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Rewiev scientific article

METHODS FOR THE ANTIBIOTIC RESISTANCE DETECTION IN MICROORGANSIMS ISOLATED FROM FOOD

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Abstract: Resistance to antibiotics is a growing problem that the World Health Organization has declared one of the biggest threats to global health. The food chain is one of the most important ways of transmitting and spreading resistance to antibiotics between the population of resistant and populated by sensitive commensal and / or pathogenic microorganisms. Resistance to antibiotics can be inborn, or acquired by mutation or lateral gene transfer. From the aspect of the spread of resistance, only resistance acquired by the lateral transfer of the gene is significant. There are numerous methods for detecting and determining the nature of antibiotic resistance in bacteria isolated from food. The methods must be standardized and ensure the consistency of the obtained results. Methods for determining the minimum inhibitory concentration of antibiotics are used to detect phenotypic resistance in bacteria isolated from food. They include a microdilution method, an agar dilution method, and an E-test. Qualitative and semi-quantitative methods commonly used in clinical isolates are not suitable for antibiotic resistance testing in food-isolated microorganisms. In the case of microorganisms with detected presence of phenotypic resistance to antibiotics, the presence of the resistance gene is determined. Microorganisms evidenced by the presence of genetic determinants associated with acquired resistance to antibiotics represent a risk of resistance dissemination among the susceptible populations. Commercially used microorganisms should not possess genetic determinants of transferable antibiotic resistance.

Key words: antibiotic resistance, minimum inhibitory concentration, genetic determinants of resistance

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INTRODUCTION

The beginning of the antibiotic era in the 1940s marked the true revolution in medicine, thanks to which lethal bacterial infections became curable. Soon after the emergence of antibiotic treatment, the first microorganisms that were resistant to them appeared. Today, antibiotic resistance is one of the biggest problems of mankind. (World Health Organization, WHO, 2011). In many countries, the annual use of antibiotics in veterinary medicine is far superior to the use of antibiotics in human medicine. As a result of metaphylaxis and prophylaxis in veterinary medicine, even healthy animals are continually exposed to large amounts of antibiotics. Microorganisms derived from animals are thus subjected to selective pressure, which leads to the survival of only antibiotic-resistant bacteria. (WHO, 2011). Food of animal origin allows direct contact between the microflora of the digestive tract of humans and animals and is one of the most important ways of spreading resistance between populations of humans and animals. From the aspect of food safety, beside resistant pathogenic microorganisms, commensal microorganisms that can serve as a reservoir for antibiotic resistance gene, are also important. (European Food Safety Authority EFSA, 2008). The products obtained from raw meat and milk are especially incriminated due to the absence of thermal treatment, enabling the survival of a large number of microorganisms in them (Mathur and Singh, 2005). Resistance to antibiotics can be inborn or acquired. Inborn resistance is an inherent feature of the species or genus and has no significant role in the dissemination of resistance among the populations of microorganisms. The acquired resistance to antibiotics is specific only to certain strains within a generally susceptible species or genus and can be caused by mutations on existing genes or by acquisition of new genes by lateral transfer (Ammor et al., 2007). Inborn resistance and antibiotic resistance resulting from mutations are not important in non-pathogenic microbial species because they have no role in spreading resistance. Resistance obtained by lateral gene transfer carries the greatest risk for the spread of genetic determinants of resistance to susceptible microorganisms (Devirgiliis et al., 2011). Tracing resistance to pathogenic and commensurate microorganisms through the food chain is of the utmost importance for understanding and predicting the emergence and spread, as well as for planning adequate measures to prevent further dissemination of resistance to antibiotics. In order to successfully

monitor resistance to bacteria isolated from food, to determine its nature and to evaluate the ability to transfer to susceptible bacteria, it is necessary to apply the appropriate methods of antibiotic resistance testing.

METHODS FOR TESTING RESISTANCE TO ANTIBIOTICS

In examining the presence of resistance in microorganisms isolated from food, the phenotypic and genotypic methods are used. First, phenotypic resistance is determined using a qualitative or quantitative method. If evidence of phenotypic resistance, which could be a consequence of lateral gene transfer , is found in the isolates then it is necessary to determine the presence of genes which encode resistance.

Qualitative methods for detection of resistance to antibiotics

Disk diffusion method is the most frequently used qualitative method for determining resistance in microorganisms. A disk diffusion method is performed by applying an inoculum containing approximately 1-2 × 108 log CFU / mL of the examining microorganism to the surface of the Petri dish with the appropriate nutrient medium, and a filter-paper disk, impregnated with the compound to be tested, is then placed on it. The results are read after 16-24h by measuring the growth inhibition zone of the examined microorganism (Jorgensen and Ferraro, 2009). Although the disc diffusion method is most often used one in clinical isolates because it is cheap, simple and well-standardized, it is not suitable for determining resistance to microorganisms isolated from food because there is no possibility of quantification of the results. Isolates based on the size of zone inhibition can only be classified as susceptible or resistant (EFSA, 2012).

Quantitative methods for the determination of minimal inhibitory concentrations of antibiotics

Quantitative methods based on the determination of minimal inhibitory concentration are recommended for testing resistance to bacteria isolated from food. (Minimal Inhibitory Concentration – MIC) (EFSA, 2008; EFSA, 2007; EFSA, 2012) The minimum inhibitory concentration is the lowest antibiotic concentration that has the ability to inhibit microorganism growth under precisely defined conditions (Wiegand et al., 2008). There are several methods for determining MIC values, such as agar dilution method, broth dilution method, E-tests, and more recently automated instrumental systems (Jorgensen and Ferraro, 2009).

In the agar dilution method, a certain concentration of antibiotics is added directly to the agar, and then the examined microorganism is deposited on its surface. Results are based on the presence or absence of microorganism

growth on agar surface after incubation (Wiegand et al., 2008). For the broth dilution method, a liquid medium is used with the addition of antibiotics at a precisely determined concentration to determine the presence of resistance. The method of macrodilution is performed in the tubes, and the brothvolume into which the microorganism and the antibiotic are added should be greater than 2 mL. Although the macrodilution method is the first method developed to determine the MIC value it is no longer in use because it is more demanding and more expensive than the microdilution method (Jorgensen and Ferraro, 2009).

The microdilution method for determining the MIC values involves the use of microtitration plates, with the total volume in the well plate not exceeding 500 µL (Wiegand et al., 2008). Microtitration plates have standard 8 x 12 well configuration allowing 8 antibiotics in 12 concentrations, or 12 antibiotics in 8 concentrations to be tested.Antibiotic concentrations in the tubes or microtitration plates are usually in a series of double dilutions (e.g., 1, 2, 4, 8, etc. µg antibiotics / mL substrate) Microdilution plates can be made directly in a laboratory, but commercial plates with wells containing dehydrated antibiotics at appropriate concentrations are also available. Concentrations of antibiotics that are put into microtitration plate wells are prescribed by standards or appropriate guidelines (Jorgensen and Ferraro, 2009).

The results of the macrodilution method are interpreted on the basis of the broth cloudiness and in the microdilution method they are based on the presence or absence of the sediment in the microtitration well. The smallest concentration of antibiotics in which there is no visible cloudiness or sediment formation is the MIC value for the examined isolate (Jorgensen and Ferraro, 2009).

E-tests are thin plastic strips on the back of which antibiotics are applied in growing concentrations. On the other side, there is a scaling with numbers that indicate antibiotic concentrations. E-tests are placed on the surface of a suitable agar previously inoculated with the examining microorganism. MIC values are read as the point where the growth inhibition ellipse intersects the MIC scale on the strip. E-tests are simple and easy to perform, but they are not used in routine tests of food isolates since the price of such tests is very high (Jorgensen and Ferraro, 2009).

Recently, semi-automated and automated methods for the determination of MIC values have been developed, such as MicroScan WalkAway (Siemens Healthcare Diagnostics), BD Phoenix Automated Microbiology Diagnostics (BD Diagnostics), Vitek 2 System (bioMerieux) and Sensititre Aris 2X (Trek Diagnostic Systems) . Automated methods allow rapid and standardized reading of results, but their use is still limited to clinical isolates (Jorgensen and Ferraro, 2009).

Specificity of resistance testing on antibiotics in microorganisms isolated from food

Microorganisms isolated from food are different from clinical isolates, so the resistance monitoring methods differ from the methods applied in clinical practice. Determination of resistance of a strain isolated from food is based on microbiological (epidemiological) MIC limit values, unlike clinical isolates for which resistance determination is based on clinical limit values (EFSA, 2008a). Microbiological limit values are set in relation to the distribution of MIC values in the bacterial population, and strains whose MIC values differ significantly from the distribution of MIC values within that species or genus are labeled as microbiologically resistant. Microorganisms that are microbiologically susceptible are not genetic carriers which encode the acquired resistance, whether it is a consequence of mutation or is caused by lateral gene transfer.

In contrast to microbiological limit values, clinical limit values are aimed at examining the possibilities of treating a bacterial infection with an antibiotic, taking into account clinical studies of efficacy, antibiotic dosing, pharmacodynamics and pharmacokinetics. When reading the results with microbiological limit values, there are only categories of resistance and susceptibility in microorganisms, but not intermediate sensitivity / resistance (Silley, 2012).

Resistance monitoring programs for bacteria isolated from food set monitoring of resistance in Salmonella and Campylobacter as a minimum condition (WHO, 2011). In addition to these two genotypes, Escherichia coli was most commonly included as a marker of Gram negative commensal microflora and Enterococcus spp. as a marker of Gram positive commensal microflora (Founou et al., 2016; WHO 2011). According to the recommendations of the European Food Safety Authority (EFSA), testing of MIC values in Salmonella spp, Campylobacter spp, Enterococcus spp. and E. coli should be done according to instructions issued by the European Comittee on Antimicrobial Susceptibility Testing (EUCAST, 2017), but only using quantitative methods for determining MIC values, since the disk diffusion method is marked as inadequate (EFSA, 2008b; EFSA, 2007). The most commonly used medium for antibiotic resistance testing is Miler-Hinton Broth. However, it is not adequate for testing Campylobacter susceptibility because of their nutritional requirements. In this case Cation-Adjusted Mueller Hinton Broth (CAMHB) supplemented with 2-5% lysed horse blood is used. (McDermott et al., 2005).

According to EFSA recommendations, all microorganisms deliberately added to food, most commonly as a culture starter or probiotics, should not be the carriers of genetic determinants of resistance acquired by lateral gene transfer (EFSA, 2012). Since microorganisms are not

clinically relevant, the methods for testing MIC values are not given in the EUCAST guidelines. The problem of antimicrobial resistance testing in lactic acid bacteria was to select an adequate nutrient medium for bacterial growth in microtiter plates, as the commonly used Miller-Hinton and Iso-Sensitest broths did not prove to be adequate for all members of this group of microorganisms (Huys et al. , 2002) Recently, the ISO standard (ISO 10932, 2010) was issued, which defines the method for testing antimicrobial resistance in bifidobacteria and lactic acid bacteria, except enterococcus. The medium used to grow microorganisms in microtitration plates is a Lactic Acid Bacteria Susceptibility Medium (LSM) consisting of 90% Iso- Sensitest Broth with addition of 10% MRS Broth (de Man, Rogosa, Sharpe - MRS). For testing resistance of Bifidobacterium spp. LSM, the cysteine (International Standardization Organization ISO 10932, 2010) is also added.

Table 1 shows the basic antibiotic groups to investigate the resistance of microorganisms isolated from food according to EFSA recommendations

Table 1 - Standard antibiotics (EFSA 2007, EFSA 2008b, EFSA 2012)

Microorganism	**Antibiotics**
Salmonella spp, E. coli	Cefotoxime, nalidic acid, ciprofloxacin, ampicillin, tetracycline, chloramphenicol, gentamicin, streptomycin, trimethoprim, sulfonamides
Campylobacter jejuni and *Campylobacter coli*	Erythromycin, ciprofloxacin, tetracycline, streptomycin, gentamicin
Enterococcus faecalis and *Enterococcus faecium*	Streptomycin, gentamicin, chloramphenicol, ampicillin, vancomycin, erythromycin, kvinpristin / dalfopristin, tetracycline, linezolid
Lactic acid bacteria and *Bifidobacterium* spp.	Ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol

* If Enterococcus faecium is added to the food in addition to these antibiotics, the resistance to tylosin should also be tested

Determination of antibiotic resistance gene

If the MIC value for some strain is above the set limit values, it is necessary to further investigate the nature of the resistance to determine whether it is inborn or acquired.

Inborn resistance is the specificity of the species or genus and accurate identification of the taxonomic affiliation of the strain tested is a basic precondition for determining the genetic basis of resistance (EFSA, 2008a). The genetic basis of resistance is determined by molecular-biological methods,and the most commonly used is PCR technique. All the data necessary for the design of primers, the data on resistance genes most commonly present in the examined microorganism, as well as data on their localization and transferability can be found in literature. There are also databases , such as the ARDB (Antibiotic Resistance Database) database (ARDB, 2017), on antibiotic resistance genes available on the Internet.

Although PCR is the most commonly used method for proving the resistance gene, it requires the isolation of bacteria and their DNA and depends on the culture techniques and their limitations in bacterial isolation,that is why advanced, culture- independent techniques, such as meta-genomics and sequencing of whole genome are being improved. (Founou et al., 2016) These methods enable the detection and testing of the whole bacterial genome, the identification of new genetic traits and the identification of unknown genetic elements, which is not possible with PCR methods .(Allen, 2014; Thanner, 2016).

CONCLUSION

The great importance of monitoring antibiotic resistance through the food chain is becoming more and more recognized throughout the world. Resistance monitoring programs for microorganisms isolated from food are constantly being developed and improved. Continuous advances in methods applied to food isolates are necessary in order to obtain the relevant results that are required for antibiotic resistance to be predicted, monitored and to prevent its spread in time.

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