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EDITORIALS

A *One Health* perspective on antimicrobial resistance

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Abstract

The paper briefly compares how the European Union (EU) and the United States (US) approached the growth-promoting antibiotic avoparcin, the emergence of vancomycin-resistant Enterococcus faecium *(VRE), and growth-promoting antibiotic use policies in livestock. In 2006, the European Union (EU) banned all growth-promoting antibiotic use in livestock. The U.S. issued a voluntary ban in 2017. The sequencing of VRE's genome yielded unexpected findings that should impact antibiotic resistance surveillance policies*

Key words: *Antimicrobial resistance, One Health, vancomycin-resistant* Enterococcus faecium

We take for granted the many therapies of modern medicine such as elective surgeries, immunosuppressive treatments for autoimmune diseases, and cancer treatments. Unfortunately, these treatments might not be feasible if safe and effective antibiotics become no longer available. Part of the challenge with antimicrobial resistance has been the blame game between medicine and agriculture regarding who is most at fault for causing the crisis. Modern medicine and modern agriculture both rely on antibiotics to prevent disease and treat infections. But agriculture has also used antibiotics as growth promoting agents in food animals, a practice generating significant controversy. The use of avoparcin, a growth promoting antibiotic, and the rise of vancomycin-resistant *Enterococcus faecium* (VRE) in hospitals drove the European Union (EU) to ban avoparcin and eventually all growth promoting antibiotics. How the EU and the US addressed avoparcin and VRE is the focus of this paper.

Vancomycin-Resistant *Enterococcus faceium* (VRE)

The emergence of vancomycin-resistant *Enterococcus faecium* (VRE) in European hospitals in the late 1980's, and the subsequent discovery of VRE in Danish pork and poultry generated widespread concern [1]. Avoparcin was assumed to be the cause of VRE; it was a

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growth-promoting antibiotic, chemically related to vancomycin, and used in European livestock production [2].

Denmark relied on avoparcin, accounting for more than 20 percent of the antibiotics used in livestock. The primary recipients of the drug were pigs. Danish pork producers voluntarily stopped using avoparcin in 1995 [3]. The European Union followed Denmark's lead and banned avoparcin in all member countries in 1997.

Denmark launched several monitoring systems including the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) which started publishing annual reports in 1997 [4]. The avoparcin ban had a tremendous impact on VRE on Danish farms, causing a 90 percent reduction of VRE in pigs and broiler chickens over ten years, from 1997 to 2007 [5].

The avoparcin ban did not, however, reverse the increasing rates of VRE in Danish hospitals. Less than 1 percent of *E.faecium* isolates in hospitals in 2008 were resistant to vancomycin, but subsequently, the rates increased: 1.6 percent in 2009, 1.8 percent in 2012, and 3.4 percent in 2013 [6]. A plausible explanation was the increased use of vancomycin to treat ampicillin-resistant *E. faecium* hospital infections [7].

An overall decrease in VRE rates in EU hospitals would be expected during the subsequent years after the EU-wide avoparcin ban. But that is not what the data showed. Instead, VRE rates in hospitals across the EU varied dramatically according to 10 year (2003-2013) data from EARS-Net, an EU-wide surveillance system of antimicrobial resistance rates [8]. The Netherlands and Sweden showed low VRE rates whereas other countries, such as Belgium and Greece, fluctuated and increased in the years after the avoparcin ban. Italy had an 83 percent decrease in VRE rates, whereas, Ireland had a two-fold increase. There was no obvious trend in the EU hospitals [9].

VRE reporting in livestock was voluntary, resulting in little data for analysis [10]. Only ten countries reported VRE rates from broiler chickens. Of these, Belgium stood out with a resistance rate of 9.1 percent. The other countries reported resistance rates less than 1.5 percent. Of the seven countries reporting VRE rates in pigs, Denmark and the Netherlands reported rates of 0.9 and 0.5 percent, respectively [10].

In contrast to the EU, the U.S. never approved avoparcin, not because of concern about antimicrobial resistance, but rather, because of its potential carcinogenicity [11]. Consequently, VRE epidemiology in the U.S. has been different compared to the E.U. Since 2002, the National Antimicrobial Resistant Monitoring System (NARMS) and the National Animal Health Monitoring System (NAHMS) have been monitoring the rates of VRE in chickens, pigs, chicken meat, and pork chops. In all the years tested, the VRE rates have been zero [12,13]. Despite the absence in U.S. livestock, VRE has been a serious problem in U.S. hospitals. In one year, the CDC estimates 10,000 vancomycin-resistant *Enterococcus faecium* infections and at least 650 deaths [14].

Genomic data might explain the discrepancies between avoparcin growth-promoting use in livestock and VRE rates in hospitals. VRE from hospitalized patients and from European farm animals were separate populations. A clone, designated as VRE CC 17, was responsible for the hospital infections, and it might have a genetic link with ampicillin-resistant *Enterococcus faecium* (AREF) CC 17 from dogs. About a decade before VRE emerged, AREF appeared in U.S. hospitals [15,16,17].

CONCLUSIONS

The VRE story highlights the complexity of antimicrobial resistance – the origin of resistant microbes should not be assumed. Companion animals, such as dogs, might be playing a major role in the emergence and spread of antibiotic resistant bacteria. These animals receive antibiotics when they get sick. They also share people's food and sometimes sleep in their owner's beds. Antimicrobial resistance surveillance systems do not include them. Genomic surveillance should be widely implemented and conducted in hospitals, veterinary clinics, farms, homes, and soils in order to better understand microbial ecology, evolution, and epidemiology. A *One Health* approach is essential to fully understand antimicrobial resistance.

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One Health approach to wicked public health problems

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Abstract

Food safety, antimicrobial resistance, and emerging diseases are public health problems found in every country of the world. Such problems often require the collaboration of public health, veterinary health, environmental health, and agricultural business professionals in their prevention and control. Increasingly, due to modern transportation and agricultural techniques, once local or regional public health problems are now becoming international in scope, affecting thousands of people and animals. We must find ways to work across disciplines and across geographical borders to prevent and control these problems. Many organizations have embraced the One Health approach as a way forward in developing the necessary multidiscipline and international collaborations.

Key words: *One Health, zoonotic diseases, food safety, antimicrobial resistance*

As the world human population approaches nine billion, international travel and transportation become more accessible, and global movements of goods increase, very complex public health problems are emerging.

One such challenge is the antimicrobial resistant bacteria, which now pose a serious threat to human health, causing a number of medications to be ineffective. The cause of this resistance has been linked not only to improper use of antibiotics in humans, but also to the common practice of using subtherapeutic doses of antibiotics in animal feed [1,2]. Further, emerging infectious diseases now quite readily move with people, animals, or food products. The SARS outbreak of 2003, the H1N1 pandemic of 2009, and the *Ebola* outbreak of 2014, to name only a few, are emerging diseases of the 21st century that resulted in an estimated 774 [3], over 151,700 [4], and 11,310 deaths [5], respectively. Another large, complex, public health concern is food safety, with an estimated 600 million people suffering food-borne illnesses each year [6]. As such, investigation and development of systems to ensure consumer safety are required at all stages of production. Such complex problems require the involvement

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of multiple disciplines and a range of methodologies to control. As more of these dynamic complex problems arise, developing innovative approaches to combat these challenges becomes a greater priority.

The One Health *Approach*

The One *Health* approach aims to tackle complex problems by coalescing the efforts of public health officials, veterinarians, physicians, environmental professionals, scientists, and so on. Broadly defined, *One Health* is, *"the collaborative effort of multiple disciplines working locally, nationally, and globally, to attain optimal health for people, animals, and our environment"* [7]. These multidisciplinary collaborations connect different perspectives, create new research strategies founded on academic breadth, and foster the kind of holistic and innovative problem solving necessary to manage these complex problems.

While the idea of *One Health* is not new [8], the concept of *One Health* approaches has gained much momentum during the last 10 years. In 2006, the American Veterinary Medicine Association (AVMA) established a *One Health Initiative Task Force* with a goal of assessing the possibility of collaboration between academic institutes, government bodies, and health science professionals in an effort to assess, treat, and prevent the problems that were too complex for one discipline to solve [7]. In an April 2016 search, there were at least 57 academic institutions worldwide with some sort of a *One Health* program; 13 percent having a *One Health* certificate, masters, or PhD program[9]. Additionally, in that same search, 68 non-academic organizations had some sort of a *One Health* program [9].

Articles citing *"One Health"* on PubMed have increased nearly 8 times from 2006 to 2016 (50 to 396) (fig.1) [10]. Additionally, dedicated *One Health* journals are beginning to emerge. *EcoHealth (Chief editor in the USA)*, *Infection Ecology and Epidemiology (Chief editor in Sweden)*, the journal *One Health (Chief editors in Australia and Germany)*, *International Journal of One Health (Chief editor in India)*, and the *One Health International Journal (Chief editor in Romania)* are each promoting the *One Health* approach. One Health research is also disseminated at dedicated conferences, such as the International Symposium on *One Health* Research, the *One Health EcoHealth* Conference, the *One Medicine* Symposium, and the *One Health* European Interregional Conference.

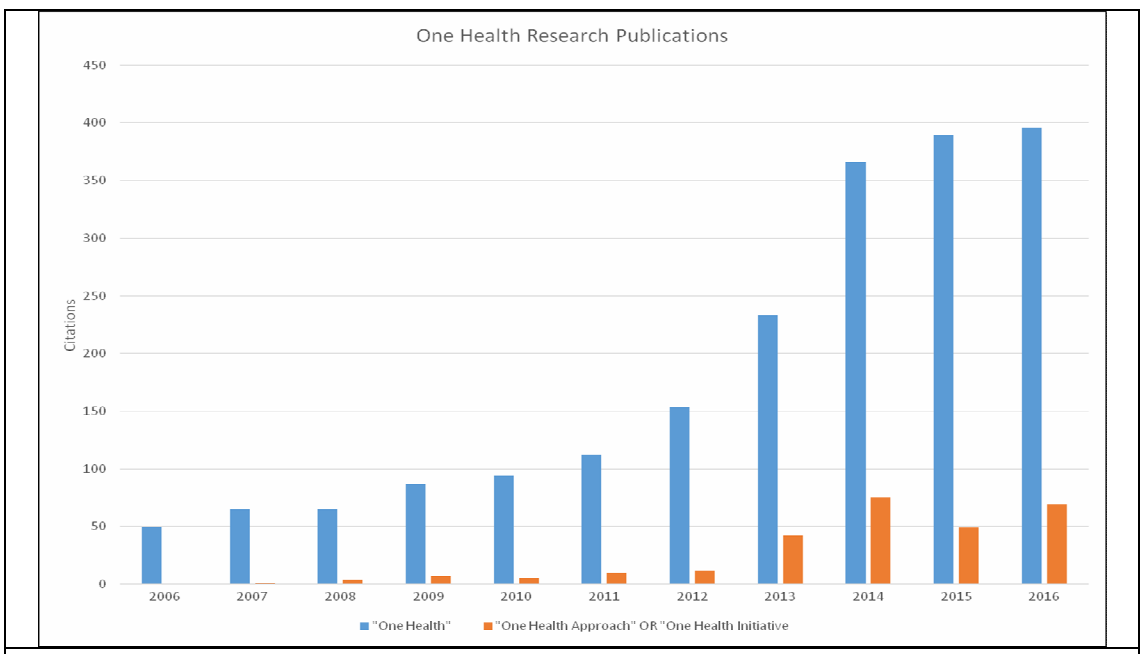

Fig. 1. Number of publications including *One Health* (blue) or *One Health Approach* OR *One Health Initiative* (orange) via PUBMED search on April 24, 2017 [10]

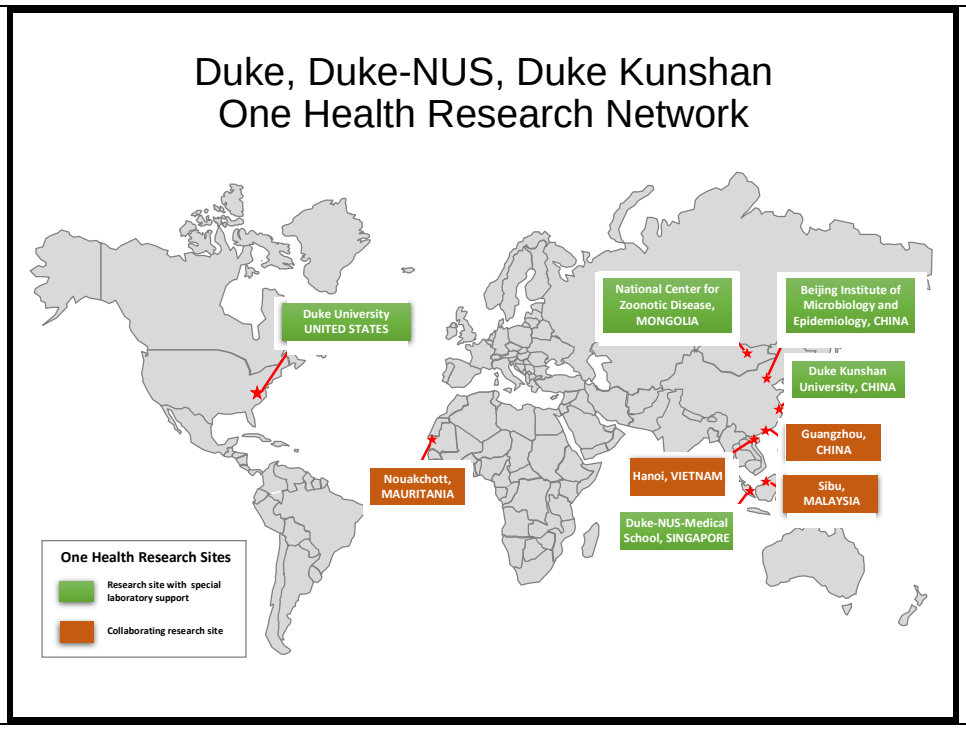

Fig.2. Duke One Health Research Network

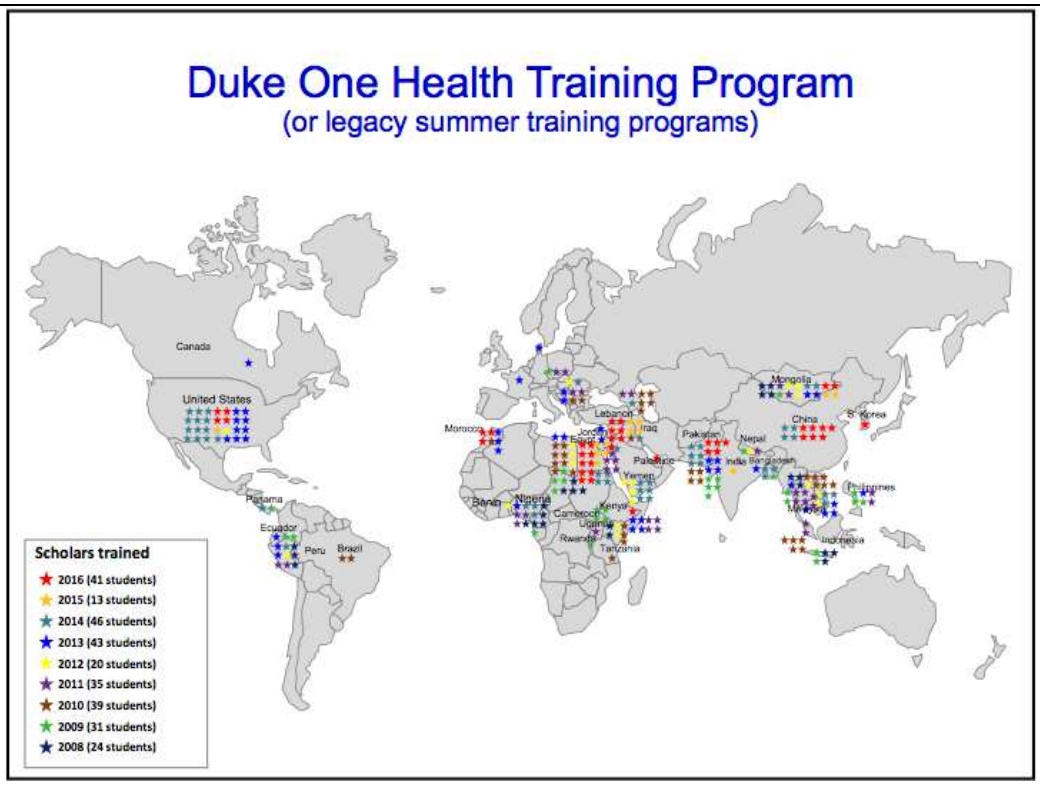

Fig.3. Trainees by country who have attended our summer short courses, now called the Duke One Health Training Program. More information may be found at: http://sites.globalhealth.duke.edu/dukeonehealth/trainingprogram/

Duke *One Health*

Taking a closer look at our program dedicated to this collaborative approach, the Duke *One Health* team, a network of researchers and professionals from more than nine different countries (fig.2), aims to understand the emergence and transmission of zoonotic pathogens. Zoonotic diseases, diseases that are transmitted from animals to humans or vice versa, account for three out of five new human illnesses [11]. The aforementioned emergent virus H1N1, for instance, originated as a reassortment of human, swine, and avian influenza viruses [12].

The Duke *One Health* team conducts surveillance for various novel respiratory viruses at the human-animal interface in multiple countries across the world. One prime example is our *US National Institute of Health R01* study with partners in China which has the following aims:

1) to identify and characterize enzootic and emergent swine influenza viruses (SIVs);

2) to employ aerosol, fecal, environmental swabs, and water sampling to identify environmental areas with a high prevalence of SIVs;

3) to identify occupational risk factors for SIV infection, and

4) to identify serological and mucosal immunity biomarkers of protection against prevalent and emergent SIVs.

Similarly, the Duke *One Health* team is also conducting surveillance for novel human and animal viruses through US Department of Defense grants for work in Malaysia, Mauritania, Singapore, and Vietnam and soon we will train other partners to do the same in Egypt, Iraq, and Pakistan. We are also training US and Mongolian professionals to conduct *One Health* research in Mongolia under a US National Institute of Health grant. This program has engaged teams of scientists conducting important multidisciplinary work in tick-borne diseases, equine zoonoses, and zoonotic parasitic diseases. These efforts will help us understand the ecology of zoonotic pathogens and multidrug resistant bacteria which are often associated with modern agriculture.

Our network is also heavily engaged in training US and international scholars continuing the tradition of the *One Health* approach through the summer Duke *One Health* Training Program. This three-week program assembles professionals from multiple countries (fig.3) to learn about *One Health* problem solving, global disease surveillance, and interventions to reduce infectious disease threats to public health. The four course offerings include:

- Introduction to the *One Health* Approach;
- Public Health Laboratory Techniques;
- An Introduction to Entomology, Zoonotic Diseases, and Food Safety; and
- An Introduction to Environmental Health.

This course work ultimately affords the scholars of *One Health* the opportunity to become well-versed in the global surveillance and public health threats while fostering multidisciplinary research skills.

CONCLUSIONS

In summary, as the human population grows, and agriculture and transportation follow suit, it is imperative that once local or regional public health, veterinary health, and environment health concerns become global concerns engaging the multiple disciplines involved. The multi-disciplinary *One Health* approach is gaining traction as the key way forward in responding to these modern complex problems that affect human, animal, and environmental health. Antimicrobial resistant bacteria, emerging diseases, and complex problems surrounding food production are challenges that are not going away, but perhaps a more concerted effort, such as the *One Health* approach, can mitigate their devastating effects on the health of humans, animals, and the environment.

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SHORT REVIEWS

Health education and prevention – strategic purposes of the health system

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Abstract

We live in a global society, where isolation is no longer possible, a world where threats to public health are multiple and complex. In modern society, the most prevalent diseases (cardiovascular and cerebrovascular diseases, diabetes, some forms of cancer) could be avoided through prevention. The understanding of the importance of the primary prevention since childhood, the health education and health promotion will have, in the long term, a major impact on the improvement of health status and on the life expectancy growth in Romania. Prevention should be the milestone of any medical system and, of course, of the Romanian medical system, and the one health concept constitutes itself the best way to achieve this goal.

Key words: *health system, One Health, prevention, health education*

We live in a global society, where isolation is unfeasible, a world where threats to public health are multiple and complex. Risk factors include: food, particularly of animal origin, stress, pollution and environmental disasters, accidents with radiological, chemical or biological consequences and so on. Population growth, increased internal and international migration, global warming and climatic changes have made the threats to public health to be progressively diverse. Antimicrobial resistance, zoonotic diseases, disaster management, safety along the food chain, ecological disasters, are arguments for the necessity to adopt the concept of health in all policies, from environmental to veterinary and human health.

The approach must be a multidisciplinary and integrated one, involving all actors in the system, providing prevention and response to public health risks. Economic and cultural development, better research and better health care are behind the new challenges. It is well known that the gap between those who received the best health care in the world and those who receive the worst is staggering [1].

Of the many barriers that may hinder health service access and service affordability, disproportionately affect people from lower social-economic groups [2].

There are three main particularities of the healthcare system that inform the policy makers in many nations: the healthfulness of the population, the degree of satisfaction citizens have with the performance of the healthcare system and the grade in which the system provides financial protection. There are also equity issues that must be solved such as better access to healthcare services for poor and vulnerable

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groups. Beyond of ethical and philosophical positions, making decisions about priorities in health system is not a technical matter. The political commitments are likely to lead the differences in priorities.

As country after country begins going through the "epidemiological transition" from infectious illnesses to noncommunicable diseases (NCD), NCDs have become an ever-increasing cause of their disease burden. Even for poorer nations, the dual burden of disease has forced them to look at health system issues beyond vector control, immunization, providing anti-infective medicines, safe motherhood and primary care.

When it comes to preventing NCDs, Ministries of Health realize that much of what needs to be done is outside their sphere of competences: How do they get the taxes on tobacco raised? How do they encourage primary schools to emphasize healthy lifestyle with regard to diet and exercise in their teaching?

Reformers trying to develop NCD policies will discover three distinct system design problems to confront with:

• Primary prevention – this involves intersectorial action – collaboration in policy making with non-health ministries;

• Secondary prevention – impede the evolution to acute complication after a diagnosis;

• Tertiary prevention – to avoid acute episodes of noncommunicable diseases.

Improving primary prevention: Actions taken before patients have developed NCDs may involve a significant and cost-effective impact on reducing a NCDs disease burden: tobacco control – lower heart disease and cancers, reduce alcohol consumption – reduce cirrhosis and liver failure, lower trans-fat and salt intake- lower cardiac disease, lower obesity and increase physical exercise – decrease cardiac disease and diabetes, adopt vaccinations (for example for cervical cancer and hepatitis B).

Improving secondary prevention means that: populations have to be screened to identify those suffering from various chronic conditions; once patients are detected, appropriate medications have to be prescribed; patients have to be educated about change in their lifestyles, to actually carry thought on such changes; patients need to cooperate with on-going monitoring and must identified as needing further assistance require treatment's adjustment.

Improving tertiary prevention: The best example is for cancer care: The heterogeneity of cancer diagnosis and prognosis means that countries cannot sensibly have a single cancer care policy or clinical pathway for cancer care. Where cost-effective screening techniques are available, the first major task of the healthcare system dealing with cancer treatment is providing effective systems in order to ensure that populations at-risk receive the adequate screening.

There are several health system challenges to improved diseases outcomes [3]:

1. Developing sufficient political commitment
2. Strengthening inter-agency cooperation
3. Creating explicit processes for setting priorities and limits
4. Creating the right incentive systems
5. Improving health systems management in all regions
6. Creating more useful information systems
7. Perfecting the incorporation of evidence in clinical practice
8. Establishing appropriate models of services delivery
9. Taking advantage economies of hierarchy and specialization
10. Improving coordination across providers
11. Enhancing citizen empowerment
12. Growing the reserve of human resources
13. Assuring access to quality chronic disease medicines
14. Reducing financial barriers to access
15. Overcoming resistance to change.

All these can be considered strategic purposes of the health system.

The importance of prevention

Prevention is the medicine of the future. In modern society, the most prevalent diseases like cardiovascular diseases and some forms of cancer could be avoided through prevention. The comprehension of the major role of the primary prevention since childhood, the health education and health promotion will reflect, in the long term, on the improvement of health status and on the life expectancy growth in Romania.

The decision makers must be informed of instruments of political and ethical analysis thus they may participate at the resolution of disagreements. Moreover, the international experience could play an important role in the decision making process, but the examples of best practices must be adapted to our country's own context.

Prevention should be the milestone of any medical system and, of course, of the Romanian medical system, and the one health

concept constitutes itself the optimum mode to reach this objective. The implementation of biotechnology in medicine, the progress in science and technology, the promotion of prevention and health education are all realities of 21st century. As a synthesis of these goal Health 2020, the new European health policy, aims to improve the health and well-being of populations, reduce health inequities and ensure people-centred health systems [4].

Nowadays, health is no more examined in terms of each person or the whole population, but in light of the ecosystem in which we all live and work. Basically, the health of the ecosystem must be the landmark of this new approach. The achievement of such initiative could be assured through an integrated approach and intersectional cooperation and at the same time through information sharing and cooperation between states and international organizations. Policy makers must decide whether and how to involve representatives of the main interested parties in the health sector reform process. Strong political commitment within and across government is necessary to reduce inequities in health through action on social determinants, extending from clear leadership through to political authority to pursue the agenda [5].

The One Health Concept

The One Health concept relies on a profound comprehension of the interrelationships between zoonotic diseases, the habitat and the public health, the fundamental purpose being the improvement of the health of the ecosystem [6]. There are numerous examples of diseases transmitted from animals to humans: rabies, salmonella infections, West Nile virus, Ebola, Zika virus, etc. Also, many epidemics are the effect of various natural hazards and climatic changes. The spread of these diseases is possible especially due to the growing interchanges between the people, animals and the environment, due to population growth, the enlargement of the surface inhabited by humans, climatic changes and extreme phenomena, as a consequence of the urbanization, internal and international migration, the intensification of international tourism and international trade.

Ensuring safety along the food chain must be done for impeding the emergence of epidemics. In the event of a risk to public health, a quick and effective response to avoid the spread of the epidemic is extremely significant. In the event of a natural hazard, rapid intervention measures are imperative to minimize the consequences on humans and the environment. Thus a good collaboration between countries and institutions is required for the progress of tools and action plans, in order to impede the extension of epidemics and also control the risk prediction instruments for preventing outbreaks. The One Health concept aims to accomplish all the objectives. The final goal must be the amelioration of the health status of the whole population.

Our country must respond with professionalism to the actual problems concerning public healthcare, in line with the Europe 2020 Strategy and the One Health concept.

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Energetic medicine as a common approach in animal and human healing

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Abstract

The paper tries to synthesize the existing data regarding new medical concept of Energetic / Holistic Medicine (EM), underlying the real role of EM in screening of some medical conditions, the support of EM as Complementary Medicine in health evaluation, as preventive medicine or even qualitative diagnosis of some incipient maladies.

The paper emphasis the necessity of a large multicenter study, according to GCP rules, to demonstrate the role of EM (SCIO-biofeedback) as Complementary approach in screening or quick diagnostic of some medical conditions to human and animals, and the similitudes of some illness and simultaneous presence of specific acute or chronic maladies.

Key words: *One Health, energetic medicine, complementary medicine, neuro-biofeedback, psycho-neuro-immunology, neuroscience, GCP, screening, quick diagnosis*

The field of Energetic Medicine

Energetic Medicine includes all concepts of energy: light, sound, electromagnetism, body, mind and spirit [1].

Holistic medicine is a term used to describe therapies that attempt to treat the patient as a whole person. That is, instead of treating an illness, as in orthodox allopathy, holistic medicine looks at an individual's overall physical, mental, spiritual, and emotional wellbeing before recommending the treatment [2].

Holistic / energetic medicine has existed in various forms for centuries; the current field does not have one absolute definition.

The answer to the question: **What is holistic / energetic / vibrational medicine?** varies according to the practitioner.

There is no standard scientific understanding or precise meaning of these ideas in the Western scientific paradigm, although various explanations are offered for holistic/energetic medicine in terms of a vital force or life energy.

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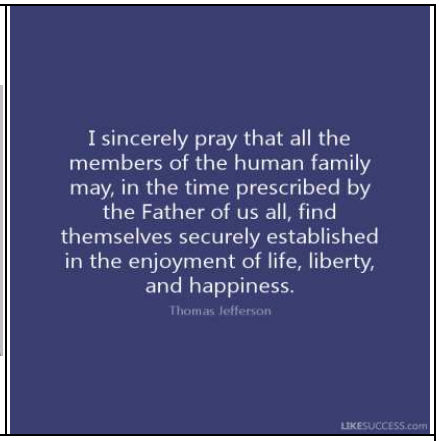

Fig.1. Meditation, Prayer

Bio-field Science and Healing: an Emerging Frontier in Health and Medicine [3]

We live in an age of unparalleled technological and scientific progress, juxtaposed with a cascading array of poor social, health, and environmental choices that could bring our species to the brink of catastrophe.

Within the past 100 years alone, we have created significant advances in technologies to better control disease outbreaks, extend our lifespan, enhance global communication, increase our work productivity, and improve our overall quality of life.

In the same time, we face major healthcare crises including diabetes, cardio-vascular diseases, cancer, and mental illness.

Despite our best efforts and technological advances, we have not yet conquered these and other life and health challenges.

Bio-field Science: the Missing Link in Understanding Health and Healing

A key ingredient in the recipe for advancing the evolution of human health is self-empowerment, which can only emerge with a clear recognition of one's own capacity for healing and thriving. Examples from clinical and research areas such as **mind-body medicine, placebo, psycho-neuro-immunology, and neuroscience, remind us that our capacity to activate our own internal healing response is within our human capabilities.**

Energetic Medicine

A new paradigm for researching biology and medicine based on energy information is required to define holistic/energetic medicine [4].

There are many key philosophies behind Energetic Medicine. The first is responsibility. The patient is encouraged to accept responsibility for their body and any disease or discomfort. The disease *might have been caused by someone else or some outside imposition*, but healing can only take place inside the body. Obsessing on someone else or blaming someone else is unproductive and sometimes damaging. Separation from a cause of disease is the responsibility of the diseased patient. If there is a cause of disease in your environment you can choose to change or reduce the cause, move to a new environment, or accept the conditions. Responsibility for healing is with the patient.

Today, holistic/energetic medicine is practiced in many forms. The following is a brief list of holistic/energetic medicine specialities.

Meditation, Prayer (fig.1)

Guided visualization, deep breathing, focusing on a word or sound, and being mindful of your thoughts are some of the current applications of the ancient arts of meditation and prayer; some benefits researched by Western scientists include lowered ***blood pressure, stimulation of the body's immune system, and stress release***.

Acupuncture/Acupressure

Acupuncture is an ancient form of Chinese medicine. The acupuncturist identifies blockages to the energy flow and opens up the pathways to increase circulation. This is done by inserting small needles into the skin at specific energy points along the meridians (fig.2).

Electro - acupuncture uses the same method with the addition of a regulated low - voltage

electric current sent through each needle into the meridians. Acupressure uses no needles, the trained hands of a therapist applies pressure to the nodal points on the meridians.

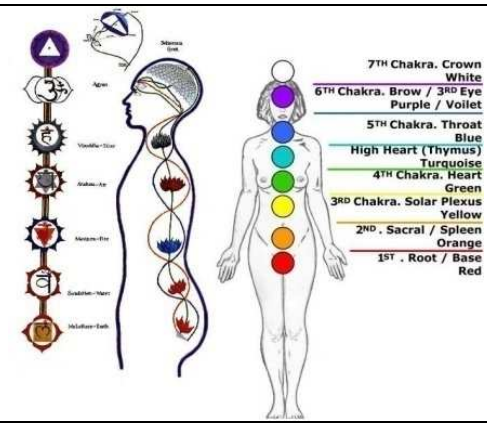

Fig.2. Human body's Chakra positions

Fig.3. Everything is Energy – chinese master

Chi Gung/Qi Gong

The movement meditation of Chi Gung sprouted the Western version called, Tai Chi Chaun; this practice is a dance like movement of the hands, body, and feet aligned with breathing techniques (fig.3).

The practitioner focuses on feeling and moving the Chi energy through the meridians of the body. Medical Qi Gong is the root from which many hands-on healing techniques branched out into the West.

Reiki/Therapeutic Touch

Reiki is a Japanese practice that trains practitioners to access the universal healing energy. This ancient modality is passed on from master teacher to student and involves specific symbol and energy transference. It is an easily accessible form of energy healing to people of all walks of life. This is a hands-on healing modality where a practitioner touches specific locations in a patient's body.

In the early 1980s, Dolores Krieger, R.N., created an American version called Therapeutic Touch. She learned some basics of spiritual healing from theosophy. She introduced this concept of spiritual healing energy, which she referred to as "prana," to thousands of nurses [5].

Homeopathy

Homeopathy was formulated 200 years ago by Samuel Hahnemann, a German physician. Remedies are prescribed according to the classical Greek Law of Similar, namely "that which makes sick shall heal". This means that the symptoms caused by an overabundance of a substance can be cured with a small dose of that substance. The homeopathic remedies are diluted so greatly that no chemical trace of the original substance remains. This process apparently imprints the energy pattern into a container of water in which it is diluted. (The Society of Homeopaths, 1998).

Flower Essences

Flower essences are vibrational preparations which were originally created by Dr. Edward Bach, a British physician, early in the 20th century. Many practitioners believe that the Bach flower remedies or flower essences are among the most popular of the homeopathic approaches (fig.4).

Fig.4. Bach Rescue Remedy

Magnetic Therapy

Studies in the application of magnets to the body have reported significant reduction of pain with applications of 11,000 gauss magnets to specific areas of the body. These magnets, which have been demonstrated to be of some benefit physiologically and psychologically, produce an electromagnetic field which creates a

differing electromagnetic response in the area of the body where it is placed.

Research has been done with 2500 gauss magnets placed six inches above the top of the head which results in increasing Delta activity deep inside the brain.

Color and Light Therapy

Colour therapy has scientific evidence to support that the application of different colored lights can markedly change body chemistry, including **oxytocin, prolactin, beta endorphins**, etc. Flashing red lights have been used successfully to treat migraine headaches. The Lucia Color Test is a psychological diagnostic based upon one's preferences of up to 156 colours.

Sound, Music and Tone

The brain can be entrained by sound frequencies just as it can be by light frequencies. The late Robert Monroe has done the best work in this field at his Monroe Institute in Virginia. The Institute has reports of research of marked **reduction in pain**, as well as many health improvements using pulse frequencies of sound. Music is well documented as an **emotional calming agent**, as well as can be used in psychotherapy to assist with emotional catharsis.

Biofeedback

Developed at the Menninger Clinic in Kansas, by **Elmer Green**, the biofeedback machine operates via electrodes connected to a patient's head and fingers. People learn to control muscle tension, skin temperature, brain wave activity, and blood pressure. A widely used modality and accepted in many medical institutions for its proven efficacy in treating migraines, high blood pressure and other illness.

Electro-Physiological Measures

Another technique was discovered by **Reinhold Voll, MD**. Dr. Voll is a German doctor who found in 1940 that the **electrical resistance of the skin decreases dramatically at the acupuncture points** when compared to the surrounding skin. This led the Western founders of the current approach to energetic medicine. These discoveries created a new field of energy medicine instruments (e.g., Voll Meter, **Meridian Stress Analyzer**) that have been developed both for assessment and treatment (fig.5).

Fig.5. Energetic medicine precursors

Energetic Medicine – Biofeedback

Franz Morrel, MD, a colleague of Dr. Voll, created another treatment instrument called the Mora. Dr. Morrel believed that **all biological processes are essentially a matter of electromagnetic signals that can be described by a complex waveform**. Health can be considered as a smooth wave, while disease is identified by unwanted variations on this wave, both higher and lower. Dr. Morrel had the idea of taking the electromagnetic signals directly from the body and manipulating the aberrant waveforms by raising or lowering them to create normal waves. These corrected waves are then fed from the device **back into the patient** at the corresponding **acu-points**. The signals can be taken from any area of the body, modified, and then returned to the specific area.

Holistic medicine in mainstream health care

In the past decade, holistic medicine has become a recognizable presence in the health-care field [6]. Holistic medicine modalities are being taught to hospital staff at California Pacific Medical Center, Health and Healing Clinic in San Francisco.

Medical Intuitives are sitting in surgery rooms at Stanford Medical Center in Palo Alto, California.

Energy medicine documentation forms for insurance claims have been available on the Internet since January 1999. [7]

Biofeedback is:

1. The most published and researched energetic medical technology in history
2. Scientifically validated and legally registered
3. Taught in medical universities
4. Natural medicine with a high tech edge.

5. Biofeedback is paid for by Medicare, Medicaid and major insurance companies in USA and Europe (Switzerland, Germany, and France) [8].

The Science of Holistic Medicine

At its essence, the practice of holistic medicine embraces a spirit of interdisciplinary and physician – patient cooperation; balances the mitigation of causes with relief of symptoms; integrates conventional and complementary therapies; and facilitates the experience of being fully alive.

Reinventing Ancient Healing Wisdom

Holistic physicians are listening to their patients, as well as their own hearts and spirits, and changing the practice and the science of medicine by the complementary inclusion of the art and science of energy medicine.

Holistic practitioners are being educated in the protocol of integrating their ancient practice of healing arts into the current western model of medicine.

Stress

Dr. Hans Selye defined a new type of medical philosophy based on accumulated stressors. "I worked with Dr. Selye and I am enclosing a paper he wrote mentioning me before his death. Stress reduction and lifestyle education is the heart of our philosophy of health care at our university. You can review our one hundred plus journals".

Animals and Spiritual Evolution.
Animals aren't just here to serve us.

Animals play an essential part in the grand plan of life. Yet too often, we think of animals simply as things to serve us when in fact, they have souls and are on an evolutionary path just as we are. Animals look up to humanity for love and inspiration.

We in the human kingdom have a sacred duty to help them in their spiritual development. Evolution unfolds at every stage of life / mineral, vegetable, animal as well as human.

Animal Energetic Healing

Quantum Biofeedback allows the practitioner to energetically communicate with your animal via resonant frequencies coming from your animal to the device [9]. By reading these frequencies, we can assess emotions, areas of inflammation, toxicity, any foods that are creating a reaction and much more.

This technology educates the animal owner of the various stresses before they develop into health challenges. It is a holistic approach that receives energetic frequencies of physical, mental, emotional and spiritual balances and imbalances.

Biofeedback is a technology that scans your body physically, mentally, emotionally and spiritually (much like a virus scan on a computer) detecting the physical and emotional stresses related to the 10,000 most common stress factors, from everyday allergies to chronic illness. The system then delivers balancing frequencies back to your pet's body to correct these imbalances and suggests lifestyle changes for your pet to maintain their new state of health. Biofeedback can be used with just about any health condition.

Studies show that 83% of degenerative chronic illness is caused by emotional stress!

Biofeedback identifies the specific stressors that are blocking the natural flow of energy through the body allowing the body to heal and strengthen your pet's overall health and vitality.

Having in mind all related information we decide to start a comprehensive study regarding this useful tool (SCIO-biofeedback) in prevention and even earlier diagnosis of acute or chronic diseases, transforming this non-invasive method in an instrument of analyzing and screening the physical, mental-emotional/affective modification of an individual (human being, animal) allowing to user (GP, family doctor, Occupational physician, veterinarian) to study the relation and interaction of humans and environmental sciences.

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GENERAL PAPERS

Xenografts in orthopedics. The necessity of developing the use of xenografts

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Abstract

Purpose

This article has the purpose of presenting the importance of developing the use of xenografts considering the need of more and more surgical grafts as well as the difficulties and limits in both quantity and quality that are faced in autografts and allografts transplants.

Patients and Methods

In the last 12 months (June 2015 – June 2016), 13 patients needed the use of morselized graft or structural graft during the surgical interventions they suffered (primary and secondary joint replacement, conversion from partial joint replacement to total joint replacement, nonunions, vicious calluses, filling spaces after bone tumor ablation).

Four of them were treated with autografts, while the remaining nine benefited from allografts from the National Bank of Bone Transplant or morselized lyophilized graft.

For three of the patients who received allografts there was the need of rescheduling the surgery due to the great volume of the needed graft. All patients were supervised after surgery in consistency with the settled protocol.

Results

The functional results were very good, the grafts integrated in all cases in reasonable periods of time, except for one case, that needed a longer period of time due to an immunosuppressive treatment.

Conclusions

There is a need of developing tissue engineering in order to have access to unlimited quantities of grafts on time. Plus, there is the need of comparative studies between the use of autografts, allografts and xenografts in order to better understand the advantages and downsides of every option.

Key words: *xenografts, allografts, autografts, tissue engineering*

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Every day, thousands of people undergo surgical interventions for repairing or replacing tissues that suffer from trauma or various affections. The treatment often consists in transplanting tissue from a donor site to a receiver (same patient – autograft or from one person to another – isograft or allograft).

Although the surgical techniques and the technology have both evolved, there are major problems with both types of grafts [1]. It is for this reason that an osteoligamentous graft – in order to have the most chances of integration – it has to be well tolerated by the host, infection resistant and fast vascularized and populated by the host's cells. It has to protect the host and accept the healing process that the body starts; it has to be nontoxic and non-immunogenic for the host, easy to sterilize and to not compromise the mechanical stability in the host. By using different types of grafts (autografts, allografts – grafts from the Bone Bank or morselized lyophilized bone graft) we surgically treated 13 patients that needed – in consistency with the pre surgery planning – the correction of bone defects or of some bone healing deficiencies. A 14th patient, which represented the most complex case (fig.1, fig.2), will only be shortly presented, because he couldn't be included in this study since his surgery had only took place 30 days before writing this paper.

PATIENTS AND METHODS

Ethics considerations

All described procedures took place and will be presented with the patients' agreement.

The patients

In the last 12 months (June 2015 – June 2016), 13 patients needed the use of morselized graft or structural graft during the surgical interventions they suffered (primary and secondary joint replacement, conversion from partial joint replacement to total joint replacement, nonunions, vicious calluses, filling bone tumor post ablation). Four of them were treated with autografts, while the remaining nine benefited from allografts from the National Bank of Bone Transplant or from morselized lyophilized graft. For three of the patients who received allografts there was the need of rescheduling the surgery due to the great volume of the needed graft. All patients were supervised after surgery in consistency with the settled protocol. A 14th patient (septic nonunion of femoral shaft with loss of bone substance of 12 cm) needed rescheduling two times because of the great defect and couldn't take part in this study, since his intervention had only took place 30 days before writing this paper (fig.1, fig.2).

The history of the xenografts

Animal models have had a very important role in modern medicine in the study of the molecular biological mechanisms and the developing of new drugs. A lot of these animal models were in fact called important evaluation and prediction methods of carcinogen substances and investigation of carcinogen mechanism, while others were used to develop tissue grafts that would later be successfully transplanted to human subjects for treating various diseases and affections.

With the development of technology there came the shrinking of the production costs and of course of the total costs paid by the state or by the patient for this type of graft. This changed how both doctors and patients accepted and used the xenografts.

The use in orthopedics

The use of grafts in orthopedics has known a great growth in the last 20 years due to the development of the harvesting, preparing, sterilizing and storing technologies. Thus, nowadays we can easily and faster treat bone affections (delays in fractures consolidation, vicious calluses, nonunions, arthrodesis or spine mergers, congenital bone defects or defects caused by trauma or tumors, bone cysts or areas of osteolysis in primary and secondary joint replacement [2]), cartilage affections (osteochondral fractures, chondromalacia, osteochondritis dissecans) and soft tissue affections (tendon ruptures – acute or chronic, ligament ruptures, meniscal tears).

Bone graft

Bone tissue in the body is the second in number of transplants performed. The bone graft is the material implanted alone or in combination with other materials that stimulates bone healing by osteoinductive, osteoconductive and bone formation.

The bone has the ability to repair and regenerate itself, but there are still situations (pathological fractures or large bone defects – fig.3, fig.4) when the repair and healing fail.

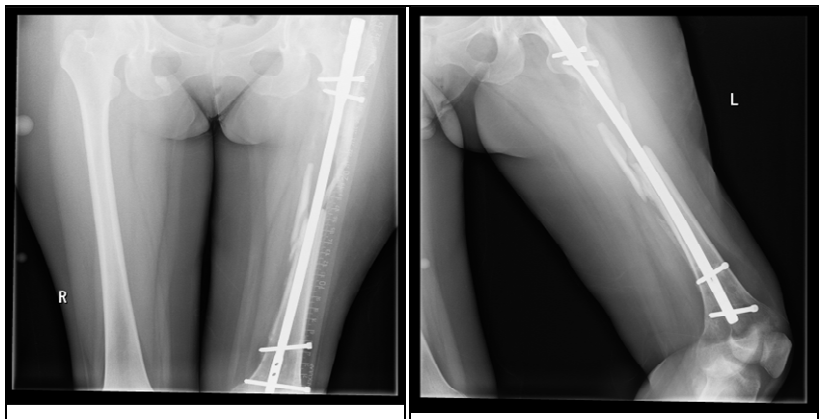

Fig.1. Patient #14 – before surgery
Atrophic nonunion of the femoral shaft due to bone loss and the degradation of the fixation assembly

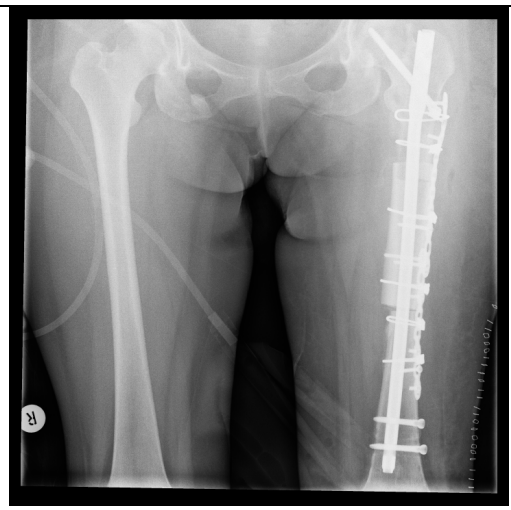

Fig.2. Patient #14 – after surgery
We used femoral shaft graft (12 cm) – from the bone bank

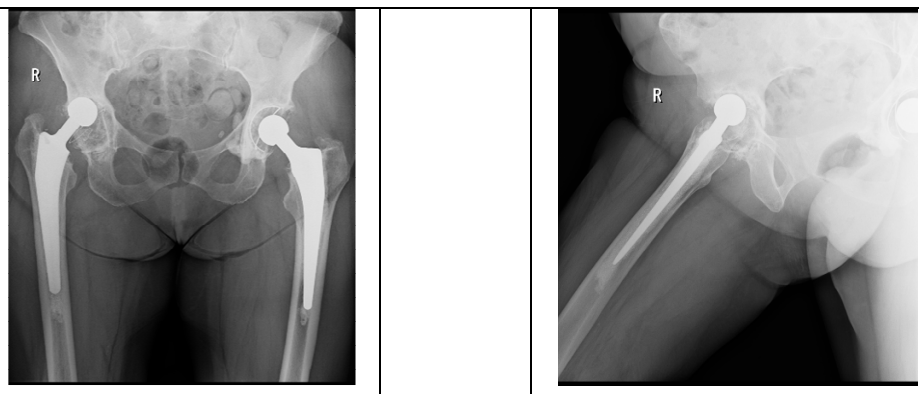

Fig.3. Patient #11 – before surgery
Bone defect following degradation of acetabular implant

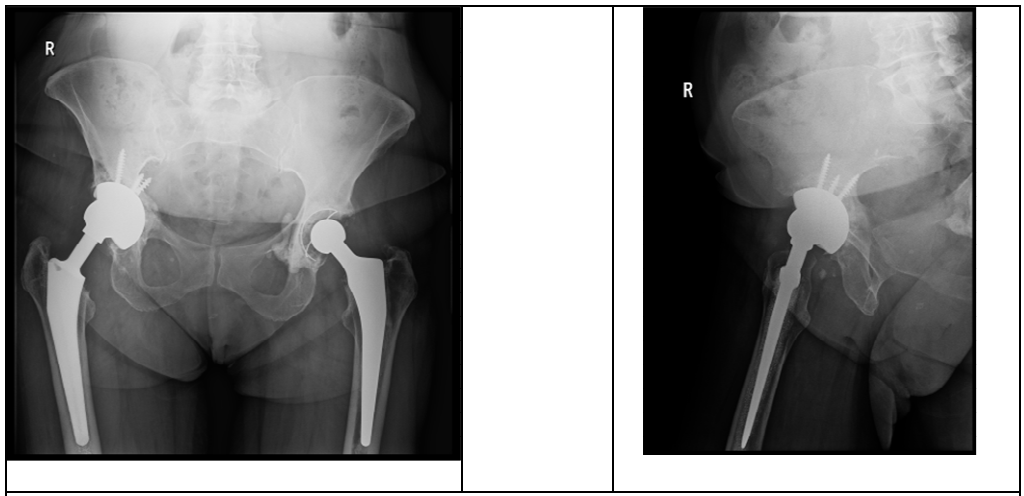

Fig.4. Patient #11 – after surgery
We used a femoral head (55mm diameter) from the bone bank

Poor visualization, tumors, bone or tissues infection and systemic diseases affected bone healing, resulting delays in bone strengthening, nonunion or even pathological bone fractures. Besides those listed, another cause involved in the bone defects is aseptic osteolysis around the acetabular implants in total hip joint replacement due to the particles disease or the abnormal mobility of the cup.

The selection of bone graft

For selecting the type of the bone graft that was used in each medical case, we had to have a very precise preoperatively planning. We established the exact size of the defect that was going to be filled/repaired, as well as the characteristics of the future graft, biomechanical and/or biological (fig.5, fig.6). Once the established shape, volume and size of the necessary graft and taking into account the availability of the tissue, the cost and the possible secondary complications linked to the harvesting location and the implanting part, we chose the type of the graft of the available autograft, allograft, xenograft, synthetic graft or biomaterials resulted from engineered tissue (Table 1).

Advantages and disadvantages of different forms of bone grafts are summarized in Table 2.

The harvesting procedure of the allografts [3]

After the notification of the death and getting the consent of the caregivers or after the donor himself has given consent we further determine the eligibility. Then, the harvesting team is informed in order to start the procedure and the needed graft is registered under a unique identification code.

Table 1. Bone grafts sources

Autografts	Allografts	Xeno-grafts	Others
Iliac crest	Corpse	Swine	Synthetic grafts
Fibula	Amputees	Cattle	Grafts resulted out of bio-engineered tissue
Ribs	Bone fragments from living donors	Horse	
Mandible			
Bones of the skull			

In the case of deceased donors it can be continued with determining additional eligibility and the tissue harvesting will be made within 12h after the confirmation of death. As additional screening it can be practice also autopsy.

The last stage of the harvesting procedure is the suitable transport to the bone bank.

The preparations of allograft

For a bone graft to be used there must undergo a process of preparation which consists of several steps. First, soft tissues must be removed (muscles, tendons, ligaments, capsule, synovium, etc), then cortical bone can be broken down into particles ranging between 500 micrometers and 5 mm (morselized grafts) or can be left in an unique fragment (structural grafts). The process of fragmentation increases the efficiency of the degreasing process of the bone.

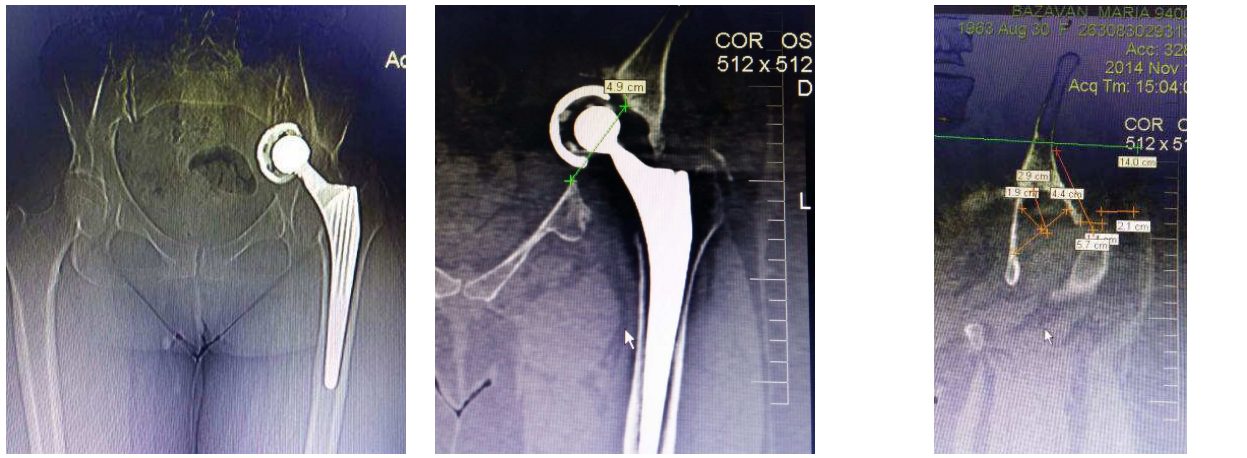

Fig.5. Patient #8 – before surgery
Acetabular bone defect after mechanical complication of the acetabular component of the total hip joint replacement

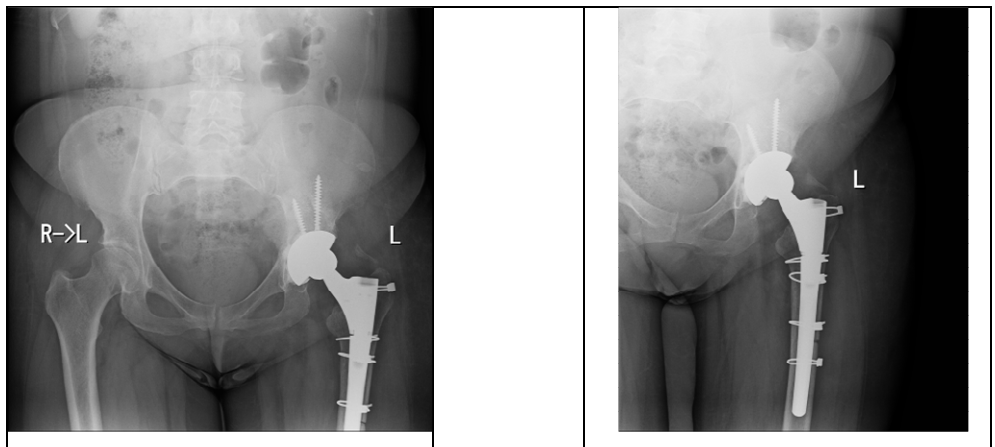

Fig.6. Patient #8 – after surgery
We used two femoral heads (56mm/52mm diameter) from the bone bank

Table 2. Advantages and disadvantages in the use of bone grafts [4]

Type	Advantages	Disadvantages
Autografts	• ensures cells and growth factors responsible for tissue repair and regeneration; • there is no risk of disease transmission; • there is no risk of graft rejection	• a second surgery is necessary; • reduced available quantity; • the risk of pain or other complications in the donor area; • not all patients have suitable donor areas
Allografts	• osteoconductive, but weak osteoinductive; • no longer need a harvesting surgery, thus avoiding the possible complications related to one; • provide immediate structural support after implant	• they can cause an immune response from the host; • there is a risk of disease transmission; • after processing, the allografts lose up to 50% of the structural integrity and mechanical strength; • increased integration time needed; • very few studies available; • reduced availability; • high costs of processing and storage
Xenografts	• unlimited availability in both number and size; • immediate availability	• the low penetration of fibroblasts, chondroblasts or osteoblasts; • there is a risk of disease transmission; • evolution of local remodeling; • the human host immune response after implantation

The graft is then introduced into ethyl alcohol (100%) for an hour, in order to eliminate the adipose tissue which might inhibit the osteogenesis and to inactivate the potential viruses. Then, the graft is frozen at -80°C for 1-2 weeks, so that the degradation process is stopped and the water in it's' structure is eliminated with the aid of a lyophilizing process. During all this time, the results from the anti-biogram are analyzed, together with the serologic tests and the measuring of antibody and antigens. Any positive result determines the removing of the bone graft. By removing the water from the bone graft the antigenicity is reduced and the bedding is enabled. The preparing process ends by storing up at low temperature for the structural grafts and at room temperature for the morselized grafts in voided recipients (in this situation, additional steps are required).

Diagnosis protocol

All the patients have come after the intervention for further analysis, adequate to every particular case, so that the diagnostic routine is customized.

After surgery rehabilitation

As soon as the surgery is over, during the hospitalization period, the patients should start the rehabilitation protocol, under the supervision of a specialist; the protocol is continued after the hospitalization, following the customized received indications, in a dedicated kinetotherapy centre.

After surgery assessments

All patients were clinically and radiologicaly assessed after surgery at 45, 90 and 180 days. Further checks up were performed annually.

Graft integration

Integration into the host organism depends on the graft characteristics (osteoconduction, osteoinduction, osteopromotion and osteogenesis) which may vary depending on the source (autograft, allograft, xenograft) or their structure (cortical or cancellous).

A rapid integration and healing of the bone defect is obtained in the context of an optimal revascularization process. In case of an avascular graft, blood vessels penetrate graft from its vicinity, so the healing time is prolonged (neovascularization).

Table 3. Synthetic results (part 1)

Patient	Diagnostic	Surgical intervention	Graft type	Graft integration		
				45	90	180
1	Nonunion of the medial cubital shaft	• nonunion excision • vascularized fibular bone graft • plate and screws fixation	**AUTOGRAFT** • vascularized fibular bone graft (fig.7, fig.8)	--+	-++	+++
2	Carpal scaphoid non union	• nonunion excision • autologous bone graft from distal radius epiphysis; • Herbert screw fixation	**AUTOGRAFT** • autologous bone graft from distal radius epiphysis	-++	+++	+++
3	Hypertrophic nonunion of the radius shaft due to fixation assembly degradation	• nonunion excision • autologous bone graft from the iliac crest • plate and screws fixation	**AUTOGRAFT** • autologous bone graft from the iliac crest (fig. 5-6)	---	--+	--+
4	Nonunion of the femoral shaft due to fixation assembly degradation	• nonunion excision; • intramedullary rod fixation; • morselized bone graft – lyophilized bone fragments with autologous bone marrow from the iliac crest	**ALLOGRAFT+ AUTOLOGOUS BONE MARROW** • morselized bone graft – lyophilized bone fragments with autologous bone marrow from the iliac crest	--+	-++	-++
5	Proximal tibia epiphysis tumor – risk of fracture	• curettage and filling the bone defect with morselized allograft; • plate and screws fixation;	**ALLOGRAFT** • morselized bone graft – femoral head from the bone bank	--+	-++	-++
6	Proximal humeral metaphysis tumor	• curettage and filling the bone defect with morselized allograft • plate and screws fixation;	**ALLOGRAFT** • morselized bone graft – lyophilized bone fragments	-++	+++	+++
7	Acetabular revision after mechanical complication of the acetabular component of the total hip joint replacement	• morselized bone graft for the acetabular bone defects • revision of the total hip replacement;	**ALLOGRAFT** • morselized bone graft – femoral head from the bone bank	-++	+++	+++
8	Acetabular revision after mechanical complication of the acetabular component of the total hip joint replacement	• morselized bone graft for the acetabular bone defects • revision of the total hip replacement;	**ALLOGRAFT** • morselized bone graft – femoral head from the bone bank	-++	+++	+++

Table 3. Synthetic results (part 2)						
Patient	Diagnostic	Surgical intervention	Graft type	Graft integration		
				45	90	180
9	Nonunion of proximal humeral metaphysis	• morselized bone graft – lyophilized bone fragments with autologous bone marrow from the iliac crest	**ALLOGRAFT + AUTOLOGOUS BONE MARROW** • morselized bone graft – lyophilized bone fragments with autologous bone marrow from the iliac crest	-++	+++	+++
10	Ankle osteoarthritis due to vicious talus consolidated fracture (fig.11, fig.12)	• ankle arthrodesis using autograft and tricalcium phosphate bone substitution • screw osteosynthesis	**AUTOGRAFT** • autograft and tricalcium phosphate bone substitution	--+	-++	+++
11	Acetabular revision after mechanical complication of the acetabular component of the total hip joint replacement	• morselized bone graft for the acetabular bone defects • revision of the total hip replacement;	**ALLOGRAFT** • morselized bone graft – femoral head from the bone bank	-++	+++	+++
12	Acetabular revision after mechanical complication of the aetabular component of the total hip joint replacement	• morselized bone graft for the acetabular bone defects; • revision of the total hip replacement;	**ALLOGRAFT** • morselized bone graft – femoral head from the bone bank	-++	+++	+++
13	Acetabular revision after mechanical complication of the aetabular component of the total hip joint replacement	• morselized bone graft for the acetabular bone defects; • revision of the total hip replacement;	**ALLOGRAFT** • morselized bone graft – femoral head from the bone bank	-++	+++	+++
14	**Atrophic nonunion of the femoral shaft due to bone loss and the degradation of the fixation assembly**	• **non union excision** • **intramedullary rod fixation;** • **femoral shaft graft (12 cm);**	**ALLOGRAFT** • **femoral shaft graft (12cm) – from the bone bank**	---	?	?

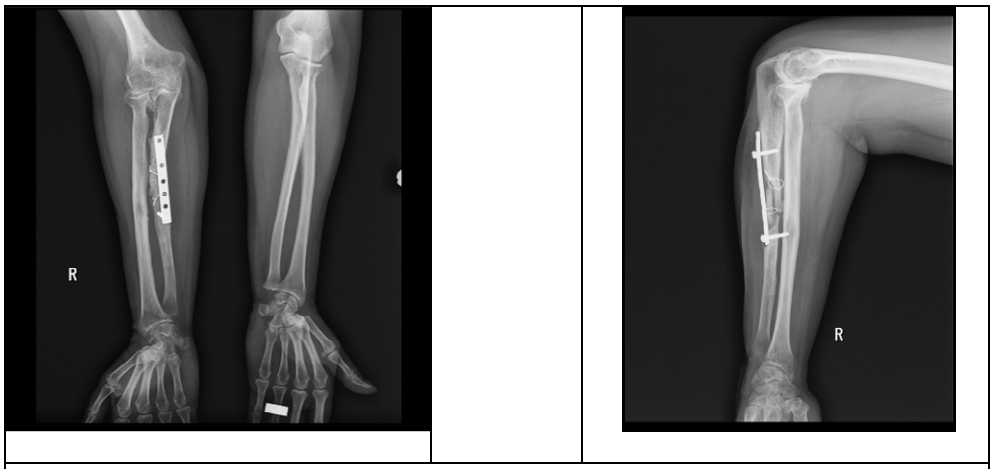

Fig.7. Patient #1 – preoperatively
Nonunion of ulna shaft

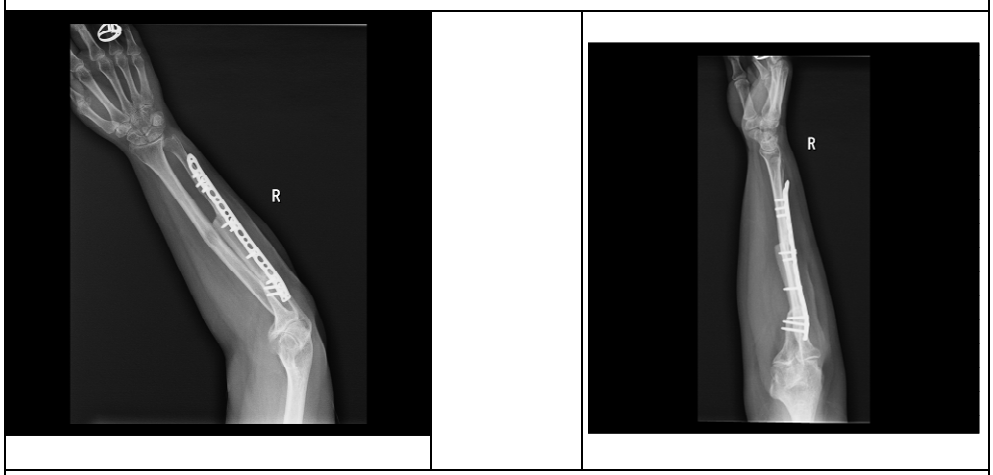

Fig.8. Patient #1 – after surgery
We used vascularized fibular bone graft

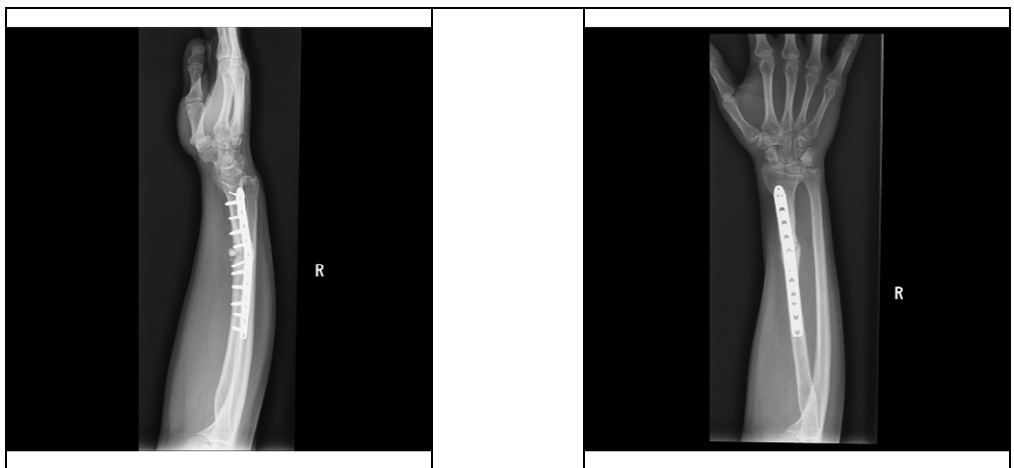

Fig.9. Patient #3 – after surgery
Hypertrophic nonunion of the radius shaft due to fixation assembly degradation

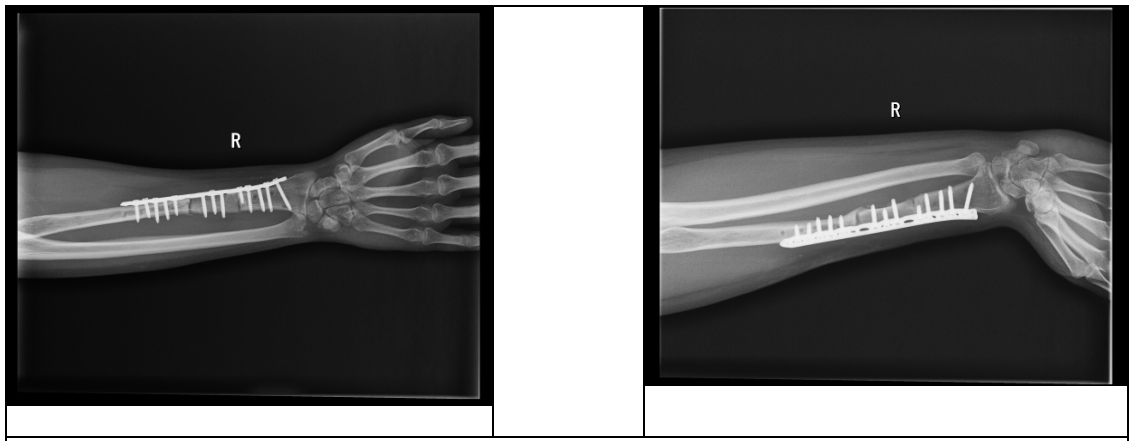

Fig.10. Patient #3 – after surgery
We used iliac crest bone graft.

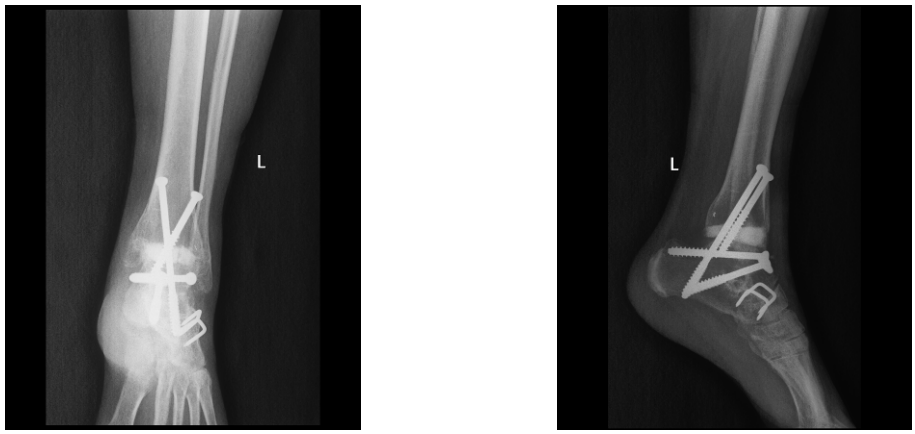

Fig.11. Patient #10 – after surgery
Ankle osteoarthritis due to vicious talus consolidated fracture - ankle arthrodesis using autograft and tricalcium phosphate bone substitution

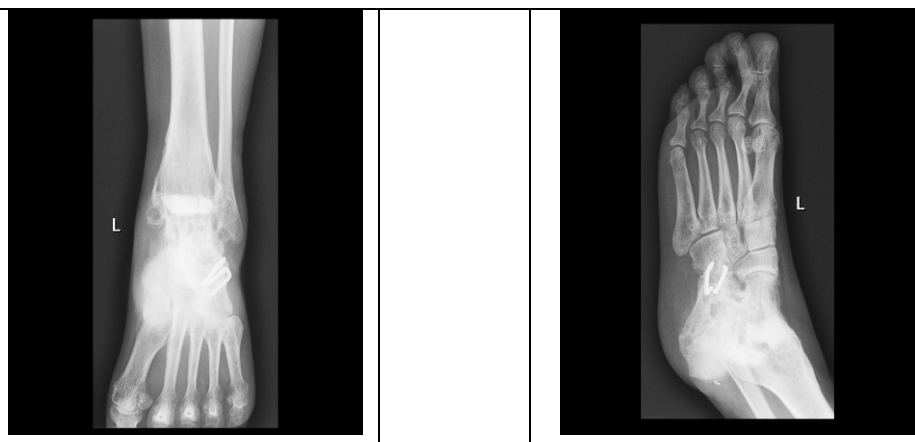

Fig.12. Patient #10 – after second surgery for implant removal

Whatever type of graft is used, the integration process is considered to pass consists of five stages: inflammation, revascularization, osteo-induction, osteoconduction and a final remodeling.

Host response

The host response to the received grafts occurs sometimes in despite of respecting the step by step protocol of preparing them. The incidence and importance of immune response to the xenografts is still smaller when compared to the one in case of using the synthetic grafts (which are non-absorbable) where there is a strong reaction [5].

RESULTS

All patients were available for consultations and reassessment. Radiologicaly, there were no problems regarding the accuracy of the surgical technique used and there were no signs noticed of endoprosthetic device or implant degradation. All bone grafts showed evidence of integration in all 13 cases at the last reassessment. All patients reported an improved life comfort after the surgery and rehabilitation (Table 3).

CONCLUSIONS

Although currently there are on the market a variety of natural and synthetic bone grafts, pseudarthrosis and bone defects constitutes an important challenge to the orthopedic surgeons. Eventhough the "gold standard" treatment of bone healing problems is still represented by autografts (biological mechanisms are clearly in their favor) there should be taken into account both the efficiency and the importance of allografts (from the national bone bank) and xenografts. For this reason studies of bone metabolism are required in research for each type of bone graft.

The high cost of xenografts compared to the lifestyle in our country determine sometimes some patients to make compromises, choosing for their cases inadequate therapeutic solutions [6,7]. Three patients in this study had to be rescheduled for surgery because of the high volume of the necessary graft and also for the reduced availability of allografts at the bone bank.

Given the qualitative and the quantitative limitations of the autografts and allografts, the development of an ideal bone graft that has osteogenic cells, growth factors and osteo-inductive and osteoconductive matrix represents a binding target to achieve. It is noticeable the need for development in the medical environment in our country of this type of 'industry', which should be financially and logistically sustain by similar institutions or by third parties in order to obtain and to make available to treating physicians the both qualitative and quantitative sufficient graft of each medical case separately. Also the idea of xenograft should be promoted among the population using medical education, scientifically arguing their importance in order to eliminate the misconceptions regarding the use of animal products.

Xenografts can be used both individually or impregnated with antibiotics in the treatment of chronic osteomyelitis with bone cavities or in combination with other products such as: autologous bone marrow, PRP (or PRF, PRGF, A-PRF, I-PRF), BMPs (Bone Morphogenetic Proteins) [8].

Bone morphogenetic proteins are osteo-inductive and induce in vitro osteo-blastogenesis and in vivo bone formation, and in the future gene therapy might be the next solution.

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Comparative analysis of antimicrobial resistance in *S.aureus* strains associated with *Staphylococcal Food Poisoning* investigated at the *National Reference Laboratory for Staphylococcus, Cantacuzino* National Institute of Research, 2009-2013

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Abstract

Antibiotic resistance can be considered a zoonotic public health problem. Our study aimed at comparing antimicrobial resistance patterns of S.aureus *strains isolated from food and human hosts, in order to evaluate potential sources of resistant strains or resistance genes of animal and/or human origin.*

We analyzed at the National Reference Laboratory for Staphylococcus, Cantacuzino *National Institute of Research, 95 strains associated in the 2009-2013 interval with 9 Staphylococcal Food Poisoning (SFP) outbreaks, of which 34 from patients (vomit liquid, coproculture, 31 from food (dairy products, creams, meet products, vegetables) and 30 from human hosts involved in the food chain (nose, throat or skin). Antimicrobial susceptibility testing was performed by using the Kirby Bauer disc diffusion method, according to the CLSI standard.*

Antimicrobial Susceptibility Testing to Vancomycin was performed by using the E-test.

We compared rates of resistance in every strain category for each antibiotic. Zone diameters values have been analyzed by using the 19.0 variant of the SPSS program. By using the Ward minimum variance method, we obtained a dendrograme which allowed us to visualize the resistance clusters.

We noticed significant differences between strains isolated from patients, staff and food, regarding rates of resistance to Penicillin (97%, 90%, and 71% respectively). Strains isolated from food showed obvious higher resistance rates than human strains to Tetracycline (64,5% versus approx. 30%), Erythromycin (74 % versus approx. 27%) and Clindamycin (71% versus approx. 25%). We obtained 11 resistance clusters, with strains isolated from food clearly grouping in one of these clusters.

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Conclusions: Food represents a distinct source of antimicrobial resistant strains and/or antimicrobial resistance genes that may interfere with antimicrobial resistance background of human origin bacteria. Integrative surveillance of antimicrobial resistance may contribute to better understanding of AR transmission dynamic, which could fundament better public health actions to prevent and/or curbe antimicrobial resistance phenomenon both in animals and humans in our country.

Key words: *antimicrobial resistance, Staphylococcus aureus, One Health approach*

Background

Antibiotic resistance (AR) can be considered a „zoonotic" public health problem [1,2,3,4,5].

AR epidemiology is one of the most complex recognized by a public health transmissible problem. (fig.1) [2].

Antibiotics are used in therapeutic purposes both in humans and animals, in the last case both in livestock and in pets.

Besides therapeutic use, antibiotics have been used as growth promoters in food animals or chickens, which may lead to presence of bacteria and/or AR genes in food [5, 6, 7, 8, 9, 10].

There are several scientific proofs on the effect of antibiotic consumption on selecting antibiotic resistant bacteria, which may spread bi-directionally between human hosts or humans and animals, being able additionally to contaminate the environment, including plants, aquatic life creatures etc. [11,12,13].

In spite of legal provisions banning the use of antimicrobials as growth promoters, particularly drastic in Europe [4,9,14,15,16], not all countries took constant action in order to reduce this practice.

Another sequence of food chain that could be involved in AR spread is processing of meat, milk, fish, vegetables, seeds, fruits, cereals etc. [2].

All mentioned mechanisms favor both presence of antimicrobials in raw animal products and/or in food and selection of resistant bacteria which may reach human host.

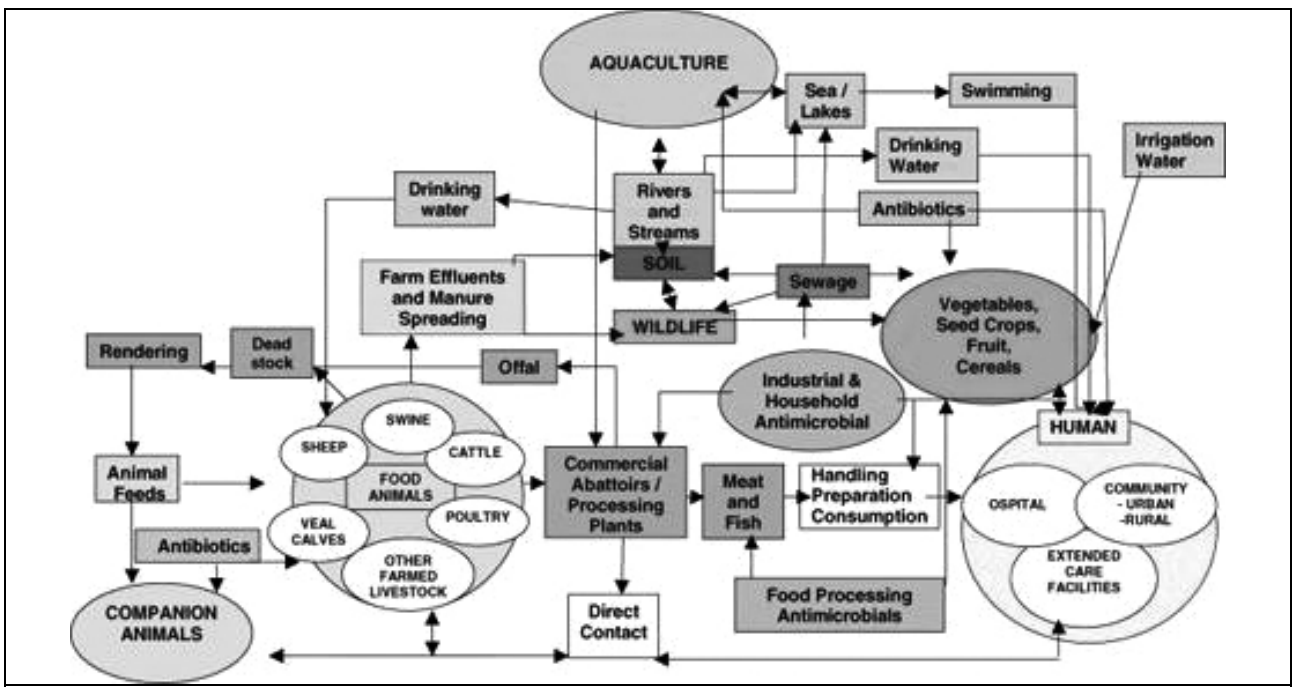

Fig.1. Schematic representation of AR epidemiology – adapted after ref. [7]

Table 1. Distribution of *S aureus* strains by sample types

Sample	Source	Number of strains / %
stool	patients	23 (24%)
vomit liquid	patients	11 (12%)
pharyngeal exudate	food staff	5 (5%)
nose exudate	food staff	24 (25%)
skin	food staff	1 (1%)
food	food	31 (33%)

Food consumption followed or not by a food or water born infection, including Staphylococcal Food Poisoning (SFP), represents one of the most frequent modalities to introduce antimicrobial resistant bacteria and/or antimicrobial resistance genes inside the human host [6].

Our study aimed at comparing antimicrobial resistance patterns of *S.aureus* strains isolated from food and human hosts, in order to evaluate potential sources of resistant strains or resistance genes of animal and/or human origin.

MATERIALS AND METHODS

We analyzed at the *National Reference Laboratory* for Staphylococcus, *Cantacuzino* National Institute of Research, 95 strains associated with 9 Staphylococcal Food Poisoning (SFP) outbreaks which evolved in the 2009-2013 interval in 6 counties: Prahova, Maramures, Iassy, Vrancea, Satu-Mare, Constantza.

Of the analyzed strains, 34 have been isolated from patients, 31 from food and 30 from human hosts involved in the food chain (Table 1).

S.aureus strains have been confirmed by using a PCR for detecting the *nuc* gene, coding for *staphylococcal thermonuclease* [17].

Antimicrobial susceptibility testing was performed by using the Kirby Bauer disc diffusion method, according to the CLSI standard, to the following antibiotics: Penicillin G (10 UI), Cefoxitin (30 µg), Erythromycin (15 µg), Clindamycin (2 µg), Kanamycin (10 µg), Tobramycin (10 µg), Gentamycin (10 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Rifampicin (5 µg), Trimethoprim-sulfamethoxazole (25 µg), Vancomycin (30 µg), Quinupristin-Dalfopristin (15 µg), Linezolid (30 µg), Amoxicillin-clavulanic acid (20/10 µg) and Cephalothin (30 µg).

Methicillin Resistant *Staphylococcus aureus* strains (MRSA) have been confirmed by disc diffusion, by using the Cefoxitin disc, according to CLSI standard and by PCR targeting the *mec*A gene [18,19].

Table 2. Patterns of AR in *S.aureus* strains associated with SFP outbreaks

Resistance patterns	No. resistant strains (n = 90)	% resistant strains
P	44	49
P, TE	3	3
P, SXT	1	1
P, E, TE	1	1
E, DA (MLS_Bi), TE, QD	8	9
P, E, DA (MLS_Bi), QD	3	3
P, E, DA (MLS_Bi), K, QD	1	1
P, E, DA (MLS_Bi), TE, QD	18	20
P, FOX, TE, KF, AMC	1	1
P, FOX, K, KF, AMC	1	1
P, E, DA (MLS_Bi), K, TE, QD	6	7
P, FOX, K, TE, KF, AMC	1	1
P, FOX, E, DA (MLS_Bi), K, TE, QD, KF, AMC	2	2

Table 3. MRSA strains – AR patterns

No. of strain	Year of isolation	County	Sample	AR pattern
8393	2012	Prahova	Vomit liquid	R: P, FOX, E, DA(MLSBi), K, TE, QD, KF, AMC
9205	2012	Iassy	Nose exudate	R: P, FOX, TE, KF, AMC
7499	2011	Prahova	Throat exudate	R: P, FOX, K, TE, KF, AMC
7500	2011	Prahova	Nose exudate	R: P, FOX, K, KF, AMC
8062	2011	Maramures	Vomit liquid	R: P, FOX, E, DA(MLSBi), K, TE,QD, KF, AMC

Table 4. S.aureus strains – AR patterns by sample type

Antibiotic	% resistance $n = 95$	% resistance/ Food $n = 31$	% resistance / Food chain staff $n = 30$	% resistance/ Patients n = 34
Penicilline G	86	71	90	97
Tetracycline	42	64.5	33	29
Erythromycin	41	74	27	26.5
Clindamycin	40	71	27	23.5

Amplification of *mecA* gene was done in an Applied Biosystems thermocycler, by using the following primer pair:

- *mec*A 1: 5'AAA ATC GAT GGT AAA GGT TGG C 3'
- *mec*A 2: 5' AGT TCT GCA GTA CCG GAT TTG C 3'

Antimicrobial Susceptibility Testing to Vancomycin was performed by using the E-test.

Zone diameters values have been analyzed by using the 19.0 variant of the SPSS program.

By using the Ward minimum variance method, we obtained a dendrogram which allowed us to visualize the resistance clusters.

RESULTS

Ninety from the 95 *S.aureus* strains showed resistance to at least 1 antibiotic. Resistance patterns of the studied strains are recorded in Table 2.

MRSA strains have been isolated from patients (2 cases) and from food chain staff (3 food staff).

Methicillin resistance is usually considered as a marker of multiple resistance to antibiotics and results of our study are confirming this hypothesis. All the 5 MRSA strains showed resistance to other classes of antibiotics: aminoglycosides, tetracyclines and/or inducible resistance to macrolides, lincosamides and streptogramins B (MLSBi) (Table 2 and 3).

Analysis of resistance rates of *S.aureus* strains associated with SFP outbreaks by sample type revealed significant differences between strains isolated from patients, food chain staff and food (Table 4).

We noticed significant differences between strains isolated from patients, staff and food, regarding rates of resistance to Penicillin (97%, 90%, and 71 % respectively).

Strains isolated from food showed obvious higher resistance rates than human strains to Tetracycline (64,5% versus 33/29%), Erythromycin (74 % versus approx. 27/26.5%) and Clindamycin (71% versus 27/23.5%).

Analysis of zone diameters values by using the 19.0 variant of the SPSS program, the Ward minimum variance method, resulted in a dendrogram which allowed visualization of the resistance clusters corresponding to food strains (fig. 2).

Ward minimum variance method (Ward, 1963) best reflects successive changes in inhibition zones, being considered as the method with the best applicability in analyzing antimicrobial resistance phenotypic data [20] by minimizing the intracluster variability [21].

Comparative analysis has been done by using the ANOVA test. Eleven clusters have been obtained, with strains isolated from food clearly grouping in one of these clusters indicated inside of the oval shape (Fig. 2).

Our results are concordant with others authors findings, showing high resistance in *S.aureus* strains isolated from different food products in different geographical areas [22, 23, 24].

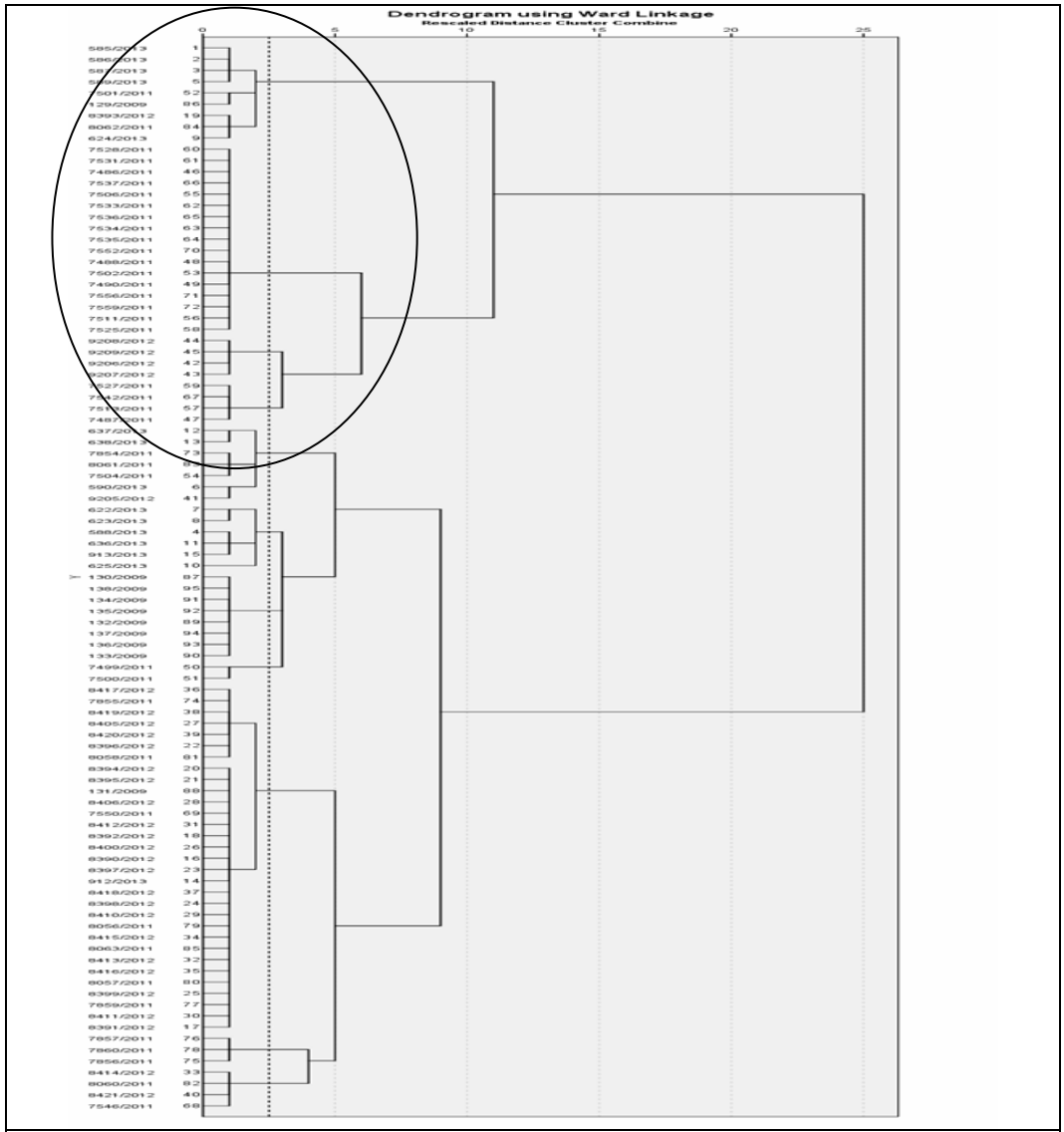

Fig. 2. Dendrogram of AR resistance patterns of *S.aureus* strains associated with SFP outbreaks – Ward method (SPSS 19.0 program), median 2.87

More than this, by analyzing a set of 90 *S.aureus* strains from Belgian poultry collected in 1970-1972 interval comparatively with 81 strains collected in 2008, Nemati et al. found that the percentage of resistance was significantly higher for the recent isolates than for the old isolates for all antimicrobial agents tested, except for Enrofloxacin, Sulfonamides, and Tetracycline, where no significant difference was found between results for old and recent isolates, and for Penicillin, where the percentage of resistance for the old isolates was significantly higher than that for the recent isolates [23].

All authors emphasized the importance of food as vehicle of antimicrobial resistant strains and resistance factors and need of public health surveillance and interventions.

CONCLUSIONS

Our results support the hypothesis that food represents a distinct source of antimicrobial

resistant strains and/or antimicrobial resistance genes that may interfere with antimicrobial resistance background of human origin bacteria.

Integrative surveillance of antimicrobial resistance may contribute to better understanding of AR transmission dynamic, which could fundament better public health actions to prevent and/or curve antimicrobial resistance phenomenon both in animals and humans in our country.

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Advanced research on the influence of industrial contaminants on health

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Abstract

The paper presents the studies performed in the frame of research theme One Health*, promoted in priority program of the Romanian Academy* Efficient valorization of the natural resources national patrimony. *There are analyzed the classification criteria for industrial contaminants in relation to the concept of integrated health for environment, population, as well as rent animals. There legal regulations on the specific aspects of the field are analyzed in terms of Romanian Constitution regarding protected environment, the right to the health and food security. A special chapter is dedicated to the actions of harmful contaminants powdered substances particularly dangerous for people and rent animals. There are given the results of studies regarding the powders by cosmic, volcanic and telluric origins and those resulting from human actions (radioactive dumps, cement factories, waste storages, etc.). There are discussed general and specific actions of industrial contaminants in clinical diseases at the digestive and respiratory apparatus as well as skin and eye irritation. There are described the methods of removing harm effects by industrial sites greening and improving the quality life of populations and rent animals. Preliminary proposals are presented by the authors about the future development of the field of* One Health.

Key words: *industrial contaminants, greening, One Health*

Based on the real requests of the civil society and state budget institutions regarding the health of people, animals and environment, in the development of sustainable life system, in Romania was created a professional association ONE HEALTH- NEW MEDICAL CONCEPT in order to promote integrative actions [1] for comparative medicine, comparative oncology, nutrition and food studies, checking of the zoonoses as well as occupational items. In this respect, the research in Romanian Academy included a task referring to the implementation of the eco-economic policy developed on the ONE HEALTH field [2]. There are studies for conservation of biodiversity, the growing in semicaptivity of birds in RED LIST [3] as well

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as the rehabilitation of important ecozones of the country. The pollution influenced the behavior of the rent animals as well as the food safety of the nation.

1. Powders

The powders are one the most important element of the air contamination, they being in the category of self scattered systems made by a dispersion phase as well as one of environment. Having in mind that some properties of the selfscattered systems there are nearly by colloidal particles. Gifs proposed the name of aerosols for the powders in air [4].

The stability of aerosols in the atmosphere is selective because it depends by:

- the properties of scattering phase (items): their size, form as well as their hardness and electrical charge;
- the properties of dispersion environment as the temperature and humidity, the air streams existence and their intensity.

For these reasons, the aerosols can loose their stability in atmosphere and the phenomenon of sedimentation will appear due the terrestrial gravity. Table 1 presents the correlation between the sedimentation speed and particle size [5].

The powders in air can be produced by multiple sources, the most significant being the following:

a. cosmic powders which permanently exists in the atmosphere, characterized by a significant dispersion, a reduced sedimentation (about 70 g/km^2; consequently, they are not important from hygienic aspects;

b. volcanic powders due the volcanic eruptions, characterized by large power of scattering, being spreaded by the wind on the very long distances; they influence negatively the sun transparency on the limited time duration, the sun radiation to the soil level will be reduced. In 1995, Kuju volcano in Japan became active after 300 years sent in the atmosphere large amounts of smog and ash, up to the altitude of 2400 m, spreaded by air streams on the an area extended up to These powders (in big amounts) have a direct negative action, on the human health as well as on the animal life, but also indirect influence by feeds, limited in time and space;

c. telluric powders coming directly by the destruction of parts in terrestrial layers. This production is permanently, in appreciable quantities. These powders are different, depending by soil, rainfalls, air streams in the zone. The telluric powders in great amounts can influenced the life and the health of the rent animals, in the rural areas.

d. powders in human activities are the most important source of aerosols, due the high level of industrial development. These aerosols have a high nocivity levels.

For these reasons the aerosols can loose their stability in atmosphere and the phenomenon of sedimentation will appear due the terrestrial gravity. Table 1 presents the correlation between the sedimentation speed and particle size [5].

Table 1
The sedimentation speed of particles versus diameter

Particle diameter (μm)	Sedimentation speed
50 – 100	4 cm/min
10 – 50	0.15 cm / min
6 – 10	0.04 cm / min
1 – 6	12 cm / h
0.5 – 1,0	3.5 cm / h
0.1 – 0.5	0.1 cm / h

Such aerosols from burning (combustion processes in housing, transportation etc.) are represented mainly soot and ashes and health problems is of prime importance (especially in environmental protection). In this category falls and dust from various industries with high environmental pollution (cement, carbon black, dyes, fertilizers, power plants etc.), factories are now bound by international laws. They are equipped with filters or other means to reduce (even eliminate) emissions to air. Therefore, in large urban centers or industrialized areas there are always larger quantities of powder (over 50,000 particles for 1 m^3 air).

Food safety for a population of over 7 billion from the Earth required an intensification and livestock so as to be satisfied "protein hunger". Therefore, at present, livestock farms maintained regardless of the species, number of animals and their operating system can be considered as sources of air pollution resulting from growth and exploitation of animals. Classification of dust in the air can be achieved by several criteria, of which we will present next only those with hygienic importance [6].

1. After **predominant chemical composition** of aerosols found in various areas, powders are as follows:

a) Inorganic powder:

- metals: Pb, Fe, Zn, Cu
- minerals: silicates, SiO_2, asbestos
- synthetics: cement, soda, glass ,dyes.

b) Organic dusts:

- vegetal dust produced by wasted leaves and lignified fibers, pollen, cotton;
- animal dust resulting from chopping fibers of wool, hair and feathers;
- synthetics: pesticides used in powder form, organic dyes.

2. After **particle size**:

- dust with a diameter greater than 10μ (dust itself) that settles a uniformly accelerated speed even in free air currents;
- powder with diameter between 0.1 – 10μ (dry mist) which settles at a uniform rate over time (according to Stokes law);
- powder diameter under 0.1μ (smoke) representing aerosols do not settle and have a great power of diffusion.

Laden air entering the respiratory aerosols can cause retention of various particles according to their shape and diameter (Table 2).

Table 2
Retention of particles at different levels of the respiratory system, depending on the size

Size (μm)	Level up to entering
> 50	Not penetrate recessed; Previous airways are retained and can be easily eliminated
10 – 50	Not penetrate recessed; Previous retained airways and bronchi are being gradually eliminated
5 – 10	Can penetrate the socket exceptionally
0.2 – 5.0	Penetrate recessed; particles are most commonly found in recessed
< 0.2	Can penetrate and be retained in the socket

2. Action powders on animals

The mode of action of dust in the air is closely dependent on the nature, quantity and chemical composition of these aerosols and could act either as toxic pollutants (if they contain in their composition a toxic agent) or pollutants nontoxic, who arrived in the internal body can cause inflammation of "foreign body"[7].

The harmful dusts can be differentiated in:

1. **Action-specific or general** that is felt throughout the body and manifests itself in different ways considering general conditions:

a. **toxic action**, which is mainly due to toxic powder (containing Pb, Cu, Zn, As, etc.) that enter the body via the digestive, respiratory and skin especially causing clinical symptoms and pathological aspect specifically in the tissues that come in direct contact. If their absorption in large quantities severe poisoning can occur, usually with chronic course ("poisoning") found mainly in animals raised in industrial areas, where such items are spreading toxic atmosphere using air currents [3].

b. **allergic action** is characteristic powder whose chemical composition designed to make the allergen by repeated contact with bodies which sensitizes them [10].

Of known allergens can remember pollen grains, dust, wool, epidermal scales, these dust allergy inducing human condition (fiber weaving, asthma weavers, fiber flax, once paprika disease etc.). Manifestations allergic to animals caused by dust were less defined, can still remember his results Asaj et al. (1979), cited by C. Draghici on sensitivity to multiple allergens hens and seized most intense reaction to dust shelters with bedding in innumerable different infested with spores of Aspergillus species. In horses, SERT (1969) and Ghergaru (1984) [8] managed reproduction of asthma by administration of moldy hay. In cattle, the same researchers (C. quoted Draghici) [9,10] have established that some pneumonias allergic (extrinsic alveolitis) due the allergenic factors by dust.

c. **irritant action** that is due mostly hardness and shape of the particles and triggering sharp reaction defensive tissues that come in contact, usually causing intense itching, permanent so that the animals are restless.

d. **carcinogenic action** caused by some aerosol known for facilitating the development of neoplastic processes (aromatic hydrocarbons, 3.4-benzpirenul etc.) is a risk factor for animal health. Cancerous disease could be reproduced in laboratory animals using extracts of "smog" that envelops numerous cities of the world.

e. action is due to **infectious or parasitic microbial agents** particulate vehicle, both in

the atmosphere and especially in air animal shelters. The implications of these agents biotic pathogenic or conditionally pathogenic animal pathology, this action appears as the most important in relation to animals, because dust favors the development of infections and infestations by irritating the tissues by creating "solutions of continuity" or circulatory disorders local.

2. **Specific actions** (local or "target") which is able to be dust in the etiologic agent of diseases with well defined clinical course and symptoms that called name conioze (KONI = powder). The most common illness caused by dust in the air is found in tissue, organs or appliances that come in connection with the external environment.

a. the action powders to the **skin** (dermatoconioze) is predominantly mechanical, because particles can obstruct pores of sebum fixed in sweat or sebaceous glands, causing inflammatory reactions (acne, pyoderma) or 30-35% reduction in sweat sequence, with adverse consequences thermoregulatory mechanism. Caustic powders (lime, soda) affects on the regions with glabrata and fine skin (axilla, urogenital areas) can cause ulcers and crevices, which quickly infects and therapeutic challenge.

b. powders action on the **eye** (oftal-moconioze) is due powders that come in direct contact with the eyeball and its annexes, causing irritation in the conjunctiva with increased lacrimation (lacrimation). A prolonged action, these purulent conjunctivitis become infected and may affect the cornea (keratitis) or lens (cataract), resulting in blurred vision. Oftal-moconioze are more likely to feed animals receiving fiber (generating particles) in high barbecues.

c. the action of dust on the **digestive system** (enteroconioze) is less important for animals because they have to reach this level only indirectly through ingestion of feed containing sediment particles on them either during the growing season or during stagnation in the shelter. By ingestion, dust or toxic stomatites can cause gastric irritation, and those with infective action can contribute to digestive transmission of infectious diseases.

d. **actions on the respiratory system** (pneumoconiosis) are most important, since this devices most exposed by their forced penetration with the inspired air. By exhalation removes some of the powders are inspired, but each time the respirator is retained a quantity of particles. Retention of aerosols in the anterior and posterior airway is achieved by: impaction initial gravitational sedimentation, diffusion and direct contact of the powder with airway wall. Penetration of aerosols through the nose and their detention in the lungs depends on the size of the particles [10].

Removing dust from the respiratory system – clearance lung – is achieved through action macro-mechanics due reflexes of defense (sneezing, coughing) and through action micromechanics due appliance mucociliary (mucus in the airways and alveoli, cilia of the epithelium). Particles with dimensions of 0.2-5μ are retained in the lungs to an extent of 60-95%; they traumatizes airway mucosa, favoring the emergence of microorganisms and inflammatory processes (rhinitis, laryngitis, tracheitis, bronchitis, etc.).

Powders that reach and are retained can block the activity of alveoli (alveolitis dust) or can cross the alveolar endothelium, penetrating lung tissue. In this parenchyma organizes causing an interstitial fibrosis or may enter the lymphatic circulation inducing tracheobronchial lymph node fibrosis, pleural and mesenteric. Due common clinical symptoms in most of this pneumonitis should be distinguished from pulmonary tuberculosis by performing localized and accurate tuberculin, especially through a correct appreciation toilet air pollution aerosol.

3. The analysis on constitutional and legislative provisions regarding the protected environment, the right to health and food safety in comparative law

The analysis of such a field of law was determined by the relationship between human and subjective rights, on the one hand, and the environment on the other hand, as well as between man and subjective (fundamental) rights. These relationships, which can subsume to the last of them, paved the current ecological crisis which affected the entire environment and the ecological balance, premises that have negatively influenced, as it easily can be seen, all environmental factors, the relationship between man and the environment and between man and individual rights, as well as the whole nature to which we belong.

All these were in turn determined by a number of limitations that can be classified depending on their content, within the internal and external boundaries.

The internal limits are of human nature, and among them there may be listed exacerbating the exercise of certain rights, which led to the emergence of polluters (cars, substances),

which, through the effects of their actions have exceeded the regenerative capacity of the environment and nature in general, thus creating the foundation for pollution [11].

At the same time, external limits that are actually generated by people are the wars, nuclear testing pollution. The emergence of human society led to the relationship between these and the environment and, more widely, the nature. The awareness of this report led to the emergence of scientific theories that tried to explain the content of the relationship between man and nature [11]. In legal doctrine there are individualized three important theories, is cosmological theories, anthropocentric theories and humanistic attitude theories. Cosmological theories are specific to human beginnings, and shortly after this primitive communism, when man lived in harmony with the surrounding nature. Ideologists of the eighteenth century revealed features of such theories. Anthropocentric theories are more advanced, because, generally, they tried to justify "the human attack" on nature. There appears even antagonism between man and nature, based on biblical precepts of Genesis. Shepherds came first in Persia and ancient Greece and they were friends with nature, but only if there were pastures. Theories on humanistic attitude agree the attitude of man with nature, as man is part of nature. They stress the need of returning to nature, to which it should be given due respect. These theories claim a humanistic attitude of man to nature within the ecological crisis and it is manifested in *geo-piety, eco-centrism, techno-centrism, eco-sociology,* etc.

All of the above have resulted in the need to amend and supplement the subjective rights of the content of such rights, but also the emergence of a new fundamental right to environment.

Among the fundamental rights whose content should be amended or supplemented, if necessary, can be listed: freedom of expression, freedom of movement, right to information – which systematically must be placed above the freedom of expression in the content of the Constitution, the right to health, freedom of assembly, freedom of association, right to work and social protection of labor law, private property, the right to a decent life, the right to protection and the right of children and young people with disabilities.

But, it is important to raise the awareness of the states to proceed to the implementation of constitutional regulation of a new fundamental right to protected environment.

Terminologically, there are many names for this new fundamental right of third generation, but we consider the most appropriate name to be *the right to a protected environment*, especially because only such an environment is healthy, clean, balanced, humane, qualitatively, preserved, decent, reasonable level, allowing free access to nature etc. For instance, in supporting our idea of naming this fundamental right, we can argue that, despite the fact that the two major jungles of Earth (Congo and Amazon) do not meet any of the above qualities, nobody doesn't even think about destroying them for this reason, as they represent at least the lungs of our planet.

The content of this fundamental right, where governed by constitutions, usually in subsequent acts to constitutions and in our country in the Government Emergency Ordinance no.195 / 2005 on environmental protection, approved by Law no.265 / 2005 with its subsequent amendments.

The recognition of this fundamental right can be classified, according to several criteria in: explicit or implicit recognition; Constitutional or legal; jurisprudential or not.

As for the constitutional recognition, this fundamental right exists in Romania starting form in 2003, Portugal, Spain, Serbia, Brazil, Turkey, Peru, Paraguay, South Korea, Bulgaria, Croatia, Hungary, Russia. Other countries do not regulate it for fear of blocking the state functioning. Thus, as a result of the environmental damage, an avalanche of court cases could be brought against states that would create great problems to its functioning.

As for the implicit legal recognition, it is governed by certain obligations of the state and, as well as, of the people, whether or not citizens of that State. Such recognition is found in Canada, Holland, Florida, Great Britain, Greece, Andorra, Thailand, Colombia, Iran and Sweden.

As for the explicit legal recognition is found in most US states, Norway, Denmark, the Philippines, Indonesia .

As for the jurisprudential recognition, it is found in Canada, Ireland, Belgium, France, Italy, India, and Kenya.

This fundamental right is regulated in article 35 in the Romanian Constitution in a faulty and contradictory manner. Its content has no coherence, unity, nor the circumstances for adequate protection of nature and environment. Proof are the massive deforestation, catastrophic floods that occurred, land degradation, lack of rain and terrible droughts, tornadoes and strong winds occurrence type hurricane, deforestation and forest protection curtains and not representing

true natural biodiversity. In other words, only the recognition of such fundamental individual right or has no value unless it is applied.

CONCLUSIONS

1. The *One Health* Romania created a multidisciplinary team involved in the highest research of the European Union and USA dedicated Commissions;
2. The ontological diseases to be not fully elucidated, part of them being influenced by industrial pollution;
3. The future of mankind depends by the preservation of the animal and vegetal heritage.

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Biological products PC2. Part 3. Active immunity via passive immunity

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Abstract

The mechanisms of action of immunologically active proteins (IAP) in the human body are presented as well as some results concerning the treatment of psoriasis and epidermolysis bullosa.

Key words: *immunologically active proteins, psoriasis, epidermolysis bullosa*

Romvac has been conducting human and veterinary medicine research and production activities for over 50 years. It all started from the need for human and veterinary vaccines prepared on specific pathogen free (SPF) poultry [1]. SPF poultry were used for the production of human and veterinary vaccines on embryonated eggs and on whole SPF embryo tissue cultures, for research and control of biological products. Genetic studies were carried out at the SPF farms of Romvac regarding the role of sensitive and resistant allele in oncogenic virus-susceptible and -resistant SPF chicken. On this occasion, studies at Cornell University, Ithaca, NY. USA and Romvac demonstrated that in any genetic cross-combination the progenies with minimum one sensitivity allele are susceptible to developing the malign lymphoma called Marek's Disease. It was proven to this extent that the sensitivity allele is dominant in any type of combination passed on to the progenies [1,2,3].

These activities were carried out in collaboration with specialists from Houghton Poultry Research Station, England and from Cornell University, Ithaca NY, USA.

Studies on oncogenic viruses were conducted at the same time in humans and animals, in collaboration with famous Romanian and American professors having constant support from the WHO. The activity in this field enabled us to discover in 1989 the greatest AIDS outbreak in children throughout the world, which ended in 1989 due to the first AIDS prevention and control program in Romania that was implemented together with the WHO [4-9].

Human and animal pathology studies expanded to the matter of antibiotic resistance for which we organized a similar research program that

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was different from that of other research institutes in the world.

Such studies were based on the active immunity produced in the body of animals and humans by oral administration of IAP. The immunologically active proteins (IAP) cross the digestive mucosa and reach the protein molecules that carry the V-variable, including lymphocytes B and T which they are directly effective against by transfer of target antigenic epitopes information. This information is absorbed differently by lymphocyte B and lymphocyte T respectively, each of these cells having distinct reactions. In the bone marrow, IgY is effective on the initial embryonic structures of lymphocyte B and produces hyperactive lymphocytes B ten times more effective than the lymphocytes which have not reached IgY. The naïve lymphocyte T absorbs the negative information from the V-variable of IgY and transfers it to its own DNA which is to be cloned. This new mature lymphocyte reaches the hyperactive lymphocyte B where it transfers the negative information from the V-variable of IgY and stimulates idiotypic IgY-identical IgG production. The amount of antibodies produced in the animal organism corresponds to a vaccination with an attenuated live virus. This mechanism represents a part of the effects of immune modulators and immunologically active proteins in the human organism.

The preclinical and clinical studies conducted during 2012-2016 revealed that we possess a set of immunologically active proteins (IAP) obtained on chickens (*Gallus domesticus*). These products that have been prepared for the first time in the world, are completely different from the biological products currently used for psoriasis treatment and for other immune disorders in humans as well.

The biological products prepared on mono-clonal antibodies or their protein structures are murine or human proteins considered as "non self" by the human organism, with adverse or idiotypic reactions against them. This group of products structured on mammalian molecules (mice, humans) is considered as "non self" in the human organism and induces multiple and violent adverse or idiotypic reactions that, within 10-14 days, reduce or even destroy these products in the human body. The parenteral administration of these products was shown to be inappropriate and harmful resulting in severe local and general reactions and even anaphylactic shock, which are life-threatening for people. Such products are to be replaced as soon as possible based on the recommendations from the European Food and Drug Administration, CDC Atlanta, Ge., ECDC Europe and from the scientific observations of certain specialists from EU, Japan, USA, Canada [10-15].

IAP are products obtained from poultry, that are phylogenetically 350 million years away from humans, more immunologically active than IgG of mammals, which are 35 million years away from humans. Under these conditions, the chicken reacts distinctly from mammals, including humans. Compared to monoclonal antibodies, IAPs are able to identify a large number of epitopes on the structure of the antigen and have specific effect against bacteria, viruses, fungi, unicellular parasites and even round worms. IAPs are specific and neutralize toxins, including exotoxins A and B caused by *Clostridium difficile* (Patrascu et al., unpublished data).

In the human body, IAPs are considered as "self" and induce no adverse or idiotypic reactions. Therefore, treatments have been carried out for 14 years on daily basis in humans, without adverse or idiotypic reactions throughout the treatment period.

Modern psoriasis treatment consists of biological drugs (-ximab, -xumab, -umab, -cept) with a few deficiencies like blocking of certain cell functions and annihilation of parenterally administered cytokines, threatening the patient's health due to some severe adverse and idiotypic reactions.

The emergence of antidrug antibodies (ADAS) against biological products resulted in functional or structural damage of biological products within 14-20 days post administration. ADAS may restrict the use of biological products for treatment of psoriasis, rheumatism and other diseases.

IAPs have a number of benefits compared to biological products, such as: interaction of V-variables, mucosal absorption, immunologically active proteins, big phylogenetic distance from humans, no adverse or idiotypic reactions, possibility of long-term treatment.

Preclinical research activities at ROMVAC were carried out at the same time with studies on treatment of antibiotic-resistant germ infections in humans, obtaining very good results in the treatment of urinary infections, prevention and treatment of nosocomial infections and treatment of respiratory tract infections.

RESULTS AND DISCUSSIONS

Psoriasis vulgaris *treatment*

Within the last two years we have started a treatment program for skin infections, on which occasion we found out that we could treat

psoriasis vulgaris using IAPs (more than 300 patients). Considering the properties of these proteins and the fact that for 14 years of study they have been accepted as "self" by the human body without inducing any adverse or idiotypic reactions, IAPs represent a scientific issue of particular importance. IAPs were used in children, resulting in healing of five of the 20 psoriasis children. The other children are still under treatment and their condition has improved. Another interesting fact of psoriasis children treatment is the wellbeing state that occurs in children and their parents as well. Preventive treatment is still administered in the cured children.

It has been for the first time in the world reported that the skin of patients who have been suffering from psoriasis for 30-55 years and cured, was completely healed without any scars. This is also an important matter to discuss in order to understand the changes occurring in the wounded skin and the way it heals.

IAPs must be administered without delay as well as the methods developed at ROMVAC for the treatment of all psoriasis children and for implementation of a special preventive program concerning potential psoriatic rashes in children, teenagers and adults.

Epidermolysis bullosa (EB) treatment in children

Upon request from a few parents with *epidermolysis bullosa* children, we administered an oral and topical IAP treatment in 4 children. These treatments are ongoing and we have not been able to determine a final assessment methodology or term yet.

At this point, we can report, for the first time in the world, that the disorders due to the digestive localizations of the disease, from mouth to anus, can be successfully treated in a short time, without knowing for sure what sequelae EB causes in the digestive tube. After administering this IAP treatment, the internal and external wounds on the lips, oral and lingual mucosa, pharynx, esophagus, stomach and intestines have healed and now the children are able to eat normally and they can tolerate any kind of food, including cellulose-containing foods. These children now have a normal intestinal transit. Their health condition has significantly improved during this treatment.

At the same time, the respiratory tract and skin of 9-14 years old children born with EB have improved considerably. A 12 years old female patient with larynx wounds and changed voice, constantly coughing for 7 years and suffering from digestive disorders beginning with lips, tongue, palate, palatine velum and esophagus, received, under strict parental observation, IAPs by aerosols for the respiratory tract, orally for the general treatment and IAP solutions for topical skin treatment. Remarkable health status improvement was reported after 14 days with disappearance of cough, oral and esophageal wounds and normal voice. To this extent, the patient started eating normally and a state of wellbeing could be noticed both in the patient and her parents.

Significant improvement was recorded after 30 days, the skin on the soles healed and it became thicker, more flexible and resistant to trauma. During the following weeks, the patient reported that no more blisters had appeared on her skin.

The next step is the treatment and specialists will perform clinical examinations to describe each and every part of the body that has been affected by EB.

The small number of patients we have at present forces us to be scientifically cautious and circumspect. But we must make these results available since we are talking about the first children in the world suffering from EB, whose health condition has significantly improved. We are ready to provide information to all special medical institutions in Romania or other countries, and we can also present the entire treatment program including EB patients' families [16,17].

The most important response is the digestive tube treatment result. A study carried out in Ireland on EB patients from more continents reported that both patients and caregivers wished to firstly solve the digestive tube treatment and, secondly, to find the therapeutical means for pain relief and skin lesions.

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Immunological active proteins I-spga. The specific reaction against Colistin resistance *Klebsiella Pneumoniae* strains

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Abstract

Colistin resistance of bacteria called superbug, triggered a warning about the risks of everyone on infection with E.coli or Klebsiella pneumoniae *containing genes that make them resistant to all antibiotics including Colistin. Immunologically active proteins (PIA) made with the immunogen PC2, the group name Imunoinstant proved that can inhibit the growth of antibiotic-resistant bacteria, including* E.coli, K.pneumoniae, Staph aureus MRSA. *The new generation of PIA made by I-SPGA immunogen have been shown to act specifically on* E.coli *resistant to all current antibiotics used in hospitals in Romania, the* K. pneumoniae *resistance to Colistin, and on* Candida spp., *antibiotic resistance. PIA first generation cured urinary infection and no relapses have occurred in 17 of 19 women treated orally with IMUNOINSTANT. Preliminary results allow us to report the possibility of using passive immunity to produce the active immunity and effective use of these mechanisms in the prevention and the treatment of the antibiotic resistance.*

Key words: *pasive immunity, active immunity, antibiotic resistance, superbug, Colistin* Klebsiella pneumoniae *resitance strains, immunological active proteins*

Superbug *E.coli,* resistant to last-resort antibiotic, show up in China 2015. The drug resistance gene known as *mcr-1* – which has the capacity to move from one bacterium to another – was found in about 1% of *E.coli* bacteria and 1% of a bacteria known as *Klebsiella pneumoniae*, that can cause pneumonia, bloodstream infections, and wound infections. The *mcr-1* gene confers transferable colistin resistance. *Mcr-1*-positive Enterobacteriaceae (MCRPE) have attracted substantial medical attention, clinical associations of *mcr-1*-positive *Escherichia coli* (MCRPEC) infection, and risk factors for MCRPEC carriage. Of 17,498 isolates associated with infection,

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mcr-1 was detected in 76 (~1%) of 5,332 *E.coli* isolates, 13 (< 1%) of 348 *Klebsiella pneumoniae*, one (< 1%) of 890 *Enterobacter cloacae*, and one (1%) of 162 *Enterobacter aerogenes*. But with resistance to better drugs on the rise, Colistin has taken on increasing importance in medicine [1].

Acquired resistance due to chromosomal mutation and selection is termed vertical evolution, since the advantage will be conferred to a bacterial line. Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms through horizontal transfer, and may occur between strains of the same species or between different bacterial species or genera sharing a same ecological niche. Mechanisms of genetic exchange include conjugation, transduction, and transformation. For each of these processes, transposons facilitate the transfer and incorporation of the new resistance genes into the genome of the bacterial host or into plasmids [2].

Superbugs are present in Europe. *E.coli* resistant to the Colistin was find out in the pig meet [3] and *Klebsiella pneumoniae* strain Colistin resistance were isolated form 18 patients in Romania, and there are existing in the bacterial collection of the Bucharest Biology Faculty and Immunomedica-P collection.

Colistin resistance has been seen before, but not in this form. What makes this situation unique – and unsettling – is that the gene that makes the bacteria Colistin-resistant is contained in a plasmid, a mobile piece of DNA. Plasmids can easily move from one bacterium to another, both within a family of bacteria and to other families, as well. That means *E.coli* carrying *mcr-1* can share it with other *E.coli,* as well as pass it to bacteria like *Klebsiella pneumoniae* [5].

A report on antimicrobial resistance in bacteria by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) said some 25,000 people die from such superbugs in the European Union every year. *"Antimicrobial resistance is an alarming threat putting human and animal health in danger,"* said Vytenis Andriukaitis, the EU's health and food safety commissioner. *"We have put substantial efforts to stop its rise, but this is not enough. We must be quicker, stronger and act on several fronts."* [6].

Bacteria found in humans, animals and food continue to show resistance to widely used antimicrobials, says the latest report on anti-microbial resistance (AMR) in bacteria by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). The findings underline that AMR poses a serious threat to public and animal health. Infections caused by bacteria that are resistant to antimicrobials lead to about 25,000 deaths in the EU every year [7].

MATERIALS AND METHODS

Microorganisms

In this study strains isolated from patients form Romanian hospitals, were used for immunization of Rhode Island red chickens. The isolated strains were: seven strains of *E.coli,* seven strains of *Klebsiella pneumoniae* resitance of colistin, 7 strains of *Proteus mirabilis* (PR 31 – *Proteus mirabilis*, PR 33 – *Proteus mirabilis,* PR 34 – *Proteus vulgaris,* PR 35 – *Proteus mirabilis,* PR 36 – *Proteus vulgaris,* - PR 37 – *Proteus mirabilis*, PR 38 – *Proteus mirabilis), 7 strains of Candida (*C1 – *Candida albicans,* C2 – *Candida tropicalis;* C3 – *Candida glabrata;* C4 – *Candida albicans;* C5 – *Candida albicans;* C6 – *Candida krusei* – ATCC 14243; C7 – *Candida parapsilosis* – ATCC 22019). Stock cultures were kept at -80°C in BHI (Brain heart infusion - Oxoid) and glycerol. Prior to each experiment, the strains were streaked on BHI agar (Oxoid) plates with incubation at 37°C for 24 h.

The bacterial strains isolates form Romanian patients a resistant to following antibiotics: Cefepime, Cefixim, Trimetroprim/Sulfamethaxazol, Fosonicin, Gentamicin. Imipenem, Cefuroxim, Meropenem, Ciprofloxacina, Amoxicilina, Clavulianic acid, Ceftibuten. The *Klebsiella pneumoniae* strains are Colistin resistance, and were isolated from Romanian patients.

Immunogen I-spga

In the presented method, the strains from *E.coli, Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis* and *Candida* are grown under appropriate conditions in a liquid culture medium to a predetermined density, followed by harvesting and suspension of the bacterial culture in saline, where upon formalin is added to the suspension under slight agitation to a final concentration of 0.2 M formaldehyde,

followed by incubation under continuous agitation at 37°C, for approximately 2 hours, followed by incubation at 4°C for 24-48 hours, resulting in a formalin-killed *E.coli* strain having substantially preserved antigenic properties. The inactivated strains were was three times with distillated water, and mixed with SPGA as immuno-modulator, 10 mg/ml for each bacterial strain and mixed with QS 21 as adjuvant.

Chicken immunization

20 weeks of age Rhode Island red chickens form the Mihailesti Poultry Farm, Romania were immunizated with 2 ml of Rhode Island red hens, at 20 weeks of age, were immunized by three intramuscular injections with I-spga (200 mg/dose/animal). After the first injection, the second one was administrated after 30 days and the third intramuscularly administration was done after 14 days after the second one.

IgY and white egg proteins preparation

IgY and white egg proteins were purified from 6 eggs laid prior to immunization and from 12 eggs after 8-10 days after the first immunization. The IgY and white egg proteins as holo-ovotransferrin, ovomuicin, ovoalbumin and lysozyme were prepared by the method described previously [9-11].

RESULTS AND DISCUSSIONS

Serological assay
Rapid seragglutination assay (SAR)

The SAR was performed using "in house" reagents. The antigens were the formalin inactivated bacterial suspension at the 10 optical density. The IgY and immunological active proteins were used as undiluted or double fold dilution in PBS. It was added 30µL of antigen at 30µL of IgY or PIA samples and homogenized for 10 seconds to form a circle about two inches in diameter, after which it was kept at rest for two minutes. At the end of reaction time, the test was considered positive if it had the presence of lumps and the absence of it, the test was considered negative (Table 1, Table 2).

Agglutination assay

Each bacterial suspension was adjusted with PPBS to an optical density at 525 nm of 0.3 with a Coleman Junior spectrophotometer. This suspension was used both in the rapid or slow Agglutination assay. The agglutination procedure used was basically that developed by Schaefer [12], with some key modifications. To the culture tubes (12 mm) with 0.5-ml portions of the working dilution of IgY-poly I-spga or PIA I-spga were added, followed by 0.5-ml portions of the bacterial suspension. Control tubes containing 0.5 ml of PPBS and 0.5 ml of bacterial suspension were also included. The tubes were shaken and placed in an incubator at 37"C, and after 3 and 24 h they were removed to analyze the extent of agglutination (Table 3).

Agglutination was graded from “no agglutination” to “complete agglutination” on a scale of 0 to 4+. In most instances type-specific agglutination occurred within 3h; however, sometimes the reaction took 24h to develop.

Proteins identification

The IgY-poly I-spga, active immunological proteins-poly I-spga (ovotransferrin-apo ovotransferrin-holo, ovomucin, ovoalbumin and lysozyme extracted from hyper immune eggs I-spga) were indentified by ELISA assay. IgY-poly I-spgawas identificated by Chicken IgY ELISA Kit (ab189577) ABCAM**,** ovotransferrin by Chicken Ovotransferrin ELISA Kit (ab157694) ABCAM, ovomucine by Chicken MUC6 (Mucin 6) ELISA kit Elabscience E-EL-Ch0799, ovoalbumine by Ovoalbumina [OVA slG1) ELISA kit Blue Genue, lysosime by Lysosime (LZM) Chicken (Gallus) SEB193Ga ELISA kit Cloud-Clone Corp.

ELISA quantitative assay for IgY-poly I-spga

The samples of IgY-poly I-spga extracted from hyper immune eggs 8-10 days after first chicken immuization with the imunogen I-spga were tested by ELISA under the intruction of use of Chicken IgY ELISA Kit (ab189577) ABCAM.

The IgY-poly has the positive reaction at the concentration of 20-40 ng of IgY specific per 0.1 ml of IgY-poly for the antigen of *K. pneumoniae, Candida albicans, E.coli* and 0.80 ng/0.1 ml of IgY-poly for *Proteus mirabilis.*

ELISA quantitative assay for IgY-poly

Samples of IgY-poly I-spga extracted form hyper immune eggs 8-10 days after the first chicken immunization with immunogenic I-spga, were tested by ELISA usind the Chicken IgY ELISA Kit (ab189577) ABCAM. The IgY-poly has 320 up to 400 mg of IgY per egg extracted by the new tehnique (Patrascu unpulished data).

Table 1
Rapid seragglutination assay for the IgY-poly I-spga and immunological active proteins (PIA) with bacterial antigens

Bacterial line	IgY-polyI-spga[a]		IgY[b)]	IgYI-PC2[c)]
	Quantitative	Positive tested		Positive tested
E.coli	+++	7/7	-	1/1
K.pneumoniae	++++	7/7	-	1/1
Proteus mirabilis	+++	7/7	-	ND
Candida albicans	++++	2/2	-	1/1
Candida tropicalis	++++	1/1	-	ND
Candida glabrada	++++	1/1	-	ND
Candida krusei ATCC 14243	++++	1/1	-	ND
Candida parapsilosis ATCC 22019	++++	1/1	-	ND

a. IgY-poly I-spga extracted from eggs of hyper immunized hens, 8 days after the first injection of the immunogenic I-spga chicken contain antibiotic resistance *E.coli, Proteus mirabilis,* 7 strains of Candida and 7 strains of Colistin resistant *K.pneumoniae*

b. IgY extracted from the same eggs before the first injection of immunogenic I-spga

c. IgY-poly IMUNOINSTANT immunogenic I-PC2 as reference sample [10]

ND – not determined

Table 2
Rapid seragglutination assay for the proteins immunological active (PIA) with bacterial antigens

Bacterial line	OTf[b)]	OTf-polyI-spga[a]	IAP-poly I-spga[c)]
		Positive tested	Positive tested
E.coli	–	7/7	1/1
K.pneumoniae	–	7/7	1/1
Proteus mirabilis	–	7/7	1/1
Candida albicans	–	2/2	1/1
Candida tropicalis	–	1/1	1/1
Candida glabrada	–	1/1	1/1
Candida krusei ATCC 14243	–	1/1	1/1
Candida parapsilosis ATCC 22019	–	1/1	1/1

a. Ovotransferrin holo poly I-spga extracted from white eggs of hyper immunized hens, 8 days after the first injection of the immunogenic I-spga chicken contain antibiotic resistance *E.coli, Proteus mirabilis,* 7 strains of Candida and 7 strains of Colistin resistant *K.pneumoniae*

b. OTf extracted from the same eggs before the first injection of immunogenic I-spga

c. IAP -poly I-spga whole sample with immunological active ovotranseferrin, ovomucin, ovoalbumin and lysozyme

Grow inhibition assay for *Klebsiella pneumoniae*

In vitro grow inhibiton assay for *Klebsiella pneumoniae* strain 55,717 ESBC by IgY-poly imunogenic I-PC2. The test was done at the *Victor Babes* Hospital, Bucharest [6,9,10,13-15].

The new generation of immunologically active proteins (IAP) prepared on red Rhode Island chickens inoculated with a new I-spga immunogenic containing G-type immuno-modulators, are considered the 2nd generation IAP and in preclinical evaluations proved to act specifically, directly on antibiotic-resistant

bacteria including Colistin-resistant *Klebsiella pneumoniae* strains.

The second generation IAP (I-spga IAP), which exhibit for identification two biologic markers, G and A, were prepared using as antigens the bacterial strains resistant to all antibiotics currently used in Romanian hospitals, isolated from patients during 2016-2017, from the *Carol Davila* Nephrology Hospital collection, Bucharest, Romania and the Faculty of Biology Bucharest collections. The I-SPGA immunogenic contained also a mixture of seven Colistin-resistant *Klebsiella pneumoniae* strains from the collection of the Faculty of Biology, Bucharest, Romania. Grow inhibition assay of Klebsiella pneumoniae strain 55717 antibiotic resitance (fig.1). Experiment was done at the Hospital Vuctor Babes Laboratory (8,13).

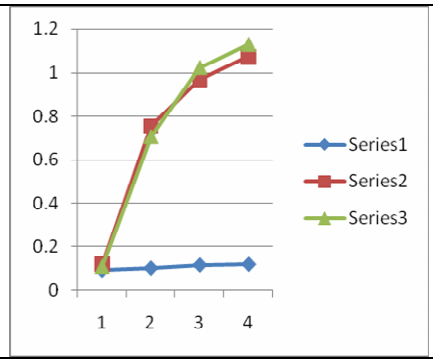

Fig.1. Grows inhibition
- blue line: K pneumoniae 55717 strain inhibition by IgY-poly I-pC2;
- red line: K pneumoniae grew titer in contact with IgY of SPF chikens;
- green line: K pneumoniae grew titer without IgY

Immunoglobulin (Ig) samples extracted from the yolk (Y) and immunologic active proteins (IAP I-spga) produced from the eggs whites obtained from hens hyperimmunized with I-SPGAimmunogen prepared from a collection of bacteria (*K.pneumoniae, E.coli, Pseudomonas aeruginosa, Candida albicans, Candida glabrata, Candida tropicalis, Candida krusei* – ATCC 14243, *Candida parapsilosis* 22019 – ATCC) resistant to all antibiotics, isolated from patients with urinary infections, reacted positively in rapid and slow agglutination tests with antibiotic-resistant pathogens corresponding to the bacteria used for immunization.

For confirmation of the specificity of the agglutination tests, the poly-IgY samples and I-spga IAP samples, containing immunologically active ovotransferrin, immunologically active ovoalbumin, immunologically active ovomucine, and immunologically active lysozyme, were tested by ELISA technique using the ABCAM kits. The poly-IgY I-spga samples were found to contain 250-500 mg of specific poly-IgY I- spga anti-*K.pneumoniae* and 250 mg specific IgY anti-pollution *C.albicans*.

Table 3
Seroaglutination assay for IgY I-spga[a] with bacterial antingens

Bacterial line	Positive reaction
Eserichia coli	1 : 320
Klebsiella pneumoniae	1 : 320
Proteus mirabilis	1 : 40
Candida albicans	1 : 160

[a] IgY poly I-spga extracted from white eggs of hyper immunized hens, 8 days after the first injection of the immunogenic I-spga chicken contain antibiotic resistance *E.coli, Proteus mirabilis,* 7 strains of *Candida* and 7 strains of Colistin resistant *K.pneumoniae*

Immunologically active proteins (I-spga IAP) and poly-IgYI-spga were retested using the exhaustion test by direct contact with the bacteria present in 18h old cultures. To this purpose, antibiotic-resistant *E. coli*, Colistin-resistant *K. pneumoniae*, antibiotic-resistant *P.aeruginosa* and antibiotic-resistant *Candida albinans. C.glabrata, C.tropicalis, C.krusei* were tested. IgY I-spga and IAP I-spga were put in contact with the pathogens directly in the culture medium, incubated at 37 °C for 60 minutes, and afterward centrifuged for 15 minutes at 3000 rpm. The supernatant was tested with ABCAM ELISA kits and compared to samples that were not in contact with the pathogenic bacteria. In the presence of specific pathogenic germs, the content in I-spga IAP and that poly-IgY I-spga decreased by two logarithms as compared to the controls.

Second generation poly-IgY, at a concentration of 2,5 mg /ml of specific poly-IgY, specifically inhibited the growth of antibiotic-resistant *E.coli, K.pneumoniae, P.aueruginosa, Acineto-bacterbaumani, Salmonella enteritidis* and *Salmonella typhimurium* [8,9].

Recent studies at *Carol Davila* Nephrology Hospital showed that the treatment with I-spga IAP and poly-IgY from the I-PC2 IMUNOINSTANT immunogenic administered orally, by gargling and afterwards swallowing, at an amount of 200 ml / 80 ml of sterile suspension in PBS, has cured the urinary infections due to antibiotic-resistant *E.coli* in

women. 17 patients healed without recurrences. Two female patients showed recurrent infections, one of which was re-infected with a new strain of a susceptible to antibiotics *E. coli* (Patrascu et al. unpublished data).

Preliminary studies concerning the direct action of I-spga IAP and poly-IgY I-spga demonstrate their ability to react specifically with pathogens resistant to antibiotics, including Colistin-resistant *K.pneumoniae* strains.

These results compel us to expand the researches concerning the *in vitro* and *in vivo* direct action of IAP and IgY I-spga on one hand, and on the other hand, the information must be taken over and checked by other specialized institutions for acknowledgment. Confirmation of these results will enable the urgent work out of special programs to prevent and treat infections with specific pathogenic germs in humans, including the prevention and treatment of urinary infections with *E.coli* and *K.pneumoniae* superbugs.

CONCLUSIONS

1. *Klebsiella pneumoniae* Colistin resistant strains isolated from Romanian patients react serologicaly by seroaglutination and by indirect ELISA test with the chicken immunolgobulins (IgY) and with immunologically active proteins I-spga form white egg of hyper immune eggs of the chickens immunized by I-spga immunogenic.

2. Immunized chickens with I-spga immunogenic with formalin inactivated *Klebsiella pneumoniae* Colistin resistant strains produce a specific immunological response. The IgY-poly and immunologically active proteins react *in vitro* with the pathogenic *Klebsiella pneumoniae* Colistin resistant strains, by rapid agglutination, slow agglutination and by competitive ELISA assay.

3. The oral passive immunological treatment with IgY-poly and immunological active proteins of patients with urinary tract infections by antibiotic resistant bacteria is a new opportunity to treat the antibiotic resistance.

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DEBUT

Surface and volume measurements of mezenchimal cell's nucleus and nucleolus – A new way of computerized morphometry

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Abstract

A new, easy-to-learn way of using the Adobe Photoshop CS3 program for computerized morphometric analysis of tumor cells is presented. It contains a step-by-step tutorial on how to use the program to accurately extract the region of interest, how to use the measurement tool offered by the program and how to convert from pixels to microns. The mathematical part of this article was built-up using the Microsoft Excel program. While the method still needs to be refined, it exhibits some advantages: it is fast, accurate and easy to use.

Key words: *nucleus, nucleolus, morphometry, morphometrics, photoshop*

By definition, morphometry represents the skill or act of measuring the external form of an object, organism, or landform. Morphometrics represent an evolutionary development of form in an organism or part of an organism

In the years 1940-1970, cellular morphometry (also known as cellular morphometrics) could be realised in two ways: either by direct measurement, using a micrometer of a microplanimeter, or via microprojection techniques. Although easy to use, some errors might occur, errors that multiply when trying to calculate the volume of the structure. Also, some of these methods are time-consuming [1].

T.Caspersson et al. described the necessary condititions for an ideal volume measurement method. This method should at least: [2]

- work in a range of dimensions where the resolution of the microscope can be used to its very limit;
- be possible to choose immediately the exact focal plane through the object where the measurement is to be made;
- be possible to make the measurement rapidly and also in an accumulating fashion, so as to get, within reasonable time, an average value.
- be possible to measure every accessible cell.

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In our days, we use computers and different software programs in order to measure a cell's various parameters, such as diameter, circumference, circularity, area, volume and so on.

The aim of our study is to try out a different approach to computerized morphometry, using ordinary software programs and a low-cost technique, as well as to determine the advantages and disadvantages of this method.

MATERIALS AND METHODS

The morphometric analysis has been performed using images of various tissues, stained using the May-Grunwald Giemsa panoptic method. Because different staining techniques lead to different morphological changes within the cell, the same staining method was used in all cases. All pictures have been taken on an analog film-camera adapted to an Olympus microscope. The reason behind choosing an analog film instead of a digital camera was the higher resolution of the film.

The computerized morphometry – the measurements of diameter, and surface of the cell's nucleus, nucleolus and cytoplasm, were conducted using the Adobe Photoshop CS3 software. In order to be loaded into the Photoshop program, the image was converted from an analog image to a digital one, that was permormed using a high performance flatbed scanner. Once the image has been loaded into the Adobe Photoshop CS3 program, we selected the area of interest (the nucleolus, the nucleus or the entire cell) using the quick selection tool. This tool selects an area based on the color gradient and a threshold that can be adapted to fit our preferences. The greatest advantage of this tool is that, for an element clearly delimited from it's soroundings, this process is highly precise and fast.

By using the Record Measurement tool, the program will return values coresponding to area, perimeter, circularity, height, width, integrated density and so on. Initially we computed the number of pixels, that later were converted to the metric system; a way to perform such conversions is based on the formula:

$$B = (A \times 264) / Ob \quad (1)$$

where:

- A – value measured in pixels;
- Ob – magnifying power of the microscope;
- B – value in the metric system (in μm).

It is important to mention that, in order to obtain volumetric values, certain logistics had to be made. Volume is characteristic for tri-dimensional objects. Morphometry is applied on pictures, which are bi-dimensional. The used mathematical formula was suggested by Tasca C [2]:

$$V = (\Pi \times LB^2) / 6 \quad (2)$$

where L is length and B is the base. The formula offers great results for an elongation (L/B) < 1.14 and satisfactory results for an elongation < 2 [1].

The obtained values were then inserted in the Microsoft Office Excel software program in order to put together the results in the form of tables, as well as for the mathematical formulas.

RESULTS

The obtained results are synthesized in Tables 1 – 4.

Table 1.
Morphometric values for the cell's nucleolus

Big axis (B)	Small axis (S)	Elongation	Surface	Volume
μm	μm	Ratio B / S	μm^2	μm^3
8.94	7.63	1.17	31.81	272.65
8.15	6.84	1.19	44.33	199.61
6.31	6.05	1.04	29.25	120.93
10.52	7.89	1.33	111.08	342.90
8.94	5.52	1.62	32.09	142.82
9.21	9.21	1.00	54.71	408.39
7.36	6.58	1.12	34.51	166.69
6.84	6.58	1.04	57.54	154.78
9.21	8.94	1.03	120.07	385.38
10.78	9.99	1.08	100.08	563.92
9.47	9.21	1.03	48.55	420.05
7.36	7.10	1.04	24.76	194.42
6.58	4.21	1.56	28.29	60.96
9.21	6.05	1.52	86.87	176.36
7.36	6.84	1.08	108.66	180.29

Table 2.
Morphometric values for the cell's nucleus

Big axis (B)	Small axis (S)	Elonga-tion	Surface	Volume
μm	μm	Ratio B / S	μm^2	μm^3
26.30	24.20	1.08	445.79	7,789.32
25.77	22.62	1.14	455.82	6,704.20
25.77	24.99	1.03	481.62	8,303.47
48.66	37.61	1.29	1,413.05	35,690.87
34.19	19.46	1.76	507.65	6,780.67
33.14	29.19	1.14	748.96	14,787.08
26.83	24.20	1.11	487.71	8,223.22
35.51	28.67	1.24	767.36	15,122.73
41.55	41.03	1.01	1,327.97	36,239.16
43.13	38.40	1.12	1,238.85	32,733.87
29.19	27.88	1.05	616.64	11,459.51
26.83	21.04	1.28	428.70	6,023.51
34.19	20.25	1.69	397.09	7,280.64
40.77	38.40	1.06	1,046.94	31,294.12
41.82	35.24	1.19	1,129.11	27,013.68

Table 3.
Morphometric values for the cell's cytoplasm

Big axis (B)	Small axis (S)	Elonga-tion	Surface	Volume
μm	μm	Ratio B / S	μm^2	μm^3
72.06	52.60	1.37	1,516.19	96,604.88
69.17	48.20	1.44	1,242.97	77,429.14
63.91	52.86	1.21	1,539.70	85,207.86
82.06	75.74	1.08	2,791.66	210,802.61
61.54	50.23	1.23	1,420.93	74,530.17
76.27	59.18	1.29	2,458.26	125,052.11
89.16	59.96	1.49	1,335.10	159,632.16
144.91	102.04	1.42	2,855.29	774,975.19
126.50	79.16	1.60	5,191.20	378,852.44
125.71	91.00	1.38	4,789.61	512,328.20
49.44	46.29	1.07	958.75	44,009.32
46.29	33.66	1.38	557.57	21,442.69
52.34	40.50	1.29	1,022.88	37,672.54
61.81	52.34	1.18	1,440.03	57,347.95
54.70	53.13	1.03	856.93	53,827.37

Table 4.
Surface and volume ratios

Nucleus Area / Nucleolus Area	Nucleus Area / Citoplasma Area	Nucleus Volume / Nucleolus Volume	Nucleus Volume / Citoplasma Volume
14.01	0.29	28.57	0.08
10.28	0.37	33.59	0.09
16.47	0.31	68.66	0.10
12.72	0.51	104.09	0.17
15.82	0.36	47.48	0.09
13.69	0.31	36.21	0.12
14.13	0.37	49.33	0.05
13.34	0.27	97.70	0.02
11.06	0.26	94.03	0.10
12.38	0.26	58.05	0.06
12.70	0.64	27.28	0.26
17.31	0.77	30.98	0.28
14.04	0.39	119.43	0.19
12.05	0.73	177.45	0.55
10.39	1.32	149.84	0.50

DISCUSSIONS

Since the method used for converting pixels to the metric system isn't 100% precise, the values obtained for diameters, area and volume are slightly different from the real ones. We can obtain more precise data if we consider an error coefficient E; in this situation:

- the diameters, would be D x E,
- surface would be A x E^2, and
- volume would be V x E^3.

The problem of true values is difficult to solve, because the value of E is only estimated. Fortunately, when we calculate the ratio, the value of E doesn't matter, because it's reduced in the final results.

One interesting element can be observed when comparing the Nucleus Area / Nucleolus Area ratio with the big and small axis of the nucleus and nucleolus respectively. Despite the various sizes of the nucleus and nucleolus, their ratio remains relatively constant.

CONCLUSIONS

Despite the fact that some differences in regards to the real values can occur, the presented method of morphometric analysis has many advantages. One of the most important is based on the gradient of color between the measured elements that prove to be a quick selection tool for dimensions and areas of interest. The disadvantage is encountered when we try to convert the recorded values from pixels to the metric system, but a calibration could solve the conversion issue. Even if this calibration value is difficult to be determined, the ratios between the values are correct.

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