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MOLECULAR DETERMINANTS OF VIRULENCE GENES OF *SALMONELLA* ENTERITIDIS PREVAILING IN ARMENIA

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The main goal of this study was to establish genetic heterogeneity of virulence genes of *Salmonella enterica serovar*, which causes salmonellosis with different clinical presentation. With the use of PCR screening the prevalence of virulence genes located on pathogenicity islands and plasmid-encoded virulence factors were revealed. The results indicate about genetic heterogeneity of spv-operon genes of *S. Enteritidis* clinical isolates.

***Keywords*:** Salmonella, virulence genes, PCR typing, genetic heterogeneity.

Introduction. Salmonella infections remain one of the leading causes of gastrointestinal disorders in the world, resulting in significant morbidity and mortality rates [1]. The human food chain is recognized as a principal source of Salmonella infections. Although the species in the Salmonella genus are genetically close, they show wide variations in host-specificity, virulence and disease manifestations [2].

Hosts and bacteria have coevolved over millions of years, during which pathogenic bacteria have modified their virulence mechanisms to adapt to host defense systems [3].

Clinical presentation of salmonellosis also depends on many other factors such as the immune status of the host, the serotype of Salmonella and the specificity of the interaction of certain serotypes with the host [4].

Reflecting a complex set of interactions with its host, Salmonella spp. employs multiple genes for the full virulence expression. Although some of these genes are found on virulence plasmids common to many Salmonella serovars, most are encoded within the Salmonella pathogenicity islands (SPI) [5].

During evolution, diverse strains of Salmonella acquired new genetic elements. In the majority of virulence factors are encoded on mobile elements and can easily transmit by horizontal transfer. New genetic elements contribute to the pathogenicity of Salmonella strains and play a major role in the clearance of disease [6].

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At the genomic level, *S. enterica serovars* are very close, with a large and stable core genome, while the accessory genome is dominated by mobile genetic elements such as phages, prophages, genomic islands, transposons and plasmids [7, 8].

Table 1

Primer pairs used for virulence characterization of Salmonella Enteritidis

Genes	Nucleotide sequence	Amplicon (bp)	Broad action
*spv*A[1]	5'- GTCAGACCCGTAAACAGT -3'	641	Promote the macrophage phase avoiding destruction by neutrophils
	5'- GCACGCAGAGTACCCGCA -3'		
*spv*B[2]	5'- ATGTTGATACTAAATGGTTTTTCA-3'	1776	Growth within host
	5'- CTATGAGTTGAGTACCCTCATGTT -3'		
*spv*C[3]	5′-CGGAAATACCATCTACAAATA-3′	669	Intracellular survival and replication
	5′-CCCAAACCCATACTTACTCTG-3′		
*spv*R[2]	5'- ATGGATTTCATTAATAAAAAATTA -3'	894	Regulation of expression of spv-genes
	5'- TCAGAAGGTGGACTGTTTCAGTTT -3'		
*spi*C[2]	5'- CCTGGATAATGACTATTGAT -3'	301	Survival in macrophages
	5'- AGTTTATGGTGATTGCGTAT -3'		
*pef*A[3]	5'-AGGGAATTCTTCTTGCTTCCATTCCATTATTGCACTGGG-3'	520	Movement
	5'- TCTGTCGACGGGGGATTATTTGTAAGCCACT-3'		
*sop*E[2]	5'- TCAGGGAGTGTTTTGTATATATTTA -3'	720	Invasion of macrophages
	5'- GTGACAAAAATAACTTTATCTCCCC -3'		
*peg*D[2]	5'-TATGTGGCAAAGACAGGAA-3'	524	Putative fimbrial-like adhesion protein (fimbriae)
	5'-GCAAAGAATCAATGGAGCA-3'		

NB: annealing temperature: [1] – $t = 54$; [2] – $t = 55$; [3] – $t = 50$ °*C*.

More Salmonella genomic information, however, is needed to uncover the set of genes that drives the differential immune responses that result in different clinical outcome of the disease. Despite the substantial efforts toward the genomics of Salmonella that has allowed a better understanding of the virulence and invasiveness mechanisms, the range of the corresponding mechanisms may be much broader than anticipated, especially in Salmonella populations from geographically distant locations.

Worldwide Salmonella-induced gastroenteritis is most frequently caused by *S. enterica serovar Typhimurium* (*S. Typhimurium*) and *S. enterica serovar Enteritidis* (*S. Enteritidis*), which are also prevalent in Armenia [9].

Previously we reported, that salmonellosis caused by *S. Enteritidis* circulating in Armenia, is characterized by such host-pathogen specificity, like the Th17 pathway of Salmonella induced inflammation, with induction of IL-17, which level remains abnormal for a few months after the acute stage of disease [9]. Furthermore, we observed a significant positive correlation between the concentrations of IL-17 and IgE suggesting a possible role played by this cytokine in triggering the production of IgE in response to *S. Enteritidis* infection, causing sensitization in infected subjects [10].

The aim of this study is to detect the prevalence of virulence genes and genetic diversity of *S. Enteritidis*, which causes salmonellosis with different clinical presentation.

Materials and Methods. Salmonella isolates (n=24) used in this study were isolated from fecal samples of *S. Enteritidis* infected patients admitted to the infectious disease hospital "Nork" in Yerevan, RA.

Within the survey, we examined plasmid DNAs extracted from S. *Enteritidis* isolates. The extraction of plasmid DNA was implemented by commercial kit GenElute Five-Minute Plasmid Miniprep ("Sigma-Aldrich", USA), according to the manufacturer's protocol. The genetic research was performed by PCR screening, with the use of BIO-RAD thermal cycler. The list of used primers is presented in Tab. 1.

Amplification products were separated by electrophoresed on 1.5% agarose gel stained with ethidium bromide with a 3000–100 bp DNA ladder as a molecular weight marker.

Results and Discussion. Eight Salmonella virulence genes were screened in all the isolates, including genes located on pathogenicity islands and plasmid-encoded virulence factors (see Tab. 2; Figure a, b).

The plasmid *spv*ABCD genes are arranged in an operon positively regulated by the upstream *spv*R gene. The spv region is represented by three genes required for the virulence phenotype in mice: the positive transcriptional regulator *spv*R and two structural genes *spv*B and *spv*C. SpvB and SpvC are translocated into the host cell by the Salmonella pathogenicity island-2 [11].

In our survey the presence of all examined genes was detected in 25% (6 of 24 isolates), in 75% *spv*B were absent (18 of 24), however, 16 of which contains *spv*C gene. In two isolates the spv locus were not detected.

SpvB and SpvC have been identified as essential effector proteins for the spv virulence phenotype. Biochemical activities for SpvB and SpvC have been identified [12]. SpvB exhibits a cytotoxic effect on host cells and is required for delayed cell death by apoptosis following intracellular infection. In the absence of *spv*C, *spv*B does not have a detectable virulence phenotype. The exact mechanisms, by which SpvB and SpvC act together to enhance virulence, are still unclear [11].

R. Käppeli and coauthors found that *spv*B (and possibly *spv*C) contribute to the alternative pathway of gut inflammation on murine model [13].

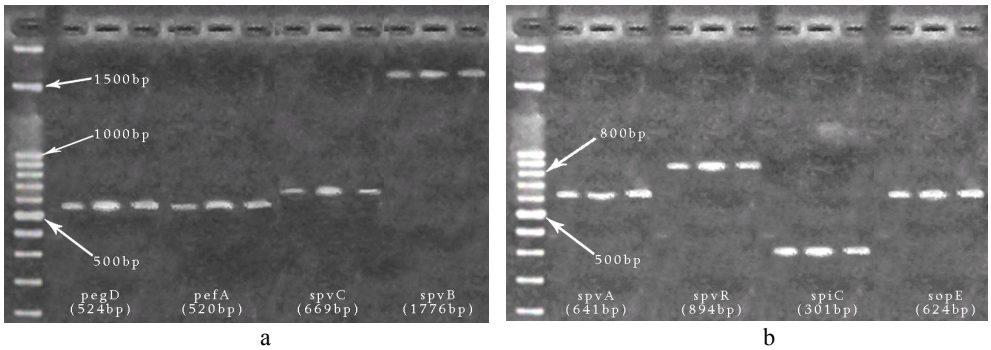
In the current study, we investigated one of the most important genes in this operon, *spv*A virulence gene, which is associated with multidrug resistance [14].

All tested isolates, which were heterogeneous by antimicrobial resistance (unpublished data), contents *spv*A gene (Tab. 2).

Table 2

Virulence genes present in different isolates of S. Enteritidis

Clinical isolate №	Genes							
	*spi*C	*spv*C	*pef*A	*spv*B	*sop*E	*spv*A	*spv*R	*peg*D
3017	+	+	+	-	+	+	+	+
5962	+	-	+	-	-	-	-	+
6892	+	+	+	+	+	+	+	+
3972	+	+	+	-	+	+	+	+
3973	+	+	+	-	+	+	+	+
7160	+	+	+	-	+	+	+	+
7686	+	+	+	+	+	+	+	+
422	+	+	+	-	+	+	+	+
884	+	+	+	-	+	+	+	+
1039	+	-	+	-	-	-	-	+
1266	+	+	+	-	+	+	+	+
2977	+	+	+	-	+	+	+	+
3784	+	+	+	-	+	+	+	+
4492	+	+	+	-	+	+	+	+
4541	+	+	+	-	+	+	+	+
5143	+	+	+	-	+	+	+	+
5330	+	+	+	-	+	+	+	+
5341	+	+	+	+	+	+	+	+
5791	+	+	+	+	+	+	+	+
5793	+	+	+	+	+	+	+	+
5887	+	+	+	-	+	+	+	+
6356	+	+	+	-	+	+	+	+
6497	+	+	+	-	-	+	-	+
7484	+	+	+	+	+	+	+	+



a b

Visualization of investigated genes' electrophoresis:

a) lane 1: 3000–100 bp molecular weight marker; lanes 2 to 4: fragment from *peg*D; lanes 5 to 7: fragment from *pef*A; lanes 8 to 10: fragment from *spv*C; lanes 11 to 13: fragment from *spv*B;

b) lane 1: 3000–100 bp molecular weight marker; lanes 2 to 4: fragment from *spv*A; lanes 5 to 7: fragment from *spv*R; lanes 8 to 10: fragment from *spi*C; lanes 11 to 13: fragment from *sop*E.

The *spi*C gene, which is located on SPI-2 and is responsible for adhesion, cell invasion and *sop*E, which is responsible for intra-cellular survival [15, 16], both were tested positive in all examined isolates (Tab. 2).

Results presented in this study suggest that the two fimbrial genes namely *pef*A and *peg*D, which exist in all isolates (Tab. 2), probably play a role in the development of clinical manifestation. In a recent study by genome analysis, was revealed, that there is a novel fimbrial cluster specific only for *S. Enteritidis*, which have been termed *peg* [7]. The role of the Peg fimbriae, in colonization and virulence has been established in a number of model experiments [17].

Conclusion. The PCR based screening allowed the identification of *S. Enteritidis* plasmid genes playing an important role in the virulence and development of pathogenicity. In this study we could observe genetic heterogeneity in the plasmid virulence genes of *S. Enteritidis* isolates, the majority of which are highly virulent strains. This diversity among isolates, possibly, can explain the wide variation in the clinical manifestation of disease. The presence of fimbial genes *pef*A and *peg*D were observed in all isolates, and the latter one have not been described for other serovars of the genus Salmonella.

This work is a first step of creation of molecular epidemiological map of Salmonella, circulating in Armenia.

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