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Efflux Pumps in Non-Typhoidal *Salmonella* Isolates Recovered from Patients in Armenia

(Submitted by corresponding member of NAS RA R. M. Aroutiounian 17/IX 2021)

Keywords: *non-typhoidal Salmonella, antimicrobial resistance, WGS, efflux pumps, ethidium bromide cartwheel method.*

Non-typhoid *Salmonella* (NTS) infection is considered as one of the most frequent foodborne diarrheal diseases that cause morbidity and mortality worldwide [1]. Third-generation cephalosporins for children, and fluoroquinolones for adults are recommended in complicated cases [2]. The emergence and dissemination of resistant bacteria hampers the use of conventional antibiotics, and growing resistance to new antimicrobial (AM) agents as well as widespread dissemination of multidrug-resistance (MDR) among NTS isolates is aggravating the situation and complicated control and treatment of salmonellosis [2].

Active efflux of antibiotics is considered as one of the most important mechanisms of resistance in NTS strains [3]. Efflux pumps (EPs) are proteins localized in plasma membrane in bacterial cell, which are known to efflux a wide range of compounds (antibiotics, dyes, detergents, disinfectants, etc.) from within the bacterial cell to the external environment. Expression of EP is strongly regulated [3].

Salmonellosis is one of the major foodborne infections in Armenia [4]. According to the Statistical Committee of the Republic of Armenia [4] salmonellosis became a leading foodborne bacterial infection in Armenia in 2019 and 2020. In our previous studies the tendency toward the MDR phenotype among the human NTS isolates collected in Armenia was identified [5] and acquired AM resistance genes were characterized based on whole genome sequencing (WGS) data analysis [6]. However, there is a lack of information on the contribution of active efflux mechanisms in the development of AM resistance in human NTS isolates circulating in Armenia. Thus, the main objectives of this work were: (i) *in silico* identification of known EP genes in

MDR and non-MDR NTS isolates recovered from patients in Armenia using WGS data, (ii) exploration of EP activity in these isolates by the Ethidium bromide cartwheel method.

Materials and methods. The study included a total of 44 NTS strains isolated from patients with salmonellosis over the period from 1996 to 2016 at the "National Centre for Infectious Diseases" (MH, Armenia). All the strains isolated from fecal samples confirmed to be *Salmonella enterica* by standard biochemical tests. Serotypes of *Salmonella* were determined using the standard Kauffman-White scheme [7].

Antimicrobial susceptibility determination. Susceptibility to 14 AM agents belonging to 9 different classes was tested according to the guidelines of the Clinical and Laboratory Standards Institute by disk diffusion assays [8]. *Escherichia coli* ATCC 25922 strain was used for quality control. Isolates that were resistant to at least three different classes of AMs were considered as MDR.

WGS and annotation. DNA samples of NTS strains were extracted using MO BIO Laboratories Inc. UltraClean® Microbial DNA Isolation Kit in accordance with the manufacturer's instructions. WGS of NTS isolates was performed by WGS provider, MicrobesNG (https://microbesng.uk/), within the frames of the ISTC project A-2140. Whole genome sequences of 44 isolates of NTS are available in the European Nucleotide Archive database (Project PRJEB36290). Resistance Gene Identifier tool (RGI [9]) was employed for *in silico* prediction of EP genes.

Ethidium bromide cartwheel (EtBrC) method. The EPs activity in NTS isolates was assessed by ethidium bromide EtBrC test as described [10] with slight modification. Briefly, overnight cultures of the bacterial isolates adjusted to 0.5 of a McFarland standard were streaked as cartwheel pattern on freshly prepared Trypticase soy agar (TSA) plates containing concentrations of EtBr ranging from 0.0 to 2.0 mg/L. The TSA plates were incubated at 37ºC for 16 hours and then examined under UV light. The minimum concentration of EtBr that produces fluorescence for each isolate was recorded and the TSA-EtBr plates photographed. Isolates without fluorescence indicated EP activity, whereas those that fluoresced lacked EP activity.

Statistical analyses. P value (two-tailed) from Fisher's exact test was calculated to evaluate statistical differences between the compared groups. *P* values $\leq$ 0,05 were considered to be significant.

Results and discussion. A total of 44 NTS isolates recovered from fecal samples of patients with salmonellosis were included in this study. Of these, 33 isolates (75%) were resistant to 3 or more classes of AMs, i.e., displayed the MDR phenotype. The small set of highly virulent isolates that exhibited a non-MDR phenotype (11 isolates, 25%) was also included in the study for comparative analysis. The most predominant serotype in this study is *S*. ser. Typhimurium (*S*. Typhimurium; 70.45%, 31/44), represented by 25 MDR isolates and 6 non-MDR isolates. The second most represented serotype is *S*. ser. Enteritidis (*S*. Enteritidis): 4 MDR and 3 non-MDR isolates. In addition, 5

isolates belonging to NTS serotypes with lower prevalence in Armenia (2 *S*. ser. Derby, 1 *S*. ser. Kentucky, *S*. ser. Agona, and 1 *S*. ser. Newport isolates) were subjected to WGS given the importance of the AM resistance phenotypes detected.

The most frequent AM resistance profile that includes resistance to ampicillin, amoxicillin-clavulanic acid, ceftriaxone, ceftazidime, and nalidixic acid, was encountered in 48.48% of MDR isolates (16/33). Remarkably, an alarming number of MDR isolates, 12 isolates (36.36%), displayed simultaneous resistance towards third-generation cephalosporins and fluoroquinolones that are most commonly used to treat *Salmonella* infections. Among the 11 non-MDR isolates, 4 isolates (3 *S*. Typhimurium and 1 *S*. Enteritidis) had no resistance to any of the tested AM agents. Resistance to one AM agent was identified in 2 isolates and the remaining 5 non-MDR isolates exhibited resistance to 2 AM agents.

Genomes of all NTS isolates in this study were interrogated for *in silico* prediction of genes encoding for EPs using RGI tool [9]. The presence, diversity and prevalence of EP genes as well as their correlation with the AM resistance phenotype was explored.

The results indicated that all NTS isolates in this study, regardless of serotype and AM resistance phenotype, had the following EPs: AcrAB, AcrD, AcrEF, EmrAB, KpnEF, MdfA, MdsAB, MdtAB, MdtK, MdtM, MsbA, and YojI. The lower prevalence was found for EPs conferring resistance to tetracycline: TetA (13.64%, 6/44) and TetB (15.91%, 7/44). It should be noted, that the presence of TetA or TetB EPs was detected in all tetracycline-resistant isolates (12/12), whereas among susceptible to tetracycline isolates only one isolate (non-MDR *S*. ser. Derby K89) was positive for TetB (1/32, $p<0.0001$). Besides, the presence of the EP encoded by *qacEΔ1* gene that mediates antiseptic resistance was identified in 29.55% of isolates (13/44).

Genomes of all isolates were also inspected for genes encoding for outer membrane proteins. The *tolC* and *mdsC* genes were predicted in all NTS isolates.

The occurrence of genes involved in regulation of EPs was also explored in the genome sequences. According to CARD, the uniform presence of *acrR*, *acrS, baeR*, *baeS*, *cpxA*, *crp*, *emrR*, *golS*, *h-ns*, *kdpE*, *marA*, *marR*, *mdtG*, *ramA*, *rsmA*, and *sdiA* was identified in all NTS isolates, irrespectively of their AM resistance phenotype. Besides, the high prevalence was observed for *soxR and soxS* genes that were detected in all but one isolates (97.73%, 43/44). Notably, the *tetR* gene was found in all isolates that were positive for *tetA* or *tetB* genes.

The efflux activity of NTS isolates was assessed by the ability to pump out EtBr out of the cell. The results indicated that *E. coli* ATCC 25922 strain (negative control) demonstrated maximum fluorescence, starting with the lowest concentration of EtBr, 0.5 mg/L (Fig. 1, J). All NTS isolates in this study showed well distinguishable EPs activity compared to the negative control strain at all EtBr concentrations used. The results are summarized and

the representative fluorescence patterns of clinical NTS isolates (MDR and non-MDR) that were identified are presented in Fig. 1.

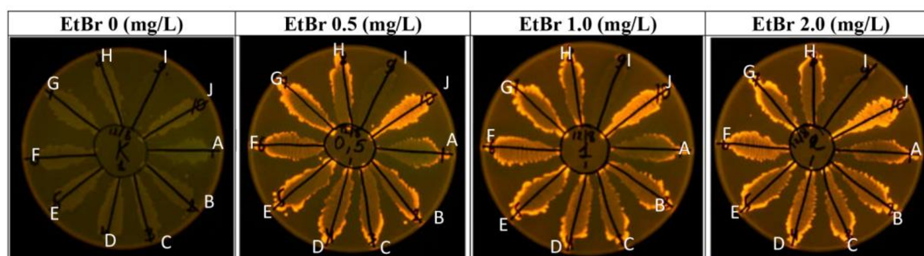

Fig. 1. Determination of efflux activity of NTS isolates by EtBrC method: A, B, C, and I – MDR *S*. Typhimurium; D, E – non-MDR *S*. Typhimurium; F – non-MDR *S*. Derby K89, G – MDR *S*. Enteritidis, H – non-MDR *S*. Enteritidis; J – *E. coli* ATCC25922 strain (negative control).

It should be emphasized that we detected two MDR *S*. Typhimurium isolates showing overexpression of EPs. These isolates did not fluoresce at all concentrations of EtBr used (Fig. 1, I). On the contrary, we detected non-MDR *S*. ser. Derby K89 isolate, displaying higher fluorescence, compared to all other NTS isolates at EtBr concentrations 1.0 mg/L and 2.0 mg/L (Fig. 1, F). However, the latter isolate did not reach the fluorescence level of the negative control strain even at the high concentrations of EtBr. The most common phenotype of fluorescence (Fig. 1; A-E, G, and H) was identified in 41 isolates of NTS: 31 MDR and 10 non-MDR. The subsequent experiments were performed to avoid differences in fluorescence levels due to variability in growth rate among NTS isolates. In brief, all NTS isolates were grown (16 h, 37°C) in TSA broth containing the same EtBr concentrations as in EtBrC test. The cell number in all bacterial suspensions was adjusted to 4.0 of a McFarland standard and 1 ml of each suspension was spined and the pellets were resuspended in an equal volume of TSA broth, 50 μl. Then, 10 μL of each obtained sample was loaded into the wells of a plastic chamber and examined under UV light. The summarized results demonstrating the identified profiles of fluorescence are presented in Figure 2. The titers of bacterial cells grown at EtBr concentration of 1.0 mg/L were evaluated.

The results indicated the pronounced efflux activity in all clinical NTS isolates compared to the negative control strain (Fig. 2, profile 1). The overexpression of efflux activity was detected in 12.12% (6/33) of MDR isolates (profiles 7 and 8), whereas this phenotype was not identified in non-MDR isolates. The lowest efflux activity (profile 2) was found in non-MDR *S*. ser. Derby K89 isolate mentioned above, which is consistent with the EtBrC test results. All other non-MDR isolates (90.9%, 10/11) demonstrated the phenotypes (profiles 3 and 5) that were identified in MDR isolates as well (profiles 4 and 6, respectively).

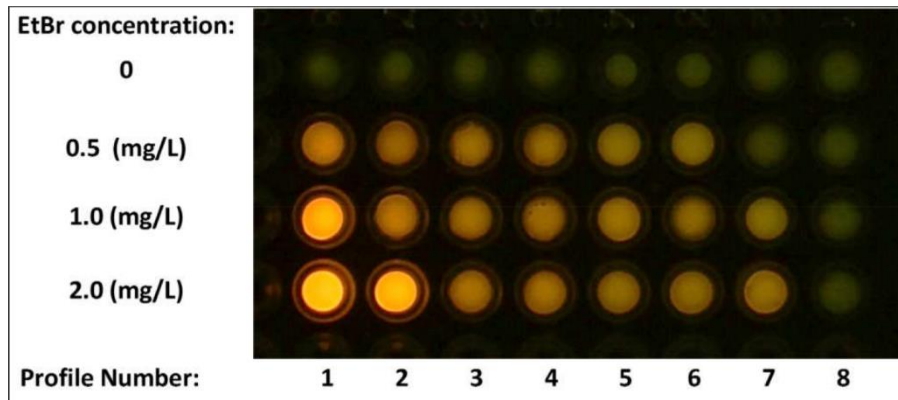

Fig. 2. Fluorescence profiles of NTS isolates in UV light after incubation with range of concentration of EtBr: 1 – *E. coli* ATCC 25922 (negative control); 2 – non-MDR *S.* Derby K89; 3, 5, 7, and 8 – MDR isolates; 4 and 6 – non-MDR isolates.

Thus, the uniform presence of major EP systems and their high activity was revealed in MDR and non-MDR isolates of NTS recovered from patients in Armenia. The results indicated a threatening potential for "awakening" of the MDR phenotype even in non-MDR strains, which is of great concern.

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Efflux Pumps in Non-Typhoidal *Salmonella* Isolates Recovered from Patients in Armenia

Efflux pumps (EPs) in non-typhoidal *Salmonella* (NTS) isolates recovered from patients in Armenia were characterized. The uniform presence of major EPs, outer membrane proteins, and genes involved in regulation of EPs was identified in all NTS isolates, multidrug resistant (MDR) and non-MDR. The results indicated association of TetAR and TetBR EPs with resistance to tetracycline ($p<0.0001$). The high activity of EPs was detected in NTS isolates by Ethidium bromide cartwheel (EtBrC) method.

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Ա. Մ. Սեդրակյան

Հայաստանում հիվանդներից անջատված ոչ տիֆոիդ սալմոնելաների իզոլյատների արտազատման պոմպերը

Բնութագրվել են Հայաստանում հիվանդներից անջատված ոչ տիֆոիդ սալմոնելաների (ՈՏՍ) իզոլյատների ակտիվ արտազատման (ԱԱ) համակարգերը: Հակամանրէային դեղամիջոցների նկատմամբ բազմակայունությամբ օժտվածն զգայունություն

ցուցաբերող բոլոր հետազոտված ՈՏՍ իզոլյատների գենոմներում բացահայտվել է հիմնական ԱԱ համակարգերի, արտաքին թաղանթային սպիտակուցների և ԱԱ համակարգերի աշխատանքը կարգավորող գեների միատեսակ առկայություն: Բացահայտվել է TetAR և TetBR ԱԱ համակարգերի կապակցվածությունը տետրացիկլինի հանդեպ կայունության հետ (p<0.0001): Բացահայտվել է ՈՏՍ իզոլյատների ԱԱ համակարգերի բարձր ակտիվություն՝ էթիդիում բրոմիդի ԱԱ մակարդակի գնահատման մեթոդի (EtBr cartwheel method) կիրառմամբ:

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Эффлюкс системы изолятов нетифоидных сальмонелл, выделенных от больных в Армении

Охарактеризованы эффлюкс системы (ЭС) изолятов нетифоидных сальмонелл (НТС), выделенных от больных в Армении. В геномах всех исследованных изолятов НТС, независимо от фенотипа множественной лекарственной устойчивости, обнаружен идентичный состав основных ЭС, белков наружной мембраны, а также регуляторных генов ЭС. Выявлена ассоциированность ЭС ТетAR и ТетBR с устойчивостью к тетрациклину (p<0.0001). С применением метода, основанного на детекции уровня флуоресценции бактериальных суспензий в присутствии этидиум бромида (EtBr cartwheel method), выявлена повышенная активность ЭС в изолятах НТС.

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