Effects of Gentamicin, Amikacin and Netilmicin on the Pathogenic Factors and Two Extracellular Quorum Sensing Systems of *Pseudomonas aeruginosa* Strains

Pseudomonas aeruginosa Suşlarının İki Hücre dışı "Quorum Sensing" Sistemi ve Patojenik Faktörleri Üzerinde Gentamisin, Amikasin ve Netilmisinin Etkileri

Hüseyin BASKIN,[a]
Vahide BAYRAKAL,[a]
İsmail Hakkı BAHAR[a]

[a]Department of Medical Microbiology,
Dokuz Eylül University
Faculty of Medicine, İzmir

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Yazışma Adresi/*Correspondence:*
Vahide BAYRAKAL
Dokuz Eylül University
Faculty of Medicine,
Department of Medical Microbiology,
İzmir,
TÜRKİYE/TURKEY
vahide.bayrakal@deu.edu.tr

ABSTRACT Objective: *Pseudomonas aeruginosa (P. aeruginosa)* is a human opportunistic pathogen that colonizes biotic and abiotic surfaces and has been emerging as an important source of nosocomial infections. Quorum sensing (QS) systems are interbacterial signaling systems and have a role in the regulation of virulence factors. These systems are efficient with extracellular signaling molecules and are used to detect the local concentration of bacteria. **Material and Methods:** Minimum inhibitory concentrations (MICs) for aminoglycosides (gentamicin, amikacin and netilmicin) of two clinical isolates from catheter site and (C1, C2) an ATCC 27853 *P. aeruginosa* isolate were determined. Then the relationship between las and rhl QS systems and biofilm formation, gelatinase, alkaline protease, oxidase, catalase, which are extracellular virulence factors of *P. aeruginosa* were investigated at these MIC and sub-MIC levels. **Results:** Biofilm formation, and protease-gelatinase activities were positive under all concentrations of aminoglycosides (gentamicin, amikacin, and netilmicin) at 18 hours of the experiments for all *Pseudomonas* strains. Enzymatic activities of catalase and oxidase, and the signal molecules of the QS systems were present under MIC and sub-MIC concentrations. **Conclusion:** Our study showed that different aminoglycoside (gentamicin, amikacin, netilmicin) concentrations had different effects on "bacterial phenotypic changes". According to our results, different "chemical microenvironments" have different effects on the "bacterial behavior". On the next step of translational experiments, it may be useful to organize experimental infection models in animals, against different susceptible antibiotics.

Key Words: *Pseudomonas aeruginosa*; gelatinases; alkaline protease; biofilms; quorum sensing; gentamicins; amikacin; netilmicin

ÖZET Amaç: *Pseudomonas aeruginosa (P. aeruginosa)*, biyotik ve abiyotik yüzeylerde kolonize olan ve hastane enfeksiyonlarında önemli bir etken olarak ortaya çıkan bir fırsatçı insan patojenidir. Bakteriler arası sinyal sistemleri olan "Çoğunluğu Algılama" (ÇA) sistemlerinin, virülans faktörlerinin düzenlenmesinde etkili olduğu gösterilmiştir. Bu sistemler hücre dışı sinyal moleküllleri ile etkindirler ve bakterilerin bulunduğu yerdeki yoğunluklarını tespit etmek için kullanılmaktadırlar. **Gereç ve Yöntemler:** Kataterden izole edilen iki klinik izolatın (K1, K2) ve ATCC 27853 *P. aeruginosa* izolatlarının aminoglikozitlere karşı (gentamisin, amikasin ve netilmisin) minimum inhibitör konsantrasyonları (MİK) belirlenmiştir. Daha sonra belirlenen MİK ve sub-MİK değerlerinde las ve rhl ÇA sistemleri ile biyofilm oluşumu, *P. aeruginosa*'nın hücre dışı virülans faktörleri olan jelatinaz, alkalen proteaz, oksidaz, katalaz arasındaki ilişki değerlendirilmiştir. **Bulgular:** Tüm *P. aeruginosa* suşlarında deneylerin 18. saatinde biyofilm oluşumları, alkalen proteaz ve jelatinaz aktiviteleri aminoglikozitlerin (gentamisin, amikasin ve netilmisin) tüm konsantrasyonlarında pozitif bulunmuştur. Katalaz ve oksidazın enzimatik aktiviteleri ve ÇA sistemlerinin sinyal molekülleri MİK ve sub-MİK konsantrasyonlarında gösterilmiştir. **Sonuç:** Çalışmamızda, farklı aminoglikozit konsantrasyonlarının (gentamisin, amikasin, netilmisin) "bakteriyel fenotipik değişikliklerde" farklı etkilerinin olduğu görülmüştür. Bulgularımıza göre, farklı "kimyasal mikroçevreler" "bakteriyel davranışta" farklı etkiler göstermektedir. Translasyonel deneylerin bir sonraki adımında, farklı duyarlı antibiyotiklere karşı, hayvanlarda deneysel enfeksiyon modelleri hazırlamak yararlı olabilecektir.

Anahtar Kelimeler: *Pseudomonas aeruginosa*; jelatinazlar; alkalin proteaz; biyofilmler; çoğunluk algisi; gentamisinler; amikasin; netilmisin

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Many bacteria use cell-cell communication to monitor their population density, and synchronize their behaviors by hormone like compounds called auto-inducers (AI). Bacteria achieve signaling in microbial communities to coordinate gene expression within a population and this communication is generally called quorum sensing.[1]

Bacteria growing in a biofilm are embedded within a matrix, which consists of proteins, polysaccharides, and nucleic acids. The "architecture" of this matrix protects these organisms against antibiotics and the host immune systems. When bacteria develop the biofilm, bacteria within the biofilm become 10-1000 times more resistant to antibiotics compared with the planktonic bacteria.[2,3]

Pseudomonas aeruginosa (*P. aeruginosa*) is the model of a bacterium in explaining quorum sensing mechanisms and biofilm formation. *P. aeruginosa* is an important opportunistic human pathogen, causing various persistent infections in immunocompromised hosts, hospitalized patients, and patients with cystic fibrosis. These infections are difficult to treat since *P. aeruginosa* has resistance to most antibiotics when it is present in biofilms.[4]

Quorum sensing (QS) mechanisms of P. aeruginosa are one of the most studied QS mechanisms because of the clinical importance of Pseudomonas infection therapy. Las (3 oxo C12 HSL, long-chain), PQS (for Pseudomonas quinolone signal, non AHL "non- Acyl Homoserine Lactone", extracellular signal) and rhl (C4 HSL, short chain "N-butyryl-L-Homoserine Lactone") are the major systems of P. aeruginosa.[5,6] The las system is responsible for expression of virulence genes such as elastase, protease, and biofilm formation and also for the regulation of the rhl system which is responsible for expression of virulence genes such as Las B elastase, Las A protease, pyocyanin, cyanide, and sigma factors.[7]

P. aeruginosa produces several extracellular products like alkaline protease, biofilm, gelatinase, exotoxin A, exoenzyme S which can cause extensive tissue damage, bloodstream invasion, and dissemination following colonization.[8,9] *P. aeruginosa* also produces several proteases including Las B elastase, Las A elastase, and alkaline protease which play a major role during acute *P. aeruginosa* infection. Among those proteases, although the function of alkaline protease in tissue invasion and systemic infections is unclear; however, its role in corneal infections may be substantial.[10,11] The consequence of minimum inhibitory concentrations (MICs) of antibiotics on a range of global responses in the cell has been identified, thus providing important information on metabolic interactions associated with the target node.[12]

The first part of this study was to determine the minimum inhibitory concentrations (MICs) of two clinical isolates from catheter site and an ATCC 27853 *P. aeruginosa* isolate for gentamicin, amikacin and netilmicin. Then the relationship between las and rhl QS systems and biofilm formation, gelatinase, alkaline protease, oxidase, and catalase- extracellular virulence factors of *P. aeruginosa*-was investigated at these MIC and sub-MIC levels.

MATERIAL AND METHODS

BACTERIAL ISOLATES

These isolates consisted of *P. aeruginosa* ATCC 27 853, and two clinical *P. aeruginosa* strains isolated from catheter site.

REPORTER STRAINS

In order to show the two different QS systems of *P. aeruginosa*, *Chromobacterium violaceum (CV026)* and *Agrobacterium tumefaciens (A136),* which are soil bacteria, were used to determine Las R/I and Rhl R/I consequently.

DETERMINATION OF MIC AND SUB-MIC LEVELS

MIC levels of gentamicin, amikacin, and netilmicin for each strain were studied by microdilution methods with cation added Muller Hinton Broth (MHB, Oxoid). The turbidity of bacterial suspension was prepared according to McFarland 0.5 ($1.5x10^8$ bacteria/mL). The MHB was dispensed into the ELISA micro plate wells in equal amounts followed by the addition of two-fold dilutions of antibiotics and finally the bacterial suspensions.

The plates were incubated at 37°C for 18 hours. The plates were spectrophotometrically (Multiskan FC.1.00.75, SN: 357-00097) evaluated at 450 nm.[13,14]

BIOFILM ASSAY

The turbidity of bacterial suspension was prepared according to McFarland 0.5 ($1.5x10^8$ bacteria/mL). The MHB was dispensed into the ELISA micro plate wells in equal amounts followed by the addition of MIC and sub-MIC dilutions of antibiotics (gentamicin, amikacin, netilmicin) and finally the bacterial suspensions. The plates were incubated at 37°C for 18 hours. At the end of the incubation period, the plates were washed with phosphate-buffered saline (PBS) (pH 7.2). The microtitre plates were then inverted and were allowed to dry before each well was filled with 200 µL of 1% (w/v) crystal violet (CV) solution and were incubated at room temperature for 30 minutes. Unbound CV was removed by three washes with distilled water and the plates were inverted to dry at room temperature. Cell-bound CV was released from the bacterial cells by the addition of 200 µL of 95% ethanol and after incubation at room temperature for 30 minutes on a rotary shaker the concentration of CV in each solution was determined by the optic density (OD) reading at 450 nm.[15]

MICRO-AHLS BIOASSAYS

To analyze AHL production we developed a quantitative micro plate assay. Sterile 96-well flat-bottom microplates of polystyrene were used for the assays. After adding 200 µl of temperate Louria Bertani Agar (LBA, Oxoid) to each well, the plates were dried at room temperature for 2 hours. CV026 and A136 strains were grown in Louria Bertani Broth (LBB, Oxoid) for 18 hours at 30°C, and suspensions were made in PBS to obtain an OD 600 of 1.00. Twenty microliters of CV026 and A136 suspensions were transferred from each well to the solid media and the plates were air dried for 30 minutes. Thereafter, 10 µl of strains exposed to MIC and sub-MIC levels of various antibiotics (gentamicin, amikacin, netilmicin) were dispensed equally into the wells of these ELISA micro plates and were incubated at 37°C for 36 h. Positive results in each assay were evaluated as described above and the AHL titers were expressed as the opposite of the last dilution at which pigment was observed.[16,17]

ALKALINE PROTEASE ASSAY

Alkaline protease activities were studied by inoculating these strains which were exposed to MIC and sub-MIC levels of various antibiotics (gentamicin, amikacin, netilmicin) on nutrient agar (Oxoid) containing 1.5% skim milk (Oxoid). After incubation for up to 72 h at 37°C, the production of protease was shown by the formation of a clear zone caused by casein degradation.[18]

ALKALINE PROTEASE ASSOCIATED WITH GELATINASE ASSAY

Gelatinase production was determined by using LB agar (Oxoid) containing gelatin (30 g/L). The strains used were exposed to MIC and sub-MIC levels of various antibiotics (gentamicin, amikacin, netilmicin). After inoculation, the plates were incubated overnight at 37°C and then were cooled for 5 hours at 4°C. The appearance of a turbid halo around the colonies was considered positive for gelatinase production.[18]

CATALASE TEST

With an inoculating needle or a wooden applicator, 10 µL of bacterial suspension was transferred to the surface of a glass slide, and then a drop of 3% hydrogen peroxide (Applichem) suspension was added and bubble formation was observed.[19]

OXIDASE TEST

Two to three drops of oxidase reagent (1% tetramethyl- paraphenylenediamine, Acros Organic) were placed on a piece of filter paper in a Petri dish and a small amount of bacterial suspension was added. A positive reaction was indicated by the appearance of a dark purple color on the paper within 10 seconds.[20]

RESULTS

GENTAMICIN

Oxidase and Catalase Responses

The oxidase and catalase activities were present at the 25-6.2% MIC levels of gentamicin in ATCC

TABLE 1: Biofilm, protease, gelatinase, oxidase, catalase responses of clinical and standard (laboratory) strains of *Pseudomonas aeruginosa* against MIC and sub-MIC concentrations of gentamicin, amikacin, and netilmicin.

Antibiotics	Strains		MIC	% 50 X MIC	% 25 X MIC	%12,5 X MIC	%6,2 X MIC	Control
GN	ATCC	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	+	+	+	+
		CAT	-	-	+	+	+	+
	C1	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	-	+	+	+
		CAT	-	-	-	+	+	+
	C2	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	+	+	+	+
		CAT	-	-	-	+	+	+
AK	ATCC	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	-	+	+	+
		CAT	-	+	+	+	+	+
	C1	BİO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	-	-	-	+
		CAT	-	-	+	+	+	+
	C2	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	-	-	+	+
		CAT	-	-	-	+	+	+
NET	ATCC	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	+	+	+	+
		CAT	-	+	+	+	+	+
	C1	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	+	+	+	+
		CAT	-	-	-	+	+	+
	C2	BIO	+	+	+	+	+	+
		PRO	+	+	+	+	+	+
		GEL	+	+	+	+	+	+
		OXI	-	-	+	+	+	+
		CAT	-	-	+	+	+	+

GN: gentamicin; AK: amikacin; NET: netilmicin; BIO: biofilm; PRO: alkaline protease; GEL: gelatinase; OXI: oxidase; CAT: catalase; C1: clinic isolate 1; C2: clinic isolate 2; ATCC: standard 27853 *Pseudomonas aeruginosa*; CONTROL: without antibiotics; MIC: minimum inhibitory concentration.

27853 standard *P. aeruginosa* strain. The oxidase and catalase activities were present at the 12.5-6.2% MIC levels of gentamicin in the C1. In the C2 the oxidase activity was present at the 25-6.2% MIC and catalase activity was present at the 12.5-6.2% MIC levels of gentamicin (Table 1).

Biofilm, Protease and Gelatinase Responses

In all strains, which are ATCC 27853 standard *P. aeruginosa*, C1 and C2, biofilm formation and alkaline protease and gelatinase production were present at all concentrations of gentamicin (Table 1).

Quorum Sensing Responses

The las and rhl systems of the ATCC 27853 standard *P. aeruginosa* took place at all concentrations of gentamicin. While the rhl system of C1 took place at 12.5-6.2% MIC levels, the las system did not work under the gentamicin effect. Both rhl and las systems of C2 did not work under the gentamicin effect (Table 2).

AMIKACIN

Oxidase and Catalase Responses

The oxidase activity was present at the 12.5-6.2%MIC and catalase activity was present at the 50-6.2%MIC levels of amikacin in ATCC 27853 standard *P. aeruginosa* strains. In C1 the catalase activity was present at the 25-6.2% MIC concentrations and there was no oxidase activity under the amikacin effect. Oxidase activity was present at 6.2% MIC levels and catalase activity was present at the 12.5-6.2% MIC levels of amikacin in C2 (Table 1).

Biofilm, Protease and Gelatinase Responses

Biofilm formation and alkaline protease and gelatinase production were present at all concentrations under the amikacin effect in all strains, which were ATCC 27853 standard *P. aeruginosa*, C1 and C2 (Table 1).

Quorum Sensing Responses

The las system of the ATCC 27853 standard *P. aeruginosa* took place at all concentrations of

TABLE 2: Quorum sensing (QS) responses of clinical and standard (laboratory) strains of *Pseudomonas aeruginosa*.

Antibiotics	Strains	QS	MIC	% 50 X MIC	% 25 X MIC	%12,5 X MIC	%6,2 X MIC	Control
GN	ATCC	Las	+	+	+	+	+	+
		Rhl	+	+	+	+	+	+
	C 1	Las	-	-	-	-	-	+
		Rhl	-	-	-	+	+	+
	C 2	Las	-	-	-	-	-	+
		Rhl	-	-	-	-	-	+
AK	ATCC	Las	+	+	+	+	+	+
		Rhl	-	+	+	+	+	+
	C 1	Las	-	-	-	-	-	+
		Rhl	-	-	-	-	-	+
	C 2	Las	-	-	-	-	-	+
		Rhl	-	-	-	-	-	+
NET	ATCC	Las	+	+	+	+	+	+
		Rhl	-	+	+	+	+	+
	C 1	Las	-	-	-	-	-	+
		Rhl	+	+	+	+	+	+
	C 2	Las	-	-	-	-	-	+
		Rhl	-	-	-	-	-	+

GN: gentamicin; AK: amikacin; NET: netilmicin; C1: clinic isolate 1; C2: clinic isolate 2; ATCC: standard 27853 Pseudomonas aeruginosa; CONTROL without antibiotics; Las: primary QS systems; Rhl: secondary QS systems; MIC: minimum inhibitory concentration.

amikacin and the rhl system took place at 50-6.2% MIC levels of amikacin. Both las and rhl systems of C1 and C2 did not work under the amikacin effect (Table 2).

NETILMICIN

Oxidase and Catalase Responses

Oxidase activity was present at 25-6.2% MIC levels and catalase activity was at the 50-6.2% MIC levels of netilmicin in ATCC 27853 standard *P. aeruginosa* strains. Oxidase activity was present at the 25-6.2% MIC and catalase activity was at the 12.5-6.2% MIC in the C1. In the C2 the oxidase and catalase activities were present at the 25-6.2% MIC levels for netilmicin. (Table 1).

Biofilm, Protease and Gelatinase Responses

Biofilm formation and alkaline protease and gelatinase production were present at the MIC level in the netilmicin effect in all strains which were ATCC 27 853 standard *P. aeruginosa,* C1 and C2 (Table 1).

Quorum Sensing Responses

The las system of the ATCC 27853 standard *P. aeruginosa* took place at all concentrations and the rhl system was 50-6.2% MIC in the netilmicin effect. The rhl system of C1 took place at all concentrations; however, the las system did not work under the netilmicin effect. Both rhl and las systems of C2 did not work under the netilmicin effect (Table 2).

DISCUSSION

Translational medicine is the continuum-often known as "from bedside to bench side-from bench side to bedside"-by which the biomedical community takes a focused point of view to move research discoveries from the laboratory into clinical practice to diagnose and treat patients. Defining the clinical problem, establishing the research hypothesis, determining the *in vitro* and *in vivo* results, defining the "niches", establishing clinical research, sharing the results with industry and community, and clinical practice are the steps followed. "Experimental modeling studies" were shown to have fruitful implications through these steps.[21] *Pseudomonas* infections have a special importance in the translational medicine concept since they may have a lethal effect in clinics, especially in intensive care units in hospitals. Therefore "developing" experimental systems for defining the mechanisms of the pathogenesis, and the resistance of *Pseudomonas* may be useful also for clinical practice.

It is known that the microenvironment, which surrounds bacteria, has an important effect on the behavior of the bacteria. Presence of the antibiotics constitutes a microenvironment. Therefore, the presence of antibiotics may also affect the microenvironment of *Pseudomonas*. The behavioral response of *P. aeruginosa* against antibiotics (aminoglycosides) may give important clues for treatment. In this study, pathological responses (biofilm formation, alkaline protease, gelatinase, oxidase, and catalase) of *P. aeruginosa* were questioned. Biofilm formation, and protease-gelatinase activities were positive under all concentrations of all aminoglycosides (gentamicin, amikacin, and netilmicin) at the end of the 18-hour experiments in all *P. aeruginosa* strains. Those results showed us the possible presence of different signaling systems other than quorum sensing, like cyclic dinucleotide 3′, 5′-cyclic diguanyclic acid (c-di-GMP) signaling system, which may play a role in biofilm formation.[22,23]

In addition, we suggest that "in vitro susceptible" strains become "resistant" through MIC and sub-MIC concentrations of gentamicin, amikacin, and netilmicin, because pathogenicity criteria (biofilm, alkaline protease, gelatinase, oxidase and catalase) were not affected/inhibited by the MIC and sub-MIC levels of aminoglycosides gentamicin, amikacin, and netilmicin.

Enzymatic activities of catalase and oxidase were present under different concentrations of different antibiotics;

In ATCCC-under gentamicin effect, oxidase and catalase were present at 25-12.5-6.2% MIC; under amikacin effect, oxidase was present at 12.5-6.2% MIC whereas catalase was present at 50-25-

12.5-6.2% MIC; and under netilmicin effect oxidase was present at 25-12.5-6.2% MIC, catalase 50-25-12.5-6.2% MIC.

In C1-under gentamicin effect, oxidase and catalase were present at 12.5-6.2% MIC; under amikacin effect no oxidase activity was determined-but catalase was present at 25-12.5-6.2% MIC; and under netilmicin effect oxidase was present at 25-12.5-6.2% MIC, and catalase was present at 12.5-6.2% MIC,

In C2-under gentamicin effect, oxidase was present at 25-12.5-6.2% MIC and catalase was present at 12.5-6.2% MIC; under amikacin effect, oxidase was present at 6.2% MIC whereas catalase was present at 12.5-6.2% MIC; and under netilmicin effect oxidase and catalase was present at 25-12.5-6.2% MIC. These results showed us that "aminoglycosides" might have different inhibitory effects on bacterial enzymatic activities in different strains, and in different concentrations. These differences might be the result of phenotypic changes of *Pseudomonas* strains.[24]

Las and rhl quorum sensing systems of the ATCC 27853 *P. aeruginosa* took place at all MIC and sub-MIC concentrations of gentamicin while the rhl system of C1 took place under 12.5% and 6.2% MIC levels, but the las system did not take place under all gentamicin concentrations. Both rhl and las systems of C2 did not take place under the gentamicin effect. The las system of the ATCC 27853 standard *P. aeruginosa* took place at all MIC and sub-MIC concentrations of amikacin and netilmicin, and the rhl system took place at 50%, 25%, 12.5%, 6.2% MIC of amikacin and netilmicin. Both las and rhl systems of C1 and C2 did not take place under the effect of amikacin. The rhl system of C1 started to take place under the MIC concentration of netilmicin, and continued to take place up to the 6.2% MIC of netilmicin. However, the las system did not take place under any concentration of netilmicin. In C2, both rhl and las systems did not take place under the netilmicin effect.

Gentamicin, amikacin, and netilmicin are aminoglycosides that bind to the 30S sub-unit (irreversible), and inhibit the protein synthesis in ribosomes while having different chemical structures, and different antibacterial spectrum.[25] Our study showed that different aminoglycoside (gentamicin, amikacin, netilmicin) concentrations might have different effects on bacterial phenotypic changes. It is known that changes in enzymatic activities are "phenotypic changes". According to our results, different "chemical microenvironments" have different effects on "bacterial behaviors". *P. aeruginosa* may "behave" in different patterns in different microenvironments. A study on three *P. aeruginosa* strains is a good example where although biofilm formation was detected in all cell lines, QS response in epithelial cell lines was different than the response in muscle cell line.[26] On the next step of translational experiments, it may be useful to organize experimental infection models in animals, against different susceptible antibiotics.

In further studies determining also the genotypic changes that direct the phenotypic changes against microenvironmental changes (different antibiotics-different concentrations-different periods), may give fruitful ideas that can be a subject for translational medicine in infection clinics.

Because also "the timing" of these changes direct us in future medicine, especially in infection clinics, "tailored medicine" (personal therapy) will be much healthier than today's "general antibiotic therapies-protocols". At this point, these "translational studies based on phenotypic and genotypic concepts" may inspire us to develop future medicine concepts, e.g. experimental animal studies followed by experimental clinical studies.

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