Ankara Üniv Vet Fak Derg, **67**, 215-220, 2020
DOI: 10.33988/ auvfd.539572

Distribution of *Salmonella* serovars and characterization of isolates in cattle feces and environmental samples

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Received date: 13.03.2019 - Accepted date: 30.12.2019

Abstract: In this study, it was aimed to identify the presence of *Salmonella* serovars, and investigate the antibiotic susceptibility of isolates and the presence of certain virulence factors in the samples collected from cattle feces and environmental samples. Fecal and environmental swab samples were regularly collected from five different dairy cattle farms for a period of one year, once in each season. Totally, 425 fecal samples from animals, 21 of which had diarrhea and 400 environmental samples were examined for *Salmonella* spp.. While no *Salmonella* spp. was isolated from the environmental samples, *Salmonella* spp. was isolated from three (0.36%) of the fecal samples. All isolates were isolated from a single farm and they were sampled in autumn. Two strains were serotyped as *S*. Kottbus and the other as *S*. Lindenburg. All serovars were found to be sulfamethoxazole-resistant, while susceptible to cefoxitin, nalidixic acid, trimethoprim-sulfamethoxazole, enrofloxacin, ciprofloxacin, ceftriaxone, ceftiofur and amoxicillin-clavulanic acid. While *mgt*C, *mis*L and *inv*A were detected in all isolates, no *pef*A was detected. *stn* was detected in *S*. Lindenburg and one of *S*. Kottbus, whereas the *spv*A was detected only in *S*. Lindenburg. Presence of isolation only in one of the five farms and the low isolation rates were associated with a good level of biosecurity measures in the area where the study was conducted. *Salmonella* spp. isolation from healthy animals apart from animals with diarrhea was found to be important in terms of the role that persistently infected animals can play in the spread of the agent.

Keywords: Cattle, environmental contamination, feces, PCR, *Salmonella* spp.

Sığır dışkılarında ve çevresel örneklerde *Salmonella* serovarlarının dağılımı ve izolatların karakterizasyonu

Özet: Bu çalışmada sığır dışkılarından ve işletmelerin çevresel ortamlarından toplanan örneklerde *Salmonella* serovarlarının varlığının belirlenmesi, izolatların antibiyotik duyarlılıklarının ve belirli virülens faktörlerinin varlığının araştırılması amaçlandı. Beş farklı süt sığırı işletmesinden bir yıl boyunca her mevsim döneminde bir kez olmak üzere düzenli olarak dışkı ve çevresel svap örnekleri toplandı. Yirmi bir tanesi ishalli hayvanlardan olmak üzere 425 adet dışkı ve 400 adet çevresel svap örneği *Salmonella* spp. yönünden incelendi. Çevresel örneklerin hiç birisinden *Salmonella* spp. izole edilmezken, dışkı örneklerinin üç tanesinden (%0,36) *Salmonella* spp. izole edildi. İzolatların hepsi tek bir işletmeden ve sonbahar döneminde izole edildi. İki suş *S*. Kottbus ve diğeri *S*. Lindenburg olarak serotiplendi. Serovarların tamamı sulfamethoksazol dirençli, sefoksitin, nalidiksik asid, trimethroprim-sulfamethoksazol, enrofloksasin, siprofloksasin, seftriakson, seftiofur ve amoksisilin-klavulanik aside duyarlı bulundu. *mgt*C, *mis*L ve *inv*A genleri tüm izolatlarda saptanırken, *pef*A geni izolatların hiçbirinde saptanmadı. *stn* virulens faktörü S. Lindenburg ve bir adet S. Kottbus serovarında, *spv*A virulens faktörü ise sadece S. Lindenburg serovarında saptandı. Çalışmaya dahil edilen beş adet işletmeden sadece birinde izolasyon olması ve izolasyon oranlarının düşük seviyelerde kalması çalışmanın yapıldığı bölgedeki biyogüvenlik önlemlerinin iyi bir seviyede olmasıyla ilişkilendirilmiştir. İshalli hayvanların yanında sağlıklı hayvan dışkılarından da *Salmonella* spp. izole edilmesi, persiste infekte hayvanların etkenin saçılımında oynayabilecekleri rol açısından önemli bulunmuştur.

Anahtar sözcükler: Çevresel kontaminasyon, dışkı, PCR, *Salmonella* spp., sığır.

Introduction

Salmonella species are Gram-negative, non-spore, and facultative anaerobic bacilli belonging to the *Enterobacteriaceae* family (8). They can lead to zoonotic infections that can be accompanied by high mortality in the vertebrate creatures they colonize (7). Farm animals can be a source of salmonellosis seen in humans. Increasing yields, unconscious and high rates of antibiotic use in treatments lead to rapidly increasing drug resistance among *Salmonella* serovars. This rapid spread has resulted in difficulties in the treatment of infectious diseases in humans and animals and has reached to an extent that threatens the public health (1). The severity of infections caused by *Salmonella* serovars shows variations. Virulence factors owned by serovars, each of which has a function in different mechanisms, are responsible for this. Studies on isolates obtained from farm animals often identified the *invA* gene responsible for the bacterial invasion of cells, the *pef*A gene, one of the genes encoding the fimbriae active in adhesions, the *mis*L gene, one of the non-fimbrial components active in adhesions, the *mgt*C gene responsible for the proliferation of bacteria in the intracellular environments with intense magnesium concentration, the *stn* gene responsible for toxin production and virulence factors such as the *Salmonella* virulence plasmids (*spv*A, *spv*B, *spv*C, *spv*R) that play a role in causing the agent to form systemic infections (1, 4, 16).

Most of the studies conducted in our country to detect *Salmonella* serovars in cattle focus on the distribution of *Salmonella* serovars in cattle carcasses or animal products produced as food. However, it is emphasized that the feces of livestock may also be an important source of contamination. For this reason, in this study, the presence of *Salmonella* and the distribution of serovars were investigated in fecal samples. It was aimed to investigate the antibiotic susceptibility of isolates and to obtain epidemiological data by investigating the presence of important virulence factors.

Material and Methods

In the study, enviromental swabs and fresh fecal samples were collected four times in each season within a one-year period from 5 dairy product farms in Istanbul and surrounding areas. Samples were collected once in each season. In addition to animals displaying diarrhea signs in each farm, samples of 20 healthy animal feces were also randomly collected. This study was approved by İstanbul University Animal Experiments Local Ethics Board (Decision no: 2013/120).

Enviromental samples listed in Table 1, were collected via sterile swabs and delivered to the laboratory on the same day as the transport mediums. *Salmonella* isolation from samples was carried out according to the World Health Organization's protocol of Isolation of *Salmonella* spp. from Food and Animal Faeces (ISO6579) (30). Briefly, Buffered peptone water used for preenrichment. Tetrathionate broth (TTB), Rappaport Vassiliadis broth (RVS), Xylose Lysine Deoxycholate Agar (XLD), Brillant Green Agar (BG), MacConkey Agar were used in isolation stage. In addition to the protocol, novobiocin (15 µg/ml final concentration) added Hektoen Enteric Agar (HEA) was also used in the isolation. The identification was made according to the World Health Organization's laboratory protocol for the identification of *Salmonella* and *Shigella* by shortened panel tests (31). Serovar identifications of the isolates identified as *Salmonella* spp. were performed at Veterinary Control Central Research Institute. Antibiotic susceptibility of the

Table 1. List of environmental samples.

Farm A	Farm B	Farm C	Farm D	Farm E
Cabin for preparing pulp	Fence (7)	Milk container (2)	Barn floor (11)	Wheelbarrow (3)
Tractor wheel	Brush	Metal barrel (4)	Waterer (4)	Water booster
Trailer wheel	Barn floor (5)	Pump of milking machine (2)	Fence (2)	Wall of milking parlour
Automatic surface scraper	Chain of scraper (2)	Garden rake (2)	Chain of scraper	Dredge
Dog paw (2)	Waterer (2)	Coveralls	Barn door	Pump of milking machine (2)
Boot (2)	Barn wall	Milk boiler	Shovel	Ramp of milking parlour
Pump of milking machine	Hose	Circuit breaker		Satairs of milking parlour
Ventilation	Boot	Tractor Wheel		Chain of scraper
Command of scraper		Gutter		Barn wall (3)
Broom		Tap		Fence (3)
Milk tank		Milking parlour door		Shovel
Manger (2)		Manger		Vacuum motor
Waterer (2)		Wheelbarrow		Brush
Barn door		Barn floor		
Mop				
Stairs of milking parlour				

The number of samples is indicated in parentheses. Unless otherwise indicated, only one sample was collected.

Table 2. Primer sequences and amplicon size, annealing temperatures for PCR assays.

Gene region	Primer sequence	Annealing temperature	Amplicon size	Reference
*inv*A	5'- ACAGTGCTCGTTTACGACCTGAAT -3' 5'- AGACGACTGGTACTGATCCGATAAT -3'	56ºC	244 bp	Chiu and Ou (3)
stn	5'- TTGTGTCGCTATCACTGGCAACC -3' 5'- ATTCGTAACCCGCTCTCGTCC -3'	59ºC	617 bp	Murugkar et al. (17)
*pef*A	5'- TGTTTCCGGGCTTCTGCTG -3' 5'- CAGGGCATTTGCTGATTCTTCC -3'	55ºC	700 bp	Murugkar et al. (17)
*mgt*C	5'- TGACTATCAATGCTCCAGTGAAT -3' 5'- ATTTACTGGCCGCTATGCTGTTG -3'	60ºC	655 bp	Soto et al. (25)
*mis*L	5'- GACGTTGATAGTCTGCCATCCAG -3' 5'- CAATGCCGCCAGTCTCCGTGC -3'	60ºC	986 bp	Soto et al. (25)
*spv*A	5'- GTCAGACCCGTAAACAGT -3' 5'- GCACGCAGAGTACCCGCA -3'	60ºC	604 bp	Guerra et al. (13)

isolates was investigated by the disc diffusion method according to CLSI standards (5, 6). After the serovar identification was performed, each isolate was examined by PCR for the presence of *inv*A, *mgt*C, *mis*L, *stn*, *pef*A, and *spv*A virulence factors. *Salmonella Typhimurium* ATCC 14028 from culture collection of Istanbul University Cerrahpasa Faculty of Veterinary Medicine, Department of Microbiology was used as the positive control. DNA extraction was performed according to the protocol by Eyigor et al. (11). HOT FIREPol® DNA Polymerase [Solis BioDyne, Tartu, Estonia, Cat. no. 01-02-00500; (DNA polymerase 5U/µl, 10xBuffer, 25 mM $MgCl_2$, dNTP mix (20 mM of each)] was used. The primers used in the study, their sequences, PCR programs etc. are given in Table 2.

Results

Salmonella spp. was isolated from three (0.36%) of 425 fecal samples at five farms. Two of the serovars were identified as *Salmonella enterica* subsp. *enterica* serovar Kottbus (*S.* Kottbus) and the other one as *S*. Lindenburg. Agent isolation took place only in one of the five farms examined. In terms of the total number of samples (n=88) examined in this farm, the isolation rate was found to be 3.4%. *Salmonella* was not isolated from 400 environmental samples. Diarrhea was observed in 21 cattle from which fecal samples were collected. *S.* Kottbus (D3-38) was isolated from one of these cattle (4.7%). *S*. Lindenburg (D3-26) and the other *S.* Kottbus (D3-33) serovar were isolated from healthy animals that did not display any clinical signs. When the isolation rates were examined seasonally, it was observed that all isolations occurred in autumn. Antibiotic susceptibility of serovars examined by disk diffusion is given in Table 3.

As a result of the PCR, *mgt*C, *mis*L and *inv*A genes were detected in all isolates, but *pef*A was not detected. *stn* was detected in *S.* Lindenburg and one of *S*. Kottbus, whereas the *spv*A virulence factor was detected only in a *S*. Lindenburg serovars. PCR results of serovars are given in Table 3.

Table 3. Characterization of isolates.

Serovar (Sample code)		*S.* Lindenburg (D3-26)	*S.* Kottbus (D3-33)	*S.* Kottbus (D3-38)
Antibiotic susceptibility	AMP	R	S	S
	AMC	S	S	S
	EFT	S	I	I
	CTR	S	S	S
	C	R	R	R
	CIP	S	S	S
	ENR	S	S	S
	CN	R	S	S
	S*	R	S	I
	TE	R	S	I
	SXT	S	S	S
	SX	R	R	R
	NA	S	S	S
	CX	S	S	S
Virulance factors	*mgt*C,	+	+	+
	*mis*L	+	+	+
	*inv*A	+	+	+
	*pef*A	-	-	-
	stn	+	+	-
	*spv*A	+	-	-

S: Susceptible; I: Moderately Susceptible; R: Resistant; AMP: Ampicillin (10 µg), AMC: Amoxicillin-Clavulanic Acid (20/10µg); EFT: Ceftiofur (30µg), CTR: Ceftriaxone (30µg), C:Chloramphenicol (30µg); CIP: Ciprofloxacin (5µg); ENR: Enrofloxacin (5µg); CN: Gentamicin (10µg); S*. Streptomycin (10µg); TE: Tetracycline (30µg); SXT: Trimethoprim sulfamethoxazole (1.25/23.7µg); SX: Sulfamethoxazole (25µg); NA: Nalidixic acid (30µg); CX: Cefoxitin (30µg).

Discussion and Conclusion

In this study, isolation rate of *Salmonella* spp. from 425 fecal samples was 0.36%. It is seen that these rates differ in similar studies like Warnick et al. (28) 9.3%; Donkersgoed et al. (9) 0.08%; and McEvoy et al. (15) 2%. In our study, all of the isolations were performed in a single farm. This farm was found to be far behind in terms of compliance with hygiene requirements compared to others, and it was thought that this could be affecting the isolation rates. Warnick et al. (28) and Murinda et al. (17) isolated 12.9% and 3.76% *Salmonella* spp., respectively from the environmental samples in the dairy cattle farms. In this study, *Salmonella* was not isolated from any of the 400 swab samples. This was associated with the good hygiene and biosecurity measures of the farms.

The seasonal factors, which are thought to have an effect on the isolation rates have been evaluated by many researchers. Pangoli et al. (22) pointed to the parallelism between seasonal temperature increase and isolation rates. On a seasonal basis, McEvoy et al. (15) obtained the highest isolation rate in autumn, but Nothingham and Urselmann (18) in spring. Researchers did not evaluate the temperature difference as the sole criterion, but they also referred to different factors. *Salmonella* spp. isolation was only carried out from the samples collected at the beginning of November when the average temperature was 11.7ºC in the autumn. In this period temperature was higher than winter months, precipitation and the humidity were higher than summer months. This result was supporting the researchers by whom claiming isolation rates could be higher in spring months when heat, precipitation and humidity is higher than the other seasons of the year.

One of the factors affecting isolation rates when working with contaminated materials such as feces was reported as the selectivity of the medium used. Alcaide et al. (2) reported that their isolation rates were higher than BSA and HEA, and BG agar was less effective in inhibiting competitive microorganisms. They performed most of the isolations in the study with HEA. Studies in which different mediums are used have shown that HEA medium has a more selective quality even though it does not provide higher isolation rates. Jensen et al. (14) reported that novobiocin additions into mediums increased isolation rates. In this study, all of the isolations were carried out with novobiocin added HEA and it was found to be particularly useful for increasing the isolation chance of *Salmonella* species from contaminated materials such as feces.

Palmera-Suarez et al. (21) reported that *S.* Kottbus was detected in bottled waters and that the consumption of bottled water in human cases played a role. They reported parrots as a contamination source of waters. *S.* Kottbus was isolated via several different sources, including poultry, cattle, pig, the environment and human between 2000 and2011 (26). Özsan et al. (20) isolated *S.* Lindenburg from wild rodents in Central Anatolia. *S.* Kottbus and *S.* Lindenburg have been reported as serovars obtained from both human and non-human sources (27). Isolation of *S.* Lindenburg and *S.* Kottbus serovars from cattle in this study indicates that cattle may be a source of contamination.

In the current study, all serovars were resistant to sulphamethoxazole, they were susceptible to cefoxitin, nalidixic acid, trimethoprim-sulphamethoxazole, enrofloxacin, ciprofloxacin, ceftriaxone, ceftiofur and amoxicillin-clavulanic acid. Similar to our study, Wells et al. (29) found resistance to ceftiofur, ceftriaxone and ciprofloxacin. Sulphamethoxazole resistance was found to be as low as 2.9% compared to this study. Fluckey et al. (12) and Duffy et al. (10) found sulfamethoxazole resistance at a high rate (96.08%) in parallel with this study. The data of Hygiene Center reported that *S.* Kottbus serovars were resistant to tetracycline, nalidixic acid, trimethoprim- sulfamethoxazole and ampicillin at the rates of 9.7%, 12.2%, 17.0% and 9.7%, respectively (14). However, tetracycline, nalidixic acid, trimethoprim-sulfamethoxazole and ampicillin resistance were not observed among *S.* Kottbus serovars in this study.

*mis*L is a gene region that plays an important role in adhesion and is significant for the bacteria to form systemic infections by sticking to the intestinal tract (24). *mgt*C plays a role in the formation of systemic infections by allowing bacteria to survive in low magnesium concentrations in the cell (23). *mis*L and *mgt*C virulence factors were detected in all serovars. The role of these factors in bringing about systemic infections should not be ignored in terms of public health. Many researchers have investigated the presence of the *inv*A virulence factor in *Salmonella* and found high rates (4, 7, 8). Because of this feature, they thought that this factor could be a rapid, unique and sensitive tool for the detection of *Salmonella* (8, 19). In this study, *inv*A was detected in all serovars. *pef*A is one of the genes encoding the fimbriae that play a role in adhesion (16). Murugkar et al. (16) reported that their role in the pathogenesis of the *pef* genes is still unclear. Chuanchuen et al. (4) reported that the mutation in the *pef*A gene did not bring about a significant change in virulence. No *pef*A gene was detected in this study. *Stn* is a virulence factor associated with toxin production that play a role in the development of diarrhea (16). In this study, *stn* gene was detected from cattle without diareae so it was not detected connection between *stn* gene and diarrhea. The genes found in the *spv* operon increase virulence of serovars and lead to lethal infections. There are studies suggesting a direct link between virulence plasmid and multiple antibiotic resistances In this study, *spv*A was detected only in the *S.* Lindenburg serovar.

When the resistance levels of two serovars against antibiotics were compared, it was found that *S*. Lindenburg serovar was resistant higher number of antibiotics than *S*. Kottbus. This is remarkable in terms of correlation that can be established between *spv*A and resistance level.

In this study, two serovars, *S*. Kottbus and *S*. Lindenburg were isolated. The fact that *S*. Kottbus has been reported in infections in newborn units abroad at different times shows its importance in terms of public health. *S*. Lindenburg has been isolated from wild-rodents in our country, which is worrying about the spread and localization of the agent via wild-rodents at farms with inadequate biosecurity practices. Both serovars carry virulence factors like *mgt*C and *mis*L that play a role in the development of systemic infections. This is remarkable in terms of the seriousness of infections that may occur in a possible epidemic. *spv*A virulence factor has been associated with multiple antibiotic resistance by some investigators. In the present study, *spv*A was detected in the *S*. Lindenburg serovar. According to the antibiogram result, the *S*. Lindenburg serovar exhibited a much more resistant picture than the *S*. Kottbus serovar and this is noteworthy, because it could pose significant risks to animal and human health with the way of causing infections difficult to treat by antibiotics especially in young individuals. That only one of the five farms included in the study had isolation and that isolation remained at low levels were associated with sufficient level of biosecurity measures in the Thrace region. The isolation taking place in healthy animal feces apart from animals with diarrhea show that persistently infected animals could spread the agent. Since there is limited data on the prevalence of *Salmonella* in live dairy cattle, one of the sources of foodborne *Salmonella* infections in our country, similar studies need to be continued.

Acknowledgements

This study is produced from the first author's PhD thesis. This study was presented as a poster in the 18th International Veterinary Medicine Students Scientific Research Congress, Istanbul, Turkey, 2-4 May, 2017 and in Ecology 2017 Symposium, Kayseri, Turkey, 11-14 May, 2017.

Financial Support

This research is funded by Scientific Research Projects Coordination Unit of Istanbul University-Cerrahpaşa (Project number: 40786).

Ethical Statement

This study was approved by the Istanbul University Animal Experiments Local Ethics Board (Decision no: 2013/120).

Conflict of Interest

The authors declared that there is no conflict of interest.

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