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# Single Cell Level Survey on Heterogenic Glycopeptide and $\beta$ -Lactams Resistance

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## 1. Introduction

Traditional microbiological methods, which involve the study of populations rather than individual cells, obscure heterogeneity of microorganisms. Now this phenomenon is widely reported and study of individual cells rather than populations seems to be highly reasonable. Differences between cells can be observed, especially in bacterial biofilm structure. In the case of wide variety of cells in the mixture, flow cytometry has proved to be a successful method in investigating varied populations of cells. This technique operates essentially by monitoring bacteria in suspension flowing, so that only one cell at a time passes by a sensor. The information acquired based on size and inner complexity of particles as well as fluorescence emission following previous staining can deliver great amount of information. This process also provides an immediate assessment of cells populations.

Although more than three decades ago flow cytometry has been applied to measure bacteria (Paau et al. 1977, Bailey et al. 1977), its use in microbiology is still underestimated and far from fully utilized. One of the reasons for that is that bacteria in comparison to mammalian cells are so much more difficult to work with in terms of their size and cell structure. Nevertheless with increasingly sensitive equipment, flow cytometry is finding its utility in dealing with highly heterogeneous bacterial populations.

The best known example of heterogeneity of bacteria is antibiotic resistance (Davey et al., 2003; Falagas et al., 2008). It is defined as resistance to antibiotics expressed by a subpopulation of cells within strains susceptible to antibiotics according to traditional in-vitro susceptibility testing. The heteroresistance has been observed in a range of microbes, including *Staphylococcus aureus*, coagulase-negative staphylococci, *Acinetobacter baumannii*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Enterococcus faecium* and *Cryptococcus neoformans* (Alvarez-Barrientos et al., 2000). Most observations on heteroresistance reported in the literature concern staphylococci resistant to methicillin, vancomycin, and/or inhibitors of teichoic acid synthesis (Finan et al., 2002). Recently,  $\beta$ -lactams heteroresistance of *S. pneumoniae* and vancomycin heteroresistance of enterococci have been also reported (Hasman et al., 2006; Nottasorn et al., 2005). The mechanism of  $\beta$ -lactams resistance is a change in the affinity of penicillin-binding proteins or overproduction of specific penicillin-binding proteins. Williamson et al. showed that at least five PBPs had

been found in *E. faecalis*. Vancomycin resistance is usually determined by one of the two related gene clusters, *vanA* and *vanB*, that encode a dehydrogenase (VanH or VanHB) and ligase (VanA or VanB). Expression of VanA- and VanB-type resistance is regulated by the VanRS and VanRBSB two-component regulatory systems, respectively (Arthur et al., 1992; Evers and Courvalin, 1996).

As flow cytometry involves the study of single cells, it is an excellent method for studying heterogeneous populations. While analyzing many more cells than by conventional cytometry, rare cell types are more likely to be detected and the results are accessible to statistical analysis. The multiparametric nature of flow cytometric measurements is also an advantage. These include differences in cell size, DNA content or antigenic properties. Additionally, many supplementary modifications of flow cytometry are used. An example is the study of heterogenic vancomycin resistance with the use of reporter system for single-cell analysis of *van* gene expression. In the study, plasmid containing gene coding a green fluorescent protein was constructed for fluorescence-activated cell sorter (FACS) analysis (Cormack et al., 1996).

So far flow cytometry has been found useful in general microbiology with some clinical uses, as well as in its more specific branches such as environmental (Troussellier et al., 1999) or industrial microbiology (Gunasekera et al., 2000). First attempts to use flow cytometry for bacterial detection in body fluids are dated for the 80's when a test was developed enabling *E. coli* detection in blood samples (Mansour et al., 1985). The approach using flow cytometry has been continued and was reported later to be successful in bile, pleural fluid, ascites and cerebrospinal fluid (Takashi et al., 2000). Lately new tools based on flow cytometry have been developed, providing detection of significant bacteriuria as well as enumeration of bacterial particles, appearing to be an effective screening method (Hiroshi et al., 2006).

The emergence of multi-drug resistant microbial strains has emphasised the need for rapid detection method of antibiotic resistance and in this case as well flow cytometry appeared to be an extremely attractive tool.

Population analysis, among others, is the golden standard for the identification of hetero resistant vancomycin intermediate Staphylococcus aureus (hVISA) due to the fact that a proportion of MRSA found to harbor hVISA can be up to 8.3%. Moreover, for as yet unknown reasons, virtually all clinical *E. faecalis* isolates are tolerant to  $\beta$ -lactams and glycopeptides. Minimal inhibitory concentrations (MICs) of antimicrobials remain the same but the minimal bactericidal concentrations (MBCs) increase in tolerance.

*Enterococcus faecalis* usually plays a role in commensal flora in the human gastrointestinal tract. However, it can become a pathogen involved in various infections such as endocarditis, urinary tract infections, meningitis, sepsis and intraabdominal infections. It is also a leading cause of nosocomial infections, and the emergence of vancomycin- and multidrug-resistant *E. faecalis* in the clinical setting is of particular concern (Bizzini et al., 2009).

Here, the authors will present the analysis of heterogeneity of resistance studied by flow cytometry with use of fluorescent antibiotics binding bacterial cells and fluorescent probes targeting resistance genes. The proposal of vancomycin and methicilin heteroresistant strain model will be presented. With use of the model, results obtained for clinical *E. faecalis* and *S. aureus* strain are discussed.

## 2. Material and methods

The study was performed on reference and clinical strains listed in Tab 1. Fourteen MRSA strains were isolated from patients of various wards of the University Clinical Centre in Gdańsk and 12 *Enterococcus faecalis* from patients of Kościerzyna Medical Centre. The staphylococcal strains were isolated in the hospital laboratory during the period March 2008 to March 2009 and were sent to the Microbiology Department of the Medical University of Gdańsk for epidemiological typing. One isolate per patient was included in the study. Bacteria were cultured from swabs: wound (4 isolates), nose (2 isolates) and fluids: tracheostomy tube fluid (3 isolates), blood (1 isolate), abscess fluid (1 isolate), pus (1 isolate), bronchial fluid (1 isolate), urine (1 isolate). The data about clinical recognitions were not available. The isolates were cultured on sheep blood agar and were identified as *S.aureus* by colony morphology, a positive plasma coagulase reaction and by biochemical tests (API, bioMerieux, France). Resistance to methicillin was primary tested using disc diffusion method with cefoxitin disc (Clinical and Laboratory Standards Institute, 2006) and then was confirmed by detection of *mec A* gene by the Polymerase Chain Reaction (PCR) as described previously (Barski et al., 1996). DNA of bacterial isolates was extracted according to the previous report (Barski et al., 1996). For further analyses, the isolates were subcultured on nutrient broth and stored with glycerol at -70°C.

*Spa* typing and BURP analyses. The polymorphic X region of the protein A gene (*spa*) was amplified from the isolates by PCR with primers and according to procedure described by Kobayashi (1999). *Spa* types were determined by using Ridom Staph Type software, according to Harmsen et al (2003). The *spa* types were clustered into *spa*-CCs (clonal complexes) using the algorithm BURP - based upon repeat pattern (Rupptisch et al., 2006).

Phage typing was performed according to Blair and Williams (1961). Two sets of phages were used as follows: a basic set of 23 phages with additional phages 88,89,187 and an additional set of phages MR8, MR12, MR25, 30, 33, 38, M3, M5, 622, 56B supplied by the Central Public Health Laboratory in London for use on MRSA strains (Richardson et al., 1999). The phages were used in concentrations at routine test dilution (RTD) and 100xRTD. Reactions for phages were noted as described by Blair and Williams (1961). The phage type was defined by all the phages with strong reaction.

The susceptibility of the MRSA isolates to antimicrobial agents other than cefoxitin was determined by the disc diffusion method according to the guidelines (Clinical and Laboratory Standards Institute. 2006). The following drugs were used for test: erythromycin, clindamycin, ciprofloxacin, co-trimoxazole, tetracycline, gentamycin, vancomycin, teicoplanin, fusidic acid, rifampicin, linezolid, synergid, chloramphenicol. For isolates identified as resistant to erythromycin, but susceptible to clindamycin, D-test was performed to detect inducible clindamycin resistance. For vancomycin, additionally, the minimal inhibitory concentration (MIC) was determined by E-tests as described by the manufacturer (AB Biodisc, Sweden).

Enterococci were identified by esculin hydrolysis on CoccoSel agar (bioMerieux). Species-level identification was based on rapid Strep ID (bioMerieux). Susceptibility testing was performed by following the current guidelines of the National Committee for Clinical Laboratory Standards (2002). Isolates were additionally tested for resistance to nitrofurantoin, gentamycin, amikacin, tobramycin, kanamycin, streptomycin, neomycin, penicilin, meropenem, ampicillin, piperacilin and differentiated by antibiotic resistance pattern.

No.	Clinical source	Antibiotic resistance pattern	Phage group	Phage type	Spa type
SA1	Urine	Te C	III	42E/53/75/83A	010
SA1	Nose	E Cc Cip	III	54/75/83A	003
SA3	Nose	Sensitive	I/V	55/94/96	018
SA4	Tracheostomy tube fluid	E Cc Cip	III	47/54/75/83A	003
SA5	Blood	Cip Te C/	NT	-	008
SA6	Wound	Cip Te C/	III	53/83A	2065
SA7	Pus	E Cc Cip Sxt Te Rf C/Ge	NT	-	037
SA8	Bronchial fluid	E Cc Cip Te Ge	NT	-	008
SA9	Tracheostomy tube fluid	E Cc Cip Te Ge	III	47/54/75/83A	008
SA10	Tracheostomy tube fluid	E Cc Cip C	III	47/54/75/83A	151
SA11	Wound	E Cc Cip	II	55/71	003
SA1	Abscess	E Cc Cip	III	75/83A	003
SA2	Wound	E Cc Cip	III	83A	003
SA12	Wound	E Cc Cip	NT	-	002
SA	ATCC 25923		-	-	
SA	ATCC 29213		-	-	
E.F	wound	Te,FM, Rf, E,CC,An,NN,K,S	-	-	
E.F	Bronchial fluid	Te,Rf,E,CC,NN,K,N,Me,Am	-	-	
E.F	urine	FM,C,E,CC,Ge,An,NN,K,S,N	-	-	
E.F	urine	CC,An,NN,K,S,N,	-	-	
E.F	wound	FM,Cip,Ra,C,CC,An,NN,K,S,N	-	-	
E.F	wound	Te,FM,CC,Ge,NN,K,S,N	-	-	
E.F	faeces	Te,FM,C,CC,An,NN,K,S	-	-	
E.F	wound	Te,Rf,C,CC,An,NN,K,N,P,	-	-	
E.F	faeces	Te,Cip,Ra,C,CC,NN,K,	-	-	
E.F	faeces	Te,Ra,CC,NN,	-	-	
E.F	faeces	Cip,Ra,C,CC,An,NN,K,S,N	-	-	
E.F	faeces	FM,Cip,Rf,C,CC,An,NN,K,N,P, Mem,Am,PIP	-	-	
E.F	ATTC 51299	Van-R	-	-	
E.F	ATTC 29212	Van-S	-	-	

S.A Staphylococcus aureus, E.F. Enterococcus faecalis, ICU- intensive care unit; DVA - dermatology, venerology, alergology department; E - erythromycin; Cc - clindamycin (/ -inducible resistance to macrolides); Cip -ciprofloxacin; Te - tetracycline; C - chloramphenicol; Rf - rifampicin; Sxt trimetoprim/ sulfamethoxazole; Ge - gentamicin NT - non- typable; ° - inhibition reaction with phages; Cc - clonal complex; MIC - minimal inhibitory concentration; Va -vancomycin; FM- Nitrofurantoin, , Ge- gentamicin, AN- Amikacin, NN -Tobramycin, K- Kanamycin, S-streptomycin , N- Neomycin, P- penicilin, MeM- Meropenem, AM- Ampicilin, PIP piperacilin

Table 1. List of strains used in the study.

For flow cytometry analysis, the isolates were grown overnight on Tryptic Soy Broth (TSB) at 37°C and standardized to OD 1 (600 nm). The cells were then centrifuged 5 min at 2500 rpm, washed and suspended in 200  $\mu$ l PBS buffer. The binding assay was carried out by adding solution of Bocillin @fl or vancomycin @fl respectively to the cell suspension of each strain. The reaction mixtures were incubated for 30 min at 37°C and centrifuged. The pellets were collected and washed three times in 1 ml of PBS. Fluorescence of particles was determined by a FACS Scan flow cytometer (Becton Dickinson, San Jose, CA, USA).

### 2.1 Optimalisation of bocillin@fl and vancomycin@fl binding

In the previous study the relation between fluorescence and amount of used bocillin@fl was described by equation below;

$$y = 4.469\ln(x) + 3.427, R^2 = 0.944 \quad (1)$$

Following these results (Jarzembowski et al., 2008) and similar results obtained during study of vancomycin @fl binding (Jarzembowski et al., 2009) we stained cells by addition of 1 $\mu$ g/ml vancomycin@fl. Because of inductive mechanism of vancomycin resistance, before staining strain was cultured in presence of 2 $\mu$ g/ml vancomycin (Sigma). The reaction mixtures were incubated for 45 min at 37°C and again centrifuged. The pellets were collected and washed three times in 1 ml of PBS. Fluorescence of particles was determined with the use of FACS Scan flow cytometer (Becton Dickinson, San Jose, CA, USA). Results of induction of reference resistant strain are shown on Fig 1.

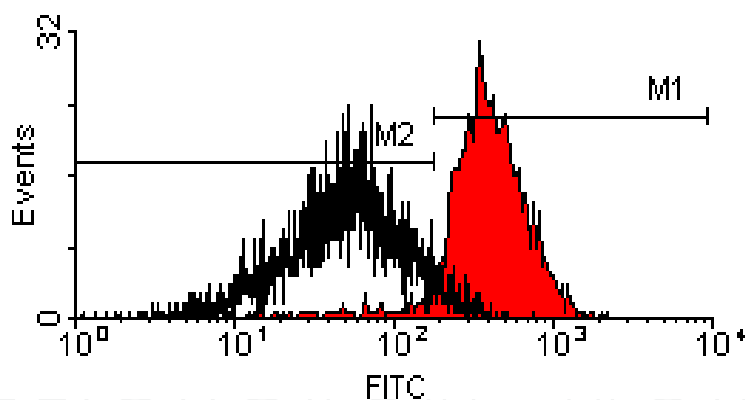


Fig. 1. Fluorescence (FL1) induced (red) and uninduced (black) vancomycin resistant reference strain after staining with vancomycin@fl

To ensure cell nature of analysed particles we have excluded from procedure particles sized below 1 $\mu$ m based on value for size standard (Polysciences. Inc) and particles with low PI (propidium iodide) binding (Fig2).

### 2.2 Modeling of vancomycin heteroresistant strain

Mixture of the reference strains in proportion starting from 1.25 up to 50 % of resistant cells were composed to create model of heteroresistant strain. Cells binding and not binding vancomycin @fl were differentiated by markers consisting of 99.69 % of susceptible (M1) and 94% of resistant (M2) cells (Fig 3)

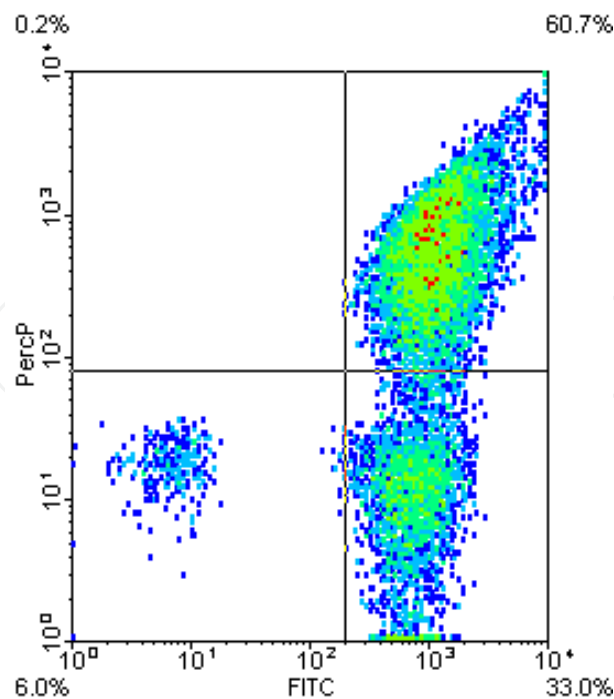


Fig. 2. Green (x axis) vancomycin@fl fluorescence and red (y axis ) PI fluorescence reference susceptible strain. Only upper regions were included into further analysis.

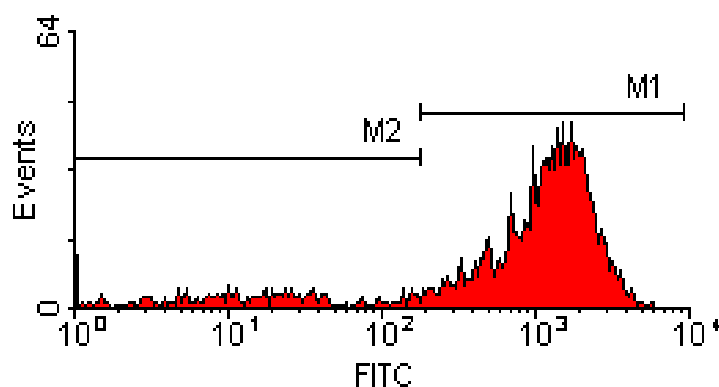


Fig. 3. Fluorescence (FL1) of mixed culture consisting of 25% resistant cells

### 2.3 Design and testing of DNA probes

Oligonucleotide probes were designed with the software package (Biosoft: Beacon Designer) and rRNA sequences were obtained from the Ribosomal Database Project (RDP) supplemented with newly deposited rRNA sequences from GenBank. As a positive control, Enfl84 probe (3'-ACGTGAGTTAACCTTTCTCC, Waar et al. 2005) targeting 16sRNA gene was used. Fluorescein-labelled oligonucleotides against selected specific target sequences (van genes) of *E. faecalis* were synthesized commercially (Metabion) and tested for specificity against the set of reference organisms. For hybridization, procedure described by Waar et al (2005) was adopted. Briefly, cell membrane was permeabilized by incubation for 30 min at 37 °C in permeabilization buffer consisting of 1 mg lysozyme ml<sup>-1</sup> (DNA Gdańsk). The cells were then centrifuged 5 min at 2500 rpm, washed and suspended in 200µl PBS buffer.

Subsequently, 200 $\mu$ l hybridization buffer containing FITC- labelled probe (1ng ) was added. The probes were hybridized at 50°C for 45 min.

### 3. Results and discussion

Heteroresistance, defined as a presence of subpopulations of resistant cells within susceptible majority was observed against  $\beta$ -lactams and glycopeptides. However, mechanism of actions of these groups of antibiotics is different.

The emergence of community acquired methicillin resistant *Staphylococcus aureus* (cMRSA) has renewed interest in the mechanisms of methicillin resistance (Okuma et al., 2002; Nunes et al., 2007). In contrast to vancomycin resistance, resistance to  $\beta$ -lactams (methicillin resistance) is constitutively mediated by a affinity change in penicillin-binding proteins (enzymes involved in cell wall synthesis). Because many genes are involved in cell wall biosynthesis, explanation of the nature of heterogeneous expression of methicillin resistance is very difficult (Markova et al., 2008). Since 2001, an increase in the number of MRSA strains with heteroresistance to oxacillin has been observed in the Netherlands. These strains can spread unnoticed, as their phenotypic heterogeneous resistance to beta-lactams affects the results of susceptibility testing (Wannet 2002). Coexistence of two subpopulations (susceptible and resistant) within a clinical isolate and expression of resistance only in a small number of cells leads to diagnostic problems in clinical laboratories (Markova et al., 2008). In methicillin-resistant *Staphylococcus* strain degree of heterogeneity is quite low: only 1 CFU of  $10^4$ - $10^7$  CFU are phenotypically resistant (De Lencastre et al., 1993).

Invention of Bocillin@fl, commercially available fluorescent penicillin, significantly increased possibility of studying heteroresistance (Jarzembowski et al., 2010). Results of flow cytometry were found to be correlated with reference heteroresistant strain properties. Furthermore, use of fluorescent antibiotics in flow cytometry proved linear nature of heteroresistance (Fig.4).

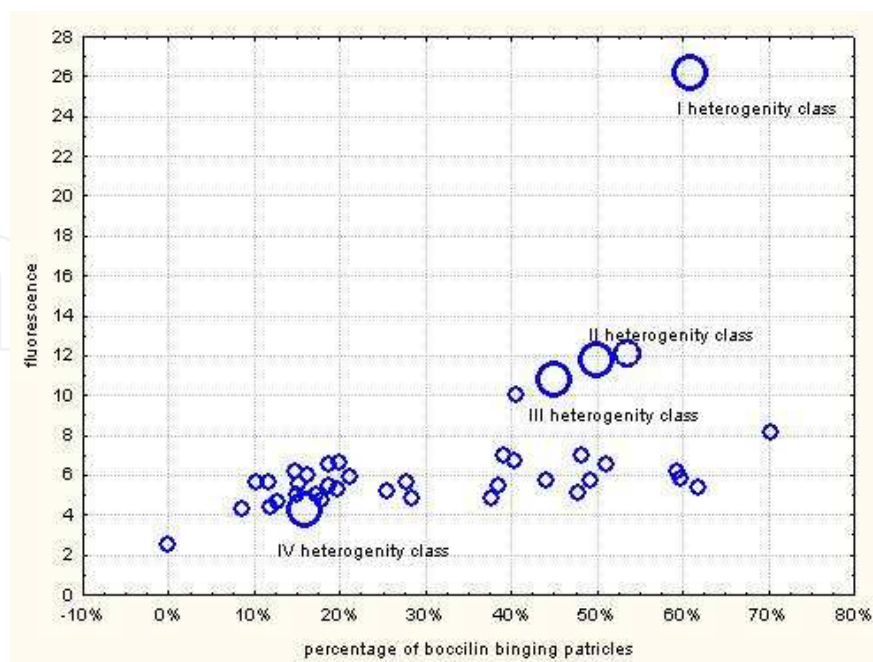


Fig. 4. Variance of bocillin @fl binding by medical isolates of *S.aureus* in relation to reference value



However, even in susceptible cell culture we always detect at least 1.47% antibiotic unbinding particles so it seems that the number of resistant cells in this method is overestimated.

Results of Markova et al study (2008) showed that also changes of cell structure can be involved in mechanism of heteroresistance. Both heteroresistant and methicillin susceptible strains (MSSA) may transform into cell wall-deficient form after exposure to sub tolerant conditions. The finding that methicillin sensitive strain and heteroresistant strain give rise to L-form colonial growth both on oxacillin-free and oxacillin-containing media appeared to be remarkable. It is known that the heterogeneous resistance phenotype of *mecA*-positive MRSA strains progresses to homogeneous resistance upon incubation with methicillin (Sakoulas et al., 2001). Study results of resistance demonstrate that differences reduction of antibiotic binding after preincubation can be proved with the use of flow cytometry (Fig.5).

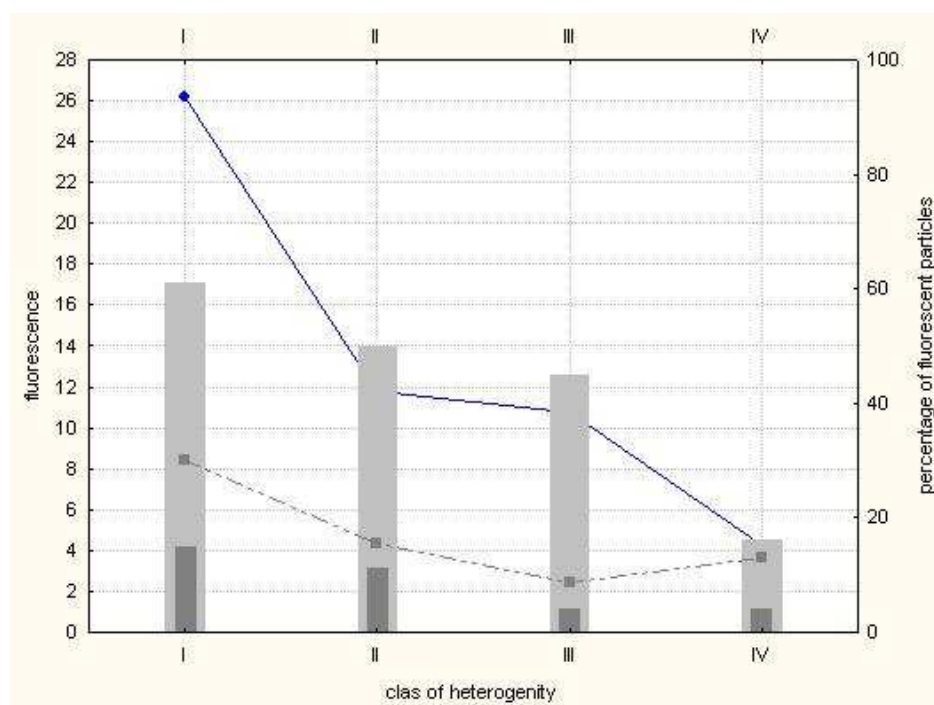


Fig. 5. Influence of incubation of heteroresistant strain with methicillin on Bocillin@fl binding

In contrast to MSSA and heteroresistant strains, homogenic MRSA strains did not convert to the cell wall-deficient forms (Markova et al., 2008).

Vancomycin resistance is an example of inductive reaction on presence of antibiotic. In presence of vancomycin high amount of cell wall precursor with low affinity to vancomycin (d-ala-d alla) is synthesized while native precursors are removed (Hasman et al., 2006). Because we aimed to detect resistant cells between susceptible majorities, the induction condition should be set to prevent inhibition of growth of susceptible strain. Thus, we set up inductive concentration below breakpoint value, at 0.016 $\mu$ g 0.8  $\mu$ g, 2 $\mu$ g and 4 $\mu$ g/ml. The results showed optimal induction with final vancomycin concentration of 2 $\mu$ g/ml (data not shown). While there are no reference strains of different degree of vancomycin heteroresistance, model obtained by combination of resistant and susceptible strain was prepared. Vancomycin@fl binding by cells in this model is presented on fig 6. The high correlation between percentage of fluorescent particles and percentage of resistant cells can be seen. (Fig. 6)

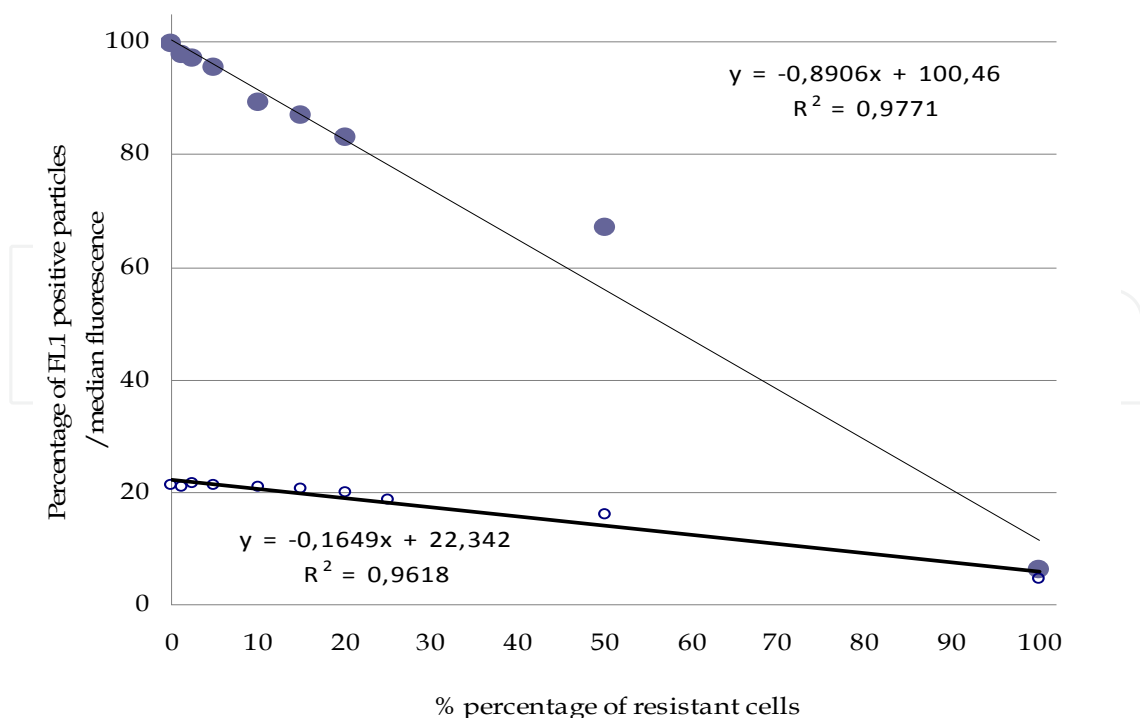


Fig. 6. Correlation between fluorescence (empty circles ), FL1 positive particle (full circles) and percentage of resistant cells

The obtained model of vancomycin heteroresistance was then used for analysis of clinical strains. The percentage of stained particles ranged from 6.07% in resistant up to 99.66% in susceptible reference strains. Percentage in resistant unindicted strains was similar to the susceptible one (94.62 %). Reference strains showed also differences in fluorescence but they were not so significant (Tab. 3.) Similar, constant divergence was observed in the study of methicillin heteroresistance (Jarzembowski et al., 2010). Despite satisfactory differentiation of heteroresistance, the results showed some limitations of the method. Even in resistant strains some particles bind vancomycin@fl according to the chosen criteria. Among clinical strains, the percentage of unstained particles varies from 46.32 to 98.53 while median fluorescence varied from 547 to 1980.96. (Tab 2.). Generally, fluorescence of staphylococci was higher than fluorescence of enterococci

	percentage of FL1 positive particles	Median fluorescence	Mean fluorescence per particle
max in staphylococci	97.77	1980.96	37.01
max in enterococci	98.53	1684.85	27.26
Min in staphylococci	46.32	572.55	10.87
Min in enterococci	90.47	547.37	9.01
resistant reference strain	6.07	239.28	4.6
Susceptible reference strain	99.69	10009.04	21.2
Resistant uninducted strains	94.62	403.15	11.5

Table 2. Comparison of estimator values in groups of strains

In contrast to results of MIC based screening of vancomycin resistance, the results obtained in flow cytometry suggest heteroresistance of some strains (Fig. 7). It is obvious if we consider that presence of heteroresistant strains in US and Europe is quite high. Nunes et al. (2007) found a prevalence of 8.5 % (9/106) of glycopeptides-heteroresistant staphylococci isolated from bacteremia while others 7.4% (Frebourg et al., 1998).

None of the tested strains had a MIC value below 4 $\mu$ g/ml, so all of them should be considered as vancomycin susceptible. On the other hand, determination of MICs have shown good accuracy only to detect vancomycin intermediate *Staphylococcus aureus* (VISA) and fail in the detection of glycopeptides heteroresistant strains (Nunes et al., 2007). Heterogeneous phenotypes of clinical strains of *S. aureus* and CNS present MIC value below 4 $\mu$ g/ml (Nunes et al., 2007). That is why heteroresistance of the strains could be missed out in routine diagnostic.

The heteroresistance has been considered responsible for failure in the treatment. Unfortunately, there is no widely accepted method to detect heteroresistant strains. The lack of a standardized method makes it difficult to determine the clinical significance in the treatment of an infection with vancomycin. In the presence of vancomycin not only van genes change expression. There is some evidence that vancomycin influences biofilm formation, at least in *S. epidermidis* culture. Changes of cell surface structure and size seen in flow cytometry as SSC and FSC values were explained by the studied effect of the glycopeptide antibiotic on cell envelope properties and biofilm formation. SEM examination of cells grown in the presence of vancomycin revealed the presence of polymorphic form. Such changes in cell morphology could be the reason of extremely high fluorescence of some strains after staining with vancomycin@fl.

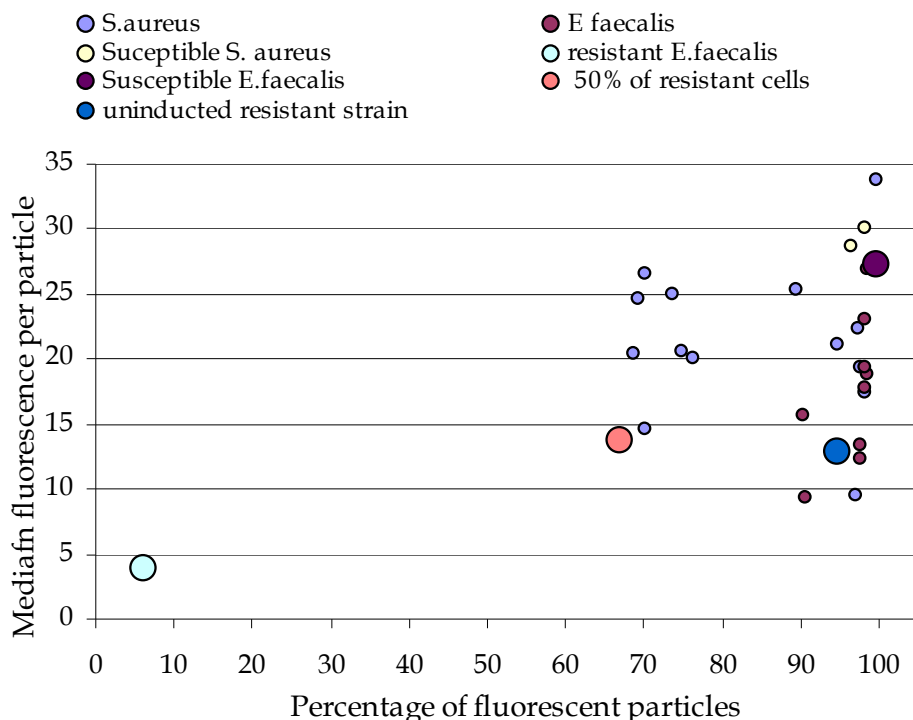


Fig. 7. Variance of vancomycin@fl binding by medical isolates of *S.aureus* and *E. faecalis* in relation to reference value

Both in study of vancomycin heteroresistance and study of methicillin resistance, median fluorescence per particle seems to be a very effective estimator (Fig. 1, 7). It is interesting to compare FCM results of “native ‘methicillin heteroresistance (Fig. 4) and “artificial’ model of vancomycin heteroresistance. Despite different resistance mechanism, changes of estimators are very similar. Both fluorescence and percentage of fluorescent particle decrease with amount of resistant cells (Fig. 7).

Detailed information about mechanism of heteroresistance can be explained by the study of resistance gene expression. At the first stage of our ongoing study we adopt protocol described by Warr (2005) to detect 16sRNA gene. In fact, Fig. 8 demonstrates successful detection of *E. faecalis* cells in flow cytometry. Furthermore, the protocol used determines the amount of cells in mixed culture.

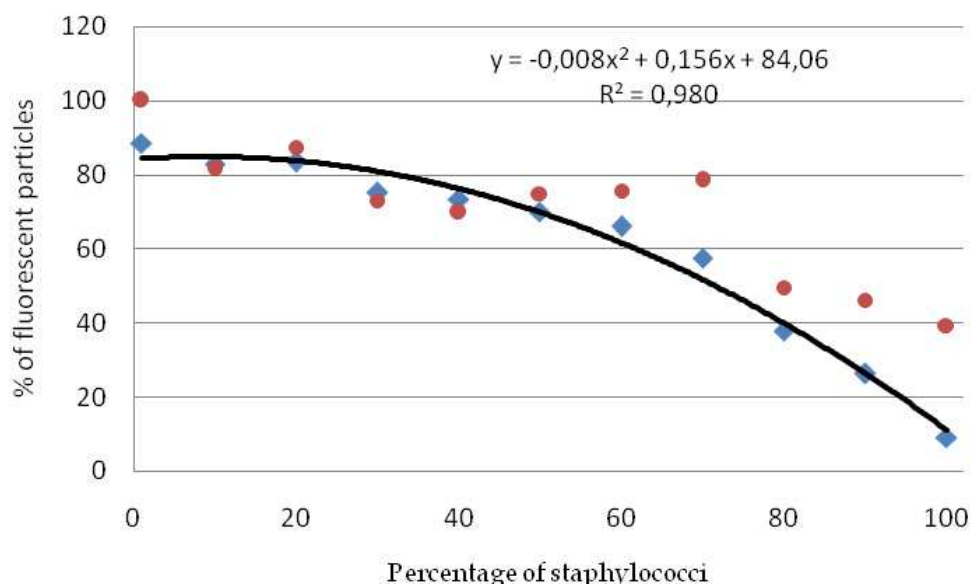


Fig. 8. Correlation between percentages of detectable particles in FLOW-FISH and proportion of *E. faecalis* cells in mixed culture stained by fluorescent 16sRNA probe

Detection of 16s RNA gene, which appeared to be successful in single cells, ranged between  $10^4$  and  $10^5$  ribosomes and, consequently, as many copies of 5S and 16S and 23S-like rRNAs (Amman et al., 1990) making it was quite easy to get satisfactory signal. Despite the proven usefulness, serious limitations of FLOW-FISH technique in its sensitivity and resolution constitute an obstacle. It is believed that probes containing nucleic acid analogs with higher affinity for DNA and RNA may have the potential to reduce these problems (Kelly et al., 2009). An example of such probe is peptide nucleic acid (PNA), which binds to DNA and RNA with high affinity.

For our study, we decided to use another class of nucleic acid analogs in which the ribose sugar is constrained by a methylene bridge between 2'-oxygen and 4'-carbon, resulting in an N-type (3'-endo) conformation, so called locked nucleic acids (LNAs) (Kelly et al., 2009).

LNA is able to hybridize with DNA and RNA according to Watson-Crick base-pairing rules and does so with unprecedented high affinity. As a result, LNAs have been shown to significantly improve the sensitivity and specificity.

This technique is expected to be particularly useful for the detection of lower mRNA transcript levels. In fact, preliminary results (Fig. 9) are promising. On the other hand, further studies are needed for validation of chosen approach.

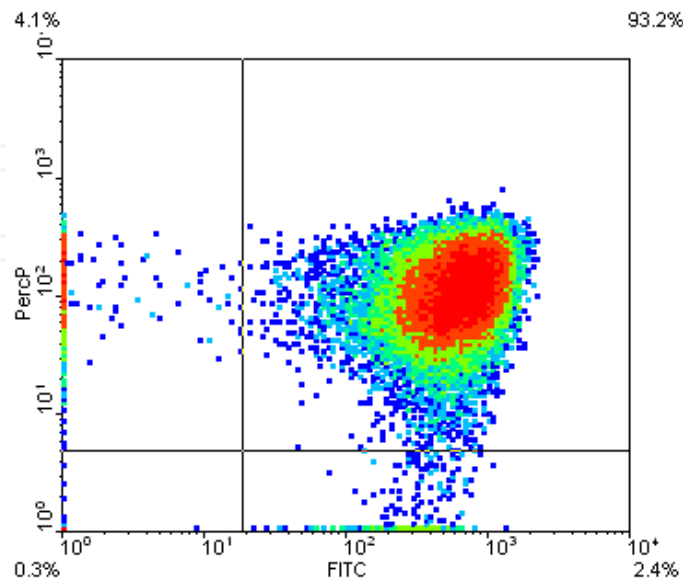


Fig. 9. Results of regulatory vancomycin gene detection with LNA probe (x axe- binding of probe, Y axe PI staining)

### 3. Conclusion

According to obtained results, fluorescent antibiotics are excellent tools both, for detection of resistance and studies of heteroresistance in flow cytometry. Model of vancomycin heteroresistance was successfully prepared and used in studies of clinical strains. Because it is believed that heteroresistance mechanism involves changes of cell structure, it is especially profitable in terms of antibiotic binding and changes in cell structure can be observed. However, overestimation of resistant cells in culture remain unsolved.

Preliminary results of FLOW-FISH are also promising. It seems, that use of LNA probes allows to express detection in single cells in culture. On the other hand, it is obvious that further studies are needed to evaluate the specificity and sensitivity of this approach.

### 4. Acknowledgment

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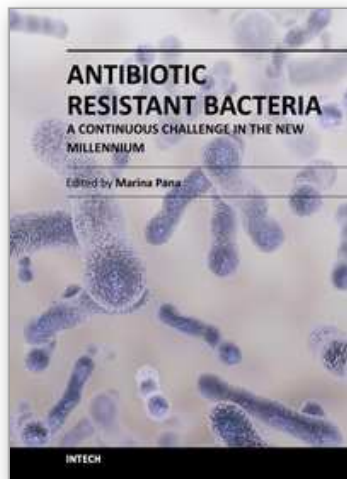
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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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