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Research Article

Change Resistance of *Salmonella enterica* and *Pseudomonas aeruginosa* against Antibacterial and Immobilization of Activities of Chloramphenicol

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Abstract

The mobility of enteropathogenic bacteria, such as *Pseudomonas aeruginosa* and *Salmonella enterica*, is an important factor in ensuring their survival and the realization of the pathogenic potential. Mobility, for example, *Salmonella*, correlates with invasiveness. We studied the adaptation of the bacteria *Salmonella enterica* and *Pseudomonas aeruginosa* to the immobilization activity of chloramphenicol. After culturing *P. aeruginosa* on the medium with high concentrations of chloramphenicol (37.5 ug/ml), bacteria have been adapted to antibiotic. We observed not only the growth of bacteria, in the presence of chloramphenicol at a concentration 75 ug/ml, but also the effect of swarming. The absence of selective pressure on the culture of *P. aeruginosa* (cultivated at low concentrations of chloramphenicol) do not initiated mechanisms providing resistance to the antibiotic. Experiments on the adaptation to chloramphenicol *S. enterica* were the same as for *P. aeruginosa*. Cultivation of *Salmonella* in the presence of various concentrations of chloramphenicol does not alter resistance to the immobilization of activity of antibiotic. However, MIC depended on the initial concentration of chloramphenicol at which *Salmonella* were cultured beforehand. There are probably some trigger mechanisms have a threshold sensitivity to certain concentrations of chloramphenicol. Thus, to a concentration of 0.3 ug/ml, MIC chloramphenicol between control and experimental cultures did not differ.

Keywords: *Mobility of the Bacteria; Salmonella enterica; Pseudomonas aeruginosa; chloramphenicol*

Introduction

Motility of enteropathogenic bacteria, such as *Pseudomonas aeruginosa* and *Salmonella enterica*, is the important factor, providing their survival and pathogenic potential realization. For example, motility in *Salmonellas* correlates with invasion [1-4]. The process of motility in the solid medium in *Pseudomonas* family bacteria is more complex than in *Salmonellas*, and includes separation of surfactant, providing slide-slip of bacteria on agar surface. It should be also noted that the loss of cells motility limits their ability to formation of biofilms [5]. According to our opinion, many power-consuming processes, such as efflux, metabolic misbalances caused by antibiotic effect, and inducible synthesis of antibiotic destructive enzymes must disorder the bacteria motility that makes its perspective target for the remedies of antibacterial therapy. Bacteria motility can be determined by the gradient of oxygen, ferrum salts, pH, amino acids, auto inducers and probiotics strains [6]. In relation to *S. typhimurium*, mutations leading to lipopolysaccharide synthesis disorder suppressed cell hive-out and also their differentiation into hiving forms [7]. Detection of the fluoroquinolones and aminoglycoside ability to suppress *S. typhimurium* cell motility *in vitro* [8] in the concentration lower MIC (minimal inhibiting concentration) expands perspective of antibiotics application and creation of the new classes of antibacterial compounds, which do not suppose the bacteria growth, but suppress their motility as a pathogenicity factor.

The aim of our studies

The study of *Salmonella enterica* and *Pseudomonas aeruginosa* bacteria to chloramphenicol, both in the aspect of immobilizing activity of this antibiotic, and in application to its bacteriostatic/bactericidal effect.

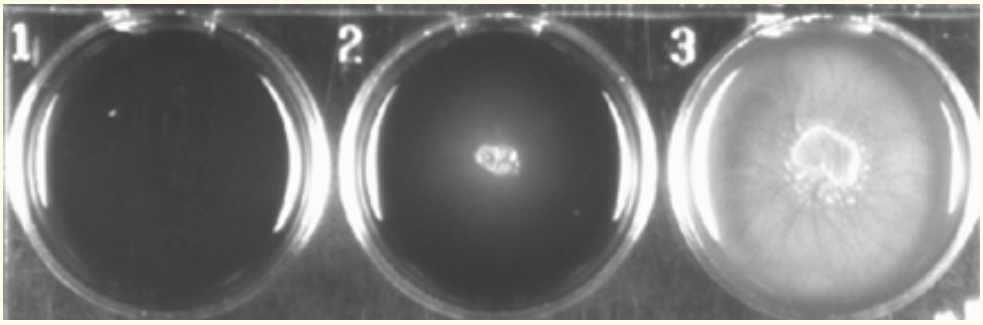
Materials and Methods

The work was executed on the basis of pharmacogenomics group of the Institute of Chemical Biology and Fundamental medicine SB RAS, the sector of molecular biology and laboratory diseases of young stock of agricultural animal breeding institute of experimental veterinary of Siberia and Far East of the Russian Agricultural Academy. For the studies *Salmonella enterica* sl.10 and *Pseudomonas aeruginosa* 668, 669, 671, strains were used, which are available in the collection of ICBFM SB RAS. Bacteria motility was estimated by means of cultivating *Salmonella* cultures and Pseudomonas aeruginosa on semi-solid agar in the presence of different concentrations of chloramphenicol. Per 2 ml of semi-solid nutrient medium MIO (Biomerieux Inc Mio Medium T3320) was injected in six-alveolar polystyrene sketch-boards, and inoculation of *Salmonella* daily cultures was conducted by wire inoculating loop in the centre of each hole. Screened cultures were incubated at the temperature 37°C during two days. The different dilutions of tested chemical compounds were injected into each hole, and dilutions, as a rule, were made with the step 1:2. The range of tested substances concentrations was determined in the preliminary tests. Bacteria antibiotic resistance was estimated also by disk-diffuse method with usage of Hottinger's agar.

Each test was executed in five replications. The data were processed by generally accepted statistic methods.

The results of own studies

Immobilizing activity was estimated according to antibacterial compound ability to suppress creeping growth of *S. enterica* and *Pseudomonas aeruginosa* by semi-solid agar. Accounting was made in two days, and the absence of creeping growth signs was estimated. In all test replications in all *P. aeruginosa* strains, MIC chloramphenicol made 75 mkg/ml (Figure 1). Immobilizing activity of chloramphenicol made 37.5 mkg/ml also in all replications and in all strains (Figure 1).



75 mkg/ml *37 mkg/ml* *18.75 mkg/l*

(chloramphenicol concentration)

Figure 1: *Suppression of P. aeruginosa motility in MIO medium at the different concentrations of chloramphenicol in the intact strains: upper row P. aeruginosa 668 strain, lower row – P. aeruginosa 671 strain.*

After *P. aeruginosa* cultivation in the medium with high chloramphenicol (37.5 mkg/ml), bacteria adaptation to antibiotic took place and we observed not only the bacteria growth at chloramphenicol concentrations 75 mkg/ml, but also the effect of cell hiving (Figure 2).

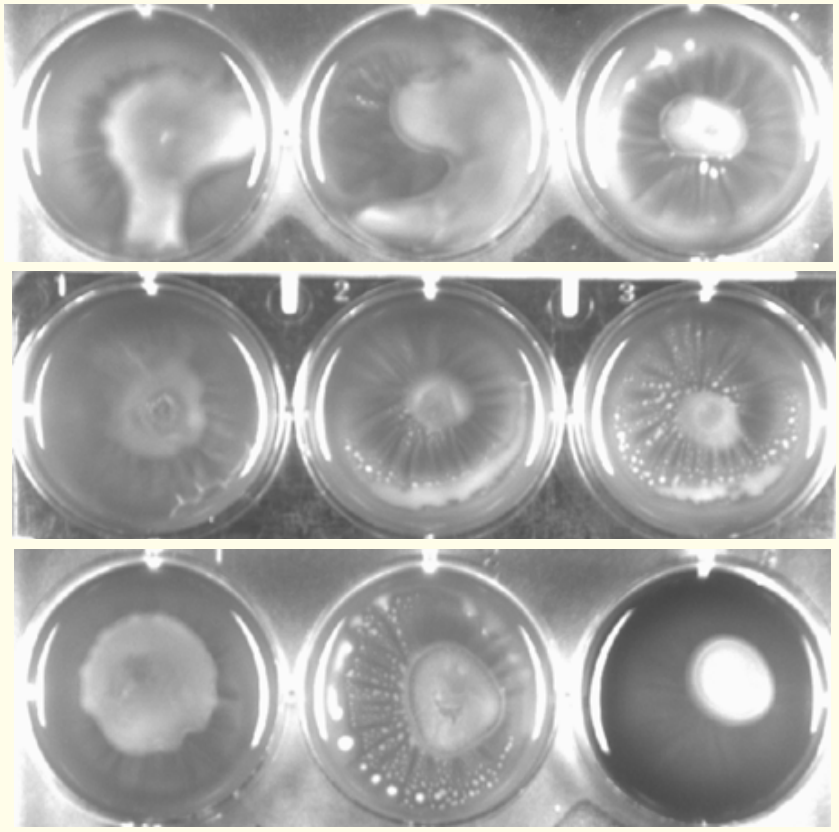


Figure 2: *Suppression of P. aeruginosa motility in MIO medium at the different chloramphenicol concentrations in strains, cultivated in the medium with chloramphenicol present 37.5 mkg/ml: upper row - P. aeruginosa 668 strain, middle row – P. aeruginosa 669 strain, lower row –P. aeruginosa 671 strain.*

After *P. aeruginosa* cultures screening, cultivated at chloramphenicol concentration 18.75 mkg/ml, chloramphenicol MIC also made 75 mkg/ml in all replications in all strains. Immobilizing chloramphenicol activity also didn't differ from such one in the source intact cultures. Thus, the absence of selective pressure on *P. aeruginosa* cultures didn't launch initiation of the mechanisms providing resistance to this antibiotic. The mechanisms of adaptation to antibiotic imply not only preservation of growth possibility at high antibiotic concentrations, but also motility preservation.

Chloramphenicol concentrations, at which *Salmonella* was tested, were less, than for cultures of Pseudomonas aeruginosa (the range of concentration from 1.175 to 0.075 mkg/ml). We didn't limit ourselves with the study of processes of Salmonella adaptation only to high and low antibiotic concentrations, and conducted the study with usage of the intermediary chloramphenicol concentrations (1.175; 0.6; 0.3; 0.15 and 0.075 mkg/ml). After Salmonella cultivation at enlisted antibiotic concentration by the method of serial dilutions, we estimated their sensibility to chloramphenicol and immobilizing antibiotic activity in relation to each of cultures in comparison with intact control. With assistance of disk-diffuse method also the change of cross antibiotic resistance change was studied.

It follows from the table 1 that *Salmonella* cultivation in the presence of different chloramphenicol concentrations didn't change their stability to immobilizing activity of this antibiotic. However, MIC depended on chloramphenicol concentration, at which *Salmonella* was

previously cultivated. Probably, there are trigger mechanisms, which have threshold sensibility to certain chloramphenicol concentrations. Therefore, MIC values between control and test cultures didn't distinguish before chloramphenicol concentration 0.3 mkg/ml.

Chloramphenicol concentration in the primary culture, mkg/ml.	MIC, mkg/ml.	Creeping growth suppression, mkg/ml.
1.175	1.175 ± 0.0	No growth
0.6	1.175 ± 0.0	0.6 ± 0.0
0.3	0.887 ± 0.14	0.6 ± 0.0
0.15	0.6 ± 0.0	0.6 ± 0.0
0.075	0.6 ± 0.0	0.6 ± 0.0
0 – intact culture	0.6 ± 0.0	0.6 ± 0.0

Table 1: *The study of stability development to chloramphenicol after cultivation of salmonella cultures with different antibiotic concentrations.*

Discussion

It comes under notice that at the primary isolation, *Salmonella enterica* sl10 strain culture was characterized by the stability to chloramphenicol MIC more than 23.5 mkg/ml, and immobilizing activity was observed at concentration 1.5. mkg/ml [9]. Strain cultivation in the conditions of laboratory increased its sensibility to antibiotic and passages in the medium with chloramphenicol didn't lead to restoration of strain stability to this antibiotic to the primary level (observed at primary isolation).

The results, obtained with using the method of serial dilutions in MIO medium are reproduced and at usage of disk-diffuse method. *Salmonella* cultivation at chloramphenicol concentration 0.6 mkg/ml was accompanied by the growth delay area reduction for 11.9% (Table 2) in comparison with intact control. The substantially reliable differences from control on resistance to chloramphenicol in cultures, preliminary cultivated in the medium with chloramphenicol concentrations 0.075, 0.15 mkg/ml were not observed. However, we observed correlation dependency of the growth delay area sizes from chloramphenicol concentration at the preliminary cultivation (Table 2).

Laevomycetin concentration in the primary culture, mkg/ml.	Antibiotics / cultures growth delay area (mm)				
	Clindamycin	Chloramphenicol	Spectinomycin	Enrofloxacin	Flumequine
0.6	17.83 ± 0.16	28.0 ± 0.14*	15 ± 0.26*	21.7 ± 0.15*	23.5 ± 0.34
0.15	18.0 ± 0.28	32.0 ± 0.28	16.2 ± 0.17	30.0 ± 0.28	27.7 ± 0.26***
0.075	18.0 ± 0.28	31.4 ± 0.35	14.8 ± 2.96	29.2 ± 0.33	30.0 ± 0.28
0 (control)	18.3 ± 0.22	31.8 ± 0.16	14 ± 0.28	29.0 ± 0.28	30.1 ± 0.27
r	-0.1485	-0.906	-0.189	-0.9644	-0.958

Table 2: *The study of salmonella stability development to antibiotics after preliminary cultivation with different chloramphenicol concentration at the usage of disk-diffuse method*

Note: r- Correlation between sizes of the growth delay area of laevomycetin concentration in the primary culture

** - P > 0.95, ** - P > 0.99, * **- P > 0.999*

Except for the resistance growth to chloramphenicol we observed the growth to cross resistance to fluoroquinolones (enrofloxacin and flumequine) and absence of such one in relation to clindamycin and spectinomycin. Earlier we showed the immobilizing activity of EDTA, some organic acids and chloramphenicol in relation to *Salmonella* [9]. Also, we detected correlation between florfenicol and chloramphenicol (r = 0.59), and the presence of similar stability mechanisms was supposed, as it is known that *floR* gene, responsible for

resistance to florfenicol, frequently is in one film-holder with genes of stability to other antibiotics, including chloramphenicol [10]. Mutation in *gyrA, gyrB, parC* or *parE* genes, coding the sub-units of topoisomerase II and IV, are the most distributed mechanism of stability to fluoroquinolones in bacteria [11]. However, these mutations must provide constitutional antibiotic resistance, as they effect on metabolic processes, constantly used by the bacterial cell, and don't effect on resistance to chloramphenicol. Nevertheless, there is efflux more – the mechanism, providing polyresistance of bacteria to antibiotic, carrying inducible character [12,13], at which the expression of genes, providing resistance, is triggered in the response to bacteria contact with antibacterial compound. In *S. enteric* serovar Hadar and *S. enterica* serovar Typhimurium the efflux of RND type pump were described: *AcrAB-TolC*, *AcrEF-TolC* and *YegMNO-TolC*, y S. Hadar is also known efflux MATE-type pump – *YdhE*. The activation of these pumps manages the global gene regulator – rma [12-14]. The German researches detected the effect of combined increase of MIC chloramphenicol and fluoroquinolones in the process of genetically modified *Salmonella* selection, which contain clones with corresponding genes of efflux-pumps and their regulators, under effect of fluoroquinolones [15,16]. We observed the analogous effect, but with using chloramphenicol in relation to "wild" *Salmonella* strains.

As it follows from the studies (Schmidt, S., P. Heisig, 2007) and Figure 3 [16,17], the inducer effect, in this case, probably antibiotic with MarR receptor launches MarA gene expression, which, in its turn, activates all enlisted *Salmonella* efflux-pump, but with different efficacy. Presumably, this process can bear self-supporting character due to activation of global gene regulator Rma. Obviously, the system of gene regulators *MarA – Rma* is capable to provide epigenetic mechanism of preservation of efflux-pump high activity and, accordingly, *Salmonella stability* to the wide range of antibiotics, during some time without repeated contacts with antibiotic-inducer.

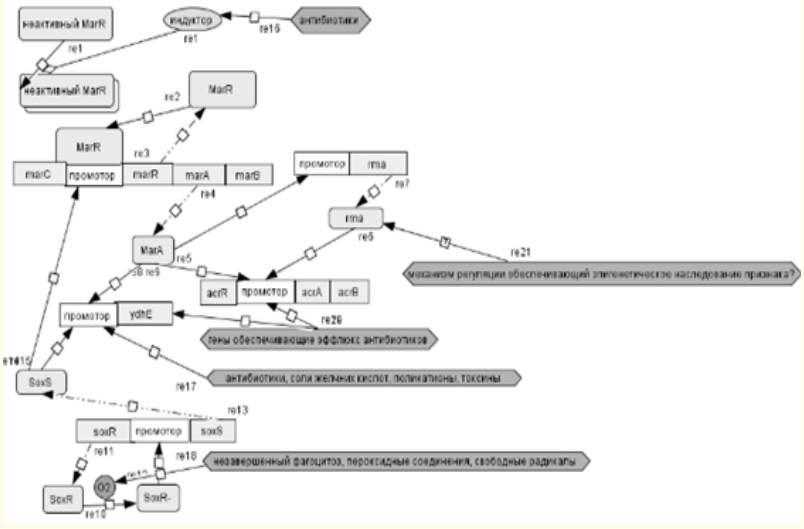


Figure 3: *The scheme of ways of regulating efflux-pump in Salmonella.*

Participation in the regulatory cascade, which launches polyresistance to antibiotics, transcription factor, which *SoxS* expression is increased in the presence of active oxygen forms, is interesting [18,19]. *SoxS* activation launches expression of the wide spectre of genes, including efflux-pumps [19]. Sensibility of this regulation system to oxygen allows supposing the reduction of efflux-pump efficacy in anaerobic conditions. In the veterinary practice, it can have value in three situations: 1. Usage of fluoroquinolones and/or chloramphenicol on the young stock of agricultural animal can be insufficiently effective, because in the gastrointestinal tract the areas with anaerobic conditions didn't manage to form. 2. The results of antibiogram, performed in anaerobic conditions, can underestimate the results of analysis in relation to the wide spectre of antibiotics. 3. The usage of peroxides indirectly before application of fluoroquinolones, chloramphenicols and the range of other antibiotics can be dangerous in the aspect of risk increase of mechanisms activation of Salmonella polyresistance.

The analysis of adaptation mechanism to chloramphenicol in the different types of bacteria, in our case *Pseudomonas aeruginosa* (PA) and *Salmonella* shows similar moments – low antibiotic concentrations don't effect on the bacteria growth and their physiological functions don't launch the adaptation or selection mechanism for stability to this antibiotic. PA and *Salmonella* cultivation in the chloramphenicol-based medium in immobilizing concentration was accompanied both by MIC and suppression of immobilizing antibiotic activity. Earlier we showed the association between bacteria adaptation to bactericidal and immobilizing antibiotic effect [9]. On the other part, if we use the lowest chloramphenicol concentrations sufficient for *Salmonella* growth suppression (1.175 - 1.5 mkg/ml), we shall not activate the protective mechanisms of adaptation to chloramphenicol in PA (18.75 mkg/ml). It is logical to suppose that the usage of antibiotic maximally low doses reduced the quantity of opportunistic bacteria types, which activated their mechanism of stability that can have value for the selection of antibiotic resistance bacterial strains [20].

Conclusions

1. The usage of chloramphenicol low concentrations, which provide only immobilizing effect on bacteria, is accompanied by activation of the resistance mechanisms both in *Salmonella enterica* and *Pseudomonas aeruginosa*, which include, above other things, MIC increase;
2. The usage of chloramphenicol low concentrations in relation to the sensible strains doesn't touch activation of antibiotic resistance mechanisms in the strains with primarily higher MIC values that reduces the risk of antibiotic resistance development.

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