

ANALELE ȘTIINȚIFICE
ALE
UNIVERSITĂȚII „ALEXANDRU IOAN CUZA”
DIN IAȘI
(SERIE NOUĂ)
SECȚIUNEA II

a. GENETICĂ ȘI
BIOLOGIE
MOLECULARĂ

TOMUL XVII, Fascicula 2

2016

Editura Universității „ALEXANDRU IOAN CUZA” Iași

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CONTENT

Cristian S. Cîmpeanu, Mirela M. Cîmpeanu – The complex organization of eukaryotic cell nucleus (IV): The nuclear envelope	43
Csilla Iuliana Băra, Elena Luca – Research regarding the frequency of ABO blood groups in a population of pupils from Râucești, Neamț county	53
Dragoș Crauciuc, Ovidiu Toma, Eduard Crauciuc – Assessing progression of cervical pre-cancer lesions	61
Simona Constantinescu, Norina Consuela Forna, Cristina Gabriela Tuchiluş, Aida Corina Bădescu, Claudia Elena Pleşca, Eugen Ungureanu, Luminița Smaranda Iancu – Risk factors in sepsis with oro-maxillofacial portal of entry	67
Antonia Poiată, Cristina Lungu, Bianca Ivănescu – Antimicrobial effects of the different extracts from <i>Amaranthus retroflexus</i> L.	75
Aykut Topdemir, Nazmi Gür – The quantitative evaluation of alternaria toxins in apple and tomato juices	81
Ionel Andriescu – <i>Academician Professor PETRE JITARIU, a visionary in the scientific research and Romanian education (May 11, 2016, 111 years after the birth of Acad. Prof. Dr. PETRE JITARIU)</i>	89
Instructions for Authors	97

THE COMPLEX ORGANIZATION OF EUKARYOTIC CELL NUCLEUS (IV): THE NUCLEAR ENVELOPE

CRISTIAN S. CÎMPEANU^{1*}, MIRELA M. CÎMPEANU²

Received: 13 April 2016 / Revised: 25 April 2016 / Accepted: 24 May 2016 / Published: 13 July 2016

Keywords: nuclear envelope, nuclear pores complexes, nuclear transport, envelopopathies

Abstract: The nuclear envelope (NE), a double membrane structure, separates the nucleoplasm from cytosol. Each of the two membranes of the NE (the inner nuclear membrane, INM and the outer nuclear membrane, ONM) contain a particular protein complement, with specific domains, which accomplish various and critical functions: the lamin and chromatin anchoring at NE, the localization and movement of nucleus within cells, the control of transcription, etc. The nuclear pores complexes (NPCs) of the NE are large and complicated proteic structures, essentially involved in bidirectional transport of molecules between nucleus and cytoplasm. Some nuclear envelope molecular components are subjected to various genetic disorders known as envelopopathies, which result in general syndroms, more or less severe.

INTRODUCTION

In the previous parts of this mini-review we tried to summarize the present knowledges regarding the structure and functions of some nuclear components: the nuclear bodies, the chromosome territories, the interchromatin domains, the nuclear matrix and the nuclear lamina. Although these components, defined as nucleoplasm (nuclear content), are essential, they could not properly function in eukaryotic nuclei without the presence of the nuclear envelope, which, primary, represent a physical barrier which separates the nucleoplasm from cytosol, but also interconnect them, allowing complex molecular exchanges. The advent of nuclear envelope in eukaryotic cells was a crucial landmark in cellular evolution and had enormous consequences in general systematics of all living beens.

THE NUCLEAR ENVELOPE

As it was shown in the first part of the minireview (*Overview on general organisation of the cell nucleus*) the nuclear envelope (also known as nucleolemma or karyotheca), is a complex structure consisting of two lipoproteic membranes – *the inner nuclear membrane* (INM) and the *outer nuclear membrane* (ONM).

These membranes are fused together at the site of *nuclear pores complexes* (NPCs), which create a discontinuous surface of nuclear surface. The INM and the ONM are parallel one to another and are separated by a 10-50 nm wide space (*perinuclear space*), which expands inside the endoplasmic reticulum (the lumen of ER).

The outer membrane, facing the cytosol, is continuous with the membrane of *rough endoplasmic reticulum* (RER) and, similar to this, carries tightly bounded ribosomes; the INM indirectly contacts the nuclear chromatin by means of *nuclear lamina*.

As specified in the previous part of this minireview (*The Nuclear Matrix and the Nuclear Lamina*) the inner nuclear membrane contains a large variety of peripheral and integral membrane proteins (about 60 proteins), most of which are poorly characterized. Yet, few of these proteins are better known, such as: *emerin*, *lamina-associated polypeptides 1 and 2* (LAP1 and LAP2), the *lamin B receptor* (LBR), the *LEM domain-containing protein 3* (MAN1) and *nurim*. Many of the INM proteins are *lamin-binding proteins*, interacting with lamins; some of them are related with chromatin. Some data suggest that these proteins could also be involved in gene regulation and, possibly, in sterol metabolism (Holmer and Worman, 2001).

All the lamina-associated protein 2 (LAP2) isoforms (β , γ , δ , ϵ), *emerin* and *MAN1* have a homologous N-terminal domain called the *LEM domain*, which is a 45 AA residue motif that

folds as two α -helices (Laguri et al., 2001). The LEM domains bind to BAF (the **B**arrier to **A**utointegration **F**actor), a chromatin-associated protein, linked to nuclear lamina and to nuclear envelope proteins mentioned above, as well.

The *Lamina-associated polypeptide 1* (LAP1) is a single-spanning integral membrane protein (isoforms A,B,C) expressed in most cells and tissues. LAP1 physically interacts with lamins, torsin A and emerin in nuclear envelope, suggesting that it may act as a crucial node in signal transduction across the inner nuclear membrane (Ji-Yeon-Shin et al., 2014).

The *Lamina-associated polypeptide 2* (LAP2) *isoforms*, via their LEM domains, which binds to BAF, interact both with B-type lamins and chromatin inside nucleoplasm. LAP2 is a single-spanning integral membrane protein, too, and possesses a large nucleoplasmic domain with multiple distinct regions; because the association of the AA situated at the second half of its N terminus (nucleoplasmic) with B-type lamins, LAP 2 may modulate the assembly of nuclear lamins (Furukawa and Kondo, 1998).

Similar to LAP 1 and LAP 2, *emerin* is a single-spanning integral membrane protein, rich in serine, composed of 254 AA. It is highly expressed in skeletal and cardiac muscle. Together with several other proteins of INM, emerin may be involved in some important processes, such as: the regulation of certain genes activity, the control of cell division cycle, the reassembly of nucleus in telophase and the conservation of nuclear structure and stability. In humans, emerin is coded by the EMD (STA) gene; mutations in EMD gene produce the Emery-Dreifuss muscular dystrophy and heart disorders (dilated cardiomyopathy and cardiac conduction abnormalities).

The *lamin B receptor* (LBR) is a multi-spanning membrane protein of INM. It has eight segments of hydrophobic amino acids (transmembrane domains), weights around 73,375 Da (Worman et al., 1990) and is coded by the LBR gene in humans. Its N-terminal end is located in nucleoplasm and binds to lamin and heterochromatin, while the C-terminal end is situated within the inner nuclear membrane and still attached at ER membranes after NE breakdown during mitosis (Olins et al., 2010).

Functionally, the LBR could mediate the interactions between lamin B and chromatin; mutations in LBR gene are associated with autosomal recessive Greenberg skeletal dysplasia (an abnormal cholesterol biosynthesis) and with Pelger–Huët anomaly (blood laminopathy).

LEM domain-containing protein 3 (LEMD3 or MAN1) is a 82,3 KDa integral protein with two transmembrane segments, a nucleoplasmic N-terminus which contain a LEM domain and a C-terminal domain also facing the nucleoplasm (Lin et al., 2005). Through its LEM domain, MAN1 attach to BAF and indirectly interact with the chromatin, whereas through the RNA recognition motif (RRM), present at the C-end, MAN1 interacts with Smad protein family members (Smad2 and Smad3), thereby mediating the cellular response at several cytokines, such as the transforming growth factor beta (TGF- β). Thus, it regulates the expression of several fundamental downstream genes. Mutations in MAN1 gene (LEM3) are involved in several genetic diseases - osteopokilosis, melorheostosis and Buschke-Ollendorff syndrome.

Nurim is a six transmembrane-spanning protein, with the N- and the C- termini residing on the same side of the membrane, containing 262 amino acids residues; it lacks an N-terminal domain, characteristic to other INM proteins (Hofemeister and O'Hare, 2005). Some experimental evidences (the expression of nurim in a broad range of cancers, correlated with tumour severity, the emergence of NE abnormal shapes and increases of apoptosis in HeLa cells, caused by its knock-down,) suggest that nurim has an important role in the suppression of apoptosis (Chen et al., 2012).

After their synthesis on RER, the integral proteins reach the INM membrane by lateral diffusion (because of the continuity of ER with nuclear membranes) and are retained here by means of the association with nuclear ligands (Holmer and Worman, 2001).

Although the majority of the NE proteins resides in the INM, the *outer nuclear membrane* (ONM) includes several specific proteins,.

Some of these proteins share different common features, such as: the presence of a single transmembrane domain followed by a short luminal sequence, the interaction with cytoskeletal elements and the existence of a conserved C-terminal KASH domain.

Among these proteins are: members of the mammalian *nesprin* family, *Klarsicht* and *Msp-300* in *Drosophila melanogaster*, *Anc-1*, *Zyg-12* and *Unc-83* in *Caenorhabditis elegans* and *Kms2* in the fission yeast *Schizosaccharomyces pombe* (Rouxet et al., 2009).

Nesprins (Nuclear envelope spectrin-repeat proteins) represent a family of proteins originally described as components of mammalian cell ONM, which connect the nucleoplasmic and cytosolic cytoskeleton elements, but encompassing a large diversity of tissue-specific isoforms localised in various cellular compartments (Rajgor and Shanahan, 2013).

More specific, different types of nesprins, through their C-terminal CASH domain connected with the SUN domain of some INM proteins (forming LINC complexes), associate the nuclear lamin network with various cytoskeletal fibrils: the *nesprin1* (synaptic nuclear envelope protein1, syne-1, enaptin, encoded by the SYNE1 gene) and the *nesprin2* (synaptic nuclear envelope protein2, syne-2, encoded by the SYNE2 gene) bind to actin filaments; the *nesprin3* associates with the intermediate filaments (IF) linker plectin; the *nesprin4* (*nesp4*) interacts with kinesin1 and can induce kinesin-mediated cell polarization. Due to their structure, cellular localisation and connections, the nesprins play important roles as intracellular scaffolds and linkers, interfering in nuclear localisation and nuclear movements within cells and in the maintenance of cellular spatial organization.

The SUN domains, which characterize the *SUN - domain* protein family, are a few hundred amino acids long regions, located at the C-terminus and conserved in several proteins thought to localize in the INM. Usually, the SUN regions follow a transmembrane domain and a less conserved AA region. A large variety of SUN proteins, from very different organisms, is now known: *SUN-1/matefin* and *UNC-84* (*Caenorhabditis elegans*), *Klaroid* and *Spag4* (*Drosophila melanogaster*), *SUN1*, 2 and 3 and *SPAG4* (mammals), *Sad1p* (*Schizosaccharomyces pombe*) and others.

The KASH domains (**K**larsicht **A**NC-1 **S**yne **H**omology) of *KASH- domain* protein family, similar to SUN domains, are C-terminal protein regions which follow a transmembrane domain and contain ~ 30 AA. The large majority of KASH proteins are situated in ONM, although some of them are reported to be components of the INM. Within perinuclear space, the proteins with KASH domains interact with SUN domain proteins, giving rise to a *LINC complex* (Linker of Nucleoskeleton and Cytoskeleton). Associating the nucleoplasmic and cytosolic cytoskeleton elements, via the nuclear envelope, the LINC complexes participate in many cell activities: nuclear relocations and movements, the attachment of centrosomes to the ONM, the response to mechanic extern stimuli, etc.

Whereas the ONM protein quality control is carried out by endoplasmic-reticulum-associated protein degradation pathways (ERAD), with the help of E3 ubiquitin ligases Hrd1 and Doa10 and E2 ubiquitin-conjugating enzymes Ubc6 and Ubc7 (in yeasts), the same processes are less known for INM proteins. However, it was demonstrated that in yeast (*Saccharomyces cerevisiae*) the degradation of INM soluble and integral membrane proteins is mediated by the Asi

complex (the RING domain proteins Asi1 and Asi3) which functions in conjunction with ubiquitin-conjugating enzymes Ubc6 and Ubc7; this pathway is distinct from ERAD pathway, but complementary to it (Khmelninskii et al., 2014).

The Nuclear Complexes (NPCs) and the Transport Through NPCs

The nuclear pores are not simple apertures or discontinuities of nuclear surface at the fusion sites of INM and ONM; in fact, the nuclear pores are large protein complexes (*nuclear pore complexes*, *NPCs*), that cross the nuclear envelope and are of crucial importance in the bidirectional transport of molecules between nucleoplasm and cytosol.

Observed in electron microscopy, the NPC appears as a complex structure with cylindrical shape and octagonal symmetry; it measures between 100-150 nm in diameter and 50-70 nm in thickness, depending of organism (Wente and Rout, 2010). The number of NPC per nucleus is submitted to large variations (from a few hundred in glial cell to almost 20,000 in some neurons, with an average of 2,000 NPCs in the NE of the vertebrates cells), depending mainly on the cell type and the stage of the cell cycle. The total mass of NPCs ranges, for instance, between 66 MDa in yeast (*Saccharomyces cerevisiae*) to about 124 MDa in mammals.

The NPCs are composed of about 30 different species of proteins called *nucleoporins* (Nups), each of them present in multiple copies (numerous nucleoporins have 8, 16 or 32 copies); in total, a mature NPC could contain between 500 and 1000 protein molecules. In fact, the NPCs proteins contain just a few different types of repetitive domains, generated through extensive gene duplication: *solenoid domains* (alpha solenoid or beta-propeller fold) – about a half of them and *intrinsically disordered domains* – the other half (highly flexible proteins devoided of ordered secondary structure).

Beside its composition and molecular weight, the complexity of NPC also express by its double symmetry: an *eightfold rotational symmetry*, visible on both sides of the NE (nucleoplasmic and cytosolic) and a *twofold transverse symmetry*, as a result of the symmetric orientation of the central portion of NPC proteins, which leads to the identity of nuclear and cytosolic NPC parts.

According their position toward the two nuclear membranes and the relative localization from outside to inside of the NPCs, the nucleoporins could be classified in three distinct types (Alberts et al., 2015):

- *Transmembrane ring proteins*, that anchor the NPC to the nuclear envelope;
- *Scaffold nucleoporins*, with a multilayered transversal architecture and ring morphology; they possess solenoid domains and some of them are membrane-bending proteins, involved in the stabilisation of NE membrane curvature at the site of nuclear pores; from each of the eight subunits of the outermost and innermost scaffold nucleoporin layers emerge microfibrils: since the fibrils facing the cytosol are free-ended, those who face the nucleoplasm converge at their distant end and form a structure similar to a basket;
- *Channel nucleoporins*, which occupy the innermost position and line the central pore of the NPC. These proteins have folded anchoring domains; in addition, some of them present intrinsically disordered domains, with unstructured polypeptide chains; the central NPC channel is filled with a mesh of these unstructured chains, which behave like a filter against the passage of large macromolecules (passive diffusion). The central region of the NPC is in fact an aqueous

channel between the nucleoplasm and the cytoplasm, with a diameter ranging from 5.2 nm in humans to 10.7 nm in *Xenopus laevis*.

The primal function of the NPCs is the control of *NE permeability*: they must to allow the free diffusion of small molecules (water, ions, sugars), the passage of macromolecules (proteins, RNAs, ribosomal subunits) and, at the same time, to prevent the passage of nonspecific molecules. All these exchanges are multi-level and highly regulated.

The measured particles diffusion rates at NPCs varies mainly according the size of particles: for instance, if the NE is freely permeable for small molecules (metal ions, small metabolites, etc., weighting 4,000 Da or less), the diffusion rate decreases for larger proteins and, for proteins larger than 60,000 Da, the passive diffusion stops. Yet, for more complex particles (including DNA polymerases and RNA polymerases, ribosomal subunits, etc.), with molecular masses up to 200,000Da, the transport through nuclear pores relies on binding to specific protein receptors that actively pass the molecules through NPCs (Alberts et al., 2015). Overall, the NPCs are capable of transporting particles up to 39 nm in diameter (Wente and Rout, 2010).

Similar to all permeable biologic membranes, the transport across the NE consists of two opposite bidirectional and continuous processes: the *import* of molecules (mainly proteins, such as histones, DNA and RNA polymerases, transcriptional regulators, carbohydrates, lipids, signaling molecules, etc.) and the *export* (including different types of RNA and ribosomal subunits). The import of molecules from cytoplasm, as well as the export of molecules synthesized into nucleus in cytoplasm are highly selective processes.

The *import of proteins*, even the very large ones, requests the fulfillment of several essential conditions: the presence of a particular sorting sequence in transported proteins (cargo), called *nuclear localization signal (NLS)*, involved in the selection of molecules which reach the nucleoplasm and the existence of *nuclear import receptors* (called *karyopherins* in general and, more specific, *importins*, in vertebrates), which recognize and bind to both NLS and NPC proteins.

A NLS, which designates a protein for import, is, typically, a single (or double) short sequence of amino acids, rich in lysine and arginine, positively charged and possibly forming loops or patches on the molecule surface; it can be located anywhere in the polypeptidic chain, the precise location of the NLS not being important in its functions. Different nuclear proteins share identical NLSs.

The first NLS sequence discovered was PKKKRKV (of the SV40 Large T- antigen), which is considered a classical NLS. Now, many classical (monopartite or bipartite) and non-classical NLSs are known in various nuclear proteins.

The nuclear import receptors (generally called *Kap* in *S. cerevisiae* and *importins* in vertebrates) ensure the import of proteins by their double recognition and binding ability: to the NLSs of cargo proteins and to the phenylalanine-glycine repeats (FG) of the unstructured regions of channel nucleoporins. The importins are soluble cytosolic proteins.

The cargo proteins do not always directly interact with the appropriate importins: in some cases the interactions between the two types of molecules are mediated by an additional class of proteins – the *adaptor proteins*, which recognize and bind to the NLS of cargo, activates and expose their own NLSs, which, in turn, bind to the nuclear import receptors. The combinations of import receptors and adaptors provide the cell recognition capacity of a large number of NLSs and thus, the nuclear import of various proteins.

Two main types of importins are known: the *importin-β family*, which bind and can transport the cargo proteins alone or can react and form heterodimers with the other type, the *importin-α*. As part of a heterodimeric complex, the importin-β play the role of a typical importin,

while the importin- α functions as an adaptor protein; in the last case, a trimer NLS-importin- α -importin- β forms.

The translocations of NLS proteins through the NPC into the nucleoplasm (the protein import cycle) could be divided in three main steps:

- The cargo proteins *bind* to the appropriate importin, directly or via an adaptor protein;
- The complex cargo-importin- β (or the trimer cargo-importin- α -importin- β) is *translocated* into cytoplasm, with a rate of diffusion depending mainly of concentration and binding affinity of importin- α . In this stage, the presence of Ran-GTP, a Ras family GTP-ase is of particular importance: the gradient of the two conformational forms of Ran (Ran-GTP and Ran-GDP) in cytosol and nucleoplasm directs the protein transport through NE (import and export). The Ran-GTP is more concentrated inside the nucleus, while Ran-GDP concentrates outside nucleus, following the GTP hydrolysis by Ran in cytosol.

When the cargo-nuclear import receptors complexes reach the nuclear side of the NPC, after passing through the poral channel, the Ran-GTP binds to them;

- The attachment of Ran-GTP modifies the conformation of importin- β of transporting complexes, leading to dissociation of the complex and the releasing of the cargo inside the nucleus; the importin- β stills bounded to the Ran-GTP, which is ready to be recycled: the complex is transported back in cytosol through the NPC and here, under Ran-GAP catalysis, the Ran-GTP is transformed in Ran-GDP, by GTP hydrolysis. The Ran-GDP dissociates from the carrier importin and the receptor became available for another import cycle.

The *export of proteins* from nucleus (e.g. those who compose the ribosomal subunits) is a process which works in a reverse order to protein import. Similar to proteins import into nucleus, the export of cargo proteins in cytosol relies on the existence of the *nuclear export signals (NESs)* on macromolecules to be exported, on *nuclear export receptors* (also called *exportins*), which are karyopherins, and on Ran-GTP enzymatic transport system. Generally, the nuclear export receptors are related to nuclear import receptors and are coded by the same gene family (with a large number of members in animal cells). The nuclear export receptors have double binding affinity: for nuclear proteins NESs and for NPC proteins, leading the passage of their cargo through the NPC to the cytoplasm.

It was demonstrated that, in some cases, the export of proteins containing leucine-rich NESs (for instance the human immunodeficiency virus type 1 (HIV-1) Rev-mediated nuclear export and Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE) - mediated nuclear export in *Xenopus laevis* oocytes), carried by the export receptor CRM1/exportin1, needs additional protein factors which interact with these specific NESs (Hofmann et al., 2001).

In proteins nuclear export cycle, Ran-GTP associates with nuclear export receptors in nucleoplasm and stimulates the association of cargo carrying the appropriate NES to these receptors. The complex travels then via NPCs into the cytosol, encounters the Ran-GAP, whose GTP is hydrolyzed in GDP; following hydrolysis, the cargo and Ran-GDP are released from nuclear export receptors and the free receptors return into nucleus.

The *export of RNA* from nucleus have some general characteristics:

- The export pathways differs for each class of exported RNA,
- The extranuclear transport of cellular RNAs ((tRNA, rRNA, U snRNA, microRNA) and viral RNAs relies on Ran-GTP cycle;
- For some mRNAs transport, export factors are necessary; for example, in higher eukaryotes, the splicing of mRNA recruits a protein complex – TREX, which function as an adapter

for mRNA binding protein TAP; for other mRNA molecules, the export does not need splicing events.

The *assembly of NPCs* has a variable dynamics during the cell cycle stages and intensifies at the beginning of telophase.

There are several theories that try to explain the main events of NPCs formation.

- One theory affirms that a complex of nucleoporins (Nups), connected with chromatin, inserts in the double nuclear membrane and determines the fusion of the two membranes at the insertion site; gradually, other nucleoporins bind to the initial Nup complexes, until a full structured NPC is made;

- Other theory states an opposite order of NPC assembly events: first, a prepore formed by several Nup complexes attached at chromatin appears; later, the double nuclear envelope forms around the prepore complexes.

The *disassembly* of NPCs during mitosis is a multiple step process, initiated by the peripheral nucleoporins dissociation (such as Nup 153, Nup 98 and Nup 214), a step thought to be driven by the phosphorylation of Nups. The scaffold nucleoporins of NPCs, which constitute cylindrical ring complexes within NE, remain stable.

More specific, during the close mitosis of *Aspergillus nidulans*, the partial NPCs disassembly consists in dispersion of at least five nucleoporins throughout cytoplasm, while at least three nucleoporins, with structural function, remains at the NPCs. These mitotic changes in NPCs architecture requires the activation of NIMA and Cdk1 kinases (De Souza et al., 2004).

Considering the lamina as a structural and functional part of the nuclear envelope, a series of human diseases are linked with NE protein defects (generally known as *envelopathies* and particularly *laminopathies*).

For instance, mutations in LMNA genes cause many clinical disorders which can be classified into several groups, according the predominantly affected tissues (Worman et al., 2010): the *striate muscle* (Emery-Dreifuss muscular dystrophy, cardiomyopathy dilated 1A, limb-girdle muscular dystrophy type 1B, etc.), the *adipose tissue* (Dunnigan-type familial partial lipodystrophy, lipodystrophy with diabetes and other features of insulin resistance, mandibuloacral dysplasia), the *peripheral nerve* (Charcot-Marie-Tooth disease type 2B1) and *multiple tissues* (Hutchinson-Gilford progeria syndrome, atypical Werner Syndrome, variant progeroid disorders etc.).

Defects in other NE proteins could result in other laminopathies: for example, mutations in emerin (or Lamin A/C) may cause Emery-Dreifuss muscular dystrophy while mutations in lamin B receptor (LBR) cause the Pelger-Huët anomaly (PHA), an autosomal dominant disorder. Of all the laminopathies, the Hutchinson-Gilford progeria syndrome (HGPS) is considered one of the most severe syndromes, because the life expectancy of the affected individuals is very low, around 13 years (Chi et al., 2009).

CONCLUSIONS

Since its discovery, at the beginning of the nineteenth century, the nucleus appears as a major rank organelle in all cells life. Since then, countless microscopy, biochemical, functional and genetic studies of nuclei were made. All these researches conclude that nuclei are extremely complex structures, with a high ordered internal organisation and coordination of their subcomponents activities and which play a major role in all cellular activities control, by expressing the genetic information they encompass.

REFERENCES

- Alberts B., Johnson A., Lewis J., Morgan D., Raff M., Roberts K., Walter P.** (2015) *Molecular Biology of the Cell*, 6th ed. (with problems by John Wilson and Tim Hunt), Garland Science, Taylor & Francis Group, 649-657
- Chen H., Chen K., Chen J., Cheng H., Zhou R.** (2012) *The integral nuclear membrane protein nurim plays a role in the suppression of apoptosis*, *Curr Mol Med.*, 12(10), 1372-82
- Chi Ya-Hui , Chen Zi-Jie, Jeang Kuan-Teh** (2009) *The nuclear envelopathies and human diseases*, *Journal of Biomedical Science*, 16(96)
- De Souza C. P.C., Osmani Aysha H., Hashmi S. B.** (2004) *Partial Nuclear Pore Complex Disassembly during Closed Mitosis in *Aspergillus nidulans**, *Current Biology*, 14 (22), 1973-1984
- Furukawa K., Kondo T.** (1998) *Identification of the lamina-associated-polypeptide-2-binding domain of B-type lamin*, *European Journal of Biochemistry*, 251 (3), 729-733
- Hofemeister H., O'Hare P.** (2005) *Analysis of the localization and topology of nurim, a polytopic protein tightly associated with the inner nuclear membrane*, *J Biol Chem.*, 280(4), 2512-21
- Hofmann Wilma, Reichart Beate, Ewald Andrea, Müller Eleonora, Schmidt Iris, Stauber R. H., Lottspeich F., Jockusch Brigitte M., Scheer U., Hauber J., Dabauvalle Marie-Christine** (2001) *Cofactor Requirements for Nuclear Export of Rev Response Element (Rre)–And Constitutive Transport Element (Cte)–Containing Retroviral Rnas; An Unexpected Role for Actin*, *J Cell Biol.*, 152(5), 895-510
- Holmer L., Worman H.J.** (2001) *Inner nuclear membrane proteins: functions and targeting*, *Cell Mol Life Sci.*, 58 (12-13), 1741-7
- Ji-Yeon-Shin, Dauer W. T., Worman H. J.** (2014) *Lamina-associated Polypeptide 1: Protein Interactions and Tissue-selective Functions*, *Seminars in Cell and Developmental Biology*, 29
- Khmelninskii A., Blaszcak Ewa, Pantazopoulou Marina, Fischer B., Omnus Deike J., Le Dez Gaelle, Brossard Audrey, Gunnarsson A., Barry J.D., Meurer M., Kirrmaier D., Huber W., Rabut Gwenael, Ljungdahl P.O., Knop M.** (2014) *Protein quality control at the inner nuclear membrane*, *Nature*, 516, 410-413
- Laguri C., Gilquin B., Wolff N., Romi-Lebrun R., Couchay K., Callebaut L., Worman H.J., Zinn-Justin S.** (2001) *Structural characterization of the LEM motif common to three human inner nuclear membrane proteins*, *Structure*, 9, 503–511
- Lin F., Morrison Juliet M., WuWei , Worman H. J.** (2005) *MAN1, an integral protein of the inner nuclear membrane, binds Smad2 and Smad3 and antagonizes transforming growth factor- β signaling*, *Human Molecular Genetics*, 14 (3), 437-445
- Olins Ada L., Rhodes G., Welch David B. M., Zweger Monica, Olins D.E.** (2010) *Lamin B receptor Multi-tasking at the nuclear envelop*, *Nucleus*, 1(1), 53-70
- Rajgor Dipen, Shanahan Chaterine M.** (2013) *Nesprins: from the nuclear envelope and beyond*, *Expert Reviews in Molecular Medicine*, 15, e5 doi: 10. 1017/ 2013.6, <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3733404/>
- Roux K. J., Crisp Melissa L., Liu Q., Kim D., Kozlov S., Stewart C. L., Burke B.** (2009) *Nesprin 4 is an outer nuclear membrane protein that can induce kinesin-mediated cell polarization*, *Proc Natl Acad Sci U S A.*, 106(7), 2194–2199.

Wente Susan R., Rout M. P. (2010) *The Nuclear Pore Complex and Nuclear Transport*, Cold Spring Harbor Perspect Biol 2010, 2(10), a000562, <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2944363/>

Worman H. J., Ostlund Cecilia, Wang Yuexia(2010) *Diseases of the Nuclear Envelope*, Cold Spring Harb Perspect Biol 2010; 2:a000760, <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2828284/>

Worman H.J., Evans C.D., Blobel G. (1990) *The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential transmembrane domains*, JCB, 111, (4) 1535-1542

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RESEARCH REGARDING THE FREQUENCY OF AB0 BLOOD GROUPS IN A POPULATION OF PUPILS FROM RĂUCEȘTI, NEAMȚ COUNTY

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Received: 01 June 2016 / Revised: 03 June 2016 / Accepted: 27 June 2016 / Published: 13 July 2016

Key words: AB0 blood groups, frequency, population genetics.

Abstract: We have studied the frequency of AB0 blood groups in Răucești, Neamț County, as part of a larger study regarding the genetic polymorphisms present in the human population of Romania and particularly in Neamț county. The blood groups frequency were: O = 34.86; A = 42.91; B = 15.88; AB = 6.34. As controls were used data obtained from Blood Transfusion Centre from Neamț county, determination made between 2010-2013. These values are in accordance with the values registered for all Romanian population and particularly in Neamț county: in Roman town between 2001-2004, and in Piatra Neamț for 2008-2009 the frequency of blood groups are also, in accordance with our results. The blood groups O, A and AB are more frequent in females, and B is more frequent in males.

INTRODUCTION

AB0 blood groups, due to their monogenic determinism, are ones of the most studied pure inherited traits. AB0 blood groups are fulfilling all the criteria for the optimal genetic study: high frequency, easy to be determined and statistically analysed (Tudose et al., 2000).

It is still a question if Mendel's laws, discovered and formulated on pea, with validity proved at the beginning of XX-th century also for animals including human beings, are universal. For *Homo sapiens sapiens*, because of ethical and moral reasons, it can not be done controlled cross experiments and consanguinisations, offspring is reduced as number for each genitor pair, carrier of any genetic maladies can not be excluded from reproduction, this limitations making investigations more difficult comparing to those regarding plants, animals or microorganisms, leading to specific working methods (populational studies, mono- and dizygote twins investigations).

Part of a larger study regarding the genetic polymorphisms present in the human population of Romania, followed in the future by the elaboration of a map illustrating the situation of AB0 system blood groups frequency for the whole country, we have continued to study the frequency and transmission of AB0 blood groups in a scholar population at regional level: Neamț County.

Our researches started in Neamț County in Roman in 2001-2004 (Băra et al., 2007) continued in Piatra Neamț for 2008-2009 (Băra and Greșanu, 2010) and are directed on: processing data for 117 pupils from School Nr.1, Răucești, Neamț County, processing data regarding AB0 blood groups, determined at Blood Transfusion Center Piatra Neamț between 2010-2013 and comparing them with those obtained for Roman in 2001-2004 (Băra et al., 2007) and Piatra Neamț for 2008-2009 (Băra and Greșanu, 2010). From the investigated group, we obtained data regarding AB0 system blood groups frequency for scholar population and for the two sexes.

MATERIALS AND METHODS

The data, regarding blood groups, were obtained from 117 pupils, 53 boys and 64 girls, born between 1999-2002, registered in scholar year 2012-2013 at School Nr.1, Răucești, as it follows: 7 fellows in the 5th class, 28 in the 6th class, 37 in the 7th class, 45 in the 8th class. Data were processed based on a filled out printed form, regarding the blood groups of them and of their families members (parents, brothers or sisters, grandparents), were grouped in 4 files, base on year of birth (1999-2000-2001-2002), and then reported to recorded data from Blood Transfusions Center (CTS) Piatra Neamț, for 2010-2013. Based on this, it was possible to elaborate 15 pedigrees, using the international symbols.

RESULTS AND DISCUSSIONS

In Romania, for AB0 blood groups, the repartition is: O Group= 34%; A Group= 41%; B Group= 19%; AB Group= 6% (Băra et al., 2007).

For Neamț County, Roman region, Băra et al, 2007, noticed the next mean frequency repartition: 0 Group= 33%; A Group= 43%; B Group= 16%; AB Group= 8%, and for Piatra Neamț region, for 2008-2009, noticed the mean frequency was: 0 Group = 31%; A Group = 44%, Group B = 16%, Group AB = 9% (Băra & Greșanu, 2010).

Table 1. Frequency of the AB0 blood groups at CTS Piatra Neamț between 2010 and 2013

Blood Group	2010(%)	2011(%)	2012(%)	2013(%)	Average (%)
0	36.69	35.41	34.37	32.99	34.86
A	43.31	42.32	42.54	43.47	42.91
B	14.76	15.81	16.54	16.42	15.88
AB	5.24	6.46	6.55	7.12	6.34

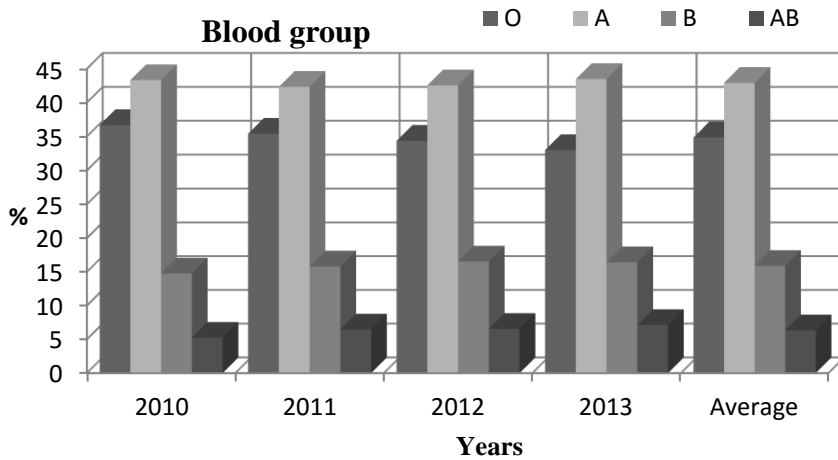


Figure 1. Frequency of the AB0 blood groups at CTS Piatra Neamț between 2010 and 2013

Data from Blood Transfusions Center (CTS) Piatra Neamț, for 2010-2013 show a mean frequency repartition of 0 Group = 35%; A Group = 43%, Group B = 16%, Group AB = 6%, very similar comparing with previous data (for 2008-2009) in Piatra Neamț.

Table 2. Average frequency of the AB0 blood groups at CTS Piatra Neamț between 2008-2009 (Băra & Greșanu, 2010) and 2010-2013 (this study), and 2001-2004 at CTS Roman (Băra et al, 2007)

Blood Group	Average (%) 2001-2004 Băra et al, 2007	Average (%) 2008-2009 Băra & Greșanu, 2010	Average (%) 2010-2013 This study
0	33	31	35
A	43	44	43
B	16	16	16

AB	8	8	6
Total	100	100	100

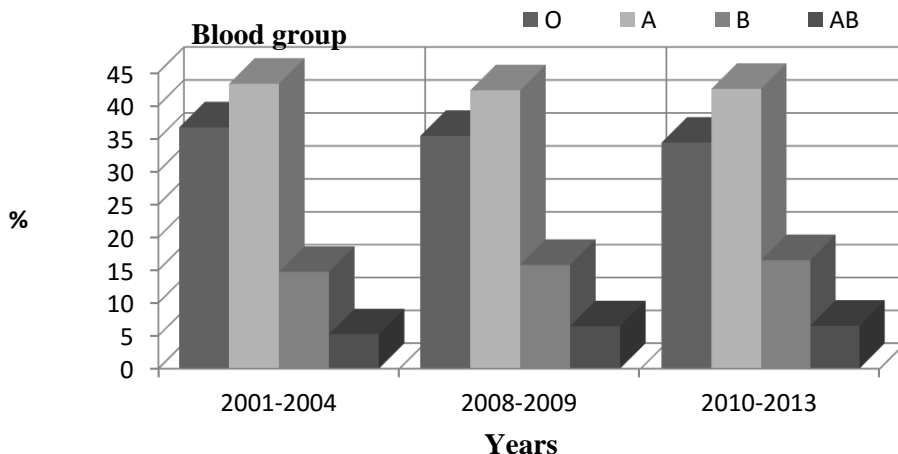


Figure 2. Average frequency of the AB0 blood groups in Neamț County, for this study data for 2010-2013, compared with data for 2008-2009 (Băra & Greșanu, 2010) and data for 2001-2004 (Băra *et al.*, 2007).

Comparing this data with AB0 blood system frequency at the level of whole country, it can be concluded that, for Piatra Neamț, blood groups frequency in normal limits, characteristic for Romanian population, and very similar to results showed by Băra & Greșanu, 2010, and Băra *et al.*, 2007, for Neamț County after a similar study made in Roman.

THE FREQUENCY OF AB0 BLOOD GROUP SYSTEM, IN THE INVESTIGATED SCHOLAR POPULATION

As shown in table 3, from the total of 117 investigated pupils (53 boys and 64 girls), from School Nr.1, Răucești, Neamț County, 39 (15 boys and 24 girls) belonged to O blood group (33.33%), 49 (22 boys and 27 girls) to A blood group (41.88%), 22 (13 boys and 9 girls) to B group (18.80%) and 7 (3 boys and 4 girls) to AB group (5.98%). The percentage is shown in Figure 3.

Table3. Distribution of the AB0 blood groups in the investigated population

Blood Group Type															
OI				AII				BIII				ABIV			
Nr.		%		Nr.		%		Nr.		%		Nr.		%	
39		33.33%		49		41.88%		22		18.80%		7		5.98%	
B	Gi	Bo	Gi	Bo	Gi	Bo	Gi	Bo	Gi	Bo	Gi	Bo	Gi	Bo	Gi
o	rls	ys	rls	ys	rls	ys	rls	ys	rls	ys	rls	ys	rls	ys	rls
y		s	s	s	s	s	s	s	s	s	s	s	s	s	s
1	24	12.8	20.5	22	27	18.8	23.0	13	9	11	8	3	4	2.5	3.4
5		2%	1%			0%	7%			%	%			6%	1%

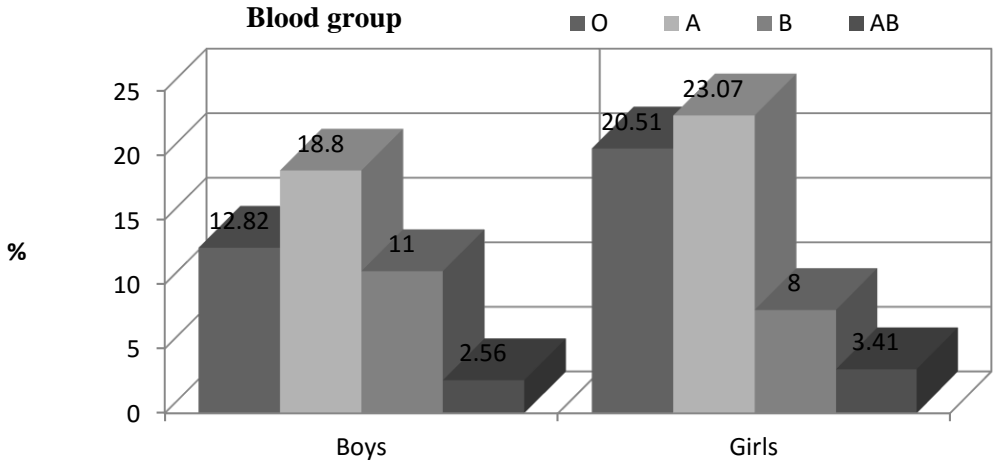


Figure 3. General frequency of the AB0 blood groups and gender distribution in the investigated population

Data regarding number of investigated pupils belonging to each blood group type grouped by the year of birth are presented in table 4. It can be observed the similar distribution characteristic for Romanian population.

Table4. Number of investigated pupils belonging to each blood group type, based on year of birth

Year of birth	Blood group type							
	OI		AII		BIII		ABIV	
	n	%	n	%	n	%	n	%
1999	16	39.02	21	42.86	7	31.82	1	14.29
2000	11	26.83	14	28.57	8	36.36	4	57.14
2001	9	21.95	11	22.45	6	27.27	2	28.57
2002	5	12.20	3	6.12	1	4.55	0	0.00
TOTAL	41	100.00	49	100.00	22	100.00	7	100.00

Results regarding AB0 blood groups frequency in the investigated scholar population (experimental lot), are very similar with the mean of results obtained from CTS Piatra Neamț (Controle), for period 2010-2013 (Fig.4).

It can be noticed a slight increase of BIII group frequency, compared also with the mean value obtained from CTS Piatra Neamț in 2010-2013, as well as in 2008-2009 (Băra & Greșanu, 2010). The community of Răucești village is quite isolate, so it is interesting to follow in time the evolution of the gene pool. In this study the investigated population is not large enough to lead to a conclusion.

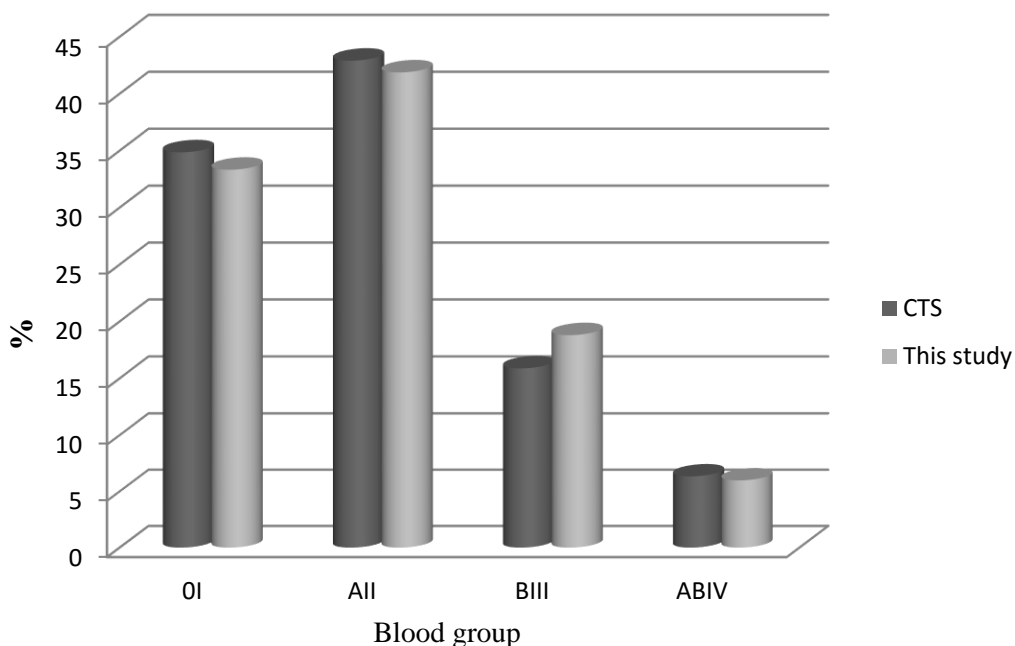


Figure 4: Frequency of AB0 blood groups for the experimental lot compared with mean frequency at CTS Piatra Neamț (Control)

Comparing blood group types depending of year of birth, it was noticed that results do not differ very much. So, for the 45 children born in 1999, determinations showed that 16 have O group, 21 have A group, 7 B group, 1 AB group. (figure 5, table 4).

For those born in 2000, results showed that: 11 have O group, 14 have A group, 8 B group, 4 AB group, and for those 28 born in 2001: 9 have O group, 11 have A group, 6 B group and 2 AB group. From the 9 investigated children born in 2002, no group ABIV was found. There were 5 with O group, 3 with A group and 1 with B group (figure 5, table 4).

Even if it is a quite isolated community, it can be observed that the blood groups frequency has the same repartition as for whole Romanian population and Neamț County. The predominant blood group is A, followed by Group O, Group B, and on the last place Group AB.

Even if number of boys were lower than of girls, regarding sex ratio, we observed that group BIII is more frequent at males. For AB group, the frequency is almost the same for both sexes. Groups A and O is more frequent at females, but because we have not investigated the same number of boys and girls, results are not conclusive.

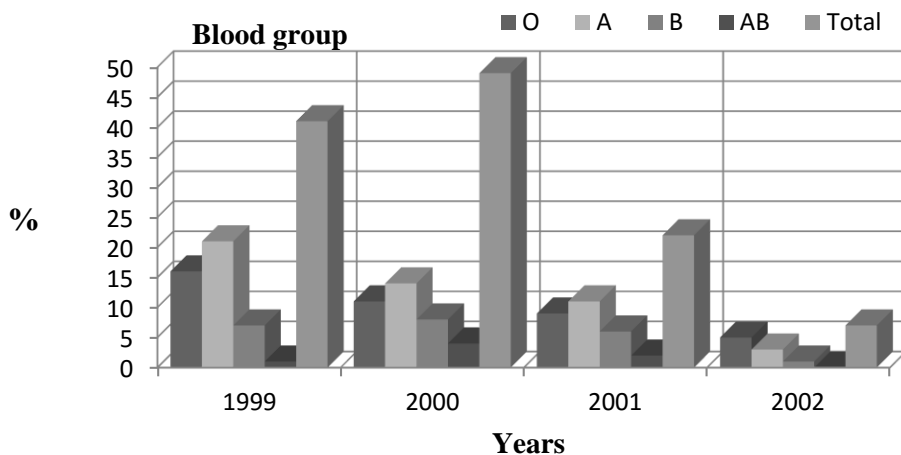


Figure 5: Frequency of AB0 blood groups for the investigated pupils, based on year of birth

CONCLUSIONS

The investigated population sample was composed of 117 children, born between 1999-2002, learning at at School Nr.1 Răucești, Neamț County, which determined blood group type between 2012 - 2013.

Results regarding AB0 blood groups frequency in the investigated scholar population, are very similar with the mean of results obtained from Transfusion Centre Piatra Neamț, for period 2012 – 2013.

Even if it is a quite isolated community, it can be observed that the blood groups frequency has the same repartition as for whole Romanian population and Neamț County.

ABO system blood groups frequency for Piatra Neamț, joins the normal parameters characteristic for the Romanian population, which is also in accordance with the general European values.

The predominant blood group is AII, followed by Group OI, Group BIII, and on the last place Group AB, not depending on sex of investigated person.

Regarding sex ratio, we observed that group BIII is more frequent at males even if number of boys were lower than of girls. For ABIV group, the frequency is almost the same for both sexes. Groups AII and OI is more frequent at females, but because we have not investigated the same number of boys and girls, results are not concludent.

REFERENCES

Băra, I.I. Câmpeanu, Mirela Mihaela., 2003- Genetică, Editura Corson, Iași, 139-183

Băra, I.I., Ivaș, Manuela Gabriela, Tudose, Cr., Băra, Csilla Iuliana, 2004. A populational research regarding the frequency and transmission of ABO blood groups in the Romanian region Bârlad. Analele Științifice ale Universității „Alexandru Ioan Cuza” din Iași, secțiunea II, GENETICĂ ȘI BIOLOGIE MOLECULARĂ, tomul VI, 91-93.

Băra, I.I., Emilia Rândunică, Cr. Tudose, Csilla Iuliana Băra, 2007. Research regarding the frequency and transmission of ABO blood groups in a population of pupils from Roman, Neamț county. Analele Științifice ale Universității “Al.I.Cuza” din Iași (serie nouă), Secțiunea I, a.Genetică și Biologie Moleculară, tom VIII, Fasc. I, 167-175.

Csilla Iuliana Băra, Camelia Gresanu, 2010. Research regarding the frequency of ABO blood groups in a population of pupils from Piatra Neamț, Neamț county. Analele Științifice ale Universității “Al.I.Cuza” din Iași (serie nouă), Secțiunea I, a.Genetică și Biologie Moleculară, tom XI, 223-228.

Raicu P., 1997. Genetica generală și umană, Editura Humanitas, București, 56-71.

Stine J., 1999. The new human genetics. Wilkins and sons, New York, 34-50.

Tudose Cr., Maniu Marilena., Maniu C.L., 2000. Genetica umană, Ed. Corson, Iași, 23-46.

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ASSESSING PROGRESSION OF CERVICAL PRE-CANCER LESIONS

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Received: 30 May 2016 / Revised: 02 June 2016 / Accepted: 27 June 2016 / Published: 13 July 2016

Key words: p16 protein, L1 major capsid protein, human papilloma virus, immunohistochemistry

Abstract. The purpose of this study was to accomplish a comparative assessment between the immune histochemical and the immunocytochemical expression of p16 protein and L1 major capsid protein of HPV respectively, in cervical squamous intraepithelial lesions with low and high grade, in order to determine, through morphological and clinical correlations, their applicability into practice when diagnosing and further monitoring the patients. There were 119 patients included in the study, having a mean age of 40, cytologically and histopathologically diagnosed in the Laboratory of Pathologic Anatomy of “Elena Doamna” Third Clinic of Obstetrics and Gynaecology in Iași. 42 of these patients were diagnosed with LSIL (low grade squamous intraepithelial lesion) and 51 with HSIL (high grade squamous intraepithelial lesion). The cervical-vaginal smears were interpreted using the Papanicolaou method. The conventional smears were assessed for the immunexpression of L1 capsid protein HPV, and the corresponding biopsies for p16 immunexpression. The colouring pattern of p16 protein was predominantly nuclear, with an occasional cytoplasmic positivity. P16 biomarker was positive in cytological smear prepared in a liquid environment for 71.6% of the patients, without significant differences for those over 40 years old (69.6% vs 69.0%; $p=0.887$), with an increase in positivity from 54.8% in LSIL to 98% in HSIL ($p=0.05$); the oncogenic risk was 1.55 higher ($RR=1.55$; $IC95\%: 1.19\div 2.01$). L1 protein was detected in 34.5% of the patients, the expression tending to increase in parallel with the increase in the severity of the lesions (66.7% LSIL; 17.6% HSIL). The presence of L1 protein in the patients with an increased risk of malignant transformation of HPV seems to be a protective factor ($RR=0.42$; $IC95\%: 0.27\div 0.66$). The immunexpression of L1 HPV protein has clinical applications in assessing the progression of cervical pre-cancer lesions. The analysis of p16 status, in parallel with the expression of L1 HPV protein, can be very useful in assessing the risk of progression for cervical intraepithelial squamous lesions. The preventive conduct supported by a primary care screening, leads to a decrease in the morbidity by pre-invasive lesions and an evolution with a favourable prognostic.

INTRODUCTION

It is generally accepted nowadays that invasive cervical squamous tumours and the corresponding preceding lesions are caused by specific types of human papilloma virus (HPV), especially by the types with oncogenic risk that infect the anogenital tract. Now, the proportion of cervical carcinoma attributed to HPV infection is estimated at 99%. Many observations showed the importance of the immune answer in HPV infection. Previously there have been studies on the antibodies against capsid protein of different HPV types, using bacterial fusion proteins or chemically synthesized peptides (Achim, R., 1998; Alexandrescu, D., 1984). As an essential condition of these studies, we had to identify the type of infecting HPV. Until recently, the detection of HPV type in a certain tissue has been done through methods of hybridization with nucleic acids. The polymerase chain reaction (PCR) was introduced as a more effective and sensitive method of amplifying the DNA of HPV, being used both for general detection and for finding the type of HPV, particularly in genital infections. The problem of quantification is the main limit of technique, being difficult to distinguish between a latent infection (subclinical) and the obvious clinical lesions. As an alternative to hybridization and PCR, the immunological detection of viral capsid antigen can be used for diagnosing the productive HPV infections.

P16 tumour suppressor protein is a cyclin-dependent kinase inhibitor that regulates the transition from phase G1 to phase S in the cellular cycle (Altekruse, S.F., et al., 2003; Ancar, V., 1999).

The intense immunexpression of p16 was previously reported as being characteristic to the dysplastic and neoplastic cervical epithelium (Anderson, M. et al., 1992; Anderson, M. et al., 1996; Anderson, N.H., 2000). Overexpression of p16 slows down the cell cycle by inactivating the cyclin-dependent kinases that phosphorylate the retinoblastoma protein (pRb) (Andre, F.E., 2003; Anhang, R., et al., 2004). The viral oncogenes of HPV - E6 and E7, whose expression is associated with the malignant transformation of cervical epithelial cells (An, H.J., et al., 2003; Anghel, R. & Bălănescu, I., 1996), can tie to and inactivate pRb which, in turn, influences the expression of p16 protein in cervical intraepithelial squamous lesions (SIL) (Anton, G. & Socolov, D., 2000). Recent studies have concluded that p16 is a useful marker for the high risk HPV cervical neoplasia (9, 13) and also for assessing the progression of SIL (Arbyn, M., et al., 2010; Ardeleanu, C, et al., 1999). The behaviour of cervical intraepithelial squamous lesions is unpredictable, many of them, particularly the low grade ones being able to disappear without treatment. Invasive cervical carcinoma appears in about 10% of the intraepithelial lesions

preceding cancer, being strongly associated with HPV infection (Baccard-Longere, M., 1999; Badea, M. & Virtej, P., 2002; Baldauf, J.J., et al., 1997).

PURPOSE AND OBJECTIVES

The purpose of this study was to accomplish a comparative assessment between the immunohistochemical and immunocytochemical expression of p16 protein and L1 capsid protein of HPV respectively, in the cervical intraepithelial squamous lesions of low and high grade, in order to determine, through morphological and clinical correlations, their practical applicability in diagnosing and further monitoring the patients.

MATERIAL AND METHODS

There were 119 patients included in the study, all of them diagnosed cytologically and histopathologically in the Laboratory of Pathological Anatomy of “Elena Doamna” Third Clinic of Obstetrics and Gynecology in Iasi. 42 of these cases were diagnosed with LSIL (low grade squamous intraepithelial lesion) and 51 cases with HSIL (high grade squamous intraepithelial lesion), which needed further biopsy. The cervical-vaginal smears were fixed and coloured using the Papanicolaou method. The conventional smears were assessed for the immunoeexpression of L1HPV capsid protein, and the corresponding biopsies for the p16 immunoeexpression.

After establishing the cytodiagnostic, the cervical-vaginal smears were used for detecting the L1 HPV capsid protein through immunocytochemistry, using monoclonal antibodies (Cytoactiv HPV L1 High Risk Set REF SCA0850, Cytoimmun Diagnostics GmbH), following a standard protocol. Epithelial cells with a positive nuclear colouring received a positive score, considering that one coloured nucleus is enough for accomplishing the score.

Cervical biopsies were investigated through a histopathologic and immunohistochemical routine examination, using p16-D25 antibodies. The collected tissues were fixed for 24 hours in buffered formalin and were processed for inclusion in paraffin. Serial sections of 4-5 µm were removed the paraffin and were coloured with haematoxylin-eosin. After the standard histopathologic examination, we made further sections for the immunohistochemical examination. HIER (Heat-induced epitope retrieval) technique was used with a solution of Target Retrieval with pH 6 (cod S1700, DAKO, Denmark). After being blocked with endogene peroxidase and being non-specific linked, the sections were incubated with one of the primary antibodies, a monoclonal anti-p16 mouse (clone D25, cod sc-81613, Santa Cruz, USA) antibody, with a dilution of 1:100. The immune reaction was amplified using the corresponding secondary antibody and the Streptavidin–Biotin–Peroxidase HRP (cod K5001, DAKO, Denmark) complex. The sections were afterwards developed, using 3,3'-diaminobenzidine tetra hydrochloride (DAB) (cod K5001, DAKO, Denmark) chromogen, under microscopic control. The sections were finally counter-coloured with Mayer haematoxylin. There was also a negative control performed.

The quality control represented by external and internal negative and positive controls was necessary for monitoring the accuracy of tissue processing, colouring procedures and efficiency of reactives. The specificity of the primary antibody must be assessed through its negative controls. P16 protein was given a score, considering the estimating proportion of immunopositive cells (table 1).

Table 1. p16 - immunohistochemical score

Score	p16
0	absent
1	Weak (<25% immunopositivity)
2	Moderate (25-75% immunopositivity)
3	Intense (75-100% immunopositivity)

RESULTS AND DISCUSSION

The positivity of **p16 biomarker** in cytological smear prepared in a liquid environment showed in 71.6% of the patients, with no significant differences for those over 40 years old (69.6% vs 69%; p=0.887).

In the cytological smears that were analysed, the distribution showed an increased p16 positivity from 54.8% in LSIL to 98% in HSIL (table 2.).

Table 2. The detection rate of p16 biomarker in cytological smear in liquid environment

Cytological diagnostic	Total number of cases	p16 positive biomarker	
		n	%
Negative	15	0	0.0
ASCUS	11	5	45.5
LSIL	42	23	54.8
HSIL	51	50	98.0
Total	119	78	65.5

79,6% of the patients with an increased risk of malignant transformation showed a positivity of the p16 biomarker, while the patients with a low risk of malignant transformation of HPV showed no case with a positive p16 ($p < 0.001$). The association of an increased risk of HPV with p16 positivity induces a relative viral oncogenic risk 1,55 higher (RR=1.55; IC95%: 1.19÷2.01).

L1 HPV capsid protein. In the smear prepared in a liquid environment, L1 protein was detected in 34,5% of the patients. The distribution of the patients on age groups showed the presence of L1 in 31/73 patients under 40 years old (42.5%), compared with 10/36 patients over 40 years old (27.8%), but this distribution is not significant from the statistic point of view ($p = 0.201$).

The study group showed an anti-L1 HPV immunoreactivity in 18.2% of the cases of ASCUS, 66.7% LSIL and 17.6% HSIL, this indicating that the expression of L1 protein tends to decrease in parallel with an increase in the severity of the lesions (table 3.).

Table 3. The detection rate of L1 capsid protein in the cytological smear in liquid environment

Cytological diagnostic	Total number of cases	positive L1 capsid protein	
		n	%
NEGATIVE	15	2	13.3
ASCUS	11	2	18.2
LSIL	42	28	66.7
HSIL	51	9	17.6
Total	119	41	34.5

29.1% of the patients with an increased risk of malignant transformation were detected with the presence of L1 capsid protein, which is significantly lower than the 100% patients with a low risk of malignant transformation of HPV ($p = 0.00003$).

It was statistically proven that the presence of L1 protein in the patients with an increased risk of malignant transformation of HPV is a protective factor, because the relative risk is below 1 (RR=0.42; IC95%: 0.27÷0.66).

HPV infection was confirmed morphologically through the presence of the cytopathic effect of the virus (koilocytes) on smears and biopsies.

The colouring pattern of p16 protein was predominantly nuclear, with an occasional cytoplasmic positivity. Most cases showed heterogeneous colouring, with positive and negative cells.

From all cervical biopsies, p16 was positive in 54.8% in LSIL and 98% in HSIL. The ratio of biopsies with an intense immunoexpression of p16 increased in parallel with the severity of cytologic anomalies. In HSIL cases, the distribution of colouring was as follows: 65% of the whole epithelium (fig. 3), 35% of the base and intermediary layers. The intensity of colouring for HSIL cases was intense in 80% (fig. 1, 2), moderate in 16% and weak in 4%. In the case of LSIL category, the distribution of colouring was as follows: basal in 75% of the cases and occasional in 25%. There was no case with LSIL that showed a positive colouring of p16 all over the epithelium. The intensity of colouring in the cases diagnosed with LSIL was intense in 20% (fig. 3, 4, 5), moderate in 13% and weak in 67%.

From all cervical smears, L1 HPV capsid protein was shown in 66.7% of LSIL and 17.6% of HSIL. The expression of L1 capsid protein was significantly reduced for the cases diagnosed with HSIL, HPV positive. In the cases of LSIL, that were HPV positive we could not show a significant decrease in the expression of capsid L1 protein.

The positive reaction was characterized through an intense colouring of the whole nucleus, surrounded by cytoplasm without background colouring. In most cases, the positive reaction for HR-HPV L1 was positive in the typical koilocytes and in diskeratocytes, showing nuclear characteristics for HSIL (CIN 2 or CIN 3). In the cases of LSIL, the positivity of nuclei was present only in the koilocytes with characteristic morphology (fig. 6).

All the cases that were HPV positive (with morphological signs of HPV infection) were also p16-positive, without identifying any significant relation between the immunopositivity of HPV infection (active infection) and the intensity and distribution of p16.

L1 capsid protein is expressed in the active phase of HPV infection and is necessary in completing the viral cell cycle. Hence, the detection of the viral protein, through an immunohistochemical reaction, represents the proof of HPV infection in the examined tissues (Ball, C. & Madden, L.E., 2003). L1 viral capsid protein is considered a major target for the cell immune response (Baseman, J.G. & Kautsky, L.A., 2005). Moderate LSIL and SIL, without the immunohistochemical detection of L1 protein, are correlated in more than 80% of the cases, with progression of dysplasia. Griesser and colab. certify these aspects, underlining the fact that minor and moderate lesions without the expression of L1 capsid protein are significantly more exposed to progression, in comparison with the cases of positive L1 (Basen-Engquist, K., et al., 2003; Benagiano, G., et al., 2006). Most likely, the lack of HPV antigen is caused by a weak proteic synthesis, below the minimum level of immunohistochemical testing. Considering the fact that L1 represents the major target of the immune cellular response (Bibbo, M., et. al, 2002), a deficitary translation can lead to an inefficient depuration of the infected cells, promoting the integration of viral ADN in the genome of the host cell and transformation of the immature epithelial cells. The observation that a decreased positivity of HPV16 capsid protein in the serum of the patients who were diagnosed with cervical cancer is a reserved prognostic indicator, supports the importance of the specific umoral response. The immunocytochemical detection of L1 capsid, on conventional smears, can show the defence status that is locally induced on HPV infection and can offer prognostic information, especially for LSIL lesions.

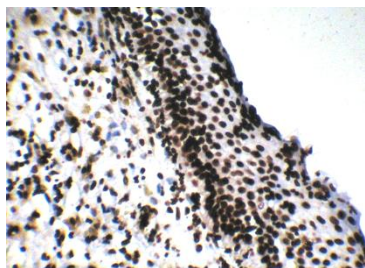


Fig. 1. HSIL (CIN2), p16, colouring intense immune colouring (x10)

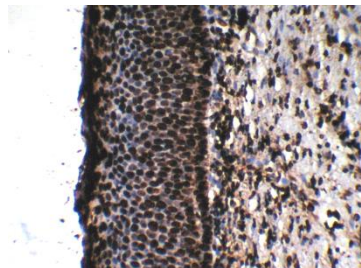


Fig. 2. HSIL (CIN3), p16, intense immune colouring for the entire thickness of the epithelium

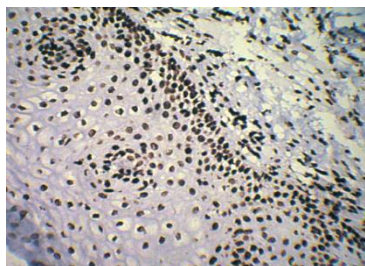


Fig. 3. LSIL, p16, intense immune colouring (x10)

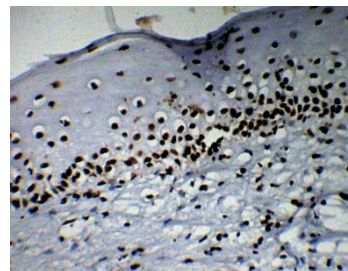


Fig. 4 LSIL, p16, intense immune colouring

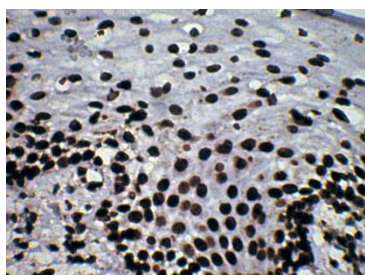


Fig. 5. LSIL, p16, intense immune colouring (x20)

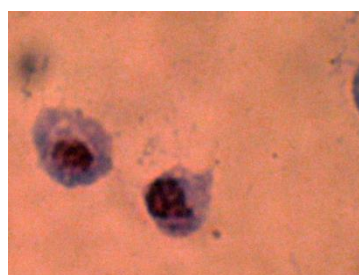


Fig. 6. LSIL, positive L1 HPV (x100)

CONCLUSIONS

In our study, from all the cervical biopsies, p16 was positive in 54.8% of LSIL, 98% of CIN2 and of CIN3.

From all cervical smears, L1 HPV capsid protein was present in 68.8% of LSIL and 29.1% of HSIL.

The expression of L1 capsid protein was significantly reduced for the cases of positive HSIL, HPV. In the cases of positive LSIL, HPV, we could not demonstrate a significant decrease of the expression of L1 protein.

The analysis of p16 status, in parallel with the expression of L1 HPV protein, can be very useful in assessing the risk of progression for the cervical intraepithelial squamous lesions. A preventive conduct that is also supported by primary care screening, leads to a decrease of morbidity by preinvasive lesions and an evolution with a favourable prognostic.

REFERENCES

1. **Achim, R.** (1998) *Identification and typing of human papillomaviruses using PCR and restriction enzyme analysis*. Balkan J Med Genetics; 1:106.
2. **Alexandrescu, D.** (1984) *Colposcopia*. Ed. Medicală București.
3. **Altekruse, S.F., Lacey, J.V., Brinton, L.A. et al.** (2003) *Comparison of HPV genotypes, sexual and reproductive risk factors of cervical adenocarcinoma and squamous cell carcinoma*. Am J Obstet Gynecol; 188: 657-663.
4. **Ancar, V.** (1999) vol: *Ginecologia*. Ed. Natională.
5. **Anderson, M., Jordan, J., Morse, A., Sharp, F.** (1992) *Integrated colposcopy*. Chapman & Hall Medical London.
6. **Anderson, M., Jordan, J., Morse, A., Sharp, F.** (1996) *Integrated colposcopy*. Chapman & Hall Medical London.
7. **Anderson, N.H.** (2000) *Automated prescreening and rescreening*. In: International Consensus Conference on the fight against cervical cancer, IUAC, Chicago, 2000.
8. **Andre, F.E.** (2003) *Vaccinology: past achievements, present roadblocks and future promises*. Vaccine;30:21[7-8]593-5.
9. **Anhang, R., Goodman, A., Goldie, S.** (2004) *HPV communication: review of existing research and recommendations for patient education*. CA Cancer Clin, 54: 248-259.
10. **An, H.J., Chom, N.H., Lee, S.Y., et al.** (2003) *Correlation of cervical carcinoma and precancerous lesions with human papillomavirus (HPV) genotypes detected with the HPV DNA chip microarray method*. Cancer; 97:1672.
11. **Anghel R, Bălănescu I.** (1996) *Cancerul colului uterin*. Ed Med Amaltea București.
12. **Anton, G., Socolov, D.** (2000) *The HPV infection in the cervical neoplasia in North-East Romania*. In: International Consensus Conference on the fight against cervical cancer, IUAC, Chicago .
13. **Arbyn, M., Antoine, J., Mägi, M., Smailyte, G.** (2010) *Trends in cervical cancer incidence and mortality in the Baltic countries, Bulgaria and Romania*. Int J Cancer.
14. **Ardeleanu, C., Comănescu, V., Zaharia, B.** (1999) *Imunohistochimie – Principii generale și aplicații în diagnosticul histopatologic*. Editura Sitech.
15. **Baccard-Longere, M.** (1999) *Typage des papillomavirus humains. Méthodes et intérêts*. Gynécologie; 41(5): 311.
16. **Badea, M., Vîrtej, P.** (2002): *Sinopsis de patologie cervicală preinvazivă*. Ed. Infomedicală, București 2002.
17. **Baldauf, J.J., Dreyfus, M., Ritter, J., Meyer, P., Philippe, E.** (1997) *Screening histories of incidence cases of cervical cancer and high grade SIL. A comparison*. Acta Cytologica, 41(5):1431-1438.
18. **Ball, C., Madden, L.E.** (2003) *Update on cervical cancer screening, current diagnostic and evidence-based management*. Protocols; 113(2): 120-121.
19. **Baseman, J.G., Kautsky, L.A.** (2005) *The epidemiology of human papillomavirus infection*. J Clin Virol; 32 Suppl 1:516-524.
20. **Basen-Engquist, K., Paskett, E.D., Buzaglo, J., et al.** (2003) *Cervical cancer*. Cancer; 98:2009.
21. **Benagiano, G., Bastianelli, C., Farris, M.** (2006) *Contraception today*. Ann N Y Acad Sci; 1092:1.
22. **Bibbo, M., Klump, W.J., DeCecco, J., Kovatich, A.J.** (2002) *Procedure for immunocytochemical detection of P16INK4a antigen in thin-layer, liquid-based specimens*. Acta Cytol; 46 (1): 25–29.

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RISK FACTORS IN SEPSIS WITH ORO-MAXILLOFACIAL PORTAL OF ENTRY

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Received: 31 May 2016 / Revised: 02 June 2016 / Accepted: 28 June 2016 / Published: 13 July 2016

Key words: sepsis, oro-maxillofacial portal of entry

Abstract. Prospective study aimed at establishing the incidence of sepsis with oro-maxillofacial portal of entry and of the causal relationship between the disease and the incriminated risk factors (factors in the living environment, collectivity, occupational environment, behavioral factors). The study group included 200 patients admitted to the Oro-Maxillofacial Surgery and ENT Clinics of the Iasi "St. Spiridon" Emergency Hospital in the interval 2012-2015. *S. aureus* was the main causal agent incriminated in the development of oro-dental sepsis, about ½ of the isolates strains being methicillin-resistant. Age under 50 years, male gender, urban, immunosuppression, recent medical history (previous hospitalization and antibiotic therapy) were significantly correlated with the oral-maxillofacial involvement, which draws attention on the outpatient follow-up of moderate and severe oral infections. The insidious onset described in 54.6% of patients did not raise the suspicion of a potentially life-threatening disease, such as sepsis, but the severe respiratory (43.1%) and neuromeningeal manifestations (25.7%) contributed to the early seeking of expert advice from an infectious diseases specialist. Over 92% of the study patients were at high risk for staphylococcal infection with multidrug-resistant (MDR) strains, significantly more common in men and in patients in whom MRSA was identified as the sepsis pathogen. Depending on the MDR agent involved in the causation of oral-maxillofacial sepsis, the risk of developing severe infections is attributable to methicillin-resistant *Streptococcus mutans*, *Streptococcus anginosus*, *S. epidermidis*, *P. aeruginosa*, *Klebsiella*, β-hemolytic streptococcus, and *Streptococcus salivarius*.

INTRODUCTION

The etiologic spectrum of potentially severe infections has expanded considerably in recent years due to the inadequate administration of antibiotic therapy, widespread use of invasive medical maneuvers, improved techniques for identifying infectious agents, increasing life expectancy and immunosenescence. Of the frequently involved pathogenic microorganisms, Gram-positive bacteria (especially staphylococci) currently rank first (Zinderman C., et al., 2004), followed by fermenting (*E. coli*, *P. aeruginosa*, *Proteus*) and non-fermenting Gram negative bacilli (*Acinetobacter*) and fungi (mainly *Candida* species).

In some epidemiological conditions the colonizing staphylococcal strains may become responsible for the occurrence of a variety of diseases: from localized infections to severe forms of sepsis. Polymorphism and the lack of specificity of symptoms make it difficult to differentiate between different disease categories and create an inventory of associated comorbidities. Within this context, a thorough history and physical exam provide additional information for identifying the patients at risk.

The favorable course of the disease depends on the early etiologic diagnosis and initiation of appropriate therapy. Considering these aspects, current research is aimed at developing new laboratory techniques to reduce the time required for the isolation of infecting strains and performance of antibiotic susceptibility testing. The alarming increase in the rate of resistant staphylococci also requires a constant re-evaluation of the treatment regimens used. In immunosuppressed patients the emergency care consists in both the control of infectious process and correction of associated imbalances (treatment of the underlying diseases) (Elliott R.A., et al., 2010; Singer A.J., et al., 2014).

Implementation of global strategies to prevent of bacterial infections continues to provoke much controversy. Data in the literature on risk factors and management options are limited and often difficult to interpret (Bratzler D.W. & Houck, P.M., 2004; Granny G., et al., 2007; Antibiotic Expert Group, 2010).

Today, one of the major issues facing clinicians is resistance to methicillin of *S. aureus* strains and coagulase-negative staphylococci, which was subsequently extended to other antibiotics. If in the past staphylococcus was regarded as "exclusively nosocomial pathogen" currently we are witnessing the emergence of community-acquired panresistant strains (Kuriyama T., et al., 2005; Nathwani D., et al., 2008; Klevens M., et al., 2006).

PURPOSE AND OBJECTIVES

The aim of this study was to establish a series of correlations between elements of epidemiology and diagnosis of sepsis with oral-maxillofacial portal of entry, highlighting the commonly found risk factors; to describe the clinical course particularities depending on the pathogenic mechanism, associated comorbidities, seeking medical advice, how early diagnosis was made and treatment initiated; study of the clinical course of sepsis, by checking the correlation between the causal agents involved and poor prognosis; selection of a therapeutic regimen according to the local resistance pattern, relying on the best evidence for susceptibility testing; exploitation of the results will lead to a proposal for an effective screening methods in order to reduce morbidity and mortality from sepsis with oral-maxillofacial portal of entry.

MATERIAL AND METHODS

Prospective study conducted in a sample of 200 patients admitted to the Oro-Maxillofacial Surgery and ENT Clinics of the Iasi "Sf. Spiridon" Emergency Hospital in the interval 2012-2015. **Inclusion criteria:** age over 18 years; diagnosis confirmed by positive findings on clinical exam, laboratory tests (bacteriological, hematological, biochemical) and imaging. The strains were isolated from different biological or pathological products: blood (42%), cerebrospinal fluid (25.7%), urine (5.5%), sputum (3.2%), pus (5.3%) or seeding on catheters (5.7%). To assess the risk of infection with MDR staphylococcus strains Carmeli score was used and the patients were stratified into three risk groups: **low risk** (Carmeli score 1- community-acquired infection) - 7 patients (63.6%); **medium risk** (Carmeli score – healthcare-associated infection) - 1 patient (9.1%); **severe risk** (Carmeli score 3 - nosocomial) - 3 patients (27.3%).

For the statistical analysis both descriptive and analytical methods were used, at 95% significance threshold. Data were entered into SPSS 18.0 databases and processed using its statistical functions, Student t-test, ANOVA F, Chi2 test, linear trend.

RESULTS AND DISCUSSION

The prevalence of sepsis with oral-maxillofacial portal of entry during the study period was bimodal, with a peak frequency in 2015 (35% of all cases) and in 2013 (22.5% of all cases).

The increasing trend in the incidence of this disease was due on the one hand to the improved techniques for causal agent identification and on the other hand to the involvement of an increasing number of risk factors.

During the study interval the disease was more common in men (70%), M/F ratio 2.3/1, mainly living in rural areas (62%). Age of patients included in the study ranged from 18 to 85 years, mean age 49.75 ± 18.15 years.

Chronic underlying disorders have a major impact on the outcome of patients with sepsis. Of the chronic diseases in the medical history of our patients we mention in order of their frequency: cardiovascular diseases (36.5%), obesity (45%), liver diseases (32%), malignancies (25%), kidney diseases (15%) and diabetes mellitus (10.5%).

Systemic infection was community-acquired in 120 (60%) of the study patients, being more common in women (58.3%; $p = 0.884$), mean age approximately 50 years ($p = 0.902$), and in rural patients (65%; $p = 0.833$). The remaining patients had hospital-onset sepsis.

In 38 cases (19%), the causal agent responsible for the disease was *S. aureus*, 1.5% being methicillin-resistant (MRSA) strains. Of the *Viridans* group of streptococci, the most frequently isolated was *Streptococcus salivarius* (15%) - 5% MDR strains, *Streptococcus mitis* (13%) - 2% MDR strains and *Streptococcus anginosus* (12%) - 2% MDR. β -hemolytic streptococcus was identified in 15.5% of cases. *Candida* sp and *Pseudomonas aeruginosa* were identified in 9% of patients. Anaerobic flora was positive for cocci/bacilli in 7% of subjects. Of the *S. epidermidis* strains (5.5% strains), 1% were MDR. Also identified were 6.5% positive *Acinetobacter* strains. Five percent of patients tested positive for *Klebsiella* and *E. coli*. The other causal agents were detected in low percentages (table1.).

Table I. Distribution of sepsis cases by causal agent

INVOLVED SPECIES	Susceptible		Resistant	
	No.	%	NR.	%
<i>S. aureus</i>	35	17.5	3	1.5
<i>S. epidermidis</i>	9	4.5	2	1.0
<i>Acinetobacter</i>	13	6.5		
<i>Pseudomonas aeruginosa</i>	15	9.0	3	1.5
Streptococ β hemolytic	28	14.0	3	1.5
<i>Streptococcus mutans</i>	16	8.0	5	2.5
<i>Streptococcus salivarius</i>	20	10.0	10	5.0
<i>Streptococcus mitis</i>	22	11.0	4	2.0
<i>Streptococcus anginosus</i>	20	10.0	4	2.0
<i>Klebsiella</i>	10	5.0	3	1.5
<i>Proteus</i>	3	1.5		
<i>E. coli</i>	10	5.0		
<i>Clostridium</i>	1	0.5		
<i>Candida</i> sp	18	9.0		
Anaerobic flora: cocci/bacili	14	7.0		

Most systemic infection with streptococci *mutans* (47.6%), *mitis* (42.3%) and *salivarius* (47.6%) were recorded in patients aged 40-59 years, while β -hemolytic streptococcus had a peak frequency in the age group 50-59 years (35.5%), but frequency distributions (table 2.) were not statistically significant ($p = 0.500$).

Table 2. Age-group distribution of sepsis cases according to the causal streptococcal species

Age group (years)	β hemolytic streptococcus		<i>Streptococcus mutans</i>		<i>Streptococcus salivarius</i>		<i>Streptococcus mitis</i>		<i>Streptococcus anginosus</i>	
	n	%	n	%	n	%	n	%	n	%
< 20	2	6.5	-	-	-	-	-	-	2	8.3
20-29	1	3.2	-	-	5	16.7	-	-	2	8.3
30-39	4	12.9	4	19.0	7	23.3	6	23.1	3	12.5
40-49	5	16.1	10	47.6	10	33.3	11	42.3	7	29.2
50-59	11	35.5	7	33.3	8	26.7	5	19.2	4	16.7
60-69	7	22.6	-	-	-	-	4	15.4	3	12.5
70-79	1	3.2	-	-	-	-	-	-	2	8.3
80-89	-	-	-	-	-	-	-	-	1	4.2

When analyzing the epidemiologic characteristics of patients according to the source of infection we found the following:

- male gender (43.8%; $p=0.884$), age over 50 years ($p = 0.902$), urban residence (37.5%; $p=0.883$) and antibiotic therapy (50%; $p=0.108$) were not statistically significantly correlated with nosocomial infection;

- previous hospitalizations (50%; $p=0.027$) and MDR (11.3%; $p=0.009$) were significantly more common in patients with nosocomial infections.

In the studied cases, *S. aureus* was sensitive to vancomycin (100%), imipenem (90%) and tobramycin (80%).

(fig.1).

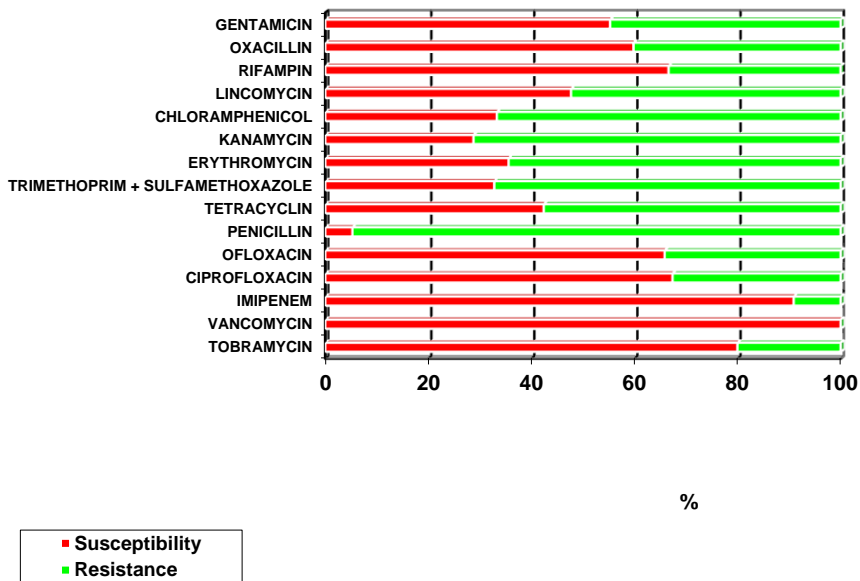


Fig. 1. Antibiotic susceptibility and resistance of *S. Aureus* strains

Antibiotic susceptibility of β -hemolytic staphylococcus strains isolated from patients diagnosed with sepsis showed: increased resistance to erythromycin (65%), gentamicin (63%); increased susceptibility to trimethoprim-sulfamethoxazole (90%); 100% susceptibility to vancomycin, teicoplanin, linezolid (Ifig. 2.).

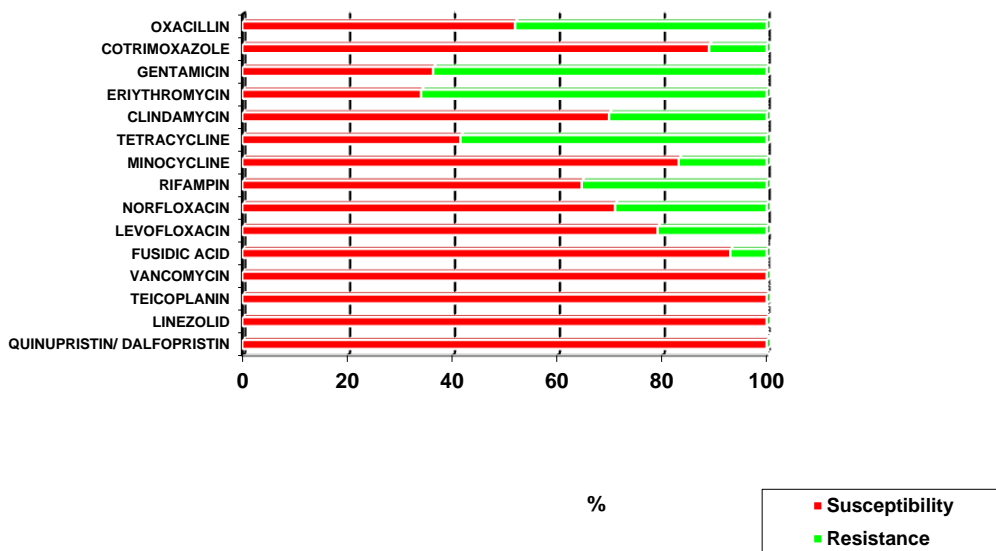


Fig. 2. Antibiotic susceptibility and resistance of β hemolytic staphylococcus strains

Cardiovascular involvement (organic and functional) was the most frequent site of secondary sepsis (36.5%), more common in men older than 50 years, and of staphylococcal etiology.

Facial skin involvement (organic and functional) was found in 33% of patients, most commonly women younger than 50 years, and of streptococcal etiology.

Liver involvement (organic and functional) was recorded in 32% of patients, most commonly men under 50 years of age, and of streptococcal etiology.

Sepsis-associated malignancies were identified in 25% of patients, most commonly in women younger than 50 years and of staphylococcal and streptococcal etiology.

Kidney involvement was present in 15% of patients, most commonly men over 50 years, and of staphylococcal etiology.

Ear involvement was detected in 10.5% of patients in the form of acute suppurative otitis media (20 patients) and unilateral sensorineural hearing loss with the contralateral ear possessing normal audiometric function (1 case). It was more common in women aged 50 years and was of streptococcal etiology.

Involvement of oral cavity was recorded in 16% of patients, most often in the form of lesions that extended beyond the confines of the floor of the mouth (7.5%) and maxillary sinusitis (5.5%). According to epidemiologic characteristics it was more common in women younger than 50 years and of streptococcal etiology.

Gingivitis and periodontitis patients were at significantly higher risk for sepsis (OR = 1.58, 95% CI = 1.14 to 2.19, $p = 0.01$), bacterial infections (OR = 2.15, 95% CI = 1.51 to 3.07; $p < 0.01$), fungal infections of the oral cavity (OR = 2.16; 95% CI = 1.43 to 3.28; $p < 0.01$) or other infectious complications (OR = 2.10; 95% CI = 1.63 to 2.84; $p < 0.01$).

Focal infections of oro-dental origin signify the fact that an oral focus of infection can act as the site of origin for dissemination of pathogenic organisms to distant body sites. This concept is controversial since it is difficult to prove the oral origin of germs responsible for an extra-oral infection (Persac S., et al., 2011).

In our prospective study, *S. aureus* was the main causal agent incriminated in the development of oral-dental sepsis.

According to the world literature, oral *Viridans* group streptococci are the species involved in the genesis of infective endocarditis and dental caries: *S. mutans*, *S. mitis*, *S. anginosus*, *S. sanguinus* and *S. salivarius* (<http://www.aae.org> / Colleagues).

Most of the study patients received antibiotic therapy prior to surgery (87.5%). It was found that antibiotic prophylaxis was not effective against the isolated pathogenic strains, particularly MRSA, enterococcus species and Gram-negative bacilli, additional prophylactic measures being required. MRSA was isolated in 45% of all periarticular infections.

CONCLUSIONS

Age under 50 years, male gender, urban residence, immunosuppression, recent medical history (previous hospitalization and antibiotic therapy) correlated significantly with the oral-maxillofacial involvement, which underlines the importance of outpatient follow-up of moderate and severe oral infections.

The insidious onset described in 54.6% of patients did not raise suspicion of a life-threatening condition such as sepsis.

A significantly higher percentage of patients with healthcare-associated infections developed MRSA sepsis than those with community-acquired infections, which is not a negligible percentage (38.5%).

The severe respiratory (43.1%) and the neuromeningeal manifestations (25.7%) were factors contributing to timely consultation with an infectious disease specialist.

Secondary involvement of the central nervous system (33.3%) was significantly more common in the immunosuppressed patients and in those with healthcare-associated infections and secondary cardiovascular involvement more frequent in patients with healthcare-associated infections.

In the patients with risk factors for methicillin resistance the first-line therapeutic options should be reconsidered: clindamycin, trimethoprim-sulfamethoxazole, new cyclins. The alternatives include vancomycin, teicoplanin and linezolid.

REFERENCES

- Zinderman, C., Conner, B., Malakooti, M.A. (2004) *Community-acquired methicillin-resistant Staphylococcus aureus among military recruits*. Emerg Infect Dis; 10: 941-944.
- Elliott, R.A., Weatherly, H.L.A, Hawkins, N.S. et al. (2010) *An economic model for the prevention of MRSA infections after surgery: non-glycopeptide or glycopeptide antibiotic prophylaxis?* Eur J Health Econ; 11: 57-66.
- Singer, A.J., Merry, Taylor R.N., et al. (2014) *Diagnostic characteristics of a clinical screening tool in combination with measuring bedside lactate level in emergency department patients with suspected sepsis*. Academic Emerg Med; 21(8): 853-857.
- Bratzler, D.W., Houck, P.M. (2004) *Antimicrobial prophylaxis for surgery: an advisory statement from the National Surgical Infection Prevention Project*. Clin Infect Dis; 38: 1706-1715.
- Granny, G., Elliott, R.A., Weatherly, H., et al. (2007) *A systematic review and economic model of switching from non-glycopeptide to glycopeptide antibiotic prophylaxis for surgery*. Health Technol. Assess; 12: 1-147.
- Antibiotic Expert Group** (2010) *Therapeutic guidelines: antibiotic*, 14th ed Therapeutic Guidelines Limited, Melbourne, Australia.

Kuriyama, T., Absi, E.G., Williams, D.W., Lewis, M.A. (2005) *An outcome audit of the treatment of acute dentoalveolar infection: Impact of penicillin resistance*. Br Dent J; 198: 759–63.

Nathwani, D., Morgan, M., Masterton, R.G., et al. (2008) *Guidelines for UK practice for the diagnosis and management of methicillin-resistant Staphylococcus aureus (MRSA) infection presenting in the community*. J Antimicrob Chemother; 61(5): 976-994.

Klevens, M., Edwards, J.R., Tenover, F., McDonald, L.C. (2006) *Changes in the epidemiology of methicillin-resistant Staphylococcus aureus in Intensive Care Units in US Hospitals, 1992-2003*. Clin Infect Dis; 42 : 389-391.

Persac, S., Prévost, R., Hardy, H., et al. (2011) *An update on focal infection of oral origin*. Revue de Stomatologie et de Chirurgie Maxillo-faciale 2011; 112: 353-359.

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ANTIMICROBIAL EFFECTS OF THE DIFFERENT EXTRACTS FROM *AMARANTHUS RETROFLEXUS* L.

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Received: 12 May 2016 / Revised: 17 May 2016 / Accepted: 20 June 2016 / Published: 13 July 2016

Keywords: *Amaranthus retroflexus*, antifungal and antibacterial activity.

Abstract: The emergence of drug-resistant bacteria strains encouraged the study of natural products from plants with potential promise for clinical use. The purpose of this study was to investigate *in vitro* the antibacterial and antifungal effects of aqueous, ethanol and chloroform extracts from different parts of *Amaranthus retroflexus* L.. The antimicrobial activity was evaluated using the agar diffusion method. Each extract exhibited antimicrobial activity against Gram-positive strains (*Staphylococcus aureus*, *Sarcina lutea*) and *Candida albicans*. In addition, the chloroform extracts from *A. retroflexus* dry seeds had significant antibacterial (against both Gram-positive and Gram-negative species) and antifungal activity. The results of the combined action of the different antibiotics and chloroform extract did not display synergism. This study indicates that different amaranth extracts are antibacterial and mostly antifungal and may represent a future therapeutic strategy.

INTRODUCTION

Plants have been exploited for their food value and for their therapeutic effect in the traditional systems of medicine. Some plants have the potential to be useful antibacterial and antifungal agents. The development of resistance among Gram-positive and Gram-negative species to many antimicrobial agents can seriously compromise the effectiveness of antibiotic treatment and there is clearly an urgent need for the discovery of new synthetic or plant-derived drugs. Thus, a number of bioactive molecules from various plants have been exploited, due to their therapeutic effects. Over 150.000 of novel natural molecules derived from various herbs and microorganisms are mentioned in the literature (Taneja and Qazi, 2007).

Amaranthus retroflexus (redroot pigweed, *Amaranthaceae* family) is an annual herb with a shallow reddish taproot, stem up to 2 m high, simple or branched, and often hairy in the upper part. Leaves are alternate, long-stalked, ovate to rhombic-ovate and sparsely hairy. Flowers are small, green and unisexual, grouped into dense, blunt spikes. Each flower is enclosed by 1-3 stiff awl-shaped bracts, giving bristly appearance to spikes. The fruit is a capsule, transversely dehiscent, enclosing one black shiny seed, broadly elliptic, 1 mm in diameter (Costea et al, 2004). Amaranth seeds have high nutrition value due to elevated concentrations of proteins, carbohydrates, lipids and minerals (Toader et al, 2011).

Amaranthus retroflexus includes around 50 varieties, forms and sub-forms and it is considered to be among the world's worst weeds. Nonetheless, *Amaranthus retroflexus* can be used as vegetable, forage, grain crop and medicinal plant and for the phytoremediation of contaminated sites (Costea et al, 2004). Moreover, weeds are highly resistant to plant pathogens so it is possible that some plant metabolites are also active against human pathogens.

Previous studies (Zhanh et al, 2013, Tharun et al, 2012, Jin et al, 2013, Maiyo et al, 2010) reported the antimicrobial activity of the *Amaranthus* species. To the best of our knowledge, just one paper focused on the antimicrobial activity of *A. retroflexus* seeds on phytopathogenic fungi (Lipkin et al, 2005). Considering the potential application in medicine, the present study evaluates the activity of different extracts from roots, stems, leaves and seeds of *A. retroflexus* against human pathogens.

In the ongoing search for novel antimicrobial agents from Romanian plants, we report the evaluation of antimicrobial activity of different extracts from *A. retroflexus*.

MATERIALS AND METHODS

Plant material and preparation of extracts

The plants were collected at fruiting stage from the surroundings of Iasi city in October 2015. The plant material was authenticated and a voucher specimen was deposited in the Herbarium of Pharmaceutical Botany Department, Faculty of Pharmacy. The roots, stems and leaves, and the seeds were pulverized and extracted (1:10) with water, 80% ethanol and chloroform by mixing for 3 hours on a magnetic stirrer at room temperature, then filtrated. The chloroform extract was evaporated to dryness and the residue dissolved in DMSO before the antimicrobial test.

Susceptibility testing

The antimicrobial activity of different aerial parts of *A. retroflexus* was tested by the disc diffusion method in Mueller-Hinton agar, according to the guidelines recommended by the National Committee of Clinical Laboratory Standards (NCCLS, 2000).

The *in vitro* antimicrobial profile of *A. retroflexus* was determined against five reference strains: *Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 25a22, *Pseudomonas aeruginosa* ATC 27853 and *Candida albicans* ATCC 10231. Mueller-Hinton broth and Mueller-Hinton agar (respectively Sabourand media for *Candida*) were used for microbial growth and susceptibility testing.

The cylinder in plate bioassay technique was used. A standard suspension of each strain was prepared from fresh overnight culture and was mixed with 15 ml portions of molten Mueller-Hinton agar, in a sterile petri plates resulting in a final concentration of about 10^6 cells/ml. When the plates were solid, metal cylinders (6 mm diameter) were placed on the medium surface and the samples (0,2 ml) were pipetted into each well. A standard commercially disks of Ampicillin (10 μ g), Chloramphenicol (30 μ g) and Nystatin (100 μ g) were used for comparison.

The size of the zone of inhibition around the wells was measured after incubation at 37°C for 24 h. The values of diameter of the inhibition zones are expressed as mean of triplicates \pm SD.

Test of combined antibacterial action

Antimicrobial synergy and antagonism were demonstrated in agar diffusion tests that offer a visually method to detect antimicrobial interactions. In the "two disc method" antimicrobial agents are placed on the surface of seeded plates, at distances from each other that are predicted upon optimum demonstration of antagonistic or synergistic action (Acar,1981).

Results of the "two disc method" were correlated with another experiment designed to determine coactions, described by Natarajan et al. (2008). In order to detect combined antimicrobial activity in this type of test, the samples, at defined concentrations, are added into the molten agar and after the solidification, different antibiotic discs are placed on the plate surfaces. If the inhibition zone remained unchanged after incubation at 37°C for 24 h, there was no synergic effect.

RESULTS AND DISCUSSION

The agar disk diffusion procedure was one of the first methods for determining the *in vitro* microbial susceptibility to antimicrobial agents. In this microbiological assay the antimicrobial agent placed in a reservoir (sterile paper discs, cylinders) diffuses directly against seeded bacteria. We used the cylinder technique that is more sensitive by comparison with the method using paper discs (Thornsberry et al, 1977). The filter discs may act as chromatography paper and separate the components of the mixture which may result in irregular inhibition zones. We tested the ability of different extracts from *A. retroflexus* to inhibit microbial growth and the results are shown in table 1.

All the extracts obtained from different plant material showed antimicrobial activity only against Gram-positive strains. These plant extracts showed a moderate antibacterial activity against *Staphylococcus aureus* and *Sarcina lutea* when compared to the positive control ampicillin and chloramphenicol. In contrast, Gram-negative bacteria were not inhibited by either aqueous or ethanol extracts. This can be explained by the presence of the permeability barrier – the outer membrane which acts as a barrier to the penetration of antimicrobial molecules and the periplasmic space which contains enzymes that destroy the molecules introduced from outside.

It can be seen that the chloroform extract from dry seeds exhibited the strongest antifungal activity, even higher than the antimicrobial agent Nystatin. Also the aqueous extracts from leaves plus stem and root showed high activity against *Candida albicans*.

Unlike the other *A. retroflexus* tested samples, the chloroform extract from seeds exhibited at concentration of 16 mg/ml, excellent activity against both, Gram-positive and Gram-negative bacteria. The results demonstrate the similar potential between the chloroform extract from seeds and ampicillin, or chloramphenicol against standard strains of *Escherichia coli* and *Pseudomonas aeruginosa*.

Table 1. Antimicrobial activity of different extracts of *A. retroflexus* plant material

Nr. crt.	Sample (0.2 ml volume)	Diameter of inhibition zone (mm±SD)				
		S. aureus *ATCC 25923	S. lutea ATCC 9341	E.coli ATCC 25922	P. aerugi nosa ATCC 27853	C. albicans ATCC 10231
1.	Root - aqueous extract	12±0.1	16±0.3	0	0	20±0.5
2.	Leaves and stems- aqueous extract	13±0.2	13±0.1	0	0	23±0.4
3.	Leaves and stems - ethanol extract (80 %)	13±0.4	13±0.5	0	0	12±0.1
4.	Root – ethanol extract (80%)	15±0.1	15±0.3	0	0	16±0.3
5.	Seeds - ethanol extract (80%)	12±0.3	15±0.2	0	0	12±0.5
6.	Seeds - chloroform extract	18±0.4	20±0.1	20±0.3	19±0.4	28±0.4
7.	Ampicillin (10 µg)	27±0.6	40±0.5	21±0.5	19±0.5	nt **
8.	Chloramphenicol (30 µg)	25±0.3	38±0.3	24±0.3	18±0.2	nt
9.	Nystatin (100 µg)	nt	nt	nt	nt	20±0.4

*ATCC – American Type Culture Collection; **nt – not tested

The possibility that chloroform extract from *A. retroflexus* seeds may have synergistic or antagonistic interaction with different therapeutic antimicrobial agents has been explored by the *in vitro* susceptibility test methods. Analysis of our data did not demonstrate a significant relationship between these samples and different antimicrobial agents (Table 2). The enhancement of antibacterial activity of chloroform extract from *A. retroflexus* seeds by the addition of ampicillin, chloramphenicol, tetracycline and ciprofloxacin to form a potent combination in inhibiting both Gram-positive and Gram-negative bacteria, is not relevant.

The combined antibacterial effect may be greater than that which each agent alone could achieve and indicates antimicrobial synergy. Together these two compounds may potentiate each other activity at a biochemical level or one may assist the other to penetrate into the microbial cell or may protect from destruction or the agents may act separately against the microorganism. When the effect of one agent is reduced by the presence of another, the combination is antagonistic. In our case, these two agents, the chloroform extract of seeds and ampicillin, chloramphenicol, tetracycline or ciprofloxacin tested together demonstrated indifference, because the combined action was no greater than that of the individual compounds.

As show in Table 1, *A. retroflexus* extracts exhibited antibacterial and antifungal abilities. All extract types were clearly less effective than the chloroform extract from the plant seeds, which was the most active against all tested strains, with the largest inhibition zones.

Table 2. *In vitro* combined effect of chloroform extract from seeds of *A. retroflexus* and some antimicrobial agents

Organism	Diameter of inhibition zone (mm)							
	Antimicrobial agents alone				The combined effect			
	A	C	T	Cip	A+Scce	C+Scce	T+Scce	Cip+Scce
<i>Staphylococcus aureus</i> ATCC 25923	27.0	25.5	26.0	30.0	26.5	26.0	27.0	30.5
<i>Sarcina lutea</i> ATCC 9341	40.0	38.0	35.5	38.0	40.0	40.0	36.0	40.0
<i>Escherichia coli</i> ATCC 25922	21.0	24.0	24.0	32.5	21.0	24.0	24.5	32.0
<i>Pseudomonas aeruginosa</i> ATCC 27853	19.0	18.0	20.0	32.0	19.5	18.0	20.0	32.0

A- ampicillin (10µg); C-chloramphenicol(30µg); T- tetracycline (30µg); Cip – ciprofloxacin (30µg); Scce – seeds chloroform extract

Data available today confirm that plants produce different natural compounds which are constitutive or inducible, capable to protect against pathogen organisms (Morrissey and Osbourn, 1999, Boman, 2000). Various secondary metabolites, high molecular defense proteins and antimicrobial peptides (AMP) have been isolated from different plant parts and they are mostly antifungal (Broekaert et al, 1992; Broekaert et al, 1995, Selitrennikoff, 2011, Garcia-Olmedo et al, 2001, Cammue et al, 1994). The mechanisms of peptide action are diverse: fungal cell wall polymer destruction, membrane channel and pore formation, action against ribosomes, inhibition of DNA synthesis and alteration of the cell cycle (Broekaert et al, 1997).

The literature data show that from the seeds of different amaranth species have been isolated peptides with antimicrobial activity (Broekaert et al, 1992, Rivillas-Acevedo and Soriano-Garcia, 2007). The antimicrobial peptides Ac-AMP1 and Ac-AMP2 were isolated from *A. caudatus* seeds (Broekaert et al, 1992). The gene encoding Ac-AMP2 peptide was also found in *A. albus*, *A. cruentus*, *A. blitum*, *A. hybridus*, *A. retroflexus*, *A. tricolor* and *A. hypocondriacus* (Pribylova et al., 2008).

The different extracts of *A. retroflexus* tested in our study showed antimicrobial activity due probably to the presence of active constituents like antimicrobial peptides (recognized as an important component of the innate defense system). An antimicrobial peptide with significant activity against five phytopathogenic fungi has been identified in *A. retroflexus* and named Ar-AMP. This peptide has a characteristic high content of cysteine residues which have the ability to form disulfide bridges and the amino-acids sequence demonstrates its homology with previously described chitin-binding proteins (Lipkin et al.2005).

Antimicrobial peptides isolated from amaranth act probably similar to plant defensins which have the capacity to bind in a reversible way to chitin from fungal cell wall resulting in a change of membrane permeability (Thevissen et al, 1999). Besides, it has been shown that antimicrobial peptides from amaranth species inhibit the growth of different Gram-positive bacteria (Broekaert et al, 1992). Our results indicate that the active compounds from seeds of *A. retroflexus* have also the ability to inhibit *in vitro* Gram-negative strains (*Escherichia coli* and

Pseudomonas aeruginosa). At concentrations of 16 mg/ml, the seeds chloroform extract had a good antibacterial activity, while the antifungal effect was remarkable, the extract strongly inhibiting the growth of *Candida albicans*.

The results are in agreement with other reports which showed that bioactive principles of *Amaranthus* species manifest antimicrobial activity. It has been shown that *A. tricolor* leaf extracts and the whole grass extracts from *A. viridis* have significant antimicrobial activity (Tharun et al, 2012, Jin et al, 2013). The ethyl acetate extracts (40-100 mg/ml) from the leaves and stems of *A. mangostanum* inhibited the growth of *Pseudomonas solanacearum*, *Acidovorax avenae*, *Rhizoctonia solani*, *Colletotrichum capsici*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli* (Zhang et al, 2013). A good antifungal action is presented in the previous study by Rivillas-Acevedo and Soriano-Garcia (2007) for the protean extract of *A. hypochondriacus* seeds.

Mayo et al. (2010) report that different extracts from the leaves of *A. hybridus*, *A. spinosus* and *A. caudatus* showed a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, but resistance to the fungus *Candida albicans*. According to these authors, the minimum inhibitory concentrations (MICs) of amaranth extracts against Gram-negative species (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumoniae*) ranged between 129-755 mg/ml (Mayo et al, 2010). In our experiments the chloroform extract obtained from the seeds of *A. retroflexus* is active against tested strains at significant lower concentrations.

CONCLUSIONS

In summary, the main result of the present study indicates that all extract types from *A. retroflexus* exhibited antimicrobial activity against Gram-positive bacteria and *Candida albicans*. The agar diffusion method indicated the highest antifungal activity for the chloroform extract from seeds of *A. retroflexus*, which was also the most active against Gram-negative and Gram-positive bacteria species. These data, in combination with other studies on amaranth suggests that *A. retroflexus* antimicrobial compounds, probably peptides, may be a useful source for the discovery of new antimicrobial agents.

REFERENCES

- Acar, J. (1981). The disc susceptibility test in Lorian V. (Ed.), *Antibiotics in Laboratory Medicine* (24-54). Williams and Wilkins.
- Boman, H. G., (2000): *Innate immunity and the normal microflora*. Immunol. Rev., 173, 5-16.
- Broekaert, W. F., Cammue B. P. A., De Bolle, M. F. C., Thevissen, K. et al., (1997): *Antimicrobial peptides from plants*. Crit. Rev. Plant Sci., 16, 297-323.
- Broekaert, W. F., Marien, W., Terras, F. R. G., De Bolle, M. F. C. et al., (1992): *Antimicrobial peptides from Amaranthus candatus seeds with sequence homology to the cysteine glycine-rich domain of chitin-binding proteins*. Biochemistry, 31: 4308-4318.
- Broekaert, W. F., Terras, F. R. G., Cammue, B. P. A., Osborn, R.W., (1995): *Plant defensins: novel antimicrobial peptides as components of the host defense system*. Plant Physiol., 108, 1353-1358.
- Cammue, B. P. A., De Bolle, M. F. C., Schoofs, H. M. E., Terras, F. R. G. et al., (1994): *Gene-encoded antimicrobial peptides from plants*. Ciba Found. Symp., 186, 91-101.
- Costea M., Weaver S, Tardif F., (2004): *The biology of Canadian weeds. 130. Amaranthus retroflexus L., A. powellii S. Watson and A. hybridus L.* Can. J. Plant Sci., 84: 631-668.
- García-Olmedo, F., Rodríguez-Palenzuela, P., Molina, A., Alamillo, J. M. et al., (2001): *Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defence*. FEBS Let., 498, 219-222.

- Jin, Y.-Sh., Jin, Y., Li, Ch., Chen, M. et al.**, (2013): *Biological activities of the whole grass extracts from Amaranthus viridis* L. Asian J. Chem., 25(13), 7169-7172.
- Lipkin, A., Amisimova, V., Nikomorova, A., Babakov, A. et al.**, (2005): *An antimicrobial peptide Ar-AMP from amaranth (Amaranthus retroflexus L.) seeds*. Phytochemistry, 66, 2426-2431.
- Maiyo, Z. C., Ngure, R. M., Matasyoh, J. C., Chepkorir, R.**, (2010): *Phytochemical constituents and antimicrobial activity of leaf extracts of three Amaranthus plant species*. Afr. J. Biotech., 9(21), 3178-3182.
- Morrissey, J. P., Osbourn, A. E.** (1999): *Fungal resistance to plant antibiotics as a mechanism of pathogenesis*. Microbiol. Mol. Biol. Rev., 63,708-724.
- Natarajan, P., Katta, S., Andrei, I., Barbu Rao Ambati V. et al.**, (2008): *Positive antibacterial co-action between hop (Humulus lupulus) constituents and selected antibiotics*. Phytomedicine, 15(3), 194-201.
- National Committee for Clinical Laboratory Standards (2000)**: *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. Approved Standard M7-A5, NCCLS, Villanova, PA.
- Pribylova, R., Kralik, P., Pisarikova, B., Pavlik, I.**, (2008): *Detection of the antimicrobial peptide gene in different Amaranthus species*. Biologia, 63(2), 217-220.
- Rivillas-Acevedo, L., Soriano-Garcia, M.**, (2007): *Antifungal activity of a protean extract from Amaranthus hypochondriacus seeds*. J. Mex. Chem Soc., 51(3), 136-140.
- Selitrennikoff, C. P.**, (2001): *Antifungal proteins*. Appl. Environ. Microbiol., 67, 2883-2894.
- Taneja, S. C., Qazi, G. N.** (2007). *Bioactive molecules in medicinal plants: a perspective on their therapeutic action in Mukund S.C. (Ed.), Drug development (Vol. 2, 1-50)*. John Wiley and Sons.
- Tharun, K. N. R., Padhy, S. K., Dinakaran, S. K., Banji, D. et al.** (2012): *Pharmacognostic, phytochemical, antimicrobial and antioxidant activity evaluations of Amaranthus tricolor Linn leaf*. Asian J. Chem., 24(1), 455-460.
- Thevisen, K., Terras, F. R., Broekaert, W. F.**, (1999): *Permeabilization of fungal membranes by plant defensins inhibits fungal growth*. Appl. Environ. Microbiol., 65, 5451-5458.
- Thornsberry, C., Gavan, T. L., Gerlach, E. H. (1977)**: *New developments in antimicrobial agent susceptibility testing*, American Society for Microbiology.
- Toader, M., Roman, Gh. V., Ionescu, A. M.**, (2011): *Chemical composition and nutritional values of some alternative crops promoted in organic agriculture*. Scientific Papers UASVM Bucharest, Series A, 54, 1222-5339.
- Zhang, Y., Su, P., Huang, H., Liu, S., Liao, X.**, (2013): *Antimicrobial activity of various extracts from different parts of Amaranthus mangostanus*. Asian J. Chem., 25(11), 6311-6315.

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THE QUANTITATIVE EVALUATION OF ALTERNARIA TOXINS IN APPLE AND TOMATO JUICES

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Received: 30 My 2016 / Revised: 02 June 2016 / Accepted: 27 June 2016 / Published: 13 July 2016

Keywords: Alternariol (AOH), Alternariol monomethyl ether (AME), Tenuazonic acid, (TEA), tomato juice, apple juice.
Abstract: In this study we aimed to quantify the alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TEA) in 10 different brands of tomato and apple juices by HPLC (high pressure liquid chromatography). In tomato juice, it was found that AOH quantity varies in a range of $1,95 \times 10^{-3}$ - $8,30 \times 10^{-3}$ $\mu\text{g/mL}$, AME of $1,22 \times 10^{-4}$ - $4,28 \times 10^{-2}$ $\mu\text{g/mL}$ and TEA between $4,0 \times 10^{-2}$ and $2,38$ $\mu\text{g/mL}$. In apple juice, it was determined that the range of AOH quantity is $6,11 \times 10^{-4}$ - $0,25$ $\mu\text{g/mL}$, the AME quantity is $2,03 \times 10^{-4}$ - $3,92 \times 10^{-3}$ $\mu\text{g/mL}$ and the range of TEA is $0,32$ - $9,62$ $\mu\text{g/mL}$. The differences among tomato juice and apple juice samples, may have resulted from the fruit's harvest time, harvest type, transport, processing, storage and shelf life in markets.

INTRODUCTION

The effects of mycotoxins on human health can be explained by susceptibility to mycotoxins, the nutritional status of the individual and the individual's resistance. The largest source for Mycotoxicoses is determined by the inappropriate storage and packaging conditions of foods and nutrients. In Europe compared to Turkey, from many years ago there are rules and strategies to control and prevent mold growth on the seeds, plants or fruits during storage and harvesting, (Anonymous, 1979).

Microbial secondary metabolites have various biological activities and they are functioning as hormones, antibiotics, toxins, anti-migraine or anti-cancer agents and even as insecticides (Küçük et al, 2003). Because most of them are small molecules with low molecular weights, they can be spread by wind, conducting to extended infected areas. *Alternaria* species can be found in the vegetal decomposing material being normally dispersed in natural ways, affecting the aerial plants organs. Most of the *Alternaria* species are plant pathogens that could cause damage; the rest are post-harvest pathogens of a wide variety of fruits and vegetables (Barkai-Golan, 2001).

Alternaria alternata (Fr.) Keiss recorded among the most widely known agricultural products are generally known as a kind of fungi group remained unstudied, formerly known as *A. tenuis* Neesauct. These genera is known as saprophyte in food and feed products, but it is also recognized as a common pathogen in harvested fruits and vegetables in cold conditions conducting to a decreased plants growing capacity and post-harvest economic losses (Barkai-Golan, 2001). Fruits and vegetables can be contaminated by different pathogens and the many stages of pathogenesis can create a variety of toxic metabolites which has not been identified as *Alternaria spp.* (Panigrahi, 1997). Tenuazonic acid, alternariol, alternariol mono-methyl ether, altenuen and altertoksin-I are toxic metabolites produced by *alternaria* fungal infection in fruits and vegetables.

A. alternata species is considered the most important mycotoxins producer, as well as many other *Alternaria* species are known to produce these mycotoxins. The most common type of *Alternaria alternata* attack strategy on the harvested products, because cannot penetrate the plant cuticle or epidermis of the host plant, it uses calyx to get inside thru the wound or injured tissue. Such favorable environments for *Alternaria* attack often occur during harvest and collection. The calyx which started the wound infection in tomatoes, is a common pathogen for the *Solanaceae* family representatives like peppers and eggplant. (Barkai-Golan, 2001; Dennis, 1983; Morris et al, 2000)

The fungus can also enter into many fruits from tears formed in the open calyx, including the unharvested cherry fruit (Snowdon, 1990). Even if *Alternaria* core rot, in apples, is often associated with *A. alternata*, the recent studies conducted in South Africa, showed that *Alternaria* isolate *A. infectoria*, all types of *A. arborescens* and *A. tenuissi* are associated with this disease (Serdani et al, 2002).

Alternaria alternata is a major pathogenic in *Vitis sp.* causing infections which usually starts from the apical meristem after grapes harvesting (Hewitt, 1974). Was recorded in Argentina as an important pathogen in 80% of wine grapes and fruits collected (Magnoli et al, 2003). Is not considered an important pathogen for strawberries and raspberries and with an reduced influence on blueberries and gooseberries, remaining the main pathogen for grapes (Smith and Moss, 1985; Wright et al, 2004).

Alternaria is commonly found in fruit and vegetables used for human consumption, inducing a high levels of toxicity, during harvesting and storage. The analysis of the exposure degree to those hazards is required followed by a frequent monitoring of fruit juice and other fruit-derived products (Bottalico and Logrieco, 1993; Stinson et al, 1980).

The purpose of this study is to detect the amount of alternariol, alternariol mono-methyl ether and tenuazonic acid mycotoxins produced by *Alternaria spp.* in apple and tomato juices available on the market.

MATERIALS AND METHODS

In this study, in order to examine *Alternaria* 1 liter containers of 10 different brands of tomato and apple juice available on the Turkish market, a total of 20 samples were used as experimental material. Test materials were collected from Elazığ, Malatya, Antalya, Ankara and Istanbul provinces in their original packaging from different markets in February 2008 and analyzed before the expiration date. During the study, all the samples were kept in the appropriate storage conditions for 7 days.

Analytical grade methanol, chloroform, anhydrous sodium sulfate, and hepta hydrate zinc sulfate and HPLC grade methanol were obtained from *Merck (Darmstadt, Germany)*. The alternariol, alternariol monomethyl ether and Tenuazonic acid standards were purchased from *Sigma (St. Louis, MO, USA)* in February 2008.

The HPLC system consisted of a SPD-10A VP liquid chromatograph (*Shimatzu, Japan*) equipped with an UV detector (model SPD-10AVP). The analytical column was Inertsil ODS-3, 150 mmx4.6mm ID 5µm. The sample and standards solutions were sonicated for 30 seconds before injection into the chromatograph. The mobile phase was methanol/water (80:20) containing 300 mg ZnSO₄.H₂O/L, 0,7 ml/min. The wavelengths for recording chromatograms were 250nm and 280nm.

A calibration curve was constructed for quantification purposes using the toxin standards and correlating peak-area versus concentration. The peak identity was confirmed by means of comparing the spectrum of the standard with the presumptive positive peak in the sample after normalization. Quantification limits of the method were taken as the minimum amount of the toxin detected in the product that allowed for confirmation by the multiple wavelength detector. The detection limits of the pure toxins by the UV detector were measured as three times the baseline standard variation under the same conditions employed for all the analyzed products.

By using the equation $C_1 \times V_1 = C_2 \times V_2$ with dilutions at different concentrations, have been prepared and graphs of study plotted: 50ml of tomato and apple juice mixed in 150ml absolute methanol for 3 minutes and filtered. To the filtered solution, 60ml 10% ammonium sulphate were added and filtered again. In the final filtrate, at 8°C, 50ml of pure water were added. The final volume of 200ml was divided in two separation funnels and 40ml chloroform were added and shaken for 2 minutes. After the chloroform separation within funnels, the samples were incubated at 35°C on the shaking unit. 2ml absolute methanol were added on the residues and filtered on the sodium sulphate. The samples were transferred to 2ml HPLC vials for quantitative analysis performed according to the direct comparison method:

The standards were analyzed in 6 µl volumes of 50 ppm transferred in 2ml HPLC vials by HPLC ODS-3 (150 mm X 4.6 mm ID 5µm). The UV detector has a 25°C column temperature, a 280 nm detection wavelength and 1 ml/min of the mobile phase flow rate. The standard peak for Alternariol (AOH) was recorded at 3,7 minutes, for Alternariol mono-methyl ether (AME) at 7,5 minutes and for tenuazonic acid (TEA) at 1,6 minutes (Scott et al, 2001) as shown in the chromatogram presented in Figure 1 .

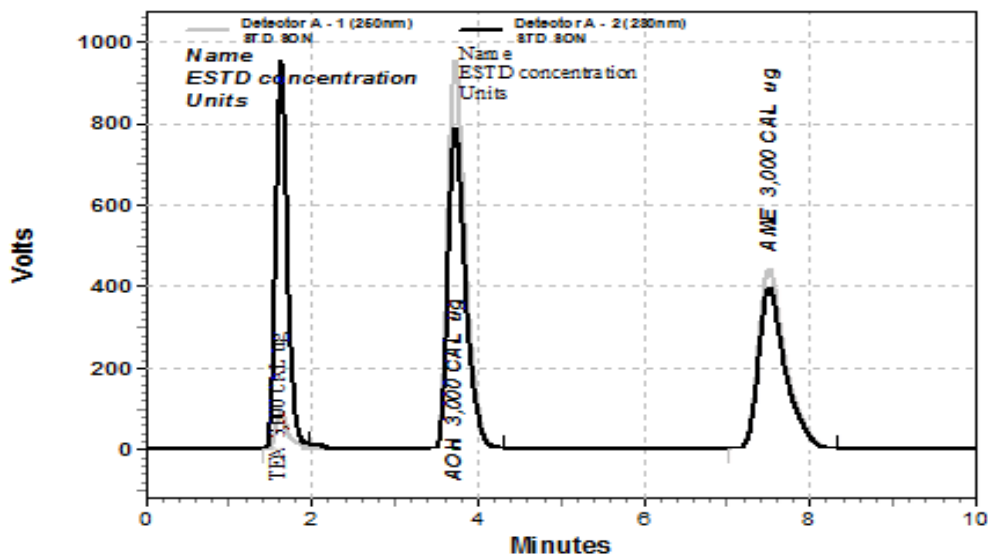


Figure 1. The standard peak for Alternariol (AOH), Alternariol mono-methyl ether (AME) and tenuazonic acid (TEA).

RESULTS AND DISCUSSIONS

The amounts of AOH, AME and TEA isolated from tomato and apple juices, are expressed in $\mu\text{g/ml}$ as follows. The Microsoft Excel 2016 program was used for the data statistics analysis (Figures 2 – 5).

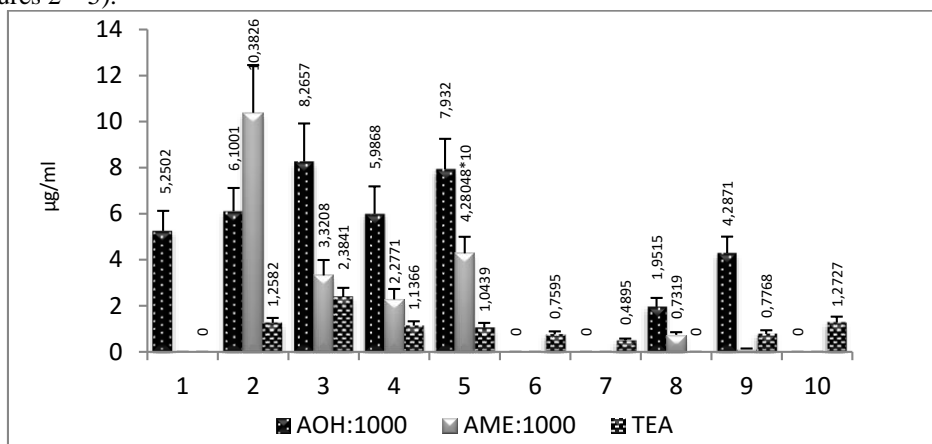


Figure 2. The arithmetic average and standard deviation of AOH, AME and TEA from tomato juices.

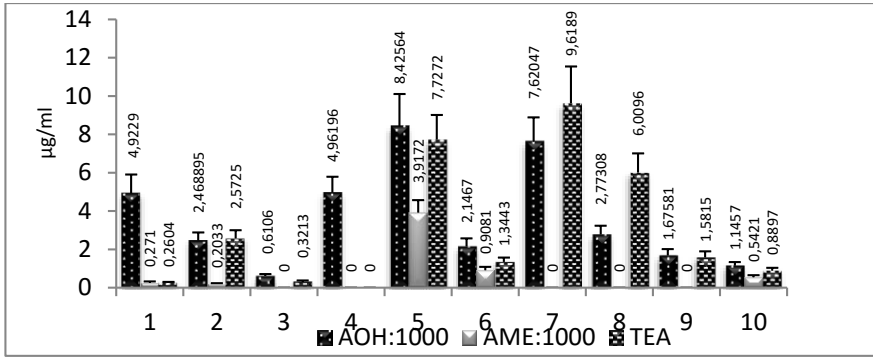


Figure 3. The arithmetic average and standard deviation of AOH, AME and TEA from apple juices.

The arithmetic mean value of mycotoxin concentrations (AOH, AME, TEA) in ten different brands of tomato juice is given in Figure 4.

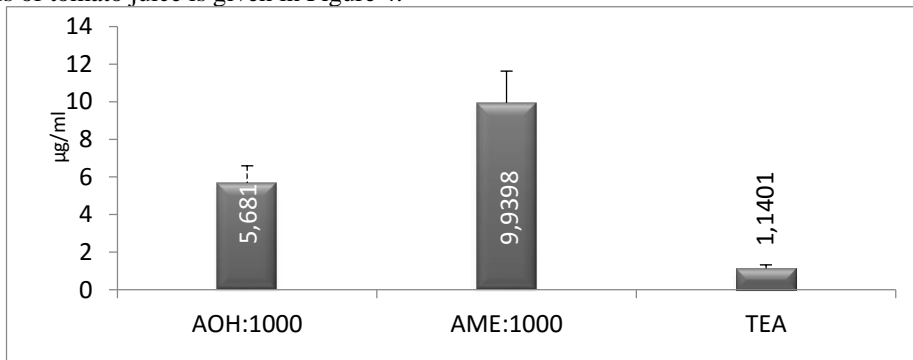


Figure 4. The arithmetic average of mycotoxin (AOH, AME, TEA) in tomato juice.

In tomato juices the AOH was detected in a concentration of $5,6819 \times 10^{-3}$ ppm, AME of $9,9398 \times 10^{-3}$ ppm and TEA of 1,1401 ppm (Figure 4.). The mycotoxin average concentrations (AOH, AME, TEA) in ten different brands of apple juice is given in Figure 5.

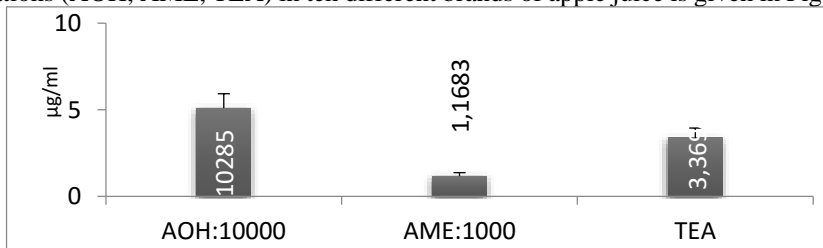


Figure 5. The arithmetic average of mycotoxin (AOH, AME, TEA) in apple juice.

According to our final results, in all 20 juice samples Alternaria toxin (Figures 2-3) was detected. In all these juices samples the concentration of AOH detected in 17 out of 20 samples (85%) has a range of $0,6106-246,8895 \times 10^{-3} \mu\text{g/ml}$, the concentration of AME detected in 11 out of 20 samples (55%) has a range of $0,1219-42,8048 \times 10^{-3} \mu\text{g/ml}$ and also, the concentration of TEA detected in 17 out of 20 samples (85%) has a range of $260,4-9618,9 \times 10^{-3} \mu\text{g/ml}$.

Fente et al., in 1998, reported that the linear study range of tomato paste about mycotoxins (AOH) in HPLC is between $5,2-196 \times 10^{-3} \mu\text{g/ml}$. Other study, indicate that the minimum amount for detection of AME and AOH in tomato paste, tomato juice and tomato purees using the HPLC method, is $2,0 \times 10^{-3} \mu\text{g/ml}$ for AME and $5,0 \mu\text{g/ml}$ for AOH (Da Motta et al, 2000).

In Argentina a study conducted on the 80 tomato products (like ketchup, tomato paste, tomato juice), the *Alternaria* toxin was detected in 39 samples (49%): TEA with concentrations in a range of $39-4021 \times 10^{-3} \mu\text{g/ml}$ in 23 samples (29%); AOH with concentrations in the range of $187-8756 \times 10^{-3} \mu\text{g/ml}$ in 5 samples (6%); and AME with concentrations in a range of $84-1713 \times 10^{-3} \mu\text{g/ml}$ in 21 (26%) samples. Six samples were contaminated with TEA and AME, two samples were contaminated with TEA and AOH, other two samples were contaminated with AME and AOH (Da Motta et al, 2000). In the present study, the detected values are, as follows: AOH ($0,6106 \times 10^{-3}-0,1971$ ppm) and AME ($0,1219 \times 10^{-3}-42,8048 \times 10^{-3}$ ppm) were found lower compared to previous studies. The TEA concentration ($0,3213-10,9377$ ppm) was found higher.

In the garden tomatoes the mycotoxin detection during harvest time in Italy, indicate $4750 \times 10^{-3} \mu\text{g/ml}$ of TEA, $600 \times 10^{-3} \mu\text{g/ml}$ of AOH and $100 \times 10^{-3} \mu\text{g/ml}$ of AME (Panigrahi, 1997; Dennis, 1983). In different studies conducted on toxin production, all three toxins (AOH, AME, TEA) were detected in 74 isolates, both AOH and AME were found in 30 isolates, and TEA and AOH in 2 isolates. The TEA was detected in 7 isolates, AME in 3 isolates and AOH 1 out of 11 isolates (Terminiello et al., 2006; Dalcero et al., 1989).

In our study it was found that in tomato juice the AOH quantity is in the range of $1,9515-8,2657 \times 10^{-3} \mu\text{g/ml}$, quantity of AME in the range of $0,1219-42,8048 \times 10^{-3} \mu\text{g/ml}$ and the quantity of TEA in range of $489,5-2384,1 \times 10^{-3} \mu\text{g/ml}$. The AOH was detected in 7 samples, AME in 6 samples and AOH was being in 8 samples out of 10 different tomato juices. In Brazil, maximum quantity of TEA in canned tomato juices, tomato essence, tomato puree, tomato paste and cooked tomato have been identified as $178 \times 10^{-3} \mu\text{g/ml}$ (Da Motta et al., 2000).

In present research, in all apple juice samples, the detected quantity of AOH is in range of $0,6106-246,8895 \times 10^{-3} \mu\text{g/ml}$, quantity of AME in range of $0,2033-3,9172 \times 10^{-3} \mu\text{g/ml}$, quantity of TEA in range of $321,3-9618,9 \times 10^{-3} \mu\text{g/ml}$. Also, it was detected that AOH was detected in all 10 different apple juices (%100), AME was detected in 5 out of 10 different apple juices (%50) and TEA was detected in 9 out of 10 different apple juices (%90).

Other studies indicate that the contamination level of maize with *Alternaria alternata*, is directly correlated with the esophageal cancer incidence (Liu et al, 1989). Also, it has been found that the foods which are mildewed with *A. alternata*, are causing tripe tumors in rats (Ohtsubo et al., 1978; Sauer et al., 1978; Younis and Al-Rawi, 1988).

Alternaria toxins presents an *in vitro* cytotoxicity in mammals and bacteria cells, fetotoxicity and teratogenicity in mice and hamsters (Visconti and Sibilina, 1994). This toxins, although not as active as fumonisin B1, they block the sphingolipids synthesis by inhibiting the rate-limiting enzymes (Gregory et al., 1983; Van der Westhuizen et al., 1998). *A. alternata* group also produce plant-specific phytotoxins (Van der Westhuizen et al., 1998). AAL (*Alternaria alternata* f. sp. *lycopersici*) toxin, is structurally similar to fumonisin B1 and causes necrotic lesions in genetically susceptible tomato lines (Otani et al, 1996; Gilchrist et al, 1992).

The present study results show that when the fungi favorable conditions are provided, fruits can be potentially contaminated with toxins. Hence consumers shouldn't buy fruits which are rotten or infected with soil. About processed fruits, if they are not cleaned from soil or the rotten ones are not removed before processing and packaging, the mycotoxins also can produce severe toxicity of human food. Thus, it's believed that people who consume fresh fruits are exposed to less quantity of

Alternaria toxin (Visconti and Sibia, 1994). Other related studies shown that *Alternaria* toxins can cause dermatologic effects, type I allergy and a weakened immune system (Morison and Weisdorf, 1993). To prevent or minimize the cytotoxic effects, new rules should be implemented about harvesting, transporting, storing and processing stages of food products.

Almost all of the molds are located in water and soil, hence, those two environments are the main sources of plants contamination. In this way, the primary products contact with the irrigation water and soil should be minimized.

Products must be carefully harvested, mainly when the process is not automatized, gloves should be worn. Fruits contaminated with mycotoxin, damaged, decayed or falling into decay shouldn't be collected, they just should be removed from the cultivation area and disposed, also, they shouldn't get in contact with healthy ones, in order to prevent contamination.

If the collected products are stored in the same containers, the risk of contamination in that containers will increase. To avoid this, disposable containers should be used, and sealed using stretch. The large scale containers should be disinfected before each use.

The products should be refrigerated during the transport and should be quickly delivered, avoiding any damage.

During storage physical conditions should be fulfilled, according to the type of product. The increase of mycotoxin should be minimized by quantity of heat, air, moisture and stack.

CONCLUSIONS

Alternaria toxin was detected in all 20 analyzed juices samples. In tomato juice were identified concentrations of $0,6106-246,8895 \times 10^{-3} \mu\text{g/ml}$ AOH, $0,1219-42,8048 \times 10^{-3} \mu\text{g/ml}$ AME and $260,4-9618,9 \times 10^{-3} \mu\text{g/ml}$ TEA. In all 10 apple juice samples, the *Alternaria* toxin it was detected as follows: $0,6106-246,8895 \times 10^{-3} \mu\text{g/ml}$ AOH, $0,2033-3,9172 \times 10^{-3} \mu\text{g/ml}$ AME, $321,3-9618,9 \times 10^{-3} \mu\text{g/ml}$ TEA. The alimentary products processing phases should be strictly controlled by the appropriate rules and measurements according to the Turkish Food Codex. These products which reached the markets should be consumed before the end of shelf life. Further studies are required to detect the levels of contaminants from different alimentary products in order to minimize the risk of toxicity induced by *Alternaria* sp.

REFERENCES

- Anonymous**, (1979): Environment Health Criteria 11: Mycotoxins, W.H.O., Geneva; 127.
- Barkai-Golan, R.**, (2001): Post-Harvest Diseases of Fruits and Vegetables, Development and Control. Amsterdam: Elsevier.
- Barkai-Golan, R.**, (2002): An Annotated Check-List of Post-Harvest Fungal Diseases of Fruits and Vegetables in Israel, 2nd ed. In Department of Postharvest Science of Fresh Produce, Bet Dagan, Israel: ARO, the Volcani Center.
- Bottalico, A. and Logrieco, A.**, (1993): Mycotoxins in *Alternaria* infected olive fruits and their possible transfer into oil, Bull. OEPP, 23, 473–479.
- Da Motta, S., Lucia M. Soares, L.M.V.**, (2000): A Method For The Determination Of Two *Alternaria* Toxins, Alternariol And Alternariol Monomethyl Ether, In Tomato Products, Brazilian Journal of Microbiology, ISSN 1517-8382, 31:315-320.
- Dalcerio, A., Chulze, S., Etcheverry, M., Farnochi, C., Varsavsky, E.**, (1989): Aflatoxins in sunflower seeds: influence of *Alternaria alternata* on aflatoxin production by *Aspergillus parasiticus*, Mycopathologia 108, 31–35.
- Dennis, C.**, (1983): Soft fruits. In Post-Harvest Pathology of Fruits and Vegetables (C. Dennis, ed.), New York: Academic Press, pp. 23–42.
- Fente, C.A., Jaimez, J., Vazquez, B.I., et al.**, (1998): Determination of alternariol in tomato paste using solid phase extraction and high performance liquid chromatography with fluorescence detection, Analyst, 123, 2277–2280.
- Gilchrist, D.G., Ward, B., Moussata, V., Mirocha, C.J.**, (1992): Genetic and physiological response to fumonisin and AAL-toxin in intact tissue of a higher plant. Mycopathologia 117, 57-64.

- Gregory, F., Griffin and Fun, Chu, S.,** (1983): Alternariol, Alternariol Methyl Ether, Altenuene, and Tenuazonic Acid in the Chicken Embryo Assay, Applied and Environmental Microbiology, No. 60099-2240/83/121420-03\$02.00/0, Vol. 46, p. 1420-1422.
- Hewitt, W.B.,** (1974): Rots and bunch rots of grapes, Bull. Calif. Agric. Street No. 868, pp. 52.
- Hiltunen, M., Söderhäll, K.,** (1992): Inhibition of poliketide synthesis in *Alternaria alternata* by the fatty acid synthesis inhibitor cerulenin, Applied and Environmental Microbiology 58, 1043–1045.
- Ito, K., Tanaka, T., Hatta, R., Yamamoto, M., Akimitsu, K. and Takashi, T.,** (2004): Dissection of the host range of the fungal plant pathogen *Alternaria alternata* by modification of secondary metabolism Molecular Microbiology 52(2), 399–411.
- Küçük, Ç., Kıvanç, M., Kınacı, E., Kınacı, G.,** (2003): Antifungal peptidler, Orlab On-Line Mikrobiyoloji Dergisi Cilt: 01 Sayı: 10 Sayfa:1-8.
- Liu, G.T., Qian, Y.Z., Zhang, P., Dong, W.H., Qi, Y.M., Guo, H.T.,** (1992): Etiological role of *Alternaria alternata* in human esophageal cancer. Chin. Med. J. (Engl.) 105, 394–400.
- Magnoli, C., Violante, M., Combina, M., et al.,** (2003): Mycoflora and ochratoxin producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina, Lett. Appl. Microbiol., 37, 179–184.
- Morris, P.F., Connolly, M.S., and St. Clair, D.A.,** (2000): Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RAPDs, Mycol. Res., 104, 286 - 292.
- Morrison, V. A., Weisdorf, D. J.,** (1993): *Alternaria*: a sinonasal pathogen of immunocompromised hosts. Clin. Infect. Dis. 16: 265 – 270.
- Ohtsubo, K., Saito, M., Ishiko, T., Umeda, M., Sekita, S., Yoshihira, K., Natori, S., Sakabe, F., Udagawa, S., Kurata, H.,** (1978): Toxicity to mice and HeLa cells and preliminary chemical examination of five fungi isolated from foods. Japan. J. Ex. Med. 48, 257–264.
- Otani, H., Kodama, M., Kohmoto, K.,** (1996): Physiological and molecular aspects of *Alternaria* host-specific toxin and plant interactions. Chapter 22. In: Mills D, Kunoh H, Keen NT, Mayama S (Eds.) Molecular Aspects of Pathogenicity and Resistance: Requirement for Signal Transduction. St. Paul, APS Press, 257-267.
- Panigrahi, S.,** (1997): *Alternaria* toxins, In Handbook of Plant and Fungal Toxicants (J.P.F.D’Mello, ed.), pp. 319–337. Boca Raton, FL: CRC Press.
- Rotem, J.,** (1994): The *Alternaria*, St. Paul, MN: APS Press.
- Sauer, D.B., Seitz, L.M., Burroughs, R., Mohr, H.E., West, J.L., Milleret, R.J., Anthony, H.D.,** (1978): Toxicity of *Alternaria* metabolites found in weathered sorghum grain at harvest. J. Agric. Food Chem. 26, 1380–1383.
- Scott, P.M., and Kanhere, S.R.,** (2001): Stability of *Alternaria* toxins in fruit juices and wine, Mycotoxin Res., 17, 9–14.
- Serdani, M., Kang, J.-C., Andersen, B., and Crous, P.W.,** (2002): Characterization of *Alternaria* species-groups associated with core rot of apples in South Africa, Mycol. Res., 106, 561–569.
- Smith, J.E., Moss, M.O.,** (1985): Mycotoxins: Formation, Analysis and Significance, John Wiley and Sons, Chichester, pp. 36-41.
- Snowdon, A.L.,** (1990): Post-Harvest Diseases and Disorders of Fruits and Vegetables, Vol. 1: General Introduction and Fruits, Boca Raton: FL: CRC Press.
- Stinson, E.E., Bills, D.D., Osman, S.F., et al., Siciliano, J., Ceponis, M. J., Heisler, E. G.,** (1980): Mycotoxin production by *Alternaria* species grown on apples, tomatoes and blueberries, J. Agric. Food Chem, 28, 9603.
- Terminiello, L., Patriarca, A., Posez, G., Pinto V.F.,** (2006). Occurrence of alternariol, alternariol monomethyl ether and tenuazonic acid in Argentinean tomato puree, Mycotoxin Research Vol 22, No. 4, 236-240.
- Van der Westhuizen, L., Shephard, G.S., Snyman, S.D., Abel, S., Swanevelder, S.,** (1998): Gelderblom WC. Inhibition of sphingolipid biosynthesis in rat primary hepatocyte cultures by fumonisin B1 and other structurally related compounds. Food Chem Toxicol. 36, 497-503.
- Visconti, A., Sibilia, A.,** (1994). *Alternaria* toxins. In: Miller, J.D., Trenholm, H.L. (Eds.), Mycotoxins in Grain: Compounds Other Than Aflatoxin. Eagan, St. Paul, M.N., pp. 315–336.
- Wright, E.R., Rivera, M.C., Esperon, J., et al.,** (2004): *Alternaria* leaf spot, twig blight and fruit rot of highbush blueberry in Argentina, Plant Dis., 88, 1383.
- Younis, S.A., Al-Rawi, F.L.,** (1988): Studies on the fetotoxic effect of *Alternaria alternata* metabolites in mice. J. Biol. Sci. Res. 19, 245–253.

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Acknowledgements

This paper is based on a PhD study titled “*The Determination of Alternaria Toxins in Apple and Tomato Juices and Tomato Paste*”. This thesis study was supported by FUBAP in 2010, Project Number: 1492.

**Academicianul Profesor PETRE JITARIU, un
vizionar în cercetarea științifică și învățământul
românesc
(11 Mai 2016, 111 ani de la nașterea Acad. Prof.
PETRE JITARIU)**

IONEL ANDRIESCU*
Prof. Univ. Dr. Emerit

Este atât de greu să cuprinzi într-o scurtă evocare o personalitate atât de complexă ca aceea a Acad. Prof. P. Jitariu, încât mă voi mărgini la momentele în care aripa tutelară, creator vizionar a atins și destinul meu.

Ca profesor nu l-am cunoscut, deoarece cursul de Fiziologie animală l-am făcut cu distinsa Doamnă profesor Matilda Jitariu. L-am cunoscut însă ca decan și om, precum și din relatările colaboratorilor apropiați. Omul înalt, frumos calm și jovial, manifestând solitudine și înțelegere, era apreciat și stimat de toți, iar calitățile sale în relațiile cu colaboratorii și subalternii între care înțelegerea constructivă și bunăvoința le-am constatat și simțit în momentele în care m-au privit direct. Astfel, primul dintre aceste momente a fost acela când la propunerea Prof. M. I. Constantineanu, și-a dat girul pentru încadrarea mea la Catedra de Zoologie a Facultății de Științe Naturale în 1955. Al doilea moment a fost acela când, în urma consultărilor din Biroul senatului Universității „Alexandru Ioan Cuza”, împreună cu D-na Prof. Elena Jeanrenaud și Prof. Pierre Jeanrenaud, Prof. Sergiu Cărăușu și Prof. M. I. Constantineanu, s-a hotărât numirea mea ca director la « Stațiunea de Biologie Marină « Ion Borcea » de la Agigea în 1966.

Al treilea moment a fost acela când, Biroul senatului împreună cu Acad. Prof. P. Jitariu, Prof. I. Gugiu și Prof. C. Martiniuc, au hotărât numirea mea ca director la Stațiunea de Cercetări Biologice, Geologice și Geografice „Stejarul” de la Pângărați în mai 1970, aflată sub egida Universității și unde, în urma trecerii cercetării în regim economic și activității pe bază de contracte (proiecte) directorul de atunci, Dr. Dan Munteanu, demisionase.

Despre „Stațiunea de la Pângărați” știam încă din faza de proiect, deoarece se vorbea în facultate și eram bucuroși că vom avea o stațiune unde să facem practică de vară, pe atunci gratuită, o lună la munte, după primul an de studiu și o lună la mare, la Agigea, după anul doi sau trei. Așa că, Stațiunea fiind înființată în toamna anului 1956, ca proaspăt asistent am făcut prima practică la

**Academician Professor PETRE JITARIU, a
visionary in the scientific research and
Romanian education
(May 11, 2016, 111 years after the birth of
Acad. Prof. Dr. PETRE JITARIU)**

IONEL ANDRIESCU*
Professor Emeritus, PhD

It is so difficult to comprise in a short evocation such a complex personality as that of the Academician Professor Petre Jitariu, so, I will confine myself to those moments when his tutelary, creative, visionary personality marked my destiny too.

I did not know him as a professor because the course of Animal Physiology was taught by the distinguished Professor Matilda Jitariu. However, I knew him as Dean and person, as well as of the relations of the close collaborators. The tall, handsome, calm and jovial man, showing solicitude and understanding was appreciated and esteemed by all. I found and felt his qualities, such as constructive understanding and goodwill, in the relationships with the collaborators and subordinates, in the moments I was directly concerned.

Thus, the first of those moments was that when, at the proposal of Professor Mihai I. Constantineanu, he gave his endorsement for my appointment to the Department of Zoology, Faculty of Natural Sciences, in 1955. The second moment was that when, after consultations within the Bureau of the Senate of "Alexandru Ioan Cuza" University, together with Professor Elena Jeanrenaud and Professor Pierre Jeanrenaud, Professor Sergiu Cărăușu and Professor Mihai I. Constantineanu, it was decided my appointment as a Director of "Ion Borcea" Marine Biology Station from Agigea, in 1966.

The third time was that when, the Senate Office together with Academician Professor Petre Jitariu, Professor Ion Gugiu and Professor Constantin Martiniuc, decided my appointment as a Director of "Stejarul Biological, Geological and Geographical Research Station" from Pângărați, in May, 1970, under the aegis of "Alexandru Ioan Cuza" University, passing research in economic regime and activity on the basis of contracts (projects), because the Director of that time, Dr. Dan Munteanu, resigned.

I knew about the Research Station from Pângărați when it was in the stage of a project

Pângărați, în 1957, în cadrul însoțit și feeric al vegetației montane, la Vila Turcan unde Stațiunea a funcționat până la darea în folosință a sediului în 1958. Al doilea contact cu Stațiunea l-am avut la aniversarea de 10 ani a Stațiunii de la Pângărați. Nu voi uita niciodată frumoasa aniversare, în acea perioadă „romantică” în cercetare, cu excursia pe lacul „Izvorul Muntelui – Bicz”, pe vasul „Emil Racoviță” nu de mult transferat de la Agigea de colegul Dr. I. Miron, când Prof. C. Motaș ne-a recitat, în calmul și liniștea absolută ce domnea între munți, lungul și frumosul poem „Lacul” de Lamartine, în frumoasa limbă a autorului.

A urmat apoi vocea inimitabilă a Prof. Gh. Hassan care a răspândit cu reverberații de catedrală, parcă mai impresionant ca niciodată, în spațiul larg și însoțit, melodia „O Sole Mio”, elogiare a bucuriei de a trăi, a naturii și a iubirii și, nu numai pentru că sunt român, dar mi s-a părut la concurență absolută cu Luciano Pavarotti. „O tempora.....”. Aici aveam să ajung, în mai 1970, în condiții cu totul diferite și complicate în ce privește relațiile interpersonale, acutizate în plus de repercusiunile social-economice ale noului regim de funcționare, economic.

Întorcându-ne la ctitorirea vizionară a Stațiunii „Stejaru”, de către Prof. Petre Jitariu, trebuie să recunoaștem amploarea acestei viziuni care avea la bază ansamblul transformărilor ce urmau să fie induse în întreaga zonă, adică pe aproape jumătatea inferioară a cursului râului Bistrița, în urma construirii barajului Izvorul Muntelui – Bicz și a formării celui mai mare lac de baraj din țara noastră. Profesorul P. Jitariu, cu largă lui deschidere biologică și-a dat seama de necesitățile și oportunitățile cunoașterii evoluției de ansamblu a acestei zone, de la aspectele geografice și geologice până la cele ecologice, sociale, economice și, cu aceași prioritate a oportunităților pentru instrucția prin practica „de teren” a studenților geografi, geologi, biologi – și nu numai – din învățământul universitar românesc.

Profesorul Petre Jitariu nu s-a oprit însă, doar la activitatea Stațiunii, pentru care a avut alături pe Prof. I. Gugiuman și pe Prof. C. Martiniuc de la Facultatea de Geografie.

D-sa a sprijinit stațiunea pe tot parcursul vieții. Astfel el s-a preocupat de alegerea și încadrarea unei bune părți a personalului biologic de cercetare, instruirea prin asistență, sfaturi, pregătirii doctorale, colaborări directe, polarizarea interesului altor specialiști asupra problemelor științifice ale stațiunii, sprijin direct la Minister și forurile

because it was an issue spoken in the faculty; we were glad that we would have a research Station for the students' summer field practice, at that time free of charge. Thus, it was one month in the mountains, after the first year of study, and one month at the Black Sea, at Agigea, after the second and third year of study. The Station being established in the autumn of 1956, as a new assistant, I did the first practical training at Pângărați in 1957, within the sunny and enchanting framework of the mountain vegetation, at Villa Turcan, where the Station functioned until the finalization of its construction in 1958.

I had the second contact with the Station at the 10th anniversary of the Station from Pângărați. I will never forget the beautiful anniversary in that "romantic" period of research, with the excursion on "Spring of Mountain - Bicz" Lake, aboard "Emil Racovita" which, not long ago, was transferred from Agigea by the colleague Dr. Ionel Miron, when Professor Constantin Motaș recited, in the absolute calm and peace surrounding the mountains, the long and beautiful poem "The Lake" by Lamartine, in the beautiful language of the author.

Then, it followed the inimitable voice of Professor Gheorghe Hassan with cathedral reverberations, even more impressive than ever, in the large and sunny space; he sang the melody "O Sole Mio", a praising of the joy of living, of nature and of love and, not only because I am a Romanian, but it seemed to me he was a genuine competitor of Luciano Pavarotti. "O tempora", I had to come here in May 1970, in totally different and complicated conditions in terms of interpersonal relationships, besides the exacerbation of the social and economic repercussions of the new functioning regime.

Returning to the visionary setting up of the "Stejaru" Station by Professor Petre Jitariu, we must recognize the magnitude of this vision that it had at the basis the ensemble of transformations that were to be induced in the whole zone, along half of the lower course of the Bistrița River, as a result of the construction of Spring of Mountain – Bicz dam and the formation of the biggest dam lake in Romania. Professor Petre Jitariu, with his wide biological opening, realized the needs and opportunities of knowing the general evolution of this zone, from the geographical and geological aspects to the ecological, social, economic ones; he also realized the importance and the opportunities

superioare în probleme științifice și administrative, etc.

Ce a devenit în timp viziunea–ctitoria profesorului Petru Jitariu și a colaboratorilor lui, ce a însemnat în știință și societatea românească a celui de a doua jumătate a secolului XX și până astăzi ?!

Putem aprecia, fără lipsă de modestie că „Stațiunea” a fost un fenomen unic în Europa și probabil în lume, dacă avem în vedere condițiile istorice în care a existat, în special până la schimbarea regimului politic, în 1989, adică în aproape 40 de ani, iar contribuția Stațiunii la viața științifică și socială a țării noastre este impresionantă și, în mare, se referă la unele domenii de mare importanță fundamentală, economică și socială:

- Cunoașterea complexă a unei importante zone geografice, Valea Bistriței și evoluția acestei zone ca urmare a formării Lacului de baraj Izvorul Muntelui – Bicaz, de la aspectele geologice, climatice, geografice, până la cele subtile, ecologice și fiziologice precum și la potențialul economic prin acvacultura păstrăvului;
- Inițierea unor cercetări geologice și geografice noi atât în zonă cât și pentru țara noastră, până la cele privind șisturile bituminoase, ca aspect economic;
- Dezvoltarea unor cercetări noi în România privind obținerea unor noi principii active farmaceutice din plante medicinale;
- Realizarea, în mediul academic universitar a primelor experimente de combatere biologică a unor insecte dăunătoare, dorite de înaintașii noștri;
- Toate aceste realizări enunțate pe scurt, au presupus organizarea unor noi laboratoare și crearea în general a unei noi logistică, iar polarizarea și disciplinarea cercetării prin noul regim de contracte-proiecte nu au avut doar efectele aparente de atingere a libertății de cercetare și independenței cercetătorului ca individ, ci a și grăbit și potențat efortul creator și publicarea unor opere de mare valoare științifică, apreciate și cu numeroase premii ale Academiei Române, în toate cele trei domenii de cercetare: Geologie, Geografie, Biologie;
- Stațiunea a devenit locul unde s-au dezvoltat și valorificat pasiunea și entuziasmul unei pleiade de tineri cercetători, marea majoritate proveniți de

the area offered for the field practice of the Geography, Geology and Biology students.

Professor Petre Jitariu did not stop himself, only at the activity of the Station where he had Professor Ion Gugiuman and Professor Constantin Martiniuc from the Faculty of Geography as close collaborators. He supported the Station during his life. Thus, he was concerned with the selection and employment of a large part of the biological research staff, the training through assistance, advices, doctoral training, direct collaboration, the polarizing of interest of other specialists on the scientific problems of the Station, direct support from the Ministry and higher forums in the scientific and administrative matters, etc.

What has become of the vision of the Professor Petre Jitariu and of his collaborators during time, what has it meant in science and in the Romanian society of the second half of the twentieth century until today?!

We can appreciate, without lack of modesty that "the Station" was a unique phenomenon in Europe and probably in the world, if we have in view the historical conditions in which it existed, particularly the change of the political regime in 1989; for almost 40 years, the contribution of the Station to the scientific and social life of Romania has been impressive and broadly it refers to some areas of great fundamental economic and social importance:

- The complex knowledge of an important geographical zone, the Bistrita Valley and the evolution of this zone due to the formation of Bicaz dam lake known as the Spring of the Mountain from the geological, climatic, geographical point of view but also ecologically and physiologically, as well as in terms of economic potential through trout aquaculture;
- The initiation of some new geological and geographical research both in the zone and Romania up to those concerning the bituminous schists, from the economic perspective;
- The Development of some new research in Romania regarding the obtaining of some new pharmaceutical active ingredients from medicinal plants;
- The realization, in the university academic medium, of the first

- la Universitatea din Iași, a noastră « Alma mater » ;
- Astfel aproape 60 de tineri și-au început sau și-au format aici cariera profesională, de la debut până la înalta specializare și deplinătate personală, atestându-se aceasta prin continuarea universitară a carierelor precum și prin înalta recunoaștere a personalităților formate în Stațiune;
 - Astfel 30 dintre cercetătorii de la Pângărați au devenit cadre didactice universitare, dintre care 26 profesori universitari la Universități de prestigiu din Iași, Bacău, Suceava, Constanța, Craiova, Cluj-Napoca și Târgu Mureș, contribuind la pregătirea a peste 30 de serii de studenți în domeniile Geologie, Geografie, Biologie, Agronomie, etc. Să mai menționăm și faptul că dintre aceștia, opt au fost decani și prodecani, trei rectori și prorectori la Bacău și Suceava, președinți de Senat, un Ministru Secretar de Stat, un membru Corespondent al Academiei Române, un membru al ASAS, mai mulți membri ai AOS, etc. Pe de altă parte aproape 20 dintre membrii Stațiunii au continuat cariera de cercetare până la treptele cele mai înalte, conducând colectivele urmașe ale Stațiunii „Stejarul” și bucurându-se de cele mai frumoase aprecieri, titluri și recompense sociale ca șefi ai unor direcții de cercetare, directori, etc. Fără să greșim, putem considera că Stațiunea de cercetări Biologice Geologice și Geografice „Stejarul” Pângărați, în prezent Stațiunea Biologică „Prof. P. Jitariu”, a fost și a rămas pentru țara noastră, un adevărat « Locus genii » al Științei Românești ;

Ținând seama de timpul pe care l-a ocupat în istoria noastră și de condițiile complexe în care a funcționat, nu avem voie să vorbim despre „o destrămare” a Stațiunii, după 1982. În afara condițiilor intrinsece, personalitățile formate la Stațiune în mod firesc trebuiau să tindă spre o împlinire superioară meritată, care era aceea universitară. Totuși, mulți dintre cei formați la Stațiune, au lăsat în urma lor colective de cercetare puternice care subzistă până în prezent și care, cel puțin parțial, și-au adunat din nou ramurile pe

experiments of biological control of some pest insects, desired by our predecessors .

All these briefly outlined achievements assumed the organization of some new laboratories and the creation, in general, of new logistics and polarization and discipline of research through the new regime of contracts-projects; the liberty and independence of the researcher as an individual was only apparently limited as creative efforts intensified and there were published works of great scientific value, that obtained numerous awards of the Romanian Academy in all those three fields of research: Geology, Geography, Biology;

The Station became the place where a pleiad of young researchers, the great majority from the University of Iași, our "Alma Mater", discovered their passion and enthusiasm and developed scientifically;

Thus, almost 60 young people started or formed here their professional career, from the debut to the high specialization and personal completeness, attesting this by university continuation of careers as well as by highly recognition of personalities formed in the Station.

Thus, 30 of researchers from Pângărați became university teaching staff among which, 26 university professors at prestigious universities in Iași, Bacău, Suceava, Constanța, Craiova, Cluj-Napoca and Târgu Mureș, contributing to the preparation of more than 30 series of students in the fields of Geology, Geography, Biology, Agronomy, etc. Let us mention also the fact that of these, eight were Deans and Deputy Deans, three Rectors and Vice-Rectors in Bacău and Suceava, Presidents of the Senate, a Secretary Minister of State, a Corresponding member of the Romanian Academy, a member of ASAS, several members of the Academy of Scientists, etc. On the other hand, almost 20 members of the Station continued the research career to the highest levels, leading the collectives that followed at „Stejarul” Station and enjoying the most beautiful appreciations, titles and social rewards as chiefs of some research directions, directors, etc. Without making a mistake, we can consider that Stejarul Station of Biological, Geological and Geographical Research, Pângărați, at present, "Professor P. Jitariu" Biological Station, was and has remained for our country, a true "Locus geniuses" of the Romanian Science.

aceiași trunchi puternic al „Stațiunii de la Pângărați”;

Cum se întâmplă în cazurile fericite, prin ceea ce a dat Stațiunea „Stejarul” Științei și Învățământului universitar ca urmare a „impulsului vital creator” al ctitorilor, s-a depășit putem spune, cu mult viziunea inițială a acestor înaintași.

Stațiunea nu s-a destrămat, sau desmembrat. Ea a fost ca o „super nova” care și-a împărțiat lumina și energia în întregul univers științific și universitar românesc, încununare a viziunii primordiale a Prof. Petre Jitariu și a colaboratorilor lui.

Destinul meu ca director s-a intersectat aproape șapte ani cu cel al Stațiunii, al istoriei alerte și oarecum zbuciumate a perioadei 1970 – 1976 dar la fel de zbuciumată pentru întreaga cercetare românească. Știam din 1967 că se pregătește și la noi după modelul occidental trecerea cercetării în regim contractual pentru orientarea acestei activități spre probleme de interes economic și aveam informații la zi despre situația din țările occidentale unde, cu cel puțin zece ani înainte se adoptase această soluție. Astfel, până în 1970 instituțiile noastre de cercetare funcționau după un regim bugetar, în care, totuși, cele aparținând de ministere cu profil economic aveau tematica orientată spre rezolvarea problemelor practice ale Agriculturii, Silviculturii, Zootehniei, Sănătății, etc. Din 1970, toate instituțiile de cercetare, în afara celor aparținând Academiei Române, au fost trecute la regimul de activitate pe bază de contracte obținute prin licitație și orientate spre probleme de importanță economică sau, cum se spunea pe atunci, spre o cercetare « fundamental-orientată ».

În cadrul instituțiilor și colectivelor mai puțin sau aproape deloc informate, această în aparență bruscă schimbare, a produs confuzie dusă până la revoltă și descurajare, manifestată uneori prin opoziție fațășă și refuzul de a contribui la găsirea de soluții.

În această situație demobilizatoare, în plus Universitatea nu mai putea să-și exercite egida asupra Stațiunii din punct de vedere economic-administrativ, decât prin sfaturi, deoarece Învățământul superior funcționa ca și în prezent în regim bugetar. „Forurile superioare” au găsit totuși o soluție temporară de compromis.

În acest sens, a fost alocată o sumă destul de consistentă prin care să se finanțeze teme de cercetare „Fundamental-orientată” cerute de institutele de profil ale Academiei de Științe

Taking into account the time occupied in our history and the complex conditions in which it functioned, we are not allowed to talk about "a teasing" of the Station after 1982. Excepting the intrinsic conditions, the personalities formed at the Station, naturally aimed at deserved superior fulfilment, which was the university. Yet, many of those trained at the Station, left behind strong research collectives that have remained up to the present, at least partially, and they have gathered again on the same strong 'trunk' of the "Station from Pângărați".

As it happens in happy cases, by what „Stejarul” Station gave (offered) to Science and University Education as a result of the „creative vital impulse” of the founders, we can say that it has by far exceeded the initial visions of these predecessors. The Station has not been broken up or dismembered. It was like a "super nova", which scattered light and energy in the whole Romanian scientific and university universe, the crowning of the primordial vision of Professor Academician Petre Jitariu and his collaborators.

My destiny as a Director was intersected almost seven years with that of the Station, of its alert and somewhat tumultuous history of the period 1970 - 1976, but equally eventful for the entire Romanian research. I knew in 1967 that, following the western model, research had to pass to a new contractual regime and focus on economic issues of great interest. Western countries had already adopted this model ten years before. Thus, until 1970 our research institutions functioned according to a budgetary regime, in which, however, those belonging to the ministries of economic profile had the themes oriented to solve the practical problems of Agriculture, Silviculture, Zootechny, Health, etc. Starting with 1970, all the research institutions, except those belonging to the Romanian Academy, passed to the activity based on contracts obtained by auction and oriented toward problems of economic importance or, as it was said at that time, toward a fundamental-oriented research.

Within the less or almost no informed institutions and collectives, this seemingly sudden change caused confusion and discouragement carried up to revolt, manifested sometimes by outspoken opposition and refuse to find solutions.

In this demobilizing situation, moreover, the University could not exercise any longer the aegis on the Station from economic-administrative

Agricole și Silvice și a unor Ministere cu profil economic. În ce ne privește, prin tematica de cercetare propusă din partea Stațiunii, am obținut o sumă consistentă care a rămas valabilă mulți ani. În acest fel, având o bază financiară asigurată, și alte contracte, s-a putut asigura activitatea și dezvoltarea absolut necesară a colectivelor și direcțiilor de cercetare din Stațiune, corespunzător noului mod de funcționare. Astfel în 1970 funcționau vreo 14 laboratoare (mai curând direcții tematice) cu doar 1-2 cercetători fiecare dintre cei 17 existenți atunci. Această situație nu putea continua pentru realizarea proiectelor, ceea ce a determinat dezvoltarea colectivelor în funcție de finanțarea obținută.

Aceasta a fost situația de ansamblu în Stațiune și pe plan național când am fost numit director la Stațiune prin Adresa nr. 53863 / 1970 a Ministerului Învățământului, în urma demisiei directorului de atunci, Dr. Dan Munteanu. Cu sprijinul majorității cercetătorilor din Stațiune am putut redresa și depăși perioada critică. Astfel, în loc să se destrame, colectivele de cercetare și baza logistică s-au dezvoltat mult și odată cu acestea, întreaga Stațiune.

Trebuie să menționez aici sprijinul continuu și constant al Prof. Petre Jitariu, director al Centrului de Cercetări Biologice din Iași, instituție în aceeași situație ca și noi, precum și al Prof. I. Gugiuman și mai ales al Prof. C. Martiniuc, foști directori ai Stațiunii, care au contribuit din plin, cu sprijinul Universității „Al. I. Cuza” din Iași, la modernizarea stării materiale a Stațiunii, dar și cu sfaturi în domeniile cercetării și administrației. Noi de altfel, începând cu 1970, am continuat și terminat unele proiecte gospodărești începute de ei.

Pe scurt, iată care au fost realizările obținute în primii cinci ani ai strădaniei subsemnatului, sprijinit de colegii și persoanele pomenite mai sus:

- Bugetul Stațiunii a crescut de la 2388 mii lei în 1970, la 4500 mii lei, adică cu 188% (sau de 1,88 ori) până în 1975;
- Valoarea contractelor care au alcătuit bugetul a crescut de la 200 mii lei în 1970, la 4500 mii lei, adică cu 2250% (sau de 22,5 ori) până în 1975;
- Dintre aceste contracte, cele cu unități (instituții economice) au crescut de la zero în 1970, la 2600 mii lei, constituind 58% din valoarea totală a contractelor (proiectelor) de cercetare ;

point of view, because higher education functioned in budgetary regime, as it still happens. However, the "Superior Forums" found a temporary compromise solution.

In this sense, it was allocated a pretty consistent sum through which to be financed "Fundamental-oriented" research themes required by profile institutes of the Academy of Agricultural and Silvicultural Sciences and of some Ministries with economic profile. As it concerns us, through the research theme proposed by the staff of the Station, we obtained a consistent sum which remained valid for many years. In this way, having a secured financial basis, and other contracts, it could ensure the activity and the absolutely necessary development of the collectives and the research directions of the Station, corresponding to the new functioning mode. Thus, in 1970, there were functioning about 14 laboratories (thematic directions) with only 1-2 researchers each among those 17 existing at that time. This situation could not continue as projects became the main activity; thus, it determined the development of collectives depending on the obtained finances.

This was the situation at the Station and at national level as well, when I was appointed Director of the Station through the address no. 53863/1970 of the Ministry of Education, following the resignation of the Director of that time, Dr. Dan Munteanu. With the support of the majority of the researchers of the Station we could straighten out and surpass the critical period. So, instead of breaking up, the research collectives and the logistic basis developed more and together with them the whole Station.

I must mention here the continuous and constant support of Professor Petre Jitariu, director of the Biological Research Centre Iasi, an institution in the same situation like us, as well as of Professor Ion Gugiuman and especially of Professor Constantin Martiniuc, former directors of the Station who contributed fully, with the support of "Alexandru Ioan Cuza" University of Iasi, to the modernization of the infrastructure of the Station, but also with advice in the research and administrative fields. We, as a matter of fact, since 1970, continued and finished some household projects started by them.

In short, these are some of the achievements obtained in the first five years of hard and dedicated efforts made by the aforementioned colleagues and the persons:

- Dar cel mai important aspect al dezvoltării a fost acela al dinamicii personalului de cercetare, viitoarele personalități formate la Pângărați. Creșterea a fost de 241 % (sau de 2,41 ori) între 1970 și 1975;
- Baza materială , în mod necesar s-a dezvoltat în aceeași măsură;
- Acestea au contribuit la o stabilitate nemăitâlnită din toate punctele de vedere a Stațiunii și activității ei;
- S-au continuat de asemenea susținerea practicii studenților de la universitățile din țară;
- În plus, în pofida greutăților epocii specifice mai ales sistemului social din România, ca și la Stațiunea de Biologie Marină „Prof. I. Borcea”, de la Agigea am dezvoltat pentru prima dată relațiile cu cercetarea și învățământul din străinătate, stabilind primele schimburi de studenți cu Universitatea Paris VII din Paris, schimburi științifice cu Universitatea din Konstanz Germania Federală –am încheiat și primul contract internațional plătit, cu Institutul Internațional de Combatere Biologică, Stațiunea de la Delémont, din Elveția, am favorizat stagii științifice personale ale cercetătorilor din Belgia, din Franța, Elveția, Venezuela, etc.

Greutățile personale pe care le-am înfruntat au provenit și din faptul că nu aveam locuință, iar salariul nu mi l-am alocat din regie, cum aveam dreptul ci din aceleași contracte cu care mi-am finanțat colectivul propriu de cercetare, contracte la care am lucrat efectiv, de la munca de teren până la redactare și predare. Am considerat activitatea mea la Stațiune ca o datorie față de Universitatea „Al. I. Cuza” al cărei student am fost și față de magistra Almei mater care mi-au acordat încrederea numind-mă director al Stațiunii, iar Prof. C. Grasu are dreptate când spune că perioada 1970 – 1976 a fost cea mai prolifică, Stațiunea atingând maximum de dezvoltare. De aceea, alăturăm eforturilor noastre, înțelegerea și eforturile colegilor de atunci care au pus umărul cu entuziasm și determinare la progresul Stațiunii, printre care au fost Dr. Ionel Miron, Dr. Gogu Gheorghită, Dr. I. Bâra, Dr. Klaus Battes și cea mai mare parte dintre colegii Geografi coordonați de Dr. I. Bojoi și dedicăm

- The budget of the Station budget increased from 2,388 thousand lei in 1970 to 4,500 thousand lei, i.e. by 188 % (or 1.88 times) until 1975;
- The value of the contracts that made up the budget increased from 200 thousand in 1970 to 4,500 thousand lei, i.e. 2,250 % (or 22.5 times) until 1975; Of these contracts, those with economic institutions (units) increased from zero in 1970 to 2,600 thousand lei, representing 58 % of the total value of the research contracts (projects);
- But the most important aspect of development was that of the dynamics of the research staff, the future personalities formed at Pângărați. The increase was 241 % (or 241 times) between 1970 and 1975;
- The necessary material resources developed in the same measure;
- These contributed to an unprecedented stability in all aspects of the Station and its activity;
- We also continued to support the field practice of the students from different universities in the country.

In addition, in spite of the specific difficulties of the epoch, especially those related to the social system in Romania, as it was the case at Professor I. Borcea Marine Biology Station, for the first time, I developed relationships with the research and education institutions from abroad, establishing the first exchanges of students with the University Paris VII from Paris, scientific exchange with the University of Konstanz, West Germany, I signed the first international paid contract with the International Institute of Biological Control, the Station from Delémont, Switzerland, I favoured personal scientific stages of researchers from Belgium, France, Switzerland, Venezuela, etc.

The personal hardship that I faced came also from the fact that I had no dwelling place and I did not receive my salary from the management, as I had the right, but from the same contracts based on which I financed my own research collective agreement (contract), contracts to which I actually worked from the field work to editing and handing. I have considered my activity at Station as a duty to "Alexandru Ioan Cuza" University whose

succesele și realizările acelei perioade, memoriei Academicianului Prof. Petre Jitaru pe care o evocam astăzi, May 11, 2016

student I was and to my masters of Alma Mater who placed confidence in me, appointing me as a Director of the Station, and Professor Constantin Grasu is right when he says that the period 1970 - 1976 was the most prolific one, the Station reaching the maximum of development. Therefore, we join our efforts, the understanding and efforts of the colleagues of that time who gave a helping hand with enthusiasm and determination to the progress of the Station, among whom, we mention: Dr. Ionel Miron, Dr. Gogu Gheorghită, Dr. Ion Bâra, Dr. Klaus Battes and most of our geographer colleagues, coordinated by Dr. Ion Bojoi; I dedicate the successes and achievements of that period to the memory of the Academician Professor Petre Jitaru that we are evoking today, May 11, 2016.

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(The translation of the text into English was made by Varvara Mircea, Professor Emeritus, PhD, and revised by Dr. Alina Vlăduț. For their work and goodwill, we address to them the sincerest and warm thanks.)

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