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Antimicrobial susceptibility and serotype distribution of *Listeria monocytogenes* isolates obtained from raw milk cheese samples sold in Nigde*

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Summary: This study was designed to evaluate public health risks in respect to listeriosis due to consumption of local raw milk cheese sold in villages and public bazaars of Nigde. In addition, this study aims to contribute to the treatment process of listeriosis by determining serotype distribution and antimicrobial susceptibility profiles of the isolates. In the study, two of cheese (1 %) samples of total 200 raw milk cheese samples (100 white cheeses and 100 tulum cheeses) were found contamined with *L. monocytogenes.* Serotype distribution of the isolates were determined as 1/2a (white cheese isolate) and 4a (tulum cheese isolate). Finally, antimicrobial resistance profiles of the isolates against 11 antibiotics (tetracycline, ciprofloxacin, gentamicin, ampicillin, erythromycin, trimethoprim, vancomycin, chloramphenicol, nalidixic acid, penicillin G and sulphanilamide) were determined and the MIC values were specified by a microdilution method. Both of the isolates were found to be resistant to nalidixic acid but sensitive to the others analysed. As a result, although low *L. monocytogenes* prevalance rates were detected in this study, it is a public health concern to isolate serotype 1/2a which is secondly incriminated serotype to cause human listeriosis. In addition, it is important in terms of public health to continue surveillance of emerging antimicrobial resistance among *L. monocytogenes* isolated especially from foods of animal origin though incidence of antibiotic resistance in *L. monocytogenes* isolates is rare.

Keywords: Antimicrobial susceptibility, cheese, *Listeria monocytogenes*, microdilution, serotype distribution.

Niğde'de satışa sunulan çiğ sütten yapılmış peynir örneklerinden elde edilen *Listeria monocytogenes* izolatlarının serotip dağılımının ve antimikrobiyel duyarlılık profilinin belirlenmesi

Özet: Bu çalışma, Niğde ili köy ve pazarlarında tüketime sunulan çiğ sütten yapılmış peynirlerin listeriozis açısından taşıdığı halk sağlığı risklerini değerlendirmek ve peynirlerden elde edilen izolatların serotip dağılımını ve antimikrobiyel duyarlılık profillerini belirleyerek tedavi sürecine katkıda bulunmak amacıyla planlanmıştır. Çalışma sonucunda 200 çiğ sütten yapılmış peynir örneğinden (100 beyaz peynir ve 100 tulum peyniri) ikisinin (% 1) *L. monocytogenes* ile kontamine olduğu bulunmuştur. *L. monocytogenes* izolatlarının serotip dağılımı 1/2a (beyaz peynir izolatı) ve 4a (tulum peyniri izolatı) olarak belirlenmiştir. Son olarak izolatların 11 antibiyotiğe (tetrasiklin, siprofloksasin, gentamisin, ampisilin, eritromisin, trimetoprim, vankomisin, kloramfenikol, nalidiksik asit, penisilin G ve sülfonilamid) karşı direnç profilleri belirlenmiş ve MIC değerleri mikrodilüsyon metodu ile ortaya konmuştur. İzolatların her ikisinin de nalidiksik asite dirençli, diğer antibiyotiklere duyarlı olduğu tespit edilmiştir. Sonuç olarak incelenen örneklerde *L. monocytogenes* prevalansının çok düşük düzeylerde bulunmasına karşın insan listeriozisinden ikinci sırada sorumlu tutulan 1/2a serotipinin belirlenmiş olması halk sağlığı açısından risk unsuru olarak değerlendirilmiştir. Ayrıca, *L. monocytogenes* izolatları arasındaki antimikrobiyel dirençlilik nadir olsa da özellikle hayvansal gıdalardan elde edilen *L. monocytogenes* izolatlarının antimikrobiyel direnç profillerinin sürekli gözetim altında tutulması halk sağlığı açısından önem arzetmektedir.

Anahtar sözcükler: Antimikrobiyel duyarlılık, *Listeria monocytogenes*, mikrodilüsyon, peynir, serotip dağılımı.

Introduction

Listeria monocytogenes is a intracellular foodborne pathogen that can cause listeriosis in humans and animals (14). As a facultative intracelluler pathogen, *L. monocytogenes* is remarkably tolerant to external stress such as extreme pH, low temperature and osmolarity, oxidative stress, carbon starvation etc… (23). A large variety of foods, particularly milk, cheeses and ready-to eat products have been implicated as vehicles of listeriosis (15).

L. monocytogenes isolates are divided in to at least 4 lineages (I, II, III, and IV) and 12 common serotype. *L. monocytogenes* isolates from different lineages differ in their virulence characteristics (18). Ninety six percent of

* This study is summarized from PhD thesis.

the human cases of listeriosis are reported to be caused only by three serotypes among the 12 serotypes that are 1/2a, 1/2b and 4b. The other serotypes (especially 4a) are reported to be found mostly in foods and animals but rarely responsible for human *L. monocytogenes* infections (12).

The use of antimicrobials at subtherapeutic levels in food-producing animals results in increased antibiotic resistance and entrance of resistant pathogens through the food chain (7). *L. monocytogenes* is usually susceptible to antibiotics that are active against Gram-positive bacteria (24). Antimicrobial resistance of *L. monocytogenes* has been conscientiously traced since the first emergence of acquired resistance in *L. monocytogenes* isolates in France in 1988 (20). Other resistant isolates to one or more antibiotics obtained from food and human listeriosis have been reported by recent studies (26, 27).

The aim of this study was to determine the presence, serotype distribution and antimicrobial susceptibility of *L. monocytogenes* in raw milk cheese sold at retail in Nigde. Developing the effective risk management strategies and enriching the data on the MIC (Minimum Inhibitory Concentration) value of the antimicrobials for the strains isolated from raw milk cheeses are also within the frame of this study.

Materials and Methods

In this study total of 200 cheese samples (100 white cheeses and 100 tulum cheeses) were collected from open-air bazaars in Nigde during April-June 2011. All samples were placed in sterile bags, numbered and transported to the laboratory inside cold portable insulated boxes and processed within 3 hours of collection.

The reference strain *L. monocytogenes* (RSKK 472, Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey) was used as a positive control.

Conventional culture method: Samples were examined in accordance with International Standardization Organization (ISO) procedure (8). Briefly, 25 g cheese samples were pre-enriched in 225 ml half Frazer Broth (Merck Frazer Broth, Germany) at 30°C for 24 h. After enrichment, 0.1 ml pre-enriched sample was inoculated in 10 ml full Fraser broth (Merck Frazer Broth, Germany) and incubated at 37°C for 24–48 h. A loopfull of enriched sample was streaked onto Oxford *Listeria* Selective Agar (Merck, Germany) agar plates which contained polymyxin, acriflavin, lithium chloride, ceftazidime, esculin and mannitol. After 48 hours, suspected colonies were purified and further identified as *L. monocytogenes* by Microbact 12L *Listeria* identification system (Oxoid, MB1128A).

DNA extraction: Total genomic DNA was extracted from control and test strains using a commercial DNA extraction kit (Axygen, Bioscience, USA) as described by the manufacturer. All the extracts were stored at -20ºC until they are used the PCR procedure.

Polimerase Chain Reaction: All *L. monocytogenes* isolates identified by phenotypic tests were confirmed by PCR using species specific primers. Primers were composed of *hly* F [5`-CCT AAG ACG CCA ATC GAA-3`] and *hly* R [5`-TAG TTC TAC ATC ACC TGA GAC AGA-3`] were used for the amplification of 840 bp region of the *hly* gene (3, 19). PCR reaction was performed in a reaction mixture of 25 µl final volume containing; 5 mM template DNA, 5 µl 10XPCR buffer A (Vivantis, 500mM KCl, 100mM Tris HCl and 1% Triton^{TM} X-100), 1 U Taq polymerase (Vivantis), 0,1mM dNTP mix (Vivantis), 1.5 mM $MgCl_2$ (Vivantis) and 0.2 µM of each primer (28). PCR amplification was performed with an initial denaturation of 94 °C for 4 min, followed by 30 cycles, each consisting of 94°C for 40 s, 62 °C for 40 s and 72 °C for 40 s. The final extension was applied 5 min at 72 °C (Techne TC-512, USA) (39).

All amplification products were detected by agarose gel (1.5%) electrophoresis performed at 100 V for 50 min (EC250-90, Thermo, USA). The gels were stained with 0.5 µl/ml ethidium bromide and inspected visually under a UV transilluminator (Vilber Lourmat, Marne La Vallee, France).

Serotyping: Serotyping was performed by commercially available serotyping kit (Denka Seiken Co., Tokyo, Japan) following the manufacturer's instructions:

Determination of the O-antigen was carried out with heat-inactivated bacteria using the slide agglutination method. Bacterial suspensions were prepared in sodium chloride (0.2% w/v) to adjust the cell concentration to about 10 mg/ml from the isolates grown in BHI (Brain Heart Infusion Agar, Merck, Germany). Then bacterial suspensions were heated 121°C 30 min and they were centrifuged at 3.000 rpm during 20 min and resuspended with small amount of sodium chloride (0.2%w/v). Suspensions placed on the slide after dropping each of I/II antiserum, V/VI antiserum and physiological salinewater (30µl) as a control. Positive agglutination pattern was observed on the mix of antiserum and bacterial suspension slide in 1 minute. Positive evaluated isolates with antiserum I/II were tested with I and IV antiserums. Isolates agglutinated with V/VI antiserums were tested with VI, VII and IX antiserums.

Determination of the H-antigen was carried out using the test tube method with the bacteria cultured in semi-liquid BHI media (Brain Heart Infusion Broth, Merck, Germany and %0.2 Agar No.2 LAB M). Bacterial cultures were placed into Craigie's tubes included semi-liquid BHI and passaged 3 times. Then they were inoculated on BHI agar and incubated overnigth at 30°C. Bacterial suspension was prepared

adding 1v/v physiological salinewater with formaline. Two drops of HA, HB, HC and HD antiserums and 0.5 ml bacterial suspension was added four sterile tubes. The fifth tube that did not include antiserum were used as control. Each tube were incubated water bath 1 h at 52°C after mixed for homogenization. At the end of the incubation, agglutination was assessed visually in possitive tubes.

Antimicrobial Susceptibility Testing: Antimicrobial resistance profiles of the isolates against 11 antibiotics (tetracycline HCl (Sigma, Lot#110M1693V), ciprofloxacin (Sigma, Lot#BCBC7322V), gentamicin (Fluka, Lot#SZBA050XV), ampicillin (Duchefa, Lot#006701.05), erythromycin (Sigma, Lot#090M1715V), trimethoprim (Sigma, Pcode: 100946080), vancomycin HCl (Multicell, Lot#400138010), chloramphenicol (Sigma, Lot#100M 0061V), nalidixic acid (Sigma, Lot#020M1562V), penicillin G (Sigma, Lot# 071M074V) and sulphanilamide (Sigma, Lot#STBB4751V) frequently used for both animal and human treatments were determined and the MIC values were specified by broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (5). Antimicrobial stock solutions were prepared by distilled water (for ciprofloxacin, gentamicin, tetracycline HCl, penicillin G, vancomycin HCl and trimethoprim), 95% ethanol (for chloramphenicol and erythromycin), phosphate buffer pH 8.0 (for ampicilin), distilled water and 1mol/l sodium hydroxide (for

Table 1. Serotype distribution of *L. monocytogenes* isolates.
Tablo 1. *L. monocytogenes* izolatlarının serotip dağılımı.

	Antiserums of O Antigen								Antiserums of H Antigen			
Serotype	I/II	V/VI	I	IV	VI	VII	VIII	IX	HA	HAB	HC	HD
4a Tulum cheese isolate	(-) [a]	(+) [b]	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(-)
1/2a White cheese isolate	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)

[a] Agglutination was observed.
[b] Agglutination wasn't observed.

Table 2. The antimicrobial susceptibility profiles, MIC values and breakpoints of the *L. monocytogenes* isolates and *S. aureus* ATCC 25923 to 11 antibiotics.
Tablo 2. *L. monocytogenes* izolatlarının ve *S. aureus* ATCC 25923'ün kırılma noktaları, MIC değerleri ve antimikrobiyel duyarlılık profilleri.

Antibiotics	Breakpoints of antibiotics (µg/ml)			Tulum cheese isolate		White cheese isolate	
	S	I	R	MIC value (µg/ml)	Scb	MIC value (µg/ml)	Scb
Ciprofloxacin [a]	≤2	2-4	≥4	1µg/ml	S	1µg/ml	S
Tetracycline HCl [b]	≤4	8	≥16	2µg/ml	S	1µg/ml	S
Penicillin G [c]	≤2	-	-	0,5µg/ml	S	0,5µg/ml	S
Vancomycin HCl [e]	≤2	4- 8	≥16	1µg/ml	S	0,25µg/ml	S
Ampicillin [c]	≤ 2	-	-	0,125µg/ml	S	0,25µg/ml	S
Nalidixic acid [f]	-	-	≥32	>32µg/ml	R	>32µg/ml	R
Chloramphenicol [b]	≤ 8	16	≥32	4µg/ml	S	4µg/ml	S
Sulphanilamide [f]	-	-	≥512	64µg/ml	S	64µg/ml	S
Trimethoprim [e]	≤2	-	≥4	<0,5µg/ml	S	0,5µg/ml	S
Erythromycin [e]	≤0.5	1-4	≥8	0,25µg/ml	S	0,125µg/ml	S
Gentamycin [g]	≤4	8	≥16	0,5µg/ml	S	1µg/ml	S

[a] Reported by Ruiz-Bolivar et al., (22).
[b] Determined by CLSI for all microorganisms except *Streptococcus* spp (5).
[c] Determined by CLSI for *Listeria* spp (5).
[d] For *S. aureus* determined by CLSI (5).
[e] For *Staphylococcus* spp. determined by CLSI.
[f] Reported by Zhang et al., (27).
[g] Determined by CLSI (5).
S: Sensitive
I: Intermediate
R: Resistance
Scb: Susceptibility profiles

nalidixic acid) and distilled water and 2.5mol/l sodium hydroxide (for sulphanilamide). Concentrations of antimicrobial agents ranged from 0.0625 to 4 µg/ml for ciprofloxacin, 0.125 to 8 µg/ml for ampicillin, penicillin G and erythromycin, 0.25 to 16 µg/ml for gentamicin, tetracycline HCl, trimethoprim and vancomycin HCl, 1 to 32 µg/ml for nalidixic acid and chloramphenicol and 8 to 512 µg/ml for sulphanilamide. Fifty µl of initial concentration of the eleven antimicrobial agents were added first and eighth wells of 96-well sterile U microdilution tray (Eren Kimya, Turkey) with 50 µl Cation-adjusted Mueller Hinton broth (CAMHB; GLB, Turkey) supplemented with 5% v/v lysed horse blood. Serial twofold dilutions were used since seventh wells and last 50 µl diluent was threw out. Eighth wells of 96-well sterile U microdilution tray was used for negative control. Bacterial inoculum preparation was emulsified in 0.45% saline solution to the equivalent 0.5 McFarland turbidity standard and 50 µl of bacterial suspension were added to each well. The final inoculum contained $5x10^5$ CFU/ml bacteria. MIC's were determined after the inoculated microdilution trays were incubated at 35 °C for 16 to 20 h. The resistance, sensitive and intermediate breakpoints of the antibiotics for *L. monocytogenes* are mentioned in Table 2. All treatments included *S.aureus* ATCC 25923 as negative control.

Results

Two *L. monocytogenes* isolates were recovered, representing 1% of total samples (n=200). The pathogen occured in 1% (1/100) of white cheese and 1% (1/100) of tulum cheese samples. According to Denka Seiken Antisera tests, white cheese isolate found to belong to serotype 1/2a while tulum cheese isolate to serotype 4a (As shown in Table 1).

Table 2 resumes the antimicrobial susceptibility profiles, MIC values and the breakpoints of *L. monocytogenes* isolates for 11 antibiotics. Each of *L. monocytogenes* isolates were found susceptible to tetracycline HCl, ciprofloxacin, gentamicin, ampicillin, erythromycin, trimethoprim, vancomycin HCl, chloramphenicol, penicillin G and sulphanilamide and were resistant to nalidixic acid. *S.aureus* ATCC 25923 were susceptible to 11 antibiotics except nalidixic acid and penicillin G.

Discussion and Conclusion

The prevalence of *L. monocytogenes* in cheese samples from some provinces in Turkey were previously reported; in Nigde as 6% (2); in Kayseri as 17.2 % (11) and in Bolu as 9.2% (1). Our results are comperatively lower (1%) when compared to other studies. This variation in isolation rate among different provinces might be due to the differences in cheese processing environment, human activity, farm management, agriculture application (silage feeding), type of cheese samples, sampling seasons and isolation methods etc…. (16).

In different studies reported around the world, isolation percentages of *L. monocytogenes* from soft and semi-hard cheeses were 15% in Iran (21) and 40% in Greece (6). Manfreda *et al*., (13) reported low *L. monocytogenes* contamination rates (2.1%) from soft cheeses which is in agreement with our results whereas several authors reported no *L. monocytogenes* in cheese samples (4, 9).

Lineage II isolates (1/2a, 1/2c and 3a serotypes) are more frequently isolated from foods and food environments compared to lineage I isolates (12, 18). One of the isolates obtained from cheese samples in this study belonged to serotype 1/2a which is in agreement with previous studies in several countries (10, 17). The occurance of 1/2a serotype in local cheese samples that are commonly consumed in this region of Turkey is an important public health concern as this serotype is one of the the most frequently incriminated serotype associated with human listeriosis.

Lineage III isolates (4a, 4b and 4c serotypes) have rarely been isolated from foods or processing plant and retail environments (18). It is suggested that lineage III isolates may be better adopted to the animal production environment than the food-processing environment (25). The isolate obtained from tulum cheese in this study belonged to 4a serotype which is in agreement with Zhang *et al*., (27) who isolated 4a serotype from other food categories. This result is thought to indicate that contamination is derived from environment or goat skin in which tulum cheese is stuffed.

Another approach to different prevalence of lineages among food and food related samples may be due to the more resistant appearance of lineage II isolates to bacteriocins than lineage I isolates which could be a selective advantage for lineage II isolates in food samples and enrichments containing bacteriocins (25). The notable diversity in the pathogenicity among *L. monocytogenes* serotypes necessitates the development of rapid and accurate laboratory procedures that readily distinguish virulent from avirulent serotypes. This information has critical importance for the effective control and prevention of listeriosis in addition to provide data on prevalence and distribution of virulent and avirulent *L. monocytogenes* strains in foods of animal origin.

In the present study MIC values were specified by a broth microdilution method. Among the other antimicrobial susceptibility tests, broth microdilution method is used as gold standard for *L. monocytogenes* (5).

In this study, both of the *L. monocytogenes* isolates showed natural resistance to nalidixic acid that is used as a selective agent during isolation of this bacterium. This

result is in aggrement with Zhang *et al*., (27). In this study, no isolate was resistant to ampicillin, gentamicin, trimethoprim, tetracycline, ciprofloxacin, erythromycin, vancomycin, chloramphenicol, penicillin G and sulphanilamide.

The standard therapy for listeriosis is ampicillin or penicillin G combined with an aminoglycoside, such as streptomycin or gentamicin (26). In this study, MIC value of penicillin G, ampicillin and gentamicin were detected 0.5 µg/ml, 0.125-0.25 µg/ml and 0.5-1 µg/ml respectively which were similar to those reported by other authors (13, 10, 15). On the other hand MIC values for penicilin G was slightly higher than those reported by Zhang *et al*., (27) as 4 µg/ml. Penicillin G, ampicillin and gentamicin resistance of *L. monocytogenes* are mentioned in several studies (1, 21, 17, 26).

The MIC ranges obtained in this study for trimethoprim and sulphanilamide were 0.5 µg/ml and 64 µg/ml which were similar to those noted for food *L. monocytogenes* isolates (10, 27). Trimethoprim and sulphonamide resistant *L. monocytogenes* isolated from various food were reported in recent studies (17, 26). This resistance is important as trimethoprim-sulfamethoxazole treatment has been successfully used for listeriosis patients with beta-lactams allergy.

Both of isolates showed susceptibility to tetracycline with MIC value of 1µg/ml (white cheese isolate) and 2µg/ml (tulum cheese isolate). Similar MIC values for tetracycline were also reported by several authors (6, 10, 15). On the other hand tetracycline resistance among *L. monocytogenes* isolates has been recently observed by some researchers (1, 10, 17, 26, 27). High prevelance of tetracycline resistance in food isolates may be partly due to the frequent use of tetracycline in animal production (26).

In the present study both isolates showed high sensitivity to ciprofloxacin, chloramphenicol, erythromycin, and vancomycin. MICs for ciprofloxacin, chloramphenicol, erythromycin and vancomycin were determined as 1µg/ml, 4 µg/ml, 0.125- 0.25 µg/ml and 0.25- 1 µg/ml and µg/ml respectively. Similar MIC values were also noticed by some authors (6, 10, 15). Resistance to these antimicrobials have been previously documented at different levels (17, 26, 27).

The results of this study suggest that the overall incidence of *L. monocytogenes* isolated from commonly consumed cheese types in Nigde is low. However, traditionally produced raw milk cheese types can potentially be the source of *L. monocytogenes* serotype 1/2a capable of causing human listeriosis. In addition, althought *L. monocytogenes* isolates obtained from cheeses have shown uniform susceptibility to antibiotics in this study, it is important to continued surveillance of MIC value for various antibiotics of *L. monocytogenes* isolates to ensure effective treatment of human listeriosis.

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