DOI: 10.29326/2304-196X-2022-11-1-7-13

Current understanding of antimicrobial resistance mechanisms in bacteria (analytical review)

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SUMMARY
Data on mechanisms of resistance to antimicrobials in bacteria are reviewed and summarized. Main causes of resistance emergence and spread in bacteria are analyzed. Mechanisms of innate resistance of pathogenic bacteria (non-specific efflux pumps, antibiotic-inactivating enzymes and mechanisms serving as permeability barriers) are characterized. Mechanisms of acquired resistance are described: antibiotic modification or degradation; active removal of an antimicrobial from a bacterial cell – efflux (draining out); sequestration; target modification (bypass). The origin of antimicrobial resistance mechanisms in pathogenic bacteria is shown to be debatable. It is noted that producer microorganisms can directly transfer antimicrobial resistance genes to pathogenic bacteria, but a reliable link between this process and antimicrobial resistance spread has not been identified and proven so far. Horizontal gene transfer, including free DNA transformation, transduction by bacteriophages and plasmid-involving conjugation, is believed to play an important role in antimicrobial resistance spread. All three mechanisms are widespread in nature, although some bacterial species use one mechanism to a great extent than the other two. Transduction is supposed to play an important role, in particular, in the antibiotic resistance gene transfer, but the significance of transformation or transduction in the resistance gene transfer under the laboratory or environmental conditions has not been clarified so far due to the difficulty of naturally emerging recombination detection. Data on the role of conjugation in the antimicrobial resistance gene spread in nature, in particular carbapenem- and quinolone-resistance genes in Gram-negative and Gram-positive bacteria are presented. New trends in the antimicrobial resistance gene spread are indicated.

Keywords: review, antimicrobial resistance, antibiotics, mechanisms of antimicrobial resistance, bacteria, microorganisms

For citation: Pruntova O. V., Russaleyev V. S., Shadrova N. B. Current understanding of antimicrobial resistance mechanisms in bacteria (analytical review). *Veterinary Science Today*. 2022; 11 (1): 7–13. DOI: 10.29326/2304-196X-2022-11-1-7-13.

Conflict of interest: The authors declare no conflict of interest.

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УДК 619:579.8:615.33

Современное представление о механизмах антимикробной резистентности бактерий (аналитический обзор)

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РЕЗЮМЕ
Проведен анализ и обобщены сведения о механизмах резистентности к антимикробным препаратам у бактерий. Рассмотрены основные причины возникновения и распространения устойчивости у бактерий. Охарактеризовано действие механизмов естественной резистентности патогенных бактерий (неспецифические эффлюксные насосы, инактивирующие антибиотики ферменты и механизмы, которые служат барьерами проницаемости). Описаны механизмы приобретенной устойчивости: модификация или разложение антибиотика; активное выведение антимикробного препарата из бактериальной клетки – эффлюкс (отток), секвестрация, модификация мишени (байпас). Показана дискуссионность вопроса о происхождении механизмов устойчивости к антибиотикам у патогенных бактерий. Отмечено, что прямая передача генов устойчивости к антимикробным препаратам может проис-

ходить от микроорганизмов-продуцентов к патогенным бактериям, но достоверная связь между этим процессом и распространением антимикробной резистентности в настоящее время не выявлена и не доказана. Роль горизонтальной передачи генов, включающей трансформацию свободной ДНК, трансдукцию бактериофагами и конъюгацию с участием плазмид, считают важной в распространении антимикробной резистентности. Все три механизма широко распространены в природе, хотя некоторые виды бактерий используют один механизм в большей степени, чем два других. Полагают, что трансдукция играет важную роль, в частности, в переносе генов устойчивости к антибиотикам, но до настоящего времени нет ясности в вопросе о значении трансформации или трансдукции в переносе генов резистентности в условиях лаборатории или в окружающей среде из-за сложности обнаружения рекомбинаций, возникших в естественных условиях. Представлены данные о роли коньюгации в распространении генов антимикробной резистентности в природе, в частности генов устойчивости к карбапенемам и хинолонам у грамотрицательных и грамположительных бактерий. Отмечены новые тенденции в распространении генов антимикробной резистентности.

Ключевые слова: обзор, антимикробная резистентность, антимикробный препарат, механизмы антимикробной резистентности, бактерии, микроорганизмы

Для цитирования: Прунтова О. В., Русалеев В. С., Шадрова Н. Б. Современное представление о механизмах антимикробной резистентности бактерий (аналитический обзор). *Ветеринария сегодня.* 2022; 11 (1): 7–13. DOI: 10.29326/2304-196X-2022-11-1-7-13.

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

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INTRODUCTION

Antimicrobial resistance (AMR), the resistance of bacteria to antimicrobials, is currently one of the most serious global problems. The long-term use of antibiotics to control animal and human disease agents resulted in that some bacteria have become resistant to drugs, and diseases have become non-responsive to treatment. According to the World Health Organization (WHO), already today many infections are caused by pathogenic microorganisms that are resistant to antimicrobials [1, 2].

The emergence and widespread of antibiotic-resistant forms of the bacteria that are non-susceptible to many antimicrobials are accompanied by a decrease in the therapy effectiveness, an increase in the treatment duration and in lethality. All this dictates the need to monitor animal bacteriosis agents, their structure and drug resistance level, and empirical antibiotic therapy of the disease, currently practiced by veterinarians, should take into account the actual data of epizootological monitoring of antibiotic resistance of the bacteria circulating in the particular livestock holdings.

This challenge has gone beyond the competence of the WHO and the World Organization for Animal Health (OIE) due to its complexity. Currently, it is recognized that no country or organization can alone cope with the AMR challenge [3, 4].

At first, the strategy for AMR prevention and containment including several directions shall be developed to address the AMR challenge. The key of which is the implementation of measures aimed at limiting and rational use of antimicrobials owing to the knowledge about antimicrobials resistance mechanisms in bacteria acquired by a wide range of veterinarians.

The Russian Federation participated in the drawing up of the Resolution on global strategy and global action plan for antimicrobial resistance adopted by the WHO Assembly in 2015. The said Resolution brought in force, urged all countries to carry out monitoring of drug-resistant bacterial infections and to ensure control of antimicrobials use in veterinary medicine, human medicine and agriculture as well as to strengthen international cooperation and funding in this field. In addition, international organizations have committed themselves to tighten legislative regulation of antimicrobials use, to search for their rational use (improvement of laboratory diagnostics of bacterioses, taking into account their susceptibility to antimicrobials) and to widely implement measures for infectious disease prevention, including vaccination, water purification, sanitary and hygiene measures [5].

In September 2017 the Government of the Russian Federation approved the 'Strategy for preventing of antimicrobial resistance in the Russian Federation for the period to 2030' [6] developed by the RF Ministry of Health. The Strategy lays down tasks for containment of the biological hazard associated with AMR spread and is aimed at prevention and limiting the resistance of microorganisms to antimicrobials.

Considering the significance of the above-said problem the paper is aimed at reviewing of national and foreign literature and description of mechanisms of antimicrobial resistance emergence and spread in bacteria.

MAIN CAUSES OF AMR EMERGENCE AND SPREAD IN BACTERIA

The phenotypic manifestation of AMR in bacteria is mediated by genetic properties, but not all and not always resistance genetic determinants manifest phenotypically. Resistance of bacteria to antimicrobials emerges and spreads due to the following:

– emergence of *random mutations* in genes capable of modifying activity spectrum of bacterial enzymes degrading antimicrobials;

– *exchange of genetic material between cells*, that is, the transfer of genes from resistant to less resistant or

susceptible microorganisms through the transfer of chromosomes, plasmids, phages, translocating elements;

– *selection of new resistant strains brought about by selective pressure of antimicrobials* associated with their uncontrolled use in various fields [7].

Traditionally, AMR mechanisms are considered only in relation to pathogenic microorganisms that have to protect themselves from the effects of medicinal products and disinfectants. And, accordingly, the main cause of AMR development is believed to be an anthropogenic impact on microorganisms. However, in the environment, antimicrobials- producing microorganisms that have to protect themselves from their metabolic byproducts are the primarily source of AMR genetic determinants rather than pathogenic microorganisms [8].

Antimicrobials-producing microorganisms, as a rule, have not one, but many complex self-protecting mechanisms that provide complete protection from the biologically active molecules that they produce. Moreover, some researchers have shown that self-resistance determinants are mostly linked to antimicrobials biosynthetic genes and their expression is co-regulated [9]. Therefore, natural reservoirs of resistance genes that may include the determinants conferring self-resistance to antimicrobials-producing microorganisms should be taken into account in addition to the often-mentioned AMR causes for full understanding of the antimicrobial resistance development in pathogenic microorganisms. Despite the fact that these resistance determinants in the environment microflora do not pose a threat to animal health, the transfer of these determinants to plasmids and integrons in pathogenic bacteria in the future may result in increase in the number of such determinants in pathogenic bacteria populations and the emergence of huge problems. That is, AMR spread prevention requires studies and control of the resistance determinant distribution in bacterial populations, clarification of the resistance mechanisms and determination of the environmental factors that contribute to their spread [8].

AMR MECHANISMS IN PATHOGENIC BACTERIA

As mentioned above, microorganisms have intrinsic and acquired AMR. Mechanisms of intrinsic resistance include nonspecific efflux pumps (that are supposed to emerge as a general response to environmental toxins), antibiotic-inactivating enzymes and mechanisms that serve as permeability barriers [10, 11]. These mechanisms are encoded by the main genetic structure – chromosome of bacterial cell. Well-studied efflux AcrAB-TolC pumping out system in *Escherichia coli* having broad substrate specificity and capable of outflow of antimicrobials and disinfectants of various classes is an example of intrinsic AMR [12]. Vancomycin-resistance in *E. coli* and Gram-negative bacteria is also a well-known example of intrinsic resistance emerging due to permeability barriers created by outer membrane [13]. Despite the fact that the intrinsic AMR mechanisms provide a low level of antimicrobials resistance, the normal commensal microflora of animals or environmental bacteria (water bodies, pastures) having intrinsic resistance mechanisms, can become opportunistic microorganisms in the animals with compromised immunity [14]. On the other hand, the mechanisms of acquired resistance in bacteria usually emerge as a result of horizontal gene transfer and also include specific efflux pumps encoded by a plasmid, for example, such as TetK and TetL in *Staphylococcus aureus*, as well as enzymes that can modify an antibiotic or an antibiotic target [15, 16]. These mechanisms pose a far more serious threat to human and animal health due to translocation of AMR determinants from chromosome to plasmid since it results in their enhanced expression and spread. Such an example is a transfer of the chromosomal AmpC *β*-lactamase gene into a plasmid, resulted in its worldwide spread [17].

MECHANISMS OF ACQUIRED ANTIMICROBIAL RESISTANCE IN PATHOGENIC BACTERIA

Biochemical mechanisms of AMR in pathogenic bacteria are very similar to the mechanisms found in producer microorganisms. Moreover, AMR genes in pathogenic bacteria belong to the same functional families as that ones of the producer microorganisms. AMR biochemical mechanisms are divided into several groups: antimicrobials modification or degradation; active antimicrobials removal from bacterial cell (efflux, outflow); sequestration of antimicrobials; target modification or bypass [18, 19].

MECHANISM OF ANTIMICROBIALS MODIFICATION OR DEGRADATION

This mechanism is commonly used by pathogenic bacteria to resist to aminoglycosides. The aim of antimicrobials modification is to render them ineffective, especially in the case of aminoglycoside antibiotics (for example, kanamycin, gentamycin and streptomycin), chloramphenicol and *β*-lactams. A large number of aminoglycoside-modifying enzymes including *N*-acetyl transferases, *O*-phosphotransferases and *O*-adenyltransferases that acetylate, phosphorylate or adenylylate aminoglycoside antibiotic are found in producer bacteria. These enzymes were first identified in early 1970s in members of *Streptomyces* species and then in other antibiotic-resistant pathogenic bacteria [20].

In pathogenic bacteria, genes coding for modification and degradation of antimicrobials are usually located on mobile genetic elements (MGE); chromosomal determinants have been also found in the majority of non-pathogenic environmental bacteria including those of *Providencia* and *Acinetobacter* genera [20]. These bacteria are considered a source of acquired AMR determinants found on MGEs in pathogenic strains. Of the known aminoglycoside-modifying enzymes, aminoglycoside-*N*-acetyltransferases are the most prevalent and well studied among pathogenic bacteria. Moreover, according to reports, some degradation enzymes were identified in both Gram-positive and Gram-negative bacteria [21]. However, *β*-lactamases are the modification/degradation enzymes most commonly used by pathogenic bacteria. While the role of *β*-lactamases in producer bacteria life is still debatable, they are known to play a critical role in *β*-lactam-resistance in Gram-negative bacteria. In Gram-positive bacteria, both penicillin-binding enzymes and *β*-lactamases play the main role in antimicrobials modification/degradation mechanism likely due to differences in their cell wall structures. More than 1,000 *β*-lactamases have been identified in pathogenic isolates of many bacteria species and their number continues to grow because of constantly emerging new mutations that allow them to adapt to new *β*-lactams. All currently known *β*-lactamases are classified into

four molecular classes based on common properties of enzymes and certain amino acid homology [22]. The majority of clinically significant β-lactamases belongs to Class A and Class C. In particular, Class A comprises β-lactamases of *Klebsiella* spp., *Citrobacter diversus*, *Proteus vulgaris* and the majority of *Bacteroides* spp. encoded by chromosome genes as well as almost all plasmid β-lactamases.

Class B enzymes are metallo-enzymes, since they contain a zinc atom as a coenzyme; they are prevalent in plasmids of *Enterobacteriaceae* family members. These enzymes are effective against penicillins, cephalosporins and carbapenems. *Beta*-lactamases of the following groups are significant in clinical practice: extended-spectrum β-lactamases of Gram-negative bacteria, cephalosporinases of Gram-negative bacteria, metallo-β-lactamases of Gram-negative bacteria [23]. TEM-3 β-lactamase can be taken as an example that is classified to extended-spectrum β-lactamases and is able to degrade the 3rd generation cephalosporins [24] that is indicative of rapid evolution of β-lactamases genes in pathogenic bacteria. Most β-lactamases are translocated to MGE facilitating their rapid spread in populations; however, some of β-lactamase genes can be present in chromosomes, for example, in members of *Enterobacteriaceae* family where they are poorly expressed and being silent genes. It can be supposed that, as in the case of aminoglycoside-modifying enzymes, β-lactamases may also perform dual functions including housekeeping and antibiotic resistance in bacteria [25]. Besides, biological function of β-lactamases in bacterial cell is supposed to be a remodeling peptoglycan cell wall but their gene translocation to plasmid results in high resistance to antimicrobials [17].

ACTIVE REMOVAL OF ANTIMICROBIALS FROM MICROBIAL CELL (EFFLUX, OUTFLOW)

Efflux is a commonly used mechanism of Gram-positive and Gram-negative bacteria to various antimicrobials such as β-lactams, fluoroquinolones, macrolides, lincosamides and tetracyclines. This mechanism uses different systems. The first one is *disorder of microbial cell membrane permeability*; this mechanism is common for Gram-negative bacteria having outer membrane and is less specific for antimicrobials of different groups. The second system is *decrease in permeability and/or antibiotic efflux from a bacterial cell*. Decreased permeability is important for Gram-negative bacteria because of the presence of the outer membrane that forms a permeability barrier and provides an intrinsic mechanism for protection from hydrophilic antibiotics, such as vancomycin [12]. Mutations in porin genes and/or changes in their expression were shown to have a further effect on Gram-negative bacteria susceptibility to hydrophilic antibiotics [26].

In addition, many types of active efflux pumps mediated by transport proteins were described in both Gram-positive and Gram-negative bacteria. Normally transport proteins carry out import or export of only one specific substrate. However, multi-drug or polyspecific exporters were found in natural microbial communities, suggesting that polyspecificity is widespread in natural microbial communities and is of ancient origin [27].

Genes encoding efflux pumps can be either intrinsic or acquired. Examples of intrinsic genes include AcrAB-TolC in *E. coli*, NorA in *St. aureus* and LmrA in *Lactococcus lactis*. Of these tripatite RND pump AcrAB-TolC is the most studied system. Although this system carries out efflux of very broad spectrum of compounds, its biological function is believed to be export of bile salts in *Enterobacteriaceae* family members [28]. The acquired antimicrobials efflux determinants often found on MGEs in pathogenic bacteria are represented by many different types of Tet genes (at least 22 genes have been identified) located on plasmids in both Gram-negative and Gram-positive bacteria [29].

SEQUESTRATION OF ANTIMICROBIALS

Sequestration involves proteins that bind to antimicrobials and prevent them from reaching their targets. This mechanism is more typical for producer microorganisms, for example, bleomycin producers – members of *Streptoalloteichus hindustanus*, *Streptomyces verticillus* and *Streptomyces flavoviridis* species which primary mechanism of resistance involves sequestration of the metal-bound or metal-free antibiotic [30].

TARGET MODIFICATION/BYPASS

This mechanism involves generation of additional targets or subunits in antimicrobials that that prevent them from binding, for example, methylation [18, 19]. Target modification acts as a self-resistance mechanism against several classes of antibiotics including β-lactams, glycopeptides, macrolides, lincosamides, streptogramins and aminoglycosides. A large number of such mechanisms were found in pathogenic bacteria. Methicillin-resistant *St. aureus* strains where β-lactam resistance is mediated by exogenous penicillin-binding protein which transpeptidase domain is not susceptible to several different β-lactams is a classical example of target modification. For example, β-lactam antibiotic has a similar structure to the substrates-peptidoglycan precursors that allow the antibiotic to associate and cause acetylation of active site serin resulting in its inhibition [31]. Resistance to vancomycin resulting from acquisition of Van gene cluster and being a typical cause of AMR in enterococci is another example of target modification [32]. In particular, VanA *и* VanB genes out of many known genes in this cluster determine AMR in pathogenic bacteria since they are found on MGEs [33].

Other examples of the target modification in pathogenic bacteria include point mutations or enzymatic alterations of the target [34]. Enzymatic alteration of the target is best understood in the case of macrolide resistance conferred by a large group of erythromycin ribosomal methylation (Erm) genes. These enzymes methylate a specific adenine in the 23S rRNA [35]. In pathogenic bacteria, Erm genes are present on MGEs and are widespread in both Gram-positive and Gram-negative bacteria [35, 36]. The most known examples of target protection in pathogenic bacteria include Tet(M) and Tet(O) proteins encoded by genes located on MGEs in *St. aureus*. These proteins were shown to be homologous to elongation factors, EF-G and EF-Tu, and their binding to ribosome facilitates removal of tetracycline from a bacterial cell in GTP-ase activity-dependent manner [37]. Thus, it can be concluded that that the most AMR mechanisms in bacteria appear to emerge from intercellular mechanisms of resistance to environmental conditions and it is the incorporation of AMR genetic determinants in MGEs in pathogenic bacteria that poses a serious threat to animal and human health.

THE ORIGIN OF ANTIMICROBIAL RESISTANCE IN PATHOGENIC BACTERIA

The question of how genes of resistance to antimicrobials emerge in pathogenic bacteria remains debatable. The idea that pathogen resistance genes could be obtained from antimicrobials –producer microorganisms by horizontal transfer was first proposed in the 1970s [38]. Despite sound evidence that their transfer from producer organisms to pathogenic strain might occur, a direct link between producers and pathogens has not been established and demonstrated so far. This is primarily due to the fact that resistance genes in producers demonstrate high sequence divergence and very different G + C content as compared to determinants in pathogens even when they use similar mechanisms. However, evolutionary link between determinants and pathogens is not denied [39]. Analysis of the data available in literature suggests that transfer of these determinants from producers to pathogenic bacteria could occur through a series of closely related non-producing actinomycetes in soil and then, finally, to proteobacteria and distant (non-related) pathogenic species [18].

ROLE OF HORIZONTAL GENE TRANSFER (HGT) IN BACTERIA AMR

Transfer of AMR genetic determinants between bacterial populations occurs by the mechanism including transformation with free DNA, transduction by bacteriophages or conjunction involving plasmids [14], collectively referred to as HGT mechanisms. All three HGT mechanisms are widely used in nature, although certain species of bacteria employ one mechanism more heavily than the other two [40]. Streptococci, for example, employ transformation whereas enterococci employ conjugative plasmids for exchange of genetic information. Transformation is best characterized in Gram-positive *Streptococcus pneumoniae* and *Bacillus subtilis*, although it also occurs in many Gram-negative bacteria. Although the role of transformation in bacteria physiology is still debated, its main purpose is believed to be DNA repair or genetic diversification for bacteria adaptation enhancement [41]. Indeed, transformation seems to have played important role in evolution of antibiotic-resistant members of *Streptococcus* and *Neisseria* genera. It is commonly thought that transduction also play a major role in AMR evolution in *St. aureus*, although it has been shown to occur in many bacteria at a low frequency ranging between 10^{-6} and 10^{-9} transductants/plaque-forming unit [42]. *St. aureus* species members are highly variable bacteria and have a large accessory genome consisting of phages, plasmids, transposons, genomic islands, etc. Traditionally, it is believed that HGT in general and transduction in particular play a major role in antimicrobials-resistance gene transfer [43], but due to the difficulty of recombinant event detection in natural conditions (outside the laboratory) the contribution of transformation or transduction to the AMR genes transfer in the clinic or in the environment remains unclear. Plasmid-mediated conjugation is still considered more important mechanism for AMR gene dissemination in nature than transformation or transduction because plasmids are capable of autonomous transfer both in the environment and in the laboratory [44]. This is confirmed by the most well known plasmids that have resulted in dissemination of carbapenem- and quinolone-resistance genes in Gram-negative bacteria over the very long geographical distances [45]. Other DNA elements in Gram-positive bacteria known as conjugative transposons or integrative conjugative elements can also mediate conjugation. These elements can both integrate into and excise from the chromosome and transfer themselves to other bacteria through conjugation [46]. Resistance gene transfer by conjugation requires high-density bacteria settings such as the human and animal gut, biofilms in the environment, animal keeping facilities [45, 46]. According to the generally accepted concept, some resistance determinants have been plasmid-associated for a long time, while other are mobilized to plasmids from chromosomes and rate of such gene mobilization has increased for the last 70 years, that is accounted for the widespread use of antibiotics [47].

New trends for AMR gene dissemination are as follows:

– increase in rate of resistance determinant mobilization from chromosomes to plasmids;

– clustering of antimicrobials-resistance genes in plasmids probably in response to selective pressure in the environment. A well-characterized mechanism of clustering is demonstrated by *St. aureus* pSK41 conjugative plasmid containing an insertion sequence IS 257 that promotes capture of small resistance plasmids [43].

CONCLUSION

Use of antimicrobials as medicines, disinfectants and feed additives in various industries is considered to be one of the major causes of challenges associated with AMR emergence and spread in bacteria. Natural sources of AMR are usually not taken into account.

Analysis of domestic and foreign literature allows us to conclude that:

1. Natural antimicrobials-producing microorganisms have been and continue to be the primary sources of AMR genetic determinants. Despite of barriers to the exchange of genetic information between different genera of bacteria, widespread transfer of AMR genes from chromosomes of environmental bacteria to mobilized elements of pathogenic bacteria has occurred.

2. Antimicrobials – producing microorganisms, as a rule, have not one, but many complex self-defense mechanisms conferring complete protection from biologically active molecules produced by them, and the genetic determinants of self-resistance are almost always clustered with the antimicrobials biosynthesis genes and their expression is co-regulated. Therefore, to better understand AMR evolution, natural reservoirs of resistance genes should be considered in addition to frequently mentioned AMR causes that may comprise self-resistance determinants of antimicrobials-producing microorganisms since such resistance determinants in environmental microflora could result in further increase in number of such determinants in pathogenic bacteria populations and tremendous challenges. That is, studies and control of AMR determinant distribution in bacterial populations, clarification of resistance mechanisms identification of environmental factors that contribute to their spread are required to prevent AMR spread [8].

3. The majority of AMR mechanisms in bacteria appeared to emerge from intercellular mechanisms of resistance to the environment and the inclusion of AMR genetic determinants in the MGE of pathogenic bacteria poses a serious threat to animal and human health. Based

on the analysis of data in the available literature, it can be assumed that the transfer of these determinants from producers to pathogenic bacteria could occur through a number of indelibly linked closely related non-producing actinomycetes in the soil and only then to proteobacteria and distant (non-related) pathogenic bacteria species.

4. New trends for AMR gene dissemination are as follows: increase in rate of resistance determinant mobilization from chromosomes to plasmids observed during the last 70 years and clustering of antibiotic-resistance genes in plasmids probably in response to selective pressure in the environment. A well-characterized mechanism of clustering is demonstrated by *St. aureus* pSK41 conjugative plasmid containing an insertion sequence IS 257 that promotes capture of small resistance plasmids.

Prompt detection of changes in antimicrobials resistance dissemination in bacteria is of great practical and theoretical importance, as it allows update of the recommendations for antibacterial therapy in animal farming industry, development of express molecular methods for antimicrobial resistance detection and provides important information for the creation of new medicinal products that can overcome the resistance.

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Received 24.12.2021
Revised 11.02.2022
Accepted 03.03.2022

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