Construction and in vitro characterisation of *aroA* defective (*aroAΔ*) mutant *Salmonella* Infantis

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Abstract

Poultry vaccine programs are important for control of *Salmonella* infections. Although there are vaccines for *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Typhi, there are no vaccines for *Salmonella* Infantis which has an increased rate in the world. In this study, it was aimed to generate *aroA* gene deleted mutant bacteria for the constitution of *S. Infantis* vaccine prototype and the in vitro characterisation of this bacterium. *S. Infantis* auxotrophic mutant which has a block at any step of chorismate pathway has been constituted for the first time in the world and it was determined that this bacterium gets susceptibility against some antibiotics and antimicrobial substances. It was also observed that the adhesion and invasion rate of mutant strain tenfold decreased in comparison with the field strain in cell culture assay. It is understood from the in vitro evaluation of this mutant strain that it can be used as a vaccine candidate in further vaccine development studies.

Keywords *aroA* gene · Auxotrophic mutant · Recombinant vaccine · *Salmonella* Infantis

Introduction

Salmonella infections, which cause serious losses in the poultry industry, have been seen as a major problem since the 1900s. Although *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most isolated serotypes in poultry, *Salmonella* Heidelberg, *Salmonella* Kentucky and *Salmonella* Infantis serotypes are also frequently seen. These serovars are capable of producing a variety of infections both in humans and in animals (Barrow et al. 2010).

According to the worldwide prevalence of *Salmonella* serotypes observed in humans and various foodstuffs, an epidemiological link has been established between poultry products and Salmonellosis seen in humans, and, it has been shown that the serotypes isolated from poultry meat products (chicken and turkey meat) are similar to the serotypes

seen in humans. In Europe, the frequency of *Salmonella* serotypes that cause infections in humans has been reported and, in some cases, these serotypes have been similar to the serotypes observed in poultry. According to the report in Europe in 2013, there is an increase in the proportion of *Salmonella* serotypes that cause infections in humans due to consumption of poultry meat (chicken and turkey). Among these: *Salmonella* Infantis, *Salmonella* Stanley and *Salmonella* Kentucky were found to be the most frequently isolated serotypes. The proportion of *S. Infantis* causing infections in humans in Europe between 2011 and 2013, particularly broiler chickens and broiler chickens' meat origin, increased by 26.5% (Antunes et al. 2016).

From 2001 onwards in Europe and especially in Germany, *S. Infantis*, which is not host dependent, is the top serotype among *S. enterica* serotypes isolated from human cases. Since 970s this serotype has been increasingly reported in Argentina, Australia, Brazil, Netherlands, Finland, Canada, Hungary, Japan, New Zealand and Russia. Animals and especially chickens are reported as the most important reservoir of this *S. Infantis* Salmonellosis in humans (Miller et al. 2010).

Since the early 1980s it has been known that virulence of auxotrophic *Salmonella* mutants, lacking aromatic amino acids, is low for animals (Hoiseth and Stocker 1981). Since then mutations in genes responsible for the biosynthesis

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of aromatic amino acids in different *Salmonella* serotypes have been used to reduce virulence. It has been determined that *aroA* and *aroD* mutants of *Salmonella enterica* serovar Typhi are successful as a vaccine against typhoid in humans (Tacket et al. 1997; Tacket et al. 2000). Similar mutations have been used to generate avirulent strains for immunization of different farm animals (Robertsson et al. 1983; Cooper et al. 1994b; Everest et al. 1999). *Aro* mutants were found to be avirulent even in gnotobiotic mice known to be quite sensitive. The decrease in virulence seen in *aroA* mutants is mainly explained by the lack of production of aromatic amino acids and aromatic metabolites such as phenylalanine, tyrosine and tryptophan.

The *aroA* is the gene located in the first step of the aromatic amino acid biosynthesis pathway and is required for the synthesis of paraaminobenzoic acid (PABA) and is a part of the shikimate pathway which is directly related to the glycolysis required for aromatic amino acid synthesis (Sebkova et al. 2008; Felgner et al. 2016).

In bacteria, the synthesis of aromatic amino acid starts in common. In this biochemical pathway, degradation of the pre-chorismate pathway by mutation of *aroA*, the gene responsible for 5-enolpyruvylshikimate 3-phosphatase synthesis from seven enzymatic reactions, is used as a common strategy in many attenuation studies (McArthur et al. 2003).

The *aroA* gene mutation has been reported as a potential live vaccine candidate in animals for the past 30 years with success for many Gram-negative bacteria, especially respiratory pathogens such as *Salmonella* sp., *Bordetella pertussis*, *Pasteurella multocida* and *Mannheimia haemolytica* (Gar-side et al. 2002).

In this study, it was aimed to develop a vaccine candidate against *S. Infantis* frequently isolated from poultry by the previously used *aroA* gene mutation technique against *S. Enteritidis* and *S. Typhimurium*, and to characterise and compare the mutant strain generated with the wild-type strain under in vitro conditions.

Materials and methods

Strains

Salmonella *Infantis* strains focused on in this study were obtained from departmental archive of Department of Microbiology, Faculty of Veterinary Medicine, Ankara University. The strains were isolated from poultry flocks (base material and dust) as described in ISO 6579 and serotyped according to Kauffmann–White–Le Minor scheme (Grimont and Weill 2007). Among these strains, *S. Infantis* strains with the most observed ribo-pattern type (ribotyping banding patterns) in the ribotyping library were used. Ribotyping is a molecular technique used for the identification of

bacteria by cutting the rRNA at specific sites, resulting in pieces of rRNA of different lengths unique for each strain. Ribotyping was performed by RiboPrinter System (DuPont, USA) based on the analysis of the band profiles resulting from cutting the rRNA operon with the *PvuII* restriction enzyme (Thermo Scientific, USA).

Bacterial genomic DNA extraction

One or two colonies (depending on size of the colony) from cultured *S. Infantis* strains were harvested in Eppendorf tubes containing 200 µl of nuclease-free buffer and were centrifuged at 5000×*g* for 10 min. The supernatant was discarded and genomic DNA (gDNA) extraction from pellet was performed with the GeneJet Genomic DNA Purification kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Construction of cloning vector for *aroA* gene deletion

The *aroA* gene including approximately 200 nucleotides up- and downstream flanking sequences were amplified from gDNA of selected *S. Infantis* strains with conventional PCR using the primers designed (Table 1) based on the sequence of *S. Infantis* isolated from healthy broilers in Hungary and UK (GenBank Acc. Number: LN649235). Approximately 2000 bp genomic region including *aroA* gene was amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). The PCR product was then purified with the QIAquick PCR Purification kit (Qiagen, Germany) and final product was inserted into the pJET1.2/blunt cloning vector with the CloneJet PCR Cloning kit (Thermo Scientific, USA) according to the manufacturer's instructions. The resulting construct was named as pJETaroA (approximately 5000 bp in size). The entire *aroA*-coding region was deleted from pJETaroA by digestion with *SalI* restriction endonuclease (Thermo Scientific, USA) and the resulting large fragment (4000 bp) was gel-extracted (QIAquick Gel Extraction kit, Qiagen, USA) and blunt-ended with T4 DNA polymerase (Thermo Scientific, USA). The plasmid generated was named as pJETaroA-Δ. As a marker gene (for tracking mutant *S. Infantis*) GFP coding gene was inserted into pJETaroA-Δ. In order to do this, pGFPuv vector

Table 1 Primers used in this study

Name	Primer sequence (5'→3') (start nt)	Purpose of use
<i>aroA</i> -F	tctttccggtctggtgtt (311)	Gene amplification
<i>aroA</i> -R	gacgtttggtgtatttgctt (2361)	
<i>MaroA</i> -F	ctccttaatatccctccaac (551)	Proof of <i>aroA</i> deletion
<i>MaroA</i> -R	atcaccttcaccctctcc (1887)	

(Clontech, USA) was digested with *EcoRI* and *PvuII* restriction enzymes (Thermo Scientific, USA) and the resulting small fragment (approximately 950 bp in length) was gel-extracted as described before and inserted into pJETaroA(–) using the enzyme T4 DNA Ligase (Thermo Scientific, USA) (Fig. 1a, b). The positive clones green fluorescing under UV-light were selected from agar surface. The resulting cloning vector was finally confirmed by nucleotide sequencing bidirectionally using the first amplification primers and the final construct named pJETaroA-Δ/GFP.

Production of *aroA* defective bacteria

Confirmed pJETaroA-Δ/GFP plasmid was first linearized with *NotI* restriction enzyme (Thermo Scientific, USA). Subsequently, the plasmid was transformed to *S. Infantis* strain, which is selected in the appropriate replication period (OD_{600} 0.4–0.6), according to the method of Sambrook and Russell (2001) (Fig. 1c). Recombinant bacterium was subjected to PCR analysis, plasmid elimination, sequence analysis and Western blot for further analysis. PCR analysis was performed with confirmatory primers (*MaroA*) in order to amplify approximately 1400 bp of the gDNA region, which included inserted reporter GFP gene surrounded by the phosphoserine aminotransferase (*PSAT*) gene located in front of the deleted *aroA* gene.

The generated *aroA*-defective *S. Infantis* strain, apart from the wild strain from which it originated, had the ampicillin-resistance gene from the plasmid transferred. Thus, the plasmid was eliminated using the method described by Carlton and Brown (1981). According to this curing technique, the strains were incubated at 5–7 °C above the optimal growth temperature. The culture was re-incubated at the elevated temperature until it became ampicillin-sensitive. Curing of the plasmid was confirmed by culturing the strains on Luria–Bertani agar media with ampicillin. Confirmation of the GFP gene in *aroA*-defective *S. Infantis* strain's gDNA after plasmid removal was performed by PCR analysis using *MaroA*-primers as before. Then, plasmid-eliminated mutant bacteria was sequenced. The Western Blot (WB) analysis was performed according to the method of Sambrook and Russel (2001) to reveal the expression of the GFP gene in the recombinant bacteria using anti-GFP-tag polyclonal antibody (Thermo Scientific, USA).

Characterisation of recombinant bacteria

To show the auxotrophy, *aroAΔ S. Infantis* and wild-type *S. Infantis* strains were cultured on M9 minimal medium with and without aromatic amino acids (phenylalanine, tryptophan and tyrosine) which were prepared according to the method of Sambrook and Russel (2001) and incubated at 37 °C for 18–24 h.

Generated *aroA*-defective *S. Infantis* (*aroAΔ S. Infantis*) strain was compared to *S. Infantis* wild-type strains, *S. Enteritidis* wild-type strains and *S. Typhimurium* wild-type strains in terms of some phenotypic and genotypic methods such as H₂S formation, colony morphology, several biochemical tests, reproduction profiles, antibiotic resistance profiles and antimicrobial susceptibilities. For these tests, MacConkey, XLD, Nutrient, TSI and O/F media were used. Fully automated biological screening device (BioScreen C MBR, Finland) was employed to determine the growth curve and motility test was performed based on the method of Tittsler and Sandholzer (1936).

For antibiotic resistance profiling, Mueller–Hinton agar cultures were used and antibiotics indicated in Table 2 were tested according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria by disk diffusion method (EUCAST 2014). EDTA susceptibility test was conducted to investigate the properties of bacteria against antimicrobial agents. For this reason, the MIC test was performed.

Pulsed Field Gel Electrophoresis (PFGE) was also used for genotyping. Recombinant *S. Infantis* strains with and without plasmid elimination, *S. Infantis* wild-type strain, *S. Enteritidis* and *S. Typhimurium* strain were used for the test. Each strain was cultured on Nutrient agar media and incubated for 18–24 h at 37 °C. Gel electrophoresis was prepared according to the Centers for Disease Control and Prevention (CDC, USA) protocol (CDC 2018).

Whole genome sequencing

Library preparation was done using TruSeq Nano DNA Low Throughput Library Prep Kit (Illumina, San Diego, CA, USA). Whole genome sequencing was performed as paired-end (150 bp × 2) sequencing on Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). The aim of coverage was > 30×. FastQ file was uploaded to the Galaxy platform (<https://usegalaxy.org/>) and the sequences of adaptors and barcodes were removed. Mapping of the sequences was done using Geneious Prime 11.0.5 (www.geneious.com) software. The genome sequences of the wild-type and mutant *S. Infantis* strains were submitted to the GenBank under the access numbers CP040600 and CP040601, respectively.

In vitro adhesion and invasion tests

Caco-2/An1 (human epithelial colorectal adenocarcinoma) cell culture was used to determine in vitro adhesion and invasion abilities of mutant and wild-type *S. Infantis* strains. Tests were performed as described elsewhere (Giannasca et al. 1996; Salehi et al. 2017).

Fig. 1 **a, b** Schematic figure of constructing cloning vector (pJETaroA-Δ/GFP), **c** homologous recombination in wild-type *S. Infantis* strain. *PSAT* phosphoserine aminotransferase *yggG* putative metalloprotease

Table 2 Antibiotics and their concentrations

Antibiotic	Concentration
Ampicillin (oxoid)	10 µg
Gentamycine (oxoid)	10 µg
Streptomycine (oxoid)	10 µg
Ciprofloxacin (oxoid)	5 µg
Nalidixic acid (oxoid)	30 µg
Trimethoprim (oxoid)	5 µg
Trimethoprim sulfamethoxazole (oxoid)	300 µg
Chloramphenicol (oxoid)	30 µg
Tetracycline (oxoid)	30 µg
Cephalexin (oxoid)	5 µg

Statistical analysis

Pearson Chi square test was used to evaluate the difference between mutant and wild-type *S. Infantis* strains for rate of adhesion and invasion. Descriptive statistics for each variable were calculated and presented as 'Percentage'. A *P* value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 14.01 package program for Windows.

Results and discussion

Homologous recombination technique was used to produce an *aroA*-defected *S. Infantis* strain. Homologous recombination is well-designed and straight forward technique if carried out in highly conserved genomic regions. For this approach, up- and downstream flanking sequences (200 bp in length in both sides) were kept around target gene and homologous recombination occurred efficiently. The constructs could be generated in the first attempt by efficiency of 100% and further confirmed by colony PCR, restriction enzyme analysis and partial sequencing. The final construct (pJETaroA-Δ/GFP) was subsequently transformed into *S. Infantis* and the resulting mutant strain *aroA*-Δ/GFP-*S. Infantis* was characterised by PCR and WB at the first step. PCR attempts with *MaroA* primers revealed 1400 bp product in mutant *S. Infantis* while producing approximately 3000 bp amplicon in wild-type strain. In addition, GFP expression was shown by using WB in mutant *S. Infantis* before and after plasmid elimination. As clearly can be seen in Fig. 2, GFP expression was remarkably exist in *S. Infantis* transformed with pJETaroA-Δ/GFP. On the other hand, GFP expression did not occur in plasmid eliminated recombinant and wild-type *S. Infantis* strains.

In vitro assays: it was determined that there was no difference in the phenotypic characteristics of recombinant bacteria and wild-type *S. Infantis*, *S. Enteritidis* and *S.*

Fig. 2 Western blot analysis of plasmid-eliminated *S. Infantis* strain (lane 1), Wild-type *S. Infantis* strain (lane 2) and *S. Infantis* strain including plasmid (lane 3) was performed. After transfer of bacterial proteins to PVDF membrane, anti-GFP polyclonal antibody (from rabbit) at dilution of 1:2000 was added and incubated for 1 h at room temperature followed by three wash steps and incubation with secondary antibody of anti-rabbit IgG-HRPO (whole molecule) antibody (Sigma, USA) at dilution of 1:5000. We added ECL (BioRad, USA) and imaged using Chemi Doc MP System from BioRad (USA). The positive band of about 30 kDa was detected in the Plasmid-eliminated *S. Infantis* strain (lane 1) and *S. Infantis* strain including plasmid (lane 3)

Typhimurium strains. It was determined that the results of biochemical test analysis and culture properties were the same in all strains. Growth curves of the strains at 37 °C (Fig. 3) and 56 °C (Fig. 4) revealed no difference among the strains used. Similarly, mutant and wild-type strains showed same movement characteristics based on growth curve patterns in the semi-solid medium. The mutant *S. Infantis* strain was grown on M9 minimal medium supplemented with three amino acids, but not only M9 minimal medium.

Wild-type and *aroA*-defective *S. Infantis* strains showed varied resistance to antibiotics. Recombinant bacterium became sensitive to nalidixic acid, tetracycline, trimethoprim, and its susceptibility increased against ciprofloxacin, chloramphenicol, ampicillin and streptomycin (Table 3). The recombinant strain was found to be approximately eight times more susceptible to EDTA than wild-type strain according to the MIC values.

According to the PFGE genotypic analysis, it was determined that the gene profiles of the *aroA*Δ *S. Infantis* strain and the wild-type strain were different from each other. As a result of dendrogram analysis based on the similarity matrix obtained by UPGMA analysis, the mutated strain and wild-type *S. Infantis* strains were in different taxas

Fig. 3 Growth curves of the strains at 37 °C **Fig. 4** Growth curves of the strains at 56 °C **Table 3** Comparison of antibiotic disc diffusion test results of *aroA*Δ and wild-type *S. Infantis* strains

Antibiotic disc/zone (mm)	NA	TE	AMP	W	CTX	C	RL	CN	S	CIP
Recombinant strain	23	15	18	29	33	33	0	22	16	30
Wild-type strain	0	0	9	0	33	20	0	20	10	20

NA nalidixic acid, TE tetracycline, AMP ampicillin, W trimethoprim, CTX cefotaxime, C chloramphenicol, RL sulfamethoxazole, CN gentamycin, S streptomycin, CIP ciprofloxacin

with > 95% similarity (Fig. 5). It was thought that the similarity difference between mutant and wild-type strain was because of the new restriction site for *Xba*I enzyme that occurred in the gene inserted into the mutant strain.

According to the results of whole genome sequence analysis it was observed that a 1265-bp region (747–2012th nucleotide in *aroA* gene, 1005379–1007238th nucleotide in genome) in *aroA* gene of wild-type strain (between the 1005226 and 1007851th nucleotide in genome) was replaced by a 945-bp GFP gene sequence in mutant strain. It was observed that the constructed region was located between the 1005226_{th} and 1007536_{th} nucleotide in mutant strain. The comparison of both genome sequences

of wild-type and mutant strains exhibited no difference except this region.

The in vitro adhesion and invasion studies resulted in a mutant strain adhesion rate of 0.07%; invasion rate was 0.03%, while the rate of wild-type strain adhesion was 0.77%; invasion rate was found to be 0.4%. These results indicate that mutant strains have 10 times less invasion and adhesion ability than wild-type strain ($p < 0.001$).

Since the 1980s, mutants lacking aromatic amino acids have been studied in many different species of bacteria, but not in *S. Infantis* strains. Therefore, designing the auxotrophic mutant of a *S. Infantis* strain is performed for the first time.

Fig. 5 Dendrogram analysis of strains. *AMP* the strain without plasmid elimination, *REC* *aroA*Δ *S. Infantis* strain, *ST* *S. Typhimurium*, *SE* *S. Enteritidis*, *WT* wild-type *S. Infantis* strain

Cooper et al. (1994a) reported that the *aroA*Δ *S. Enteritidis* strain could not form H₂S in triple sugar iron (TSI) medium. On the contrary, in the *aroA* mutant *S. Infantis* strain generated in this study, H₂S formation was observed in TSI medium. Cooper et al. (1994a) associate the H₂S formation with the *aroA* mutation, but more detailed studies on this issue are needed.

O'Callaghan et al. (1988) reported that *aroA* mutant *Salmonella* strains were able to grow as wild-type strains by providing essential amino acids from the medium. Compatible to this study, it was determined that there was no difference between the strains when the growth curves of the strains were examined at 37 °C and 56 °C for 24 h in the present study.

Sebkova et al. (2008) reported that the susceptibility of *aro* mutants of some serotypes of *S. enterica* to substances known to be antimicrobial effects such as serum, albumin, EDTA, cysteine, trypsin, lysozyme increased. Sebkova et al. (2008), suggested that this increased sensitivity was due to the deterioration of cell membrane structure in *aro* mutants. In this study, when EDTA susceptibility known to affect cell membrane was examined, it was determined that the susceptibility to EDTA increased by eight times when comparing mutant strain to wild-type strain. This result supports the results of Sebkova et al. (2008).

Compared to the antibiotic resistance profile, mutant strains were found to be more susceptible or completely susceptible to some antibiotics compared to wild-type strains. It was observed that susceptibilities to aminoglycoside (streptomycin), chloramphenicol, second-generation quinolones (ciprofloxacin) and ampicillin were increased while first-generation quinolones (nalidixic acid), tetracyclin and trimethoprim were found to be completely susceptible. Felgner et al. (2016) reported an increased susceptibility to ampicillin of *aroA* mutant *S. Typhimurium* strain. The researchers thought that this sensitivity to ampicillin could be due to the deterioration of the outer membrane structure in the *aroA* mutant *S. Typhimurium* strain. In

the present study, the increase in the susceptibility to ampicillin of *aroA* mutant *S. Infantis* strain supported the result of Felgner et al. (2016). Mutant strain's sensitivity against trimethoprim is consistent with the mechanism of action of trimethoprim and *aroA* mutation. It is known that trimethoprim works as PABA antagonist. *aroA* is a gene necessary for PABA synthesis and it is thought that this mutation prevents PABA synthesis (Smith and Powell 2000). Similarly, it was thought that since PABA synthesis did not occur in *aroA*Δ *S. Infantis* strain generated in the study, the strain became sensitive to trimethoprim.

It is known that some bacteria require ubiquinone for the flagella production (Hertz and Bar-Tana 1977). Among these bacteria *S. Typhimurium* require the synthesis of ubiquinone, in the presence of aliphatic or aromatic carboxylic acids, for its bacterial growth and flagella production (Bar-Tana et al. 1980). Besides, Sebkova et al. (2008) reported that movement in *aro* mutants was impaired. In this study, motility test was carried out for the purpose of the control of synthesis of the ubiquinone, one of the end products of the same pathway as the result of extinguishing the *aroA* gene involved in shikimate pathway, in the *aroA*Δ *S. Infantis* strain. However, the results showed that there was no difference between the recombinant strain and the wild-type strain in terms of movement characteristics. This result suggests that there may be a different mechanism in *S. Infantis* strains for regulation of flagella production.

There are studies for the constitution of *aroA* mutant recombinant *S. Enteritidis* and *S. Typhimurium* strains in the literature; however, there is no such kind of research for *S. Infantis*, currently the most frequent *Salmonella* serotype isolated from poultry. This is the first study that aimed to constitute an *aroA* mutant *S. Infantis* as a vaccine candidate. It was determined that the *aroA*Δ *S. Infantis* strain produced in this study has susceptibility to some antibiotics and also has decreased adhesion and invasion abilities in comparison to the wild strain.

According to these results, it can be concluded that this recombinant strain can be used as a vaccine candidate in vaccine development research after performing further in vivo investigations on experimental animals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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