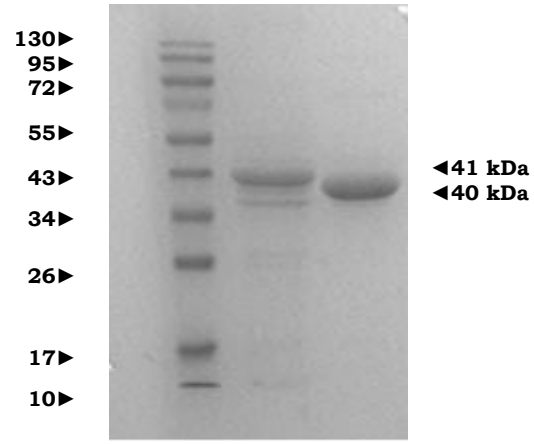

Figure 13. Verification of FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa* on the nitrocellulose membrane.

1- PageRuler Plus Prestained Protein Ladder from Fermentas (SM 0671)

2- 10µg of FtsZ *Pseudomonas aeruginosa* fraction MW= 41kDa

3- 10µg of FtsZ *Escherichia coli* fraction (MW= 40kDa)

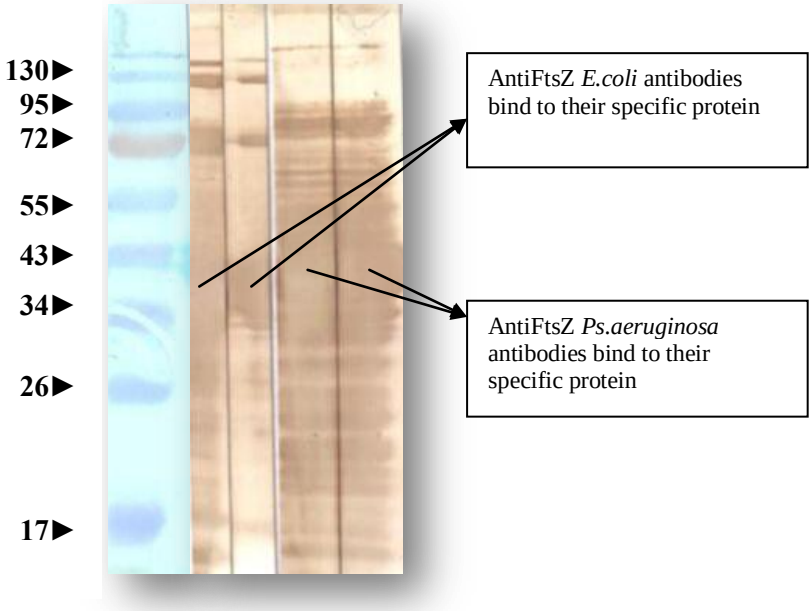

Figure 14. Immune sera collected at 8 weeks after the onset of mice immunization and diluted 100x showed positive reactivity with FtsZ *E. coli* and FtsZ *P. aeruginosa*.

5.8. Characterisation of murine antiFtsZ antibodies

By testing optimal concentrations of primary antibodies (by dilutions of immune sera obtained at 16 weeks after the onset of immunization with FtsZ *Escherichia coli*) and optimal concentrations of secondary antibodies were obtained the results from Fig.15.

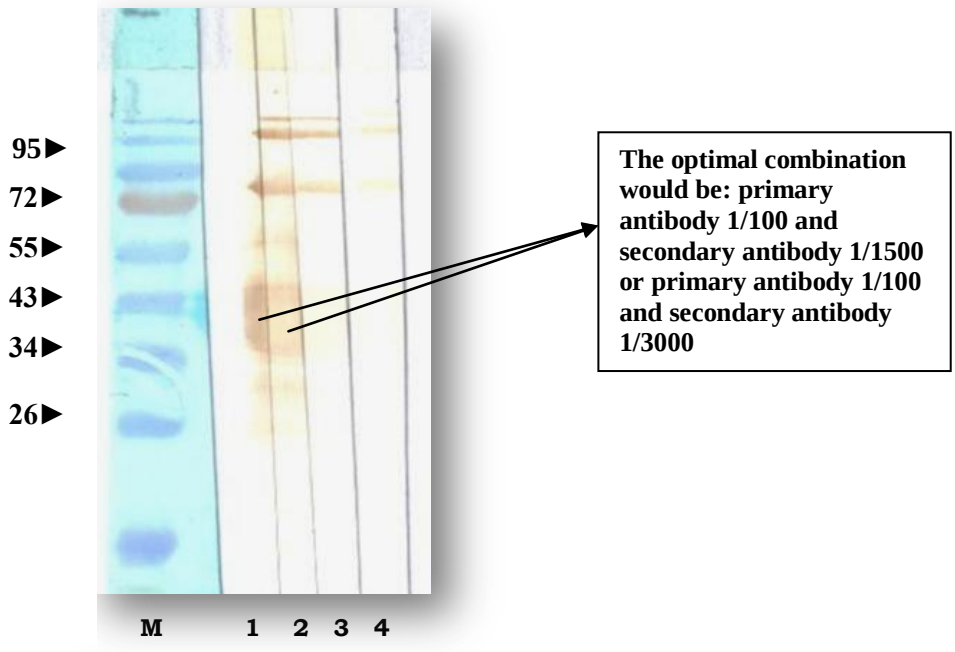

Figure 15. Analysis by Western-blot of the optimal primary antibodies concentrations from immune sera obtained at 16 weeks, from *E.coli* group; **M** is Marker PageRuler™ Prestained Protein Ladder (Fermentas SM 0671)

Table 1. Primary and secondary antibodies concentrations used to testing the optimal antibodies concentrations:

Strip Number	Substrate	Primary Ab	Secondary Ab HRP-conjugated	Obs.
1	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/100	Anti-mouse 1/1500	++++
2	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/100	Anti-mouse 1/3000	+++
3	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/250	Anti-mouse 1/1500	+++
4	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/250	Anti-mouse 1/3000	+

By testing the specific reactivity of antiFtsZ *Escherichia coli* antibodies using different concentrations of the first antibody were obtained the best reactivity detection bands at 500 x dilutions of immune sera.

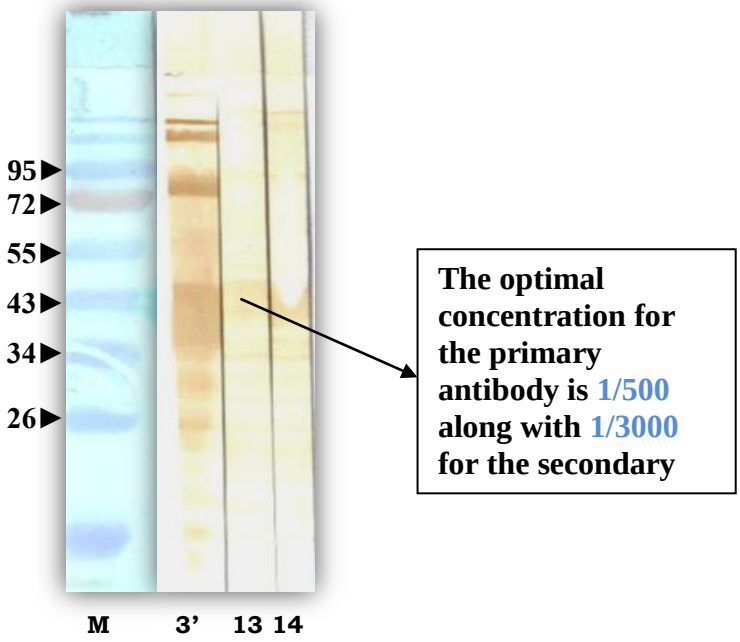

Figure 16. Testing different concentrations of the first antibody

M- Marker PageRuler™ Prestained Protein Ladder (Fermentas SM 0671)

Table 2. The different concentrations of the antiFtsZ *Escherichia coli* antibodies used to detect the optimal concentration of this antibodies:

Strip Number	Substrate	Primary Ab	Secondary Ab HRP-conjugated	Obs
3'	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/250	Anti-mouse 1/3000	++++
13	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/500	Anti-mouse 1/3000	+++
14	FTSZ E.C.	Anti E.C. (25.01.11; 16wks) 1/1000	Anti-mouse 1/3000	++

The cross – reactivity between FtsZ from the two bacterial species, *Escherichia coli* and *Pseudomonas aeruginosa*

Cross-reactivity analysis between FtsZ from the two bacterial species by Western-blot was performed with immune sera collected at 8 weeks after the onset of immunization and further with immune sera obtained after 16 and 32 weeks after first immunization.

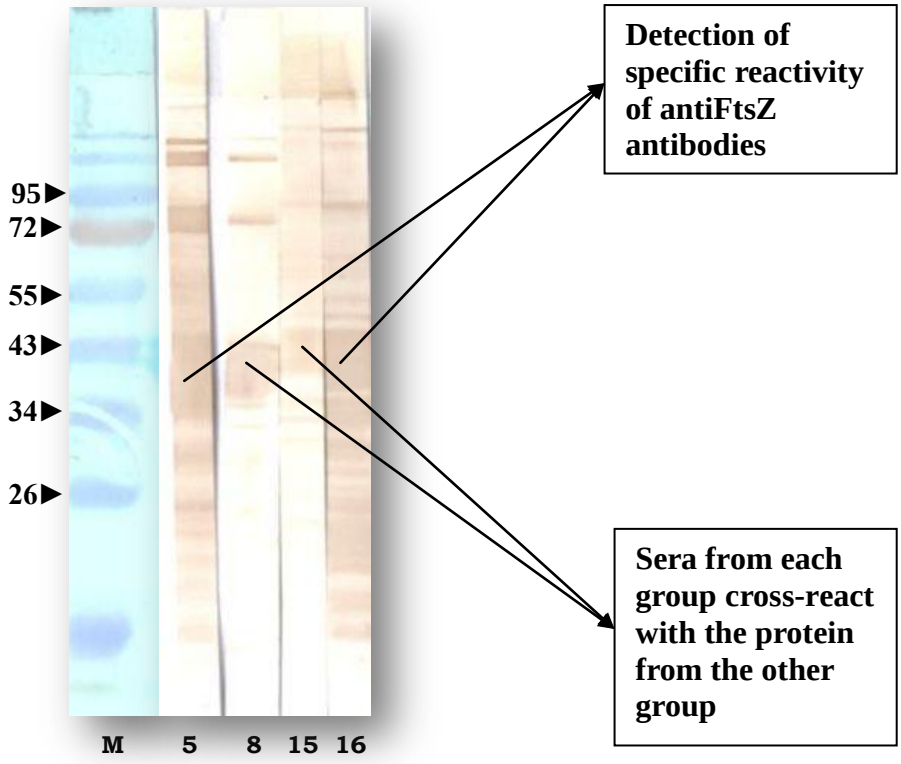

Figure 17. Cross-reactivity analysis of FtsZ from *E. coli* and *P. aeruginosa* by Western-blot using immune sera obtained at 8 weeks after the onset of immunization.

M- Marker PageRuler™ Prestained Protein Ladder (Fermentas SM 0671)

Table 3. Primary and secondary antibodies concentrations used for testing cross-reactivity between FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa*:

Strip Number	Substrate	Primary Ab	Secondary Ab HRP-conjugated	Obs
5	FTSZ E.C.	Anti E.C. (09.12.10; 8wks) 1/250	Anti-mouse 1/3000	++++
8	FTSZ E.C.	Anti P.A. (09.12.10; 8wks) 1/250	Anti-mouse 1/3000	+++
15	FTSZ P.A	Anti E.C. (09.12.10; 8wks) 1/250	Anti-mouse 1/3000	+++
16	FTSZ P.A	Anti P.A. (09.12.10; 8wks) 1/250	Anti-mouse 1/3000	++++

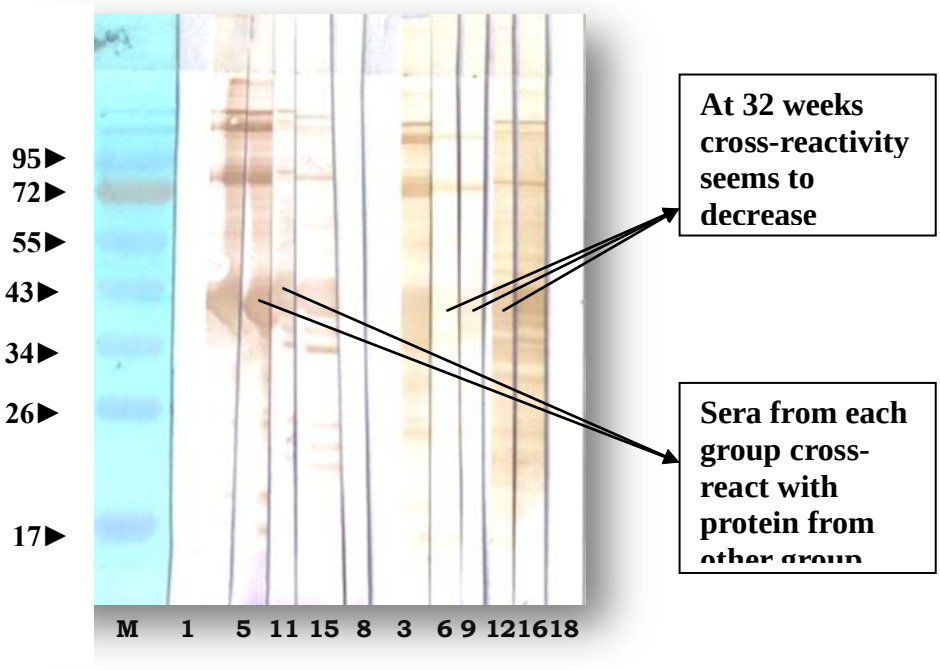

Figure 18. Cross-reactivity of FtsZ from *Escherichia coli* and *Pseudomonas aeruginosa* detected by Western-blot, using different dilutions of immune sera collected at 16 and 32 weeks after the onset of immunization.

M- Marker PageRuler™ Prestained Protein Ladder (Fermentas SM 0671)

Table 4. Primary and secondary antibodies concentrations used for testing cross-reactivity between FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa*:

Strip Number	Substrate	Primary Ab	Secondary Ab HRP-conjugated	Obs.
1	FTSZ E.C.	Anti E.C. 25.01.11; 16wks 1/250	Anti-mouse 1/3000	++++
5	FTSZ E.C.	Anti P.A. 25.01.11; 16wks 1/250	Anti-mouse 1/3000	++++ cross reactive
11	FTSZ P.A	Anti E.C. 25.01.11; 16wks 1/250	Anti-mouse 1/3000	+++ cross reactive
15	FTSZ P.A	Anti P.A. 25.01.11; 16wks 1/250	Anti-mouse 1/3000	+++
8	FTSZ E.C.	Normal Mouse Serum (NMS) 15.10.10; 0wks 1/250	Anti-mouse 1/3000	- preimmune serum
3	FTSZ E.C.	Anti E.C. 20.03.11; 32wks 1/500	Anti-mouse 1/3000	++++
6	FTSZ E.C.	Anti P.A. 20.03.11; 32wks 1/500	Anti-mouse 1/3000	+ weak cross reactive
9	FTSZ E.C.	Anti P.A. 20.03.11; 32wks 1/1000	Anti-mouse 1/3000	+ weak cross reactive
12	FTSZ P.A	Anti E.C. 20.03.11; 32wks 1/500	Anti-mouse 1/3000	+++ cross reactive
16	FTSZ P.A	Anti P.A. 20.03.11; 32wks 1/250	Anti-mouse 1/3000	++++
18	FTSZ P.A	Normal Mouse Serum (NMS) 15.10.10; 0wks 1/550	Anti-mouse 1/3000	- preimmune serum

Cross-reactivity analysis between FtsZ and proteins of eukaryotic cytoskeleton

FtsZ from both bacterial species, *Escherichia coli* and *Pseudomonas aeruginosa* were migrated on polyacrylamide gels and transferred to a nitrocellulose membrane. In order to verify the transfer, one gel was stained with Coomasie Blue.

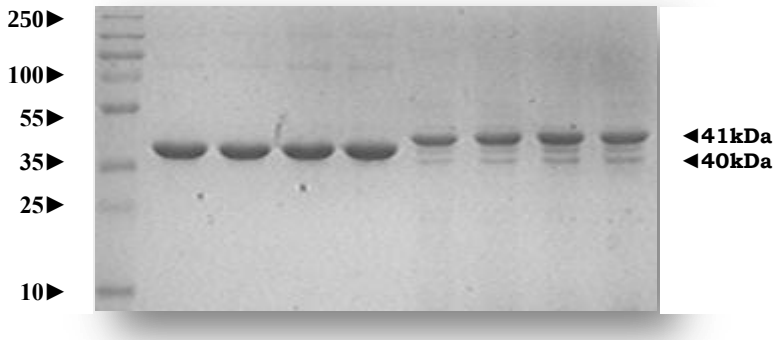

Figure 19. Transfer of FtsZ *E. coli* şi *P.aeruginosa* to a nitrocellulose membrane

1: marker PageRuler Plus Prestained Protein Ladder (Fermentas SM 1181);

2, 3, 4, 5: FtsZ *E. coli*

6, 7, 8, 9: FtsZ *P. aeruginosa*

Membrane on which were fixed FtsZ protein substrates was used for Western-blot analysis of polyclonal antiFtsZ antibodies cross-reactivity against essential proteins of eukaryotic cytoskeleton: actin and beta-tubulin.

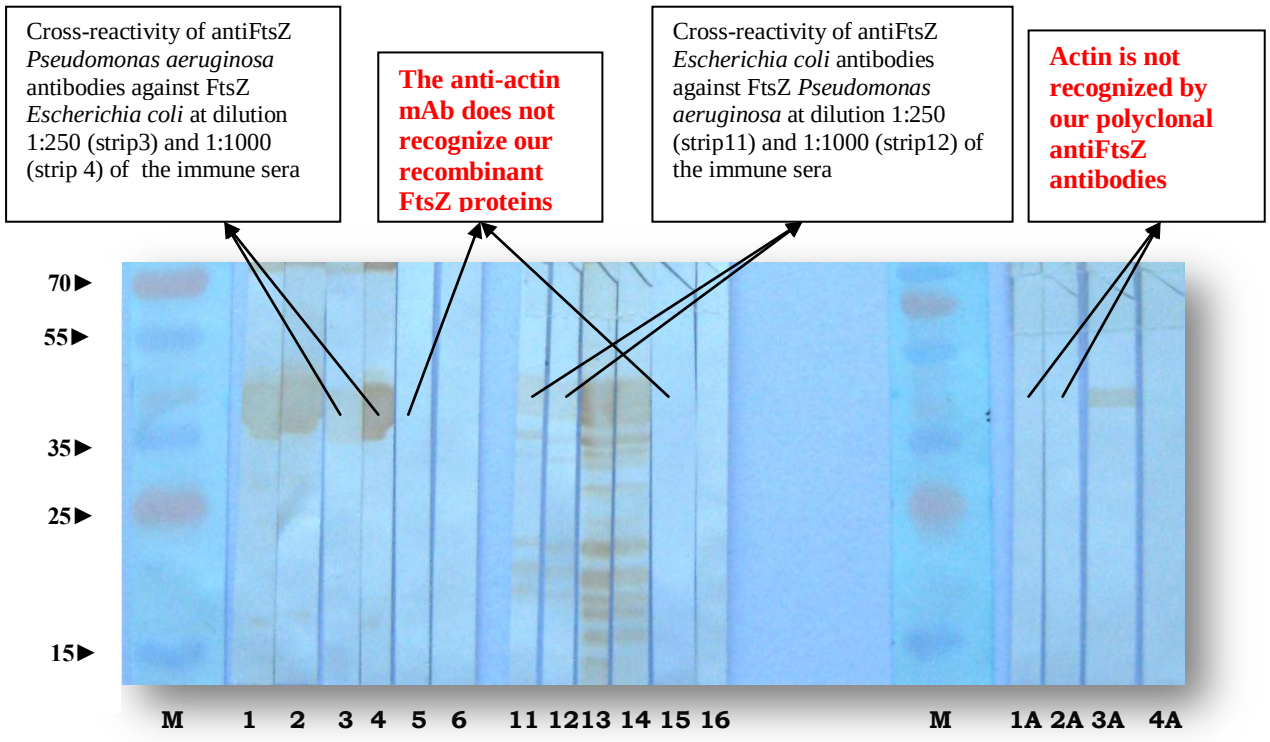

Figure 20. Analysis by Western-blot of polyclonal antiFtsZ antibodies reactivity with FtsZ *Escherichia coli*, FtsZ *Pseudomonas aeruginosa* and actin

M- Marker PageRuler Plus Prestained Protein Ladder (Fermentas SM 1181)

Table 5. The substrates and the primary and secondary antibodies concentrations used to test cross-reactivity between FtsZ from the two bacterial species and also, cross-reactivity with actin:

Strip Number	Substrate	Primary Ab	Secondary Ab HRP-conjugated	Obs
1	FTSZ E.C.	Anti E.C. 25.01.2011/16wks 1/250	Anti-mouse 1/3000	+
2	FTSZ E.C.	Anti E.C. 25.01.2011/16wks 1/1000	Anti-mouse 1/3000	+
3	FTSZ E.C.	Anti P.A. 25.01.2011/16wks 1/250	Anti-mouse 1/3000	+ cross reactive
4	FTSZ E.C.	Anti P.A. 25.01.2011/16wks 1/1000	Anti-mouse 1/3000	+ cross reactive
5	FTSZ E.C.	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	- NO cross reactivity
6	FTSZ E.C.	NMS 15.10.2010/0wks 1/250	Anti-mouse 1/3000	- preimmune serum
11	FTSZ P.A.	Anti E.C. 25.01.2011/16wks 1/250	Anti-mouse 1/3000	+ cross reactive
12	FTSZ P.A.	Anti E.C. 25.01.2011/16wks 1/1000	Anti-mouse 1/3000	+ cross reactive
13	FTSZ P.A.	Anti P.A. 25.01.2011/16wks 1/250	Anti-mouse 1/3000	+
14	FTSZ P.A.	Anti P.A. 25.01.2011/16wks 1/1000	Anti-mouse 1/3000	+
15	FTSZ P.A.	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	- NO cross reactivity
16	FTSZ P.A.	NMS 15.10.2010/0wks 1/250	Anti-mouse 1/3000	- preimmune serum
1A	Actin	Anti E.C. 25.01.2011/16wks 1/250	Anti-mouse 1/3000	- NO cross reactivity
2A	Actin	Anti P.A. 25.01.2011/16wks 1/250	Anti-mouse 1/3000	- NO cross reactivity
3A	Actin	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	+
4A	Actin	NMS 15.10.2010/0wks 1/250	Anti-mouse 1/3000	- preimmune serum

AntiFtsZ antibodies determin the appearance of detection bands of specific reactivity and cross-reactivity even at high dilutions of the immune sera (1:1000). Actin is not recognized by polyclonal antiFtsZ antibodies neither at high concentrations of these antibodies (1:250 dilutions of the immune sera) and anti actin monoclonal antibodies do not recognize recombinant FtsZ proteins.

Testing polyclonal antiFtsZ antibody reactivity over time

Subsequent bleeding performed at 40 weeks after the first immunization, 1:500 dilutions of the immune sera were prepared, starting with the immune serum from the first bleeding, in 19.12.2010. Secondary antibodies were diluted in the proportion of 1:3000.

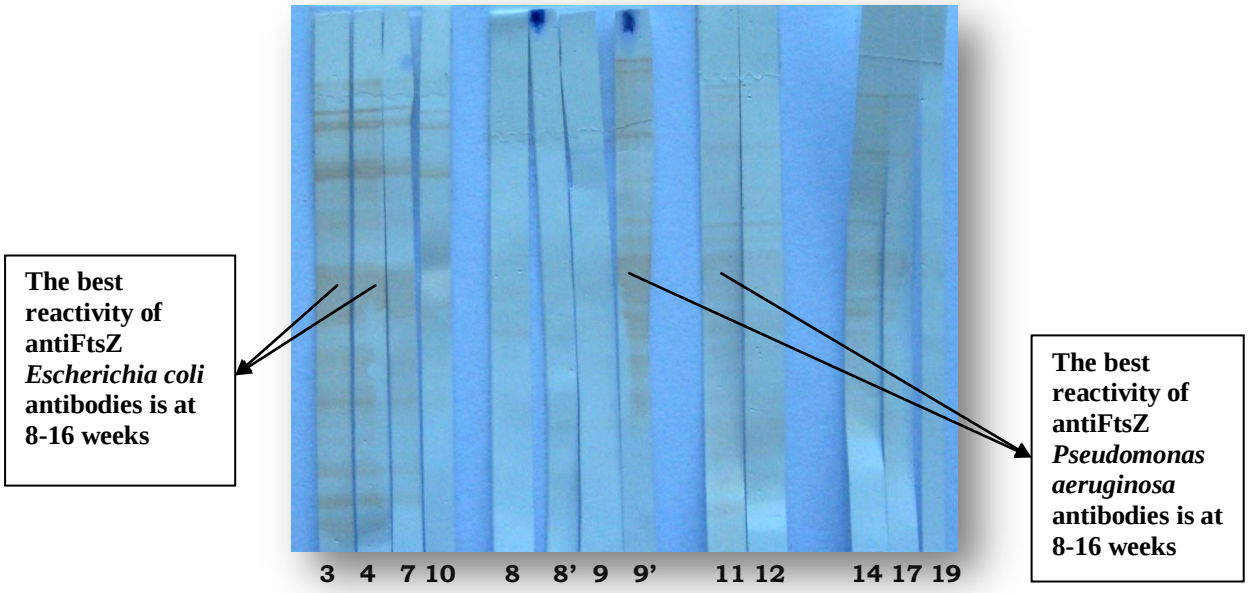

Figure 21. Analysis by Western-blot of polyclonal antiFtsZ *Escherichia coli* and antiFtsZ *Pseudomonas aeruginosa* reactivity over time.

Table 6. Primary and secondary antibodies used to test polyclonal antiFtsZ antibody reactivity over time:

Strip Number	**Substrate**	**Primary Ab 1/500** i.e. 2 microL in 1mL	**Secondary Ab HRP-conjugated**	**Obs**
3 /7.2.11	FTSZ E.C.	Anti E.C. (09.12.10; 8wks)	Anti-mouse 1/3000	++++
4	FTSZ E.C.	Anti E.C. (25.01.11; 16wks)	Anti-mouse 1/3000	++++
7	FTSZ E.C.	Anti E.C. (20.03.11; 24wks)	Anti-mouse 1/3000	++
10	FTSZ E.C.	Anti E.C. (26.05.11; 32wks)	Anti-mouse 1/3000	++
8/10.12.10	FTSZ E.C.	Anti E.C. (22.07.11; 40wks)	Anti-mouse 1/3000	+
8'	FTSZ E.C.	NMS (15.10.10; 0wks)	Anti-mouse 1/3000	- preimmune serum
9	FTSZ P.A	NMS (15.10.10; 0wks)	Anti-mouse 1/3000	- preimmune serum
9'	FTSZ P.A	Anti P.A. (09.12.10; 8wks)	Anti-mouse 1/3000	++++
11/26.1.11	FTSZ P.A	Anti P.A. (25.01.11; 16wks)	Anti-mouse 1/3000	++++
12	FTSZ P.A	Anti P.A. (20.03.11; 24wks)	Anti-mouse 1/3000	++++
14/7.2.11	FTSZ P.A	Anti P.A. (26.05.11; 32wks)	Anti-mouse 1/3000	+++
17	FTSZ P.A	Anti P.A. (22.07.11; 40wks)	Anti-mouse 1/3000	++
19	FTSZ P.A	NMS (15.10.10; 0wks)	Anti-mouse 1/3000	- preimmune serum

The best reactivity of antiFtsZ polyclonal antibodies is that of antibodies from immune sera collected at 8-16 weeks. This result correspond to data from antibodies titer determinations, performed by ELISA technique.

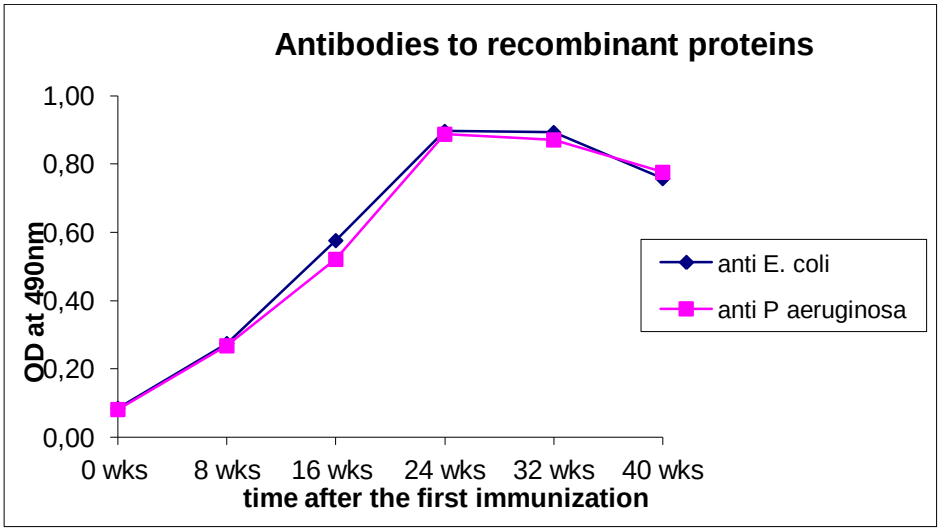

Figure 22. Antibodies titer in immune sera obtained by bleedings performed at 8, 16, 24, 32, 40 weeks after the onset of mice immunization with recombinant FtsZ proteins.

Testing cross reactivity between FtsZ and actin - tubulin

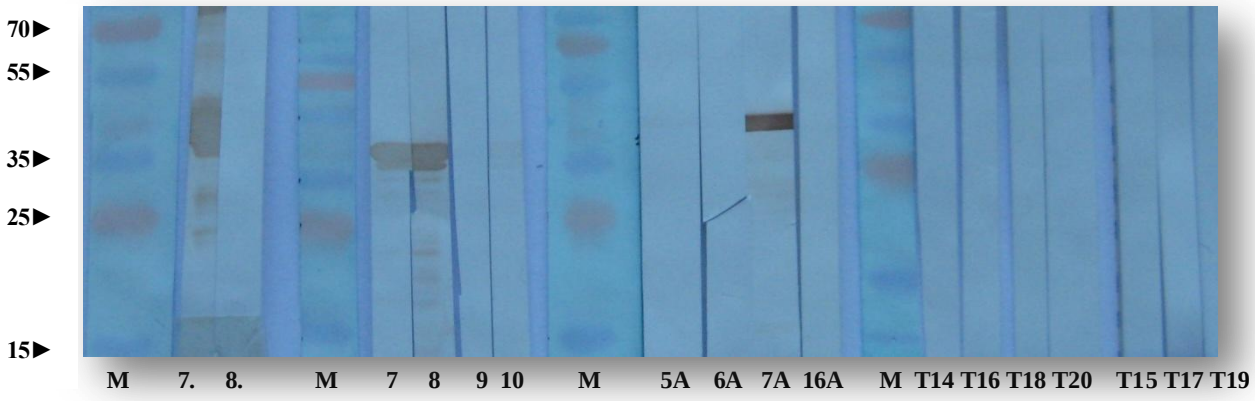

Figure 23. Analysis by Western-blot of possible cross-reactivity of antiFtsZ antibodies with actin and β-tubulin

Table 7. The substrates and the primary and secondary antibodies concentrations used to test FtsZ cross-reactivity with actin and β-tubulin:

Strip Number	**Substrate**	**Primary Ab 1/500** i.e. 2 microL in 1mL	**Secondary Ab** **HRP-conjugated**	**Obs**
7.	FTSZ E.C.	Anti E.C. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	+
8.	FTSZ E.C.	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	- NO cross reactivity
7	FTSZ P.A	Anti E.C. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	+ cross reactive
8	FTSZ P.A	Anti P.A. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	+
9	FTSZ P.A	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	- NO cross reactivity
10	FTSZ P.A	NMS 15.10.2010/0wks 1/500	Anti-mouse 1/3000	- preimmune serum
5A	Actin	Anti E.C. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	- NO cross reactivity
6A	Actin	Anti P.A. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	- NO cross reactivity
7A	Actin	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	+
16A	Actin	NMS 15.10.2010/0wks 1/500	Anti-mouse 1/3000	- preimmune serum
T14	Tubulin	Anti E.C. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	- NO cross reactivity
T16	Tubulin	Anti P.A. 25.01.2011/16wks 1/500	Anti-mouse 1/3000	- NO cross reactivity
T18	Tubulin	Anti actin monoclonal Ab 1/500	Anti-rabbit 1/2000	- NO cross reactivity
T20	Tubulin	NMS 15.10.2010/0wks 1/500	Anti-mouse 1/3000	- preimmune serum
T15	Tubulin	Anti E.C. 25.01.2011/16wks **1/100**	Anti-mouse 1/3000	- NO cross reactivity
T17	Tubulin	Anti P.A. 25.01.2011/16wks **1/100**	Anti-mouse 1/3000	- NO cross reactivity
T19	Tubulin	NMS 15.10.2010/0wks **1/100**	Anti-mouse 1/3000	- preimmune serum

Neither actin nor tubulin is recognized by our polyclonal antibodies to the FtsZ proteins; the anti-actin monoclonal antibodies does not recognize our proteins or the tubulin.

6. Analysis and discussion of results

Some studies shows the presence of the f*tsZ* gene and the protein encoded by this gene in ,,most if not all species of bacteria, cocci or bacilli shaped, Gram-negative şi Gram-positive" [Corton *et al.*, 1987]. FtsZ protein can be considered as an ideal target for antibacterial therapy, because it provided a wide spectrum of action of therapeuthic agents oriented against this protein, FtsZ being conserved and universally present in bacteria. FtsZ is an essential protein, with a central role in cell division, an event which is critical to the survival of bacteria. [Amer et al., 2008; Jeffrey et al., 2003; Löwe et al., 2004; McDevitt et al., 2002; Mingorance et al., 2010; Weiss, 2004]

Considering these issues, FtsZ *Escherichia coli* and *Pseudomonas aeruginosa* were overexpressed in order to be used to obtain their specific polyclonal antibodies.

Conditions under which PCR amplification of the *ftsZ* gene from *Pseudomonas aeruginosa* and *Escherichia coli* was performed, allowed to obtain amplification fragments wich proved to be identical in nucleotide sequence with sequences identified in databases.

Any errors in gene sequence have been avoided in order to obtain the recombinant FtsZ proteins.

Mice immunized with recombinant FtsZ proteins from *Escherichia coli* and *Pseudomonas aeruginosa* produced polyclonal antibodies at only 8 weeks after the onset of the immunization, dilutions of 500x and even 1000x of the immune sera showed reactivity with the specific antigen. The highest reactivity was presented by antibodies in sera obtained at 8-16 weeks after onset of immunization. Antibodies from sera obtained at 32 weeks after onset of mice immunization showed a noticeable decrease in titer and reactivity. Antibody titer dynamics established by ELISA are correlated with results obtained by Western-blot: concentration of antiFtsZ antibodies from immune sera decrease after reach a peak at 24 weeks and enter a plateau up to 32 weeks. The antibody levels and affinity are usually low after the first immunization. Both titer and affinity increased significantly after the second and the third immunization, consecutive secondary immune response. The distinctive features of the secondary immune response are antibodies affinity maturation and isotype switching to IgG synthesis. Affinity maturation is explained by producing of some somatic mutations of V region sequence, during proliferating differentiation of B lymphocytes, due to antigen-mediated selection of lymphocyte clones with high binding affinity to complementary epitopes.

High affinity antibody synthesis has two important consequences: antigen-antibody complexes are hard indissociable and this antibodies give cross-reactions with phylogenetic related antigens.

Protein molecules from organisms phylogenetic unrelated with laboratory animals subjected to immunization, like FtsZ proteins, have a strong immunogenic feature, produce an appropiate immune response and the appearance of a high titre of specific antibodies.[Honjo & Habu, 1985; Diaz & Casali, 2002; Neuberger et al., 2007; Or-Guil et al., 2007]

Cross-reactivity between FtsZ of the two bacterial species, *Escherichia coli* and *Pseudomonas aeruginosa* was present in the intial point of detection, at 8 weeks after the onset of immunization and then persisted the entire period of analysis, but with a visible decrease after 32 weeks from the initiation of the repeated immunization. Even in strips incubated with immune sera diluted 500 x or 1000 x, cross-reactivity could be detected. These results are not surprising, having regard to the high degree of homology between amino acid sequences of the two proteins, FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa*. AntiFtsZ polyclonal antibodies characterization and the finding of antiFtsZ antibodies cross-reactivity with antigens from the other bacterial species were followed by the study of this antiFtsZ antibodies cross-reactivity against proteins of eukaryotic cytoskeleton: actin and β-tubulin.

It is sustained the idea that a ancestral protein, containing an GTP binding domain with Rossman type folding, evolved divergently in two directions: in a family of typical GTPases and in a family of atypical GTPases wich include FtsZ proteins and tubulins. [Erickson, 1998; Löwe & Amos,1998].

Comparison between primary structure of FtsZ and tubulins revealed that this two proteins have in common ,,a rich cluster glycine sequence" [GGGTG(S\T)G], which in tubulins is known as a part of the structure of the GTP binding site, from the N-terminal domain. No other known proteins have this sequence, beside tubulins and FtsZ. This

sequence is one of the three sequences highly conserved among tubulins family, which counts more than 150 proteins. This sequence is also conserved in FtsZ proteins [Mukherjee şi colab., 1993] Alignment of tubulins and FtsZ sequences shows a total lack of identity at the C-terminal domain. Sequence homology as evidenced by alignment is less than 20%, regardless the species which were selected for the alignment of mentioned sequences. [Burns, 1998]. Sequence homology between human, bovine or murine β-tubulins and FtsZ protein from *Escherichia coli* is 13,96%, and if alignment is done with amino acids sequence of FtsZ from *Pseudomonas aeruginosa*, identity found is 13,51%.

There is a total lack of homology between actin and prokaryotic counterpart of tubulins which is FtsZ protein, although actin is a component of the cytoskeleton and the essential component of cytokinetic ring of eukaryotes and FtsZ has the same function in prokaryotic cells.

Erickson points out that practically, there was a inversion of role in cytokinesis during evolution: FtsZ proteins have a certain degree of structural homology with tubulins, but are functionally similar to actin; in reverse, eukaryotic tubulins have a similar role to that of prokaryotic proteins which have sequence and structural homology with actin. [Erickson, 2007]

These observations explain the results obtained during analysis by Western-blot, concerning the cross-reactivity between FtsZ and eukaryotic proteins, actin and β-tubulins.

Actin does not bind antibodies of immune sera collected from the mice immunized with FtsZ *Escherichia coli*. Identically, antibodies synthesized against FtsZ from *Pseudomonas aeruginosa* do not recognize actin. Actin is not recognized by antiFtsZ polyclonal antibodies even at high concentrations of these antibodies (at 1:250 dilutions of immune sera) and anti actin monoclonal antibodies do not recognize FtsZ recombinant proteins.

Sequence homology between FtsZ and tubulins ranges from 13 to 14% and is mainly due to N-terminal amino acids sequence and particularly, to GTP binding and hydrolysing site. Although the antibodies were synthesized against FtsZ proteins and the presence of some epitopes have been established in the N-terminal domain of FtsZ from *Escherichia coli* [Voskuil et al., 1994], it seems that sequence homology does not concern the epitopes, so there is not cross-reactivity between FtsZ and tubulins. Actin and β-tubulin are not recognized by antiFtsZ polyclonal antibodies. Anti actin monoclonal antibodies do not recognize FtsZ *Escherichia coli*, FtsZ *Pseudomonas aeruginosa* and β-tubulin.

Immune sera react with microbial homologous antigen, but sometimes, also react with host antigens. AntiFtsZ polyclonal antibodies recognize and bind common epitopes fom homologous FtsZ molecules of the two bacteria species, *Escherichia coli* and *Pseudomonas aeruginosa*. Protein antigens from sources which are taxonomic related often give cross-reactions. The more two species of organisms are phylogenetic closer, they are giving even more cross-reactions between homologous proteins. Antibodies are selective and specific, cross-reactivity being determined by the existence of similar spatial epitopes, with small differences in amino acid sequences, inducing slight epitope changes.

Molecules involved in prokaryotes and eukaryotes cytokinesis underwent divergent evolution, so that the reduced homology between FtsZ and tubulins sequences does not provide antigenic determinants to antiFtsZ antibodies, with a primary structure and/or a spatial conformation that would determine cross-reactivity. The binding of these antibbodies is selective and targets only epitopes of bacterial antigens, which means that it does not affect the actinic cytoskeleton or the microtubules of the host eukaryotic cells.

Polyclonal antibodies have numerous applications which include qualitative and quantitative analysis, for research, diagnostic or therapeutic purposes. Polyclonal antibodies allow localize epitopes, localization and purification of specific or closely related antigens,

emphasizing some molecules associated to antigen or highlighting of cells expressing an antigen.

First generation therapy with polyclonal antibodies involves the purification of immunoglobulin fraction from plasma of immunized animals or fromhuman donor plasma. Antibodies resulted from human donor plasma have the advantage of natural polyclonal immune response, with a specific but in the same time diverse repertoire, with a multitude of binding specificities. Increasing levels of specific antibodies for the target antigen was achieved by using hiperimmune immunoglobulins from individuals who have been recovered after a certain infection and showed an increased titer of antibodies against a specific antigen. This method of immunotherapy is limited by donor availability and by the risk that screening techniques can not detect some pathogens of the donor, so immunoglobulin products obtained from plasma present a potential risk of transmission of some diseases.

Although widely used, especially in some forms of cancer therapy, monoclonal antibodies do not have the multitude of specificities which determine in the case of polyclonal antibodies the link to multiple epitopes. Monoclonal antibody molecules are all competing for the same epitope and therefore epitopes density is a limiting factor. Therapy efficiency can be increased only by increasing the dose of therapeutic substance, wich determines side effects in the case of excess antibody binding in other tissues than that targeted by therapy. All these inconveniences lead currently to a shift in researchers concerns to polyclonal antibodies. Recombinant polyclonal antibodies currently offer an alternative in research and also in immunotherapy, being regarded a third-generation therapy. [Tengbjerg & Haurum, 2006; Rassmussen et al., 2007]

Polyclonal antibodies obtained against FtsZ from *Escherichia coli* and *Pseudomonas aeruginosa* have reactivity characteristics which allow their use in studies regarding the proteins against they were synthesised, including the evaluation of this proteins as targets for different antibacterial therapeutic agents, considering the pathogenicity and multiple antibiotic resistance of *Escherichia coli* and *Pseudomonas aeruginosa* bacteria.

7. Conclusions

1. The design of primers and the conditions of amplification by PCR ensured the efficiency and the specificity of the reaction:

- amplification products were of the desired length, without modified nucleotide sequences compared to *ftsZ E.coli* and *ftsZ P.aeruginosa* gene sequences from databases. Any errors are not desirable in these genes sequences, an essential condition for obtaining recombinant proteins.
- there were no unspecific amplifications

- amount of amplicon was high enough, which facilitated subsequent handling of the amplified sequence and obtaining the recombinant FtsZ proteins from *Escherichia coli* and *Pseudomonas aeruginosa*.

2. Recombinant proteins FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa* express soluble in *Escherichia coli* BL21(DE3).

3. Alignment of aminoacid sequences of FtsZ proteins from *Escherichia coli* and *Pseudomonas aeruginosa* revealed a high degree of homology of these sequences, about 83,76%, wich correspond to data from literature according to which, ftsZ gene is essential for for bacterial survival and conserved in all species of bacteria.

4. Mice immunized with recombinant FtsZ proteins produced antibodies as soon as 8 weeks after the first immunization. The titer and the affinity of our antiFtsZ polyclonal antibodies are high enough, so that antibodies showed a positive reaction with their specific antigens at 8 weeks after the onset of repeated immunization.

5. The optimal concentration of our antiFtsZ polyclonal antibodies used in specific reactivity determinations by Western-blot, correspond to 1:500 dilutions of the immune sera. At lower dilutions, there are many detection bands of unspecific binding.

6. It was found FtsZ cross-reactivity of the two species of bacteria at 8 and 16 weeks: antiFtsZ *Pseudomonas aeruginosa* antibodies showed cross-reactivity with FtsZ from *Escherichia coli* and antiFtsZ *Escherichia coli* antibodies also showed cross-reactivity with FtsZ from *Pseudomonas aeruginosa.*

7. The best reactivity is characteristic to the antiFtsZ antibodies from the immune sera obtained by bleedings performed at 8 and 16 weeks after the onset of repeated immunization. AntiFtsZ antibodies from immune sera, obtained at 16 weeks after the first immunization, determine the appearance of specific and cross-reactivity detection bands, even at high dilutions of immune sera of 1000 x.

8. After 32 weeks of the onset of repeated immunization, cross-reactivity of antibodies from immune sera with FtsZ antigens from the other bacteria species seems to decrease.

9. Results of alignments between the aminoacids sequences of FtsZ *Escherichia coli* or FtsZ *Pseudomonas aeruginosa* with aminoacids sequences of human, murine and bovine tubulinic isotypes from UniprotKB showed a sequence homology of 13,96% (TBB4 with FtsZ *E.coli*) and of 13,51% (TBB4 with FtsZ *P.aeruginosa*).

10. Actin and beta-tubulin are not recognized by antiFtsZ polyclonal antibodies even at high concentrations of these antibodies (at 1:250 dilutions of immune sera) and anti actin monoclonal antibodies do not recognize FtsZ recombinant proteins and β-tubulin.

11. Polyclonal antibodies obtained by inoculation of FtsZ *Escherichia coli* and FtsZ *Pseudomonas aeruginosa* at BALB/c mice have affinity characteristics and also characteristics of specific and cross-reactivity which would permit their use in research techniques and the field of antibacterial therapy or diagnostic.

12. The cross-reactivity of FtsZ proteins from the two bacterial species opens the possibility of cross-reactivity evaluation of our antiFtsZ antibodies with FtsZ proteins of other species of bacteria. An extended cross-reactivity between FtsZ from the cells of a large number of bacteria species provides to FtsZ proteins the quality of target molecules for antibacterial therapeutic agents with a broad-spectrum of activity.

13. AntiFtsZ *Escherichia coli* antiFtsZ *Pseudomonas aeruginosa* polyclonal antibodies show cross-reactivity against FtsZ of the other bacterial species, but do not recognize actin and tubulins and consequently, can be selectively used against FtsZ, because do not bind or affect proteins of eukaryotic cytoskeleton from the host cells.

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