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phoPQ carrying *Salmonella* in bile of cattle

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Summary: The objectives of this study were to determine the prevalence of *Salmonella* in bile of cattle by comparing immunomagnetic separation (IMS) based cultivation and conventional cultivation techniques, to verify the isolates by the detection of *oriC* gene, to determine *phoPQ* gene by PCR, to identify the isolates by serotyping and to find out the antibiotic resistance profiles using disc diffusion method. A total of 188 cattle bile samples were collected from two slaughterhouses near Ankara between May-September 2007. In three (1.6 %) of the samples *Salmonella* spp. were detected by IMS based cultivation technique and two (1.1 %) of the samples with conventional cultivation technique. It was shown that IMS based cultivation technique is more sensitive for the detection of *Salmonella* in bile of cattle. From the each contaminated samples three colonies were picked and coded (A, B and C). Isolates were verified by detection of *oriC* gene and *phoPQ* gene was detected using PCR. According to serotyping; two of them (A and B) were found to be *S*. Dublin and one (C) *S*. Bredeney. Disc diffusion method indicated that, *S*. Bredeney was resistant to ampicillin, cephalothin, tetracycline, nalidixic acid and sulphamethoxazole. All *S*. Dublin and *S*. Bredeney isolates were intermediately resistant to streptomycin, also *S*. Dublin to sulphamethoxazole and *S*. Bredeney to amoxicillin/clavulanic acid and cephazolin. In conclusion bile can be a site of *Salmonella* in cattle and all the isolates carried *phoPQ* gene that may play a significant role in the survival of *Salmonella* spp. in bile.

Key words: Antibiotic resistance, bile, cattle, IMS, PCR, *PhoPQ*, *Salmonella*, serotype.

Sığır safrasında *phoPQ* taşıyan *Salmonella*

Özet: Bu çalışmada, sığır safra örneklerinde *Salmonella* varlığının klasik kültür ve IMS (immuno-manyetik separasyon) yöntemleriyle tespit edilmesi, elde edilen izolatların PCR yöntemiyle *oriC* geni ile doğrulanması, *phoPQ* geninin belirlenmesi, izolatların serotiplendirilmesi ve antibiyotik direnç profillerinin belirlenmesi amaçlanmıştır. Bu amaçla 2007 yılı Mayıs-Eylül ayları arasında Ankara bölgesinde yer alan iki mezbahadan 188 sığır safra örneği toplanmıştır. Örneklerden 3 tanesi (% 1.6) IMS ve 2 tanesi (% 1.1) klasik kültür yöntemiyle *Salmonella* spp. olarak tespit edilmiştir. Sonuçlar ile sığır safrasında *Salmonella*'nın belirlenmesinde IMS bazlı kültür tekniğinin klasik kültür tekniğine gore daha duyarlı olduğu görülmektedir. *Salmonella* tespit edilen örneklerden 3'er koloni alınarak A, B ve C şeklinde kodlanmıştır. İzolatlar PCR yöntemiyle *oriC* ve *phoPQ* genleri yönünden analiz edilmiştir. Serotiplendirme işlemi sonucunda elde edilen izolatların ikisinin (A ve B) *S.* Dublin, birinin (C) *S*. Bredeney olduğu tespit edilmiştir. Disk difüzyon yöntemiyle *S.* Bredeney izolatının ampisilin, sefalotin, tetrasiklin, nalidiksik asit ve sülfametaksazole karşı dirençli olduğu tespit edilmiştir. Bütün izolatların (her iki *S.* Dublin ve *S.* Bredeney) streptomisine karşı, *S.* Dublin'in sülfametaksazole ve *S.* Bredeney'in amoksisilin/klavulanik asite karşı orta düzeyde dirençli olduğu belirlenmiştir. Sonuç olarak, sığır safralarının *Salmonella* yönünden önemli bir kaynak olabileceği ve izolatların taşıdığı *phoPQ* geninin *Salmonella* türlerinin safra koşullarında canlı kalmasında etkin rol oyanayabileceği belirlenmiştir.

Anahtar sözcükler: Antibiyotik direnç, IMS, PCR, *phoPQ,* safra, *Salmonella,* serotip, sığır.

Introduction

Salmonella, an important foodborne pathogen of zoonotic significance, has been associated with foods of animal origin (6). *Salmonella* has the ability to colonize the gallbladder where bile concentration is extremely high (4). Bile acids are derived from cholesterol in the liver and secreted into bile, which is stored in the gallbladder. Bile acids are also reabsorbed in the distal small intestine and large intestine following de-conjugation by the resident microbial flora. Bile is produced as a sterile compound, but interacts with enteric bacteria following secretion into the duodenum (19). Bile represents a major challenge to survival and subsequent colonization of microorganisms in the gastrointestinal tract. Therefore the gallbladder should be considered as a potential source of enteric pathogens such as *Salmonella* and *E. coli* O157:H7 (4; 17; 22; 27). It was reported that, after invading to macrophages in intestine *S*. Typhi can transport to the liver and it can be shed into the gallbladder (30).

Salmonella spp. encounter and must be able to resist the action of bile salts within the intestine. Enteric bacteria, including *Salmonella* spp., are resistant to the effects of bile (32). Previous studies revealed that a percentage (1 to 3 %) of individuals infected with *Salmonella* become chronic carriers and the prime location of the persistent infection is the gallbladder. In the carrier state, organisms are continuously released into the intestine and shed in the feces. It was reported that bile or gallbladder may play a role in the development of the carrier state (23).

Salmonellae are able to use bile as an environmental signal that effects its virulence by showing resistance to bile's emulsifying and antimicrobial characteristics (19). The outer membrane of Gram negative bacteria is thought to be the main barrier to bile salts (29). Also it was reported that PhoP-PhoQ (PhoPQ) regulated products play an important role in the survival of *Salmonella* spp. in the intestine and gallbladder (32) and also PhoPQ regulatory system is necessary for the virulence of *Salmonella* spp. (18; 20).

A considerable number of antimicrobials commonly used in the treatment of salmonellosis and other bacterial infections of humans are also used in veterinary practices. This may present a public health risk by the transfer of resistant *Salmonella* and other zoonotic bacterial pathogens or the resistant genes from food animals to humans through consumption of contaminated food and food products (16; 35). The increase of antimicrobial resistance in *Salmonella* and other bacterial pathogens have been a serious public health concern worldwide. Over the last two decades several multidrug-resistant *Salmonella* serotypes causing human and animal disease, have emerged (26; 33).

The aims of this study were to determine the prevalence of *Salmonella* in bile of cattle by comparing IMS based cultivation and conventional cultivation tecniques, to verify the isolates by the detection of *oriC* gene, to determine *phoP*/*phoQ* (*phoPQ*) gene by PCR, to identify the isolates by serotyping and to find out the antibiotic resistance profiles using disc diffusion method.

Material and Methods

Sample design and collection: A total of 188 bile samples of cattle were obtained using sterile syringe from undamaged gallbladders of healthy animals after evisceration of carcasses from two different slaughterhouses near Ankara between May to September of 2007. Approximately 20 ml of bile samples were taken into laboratory in an ice bag and analyzed in the same day.

Isolation and identification of Salmonella spp.: In the study conventional cultivation and immunomagnetic separation (IMS) based cultivation techniques were compared for the isolation of *Salmonella* from bile of cattle.

Conventional cultivation technique: ISO 6579 technique was used for the isolation of *Salmonella* (2). Ten ml of bile samples were weighted to sterile bags and enriched with 90 ml Buffered Peptone Water (BPW, Oxoid CM1049, Hampshire, UK) and incubated at 37°C for 24 hours. Afterwards, aliquots of 0.1 ml were transferred to 10 ml of Rappaport-Vasilliadis Broth (RVB, Oxoid CM669), and 1 ml to 9 ml of Selenite Cystine Broth (SCB, Oxoid CM0699) supplemented with sodium biselenite (Oxoid LP0121) and incubated for 24 hours at 42°C and 37°C, respectively. Following to the incubation broths were streak onto both of Brilliant-green Phenol-red Lactose Sucrose Agar (BPLS, Merck 1.07237, Hohenbrunn, Germany) and Xylose-Lysin Desoxycholate Agar (XLD, Oxoid CM0469). The plates were then incubated at 37°C for 24-48 hours. One to three typical colonies grown were picked from each medium and inoculated into Triple Sugar Iron Agar (TSIA, Oxoid CM0277), Lysine Iron Agar (LIA, Oxoid CM0381) and Urea Broth Base (Merck 1.08483) supplemented with 40 % of urea solution (Oxoid SR0020). The mediums were incubated at 37°C for 24-48 hours. TSIA positive, LIA positive and urease negative colonies were considered as suspect *Salmonella*.

The agglutination test was done with omnivalent *Salmonella* antisera (Denka Seiken 055111, Tokyo, Japan). Agglutination with antiserum was accepted as a positive reaction for *Salmonella* spp. The isolates were stored at 4°C and - 20°C for further tests.

Immunomagnetic separation (IMS) Based Cultivation Method: Ten ml of bile samples were weighted to sterile bags and enriched with 90 ml BPW (Oxoid CM1049) and incubated at 37°C for 24 hours. After the incubation period IMS was performed with 20 µl of magnetic beads coated with specific antibody against *Salmonella* (Dynabeads anti *Salmonella*, Prod. No. 710.02, Dynal, Oslo, Norway) according to the manufacturer's protocol (1).

Serotyping: Serotyping of the *Salmonella* isolates were performed with the scheme of Kaufmann-White using lam aglutination and serum neutralization tests (25).

PCR analysis: In order to determine the origin of DNA Replication (*oriC*) (37; 15; 12) and *phoPQ* (34) genes of *Salmonella* strains, PCR analysis were performed. For the PCR analysis *Salmonella* Typhimurium ATCC 14028 was used as positive control.

DNA extraction: Isolates that stored at 4ºC in Tryptone Soy Agar (TSA, Oxoid CM 131) were incubated in Brain Heart Infusion broth (BHI, Oxoid CM0225) at 37ºC for 24 h. Then 1 ml of each enrichment culture was separately transferred to microcentrifuge tubes. All tubes were centrifuged (Eppendorf Centrifuge 5417R, Hamburg, Germany) for 15 min at 5000 rcf at 10°C. The pellets were resuspended in 1ml sterile aquabidest. The suspensions were mixed by vortex (IKA MS1 Minishaker, Wilmington, USA). Then all tubes were centrifuged for 5 min at 5000 rcf at 10°C. The

pellets were resuspended with 200 µl sterile aquabidest and incubated for 20 min at 95ºC in a water bath (Memmert WB/OB 7-45, WBU 45, Schwabach, Germany) then cooled on ice.

PCR analysis for the detection of oriC gene: OriC gene specific primers (primer 1: 5'- TTA TTA GGA TCG CGC CAG GC-3'; primer 2: 5'- AAA GAA TAA CCG TTG TTC AC-3') (Promega, Madison, WI, USA) that produce a 163 bp DNA fragment were used for the verification of the *Salmonella* isolates (37; 15; 12)

PCR analysis for the detection of PhoPQ gene: Primers (337-L: 5'- ATG CAA AGC CCG ACC ATG ACG-3'; 338-R: 5'-GTA TCG ACC ACC ACG ATG GTT-3') (Promega) that produce a 299 bp DNA fragment and PCR conditions were used for the detection of *PhoPQ* gene from *Salmonella* isolates according to the Way et al. (34).

Gel electrophoresis: A 10 µl aliquot of each PCR product was subjected to 1.5 % agarose gel (SeaKem® LE Agarose, Rockland, ME, USA) electrophoresis containing 0.1 µg/ml ethidium bromide for 1 h at 100 V (Biometra, Agagel-Maxi-System B15359). Amplicon visualization and documentation was performed using gel documentation and analysis system (Syngene Ingenius, Cambridge, UK).

Antimicrobial Susceptibility Tests: The antibiotic resistance tests of *Salmonella* isolates were carried out with the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (3) in Mueller-Hinton agar (Oxoid CM0337) with ampicillin (Oxoid CT0003B), cephazolin (Oxoid CT0011B), cephalothin (Oxoid CT0010B), gentamicin (Oxoid CT0794B), amikacin (Oxoid CT0107B), streptomycin (Oxoid CT0047B), tetracycline (Oxoid CT0054B), chloramphenicol (Oxoid CT0013B), amoxicillin/clavulanic acid (Oxoid CT0223B), cefoxitin (Oxoid CT0119B), ceftriaxone (Oxoid CT0417B), ciprofloxacin (Oxoid CT0425B), imipenem (Oxoid CT0455B), trimethoprim/ sulphamethoxazole (Oxoid CT0052B), kanamycin (Oxoid CT0026B), trimethoprim (Oxoid CT0076B), sulphonamide compounds (Oxoid CT0059B), ceftiofur (Oxoid CT1751B), nalidixic acid (Oxoid CT0031B), and sulphamethoxazole (Oxoid CT0051B).

Results

In the study, a total of 188 cattle bile samples were collected from two slaughterhouses near Ankara between May-September 2007. A total of three (1.6 %) samples were found positive for *Salmonella*. Within these positive isolates, using IMS based cultivation technique three cattle bile samples, by conventional cultivation technique two samples were found to be contaminated with *Salmonella* spp. From the each three *Salmonella* positive samples three colonies were picked and coded as A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2 and C_3, respectively (Table 1). In *Salmonella* detected bile's gallbladders, any abnormalities or gallstones were not observed.

PCR assay was performed for the verification (by the detection of *oriC* gene) and detection of *phoPQ* gene. From all isolates *oriC* and *phoPQ* genes were detected (Figure 1 and 2).

Tablo 1. Sığır safrasında *Salmonella* spp. varlığında kullanılan iki izolasyon yönteminin karşılaştırılması.
Table 1. Comparison of the two isolation methods for the incidence of *Salmonella* spp. in bile of cattle.

Isolation method	Tested samples	*Salmonella* positive samples (%)	Code of positive samples and serotype distribution
Conventional cultivation method	188	2 (1.1)	131 (*S*. Dublin) 143 (*S*. Dublin)
IMS based cultivation method	188	3 (1.6)	131 (*S*. Dublin) 143 (*S*. Dublin) 147 (*S*. Bredeney)

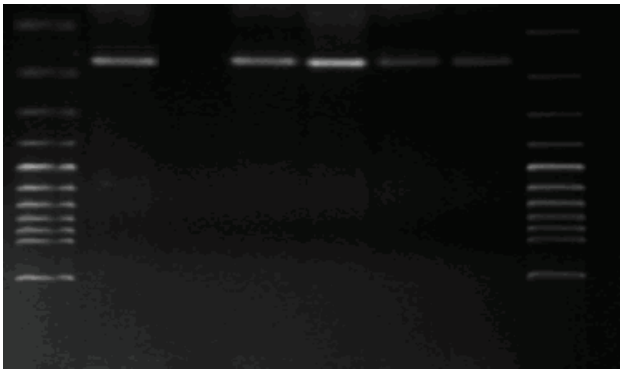

Fig. 1. *Ori*C gene detected *Salmonella* spp. isolates by PCR. *Lanes 1 and 8* 100 bp DNA marker; *Lanes 2 and 7* Positive control - *Salmonella* Typhimurium ATCC 14028; *Lanes 3* Negative control; *Lanes 4 - 6 Ori*C positive *Salmonella* spp. isolates.
Şekil 1. *Salmonella* spp. izolatlarında PCR yöntemiyle *oriC* geninin tespiti. 1 ve 8 no'lu sütunlar DNA marker; 2 ve 7 no'lu sütunlar Pozitif kontrol – *Salmonella* Typhimurium ATCC 14028; 3 no'lu sütun Negatif kontrol; 4-5-6 *oriC* pozitif *Salmonella* izolatları.

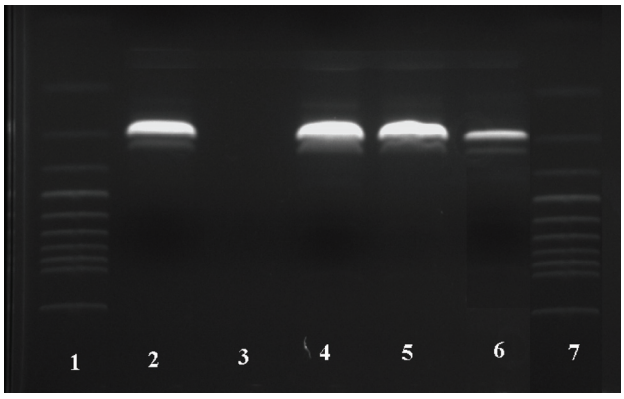

Fig. 2. *PhoP/Q* gene detected *Salmonella* spp. isolates by PCR. *Lanes 1 and 7* 100 bp DNA marker; *Lanes 2* Positive control - *Salmonella* Typhimurium ATCC 14028; *Lanes 3* Negative control; *Lanes 4 - 6 PhoP/Q* positive *Salmonella* spp. isolates.
Şekil 2. *Salmonella* spp. izolatlarında *phoP/Q* geni tespiti. 1 ve 7 no'lu sütunlar DNA marker; 2 no'lu sütun Pozitif kontrol – *Salmonella* Typhimurium ATCC 14028; 3 no'lu sütun Negatif kontrol; 4-5-6 no'lu kuyucuklar *phoP/Q* pozitif *Salmonella* spp. izolatları.

Table 2. Antibiotic resistance profiles of *Salmonella* isolates among the serotypes.
Tablo 2. *Salmonella* izolatlarının serotiplere göre antibiyotik direnç profilleri.

Antibiotics	µg disc^{-1}	*S*. Dublin			*S*. Dublin			*S*. Bredeney		
		A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3
Ampicillin	10	S	S	S	S	S	S	R	R	R
Cephazolin	30	S	S	S	S	S	S	I	I	I
Cephalothin	30	S	S	S	S	S	S	R	R	R
Gentamicin	120	S	S	S	S	S	S	S	S	S
Amikacin	30	S	S	S	S	S	S	S	S	S
Amoxicillin-clavulanic acid	30	S	S	S	S	S	S	I	I	I
Cefoxitin	30	S	S	S	S	S	S	S	S	S
Ceftriaxone	30	S	S	S	S	S	S	S	S	S
Ciprofloxacin	5	S	S	S	S	S	S	S	S	S
Imipenem	10	S	S	S	S	S	S	S	S	S
Trimethoprim-sulfamethoxazole	1.25/23.75	S	S	S	S	S	S	S	S	S
Chloramphenicol	30	S	S	S	S	S	S	S	S	S
Kanamycin	30	S	S	S	S	S	S	S	S	S
Tetracycline	30	S	S	S	S	S	S	R	R	R
Trimethoprim	5	S	S	S	S	S	S	S	S	S
Sulphonamide	300	S	S	S	S	S	S	S	S	S
Ceftiofur	30	S	S	S	S	S	S	S	S	S
Streptomycin	10	I	I	I	I	I	I	I	I	I
Nalidixic acid	30	S	S	S	S	S	S	R	R	R
Sulfamethoxole	25	I	I	I	I	I	I	R	R	R

R Resistance, *I* Intermediately resistance, *S* Susceptible

Isolates were identified by serotyping; two of them ($A_{1, 2, 3}$ and $B_{1, 2, 3}$) were found as *S*. Dublin and one ($C_{1, 2, 3}$) as *S*. Bredeney.

Disc diffusion method indicated that, *S*. Bredeney was found to be resistant to ampicillin, cephalothin, tetracycline, nalidixic acid and sulphamethoxazole. In addition, all *S*. Dublin A, B and *S*. Bredeney isolates were intermediately resistant to streptomycin, *S*. Dublin A and B to sulphamethoxazole and *S*. Bredeney to amoxicillin/clavulanic acid and cephazolin (Table 2).

Discussion

Increased prevalence in animal origin foods makes *Salmonella* very important for food safety and public health. So knowing more about its complex life cycle and identifying the regular distribution pattern of *Salmonella* in the internal organs became necessary. In the present study 1.6 % (3/188) of the cattle bile samples were found to be contaminated with *Salmonella*. In a study in Brazil, from 18 gallbladder and 16 bile samples belong to 30 cattle *Salmonella* was isolated. In 11 samples *Salmonella* was detected from both gallbladder and bile samples (10). Previous studies revealed that gallbladder of cattle and sheep may be the site and the source for fecal shedding of important enteric foodborne pathogens, such as *Salmonella* spp. and *Campylobacter* spp. (5; 14). In addition Deng et al. (9) reported that the liver and spleen may be the primary sites for setting itself up as a commensa over a long time after oral challenge and the gallbladder may be considered as a potential source of *Salmonella*. The gallbladder is a frequent and an important site of *S.*Typhi, which is generally more numerous in the bile than in feces (31). Although in various studies it was reported that, gallbladder abnormalities and gallstones often associated with chronic carriage of *Salmonella* in gallbladder (23), in this study any abnormalities or gallstones were not detected in *Salmonella* positive bile's gallbladders.

In the present study, from all isolates *phoPQ* gene was detected by PCR. Van Velkinburgh and Gunn (32) suggested that *Salmonella* spp. can respond to bile to increase resistance and that this response likely includes proteins that are the members of PhoP regulon. These PhoP-PhoQ regulated products may play an important role in the survival of *Salmonella* spp. in the intestine or gallbladder.

In the current study out of the three *Salmonella* isolates two of them were identified as *S*. Dublin and one as *S*. Bredeney. The epidemiological importance of *S*. Dublin is its ability to cause subclinical persistent infection in cattle (24). It is possible that gallbladder and bile might be the reservoir of bacterium. Accordingly, these carriers can contaminate environment and other food animals.

However, *S* Typhimurium or *S.* Enteritidis are the prevalent serotypes of invasive non-typhoidal salmonella disease, a case was reported with *S.* Dublin in Mali (28). *S*. Dublin is host adapted to cattle zoonotic bacterium can

causes illness and septicaemia in human (21). In most reference laboratories *S.* Bredeney is an uncommon human pathogen. Although *S.* Bredeney accounts for a very small proportion of overall human infection, there are indications that it may achieve local importance in particular regions at specific times. In recent years *S.* Bredeney has become the third most common *S*. Enterica serotype among isolates from human infections submitted for identification to the National *Salmonella* Reference Laboratory in Ireland (7). Also in a study conducted in Turkey, *S*. Bredeney was one of the identified serotype in spices contaminated with *Salmonella* spp. (13).

There is worldwide concern that many bacteria, including *Salmonella*, are becoming resistant to antimicrobial agents. Trends in antimicrobial susceptibility patterns of *Salmonella* isolates are being monitored in different countries. In the United States, 84 % of the *Salmonella* isolates from retail meats were resistant to at least one antibiotic, and 53 % to at least three antibiotics (36). *Salmonella* isolated from meat products in Ireland were resistant to sulfamethoxazole and streptomycin with a rate of 86.3 % and 80.9 %, respectively (11). Dias et al. (10) both cattle gallbladder and bile *Salmonella* isolates showed resistance to cephalothin, sulfazothrim and ampicillin. In the same study 50% of the *Salmonella* isolated from bile of cattle showed resistance to chloramphenicol. 6.25% of bile and 5.55% of gallbladder isolates were resistant to 12 antibiotics including amikacin, ampicillin, cephalothin, ceotaxime, ceftadizime, sulfazothrim, aztreonam, cefoxitin, ceftriaxone, chloramphenicol, gentamicin and tetracycline. In a different study between 2001 and 2004 79.6% of *S*. Dublin isolates from cattle were found resistant to ampicillin and 32.7% to trimethoprim-sulfamethoxazole (8).

In conclusion bile can be a site of *Salmonella* in cattle and all the isolates carried *phoPQ* gene which is required for increased bile resistance in *Salmonella*. Additionally, *Salmonella* isolates showed resistance to serious antibiotics. *S.* Bredeney showed resistance to antibiotics more than *S*. Dublin. Antibiotic resistant *Salmonella* where colonized in gallbladder or bile of cattle (chronic carriers) has an epidemiological impact.

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