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IMMUNOSENSORS FOR THE EXPRESS DETECTION OF ANTIBIOTIC RESISTANT BACTERIAL PATHOGENS

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Summary. Resistant microorganisms can spread rapidly over countries, regions and the world, facilitated by global trade, travel and tourism. This problem concerns all countries.

The article is devoted to the analysis of methods for the indication of bacterial pathogens. The authors compare the characteristics of the immune biosensors based on the SPR, TIRE, photoluminescence and on the ISFETs with CeO_x gate surface and conclude that they have similar sensitivity and may provide to achieve low cost of analysis.

Keywords: immunosensor, bacteria, antibiotic resistant microorganisms, determination, antibody, antigen

Introduction. The antibiotic resistance phenomenon has become a global concern as geographic borders among countries and continents have become less distinct due to increasing global trade, expanding human and animal populations, societal advances and technological developments. Because of this increasing global connectivity, now we see rapid transport of infectious agents and their antibiotic-resistant genes. Thus, the use of antimicrobials in one area, such as aquaculture, can have an impact on the resistance situation in another area, such as in human medicine, and resistance problems in one country can spread to another (GAO, 2004; FAO, 2011, 2015).

Many expert panels, including WHO consultations, national committees and independent organizations, have examined the association between use of antimicrobial agents in food animals and antimicrobial resistance among bacteria isolated from humans. WHO organized two consultations, in Berlin in 1997 and in Geneva in 1998, to qualitatively assess the risk of human health consequences associated with the use of antimicrobial agents in food-producing animals (Angulo, Nargund and Chiller, 2004; WHO, 1997, 1998, 2011a, 2011b). The WHO Consultation in Geneva focused on the human health importance of fluoroquinolones and public health concern of increasing resistance to such substance, particularly among *Salmonella* and *Campylobacter.* There is the risk of human health consequences associated with the use of fluoroquinolones in food animals too. This meeting was entitled 'Use of Quinolones in Food Animals and Potential Impact on Human Health'. It was concluded at this meeting that the use of fluoroquinolones in food animals has led to the emergence to *Campylobacter* and of *Salmonella* with reduced susceptibility to them. (WHO, 1998). Similar conclusions have been presented to two committees of the Codex Alimentarius Commission: The Codex Committee on Food Hygiene (CCFH) and Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF). A risk profile on anti-microbial-resistant bacteria in food presented to the 34th of CCFH in October 2001, stated that: Antimicrobials are used in food animals for growth promotion, prophylaxis, metaphylaxis and therapy. This use is the principle contributing factor to the emergence and dissemination of Consequences of the Use of Anti-Microbial Agents in Food Animals 377 anti-microbial resistance among bacterial pathogens and commensals that have food animal reservoirs (Codex Committee on Food Hygiene, 2001; Codex Committee on Residues of Veterinary Drugs in Foods, 2001).

Bacteria can be resisted to antibiotics as a result of the chromosomal mutation or inductive expression of a latent chromosomal gene or by exchange of the genetic material through transformation (the exchange of DNA), transduction (bacteriophage), or conjugation with plasmids (extrachromosomal DNA). The last is particularly common among the Enterobacteriaceae, *Pseudomonas* and anaerobic species. In addition to conjugative plasmids, bacteria may possess transposons, the so-called jumping genes, that have the ability to enter transmissible plasmids or chromosomes (Bryan, 1988). Resistance can be transferred horizontally by plasmids or by chromosomally located conjugative transposons that spread the resistance to other species. It has been postulated that *E. coli* transferred the ability to produce P-lactamase enzymes that destroy compounds with an I-lactam nucleus into *Haemophilus influenzae* by initially infecting *Haemophilus parainfluenzae.* Intergenus spread of resistance can occur between Gram-positive species such as staphylococci and enterococci and between

Enterobacteriaceae and *Pseudononas* or anaerobes such as *Bacteroides* (Neu, 1992).

Poor infection control in any setting can greatly increase the spread of drug-resistant infections, especially during outbreaks of disease. Rapid diagnostic and infection prevention should be very essential to curb the movement of antimicrobial-resistant organisms, starting with good basic hygiene, which limits the spread of all infections, including those that are resistant to antimicrobial medicines (WHO, 2015). The antimicrobial resistance among bacteria isolated from humans could be the result of using antimicrobial agents in food animals and is leading to human health consequences (Angulo, Nargund and Chiller, 2004).

A wide range methods are available for the antibiotic resistant bacteria identification and detection, in connection with these programs, for the prevention and identification of problems related to health and safety. The choice of the method is a key factor for the detection of pathogens and the intended use of the method, for instance whether for a qualitative or semi-quantitative screening, quantitative and/or confirmatory analysis, must be clearly defined (Stead, 2014; Pividori et al., 2016).

Identification and detection of bacteria is in general required for routine surveillance and monitoring. The conventional analytical techniques for the quality and safety analyses are very tedious, time consuming and require trained personal, therefore there is a need to develop quick, sensitive and reliable techniques for quick monitoring of food quality and safety (OIE, 2016; Buchanan, 2004). In this connection biosensor is an appropriate alternative to the conventional techniques. Biosensor/immune biosensor devices are emerging as one of the foremost relevant diagnostic techniques for food, clinical and environmental monitoring due to their rapidity, specificity, ease of mass fabrication, economics and field applicability. They obtain the specificity from biological binding reaction, which is derived from a range of interactions that include antigen/antibody, enzyme/substrate/cofactor, receptor/ligand, chemical interactions and nucleic acid hybridization in combination with a range of transducers (Thakur, Ragavan, 2013).

Aim. To compare different methods for the detection of bacterial pathogens and present different types of immune biosensors which are involved into bacteria detection and compare their efficiency.

Materials and methods. The main method was analysis of the existed literature data about the efficiency of the biosensors at the reveal of different types of microorganisms.

Results. The highest sensitivity in species identification of bacteria has been achieved with molecular methods based both on polymerase chain reaction (real-time PCR, digital PCR) and isothermal amplification methods, like rolling circle amplification (RCA), recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP). In addition to the quantitative evaluation of the amplified DNA by quantitative real-time PCR, other methods have been developed, which are based on the hybridization of target DNA with highly selective probes bound to a surface. The detection limit of DNA methods range between 10 to 100 colony forming units/ml of sample (Poltronieri, de Blasi and D'Urso, 2009; D'Urso et al., 2009; Pividori et al., 2003).

Nowadays, the developed immunoassays are based upon bacterial species-specific antibodies, aptamers (Amaya-González et al., 2013; Paniel et al., 2013) and immuno-recognition of bacterial antigens (such as bacteriophage tailspike protein) (Dutt et al., 2013). These methods require standard conditions for the optimum binding of proteins or other highly-specific affinity compounds on: beads (Luminex, Austin, TX, USA), glass slides, gold surfaces, microplates and membranes suitable for chromatographic separation of antigen-antibody complexes, combined with dipsticks, microfluidic channels on paper (μPADs) (Liana et al., 2012) or lateral flow immuno-assays (LFIA), in which the capture antibody is conjugated with a detection molecule exploiting colorimetric methods, chemiluminescence or gold nanoparticles (Cimaglia et al., 2012). We found that the sensitivity of this last method applied in *Salmonella spp*. detection was approximately 10^5 CFU/ml, thus making it unsuitable for detection in pre-enrichment broth at early stages of growth (18–24 h). Presently many biosensor-based methods are still labor-intensive, expensive, and not easily implementable in-field applications.

There are now a large number of immune biosensors available for detection of target microorganisms in a variety of food, water, clinical, and industrial samples. Ivnitski et al. (1999) provided a comprehensive overview of different physicochemical instrumental techniques for direct and indirect identification of bacteria, including infrared and fluorescence spectroscopy, flow cytometry, chromatography, and chemiluminescence techniques, as a basis for biosensor construction (Ivnitski et al., 1999). Biosensor/immune biosensor development and application are exciting fields in applied microbiology. Basically, a biosensor is a molecule or a group of molecules of biological origin attached to the detector surface. When an analyte comes in contact with this surface, the interaction will initiate a recognition signal that can be registered in an instrument. An ideal biosensor should detect target molecules directly without the use of labelled ligands

or multiple washing steps. Many types of immune biosensors have been developed, including a large variety of enzymes, polyclonal and monoclonal antibodies, nucleic acids, and cellular materials. In some applications, whole cells can also be used as a biosensor. Detected analytes include toxins (e.g., staphylococcal enterotoxins, tetrodotoxins, saxitoxin, and botulinum toxin); specific pathogens (e.g., *Salmonella, Staphylococcus*, *Pseudomonas* and *Escherichia coli* O157:H7); carbohydrates (e.g., fructose, lactose, and galactose); insecticides and herbicides; ATP; antibiotics (e.g., penicillins); and others. The used recognition signals include electrochemical (e.g., potentiometry, voltage changes, conductance and impedance, and light addressable); optical (e.g., ultraviolet, bioluminescence, chemiluminescence, fluorescence, laser scattering, reflection and refraction of light, surface plasmon resonance, and polarized light); and miscellaneous transducers (e.g., piezoelectric crystals, thermistor, acoustic waves, and quartz crystal) (Shah and Wilkins, 2003).

Optical immuno-sensing technologies can be split into two categories, namely luminescence (fluorescence) sensors and label-free sensors. In the first case sensitive elements, such as proteins, antibodies, enzymes, nano-particles are conjugated with the fluorescent labels; binding analyte molecules to such receptors causes luminescence (fluorescence) or it's quenching. As result, the response can be easily visualized either by naked eye or with a suitable photodetector. The example could be the method of ELISA, which was established as a standard bio-sensing method in analytical laboratories, and other bio-sensing methods commonly compared with it. Label-free optical methods based on the phenomenon of evanescent field or wave which appear as electromagnetic wave propagating along the interface between two materials with the different refractive indices when the light enter the material with lower refractive index at total internal reflection condition (Nabok, 2016; Qi, Gao and Jin, 2011).

Starodub with group (Starodub and Ogorodnijchuk, 2012a, 2012b; Starodub et al., 1986, 2001, 2005; Starodub, Ogorodniichuk and Novgorodova, 2016; Starodub, Ogorodnijchuk and Romanov, 2011) also studied the possibility of different substances detection using SPR based onbiosensors and *S. typhimurium* was among them. Two SPR biosensors/immune biosensors were used for the study: one was based on commercial Spreeta module and the other one 'Plasmonotest' was designed V. M. Glushkov Institute of Cybernetics of National Academy of Sciences of Ukraine. As reactive part antigen-antibody interactions were used and previous working surface preparation was occurred which included several sequential steps: a) covering of surface by polyalylamine hydrochloride (PAA); b) immobilization of protein A from *St. aureus*; c) the oriented binding of the specific antibodies; d) bovine serum albumin immobilization (BSA) for blocking free non-specific binding centers on the gold surface. Polyelectrolyts are widely used for biological material immobilization on the gold surface (Starodub et al., 2005). Thin obtained films using small charged organic molecules are common since this molecules form insoluble polymer which electrostatically sorbs molecules with opposite charge (Starodub et al., 2001).

SPR can be used for the setup of immune biosensors applied to the detection of food pathogens in enrichment broth, in liquids or in food dilutions. The SPR technique for biosensing allows real-time monitoring of chemical and bio-chemical interactions occurring at the interface between a thin gold film and a dielectric interface or transparent material, such as the liquid analyte (Sun, 2014).

One of the promising optical methods for biosensors creating is TIRE. Ellipsometry is an optical method for studying of surfaces and environments, which is based on analysis of amplitude and phase changes of a light wave during its interaction with the investigated object. Later it was studied the possibility of using phase change of reflected light in terms of total internal reflection. This method was called total internal reflection ellipsometry and was firstly described in 1976 (Abelès, 1976). Getting ellipsometry parameters in terms of total internal reflection significantly increased sensitivity and detection level comparing with conventional ellipsometry and SPR technology. Thus, the method of TIRE provides a level of determination within 5×10^{-7} RIU while for ellipsometric measurements this index is 10^{-5} RIU (Iwata and Maeda, 2007). The reflected wave is formed on the edge of optically contrasting environments therefore ellipsometric measurements provide the information about optical structure of surface area and these processes that affect to its optical properties (Arwin, Poksinski and Johansen, 2004; Baleviciute et al., 2013).

A very successful commercial immunosensing instrument was based on dual polarization interferometer (DPI). The idea of this instrument is based on interference of two waves propagating in adjacent slabs and the formation of the interference pattern. Since the upper waveguiding slab is exposed to the environment, the molecular adsorption affecting the propagating wave causes the shift of the interference pattern which can be quantified in the concentrations of adsorbed molecules. The sensitivity of this method is claimed to be of 10^{-7} RIU which is comparable with the best SPR achievements. The interpretation of the results is not model-dependent (as compared to SPR

or ellipsometry), so the outcomes can be easily interpreted as changes in refractive index or thickness, or optical density of molecular layer. The instrument is however quite expensive, bulky, and obviously laboratory based (Nabok et al., 2009; Nabok, 2016).

Recently, there is a growing interest in the studying and obtaining photoluminescent nanomaterials such as nanoscale structures and quantum dots of metal oxides. This is because nanoparticles of metal oxides acquire new qualitative changes of physical and chemical properties, catalytic ability and reactivity, which are not observed in microscopic bodies of the same chemical nature. One of these properties is PhL. It is a powerful technology for development of optical biosensors since it does not require any procedures of bioreceptors preparation, complex electrical circuits and expensive equipment. The principle of PhL based biosensor operating is to measure changes of PhL spectra of nanoparticles (intensity and peak position) caused by interaction of biological components. Many different substances were successfully detected by means of nanoparticle PhL such as ions, DNA molecules, dopamine, carbohydrate antigen, bovine leukemia virus and *S. typhimurium* etc. (Guo et al., 2012; Liang et al., 2014; Qian et al., 2014; Gu et al., 2011; Viter et al., 2014).

Graphene nanostructures with their microscale area, sensitive electrical properties, and modifiable chemical functionality are excellent candidates for such biodevices at both biocellular and biomolecular scale. Graphene has already been successfully applied in immunosensors creating for numerous substances detection such as DNA and proteins (Xue et al., 2014; Zhang et al., 2013) and pathogenic bacteria are among them. Abdelhamid et al. described graphene magnetic nanosheet decorated with chitosan as a promising biosensor/immunosensor for fluorescence spectroscopy and it can be also applied for matrix assisted laser desorption/ionization mass spectrometry (MALDI) for sensitive pathogenic bacteria detection. *P. aeruginosa* and *St. aureus* were detected in cell suspension and in blood. Limit of detection of *P. aeruginosa* and *St. aureus* in suspension using fluorescence was 5.0×10^2, 4.5×10^2 CFU/ml and for blood it was 4.0×10^2, 1.0×10^2 CFU/ml for each bacteria respectively. MALDI provided sensitivity on the level of 5.0×10^2, 4.5×10^2 CFU/ml in suspension and 6.0×10^2, 5.0×10^2 CFU/ml in blood samples (Abdelhamid and Wu, 2013).

ISFETs represent the group of semiconductor potentiometric devices and are widely applied in biosensors. The interest to these devices only grows since 70s of past century when P. Bergvald and T. Matsuo have proposed pH-FET as promising transducers for biosensors (Starodub and Starodub, 2000; Caras and Janata, 1980; Van der Schoot and Bergveld, 1987) and reported that these structures are suitable for highly sensitive detection of protons generated during the biochemical reaction and as elements for integrated multifunctional and multiparametric on-chip biosensors. Starodub and Ogorodnijchuk (2012a, 2012b) developed new type of immune biosensor based on ISFETs with CeO_x instead of Si_3N_4 gate surface, which provides high sensitivity and stability of the analysis. The biosensor was used for *S. typhimurium* detection in model solutions using immune reaction. The surface was activated twice by water solution of glutaraldehyde (GA). The sensitivity of the analysis of *Salmonella* was about 2–3 cells/ml with the maximal response up to 5×10^5 cells/ml.

Discussion and conclusion. The antibiotic abuse may lead to serious disorders in living organisms. To prevent non-desirable effect from bacterial diseases there is necessary to fulfill the constant and effective control of all types of bacteria, in particular, of pathogenic ones among of different environmental objects and a special in water, foods and feed. Today medical controlling organs use a huge set of methods but, unfortunately, as a rule they are routine, expensive and demand a lot of time for accomplishing. As alternative to them, there is a new generation development of instrumental analytical approaches based on the principles of biosensorics.

Immune biosensors are the effective tools for the express detection of microorganisms in the real time. They have shown tremendous promise to overcome the limitations of the traditional methods and to provide rapid, reliable and sensitive detection of bacteria. A special attention in this article is given to the results obtained by authors at the control of antibiotic resistant bacteria as *Pseudomonas aeruginosa* and *Salmonella typhimurium* with the different types of biosensors in particular based on the surface plasmon resonance (SPR), total internal reflectance ellipsometry (TIRE), photoluminescence (PhL) and on the ion-selective field effect transistors (IsFETs) with CeO_x gate surface. Also is described the last tendencies of biosensors creation with the application of the nanostructures.

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