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Investigation on presence of major gene for body weight, feed intake and feed efficiency using a segregation analyses in a mice population

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Abstract: Recent developments in molecular genetics and statistics have allowed the identification and use of major genes to explain the genetic variation. In this context, segregation analysis is a fast, reliable and inexpensive method that uses only phenotype and pedigree information. The aim of this study was to examine whether body weight, feed intake, and feed efficiency in a mouse population are directed by major gene in addition to polygenic and major gene effects by segregation analysis. For this purpose, previously collected dataset was used (n= 661). In this study, genetic variance, error variance, major gene variance, additive and dominant gene effects were estimated by segregation analyses. Dominant variance (1.04) was found to be smaller than the additive genetic variance (7.32) for body weight. Polygenic and major gene heritability predicted as 0.29 (± 0.63) and 0.81 (± 0.98) for body weight, 0.35 (± 0.63) and 0.96 (± 0.98) for feed intake and 0.52 (± 0.63) and 0.81 (± 0.98) for feed efficiency respectively. Existence of major gene was determined by examining the highest probability density regions. Although the major gene has been identified for body weight and feed intake, this result is not confirmed by the Mendelian transmission probabilities.
Keywords: Bayesian analyses, gibbs sampling, major gene, quantitative phenotype, segregation analyses.

Farelerde vücut ağırlığı, yem tüketimi ve yem etkinliğinden sorumlu major gen varlığının segregasyon analizi ile incelemesi

Özet: Moleküler genetik ve istatistikte elde edilen son gelişmeler sayesinde genetik çeşitliliğin açıklanmasında major genlerin belirlenmesi ve kullanılması olanaklı hale gelmiştir. Segregasyon analizi sadece fenotip ve pedigri bilgilerini kullanan hızlı, güvenilir ve ucuz bir yöntemdir. Bu çalışmanın amacı bir fare populasyonunda vücut ağırlığı, yem etkinliği ve yem tüketiminin poligenik etkilere ek olarak major gen tarafından yönlendirilip yönlendirilmediğinin segregasyon analizi ile incelenmesidir. Bu amaçla daha önceden toplanan bir veri seti kullanılmıştır (n= 661). Bu çalışmada segregasyon analizi ile eklemeli genetik varyans, hata varyansı, major gen varyansı, eklemeli ve baskın gen etkileri tahmin edilmiştir. Vücut ağırlığında; dominant etkinin (1.04) eklemeli gen etkisinden (7.32) daha küçük olduğu bulunmuştur. Poligenik model kullanılarak kalıtım derecesi tahmini 0.29 (± 0.63) ve major gen modelinde kalıtım derecesi 0.81 (± 0.98) olarak bulunmuştur. Poligenik model kullanılarak kalıtım derecesi tahmini 0.35 (± 0.63) ve major gen modelinde kalıtım derecesi 0.96 (± 0.98) olarak bulundu Poligenik model kullanılarak kalıtım derecesi tahmini 0.52 (± 0.63) ve major gen modelinde kalıtım derecesi 0.81 (± 0.98) olarak bulundu. Major gen varlığı en yüksek soncul olasılık bölgeleri incelenerek belirlenmiştir. Her ne kadar vücut ağırlığı ve yem tüketimi için major gen belirlenmiş olsada, mendelci geçiş olasılıkları bu sonucu doğrulamamıştır.
Anahtar sözcükler: Bayesçi analiz, gibbs örneklemesi, kantitatif fenotip, major gen, segregasyon analizi.

Introduction

The infinitesimal model in quantitative genetics postulate that a quantitative trait is determined by large number of loci with every locus has tiny effect (2). However genomic studies showed that in addition to polygenic effects sourced from the infinitesimal model there might be loci with also larger effects referring to the genetic architecture of the quantitative trait (5). It might be beneficial to detect such loci with larger effects (as such a major gene) for obtaining efficient selection and breeding programs in animal science. Although genotyping of an animal costs decrease however applying genomic studies at the farm level, still is not affordable at developing countries. In that regard, segregation analyses would be a cheap and accurate methodology to detect major genes (9) for animal breeding at developing countries. It would be informative to compare the results of segregation analyses with the output of genomic

analyses to evaluate if both methodologies lead to the same conclusion (15).

Recent advances in biotechnology have made it possible to investigate the genome at the molecular level. Ehsani et al. (3) and Karacaören (12) conducted genome wide association studies to infer genomic architecture of the body weight, feed intake and feed efficiency in mice using deoxyribonucleic acid (DNA), (or molecular marker) information. However, DNA information might not be available in most of the breeding populations due to the technical and/ or economical reasons especially at the developing countries. The aim of this study was to investigate existence of a major gene for body weight, feed intake and feed efficiency using only pedigree and phenotype information in a mice population in which major gene(s) have been confirmed by using DNA information in a previous study (3). Detection of major gene is important for a wide range of scientific and industrial process. Recent developments in the field of genomics have led to a renewed interest in detection of major gene(s) in DNA level. There has been no detailed investigation of comparisons for segregation and genomic analyses results. To our knowledge this is the first study to compare the output of genome wide association study with the segregation analyses.

Material and Methods

Materials: An F_2 population (n = 661; 323 males, 338 females) was formed by crossing M16 (F0; n =12) and ICR (F0; n=12) mouse lines for body weight studies at 8 weeks of age (1, 3). The pedigree file included 11 half-sib families. The M16 line was created by selecting for rapid weight gain while the ICR line was used as random control. More details about the data set could be found at (3).

Methods: A complex segregation model (9) incorporating both polygenic and major gene components was used to investigate existence of a major gene for the body weight, feed intake and feed efficiency in mice. Following mixed inheritance model was used to detect major gene in relation with body weight, feed intake and feed efficiency:

$$y = X\beta + Zu + ZWm + e \qquad [1]$$

where y is the vector of phenotypes (body weight, feed intake, feed efficiency), β is a vector of fixed effects of sex, u is a random vector of individual polygenic effects, W is a design matrix that includes the genotype of each mouse, m is a vector of genotypic means, e is a vector of residuals, and X and Z are incidence matrices connecting the phenotypes with model parameters. Two alleles are assumed at the major gene locus: by genotypes of AA, AB and BB with Mendelian transmission probabilities and associated additive (a), and dominance effect (d) (4). Poligenic model and associated inference could be obtained by omission of the ZWm term from the model [1] Distributional assumptions for polygenic effects were $u|A, \sigma_u^2 \sim N(0, A\sigma_u^2)$, where A is obtained from the relationship matrix of the pedigree file with polygenic variance of σ_u^2. Residuals were assumed to be normally distributed with error variance (σ_e^2) of $e \sim N(0, \sigma_e^2)$. Uniform prior distributions were used for the model parameters of β and m in [1]. The variances are estimated using inverse chisquare prior distributions. The Gibbs sampling strategy with blocking of parents and progeny algorithm was used to obtain the desired posterior distributions by Bayesian segregation analyses using iBay software (8). Polygenic model heritability determined by $\frac{\sigma_u^2}{\sigma_u^2 \cdot + \sigma_e^2}$ and major gene heritability determined by $\frac{\sigma_u^2 \cdot + \sigma_a^2}{\sigma_u^2 \cdot + \sigma_e^2 + \sigma_a^2}$. where σ_a^2 is major gene variance. 10 replicates run of the Markov chain for each trait consisted of 100.000 samples, and there after each 10th sample was collected to obtain 10000 samples because of the high correlation among the samples. Gelman et al. (6) propose a convergence diagnostic for markov chain monte carlo algorithms by comparing within (W) and between (B) chain (ψ_{ij} (i = 1, …, n; j=1,…,m)) variances:

$$B = \frac{n}{m-1} \sum_{j=1}^{m} (\bar{\psi}_{\cdot j} - \bar{\psi}_{\cdot\cdot})^2, \qquad \text{where}$$

$$\bar{\psi}_{\cdot j} = \frac{1}{n} \sum_{i=1}^{n} \psi_{ij} \ , \ \bar{\psi}_{\cdot\cdot} = \frac{1}{m} \sum_{j=1}^{m} (\bar{\psi}_{\cdot j})$$

$$W = \frac{1}{m} \sum_{j=1}^{m} s_j^2, \text{ where } s_j^2 = \frac{1}{n-1} \sum_{i=1}^{n} (\psi_{ij} - \bar{\psi}_{\cdot j})^2.$$

with number of iterations, n, and replicates, m, leads to variances of the chain as

$$\widehat{\text{var}}^{+}(\psi \backslash y) = \frac{n-1}{n} W + \frac{1}{n} B \, .$$

By using $\widehat{\text{var}}^{+}(\psi \backslash y)$ $\hat{R}$ test statistics could be used to investigate convergence of the chain by if the limiting value of $\hat{R}$ approach to 1 as n→ ∞.

$$\hat{R} = \sqrt{\frac{\widehat{\text{var}}^{+}(\psi \backslash y)}{W}} \, .$$

Results

The assumption of normality was tested by Kolmogrow-Smirnow test for all the phenotypes and confirmed the normality for feed intake (P> 0.05). The results of segregation analyses of body weight are given in Table 1. The polygenic variance was found to be smaller than the major gene variance (Figure 1 and Figure 2). Probably deviation from normality confounded with major gene variance hence it could have overestimated (Table 1). We applied various logarithmic and geometric data transformations to body weight to obtain normality

(results are not shown) but still, major gene variance found to be larger. Existence of the major gene for body weight is confirmed by the 95% HPDR, not including zero (Table 1). In terms of frequentist statistical inference, rejecting the null hypothesis stating no major gene for body weight at 95 % significance level. Dominance effect was found to be smaller (1.04) than the additive effect (7.32). Results of 95 % HPDR for Mendelian transmission probabilities are given in Table 2. Since 95 % HPDR is not included Mendelian transmission probabilities of 1, 0.5 and 0 it was concluded that mode of inheritance of body weight is not Mendelian. Estimates of major gene heritability for body weight were found to be around 0.81 while polygenic heritability found to be 0.29. Convergence analyses of the Gibbs sampler based on $\hat{R}$ given at Table 4. Convergence was concluded since estimates of $\hat{R}$ found to be around 1 for all model parameters.

Table 1. Posterior mean and standard deviation and left and right bounds of the 95% highest posterior density (HPDR 95 %) for body weight, and feed intake.

Parameter	Body weight				Feed intake			
	Posterior Mean	**Posterior Standard Deviation**	**HPDR 95 % left**	**HPDR 95 % right**	**Posterior Mean**	**Posterior Standard Deviation**	**HPDR 95 % left**	**HPDR 95 % right**
Error variance	11.13	1.96	6.56	17.34	142.22	37.21	129.01	272.07
Polygenic variance	4.46	2.55	0.00	18.52	132.29	58.89	111.34	423.27
Major gene variance	44.21	121.37	101.71	1401.92	919.99	2111.35	20355.53	382302.40
Additive effect	7.32	11.61	0.00	61.24	28.80	26.70	0.00	162.81
Dominant effect	1.04	6.38	0.00	37.87	24.88	20.19	41.84	125.20

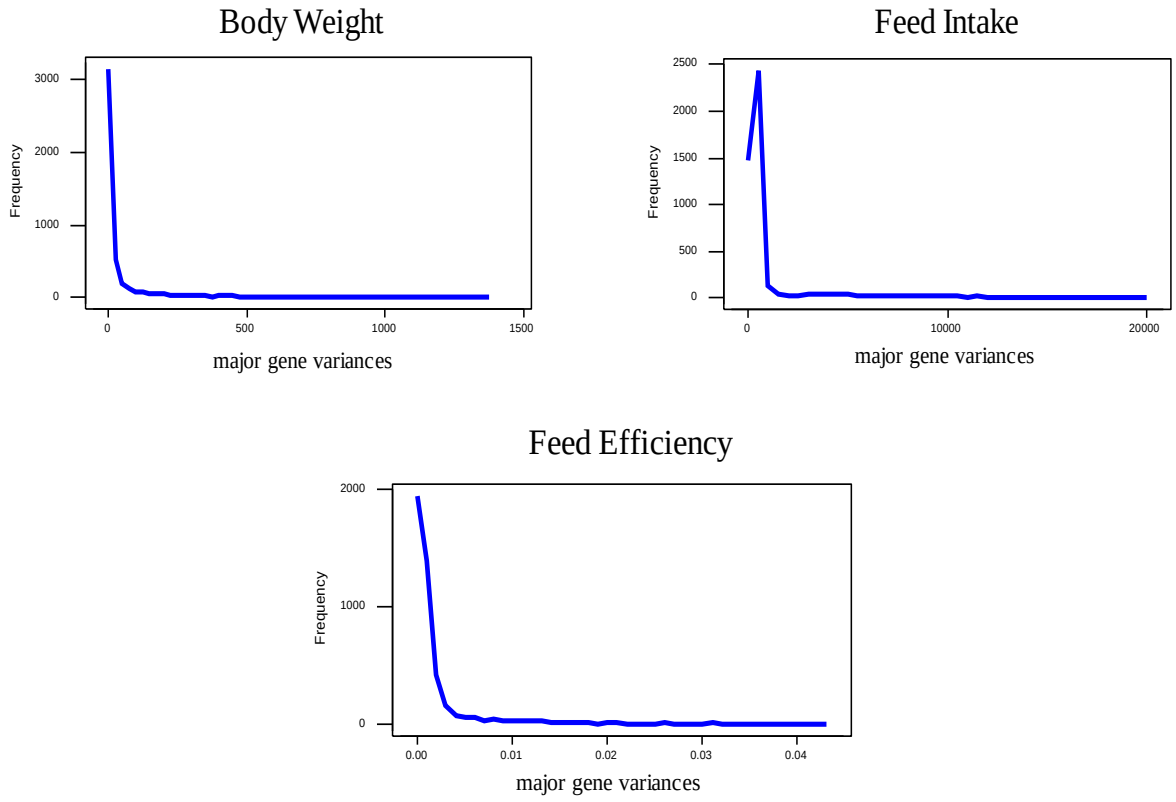

Figure 1. Marginal posterior distributions of major gene variances for body weight, feed intake and feed efficiency.

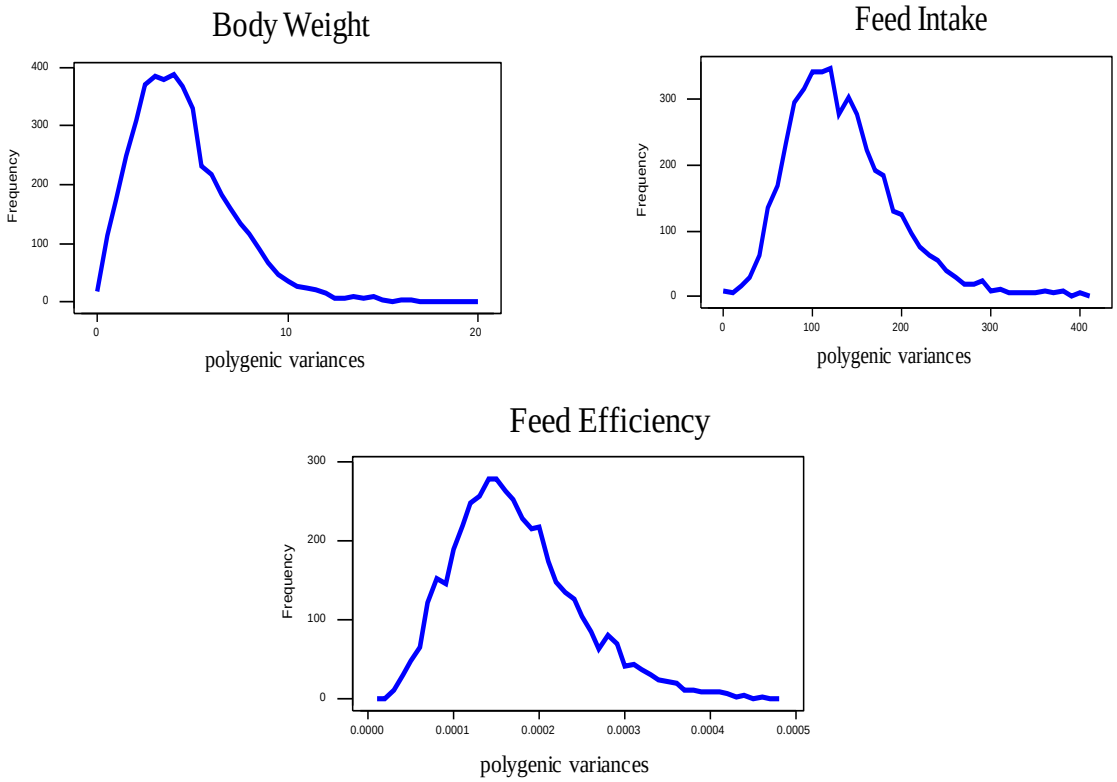

Figure 2. Marginal posterior distributions of polygenic variances for body weight, feed intake and feed efficiency.

Table 2. Posterior mean and standard deviation and left and right bounds of the 95 % highest posterior density for feed efficiency.

	Feed efficiency			
Parameter	**Posterior Mean**	**Posterior Standard Deviation**	**HPDR 95 % left**	**HPDR 95 % right**
Error variance	0.00017	0.00004	0.00000	0.00031
Polygenic variance	0.00017	0.00007	0.00001	0.00050
Major gene variance	0.00221	0.00502	0.00000	0.04408
Additive effect	0.05073	0.05327	0.00000	0.29994
Dominant effect	- 0.00347	0.05787	- 0.17884	0.31596

The results of segregation analyses of feed intake are given in Table 1. Polygenic variance (132.29) was found to be smaller than the major gene variance (919.99) (Figure 1 and Figure 2). Existence of the major gene for feed intake is confirmed by the 95 % HPDR, not including zero (Table 1). Dominance effect (24.88) was found to be nearly identical with the additive effect (24.80). Results of 95% HPDR for Mendelian transmission probabilities are given in Table 2. Since 95% HPDR is not included Mendelian transmission probabilities of 1, 0.5 and 0 it was concluded that mode of inheritance of feed intake is not Mendelian. Estimates of major gene heritability for feed intake were found to be around 0.96 while polygenic heritability found to be 0.35. Convergence analyses of the Gibbs sampler based on $\hat{R}$ given at Table 4. Convergence was concluded since estimates of $\hat{R}$ found to be around 1 for all parameters.

The results of segregation analyses of feed efficiency are given in Table 2. Polygenic variance (0.00017) was found to be smaller than the major gene variance (0.00221) (Figure 1 and Figure 2). Since 95 % HPDR included zero (Table 2) null hypothesis cannot be rejected, hence concluded that there is no segregation of major gene for feed efficiency. Dominance effect (- 0.00347) was found to be smaller than the additive effect (0.05073). Results of 95 % HPDR for Mendelian transmission probabilities are given in Table 2. Since 95 % HPDR is not included Mendelian transmission probabilities of 1, 0.5 and 0 it was concluded that mode of inheritance of feed efficiency is not Mendelian. Estimates of major gene heritability for feed efficiency were found to be around 0.93 while polygenic heritability found to be 0.52. Convergence analyses of the Gibbs sampler based on $\hat{R}$ given at Table 4. Convergence was concluded since estimates of $\hat{R}$ found to be around 1 for all parameters.

Table 3. Left and right bounds of 95 % highest posterior density regions for transmission probabilities, presented as the probabilities to inherit an A allele from AA, AB, and BB genotypes.

	Transmission probability	**HPD 95 % left**	**HPD 95 % right**
Body weight	Pr(A|AA)	0.00000	1.00323
	Pr(A|AB)	0.00000	1.00381
	Pr(A|BB)	0.00000	1.00335
	Transmission probability	**HPD 95 % left**	**HPD 95 % right**
Feed intake	Pr(A|AA)	0.00000	1.00271
	Pr(A|AB)	0.00000	0.96765
	Pr(A|BB)	0.00000	0.31410
	Transmission probability	**HPD 95 % left**	**HPD 95 % right**
Feed efficiency	Pr(A|AA)	0.00197	1.00200
	Pr(A|AB)	0.00200	1.00201
	Pr(A|BB)	0.00199	1.00383

Table 4. Investigation on the existence of convergence using $\hat{R}$ for body weight, feed intake and feed efficiency.

Parameter	**Body weight**	**Feed intake**	**Feed efficiency**
Error variance	1.000033	1.000108	**
Polygenic variance	1.000098	1.000084	**
Major gene variance	1.000923	0.99999	1.001863
Additive effect	1.002559	1.008708	1.008368
Dominant effect	1.000464	1.00132	1.010733

** There is convergence but the program could not calculate the number because it is too small.

Discussion and Conclusion

Animal breeding employs deep and complex pedigrees in breeding programs. Estimating the breeding value with high precision allows higher genetic improvement (11). Similarly, the use of deep and complex pedigrees in the analysis of major genes allows more accurate predictions and higher genetic improvements (7, 16). Gibbs sampling allows accurate statistical interpretation from deep pedigrees for segregation analyses in animal science (9).

In this study, segregation analyses were performed using an F2 mouse dataset for body weight, feed intake and feed efficiency. Ehsani et al. (3) and Karacaören (12) reported a number of genes in association with the body weight, feed intake and feed efficiency by genome wide association analyses using the same mouse dataset. Ehsani et al. (3) have shown that three QTLs are effecting body weight. In this study, we also detected a strong major gene variance (Table 1 and Table 2) for body weight but using only pedigrees and phenotype information. However, the mode of inheritance of this major gene is not confirmed to be Mendelian (Table 3). Linkage disequilibrium (LD) may cause deviation from Mendelian inheritance. Since the genome wide association studies (GWAS) exploits the LD over the genome such a result (Table 3) also expectable. Deviation from Mendelian segregation ratios could be explained by physical closeness with genes under selection pressure, mating among relatives and founder effects (13, 14,). In addition to LD, results of Ehsani et al. (3) and Karacaören (12) showed that the assumption of just one major gene for body weight is not a correct assumption.

Sanchez et al. (15) also compared the results of QTL (using interval mapping) and segregation analyses in pigs and reported that the at least presence of two QTL's could be responsible for the partial disagreement between the two approaches.

Jarvik (10) reported the relationship between normality and interpretation of the results of segregation analyzes. We applied various logarithmic and geometric transformations to obtain normality for body weight (results are not shown) but the major gene component was found to be non-significant after the transformations. Sanchez et al. (15) also reported decreased power due to

the Box-Cox transformations for the segregation analyses using a pig population.

Wolc et al. (16) found the heritability for body weight at the range of 0.25 to 0.47 using number of generations in mice with a polygenic model, whereas we found 0.42. Estimated polygenic heritabilites for body weight (h^2_{BW}=0.42), feed intake (h^2_{FI}=0.53) and feed efficiency (h^2_{FE}=0.58) was proportionally higher those obtained by Ehsani et al. (3): as h^2_{BW} =0.29, h^2_{FI} =0.35 and h^2_{FE} =0.52 respectively. Similar to our results Sanchez et al. (15) and Wolc et al. (16) reported that the results of segregation analysis overlap with the results of DNA analysis.

The present study was designed to determine if the segregation analyses and the genomic analyses (3) gave similar or contrasting results. The most striking result to emerge from the data (Table 1) is that existence of a major gene for body weight and feed intake was confirmed by both the segregation analyses and GWAS (12). Contrary to expectations this study did not detect any evidence for the Mendelian inheritance of the body weight or feed intake (Table 3). These results reflect those of (3) who also found that QTLs with major (large) and small effects at various chromosomes. In accordance with the present results, previous studies have demonstrated (2, 5) the importance of the genetic architecture of the phenotypes.

The present study provides the first comprehensive empirical assessment of genomic and segregation analyses under laboratory (controlled environment and homogenized genetic material) conditions for body weight, feed intake and feed efficiency in mice. The scope of this study was limited in terms of genetic and environmental conditions. Further studies need to be carried out in order to validate the findings of the current study at the farm level. These findings suggest (confirm) a role for segregation analyses for detection of a major gene(s) when the DNA information is not available due to economic reasons.

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Conflict of Interest

The authors are declared that there is no conflict of interest.

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Antibiotic resistance profiles of vancomycin resistant enterococci in chicken meat samples

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Abstract: Antibiotic resistance is one of the serious threats to global public health and food safety today. Acquired antibiotic resistance in microorganisms arises from prevalent use of antibiotics for human and animal medicine. Owing to the fact that Vancomycin Resistant Enteroccocci (VRE) is a vital problem for public health, determination of the antibiotic resistance profiles of *Enterococcus* spp. isolates have crucial importance as a part of the farm to fork food safety. In the study, 120 retail chicken meat samples were analyzed, and 36 (30%) of the samples were detected as *Enterococcus* spp. positive. According to the results, the most prevalent species was *E. faecalis* with a rate of 44.4% (16/36), followed by 27.8% (10/36) *E. faecium*, 11.1% (4/36) *E. durans*, 2.8% (1/36) *E. gallinarum* and 2.8% (1/36) *E. casseliflavus.* Antibiotic resistance profiles of the verified *Enterococcus* spp. isolates were determined with disc diffusion method in terms of eight different antibiotics. Among the *Enterococcus* spp. isolates, 20 (55.5%) isolates were phenotypically resistant to vancomycin, 6 isolates (16.7%) were detected as *vanA* positive, 3 isolates (8.3%) were detected as *vanB* positive, and one isolate (5%) showed high resistance to vancomycin (MIC >256 µg/ml). Even though the observed percentages are low, the observed resistance patterns are still of concern for public health.
Keywords: *Chicken meat, E. faecalis, E. faecium, vanA, vanB.*

Tavuk eti örneklerinde vankomisin dirençli enterokokların antibiyotik direnç profilleri

Özet: Günümüzde antibiyotik direnci, halk sağlığı ve gıda güvenliğini küresel anlamda tehdit eden ciddi problemlerden birisidir. Mikroorganizmalarda oluşan antibiyotik direnci, başlıca insan ve hayvan infeksiyonlarında antibiyotiklerin yaygın olarak kullanılmasından kaynaklanmaktadır. Vankomisin dirençli enterokokların (VRE) halk sağlığı açısından hayati bir sorun teşkil etmeleri nedeniyle, *Enterococcus* spp. izolatlarının antibiyotik direnç profillerinin belirlenmesi çiftlikten sofraya gıda güvenliği kapsamında kritik öneme sahiptir. Çalışmada, perakende olarak satışa sunulan 120 tavuk eti örneği analiz edilmiş ve örneklerin 36 (%30)'sı *Enterococcus* spp. pozitif olarak izole edilmiştir. İzolatlar arasında en yayın tür %44,4 (16/36) oranla *E. faecalis* olmuş, bunu %27,8 (10/36) ile *E. faecium*, %11.1 (4/36) ile *E. durans*, %2,8 (1/36) ile *E. gallinarum* ve %2,8 ile (1/36) *E. casseliflavus* olarak identifiye edilmiştir. *Enterococcus* spp. izolatlarının antibiyotik direnç profilleri sekiz farklı antibiyotik yönünden disk difüzyon metodu kullanılarak belirlenmiştir. *Enterococcus* spp. izolatlarından 20'si (%55,5) fenotipik olarak vankomisine dirençli, 6 (%16,7) izolat *vanA* pozitif, 3 (%8,3) izolat ise *vanB* pozitif olarak belirlenmiş, bir (%5) izolatın ise vankomisine karşı yüksek seviye dirençli (MİK >256 µg/ml) olduğu saptanmıştır. Belirlenen yüzdeler düşük olmasına rağmen, tespit edilen direnç profillerinin halk sağlığı açısından risk teşkil edecek düzeyde olduğu görülmektedir.
Anahtar sözcükler: *E. faecalis, E. faecium,* tavuk eti, *vanA, vanB.*

Introduction

Increasing antibiotic resistance in microorganisms arisen from prevalent and inaccurate use of antibiotics, is a serious threat to global public health and food safety. According to the Centers for Disease Control and Prevention (CDC) report in 2013, at least 2 million people are infected with microorganisms that are resistant to one or more antibiotics designed to treat those infections. In addition, it is pointed out that in the United States, at least 23,000 deaths occur as a direct cause of these antibiotic resistant infections (4).

Vancomycin resistance is the most prevalent antimicrobial resistant phenotype in enterococci. The importance of vancomycin arising from its use for the elimination of multidrug resistant strains or the treatment of patients allergic to antibiotics like ampicillin and penicillin. Besides it is used frequently in nosocomial infections as a last resort antibiotic, resistance genes can

be transferred to humans via foods. According to CDC, an estimated 66,000 healthcare-associated *Enterococcus* spp. infections occur in the United States and overall 20,000 VRE infections occurred among hospitalized patients each year, with approximately 1,300 deaths. Additionally, *E. faecalis* and *E. faecium* are the most isolated species from enterococcal infections (4, 9).

Growing concerns about risks to public health have heightened consumer awareness of safety in retail *chicken meat* consumption. Retail chicken meats are commonly contaminated with *Enterococcus* spp., and increasing interest on the epidemiology of these bacteria continues worldwide (23). In Turkey, there is a little information about antimicrobial resistance profiles, resistance gene distribution and incidence of Enterococcus spp. from poultry meat so that kind of information is a prominent need for food safety and public health.

The objectives of this study were to isolate and identify *Enterococcus* spp. from retail chicken meat samples collected from Ankara markets, to evaluate the antibiotic resistance profiles of the verified *Enterococcus* spp. isolates, to determine *vanA* and *vanB* resistance phenotypes of the species identified as *E. faecalis* and *E. faecium*, to analyze MIC (Minimum Inhibitory Concentration) values of the isolates determined as vancomycin resistant.

Material and Methods

Bacterial strains: As positive control strains; *E. faecalis* WHO3 (vanA$^+$), *E. faecalis* ATCC 29212, *E. faecium tetM* 7003, *E. faecalis* WHO14 (vanB$^+$), *E. gallinarum* C30BR, *E. hirae* (wild type), and *E. durans* (wild type) were used in PCR analysis.

Collection of samples: From different markets located at Ankara in Turkey, 120 retail chicken meat samples were obtained in unpackaged and packaged form, during the years 2013-2014. Samples were transported to the laboratory in cold chain, and examined for enterococci in two hours.

Isolation of enterococci: With the amount of 90 ml, sterile buffered peptone water (Oxoid, CM0009, Thermo Scientific) were added into the 10 g of each chicken meat samples and they were homogenized for 2 min. Afterwards, 0.1 ml of the homogenate was plated on Slanetz and Bartley medium (Oxoid, CM0377A, Thermo Scientific) and incubated for 24–48 h at 37°C. Then, typical colonies were transmitted into 0.6% yeast extract powder (Oxoid, L0021, Thermo Scientific) enriched tryptone soya agar (Oxoid, CM0131, Thermo Scientific) and subjected to biochemical tests according to Manero and Blanch (21).

Identification of enterococci: Chelex-100 resin based technique was used as to the DNA extraction and *Enterococcus* spp. isolates were identificated according to the determination of tuf gene (Table 1). PCR procedure was performed according to Kasımoğlu Doğru et al. (16).

Two different multiplex PCR were carried out for the verification of *E. faecium*, *E. faecalis*, *E. gallinarum* according to Kariyama et al. (15); *E. durans*, and *E. hirae* according to Jackson et al. (14). Primer pairs used in the PCR analysis were shown in Table 1.

Table 1. Target genes used in PCR analysis.

Target gene	Primer sequence (5′–3′)	Product size (bp)	Reference
tuf	Ent1:TACTGACAAACCATTCATGATG Ent2:AACTTCGTCACCAACGCGAAC	112	17
ddl$_{E.\ faecalis}$	ddlE1:ATCAAGTACAGTTAGTCTTTATTAG ddlE2:ACGATTCAAAGCTAACTGAATCAGT	941	15
ddl$_{E.\ faecium}$	ddlF1:TTGAGGCAGACCAGATTGACG ddlF2:TATGACAGCGACTCCGATTCC	658	15
van C$_{1E.\ gallinarum}$	C1:GGTATCAAGGAAACCTC C2:CTTCCGCCATCATAGCT	822	7
ddl$_{E.\ durans}$	DU1:CCTACTGATATTAAGACAGCG DU2:TAATCCTAAGATAGGTGTTTG	295	14
ddl$_{E.\ hirae}$	HI1:CTTTCTGATATGGATGCTGTC HI2:TAAATTCTTCCTTAAATGTTG	187	14
*Van*A	A1:CATGAATAGAATAAAAGTTGCAATA A2:CCCCTTTAACGCTAATACGATCAA	1030	15
*Van*B	B1:GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433	15

Determination of antibiotic resistance profiles: As defined by the Clinical and Laboratory Standards Institute (5), disc diffusion method was used for the determination of phenotypic antibiotic resistance of Enterococcus *spp.* isolates against eight antibiotics. Chloramphenicol (30 µg, Oxoid CT0013B, Thermo Scientific), erythromycin (15 µg, Oxoid CT0020B, Thermo Scientific), gentamicin (120 µg, Oxoid CT0794B, Thermo Scientific), penicillin (10 U, Oxoid CT0043B, Thermo Scientific), streptomycin (300 µg, Oxoid CT1897B, Thermo Scientific), vancomycin (30 µg, Oxoid CT0058B, Thermo Scientific), linezolid (30 µg, Oxoid CT1650B, Thermo Scientific), quinupristin/ dalfopristin (15 µg, Oxoid CT1644B, Thermo Scientific) were used within this context.

Determination of Minimal Inhibition Concentration (MIC) values: MIC values of the isolates were determined by E-test method against vancomycin by using swab technique. E-test strips (Oxoid, MA0102F, Thermo Scientific) were placed on Mueller–Hinton agar (Oxoid, CM0337B, Thermo Scientific) plates and were incubated at 35°C for 24-48 h. After incubation, MIC values were evaluated according to the CLSI standarts (5) as; for VRE is ≥ 32 µg/ml, for vancomycin succeptible enterococci is ≤ 4 µg/ml.

Detection of* vanA*, and* vanB *genes: Gene specific primers for *vanA*, and *vanB* used in the multiplex PCR assays are shown in Table 1. PCR procedures were performed according to Kariyama et al. (15).

Results

In the study, among 120 retail chicken meat samples, 36 (30%) of the samples were found positive for *Enterococcus* spp. Among enterococci, *E. faecalis* was the most prevalent species with a ratio of 44.4% (16/36), followed by 27.8% (10/36) *E. faecium*, 11.1% (4/36) *E. durans*, 2.8% (1/36) *E. gallinarum*. None of the isolates were confirmed as *E. hirae*, while one (2.8%) isolate was confirmed as *E. casseliflavus* with biochemical tests**.**

According to the antibiotic resistance profiles of the verified *Enterococcus* spp. isolates all of the isolates were determined as intermediate or resistant to at least one antibiotic. According to the antibiotic resistance profiles of the isolates; 72.2% (26/36) of the isolates were resistant to three and more antibiotics, 19.4% (7/36) were resistant to five and more antibiotics, and 2.8% (1/36) were resistant to all of the antibiotics and 55.5% (20/36) of the isolates were resistant to vancomycin. Antibiotic resistance profiles of VRE isolates are shown in Table 2. On the other hand, number of resistant and susceptible VRE isolates in view of vancomycin MIC values are shown in Table 3.

According to the results, *vanA* genes were detected in 16.7% (6/36) of the *Enterococcus* spp. isolates, while *vanB* genes were detected in 8.3% (3/36) of them. One of the isolates was positive for both *vanA* or *vanB* genes and was determined as *E. faecalis*.

Table 2. Antibiotic resistance percentages of the VRE isolates (%).

Number of isolates		LZD			QD			C			CN			P			S*		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Efa	14	6(43)	6(43)	2(14)	14(100)	-	-	2(14)	9(64)	3(21)	1(7)	1(7)	12(86)	2(14)	-	12(86)	4(29)	1(7)	9(64)
Efec	2	-	2(100)	-	2(100)	-	-	-	2(100)	-	-	-	2(100)	-	-	2(100)	-	-	2(100)
Du	2	1(50)	1(50)	-	2(100)	-	-	1(50)	1(50)	-	1(50)	1(50)	-	1(50)	-	1(50)	1(50)	-	1(50)
Gal	1	1(100)	-	-	1(100)	-	-	1(100)	-	-	1(100)	-	-	-	-	1(100)	1(100)	-	-
ND	1	-	100	-	1(100)	-	-	-	1(100)	-	-	-	1(100)	-	-	1(100)	-	-	1(100)
Total	20	8(40)	10(50)	2(10)	20(100)	-	-	4(20)	13(93)	3(21)	3(21)	2(10)	15(75)	3(21)	-	17(85)	6(43)	1(5)	13(93)

Efa: *E. faecalis*; Efec: *E. faecium*; Du: *E. durans*; Gal: *E. gallinarum*; ND: Not Determined; LZD: Linezolid; QD: Quinupristin-Dalfopristin; C: Chloramphenicol; CN: Gentamicin; P: Penicillin; S*: Streptomycin; R: Resistant; I: Intermediate; S: Susceptible

Table 3. Number of resistant and susceptible VRE isolates in view of vancomycin MIC values, according to the CLSI standarts.

MIC (µg/ml)	Resistance profile	Number of isolates	Isolated species	*Van* gene
≥ 32	R	1	Du	-
8-16	I	4	Efa (3), Efec (1)	*vanA* (2)
≤ 4	S	15	Efa (11), Efec (1), Gal (1), Du (1), ND (1)	*vanA* (3), *vanB* (1), *vanA* + *vanB* (1)

Efa: *E. faecalis*; Efec: *E. faecium*; Du: *E. durans*; Gal: *E. gallinarum*; ND: Not Determined

Discussion and Conclusion

The consumption of food contamined with antibiotic resistant enterococci strains, is considered a probable path of transport of this agent from animals to humans (19). In this study, 30% of the chicken samples were found positive for *Enterococcus* spp., also Pesavento et al. (23) had similar results (28.6%) in terms of *Enterococcus* spp. existence in chicken meat. Lower prevalences have been reported from Tunisia (19) and Greece (12) with the percentages of 24.5% and 21.7%, in contrast higher prevalences have been reported from Brazil (8) and Tennessee (18) with 56.8% and 82.2% of *Enterococcus* spp. contamination, respectively. Besides, according to our results, 30.8% of the packaged and 29.6% of the unpackaged samples were contaminated with *Enterococcus* spp.

In our study, the most prevalent species were determined as *E. faecalis*, followed by *E. faecium*, *E. durans*, *E. gallinarum* and *E. casseliflavus*. Similar preponderances from chicken meat samples have been reported previously from Tunisia (19), Brazil (8), USA (25), in contrast, some other studies from Scandinavia, Spain and Italy (6), Greece (12), *E. faecium* was stated as of the preponderant species. Varieties of preponderances can be linked with geographical diversities or typing techniques (14, 21).

According to our study, all the isolates were determined as resistant or intermediate to at least one antibiotic used in study and which is a significant public health concern. Goncuoglu et al. (11) also implied that on-going surveillance and antibiotic resistance of foodborne pathogens in the food chain is essential due to the public health implementations. Besides, by the reason of high antibiotic resistance profiles, it is very crucial to analyze antibiotic profiles of the isolates.

In some other studies, it is also determined that poultry isolates had similar antibiotic resistance profiles to similar group of antibiotics with that of our isolates (14-17). Outcomes of this study pointed out the crucial importance regarding high level aminoglycoside resistance in poultry originated enterococci, that is quite low in Europe (6, 24). As aminoglycosides are considered as an option for enterococcal infections treatments, the contingency of the spread of the resistance via food food chain is minacious. In contrast to Europe, high level aminoglycoside resistance in enterococci prevalent in USA is similar to our results. Hayes et al. (13) analyzed the incidence of aminoglycoside resistance of *E. gallinarum* (56%), *E. faecium* (58%), and with the highest rate of *E. casseliflavus* (86%). Although the use of avoparcin was banned in Turkey in 2002 (3), VRE can still be isolated from chicken meat. Besides, resistance to quinupristin-dalfopristin was observed in 27% of *E. faecium* isolates from chicken samples while all of the VRE isolates were resistant to this antibiotic in our study. Due to the significance of quinupristin–dalfopristin was the initial antibiotic certificated by the US Food and Drug Administration (FDA) for VRE infections, the resistance to this antibiotic has crucial importance for public health.

Linezolid is also the first commercially available drug of the group of the oxazolidinones and the other antibiotic also approved by FDA to treat infections caused by VRE (10). In our study 90% (18/20) of the VRE isolates were intermediate or resistant to linezolid and it also provides an alarming warning about treatment of VRE infections. As part of the NARMS (National Antimicrobial Resistance Monitoring System), Tyson et al. (25) evaluated several retail meat commodities from 2002 to 2014, and found 92% of enterococci contamination. According to their study, none of the isolates was resistant to vancomycin, but only one isolate was resistant to linezolid, and resistance to tigecycline was below 1%. In contrast, *E. faecalis* and *E. faecium* isolates were resistant to tetracycline with the percentages of 67.5% and 53.7%, respectively.

In studies conducted in Turkey, Kasimoglu Dogru et al. (16) analyzed 106 chicken neck skin samples and their results are similar to our results, they determined majority (90%) of enterococci isolates were high level resistant to tetracycline and erythromycin. Unlike to our results, they stated that all their isolates were susceptible against penicillin G. In another study, Yilmaz et al. (26) analyzed 105 isolates collected from chicken meat samples, they stated that the majority (96%) of the samples were resistant to at least one antibiotic among tested twelve antibiotics. For the isolates from chicken samples, resistance percentages against tetracycline, erythromycin, ciprofloxacin, and trimethoprim/sulfamethoxazole antibiotics were 89.5%, 59%, 35.2%, 34.3%, respectively. In this study, five strains were phenotypically resistant to vancomycin and also carried *vanA* gene besides, all VRE isolates were found to be resistant against trimethoprim/sulfamethoxazole, penicillin, tetracycline, ampicillin, and erythromycin. In contrast, Pesavento et al. (23) analyzed lower rates of enterococci were resistant to amoxicillin-clavulanic acid (0.32%), linezolid (0.32%), teicoplanin (2.24%), and vancomycin (3.53%). Higher rates were determined in *E. faecalis* isolates against tetracycline (60.6%) and gentamicin (21.9%). Generally, *E. faecalis* isolates stated to be more resistant that of *E. faecium*, similar with our results.

Lopez et al. (20) analyzed 129 chicken meat samples and determined 17.82% of them as VRE, indicated lower percentages (55.5%) as compared to our study. Besides, according to CDC report, in United States, about 30% of healthcare-associated infections were related vancomycin

resistant enterococci in 2013 (23). According to E-test results conducted in our study, one isolate (5%) showed high resistance to vancomycin (MIC: >256 µg/ml). On the other hand, Lopez et al. (20) stated that among VRE isolates, 7 (5.4%) isolates showed high resistance to vancomycin (MIC: 64–128 µg/ml). Besides, VRE isolates harboured five *vanA* and one *vanB* genes, while in our study, *vanA* genes were detected in six isolates and three of the isolates were harboured *vanB* genes.

In our study, twenty (55.5%) isolates were phenotypically but eight (22.2%) isolates were genotypically resistant to vancomycin. These differences of phenotypic and genotypic resistance to vancomycin in the studies can be attributed to the vancomycin resistance genotypes not limited only *vanA* and *vanB*. However, genes regarding vancomycin resistance for enterococci are defined as *vanA*, *-B*, *-C*, *-D*, *-E*, *-G*, *-L*, *-M*, and *N*, to date (2).

Messi et al. (22) analyzed 45 poultry isolates and 15 (33.3%) isolates determined as VRE, whereas 4 isolates (8.9%) contained *vanA*, 2 (4.4%) isolates contained *vanB*, 9 (20%) isolates contained *vanC* genes. In contrast, Aarestrup et al. (1) analyzed 126 chicken meat samples, and 10% of the samples identified as VRE. It is also stated that all VRE isolates were *E. faecium*. Vancomycin resistance is associated with sequences related to *vanC* genes are specific for *E. gallinarum* and *E. casseliflavus* (7). Therefore, *E. gallinarum* ve *E. casseliflavus* isolates also might be considered as harbouring *vanC* genes, in our study.

The study shows that VRE isolates from chicken meat in Turkey based on a high prevalence of resistance to antibiotics. By virtue of the fact that, VRE is a vital issue for global public health, determination of the antibiotic resistance profiles of *Enterococcus* spp. isolates have crucial importance as a part of the farm to fork food safety. Potential solutions should include efficient control survey programs for determining the enterococci in environmental sources and especially in food for preventing the spread of the pathogenic strains. Provided that the contamination with enterococci from different sources can be verified and limited, a rise in the prevalence of colonisation and infection amongst the hospitalised patients and also antibiotic resistance could be controlled. Longterm policies should be based on international survey systems in terms of monitoring the contamination in carcasses, foods, animals, and humans.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Safety aspects of *Lactobacillus plantarum* strains isolated from Siahmazgi cheese

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Abstract: In order to use lactic acid bacteria as starter and non-starter cultures in fermentative products, their safety qualities should be evaluated. The objective of the present study was to evaluate safety characteristics including antibiotic sensitivity pattern, antimicrobial effect, H_2O_2 production, and biogenic amine production by *Lactobacillus plantarum* strains isolated from Siahmazgi cheese. *Lactobacillus plantarum* strains were unable to produce tyramine except for SD6 strain. All strains in the current study were able to produce histamine but unable to decarboxylate neither lysine nor ornithine. *Lactobacillus plantarum* strains showed considerable antimicrobial activity against *Salmonella typhimurium* and *Listeria monocytogenes*. All *L. plantarum* strains showed stronger antimicrobial activity against *S. typhimurium* (3.5-42.55 mm) than *L. monocytogenes* (1.5-30.49 mm). The SC9 strain had the strongest inhibitory effect against both pathogens. After titering pH of the medium to approximately 6.5, no antimicrobial activity was noticed indicating that the antimicrobial activity of *L. plantarum* strains was contributed to their acid production and not to bacteriocin. All *L. plantarum* strains were capable of producing H_2O_2. SA32 and SD13 strains with 2.37 and 0.77 mmol/L were the strongest and the weakest strains regarding H_2O_2 production, respectively ($P< 0.05$). All *L. plantarum* strains were sensitive to chloramphenicol, erythromycin, rifampicin, and tetracycline, yet resistant against vancomycin, and norfloxacin. Four patterns of antibiotic resistance were observed among *L. plantarum* strains. Only two strains of SC9 and SE4 were resistant against four antibiotics. *L. plantarum* strains naturally found in Siahmazgi cheese do not generally possess dangerous characteristics to be used in fermentative dairy products.

Keywords: Artisanal cheese, food safety, lactic acid bacteria, *L. plantarum*, Siahmazgi cheese.

Introduction

With respect to metabolic characteristics of lactic acid bacteria (LAB), they are used to improve the taste, texture, nutritional value, and safety of fermentative foods (38). Production of traditional fermentative products is dependent on spontaneous fermentation by wild LAB. Manufacturing such products in industrial scale and under hygienic conditions necessitates formulating a new set of specifically chosen strains as a starter culture which can guarantee the quality, safety, and consistency of the product (32).

In order to use starter and non-starter cultures in fermentative products, their safety qualities should be evaluated. Indiscriminate use of antibiotics is leading to resistance, mutations in the genes of microorganisms or resistance gene transformation from other bacteria (5). Antibiotic resistance is a serious concern today due to the risk of gene transformation from LAB to human pathogenic bacteria. Numerous studies have been conducted on antibiotic sensitivity/resistance of pathogenic bacteria so far (12, 16, 26, 33). Cataloluk and Gogebakan (5) showed that manually produced cheese contained the highest number of antibiotic resistant lactobacilli.

Many researchers have proven that the LAB will lengthen the shelf life and improve the safety of the product by restriction the growth of unfavorable microorganisms (3, 29, 38). Some LAB produces metabolites (organic acids, H_2O_2, and bacteriocins) which act against bacteria including *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* species (14). LAB with ability to produce H_2O_2 restrict pathogens' growth in food and also can act as probiotic strains with beneficial health effects for consumers. Researchers have found a relationship between the presence of H_2O_2 producing Lactobacilli and decreased bacterial vaginosis, healing colitis and human immunodeficiency virus prevention (14, 36, 40).

Decarboxylation of amino acids histidine, tyrosine, ornithine, and lysine will lead to biogenic amine production including histamine, tyramine, putrescine, and cadaverine. Production of excessive amounts of such substances will stimulate secretion of adrenalin and noradrenalin, stimulate gastric acid secretion, cause

tachycardia and hypertension (10). In addition to all these, some people have histamine intolerance which causes diarrhea, headache, dizziness, cough, respiratory distress, hypotension, arrhythmia and heart rhythm disorders (17, 19, 21). The ability of biogenic amine production by LAB need to be evaluated before they are used in foods so as to make it possible to evaluate freshness or spoilage of the product by measuring the concentration of these substances (19). Biogenic amine producing a capacity of *Lactobacillus* species is not considered as a legal criterion for the selection of species used as a starter or probiotic culture (7).

Siahmazgi cheese is an artisanal cheese produced from raw sheep and goat milk without addition of starter culture in the spring. The cheese ripens for a six month period in bags made of sheepskin called Khik and ripening is carried out by wild LAB normally found in raw milk, Khik or those naturally added to the product during handling in the production process. Partovi et al. (30) studied the microbial and chemical properties of Siahmazgi cheese for the first time and identified the majority of the LAB found using biochemical methods and 16s rDNA analysis. *Lactobacillus plantarum* was the major strain isolated from Siahmazgi cheese with 41.6 % occurrence among the total LAB (34). Technological properties of *L. plantarum* strains isolated from Siahmazgi cheese have also been evaluated (31).

The objective of the present study is to evaluate safety characteristics including antibiotic sensitivity/ resistance pattern, antimicrobial effect, H_2O_2 production, and biogenic amine production by *L. plantarum* strains isolated from Siahmazgi cheese.

Material and Methods

***Strains selection and preparation*:** Ten strains of *L. plantarum* were selected from the strains isolated from Siahmazgi cheese and were identified by means of biochemical tests and 16s rDNA analysis. The strains were lyophilized and stored in the Food Hygiene department of the Faculty of Veterinary Medicine of the University of Tehran (30).

***Biogenic amine production*:** Moeller Decarboxylase broth base (Merck, Darmstadt, Germany) was used to evaluate biogenic amine production by *L. plantarum* strains. An amount of 1 % amino acid (histidine, lysine, tyrosine and ornithine) was added to the medium and sterilized at 110 °C for 10 minutes. Bacterial culture (in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 30 °C for 48h) was used in order to inoculate the medium and the surface of the medium was covered with sterile liquid paraffin. Test tubes were then incubated at 30 °C for 1-4 days and evaluated daily. A control test tube was dedicated to each *L. plantaru*m strain containing every item except the amino acid (20).

***Antimicrobial activity*:** The antimicrobial activity of *L. plantarum* strains isolated from Siahmazgi cheese was evaluated using well diffusion method. Two reference strains (*Listeria monocytogenes* ATCC 7644 and *Salmonella enterica* subsp. *enterica* serotype Typhimurium (*S. typhimurium* hereafter) ATCC 14028) were used to check sensitivity to the antimicrobial substances produced by *L. plantarum* strains. Indicator strains were grown in Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) at 37 °C for 24h. These indicator strain cultures were added to sterile MRS agar (100 µl/L) and poured into petri-dishes. Then wells were made with a sterile pipette. *L. plantarum* strains were initially grown in MRS broth at 30 °C for 24h and they were adjusted to OD_{600} = 0.5 and then 50 µl of the cultures were added to the wells. The plates were held at 4 °C for 2h and incubated at 30 °C for 24h and were subsequently examined for zones of inhibition. Then the diameter of inhibition zones (mm) around the agar wells were measured. *Lactobacillus plantarum* strains were cultured in MRS broth at 30 °C for 24h and then centrifuged at 12000 g for 15 minutes to remove cells. The supernatant fluid was adjusted to pH=6.5 with sterilized 1 N NaOH and antimicrobial activity was checked by a well diffusion assay after excluding inhibition due to organic acids (8).

***H_2O_2 production*:** In order to evaluate H_2O_2 production (mmol/L) by *L. plantarum* strains, 25 ml of MRS broth supernatant from each strain was transmitted to 150 ml flask. Twenty-five ml of newly made sulfuric acid was then added to it and tittered using 0.1 N potassium permanganate ($KMnO_4$). Becoming colorless is the end point of titration (13).

***Antibiotic susceptibility*:** Disc diffusion method was used to evaluate antibiotic susceptibility of *L. plantarum* strains (8). Müller-Hinton medium (Merck, Darmstadt, Germany) with a pH value between 7.2 and 7.4 was used. Inoculation dose of each strain was initially calculated at the wavelength of 600 nm and then a microbial suspension containing 1×10^8 cfu/ml of *L. plantarum* was prepared. Antibiotic susceptibility test was then followed by inoculating the bacteria on the medium and antibiotic discs were placed on the medium using a sterile forceps 15 min after inoculation. The plate was then incubated at 30 °C for 48h after which the diameter of growth inhibition area was measured in millimeters and the sensitivity of each strain against antibiotics was determined as sensitive, semi-sensitive, and resistant. The sensitivity of *L. plantarum* strains against 11 antibiotics chloramphenicol, gentamicin, vancomycin, ampicillin, penicillin, streptomycin, norfloxacin, erythromycin, rifampicin, tetracycline, and kanamycin was evaluated.

***Statistical analysis*:** Analysis of variance (ANOVA test) with the Bonferroni test was used to determine significant differences in H_2O_2 production between different *L. plantarum* strains. All results were expressed as Mean ± SD. Statistical analyses were performed using the SPSS version 22 software. $P< 0.05$ was considered as statistically significant.

Results

Amino acid decarboxylation activity of *L. plantarum* strains has been shown in Table 1. *L. plantarum* strains isolated from Siahmazgi cheese were unable to produce tyramine except for SD6 strain. All strains in this study were able to produce histamine. *L. plantarum* strains were unable to decarboxylate either lysine or ornithine.

As it is depicted in Table 1, *L. plantarum* strains isolated from Siahmazgi cheese showed considerable antimicrobial activity against *S. typhimurium* and *L. monocytogenes*. All *L. plantarum* strains showed stronger antimicrobial activity against *S. typhimurium* (3.5-42.55 mm) than *L. monocytogenes* (1.5-30.49 mm). The SC9 strain had the strongest inhibitory effect against both pathogens with 49.5 and 55.5 mm of inhibition area for *L. monocytogenes* and *S. typhimurium* respectively. After tittering pH to approximately 6.5, no antimicrobial activity was noticed indicating that the antimicrobial activity of *L. plantarum* strains was contributed to their acid production and that these strains were unable to produce bacteriocin.

All *L. plantarum* strains were capable of producing H_2O_2 (Table 1). There are significant differences among *L. plantarum* strains in the present study regarding H_2O_2 production (P value=0.0001). SA32 and SD13 strains with 2.37 and 0.77 mmol/L were the strongest and the weakest strains regarding H_2O_2 production, respectively and were significantly different from other strains.

Antibiotic sensitivity profile of *L. plantarum* strains against 11 antibiotics is depicted in Table 2. All *L. plantarum* strains isolated from Siahmazgi cheese were sensitive to chloramphenicol, erythromycin, rifampicin, and tetracycline, yet resistant against vancomycin, and norfloxacin. Four patterns of antibiotic resistance were observed among *L. plantarum* strains (Table 3). Only two strains of SC9 and SE4 were resistant against four antibiotics which belong to vancomycin, streptomycin, norfloxacin, kanamycin and gentamicin, vancomycin, ampicillin, norfloxacin patterns, respectively and 50 % of *L. plantarum* strains were resistant against three antibiotics vancomycin, norfloxacin and penicillin.

Table 1. Decarboxylation activity, H_2O_2 production and antimicrobial activity of *L. plantarum* strains isolated from Siahmazgi cheese against *L. monocytogenes and S. typhimurium.*

Strains of *L.plantarum*	**Decarboxylation activity***				**Antimicrobial activity****		**H_2O_2 production (mmol/L)*** **
	Ornithine	**Tyrosine**	**Lysine**	**Histidine**	***L.monocytogenes***	***S.typhimurium***	
SC9	-	-	-	+	49.5	55.5	1.45±0.04^{af}
SD6	-	+	-	+	34.7	54.5	1.90±0.01^{b}
SA32	-	-	-	+	37.5	49.5	2.37±0.09^{c}
SC6	-	-	-	+	35.1	51.2	1.93±0.04^{bd}
SD5	-	-	-	+	35.3	55.2	1.86±0.04^{ab}
SC4	-	-	-	+	43.5	47.5	1.66±0.04^{bf}
SD13	-	-	-	+	32.6	45.2	0.77±0.04^{e}
SE4	-	-	-	+	31.1	42.3	1.27±0.20^{f}
SD11	-	-	-	+	30.1	51.2	1.76±0.09^{ab}
SD12	-	-	-	+	37.7	43.7	1.21±0.11^{f}
P-value							<0.001

*+ = positive; - = negative, **average diameter of growth inhibition zone of two replicates, ***Values are means ± SD of two replicates, ****The different superscripts a, b, c, d, e, f in the same column indicate significant differences (P< 0.05).

Table 2. Antibiotic sensitivity/resistance properties of *L. plantarum* strains isolated from Siahmazgi cheese.

Strains of *L.plantarum*	**Chloramphenicol**	**Gentamicin**	**Vancomycin**	**Ampicillin**	**Penicillin**	**Streptomycin**	**Norfloxacin**	**Erythromycin**	**Rifampicin**	**Kanamycin**	**Tetracycline**
SC9	+++*	+++	R	+++	+++	R	R	+++	+++	R	+++
SD6	+++	+++	R	+++	R	+	R	+++	+++	++	++
SA32	+++	+++	R	+++	R	+	R	+++	+++	++	+++
SC6	+++	+++	R	+++	R	+	R	+++	+++	+++	+++
SD5	+++	+++	R	+++	+++	+++	R	+++	+++	+++	+++
SC4	+++	+++	R	++	R	+++	R	+++	+++	+++	+++
SD13	+++	+++	R	+++	+++	+	R	+++	+++	++	+
SE4	+++	R	R	R	+++	+++	R	+++	+++	+++	+++
SD11	+++	+++	R	+++	+++	+	R	+++	+++	++	++
SD12	+++	+++	R	+++	R	+	R	+++	+++	+++	+++

*+++: 15-20 mm, ++: 10-14 mm, +: 1-9 mm, R: resistant.

Table 3. Multiple drug resistance (MDR) patterns in *L. plantarum* strains isolated from Siahmazgi cheese.

MDR patterns	Percentage of resistant isolates (%)
Van/Str/Nor/Kan	10
Van/Pen/Nor	50
Van/Nor	30
Gen/Van/Amp/Nor	10

Van: vancomycin, Str: streptomycin, Nor: norfloxacin, Kan: kanamycin, Pen: penicillin, Gen: gentamicin, Amp: ampicillin

Discussion and Conclusion

Similar to the results of the current study, Moreno-Arribas et al. (24) and Landete et al. (18) reported that none of the *L. plantarum* strains were capable of producing putrescine or tyramine. Halasz et al. (11) showed that 20 %, 40 % and none of *L. plantarum* strains isolated from dairy products were capable of producing tyramine, cadaverine, and putrescine, respectively. A number of researchers have noticed considerable concentrations of histamine in Goda cheese which is presumably produced by the Lactobacilli in rennet (19). *Lactobacillus acidophilus* isolated from yoghurt in Nigeria possessed characteristics exactly contrary to those found in the *L. plantarum* strains in the current study as was capable of lysine, ornithine, and tyrosine decarboxylation but unable to produce histamine (27). This diversity in results regarding biogenic amine production suggests that this quality is strain dependent, and also it is affected by other factors including nutrient availability, production process, type of milk used, ripening time, growth condition, and pH (10, 19). The inability to decarboxylate amino acids is a favorable quality in selecting strains as starter or adjunct cultures.

The SC9 strain had the strongest inhibitory effect against both pathogens. As it was stated in the previous study, SC9 strain was the strongest strain regarding acid production (31). The antimicrobial activity against *L. monocytogenes* is of paramount importance as this pathogen is ubiquitously found in the environment, and is resistant to refrigeration, acidity and high salt concentrations (29). A considerable number of listeriosis outbreaks worldwide have been contributed to raw milk and cheese consumption. The environment of ripened cheeses is suitable for the growth of *L. monocytogenes* because of lactate consumption by microorganisms and amine production and also increased pH. Therefore, *L. monocytogenes* is considered a concern in cheeses such as Siahmazgi that undergo ripening for six months (28). Fifty-eight percent of *L. plantarum* strains isolated from fermented sausages showed antimicrobial effects against *L. monocytogenes* with growth inhibition areas of 2-10 mm of diameter (29). Klinberg et al. (15) and Nieto-Lozano et al. (25) also have proven the antimicrobial activity of *L. plantarum* strains against *L. monocytogenes*. One of the most important pathways for *Salmonella* infection in human is consumption of raw milk or non-pasteurized dairy products (28). All *L. plantarum* strains isolated from traditional salted meat in Tunisia had antimicrobial activity against *Staphylococcus aureus* and *S. enterica* subsp. *arizonae* and most of them inhibited growth of *E. coli* and *Pseudomonas aeruginosa* (8). Contrary to the results of the present study, Nieto-Lozano et al. (25) showed that *L. plantarum* was ineffective against *Salmonella* species. The results of the current study verified those of Essid et al. (8) while are in contrast with those of Messi et al. (23) and Aymerich et al. (2) who have proven bacteriocin production by *L. plantarum* strains. Contrary to the results of the current study, Albano et al. (1) showed that *L. plantarum* strains are more effective against Gram positive than Gram negative bacteria, because bacteriocin producing strains are ineffective against Gram negative bacteria.

All *L. plantarum* strains isolated from Siahmazgi cheese were able to produce H_2O_2. SA32 strain with 2.37 mmol/L was the strongest regarding H_2O_2 production. Sakamoto and Komagata (35) showed that *L. delbrueckii* subsp. *delbrueckii* produced up to 4.9 mmol/L of H_2O_2. *L. acidophilus* strains isolated from dairy products produced 1.62 mmol/L of H_2O_2 (6).

A significant number of strains of *Lactobacillus salivarius*, *Lactobacillus casei*, *L. plantarum*, *Lactobacillus leichmannii*, and *L. acidophilus* possess a gene causing natural resistance against vancomycin (22). In a study conducted by Beyan et al. (4) all LAB isolated from traditionally fermented milk were sensitive to penicillin and erythromycin. Beyan et al. (4) reported that 98.2% of the LAB strains isolated from traditionally fermented milk were sensitive to tetracycline and 80.7% were resistant against norfloxacin which showed significant similarity with the results of the current study. *Lactobacillus plantarum* strains isolated from raw camel milk were sensitive to tetracycline, vancomycin, erythromycin, ampicillin, kanamycin and resistant against rifampicin (9). Vancomycin is prescribed in the treatment of severe infections caused by *Enterococcus* and *Staphylococcus* species. Some *Lactobacillus* species including *L. casei*, *Lactobacillus rhamnosus*, *Lactobacillus curvatus*, *L. plantarum*, *Lactobacillus coryniformis*, *L. brevis*, and *Lactobacillus fermentum* are naturally resistant against vancomycin (42). Drug resistance can be transferred to other pathogens by transformation and conjugative plasmids or

transposons in gastrointestinal tract (34, 37, 39). It may become dangerous if there is the probability of resistance gene against vancomycin being transferred to *Enterococcus* species. Furthermore, some *Enterococcus* species possess the resistance gene against other antibiotics which may cause serious consequences in *Enterococcus* infections (42). This is not a concern per se, because these bacteria are sensitive to other antibiotics and have been safely used for long periods. The majority of *L. plantarum* strains in the current study were sensitive to most antibiotics. Absence of antibiotic resistance implies the absence of naturally occurring antibiotic resistance genes and also indicates that such genes have not been acquired by the bacteria from the environment (4). Generally, lactic acid bacteria isolated from dairy products show less commonly found antibiotic resistance in comparison to bacteria isolated from clinical or environmental sources (41).

Lactobacillus plantarum strains naturally found in Siahmazgi cheese do not generally possess dangerous characteristics to be used in fermentative dairy products. Considering that technological properties and safety aspects of *L. plantarum* strains from Siahmazgi cheese have been identified, it is recommended to produce fermented dairy products with original organoleptic properties using these strains as starter or adjunct cultures.

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Conflict of Interest

The authors are declared that there is no conflict of interest.

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The effect of tamoxifen on IGF signaling pathway in the mouse ovary

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Abstract: Tamoxifen (TAM) is one of selective estrogen receptor modulators used in breast cancer treatment and prevention. The objective of this study was to determine whether or not insulin-like growth factor-I (IGF-1) and its receptor (IGF-1R), has any role in the effect mechanism of TAM on the ovary. Experimentally, animals were divided into three groups as control group (n= 20), low dose TAM treatment group (0.5 mg/mouse/day, n= 20) and high dose TAM treatment group (1.5 mg/mouse/day, n= 20). TAM was injected 0.5 and 1.5 mg/mouse/day for 5 days. Ovarian sections were used to examine the general structure by trichrome staining method and to determine IGF-1 and IGF-1R expressions by immunohistochemical staining method. After the experiment, the presence of atretic follicles and small cystic structures in the TAM-treated animals was determined. Also, antral follicles and the corpus luteum were much less in the high dose TAM group than in the control. TAM did not change the expression of IGF-1 in granulosa cells, but increased the expression of IGF-1R. In TAM groups, IGF-1 and IGF-1R expression were increased in oocytes of follicles and in interstitial cells depending on TAM doses. However, while IGF-1 expression was unchanged in the corpus luteum, decreased in treatment group. TAM generally stimulated IGF-1 and IGF-1R expression in a dose-dependent manner. The results suggest that IGF-1 signaling pathway is involved in the mechanism of action of TAM on the ovary. We may assert that it may be useful to use IGF-1 signaling pathway regulators to adjust the effects of TAM on the ovary.
Keywords: IGF-1, IGF-1R, mouse, ovary, reproduction, tamoxifen.

Fare ovaryumunda tamoxifenin IGF-1 sinyal yolağı üzerine etkisi

Özet: Tamoksifen (TAM), meme kanseri tedavisinde ve önlenmesinde kullanılan seçici östrojen reseptörü modülatörlerinden biridir. Sunulan çalışmanın amacı, ovaryum üzerine TAM'ın etki mekanizmasında, insülin benzeri büyüme faktörü-I (IGF-1) ve reseptörünün (IGF-1R) herhangi bir rolünün olup olmadığını belirlemektir. Deney hayvanları, kontrol grubu (n= 20), düşük doz TAM uygulanan grup (0.5 mg/fare/gün, n= 20) ve yüksek doz TAM uygulanan grup (1.5 mg/fare/gün, n= 20) olmak üzere üç gruba ayrıldı. TAM enjeksiyonu 0.5 ve 1.5 mg/fare/gün olmak üzere 5 gün boyunca uygulandı. Ovaryum kesitlerine, genel yapıyı incelemek için trikom boyama, IGF-1 ve IGF-1R'in ekspresyonlarını belirlemek için immunohistokimyasal yöntem uygulandı. Deney sonrasında, TAM uygulanan hayvanlarda atretik foliküller ve küçük kistik yapıların varlığı belirlendi. Bununla beraber, yüksek doz TAM grubunda, antral foliküller ve korpus luteum, kontrol grubuna göre daha azdı. Granüloza hücrelerinde TAM, IGF-1'in ekspresyonunu değiştirmezken, IGF-1R'nün ekspresyonunu arttırdı. TAM, folliküllerin oositlerinde ve doza bağlı olarak intersitisyel hücrelerde IGF-1 ve IGF-1R expresyonunu arttırdı. Bununla birlikte deney grubunun korpus luteumunda IGF-1 ekspresyonu değişmezken, IGF-1R ekpresyonu azaldı. Genel olarak TAM doza bağlı, IGF-1 ve IGF-1R ekspresyonlarını uyardı. Bu sonuçlar, ovaryum üzerine TAM'ın etki mekanizmasına IGF-1 sinyal yolağının dahil olduğunu göstermektedir. Tamoksifen'in ovaryum üzerindeki etkilerinin düzenlenmesinde, IGF-1 sinyal yolağı regülatörlerinin kullanılmasının yararlı olabileceğini düşünmekteyiz.
Anahtar sözcükler: Fare, IGF-1, IGF-1R, ovaryum, tamoxifen, üreme.

Introduction

Tamoxifen (TAM) is a nonsteroidal triphenylethyl compound belonging to the class of selective estrogen receptor modulators (SERMs) that bind to estrogen receptors (ERs) and exhibit estrogenic or antiestrogenic effects depending on the target tissue (17, 24). At present, TAM is one of the most widely used drugs in breast cancer treatment (13). IGF-1 and IGF-1R are important factors in cell proliferation, cell differentiation, follicular development and ovulation (4, 5, 44). IGF-1/IGF-1R acts as modulators of gonadotropins at the cellular level as well as stimulating granulosa and theca cell proliferation and differentiation (4). Also, IGFs is involved to the synthesis of both estradiol and progesterone (16). The ovary is a major site of hormone-regulated IGF-1 production in mammalians (37). TAM can modulate the IGF-1/IGF-1R

system in different tissues by making a direct regulatory effect on IGF-1 secretion (26, 29). Furthermore, TAM has been shown to have a radioprotective effect and to prevent follicle loss, through increasing the local IGF-1 level (18). Also, TAM administration increases gonadotropin stimulation in ovarian follicles and causes hyperestrogenic effect (31). On the other hand, TAM causes formation of persistent follicular cysts in the premenopausal women (7, 22, 32) and increase of IGF-1 expression in interstitial cells has been directly related to the polycystic ovary syndrome (30). It was reported that TAM administration in ovarian and ER positive breast cancer models prevents breast cancer but was not effective on experimental ovarian cancer (36).

Nevertheless, there was not a sufficient number of studies on the role of IGFs in the mechanism of action on ovaries of TAM. The aim of this study is to determine whether or not IGFI signaling pathway has any a role in the effect mechanism of TAM on the ovary.

Material and Methods

Experimental protocol of animals: Sixty mature (8 week old) female inbred BALB/C mice obtained from the Experimental Animals Breeding and Research Center, Uludag University, Turkey, were used throughout the experiments. The experimental animals were examined under controlled conditions with humidity of 60-70% and 12 hourlight / 12 hour dark cycle at a temperature of 20-24° C with 5 experimental animals per cage. Feed and water were provided *ad libitum* throughout the experimental period. The experimental protocols were approved by the Animal Care and Use Committee of the Uludag University and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (2012- 09/04).

Mice were randomly divided into 3 groups. The first treatment group (0.5 TAM group, n = 20) was injected subcutaneously (s.c.) 0.5 mg/mouse/day with TAM dissolved in 10% ethanol: 90% corn oil. The other treatment group (1.5 TAM group, n = 20) was injected (s.c.) 1.5 mg/mouse/day with dissolved TAM. Control group mice ($n = 20$) were injected (s.c.) daily with only 10% ethanol: 90% corn oil (vehicle). The dissolved TAM and vehicle were injected for 5 days in a total of 0.1 ml s.c. to the animals (23). TAM was freshly prepared everyday before injection. At the end of the treatment the animals were euthanized under inhalation anesthesia and abdomens were opened. The ovaries were fixed with 10% neutral buffered formalin. The ovaries were used routine histological procedures and embedded in paraffin and blocked. Five µm thick sections were obtained from the paraffin blocks and cuts were taken to lysine slides. After deparaffinization and rehydration, sections were stained with immunohistochemistry for IGF-1 and IGF-1R expression and with Crossman's modified trichrome stain (8) for ovary morphology.

Reagents: Antibodies against IGF-1 (G-17) (sc-1422) and IGF-1R (C-20) (sc-713) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody, ImmPRESS anti goat Ig Peroksidaz (MP-7405) and ImmPRESS anti rabbit Ig Peroksidaz (MP-7401) were purchased from Vector laboratories. DAB (3-3 Diaminobenzidin) (Steady DAB/Plus (ab103723).

Immunohistochemical analysis: The standard streptavidin biotin peroxidase complex technique was applied. For antigen retrieval, the boiling step was performed in a microwave oven of 750 W with sodium citrate buffer (1 M, pH 6.1) for 3 x 5 minutes. After the sections were washed with PBS, the sections blocked for 10 minutes for endogenous peroxidase activity. To reduce nonspecific antibody binding, horse serum was applied to sections for 20 min. After that sections were incubated overnight 4°C with anti IGF-1 primary antibody diluted 1:150 and anti IGF-1R primary antibody diluted 1:100 as recommended by manufacturer. Samples were then washed three times with PBS and incubated with ImmPRESS reagent for 30 min at room temperature. After washing again with PBS for 3 to 5 minutes, the tissues were washed with distilled water and incubated with 5 minutes DAB for imaging. Hematoxylin was used as counterstaining. Sections were cleared with xylol and covered with the entellan. Negative controls were incubated with the antibody diluent without using the primary antibody. The intensity and localization of IGF-1 and IGF-1R expressions were assessed by two independent observers with scoring system: 0, no immunoreaction; 1, weak immunoreaction; 2, moderate immunosuppression; 3, strong immunoreaction (10).

Follicle classification: Primordial follicle (PO) contains single-layered squamous pre-granulosa cells and at maximum one cubic granulosa cell. Primary follicle (PR) contains single-layer with two or more cubic granulosa cells. Secondary follicle (S) contains two or three-layer cubic granulosa cells. Pre-antral follicle (PA) contains more than three layers of granulosa cells, and antrum is not formed. Antral follicle (A) contains the multi-layered granulosa cells and the antrum is formed.

Statistical analysis: Among the experimental and control groups, IGF-1 and IGF-1R expressions were investigated by using the non-parametric Kruskal Wallis test. The Mann-Whitney U test was used to analyze statistical significance between two groups. Symbols were used for showing confidence level ($P< 0.5$, $P< 0.001$). Statistical analysis of the study was performed using SPSS 23.0 (Statistical Package for Social Sciences).

Results

***Ovarian histology*:** In the both 0.5 and 1.5 TAM groups, it was observed that the cortex and medulla distinction on the ovary was distinctive and the interstitial area was wide (Figure 1b, 1c). Especially antral follicles were less in treatment groups than control group (Figure 1a, 1b, 1c). Furthermore, it was determined that the corpus luteum was very few in the ovarium depending on doses of TAM. In addition to, especially the presence of cystic structures and atretic follicles were observed in the medulla region of the experimental groups (Figure 1d).

***IGF-1 expression*:** IGF-1 expression was observed in oocyte cytoplasm of all follicles, granulosa and ten cells, interstitial cells and corpus luteum (Figure 2). Immunohistochemical IGF-1 scores in the ovarian of groups are presented in Figure 3. In general, IGF-1 immunoreactions changed from weak to moderate in the oocytes cytoplasma of the all follicle stages. Statistical significance was determined in preantral follicle oocytes of all groups ($P< 0.001$; $P< 0.05$) (Figure 3a). In granulosa cells, IGF-1 expression was weak in all follicle stages of all groups (Figure 2). No statistical significance was found between groups (Figure 3b). IGF-1 expression in interstitial cells increased after high dose TAM administration ($P< 0.001$; $P< 0.05$) (Figure 2b, 2c, 2d, 3c). However, in theca cells were determined a weak expression in the all groups ($P> 0.05$) (Figure 2c). IGF-1 expression in corpus luteum changed from weak to moderate in the all groups ($P> 0.05$) (Figure 2b, 2c, 3c).

IGF-1R expression: IGF-1R expression was detected in the oocytes and granulosa cells of all follicles stages, intersititial cells, and corpus luteum (Figure 4a). Immunohistochemical IGF-1R scores in the ovarian of groups are presented in Figure 5. For oocytes cell staining, the strongest immunoreactions were observed in the treatment groups. IGF-1R immunreaction in the oocyte of the all follicles except for primordial and secondary follicles was significantly increased in the treatment compared to control group ($P< 0.05$) (Figure 5a).

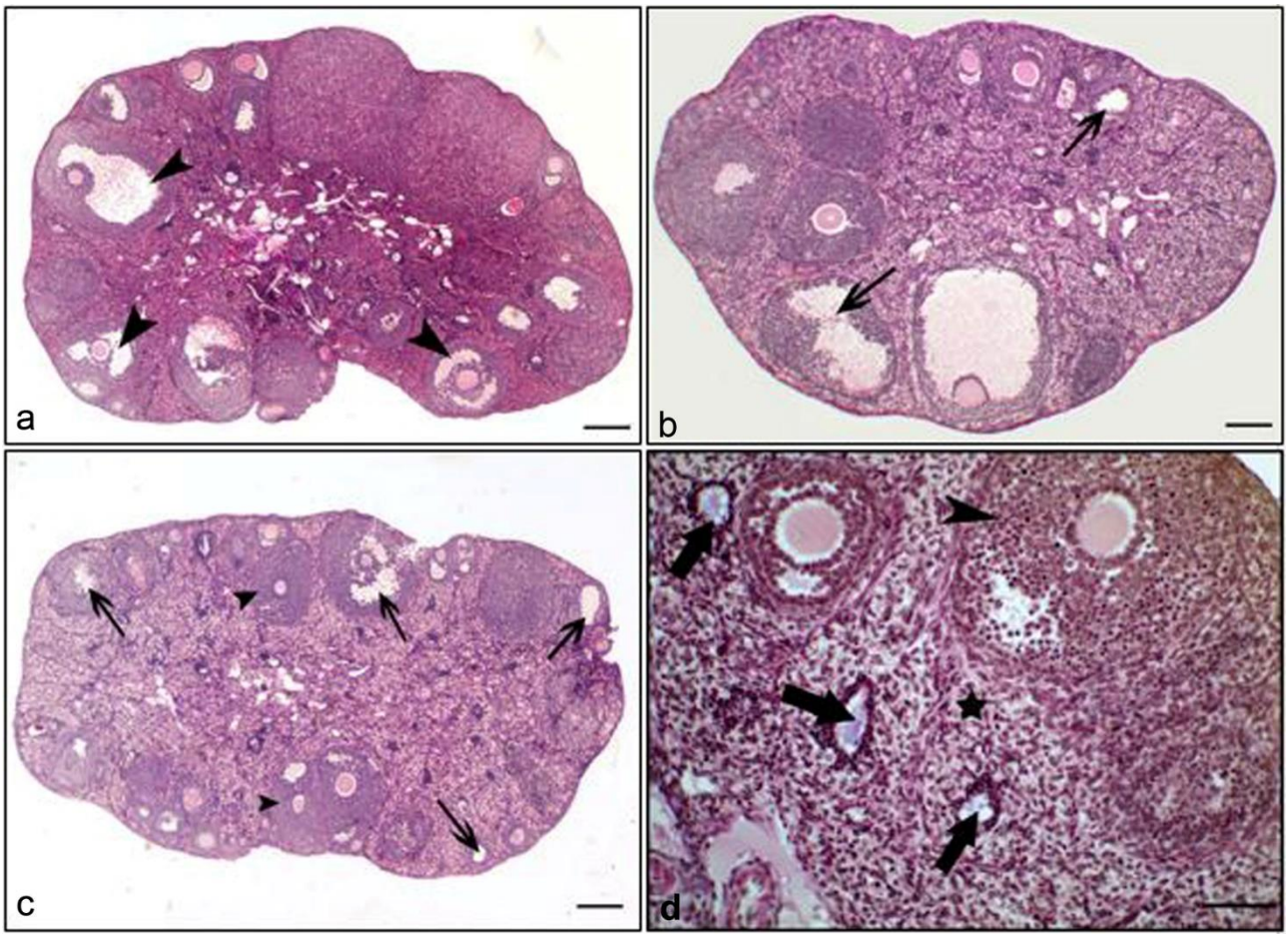

Figure 1. Ovarian histology in control and treatment groups. (a) Control group, (b) Low dose (0.5 TAM) treatment group, (c-d) High dose (1.5 TAM) treatment group. Healthy follicles (arrow head), cystic structures (thick arrows), atretic follicles (thin arrows), interstitial cells (star). Bar (a,b,c) 25 μm., (d) 50 μm.

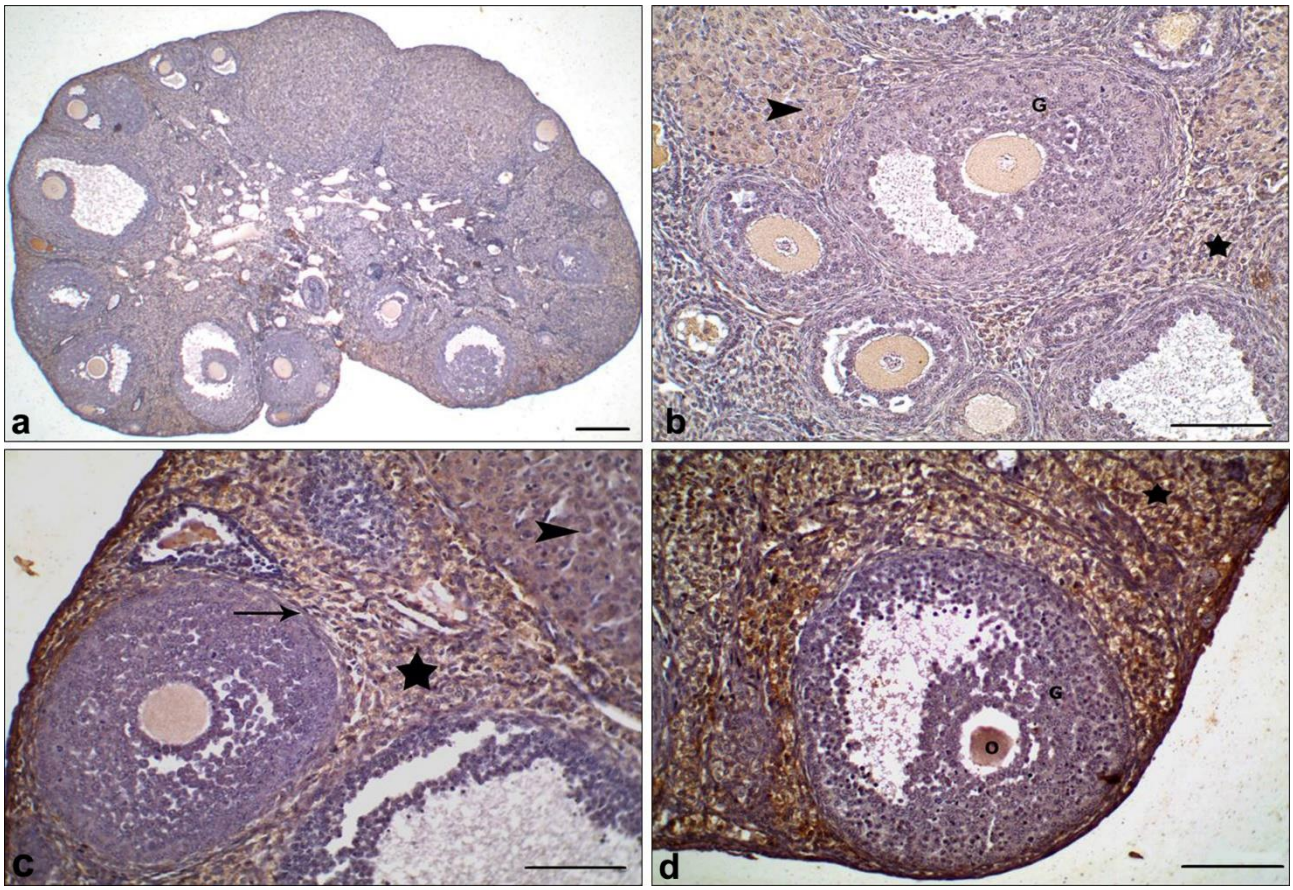

Figure 2. IGF-1 expression in ovaries of control and treatment groups; (a-b) Control group, (c) Low dose (0.5 TAM) treatment group, (d) High dose (1.5 TAM) treatment group. Oocyte (O), granulosa cell (G), corpus luteum (arrow heads), interstitial cells (stars), theca cells (arrow). Bar: (a) 25 µm., (b,c,d) 200 µm.

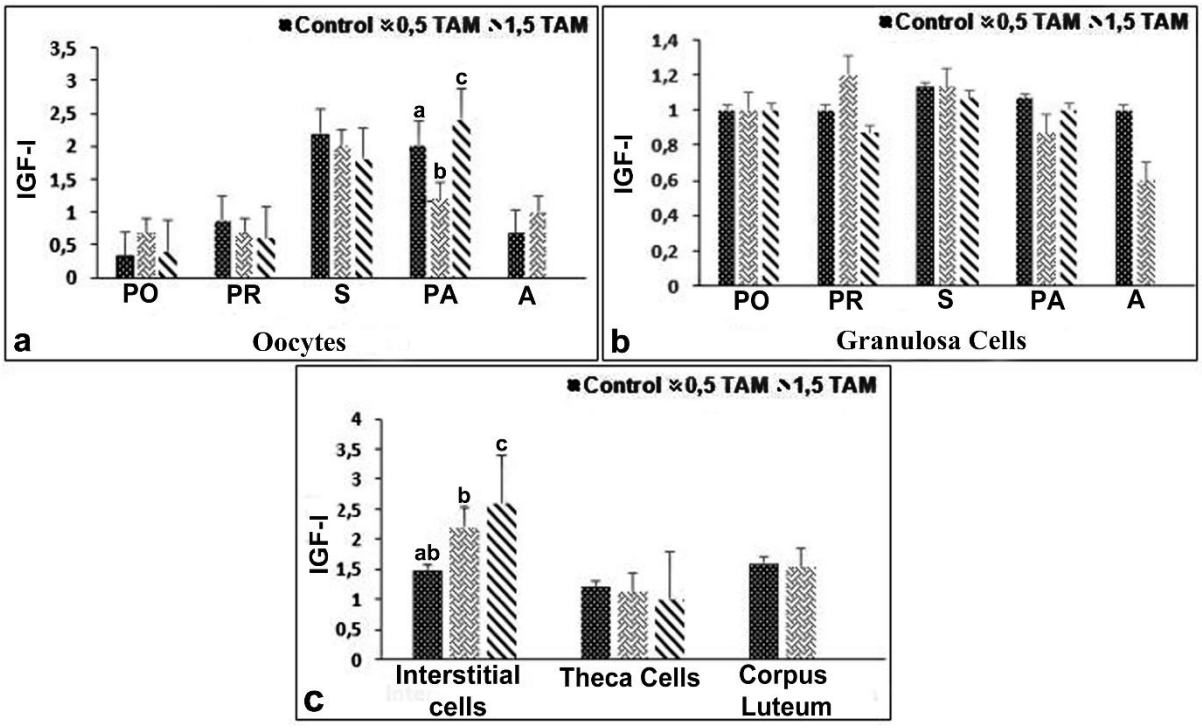

Figure 3. IGF-1 immunoreactivity in (a) oocytes, (b) granulosa cells, (c) interstitial cells, theca cells, and corpus luteum. Primordial follicle (PO), Primary follicle (PR), Secondary follicle (S), Pre-antral follicle (PA), Antral follicle (A). Different letters indicate statistically significant between groups.

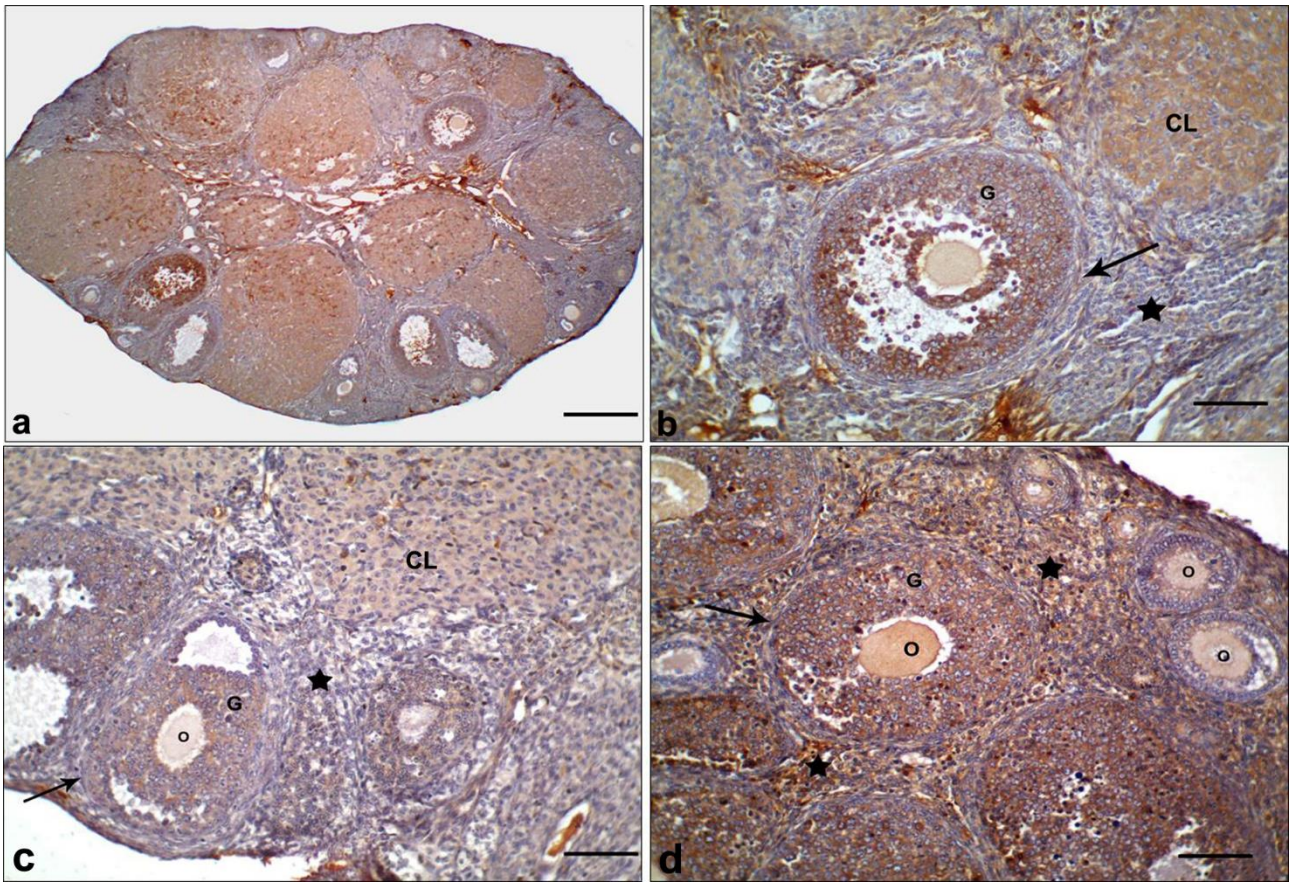

Figure 4. IGF-1R expression in ovaries of control and treatment groups; (a-b) Control group, (c) Low dose (0.5 TAM) treatment group, (d) High dose (1.5 TAM) treatment group. Oocyte (O), granulosa cell (G), corpus luteum (CL), interstitial cells (stars), theca cells (arrows). Bar: (a) 50 µm., (b,c,d) 100 µm.

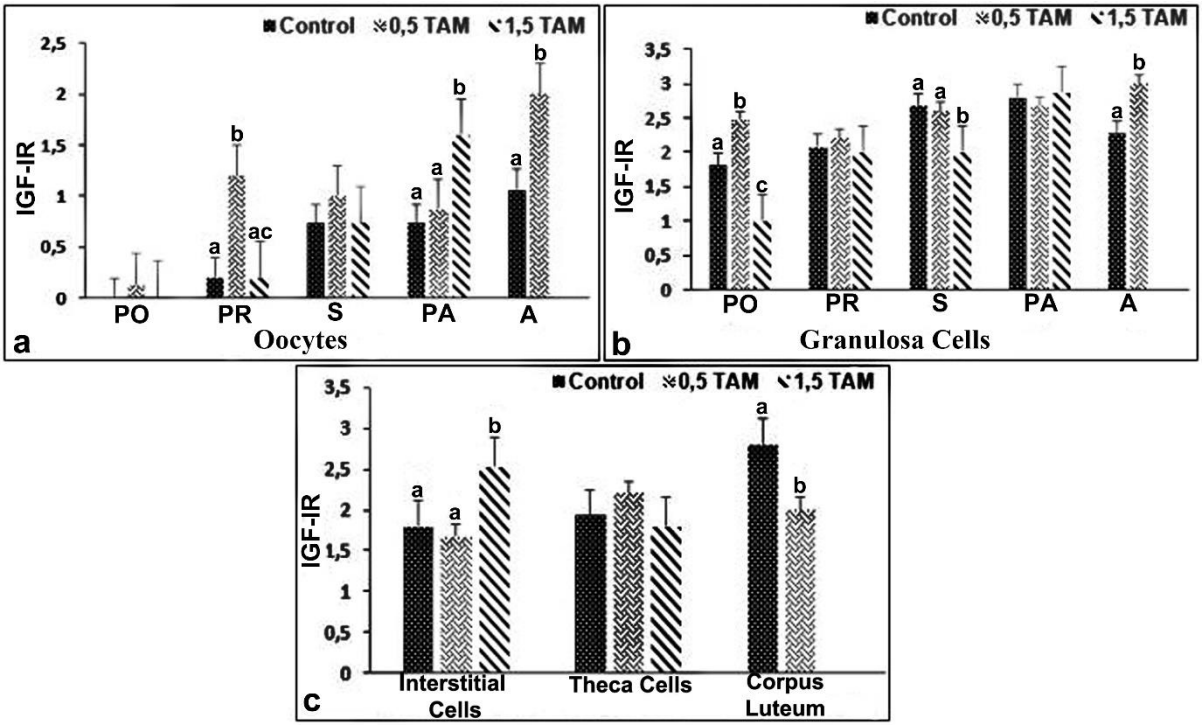

Figure 5. IGF-1R immunoreactivity (a) oocytes, (b) granulosa cells, (c) interstitial cells, theca cells, and corpus luteum. Primordial follicle (PO), Primary follicle (PR), Secondary follicle (S), Pre-antral follicle (PA), Antral follicle (A). Different letters indicate statistically significant between groups.

IGF-1R expression was increased moderate to strong depending on follicle development in granulosa cells. Statistical significance was determined in granulosa cells of primordial, secondary and antral follicles between experimental and control groups ($P< 0.001$; $P< 0.05$) (Figure 5b). The strongest immunoreactions were observed in granulosa cells of the antral follicles of the low dose treatment groups ($P< 0.001$) (Figure 5b). Due to there are not enough antral follicles in the high dose TAM groups, so it couldn't be evaluated. IGF-1R expression in both theca cells and interstitial cells was showed weak to moderate reaction in all groups. IGF-1R expression in intersititial cells was significantly increased in the high dose treated group compared to the other groups ($P< 0.05$) (Figure 4d, 5c). Expression of IGF-1R in corpus luteum showed moderate to strong reaction. IGF-1R immunoreactions in corpus luteum were significantly decreased in the treatment group compared to the control group ($P< 0.05$) (Figure 4b, 4c, 5c). There are not observed enough corpus luteum in the high dose TAM group, so it couldn't be evaluated.

Discussion and Conclusion

In the present study, we observed the presence of small cystic structures in the interstitial area in the TAM administered groups. However, cortex-medulla distinction was not apparent in the control group and all follicles were seen to be spread across the ovarian surface. Furthermore, we found that the corpus luteum and antral follicles were fewer in the TAM administered groups compared to the control group. Also the atretic follicles were abundant in treatment groups. In agreement with our study, Tsujioka et al. (40) reported that TAM induces large atretic follicles, increases interstitial space and decreases corpus luteum in female mice. Similarly, Akduman et al. (1) also found that after TAM administration, the numbers of corpus luteum and antral follicles decreased significantly compared to the control group and that the atretic follicles increased significantly but the follicle reserve was not affected. Also, Tucker et al. (41) reported that cystic structures were increased in female mice treated with 5 and 50 mg / kg TAM.

We also examined the protein expression of IGF-1 and IGF-1R in ovarian sections in order to determine whether the IGF signaling pathway could have a role in TAM's effect on the ovary. In the present study, expression of IGF-1 was detected in the oocyte cytoplasm, granulosa cells and theca cells of all follicles stages, interstitial cells and corpus luteum in control group. However, IGF- I immunoreactivity could not be assessed in antral follicle and corpus luteum in the high-dose TAM group, as these structures were not detected in this group, consistent with other studies (1, 40). In our study, weak IGF-1 immunoreactivity was observed in the granulosa cells of all groups, as in other studies (3, 21, 42, 33). In granulosa cells, IGF-1 expression decreased in the preantral and antral follicle of low dose TAM group, but no statistically significant were observed between the groups. Similar to our study, Akkaya et al. (2) reported that Metoxychloride, an endocrine disrupting pesticide, also reduced the expression of IGF-1 in granulosa cells of secondary follicles, preantral follicles and antral follicles. Mahran et al (18) have documented the molecular mechanisms of the radioprotective effect of TAM. When injected sc with either corn oil or TAM starting 3 days before irradiation and lasting 3 days after irradiation, TAM has been shown to have a radioprotective effect and to prevent follicle loss, due to its potentiation effect on IGF-1/IGF-1R-mediated antioxidant and cytoprotection (18). IGF-1 generally stimulates differentiation and proliferation of granulosa cells. (19, 20, 29) and prevents apoptosis and follicular atresia (12, 14). Therefore, according to our study, we hypothesize that decreasing IGF-1 in the granulosa cells may lead to atresia and suppress the growth of these follicles. We found that IGF-1 expression in intersititial cells was significantly increased in the high dose group compared to the other groups. Rzepczynska et al. (31) demonstrated that the increase of IGF-1 expression in interstitial cells is directly related to the polycystic ovary syndrome. We suggest that the increased IGF-1 expression in interstitial cells and presence of cystic structures in the experimental groups may be related to the polycystic ovary syndrome. We found no significant difference in IGF-1 expression in theca cells and corpus luteum, suggesting that TAM does not affect IGF-1 expression in these structures.

Also, expression of IGF- IR was detected in the oocyte cytoplasm, granulosa cells and theca cells of all follicles, interstitial cells and corpus luteum in control group. In our study IGF-1R expressions in oocytes are consistent with previous studies (44, 28). IGF-1R expression was detected in the granulosa cells of human and various animal species (15, 39, 44). We observed that IGF-1R immunoreactivity varied from weak to strong depending on follicle development in granulosa cells of all groups. In the present study, the strongest IGF-1R immunoreactions were observed in the antral follicles of the low dose TAM groups. These data suggest that TAM suppresses IGF-1 expression in the large follicles while increasing IGF-1R expression. Similar to our study, previous studies have reported an inverse correlation between IGF-1 and IGF-1R levels (9, 11, 36, 43). Our observations of IGF-1R expression are consistent with previous studies of corpus luteum (25), theca cells (34) and interstitial cells (44). The present study we observed that TAM causes an increase in the expression of both IGF-1 and IGF-1R in interstitial cells. Terada et al. (35) reported that TAM directly affects the ovary by increasing

the level of estradiol and showing estrogen agonist effect. Given that interstitial cells play an active role in estrogen synthesis (6), as reported by Terada et al. (35), we speculate that TAM may have a stimulatory function on estrogen synthesis by increasing IGF-1 and IGF-1R expressions. In the present study, IGF-1R immunoreactions in corpus luteum were significantly decreased in the treatment group compared to the control group. This decrease may be due to the hyperestrogenic effect of TAM.

As a result, we showed that TAM changes the IGF-1 and IGF-1R expressions in the ovary in a dose-dependent manner. TAM may stimulate IGFs in ovarian follicles and show an agonist effect on estrogen, leading to polycystic ovary syndrome in mice. Therefore, use of IGF-1 signaling pathway regulators may be considered to counteract the adverse effects of TAM on the ovary.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Oxidative status of colitis-associated cancer model induced by azoxymethane /dextran sulfate sodium and the effects of COX-2 inhibitor in mice

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Abstract: Natural products and anti-inflammatory agents including cyclooxygenase-2 (COX-2) inhibitors which is a type of nonsteroidal anti-inflammatory drugs (NSAIDs) are highly considerable interest for the prevention of carcinogenesis. The objective of this study is to evaluate the oxidative status of colitis-associated cancer induced by azoxymethane (AOM)/dextran sulfate sodium (DSS), and the effects of COX-2 inhibitor in mice. Totally 40 mice were randomized and divided to four groups. All animals except control and Cox-2 inhibitor alone group received AOM/DSS to establish colitis-associated cancer model as reported elsewhere. COX-2 preferential inhibitor meloxicam was used to minimize side effects such as gastrointestinal hemorrhage. Meloxicam were used (5mg/kg, intraperitoneal) three times a week with meloxicam alone and AOM/DSS + meloxicam group. Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Malondialdehyde (MDA) and Advanced Oxidation Protein Products (AOPP) which all of them are oxidative stress markers were measured by spectrophotometrically. The combination treatment of Meloxicam and AOM/DSS significantly increased ($P< 0.05$) SOD activities in mice. GPx activities were found significantly increased ($P< 0.05$) in Meloxicam and AOM/DSS combinations or alone. There were no differences between the control and treatment groups of MDA levels. AOPP levels of Meloxicam and AOM/DSS combination group were found higher than the other groups. Meloxicam and /or AOM/DSS treatment not caused lipid peroxidations, but increased the antioxidant enzymes and Advanced Oxidation Protein Products levels.

Keywords: Azoxymethane, colon cancer, COX-2, dextran sulfate sodium, oxidative stress.

Farelerde azoksimetan /dekstran sülfat sodyum ile oluşturulan kolit ile ilişkili kanser modelinde oksidatif durum ve COX-2 inhibitörünün etkileri

Özet: Anti-inflamatuar ajanlar, selektif siklooksijenaz 2 inhibitörleri ve doğal moleküllerin karsinogenezin önlenmesinde kullanılması önemli araştırma ve ilgi alanını oluşturur. Bu çalışmanın amacı, farelerde azoksimetan (AOM)/dekstran sülfat sodyum (DSS) ile oluşturulan kolit ile ilişkili kanser modelinde oksidatif durum ve COX-2 inhibitörünün etkilerini araştırmaktır. Toplam 40 fare rastgele olarak dört gruba ayrıldı. Kontrol ve COX-2 inhibitörü alan gruplar hariç bütün gruplar daha önce bildirilen yönteme göre AOM/DSS uygulanarak kolit ile ilişkili kanser modeli oluşturuldu. Gastrointestinal hemoraji gibi yan etkileri minimize etmek için COX-2 seçici inhibitörü meloksikam kullanıldı. Meloksikam 5 mg/kg dozda haftada üç kez i.p. olarak uygulandı. Oksidatif stres belirteçleri süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx), malondialdehit (MDA) ve ileri oksidasyon protein ürünleri (AOPP) spektrofotometrik olarak ölçüldü. Meloksikam ve AOM/DSS birlikte uygulaması farelerde SOD aktivitesine önemli düzeyde yükseltmiştir ($P< 0.05$). GPx aktivitesi meloksikam ve AOM/DSS tek ve kombine kullanımında önemli düzeyde artırmıştır ($P< 0.05$). MDA düzeyinde konrol grubu ile diğer gruplar arasında fark bulunmamıştır. AOPP seviyesi ise, meloksikam ve AOM/DSS kombine kullanıldığı grupta diğer gruplara göre daha yüksek bulundu. Meloksikam ve/veya AOM/DSS uygulaması lipid peroksidasyonuna neden olmazken, antioksidan enzim ve AOPP değerlerini yükselttiği görüldü.

Anahtar sözcükler: Azoksimetan, COX-2, dekstran sülfat sodyum, kolon kanser, oksidatif stress.

Introduction

Previous studies have shown that the activation of Arachidonic acid (AA) pathway plays an important role in the pathophysiological processes of inflammation and tumorigenesis (25). Membrane-released AA is immediately oxidized into the relatively unstable metabolite Prostaglandin G2 (PGG2), which is subsequently reduced to Prostaglandin H2 (PGH2) and both steps are catalyzed by the cyclooxygenase (COXs) enzymes. COX-1 and COX-2 are two important isoforms, but COX-2 is the most abundant isoform involving in various pathophysiological processes. Many tissues release COX-2 that are highly inducible by pro-inflammatory and mitogenic stimuli, including cytokines and growth factors (38). COXs and prostaglandin (PG) endoperoxide H synthases are critical enzymes of AA cascade and non-steroidal anti-inflammatory drugs (NSAIDs) and they have important functions in the inhibition of the production of primary prostanoids by leading to no access for AA to the active site of the cyclooxygenases (26).

The studies on the prevention of carcinogenesis strategies for colorectal cancer (CRC) are robust. Anti-inflammatory agents including NSAIDs and selective COX-2 inhibitors have clinically been shown that they are promising and the most interesting candidates for the prevention of CRC. Additionally, some natural compounds have been reported as chemopreventive agent for CRC (1, 11, 20).

It is well-known that chronic inflammation is associated with the increased cancer risk in different diseases or disorders such as persistent hepatitis B, Helicobacter pylori infections, or an immunopathy which has the potential for the development of liver and gastrointestinal cancers. It has been reported that chronic inflammation leads to the development of malignancies worldwide at a ratio of 15%. Thus, the association between inflammation and cancer rises the question of whether anti-inflammatory drugs such as NSAIDs can be used as antineoplastic treatment (21). Several NSAIDs have been discovered and still in use to relief the symptoms occurring in acute pain and chronic inflammatory diseases. Meloxicam is a NSAID which acts on COX-2 to inhibit the inflammation. In vivo and in vitro studies indicate that ability of meloxicam on the inhibition of COX-2 is more than on COX-1. Clinical studies showed that, meloxicam has good tolerability and less side effects (9).

Recently, number of evidence has demonstrated that oxidants or reactive oxygen species (ROS) are involved in the development of chronic inflammation and cancer (27). Indeed, tumor promoters have the ability to stimulate immune cells to generate ROS. Following an inflammatory stimulus, the initiation of carcinogenetic processes mediated by ROS may be due to the direct inflammatory stimulus. In addition of this, ROS can directly activate specific signaling pathways and thus lead to tumor development via the activation of cellular proliferation and angiogenesis as well as metastasis (31).

The objective of this study is to evaluate oxidative status of colitis-associated cancer model induced by azoxymethane (AOM)/dextran sulfate sodium (DSS) and effects of a COX-2 inhibitor, meloxicam, in mice.

Material and Methods

Animal model: Six-week-old BALB/c female mice (n=40) were housed five per cage under an environment with controlled temperature (22 ± 1 °C), and 12 h light/dark cycle. Mice were randomly divided to four groups; control group, AOM/DSS group, Meloxicam group and AOM/DSS Meloxicam group (Figure 1). All animal care and experimental procedures were approved by Adnan Menderes University, Ethical committee (Protocol number 64583101/2013/025).

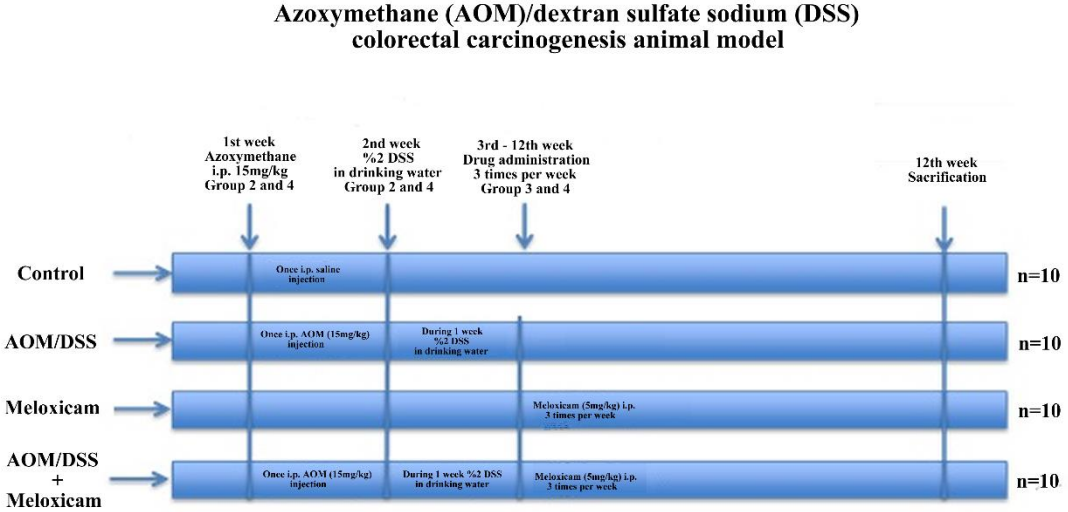

Figure 1. Experimental protocol for the colitis-associated colon carcinogenesis model.

Induction of colon carcinogenesis, treatment with Meloxicam: Mice were injected with AOM (15mg/kg, intraperitoneal) to induce colitis-associated colon carcinogenesis. After one week, mice were treated with 2% DSS (Sigma–Aldrich, USA) via drinking water for one week, followed by regular drinking water for recovery. Mice which were not treated with AOM/DSS, served as a negative control group. AOM/DSS group served as a positive control for colitis-associated colon carcinogenesis. Meloxicam and AOM/DSS Meloxicam groups were treated with Meloxicam at doses of 5mg/kg at 3 times per week for 8 consecutive weeks, starting two weeks after the AOM injection. The overall experimental schedule is depicted in Figure 1.

Measurement of oxidative stress parameters: Blood samples were collected after sacrification and stored at -80 °C until analyses. Plasma superoxide dismutase (SOD), malondialdehyde (MDA), advanced oxidation protein products (AOPP) levels and Glutathione peroxidase (GPx) activities were performed spectrophotometrically. SOD activities were determined using the SOD determination kit (Catalog no. 19160, Sigma-Aldrich, USA) according to the manufacturer's instructions. MDA levels was measured as a thiobarbituric acid (TBA) reactive substances with a spectrophotometer at 535 nm according to the method described by Yoshioka et al. (42). GPx activities were measured at 340 nm according to the procedure described by Paglia and Valentine (28). AOPP is a marker of oxidative modified proteins and consecutive inflammation and the method based on the reaction of AOPP with potassium iodide in an acidic medium. AOPP were measured at 340 nm and the values were expressed in µmol/mg of protein (40). Plasma protein levels were determined by commercially available colorimetric assay kit (ERBA) using autoanalyzer (ERBA XL 600, Meinheim, Germany).

Statistical analysis: Statistical comparison between Meloxicam alone, AOM/DSS, Meloxicam and AOM/DSS and control groups were performed using one-way ANOVA with post hoc Duncan test. $P < 0.05$ were considered statistically significant.

Results

The combination treatment of Meloxicam and AOM/DSS significantly increased ($P<0.05$) SOD activities (Table 1). GPx activities were found significantly increased ($P<0.05$) in mice treated with Meloxicam and AOM/DSS combinations or alone (Table 1). There were no differences between the control and treatment groups of MDA levels (Table 1). Advanced Oxidation Protein Products levels of Meloxicam and AOM/DSS combination group were found higher than the other groups ($P< 0.05$) (Table 1).

Discussion and Conclusion

In developed countries, CRC is one of the most commonly diagnosed and extensively studied gastrointestinal cancers and one of the most leading cause of cancer death even though risk factors and pathologic mechanisms of CRC are well-known. The goals of cancer treatment are to prevent and eventually reverse the whole carcinogenic processes by natural or synthetic agents. Despite modern medical technology in oncologic therapy, cancer still remains the most common malignancy over the last decade (16, 30).

Meloxicam, a NSAID, selectively inhibits COX-2. COX-2 inhibitors may have the anti-tumoral effects by causing reductions in prostanoid and free radical synthesis, as well as by exerting changes in arachidonic acid metabolism via alternate metabolic pathways. One of the most common result of ROS production is inflammation, which plays important roles in the development of cancer. Several studies showed that selective COX-2 inhibitors might have been antitumor activity (15, 39).

Table 1. Plasma glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA) and Advanced Oxidation Protein Product (AOPP) levels of control and study groups.

Groups	n	GPx (X±SD)	SOD (X±SD)	MDA (X±SD)	AOPP (X±SD)
Control	10	4.79±0.149^{a}	26.77±5.068^{a}	0.133±0.010^{a}	1.30±0.248^{a}
Meloxicam	10	6.74±0.986^{b}	36.86±5.927^{ab}	0.130±0.024^{a}	1.47±0.346^{a}
AOM/DSS	10	7.24±0.625^{b}	31.97±1.623^{ab}	0.108±0.022^{a}	1.15±0.140^{a}
AOM/DSS + Meloxicam	10	9.35±0.924^{b}	42.53±1.687^{b}	0.125±0.030^{a}	2.66±0.260^{b}

Data are presented as (Mean±SD). SD: Standard deviation. Statistical comparisons were performed using one-way ANOVA with post hoc Duncan test. $P < 0.05$ were considered statistically significant. (GPx : U/g of protein; SOD: U/g of protein; MDA: nmol/g of protein; AOPP: nmol/g of protein). Different letters in the same column are statistically significant.

Many pathways on the apoptotic effect of COX-2 inhibitors have been elucidated in colon carcinoma cells. Celecoxib, a COX-2 selective NSAID, partially induces apoptosis via a COX-2-dependent pathway. Maier et al. reported that celecoxib leads to an activation in caspases 3 and 9 together with cytochrome c release of colorectal carcinoma cells in humans (23). Sulindac and ibuprofen are other NSAID drugs. Studies showed that these drugs induce Bax and Bcl-XL expressions (44).

Dextran sulfate sodium (DSS) is a chemical that has direct toxic effects on the colonic epithelium and induces experimental colitis. The combination of DSS with AOM, which is a genotoxic agent, is a rapid, cheap and effective method for modeling CRC (34). According to Lee et al. the biological response to the AOM and DSS was different depending on the sex, showing more severely and earlier tumor incidence in male than female mice (22).

In the liver and colon, AOM is metabolized to a highly reactive methyl diazonium ion, eventually eliciting oxidative stress. During oxidative stress, the transcription of genes encoding antioxidant enzymes is activated and resulted in antioxidative responses characterized by the upregulation of antioxidative enzymes and a reduction in the sensitivity to the oxidative damage. Antioxidative enzymes have the beneficial effects in the attenuation of inflammatory damage and the neutralization of the ROS (32).

Antioxidant enzymes remove toxic intermediates that are generated when oxygen reduction is being occurred and thus they provide primary defense against the cytotoxic free oxygen species. As protective enzymes against lipid peroxidation in many tissues, beneficial roles of SOD, CAT and GPx are well-known. These enzymes are directly involved in the direct elimination of ROS which is one of the most powerful ways to prevent diseases and aging (7). Ashokkumar and Sudhandiran (2008) reported that SOD activity was lower in the mice treated by AOM compared to the control mice (3). In this study, we showed that treatment with Meloxicam increased SOD level in AOM/DSS induced colitis group.

Glutathione peroxidases, selenium-dependent enzymes, detoxify hydrogen peroxide and most of lipid hydroperoxides (36). Although more than twenty-five selenoproteins have been identified glutathione peroxidase (GPx), thioredoxin reductase (TrR), and selenoprotein P (sel P) play a role in antioxidative defense (18). A reduction in the activity of these antioxidant enzymes may result in an increase in oxidative stress and can cause damage to several biomolecules, preferentially DNA, which may further cause neoplastic transformation in the affected tissues (5, 13).

In vivo and in vitro studies have confirmed the anticarcinogenic effects of several selenoenzymes. Mice with impaired selenoprotein biosynthesis show more carcinogenesis in the prostate compared to the control mice (10). Overexpression of GPx-1 and selenium supplementation into the cultured carcinoma cells or normal fibroblasts attenuate induced by ultraviolet (UV) (4). GPx-4 overexpression inhibits the development of L929 fibrosarcoma cells and increases the sensitivity of angio-destructive treatment against to the B16-BL6 melanoma cells (14). Moreover, Thirupurasundari et al. (2009) demonstrated a reduction in the concentration of reduced glutathione (GSH) of rats with colon cancer induced by AOM compared to the control rats. Overall, GSH together with GPx and glutathione-S-transferase (GST) have important protective effects on cells in terms of cytotoxic and carcinogenic agents by scavenging ROS (35).

Little is known that there is an association between genetic differences in GPx and the development of neoplasia in colon as evidenced by increasing the risk of colorectal cancer in subjects with GPx-1 (P200L) polymorphism (12, 13).

In the intestine, SOD, GPx and catalase (CAT) are main antioxidant enzymes (45). To maintain homeostasis, cells can adapt to the changes in the oxidant and antioxidant species. Excessive generation of free radicals beyond the capacity of defense leads to failure of this homeostatic process (6).

Kochi et al were found significantly higher serum hydroperoxide levels which is a marker of oxidative stress, in the AOM/ DSS treated Kyoto Apc Delta (KAD) rats than in the control rats (19). For all that, KAD rats are susceptible to inflammation and have a significantly higher incidence, multiplicity and malignancy of colon tumors compared with their genetic control (41). Treated with AOM exhibited a reduction in the colon SOD activity and an increased in the colon MDA level, as reported by Tan et al (33). Pandurangan et al were observed an increased MDA levels of colon tissues in AOM/DSS induced mice. Enzymatic antioxidants, such as SOD, CAT and GPx were decreased in the colon tissues of AOM/DSS induced mice (29). Yu et al showed that ginseng effectively suppressed AOM/DSS induced proinflammatory cytokines activation (43).

Oxidative stress is related to the pathogenesis of colitis (24). Treatment with DSS decreased the activity of SOD and increased the MDA levels of colonic tissues (29).

In this study, female BALB/c mice were used and increased serum SOD and GPx activities were observed in AOM/ DSS-induced colitis. This may be because of the serum antioxidant responses occur in AOM/DSS induced colitis.

Previous studies showed that the difference in the mean plasma MDA levels between the group with ulcerative colitis and the controls was not significant (2,

37). In the present study no differences were observed between the control and treatment groups of MDA levels. As a result, it can be said that Meloxicam and /or AOM/DSS treatment not caused lipid peroxidation.

It has been reported that AOPP can be used as a more suitable marker of oxidative stress compared to lipid derivatives (8). AOPP has also been demonstrated as an inflammatory marker and mediator in many diseases. AOPP is an indicator showing oxidative protein damage and it leads to an activation in monocytes (40). In the present study, meloxicam, a specific COX-2 inhibitor, increased AOPP level in chemically induced colitis group compare to other groups.

Alagozlu et al (2) reported that the plasma AOPP levels were not different in the patients with active ulcerative colitis compared to the patients with inactive ulcerative colitis and the control subjects. Khan and Rampal (17) reported that oral administration of meloxicam produces oxidative stress in rabbits, as indicated by their elevated MDA levels and alteration of various antioxidant parameters. Meloxicam yielded a prooxidative effect, because of the prolonged duration of drug administration. However, several studies have reported the ameliorating effect of meloxicam on oxidative stress.

In conclusion, the oxidative stress was prevented by increased antioxidant activity in mice with AOM/DSS induced colitis model. Antioxidant response due to increased SOD and GPx levels were observed in mice with AOM/DSS induced colitis model. As a result, it can be said that the serum oxidant / antioxidant balance shows a change in favor of antioxidants in mice with AOM/DSS induced colitis model. However, it is necessary to work at the tissue level to define the oxidant / antioxidant balance in the pathogenesis of colitis.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Localization of plasminogen activator inhibitor type 1 and 2 in preimplantation mouse development *in vitro*

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Abstract: Plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2) are the major endogenous inhibitors of fibrinolysis, or thrombolysis, as it is effective in blocking the conversion of plasminogen to plasmin. In mammalian embryos, both PAI-1 and PAI-2 proteins are expressed in the trophoblasts during and after implantation, suggesting their critical roles in implantation and placentation during pregnancy. However, it remains unclear how both proteins localize in the early stage embryos before implantation. In this study, 2 cell stage embryos were flushed from the oviducts and cultured to specified stages in medium at 37 ℃ in a 5% CO_2 incubator. Embryos were fixed and double immunostained with anti-PAI-1 and anti- PAI-2 antibody. We determined the critical expression and localization patterns of PAI-1 and PAI-2 proteins in murine preimplantation embryos at 2 cell, 8 cell, morula and blastocyst stages by using confocal laser scanning microscope. We found that PAI-1 and PAI-2 constantly express in the embryos during preimplantation development, and these proteins localize in both the cytoplasm and the nucleus of each blastomere regardless of their developmental stage. Our results suggest that PAI-1 and PAI-2 proteins may play roles in early embryonic development before implantation.
Keywords: Embryo development, PAI-1, PAI-2, preimplantation.

Farelerde preimplantasyon embriyo gelişiminde plazminojen aktivatör inhibitör tip 1 ve 2'nin *in vitro* lokalizasyonu

Özet: Plazminojen aktivatör inhibitör tip 1 (PAI-1) ve tip 2 (PAI-2), plazminojenin plazmaya dönüşümünü bloke etmede etkili olduğundan fibrinolizis veya trombolizisin başlıca endojen inhibitörleridir. Memeli embriyosunda, hem PAI-1 hem de PAI-2 proteinlerinin implantasyon sırasında ve sonrasında trofoblastlarda eksprese ediliyor olması gebelikte bu proteinlerin implantasyon ve plasentasyonda kritik rolleri olduğunu düşündürmektedir. Bununla birlikte, her iki proteinin implantasyondan önce erken embriyo döneminde nasıl lokalize olduğu konusu hala açık değildir. Bu çalışmada, 2 hücre evrede bulunan embriyolar oviduktlar yıkanılarak çıkarıldı ve %5'lik CO_2 inkübatöründe 37 ℃ 'de belirtilen aşamalara göre kültüre edildi. Embriyolar tespit edildi ve anti-PAI-1 ve anti-PAI-2 antikoru kullanılarak ikili immunboyama yapıldı. PAI-1 ve PAI-2 proteinlerinin murin preimplantasyon dönemi embriyolarındaki kritik ekspresyon ve lokalizasyon paternleri 2 hücre, 8 hücre, morula ve blastosist aşamalarında konfokal lazer tarama mikroskobu kullanarak belirlenmiştir. PAI-1 ve PAI-2'nin preimplantasyon gelişimi sırasında embriyolarda sürekli eksprese ettiğini ve bu proteinlerin gelişim aşamalarına bakılmaksızın her bir blastomerin hem çekirdeğinde hem de sitoplazmasında lokalize olduğu gösterilmiştir. Bu sonuçlar, PAI-1 ve PAI-2 proteinlerinin implantasyon öncesinde erken embriyo gelişimde rol oynuyor olabileceğini göstermektedir.
Anahtar sözcükler: Embriyo gelişimi, PAI-1, PAI-2, preimplantasyon.

Introduction

Plasminogen activators (PAs) are serine proteases that catalyze the activation of plasmin, which is a factor to breakdown fibrin polymers during blood clotting (22). Two mammalian PA isoforms, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are the central components of the plasmin/plasminogen activator system, which plays a

major role in benign disorders such as deep vein thrombosis, myocardial infarction, atherosclerosis, and stroke (9). Plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) are known to be the major inhibitors of this system (7, 29). PAI-1 expression is regulated by a number of intrinsic factors (*e.g.*, cytokines and growth factors) and extrinsic factors (*e.g.*, cellular stress) (29). In the early stage embryos, PAI-1 is expressed in trophoblasts, cytotrophoblasts, trophectodermal cells (5, 24) endothelial cells and placental cells (30). PAI-2 is also present in placental trophoblasts and macrophages and keratinocyte (2, 8). During the second trimester of pregnancy in humans, the concentration of PAI-1 in mother's plasma is gradually increased and it reaches a peak at 32-40 weeks of pregnancy (14). The PAI-2 concentration is also increased during pregnancy and birth (2).

Accumulating evidence suggests that PAs are implicated in oocyte meiotic maturation (16), ovulation (21), fertilization (17, 34) and embryo implantation (31). In rat embryos, uPA and tPA are expressed throughout their preimplantation development. While uPA is localized in the cell cytoplasm, tPA is detected only on cell surface and in the perivitelline space (1). In light of these studies, we hypothesize that the expression of PAI-1 and PAI-2 may be also important to maintain the normal embryogenesis. However, the critical expression patterns of PAI-1 and 2 in mammalian preimplantation embryos remain, unclear. In this study for the first time we obtained critical information about the localization of PAI-1 and 2 proteins throughout the embryo development in vitro by using immunofluorescence confocal microscopy.

Material and Methods

Animals and collection of embryos: C57BL/6J (Jackson Laboratory, Bar Harbor, ME) female and male mice were used in this study. Females at 6-8 weeks old were mated with males at 12-16 weeks old. After the mating, females with the vaginal plug were indicated as Day 1 of pregnancy. On Day 2 of pregnancy, females were sacrificed to dissociate their oviducts. Two-cell stage embryos were flushed out from the oviducts in M2 medium (MR-015P, Sigma-Aldrich). These 2-cell embryos were cultured in 20 µl drops of KSOM-AA medium (MR-121, Millipore) overlaid with mineral oil at 37ºC in 5% CO_2 in air for up to Day 5 (26). The embryos at 2-cell (Day 2), 8-cell (Day 3), morula (Day 4) and blastocyst (Day 5) stages were subjected to immunoflorescent staining. At least three pregnant females were used to collect each stage embryos. All relevant experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Hawaii with a Protocol No. 11-1160-8.

Double immunofluorescent staining: The embryos at different stages were fixed for 15 min at room temperature. After fixation, the embryos were permeabilized for 15 min and blocked with 5% bovine serum albumin. Then the embryos were incubated in the primary antibodies overnight at 4°C The primary antibodies provided were rabbit polyclonal anti-PAI-1 (1:400 dilution; sc-8979, Santa Cruz) and goat polyclonal anti-PAI-2 (1:400 dilution; sc-6649, Santa Cruz). After the first antibodies treatment, the embryos were incubated with secondary antibodies for 2–3 h at room temperature. The secondary antibodies were conjugated with DyLight 488 (1:500; Thermo Fisher Scientific, Life Technologies), namely donkey anti-goat and Alexa Fluor 568 (1:500; Life Technologies), namely, goat anti-rabbit for 3 h at 25°C. Stained samples were mounted in ProLong Gold antifade reagent containing 4′,6′-diamidino-2-phenylindole (DAPI) (Life Technologies) on a slide (25). Negative and positive control staining on embryos were done during this study. The negative control groups underwent the same staining protocol as the positive control group with the absence of the primary antibody. Moreover, the specificity of the PAI-1 and 2 secondary antibody was determined that no PAI-1 and 2 staining was observed in negative control groups.

Microscopy and image analysis: Embryos were imaged using a Leica TCS SP5 confocal laser scanning microscope. For confocal microscopy, serial optical sections were imaged at 1-2 µm intervals under a 60x objective lens with oil.

Results

Localization of PAI-1 and 2 proteins during the mouse preimplantation development: To determine the expression patterns of PAI-1 and 2, proteins during preimplantation development, mouse embryos from 2-cell to blastocyst stages were double immunostained with anti-PAI-1 and 2 antibodies. In the 2-cell stage embryos (n= 25), both PAI-1 (red) and PAI-2 (green) proteins localized in the cytoplasm and the nucleus (Figure 1). In addition, polar bodies in the 2-cell embryos showed PAI-1 and 2, staining positive (Figure 1), suggesting that PAI-1 and 2 proteins are produced not only in the embryo after fertilization, but also in the female-specific oocytes. In the advanced embryos at the 8-cell (n= 10) and the morula (n= 25) stages, PAI-1 and 2, expressions were constantly observed (Figure 2). These proteins were entirely observed in the cytoplasm and the nucleus except a few nucleoli (Figure 2). In morula stage embryos (16-32 cells) strongly stained dotty structures were observed in each blastomeres regardless of their internal or external position (Figure 2).

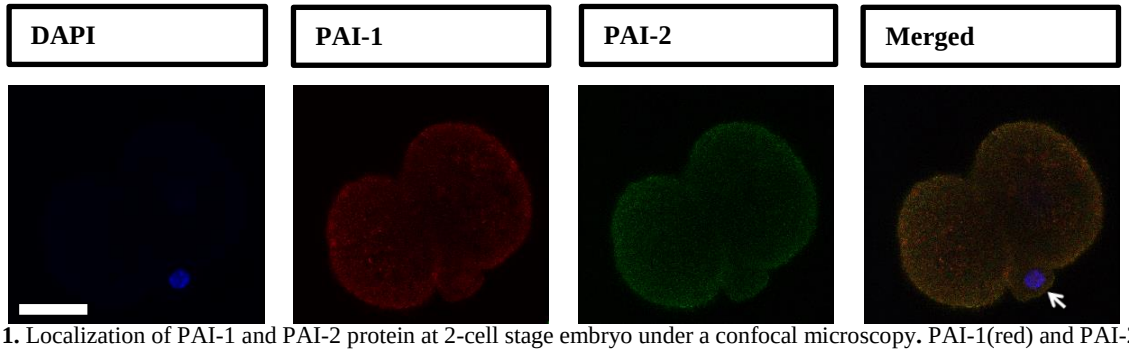

Figure 1. Localization of PAI-1 and PAI-2 protein at 2-cell stage embryo under a confocal microscopy**.** PAI-1(red) and PAI-2 (green) were localized in the polar body (arrow). Nuclei were stained with DAPI (blue). Scale bar represents 20 µm. DAPI: 4',6'- Diamidino-2-phenylindole, PAI-1: Plasminogen Activator Inhibitor Type 1, PAI-2: Plasminogen Activator Inhibitor Type 2.

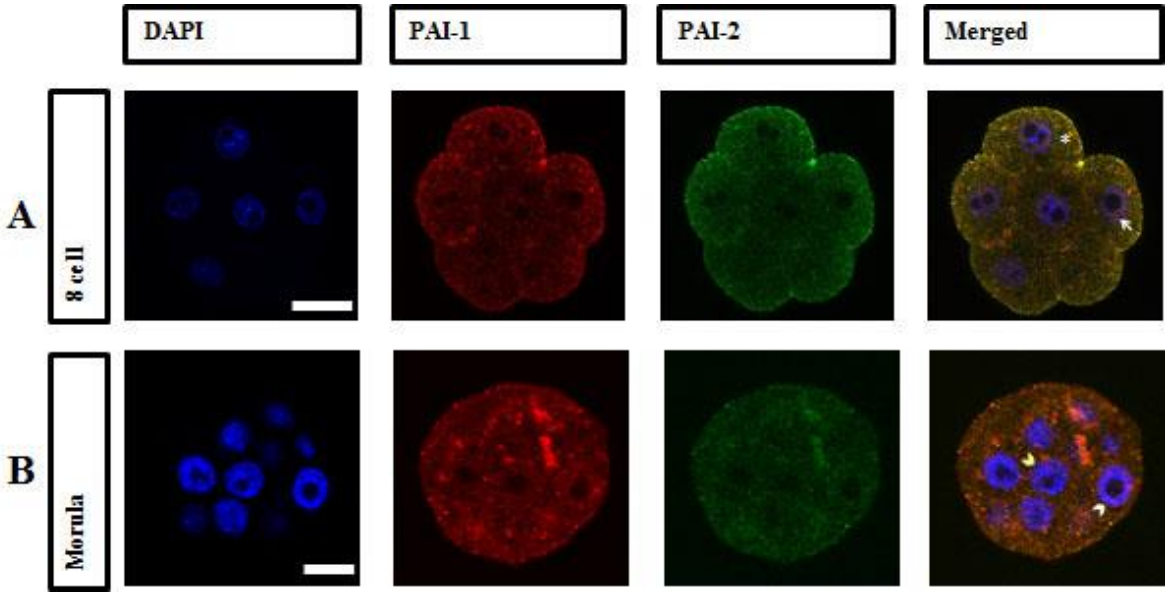

Figure 2. Localization of PAI-1 and PAI-2 in 8-cell and morula stage embryos. (A-B) PAI-1 and PAI-2 were present at the cytoplasm (asterisk) and nucleus (arrow). External (white arrowhead) and internal cells (yellow arrowhead) cytoplasm and nuclei were positively stained with PAI-1 and PAI-2. Confocal optical sections of embryos immunofluorescently stained with PAI-1 (red), PAI-2 (green) and nuclei stained with DAPI (blue). Scale bar represents 20 µm.

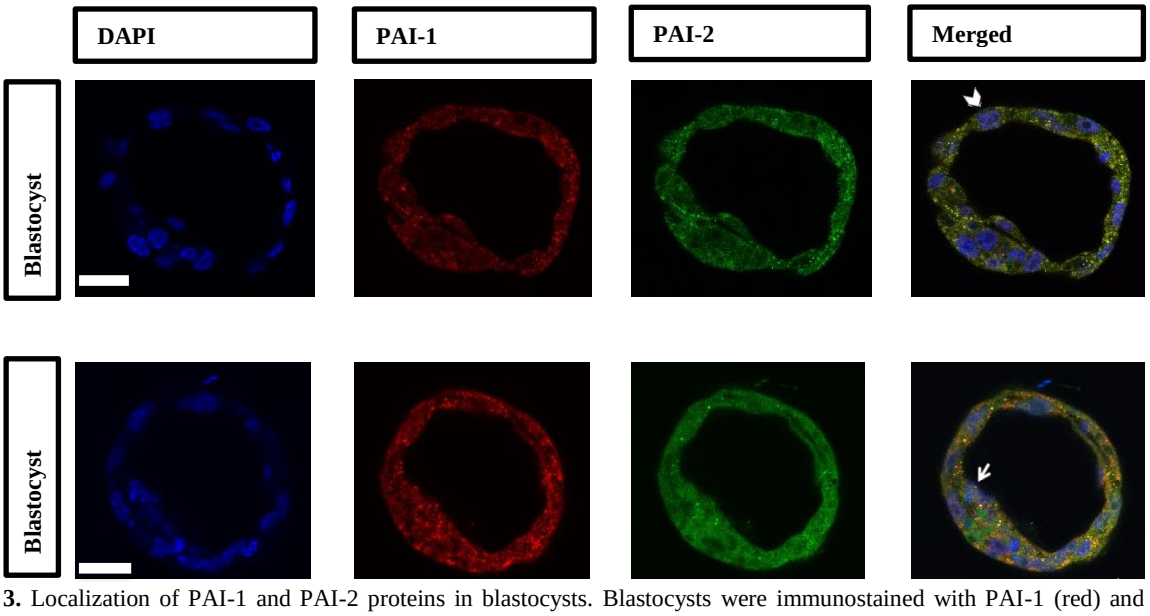

Figure 3. Localization of PAI-1 and PAI-2 proteins in blastocysts. Blastocysts were immunostained with PAI-1 (red) and PAI-2 (green). The nuclear and cytoplasmic staining on TE cells (arrowhead) and on ICM (arrow). Nuclei were stained with DAPI (blue). Scale bar represents 20 µm.

At the blastocyst stage, the embryos differentiate into two different cell lineages, trophectoderm (TE) and inner cell mass (ICM). After the double immunostaining of the blastocyst embryos, the blastomeres in TE and ICM were strongly stained with both PAI-1 and PAI-2 antibodies (Figure 3). Both proteins were localized in the cytoplasm and the nucleus (except a few nucleoli) in each blastomere at the blastocyst stage. In conclusion, PAI-1 and PAI-2 were detected in the early mouse embryos in all preimplantation stages, suggesting that PAI-1 and 2 proteins are constantly expressed in the early stage embryos before implantation in the mother's uterus.

Discussion and Conclusion

In the present study, we demonstrated that PAI-1 and 2 are constantly expressed and localized in both the cytoplasm and the nucleus in the mouse embryos throughout the preimplantation period. Our results indicate that PAI-1 and PAI-2 may contribute to normal preimplantation development essential for successful implantation and maintaining the pregnancy.

Previous studies reported that PAI-1 and 2 are expressed in human trophoblasts during and after implantation (10). Also, it was observed that both proteins are distributed in the cytoplasm of cytotrophoblasts and cytoplasm and plasma membrane of the syncytiotrophoblasts (15). In contrast, uPA and tPA expression in rat embryos has occurred during their early embryonic development, while uPA was distributed in the cell cytoplasm, tPA was appeared only on cell surface and in the perivitelline space (1). Because PAs are implicated in early stages of development and embryo implantation, we expected that the expression of PAI-1 and 2 (inhibitors of PAs) could be suppressed, or very limited during embryogenesis. However, our data indicate that both proteins are strongly and entirely expressed in the embryos at the 2-cell ~ the blastocyst stages, suggesting that PAI-1 and 2 proteins may not inhibit roles of PAs in oocyte meiotic maturation, ovulation, fertilization and embryo implantation. In fact, several studies showed that PAI-1 is a multi-functional protein that plays a role in physiological and pathological processes such as tissue remodeling, embryogenesis, regulating cell proliferation and migration, adhesion and tumor invasion, angiogenesis and metastasis (3, 19, 20, 28). Mashiko et al. (23) and Giacoia et al. (12, 13) suggested that PAI-1 inhibition promotes cell cycle arrest and apoptosis in ovarian and bladder cancer, and PAI-1 knockdown resulted in significant suppression of cell growth in cancer cells. The evidence suggests that PAI-1 may have some unique role associated with embryogenesis rather than inhibiting PAs.

It has been known that PAI-1 levels in the plasma gradually increased during normal pregnancy and reached maximum at 32-40 weeks of pregnancy, while PAI levels fall again 5-8 weeks after the birth in healthy pregnant women (14). PAI-1 may also play a role in remodeling maternal uterine spiral arteries (11). Hypoinvasion and unsuccessful placental vascular remodeling are related with intrauterine growth restriction, maternal and fetal death (18, 32). Depending on this information, previous studies demonstrated that any pathological disturbance in PAI-1 concentrations can cause pregnancy complications (27) and PAI-1 expression is increased in recurrent pregnancy losses, pre-eclampsia, intrauterine growth restriction and gestational diabetes mellitus (33). Intriguingly, Carmeliet et al. (6) indicated that PAI-1 deficient mice were viable and could generate morphologically normal offspring with normal litter sizes. PAI-2 exists in human plasma and its expression levels are increased during normal pregnancy, suggesting a possible requirement for PAI-2 during early development and/or placentation (4). However, Dougherty et al. (8) reported that PAI-2 is not required for normal murine development, survival and fertility. Our findings showed the localization of PAI-1 and 2, in cytoplasm and nucleus in all developmental stages, suggesting possible roles of these genes in preimplantation embryo development.

In this study our results indicated that, PAI-1 and PAI-2 are expressed in both cytoplasm and nucleus during all stages of preimplantation development in mice, suggesting that they may play roles in early stages of embryonic development. Especially expression of PAI-1 and PAI-2 in the trophectoderm supports the idea that these genes can be important for proper implantation and placentation. Further studies are required to fully understand the functions of PAI-1 and PAI-2 in embryogenesis.

Acknowledgements

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Determination and stability of some international screening limited drugs in equine blood by LC-MS/MS

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Abstract: Stability of some international screening limited therapeutic substances for doping control in equine blood has been investigated with a validated method by using LC-MS/MS after chemical hydrolysis with orthophosphoric acid. Whole blood samples were extracted with a WAX cartridge on auto-SPE. Stability test of the drug substances performed at the same time in working solution and matrix at +4°C, -20°C, +20°C in dark and +20°C in light conditions for 1, 2, 3 and 4 weeks. For short-time stability, the effects of 6 h storage at +55°C was also investigated. Repeated data were statistically analyzed with ANOVA. All substances in working solution were stable at -20°C and +4°C. In the blood matrix, higher temperatures (+20°C) in light and dark caused degradation of substances at the end of four weeks and short-term study ($P< 0.05$). In conclusion, these international screening limited substances in blood matrix could be unstable related to temperature and storage time, although in working solution they could be mostly stable in various temperatures for four weeks.

Keywords: Doping control, equine blood, LC-MS/MS, stability.

Uluslararası tarama limiti olan bazı ilaçların at kanında LC-MS/MS ile tayini ve stabilitesi

Özet: Doping kontrolü amacıyla uluslararası tarama limiti kapsamında olan bazı terapötik maddelerin at kanında valide bir metotla LC-MS/MS cihazı kullanılarak stabilitesi araştırılmıştır. Tam kan örneklerinde ortofosforik asitle kimyasal hidroliz sonrasında bir WAX kartuş kullanılarak otomatik katı faz ekstaksiyonuyla ekstrakte edilmiştir. Etken maddelerinin stabilite testi, çalışma çözeltisinde ve matriks içerisinde +4°C, -20°C, +20°C karanlıkta ve +20°C gün ışığında 1, 2, 3 ve 4 hafta için eşzamanlı olarak çalışılmıştır. Kısa zamanlı stabilite çalışması +55°C'de 6 saat saklamanın etkisini kapsayacak şekilde araştırılmıştır. Yapılan tekrarlı analizler ANOVA ile istatistiksel olarak değerlendirilmiştir. Çalışma çözeltisindeki tüm maddeler -20°C ve 4°C'de stabildi. Işıkta ve karanlıkta yüksek sıcaklık, dört hafta sonunda ve kısa süreli çalışmada kan matriksindeki maddelerin bozunmasına neden olmuştur ($P< 0.05$). Sonuç olarak, bu uluslararası tarama limitli maddeler sıcaklık ve zamana bağlı olarak kan matriksinde stabil olmayabilirken, çalışma çözeltisinde farklı sıcaklıklarda dört hafta boyunca çoğunlukla stabildir.

Anahtar sözcükler: At kanı, doping kontrol, LC-MS/MS, stabilite.

Introduction

Most of the doping laboratories that control the samples collected from racing horses are conducting the analysis of prohibited substances according to the International Agreement on Breeding, Racing and Wagering (IABRW) guidance document, which is determined and updated by International Federation of Horseracing Authorities (IFHA) (7). There is no limit level for prohibited substances, however, some endogenous substances in horse metabolism, feed or environmental contaminants have a threshold and therapeutic substances with international screening limits should be analyzed at their specified value as a requirement. The limits for substances are regulated based on therapeutic effects on equines health and prevent their misuse (26). Most of the samples taken from equines are urine and/or blood. When determining the limit values and substances, the type of the sample and drug metabolism are considered. The studied therapeutic substances and the international screening limits in blood are shown in Table 1.

Post-race samples are separated into two parts as called A and B (7). Sample A is transported as quickly as possible for doping control. Sample B stored under suitable conditions is analyzed if sample A is detected as positive. According to the current legislation, blood samples are not frozen and stored in the refrigerator (+4°C) unless separated to serum or plasma (7, 26). The time elapsed between the analysis of sample A and B; the storage conditions of sample B, the description of the effect of temperature changes during transport and consequently knowing the stability of the positive detected substance are critical for the proper interpretation results of the analysis (20). If sample A is identified as positive, sample B should be identifiable in the same manner. In biological fluids sample during storage and transportation, drug concentration could be decreased or increased related to temperature, pH, thermal or chemical degradation, hydrolysis, enzymatic metabolism, compound interference, microbiological or matrix effects (9, 11). Due to these effects, confirmatory analysis with sample B, reported as positive before, could be determined as negative. Especially for limited substances, the storage conditions and time are crucial for the quantitative drug presence in the sample to avoid false positive/negative results (9, 11, 24, 25). So, the stability knowledge of the substances in the sample gives vital information about reporting time.

In previous studies, short or long-term stability were conducted in urine (6, 22, 23, 24, 25), whole blood (1, 2, 5, 8, 12), plasma or serum (1, 2, 19) for doping control (10, 18, 19, 24, 25), trace element analysis (21) and toxicological researches (4, 5, 6). Nevertheless, there are few studies were focused on the threshold or limited doping agents except some anabolic steroids (10, 18), diuretics (24) and caffeine (25) in urine matrix. Beside of previous studies, we aimed to analyze the international screening limited Flunixin, Meloxicam, Dembrexine, N-Butylscopolammonium, Carprofen, Butorphanol, Lidocaine, Mepivacaine, Acepromazine, 7 deuterated internal standards (IS) with the validated method by LC-MS/MS and evaluate firstly their stability in diluted working standard and equine blood matrix at different temperatures and conditions in four week period. In addition to that, short-term stability of 6 h storage at +55°C was investigated as a simulation of hot-day storage effects.

Material and Methods

Chemicals and standard solutions: Flunixin, Flunixin d3 and Carprofen obtained from Sigma Aldrich (Schnelldorf, Germany); Meloxicam, Dembrexine, Butorphanol tartrate, Scopolamine d3 Hydrobromide trihydrate and Cortisol-9,11,12,12 d4 purchased from Chiron (Chiron AS, Trondheim Norway); Meloxicam d3, Carprofen-d3, 3-OH Lidokain-d5, 3-OH Mepivacaine-d3, Acepromazine, N-Butylscopolammonium provided from TRC (Toronto Research Chemicals, North York ON, Canada); Mepivacaine obtained from EDQM (Strasbourg, France); Lidocaine was purchased from LGC (Molsheim France). All standards purity was ≥ 98 %. Water was purified with an Elga-purelab flex water purification system (Elga-Veolia Water Solutions&Technologies, UK). n-Hexane was obtained from VWR Chemicals (VWR International Fontenay Sous Bois, France), Ethyl acetate and Methanol were obtained from J.T. Baker (Gliwice, Poland). Potassium dihydrogen phosphate and orthophosphoric acid, acetonitrile (ACN) and formic acid (FA) were purchased from Merck (Darmstadt, Germany). Acetic acid was obtained from Sigma Aldrich (Schnelldorf, Germany). All chemicals were of HPLC grade purity. The solid-phase extraction (SPE) cartridge Oasis Wax (3 cc Vac Cartridge, 60 mg Sorbent per Cartridge, 60 µm Particle Size, 100/pk**)** were obtained from Waters, USA. For sample preparation auto-pipette (Eppendorf Multipette Xstream), vortex mixer (Allsheng MTV-100), laboratory centrifuge (Thermo Scientific Heraeus Cryofuge 5500i), nitrogen evaporator (Biotage Turbo Vab LV), SPE system auto-SPE (Gilson Aspec 274) were used. For storage, Sanyo Medicool (MPR-414F) refrigerator and Sanyo Biomedical (MDF-U537D) freezer were used. Blood samples taken from race horses which were determined as negative in the doping control laboratory were used. Permission of the ethics committee was received for this study (Local Ethical Committee for Experimental Animals of Pendik Veterinary Control Institute 03/2019).

Preparation of solutions and instrument conditions: Primary stock standard solutions were prepared in methanol at a concentration of 1 mg mL^{-1} (Carprofen, Dembrexine, Flunixin, Acepromazine, Lidocaine, Mepivacaine, N-Butylscopolammonium, Butorphanol, Meloxicam d3 (IS), 3 Hydroxy Lidocaine d5 (IS), 3 Hydroxy Mepivacaine d3 (IS), Carprofen d3 (IS) and Scopolamine d3 Hydrobromide trihydrate (IS)), 100 µg mL^{-1} (Flunixin d3 and Cortisol d4) except 1 mg mL^{-1} Meloxicam in DMSO. The working standard mix solution was prepared by dilution of suitable aliquots of primary stock standard solutions and used to spike the blood samples. Cortisol d4 was used as an internal standard for Dembrexine, Acepromazine and Butorphanol. The working solution mix (10 mL) was contained 0.1 µg mL^{-1} Flunixin, 0.1 µg mL^{-1} Meloxicam, 0.1 µg mL^{-1} Carprofen, 0.002 µg mL^{-1} Acepromazine, 0.002 µg mL^{-1} Lidocaine, 0.5 µg mL^{-1} Dembrexine, 0.005 µg mL^{-1} Mepivacaine, 0.01 µg mL^{-1} N-Butylscopolammonium , 0.002 µg mL^{-1} Butorphanol. Internal standard mix solution (10 mL) was contained 5 µg mL^{-1} concentration of deuterated IS.

Table 1. International screening limits (ISL) of nine drugs recommended by IFHA's Advisory Council on Equine Prohibited Substances and Practices, March 2016 (7).

Substance	ISL (ng/mL in plasma)
Flunixin (FLU)	1
Meloxicam (MEL)	1
Dembrexine (DEM)	5
N-Butylscopolammonium (SCO)	0.05
Carprofen (CAR)	100
Butorphanol (BUT)	0.01
Lidocaine (LID)	0.05
Mepivacaine (MEP)	0.05
Acepromazine (ACE)	0.02

Table 2. Optimisation data of drug substances and internal standards in MRM mode.

Compound Name	Precursor ion	Fragmentor	Product ion	Collision energy	Polarity
Meloxicam	352.2	100	141	15	Positive
			115	16	
Flunixin	297.2	130	279	21	Positive
			264	33	
Carprofen	274.1	90	228	9	Positive
			193	31	
Acepromazine	327.3	150	254.1	20	Positive
			222.1	40	
			86.1	20	
Lidocaine	235.3	100	86.1	11	Positive
			58.1	33	
Dembrexin	378	100	262.7	25	Positive
			81	25	
Mepivacaine	247.3	110	98.1	16	Positive
			70.1	48	
N-Butylscopolammonium Bromide	360.2	150	194	16	Positive
			103	50	
Butorphanol	328.4	140	310.2	20	Positive
			157.1	46	
Cortisol D4	367.2	120	121	21	Positive
			97	41	
Flunixin D3	300.1	140	282.1	21	Positive
			264.1	35	
Scopolamine D3 Hyrobromid trihydrate	307.2	90	159.1	11	Positive
			141	27	
3-OH Mepivacaine-D3	266.2	110	101.1	17	Positive
			73.1	37	
3-OH Lidocaine-D5	256.2	100	91.2	17	Positive
			63.2	41	
Carprofen-d3	275	80	231	13	Negative
			228	41	
Meloxicam-d3	355.1	120	141	39	Positive
			115	9	

LC-MS/MS analyses were performed on an Agilent series 1200 liquid chromatography (Santa Clara, CA, USA) coupled to 6460 triple-quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source. The study was modified by Moulard et al. (14) LC MS/MS method. For separation, Sunfire C18 column (Waters, USA) with dimension 2.1x150 mm and particle size 3.5 µm was used. The gradient was applied with H_2O-0.1 % FA (phase A) and ACN-0.1 % FA (phase B). The initial conditions of the gradient kept until 5 min was 80 % A and 20 % B. The phase A was decreased to 50 % at 20 min, and at 25 min to 0 %. Such conditions were maintained until 27 min and the system was re-equilibrated with initial compositions of the mobile phase. The total run-time of the method was 31 min. The flow rate was 0.3 mL min^{-1}, the injection volume was 20 µL, and the column temperature was 35°C. The ESI interface conditions with multiple reaction monitoring (MRM) modes were; the gas temperature 325°C, gas flow 10 L min^{-1} (N_2), sheath gas temperature 350°C, sheath gas flow 12 L min^{-1}, capillary voltage 3.5 kV in negative mode and 4.5 kV in positive mode, nebulizer gas 50 psi and max. pressure limit was 600 bar. Diagnostic ions of drug substances were detected in MRM mode as listed in Table 2.

Sample preparation: The study was modified by Popot et al. (16) extraction method. 3 mL whole blood samples plasma into 15 mL polypropylene tube after centrifuge at 4400 rpm for 15 min 30 µL of IS mix, 100 µL orthophosphoric acid and 1.5 mL deionized water was added, mixed in the vortex for 10 min for hydrolysis. Then, 2.5 mL phosphate buffer (0.5 M, pH 6.5) was added and pH adjusted to 6 with 5 % HCl. The sample was mixed again and centrifuged (3500 rpm, 15 min). The tubes were placed into auto-SPE and preconditioned (2 mL deionized water, 2 mL methanol). The sample was loaded on the cartridge, washed with 3 mL deionized water/methanol (95/5) and dried for 2 min under vacuum then eluted from the cartridge with 3 mL ethyl acetate/methanol-2 % acetic acid (50/50) and evaporated to dryness (N_2, 45°C). The dry residue dissolved with 2x100 µL methanol, transferred into a glass vial and dried again. 50 µL mobile phase solution (80/20 H_2O-0.1 % FA/ACN-0.1 % FA) added and mixed for 3 min before injected to LC MS-MS.

***Stability studies*:** Stability design have been demonstrated according to 2002/657/EC Commission Decision (3, 15). For this purpose; 60 mL drug-free blood sample was collected and spiked with mix standard solution and IS mix solution on ISL levels and shaken for 10 min. After that 3 mL of blood sample was taken and analyzed as fresh solution data for matrix stability. For each storage period, 15 mL of blood sample divided into glass bottles and stored at +4±2°C (in the refrigerator), -20±2°C (deep freeze), +20±2°C in light and +20±2°C in dark at climate room and temperature controlled with a data logger. The same application was designed for the working solution mix stability with the separation of 750 µL of standard mix solutions to different storage conditions and fresh solution analyzed as initial data for stability test. The aliquots were tested with three replicates after 1, 2, 3 and 4 weeks' storage (estimated reporting time). Short-term stability was evaluated as the influence of 6 h storage at +55±2°C for simulating the transfer effects on hot days. 6 mL of drug-free whole blood spiked with 60 µL mix standard solution. 3 mL was kept in an incubator at +55±2°C for 6 h and the other 3 mL kept at +4°C. Then both of them were analyzed together (n= 3).

The stability test was designed according to 2002/657/EC, but there is no criterion for the assessment of stability in the guideline. Therefore, for the estimation of the instability of drugs while for the evaluation of degradation over a time period the ±15% precision acceptance criterion was used (20). In many other stability studies, the results were interpreted according to this criterion (9, 12, 13, 17, 23). The results outside the specified criteria are shown on the lines of the graphics. Analysis of Variances (ANOVA) was used for statistical evaluation of the effect of temperature, light-darkness and time of the storage by comparing the initial concentration and measured concentration at varying temperature using SPSS software package (SPSS version 21 for Windows).

Results

Results of the method for quantitation of some ISL drugs were shown in Figure 1 at the limit level spiked for each drug. The method was used in our laboratories routine analyses and validated according to 2002/657/EC (3). The validation summary for studied substances were shown in Table 3. In the results of validation data, CV % values were calculated as 1.5-7.2 % for precision and 1.6-19.7 % for with-in laboratory reproducibility (Table 2).

For estimation of the results, responses of substances were shown depend on temperature and time in Figure 2 and 3. Initial and end of four weeks concentrations were statistically compared in Table 4. Over four weeks, all substances in working solution were stable in conditions of +4°C and -20°C ($P> 0.05$). But N-Butylscopolammonium in the light condition of +20°C after 3rd week and Dembrexin in both of light and dark conditions of +20°C after 2nd week deviated out of the criterion of ±15% (Figure 2, $P< 0.05$).

In blood matrix, while all substances were stable at +4°C for four weeks (Figure 3, $P> 0.05$), N-Butylscopolammonium was significantly degraded at 4th week (Table 4, $P< 0.05$). There were significant differences in all substances at +20°C in both light and dark conditions when comparing initial data with 4th week data (Table 4, $P< 0.05$). In addition, N-

Butylscopolammonium, Acepromazine and Carprofen were significantly unstable at -20°C during four weeks (Figure 3, P< 0.05).

When blood samples were stored at +55°C during six hours for short-term stability, substances (except Flunixin and Carprofen) were degraded at an amount of up to 50 %. Though, Flunixin and Carprofen were increased at high temperature (Figure 4).

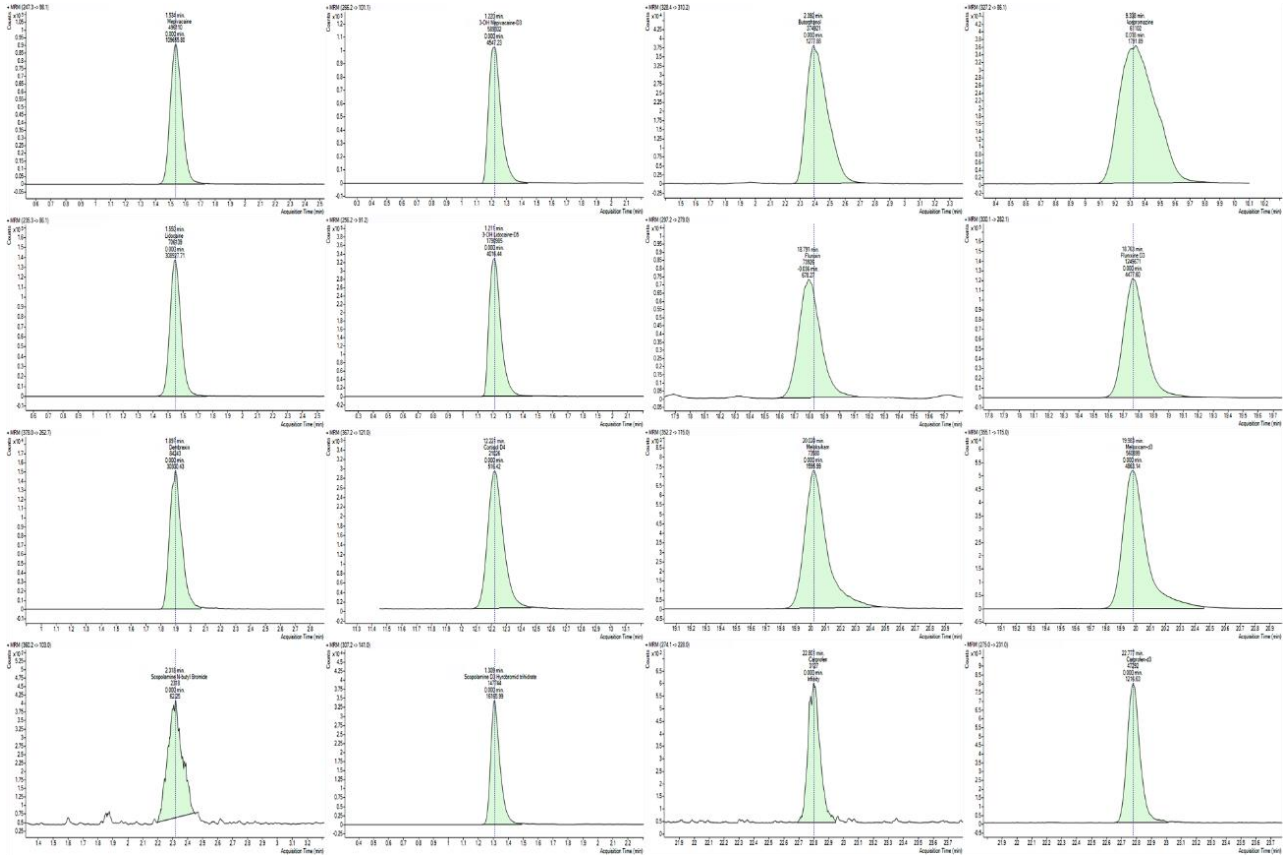

Figure 1. Chromatograms of spiked equine blood samples at ISL levels and internal standards.

Table 3. Results of method validation.

		Interday (n=6)		Intraday (n=18)			
Substances	**Conc. spiked (ng/mL)**	**Conc. measured (mean±SD)(ng/mL)**	**Precision CV (%)[a]**	**Conc. measured (mean±SD)(ng/mL)**	**Precision CV (%)[a]**	**Within lab. reproducibility CV (%)[b]**	**CCα (ng/mL) (α=1%)**	**CCβ (ng/mL) (β= 5%)**
MEP	0.05	0.0494 ± 0.0015	3.1235	0.0496 ± 0.0013	2.5585	3.0827	0.0522	0.0544
LID	0.05	0.0487 ± 0.0010	1.9965	0.0494 ± 0.0012	2.4186	2.4747	0.0520	0.0540
DEM	5.00	5.0397 ± 0.0799	1.5863	5.0243 ± 0.0841	1.6733	1.6530	5.1385	5.2769
SCO	0.05	0.0492 ± 0.0008	1.6536	0.0492 ± 0.0019	3.9028	4.7741	0.0537	0.0575
BUT	0.01	0.0108 ± 0.0006	5.4450	0.0103 ± 0.0007	7.1511	7.8057	0.0113	0.0126
ACE	0.02	0.0195 ± 0.0014	7.2780	0.0205 ± 0.0033	16.2990	19.7906	0.0250	0.0300
FLU	1.00	1.0272 ± 0.0375	3.6550	0.9970 ± 0.0404	4.0536	3.6661	1.0596	1.1192
MEL	1.00	0.9429 ± 0.0550	5.8288	0.9992 ± 0.0778	7.7836	5.5974	1.1140	1.2279
CAR	100.00	101.6593 ± 6.0112	5.9131	100.2595 ± 7.3812	7.3621	7.8057	113.9520	127.9039

[a] For ≥10-100 ng/mL CV(%) shall be not exceed 20. For mass fractions lower than 100 ng/mL the application of the Horwitz Equation gives unacceptable high values. Therefore. the CVs for concentrations lower than 100 ng/mL shall be as low as possible.
[b] For 100 ng/mL CV(%) shall be not exceed 23. For mass fractions lower than 100 ng/mL the application of the Horwitz Equation gives unacceptable high values. Therefore. the CVs for concentrations lower than 100 ng/mL shall be as low as possible.

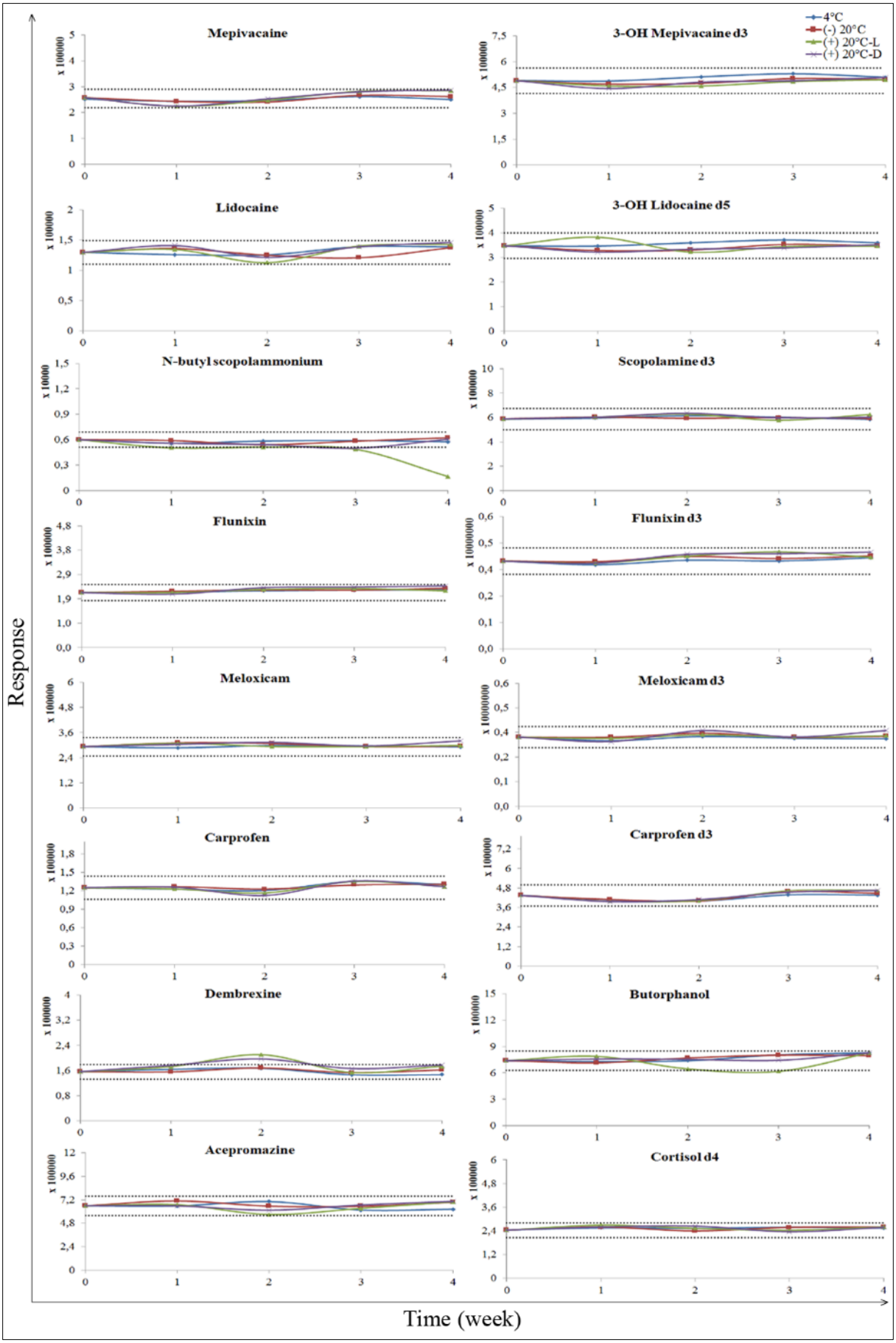

Figure 2. Stability results of working solution mix of nine ISL substances and their internal standards.

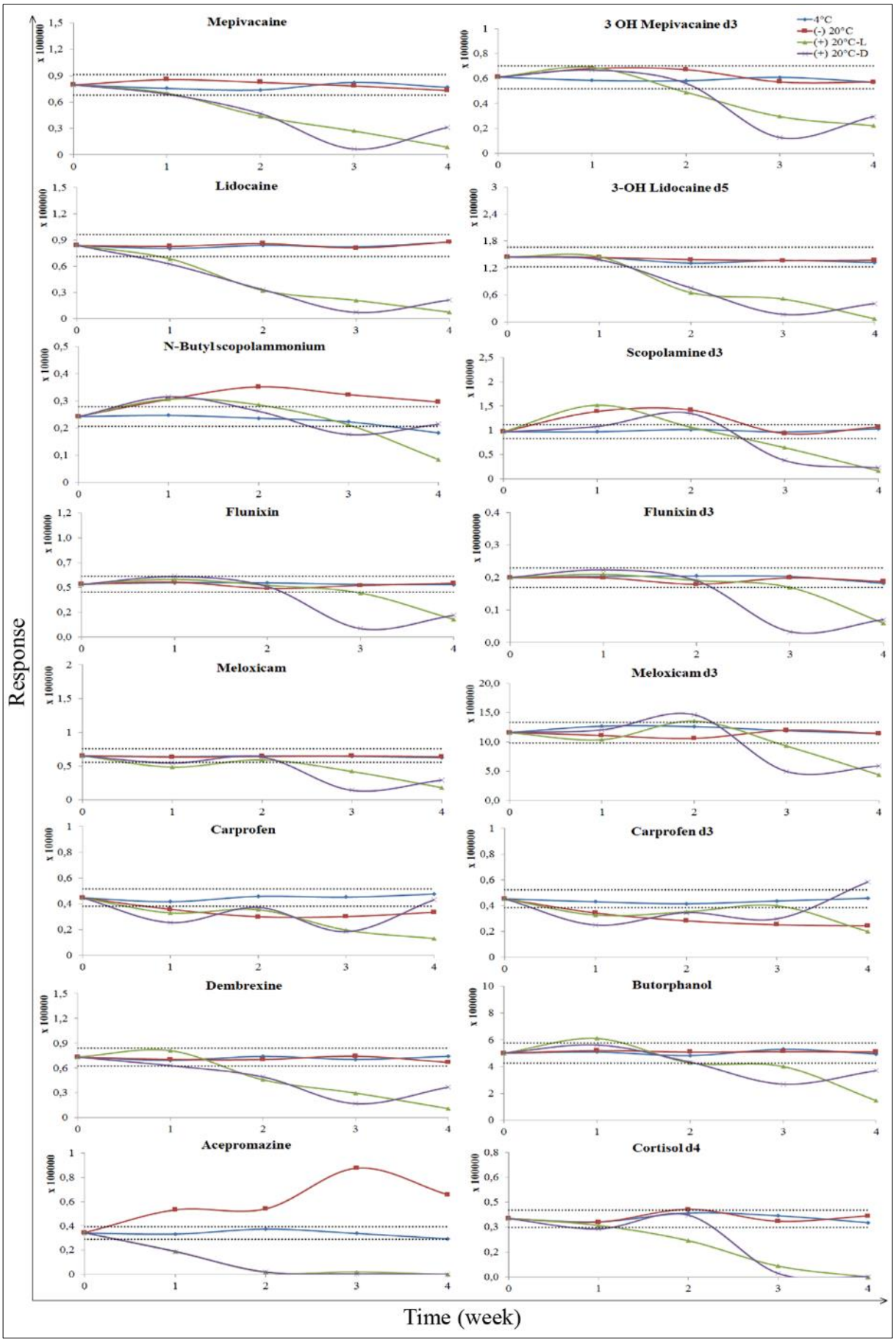

Figure 3. Nine ISL substances and their IS's stability results in blood matrix.

Table 4. The effects of different storage conditions on stability after four weeks.

	Matrix (mean±SD) (ng/mL)					Working Solution (mean±SD) (ng/mL)				
Substances	**Initial**	**+4°C**	**-20°C**	**+20°C-Light**	**+20°C-Dark**	**Initial**	**+4°C**	**-20°C**	**+20°C-Light**	**+20°C-Dark**
MEP	0057±0.008	0.062±0.001	0.063±0.002	0.029±0.001[a]	0.034±0.001[a]	0.050±0.001	0.051±0.001	0.051±0.001	0.052±0.001	0.052±0.001
LID	0.043±0.005	0.04±0.001	0.043±0.001	0.017±0.002[a]	0.024±0.001[a]	0.051±0.002	0.051±0.001	0.051±0.001	0.051±0.001	0.051±0.001
DEM	3.067±0.142	3.016±0.077	2.992±0.025	0.02±0.001[a]	0.05±0.001[a]	5.198±0.169	5.108±0.038	5.104±0.050	5.104±0.129[a]	5.222±0.069
SCO	0.066±0.006	0.047±0.003[a]	0.076±0.001[a]	0.035±0.004[a]	0.059±0.001[a]	0.049±0.001	0.051±0.001	0.050±0.001	0.035±0.001[a]	0.050±0.001
BUT	0.008±0.001	0.008±0.001	0.008±0.001	0.001±0.000[a]	0.005±0.000[a]	0.010±0.001	0.010±0.001	0.011±0.001	0.011±0.001	0.011±0.001
ACE	0.015±0.001	0.016±0.001	0.025±0.003[a]	0.008±0.005[a]	0.005±0.001[a]	0.019±0.001	0.020±0.001	0.021±0.001	0.020±0.001	0.020±0.001
FLU	0.991±0.010	0.99±±0.011	0.995±0.005	0.738±0.055[a]	0.005±±0.001[a]	1.018±0.055	1.022±0.021	1.031±0.016	1.032±0.009	1.050±0.023
MEL	1.008±0.069	1.015±0.009	1.015±0.013	0.615±0.046[a]	0.707±0.037[a]	0.978±0.012	1.001±0.017	1.001±0.012	1.007±0.021	1.036±0.021
CAR	107.430±12.180	98.592±3.215	72.473±1.874[a]	33.500±5.168[a]	85.746±1.686[a]	103.188±8.020	108.325±4.961	109.825±3.483	108.173±2.917	107.583±2.884

[a] Significant difference (P< 0.05) when compared to the initial data mean.

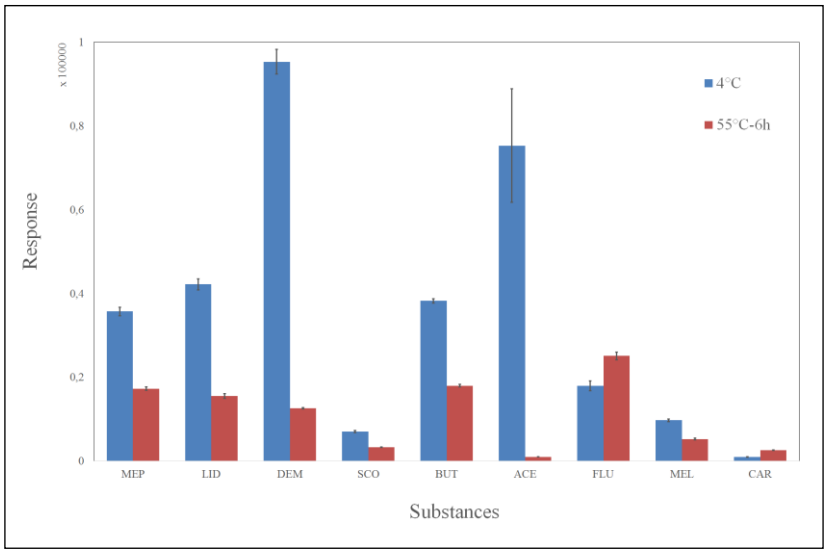

Figure 4. Short-term stability of ISL substances.

Discussion and Conclusion

The stability knowledge of standards used as references and working solutions at different storage conditions is requisite to check the analysis method and to compare the spiked sample with real sample. For this reason, the stability of both working solution and internal standards used in this study was examined and compared unlike previous studies. Results of this study found out that substances in working solution were stable and matrix effects were clearly occurred related to storage temperature and time. But, at 4th week N-Butylscopolammonium in working solution was degraded at +20°C in light. Van der Merwe et al. (22) similarly reported that substances can be affected by light.

In blood matrix stability study, reproduction of microorganisms has occurred in the samples kept in +20°C light and dark after 2nd week. The stability of the substances significantly degraded in +20°C conditions in a short time like other studies on urine and blood (19, 22, 24). It could be a result of thermal or microbiological degradation (11). Considering the short-term stability study, the samples have a negative effect if the samples are above +20°C, hence the results may be misleading.

There were deviations from the criterion value (±15%) at -20°C for Acepromazine, Carprofen and N-Butylscopolammonium. Acepromazine showed a negligible increase in concentration overtime during the storage at -20°C (Figure 3). This effect could be re-investigated. The materials used as internal standard are showed similar effects. These results demonstrated that blood samples should not be frozen as indicated on legislation (7). The previous study for 10-week stability in the blood, a decrease of Carprofen (30% at -20°C) and Meloxicam (70% at +20°C) occurred similar to our study, and it was stated that the time between the analyses of A and B samples was 4 weeks as a proposal (19). However, the instability of N-Butylscopolammonium after the 3rd week, some antipsychotic drugs after 1st week (17) and diuretics after 4th week (24) showed that the analysis time between sample A and B should be kept as short as possible because the substances were differently affected at storage conditions.

Short-term stability demonstrated the high temperature decreased all ISL substances except Flunixin and Carprofen (24). Schenk et al. (19) suggested that the temperature may increase some substances with protein participation. So, the increase of Flunixin and Carprofen might be related to thermo-hydrolysis of proteins in blood matrix.

The best of our knowledge, this is the first report about the stability of ISL substances based on limit value in whole equine blood related to different temperatures, time and light conditions. It was presented the effects of temperature and time on substances in matrix by the comparison of working solution and matrix stabilities. In accordance with results, samples might be stored and transported at +4°C in dark condition and immediately analyzed to avoid false negative/positive results. Future studies about the stability of doping agents may be increased for correct interpretation.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Antibacterial activity of partially purified enterocins from foodborne and clinical enterococci against some pathogenic bacteria

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Abstract: The purpose of the present research was to obtain enterocins from bacteriocinogenic enterococci (*Enterococcus faecalis* and *Enterococcus faecium*) in clinical and food sources, and to determine antibacterial activity of these enterocins against pathogenic bacteria including *Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Salmonella* Enteritidis. Enterocins were partially purified with ammonium sulfate precipitation from *E. faecium* and *E. faecalis.* After purification, the antimicrobial activity of enterocin was tested on Mueller Hinton Agar by disk diffusion assay. The 13, 8, 4 and 1 of 20 bacteriocins obtained by *Enterococcus* strain showed antimicrobial effect against *S.* Enteritidis, *B. cereus*, *E. coli* and *S. aureus*, respectively. One of food origin *Enterococcus* (*E. faecium*) exhibited the antimicrobial effect on all of the pathogen microorganisms used in our study. Enterocins from food and clinical isolates were very effective against *Salmonella* Enteritidis. The most active enterocins were produced by enterococci isolates from Hatay cow cheese due to their antibacterial spectrum on pathogenic bacteria used in this study. This study concluded the importance of investigating clinical enterococci besides foodborne enterococci to benefit from antibacterial properties.
Keywords: Antibacterial, bacteriocin, enterococci, enterocin.

Gıda ve klinik kaynaklı enterokoklardan kısmi saflaştırılmış enterosinlerin bazı patojen bakterilere karşı antibakteriyel aktivitesi

Özet: Bu çalışmanın amacı klinik ve gıda kaynaklı bakteriyosin aktif enterokoklardan (*Enterococcus faecalis* ve *Enterococcus faecium*) enterosin elde etmek ve bu enterosinlerin *Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Salmonella* Enteritidis gibi patojen bakterilere karşı antibakteriyel aktivitesini belirlemektir. Enterosinler, *E. faecium* ve *E. faecalis*'ten amonyum sülfat çökeltmesi ile kısmen saflaştırılmıştır. Saflaştırmadan sonra, enterosinlerin antimikrobiyel aktivitesi disk difüzyon yöntemine göre Mueller Hinton Agar üzerinde test edilmiştir. Enterokok suşları tarafından elde edilen 20 bakteriyosinin 13'ü, 8'i 4'ü ve 1'i sırasıyla, *S.* Enteritidis'e, *B. cereus*'a, *E. coli*'ye ve *S. aureus*'a karşı antimikrobiyel etki göstermiştir. Gıda kaynaklı enterokokların biri (*E. faecium*) çalışmamızda kullanılan patojen mikroorganizmaların hepsi üzerinde antimikrobiyel etki göstermiştir. Gıda ve klinik kaynaklı enterosinler *Salmonella* Enteritidis'e karşı oldukça etkili bulunmuştur. En aktif enterosinler, bu çalışmada kullanılan patojenik bakteriler üzerindeki antibakteriyel spektrumları nedeniyle Hatay inek peynirinden elde edilen enterokok izolatları tarafından üretilmiştir. Bu çalışma antibakteriyel özelliklerden yararlanmak için gıda kaynaklı enterokokların yanı sıra klinik kaynaklı enterokokların araştırılmasının önemini göstermiştir.
Anahtar sözcükler: Antibakteriyel, bakteriyosin, enterokok, enterosin.

Introduction

Nowadays, there is an increased interest to apply and investigate natural additives including antimicrobials and antioxidants in food and feed. Consumers prefer food products of high quality, prepared without artificial preservatives, safe and with long shelf-life. For this reason, researchers focused on bacteriocins known as microbial metabolites (18, 21, 30). Bacteriocins or bacteriocinogenic cultures seen as useful biocontrol agents in food preservation to ensure the microbial safety and decrease the risk of the growth of spoilage or pathogenic microorganisms such as *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Pseudomona*s spp., *Bacillus* spp., *Salmonella* spp., and *Clostridium* spp. (12, 17, 18).

The bacteriocins are small, cationic, amphiphilic, antimicrobial peptides which ribosomally synthesized by mostly lactic acid bacteria (16). *Enterococcus* spp. are

resistant to harsh or extreme conditions, such as high and low temperatures, extreme pH and salinity. These properties make it possible for bacteria and their bacteriocins to be used in any food products (6). Due to bacteriocinogenic activity of enterococci against food-borne pathogenic and spoilage bacteria, various researchers focused on novel enterocins (1). Bacteriocinogenic enterococci strains, mostly *E. faecalis* and *E. faecium*, were isolated from different sources including vegetables, fermented foods (cheese, sausages and other meat products), gastrointestinal system and various clinical specimens like urine, skin swab, pus, and blood (3, 6, 7, 18, 25).

In the previous studies, a majority of bacteriocin-producing enterococci have been obtained from food such as cheese, meat, fish, and vegetables (4, 17). While most of the papers on enterocins has related to bacteriocinogenic enterococci from food sources, less attention has been given to isolates from the clinical origins. As a matter of fact, studies concerning the use of enterocins from clinical origin are scarce compared with food sources. The isolation of novel bacteriocins will be beneficial for food and other related industries (15, 18).

The present study aimed partially purification of enterocins from bacteriocinogenic enterococci (*E. faecalis* and *E. faecium*) from clinical and food sources, and to investigate inhibition effect of these enterocins against *E. coli, S. aureus, B. cereus,* and *S.* Enteritidis.

Material and Methods

***Samples and bacterial strains*:** A total of 20 enterococcal isolates (10 of *E. faecium* and 10 of *E. faecalis*) from several sources (10 from clinical cases and 10 from foods) were collected from a stock culture in Food Microbiology Laboratory, Food Engineering Department, University of Çukurova. Isolates in stock were previously obtained from cheese, kasseri, sucuk, chicken meat (5 of *E. faecium* and 5 of *E. faecalis*) and stool or rectal specimens (5 of *E. faecium* and 5 of *E. faecalis*). Enterococci were grown in De Man, Rogosa and Sharpe broth (MRS broth; Merck, Darmstadt, Germany). Pathogenic bacteria including *Escherichia coli* O157:H7 ATCC-35150, *Bacillus cereus* isolate, *Salmonella* Enteritidis isolate and *Staphylococcus aureus* ATCC-25923 were used as indicator organisms. Pathogen bacteria were grown in Brain heart infusion (BHI) broth (Merck KGaA, Darmstadt-Germany) and stocked at -20°C in BHI supplemented with 20 % (v/v) glycerol (18).

Partially purification of enterocins from enterococcal isolates: Enterocins were partially purified from food and clinically isolates of *E. faecium* and *E. faecalis* according to modified method of Anandani and Khan (2); Savadago et al. (21); Javed et al. (13). The enterococcal isolates were incubated for 48 h at 37°C, in flask including 250 mL MRS broth. After incubation, this mix was centrifuged (10000 g at 4°C, 20 min) for separation of the cell-free culture supernatant (CFS). 10 N NaOH (Merck, CAS No.1310-73-2 pellets EMPLURA) to exclude the antimicrobial effect of organic acid was added to CFS with the adjustment of pH 6.5. Then, CFS was sterilized by using 0.45 µm membrane filter (Millipore, Carrigtwohill, Ireland). Ammonium sulphate (Merck Millipore) was slowly added to this sterile CFS suspension to reach 40 % saturation and this mixture was stirred overnight at 4°C. The centrifugation of this mixture (13000 g at 4°C, 45 min) ensured the harvesting of the surface pellicles and bottom pellets and thus, resuspension was performed in 10 mL of 10 mM sodium phosphate buffer (Merck-pH 7). The extraction procedure was performed at 4ºC for 1h by adding 15 volumes of a methanol–chloroform (Sigma-Aldrich) mixture (1:2, v/v) to one volume of the resuspended product. After the centrifugation of sample (15500 g, 4ºC, 30 min), cell-free supernatant and pellet were separated. The pellet was resuspended in 10 mL of ultrapure water (MilliQ; Millipore N.V., Brussels, Belgium). This partially purified enterocin extract was defined as a bacteriocinogenic sample and stored at +20°C. The presence of enterocins in extracts or bacteriocinogenic samples obtained from these enterococci strains was detected by antimicrobial activity test. Extracts causing inhibitory activity were evaluated as bacteriocinogenic positive (Bac +) otherwise bacteriocinogenic negative (Bac -).

The determination of antimicrobial activity of enterocins from different sources: After purification, the antimicrobial activity of enterocin was tested on Mueller Hinton Agar (MHA, Oxoid-UK) by disk diffusion assay against *Escherichia coli* O157:H7 ATCC-35150, *Bacillus cereus*, *Salmonella* Enteritidis and *Staphylococcus aureus* ATCC-25923 as target (indicator) strains with a bit modification of previous reports (14, 21). Disk diffusion assay was used for detection of antimicrobial activity from enterocins of enterococcal strains. Pathogenic indicator strain at a 10^6 CFU mL^{-1} concentration was spread on MHA and then paper disks were placed on these agar plates. Afterward, 100 µL portions of bacteriocinogenic samples were placed on these paper disks (thick, 6 mm, Oxoid-UK) and the plates were incubated at 37ºC, for 24 h. The detection of antimicrobial activity was carried out with the measurement of translucent halos in the bacterial lawn surrounding the disks. Diameters of inhibition zone around the disks were measured in millimeters. The observation of the inhibition zone has supported the presence of enterocins in partially purified extract from enterococcal strains.

Results

Antimicrobial activity of enterocins from foodborne enterococcal strains was represented in Table 1. 40 % of enterocins from *E. faecium* with food origin inhibited *B. cereus* and *E. coli*, whereas 60 % and 20 % of these had inhibition effect on *S.* Enteritidis and *S. aureus*, respectively. 60 % of enterocins from foodborne *E. faecalis* showed antibacterial activity against *B. cereus* and *S.* Enteritidis, however, none of them had inhibition effect on *E. coli* and *S. aureus.*

Inhibitory activity of enterocins from clinical enterococci was shown in Table 2. 60 % of enterocins from *E. faecium* with clinical sources inhibited *S.* Enteritidis and 20 % of these exhibited inhibition effects on *B. cereus* and *E. coli*, whereas none of them had antibacterial activity against *S. aureus*. 80, 40 and 20 % of enterocins from *E. faecalis* with clinical origin showed antibacterial activity against *S.* Enteritidis, *B. cereus* and *E. coli*, respectively, whereas none of them inhibited *S. aureus*.

As seen our results, enterocins from *E. faecium* in Hatay cow cheese showed antibacterial activity against studied all pathogenic bacteria. Enterocins from Erzincan Tulum cheese, kangal sucuk and homemade cheese were found as "Bac -". Enterocins from clinical isolates V146 and 225 did not display bacteriocinogenic effect on indicator microorganisms. Enterocins from food and clinical isolates mostly had an inhibition effect on *B. cereus* and *S.* Enteritidis. One of the food isolates and none of the clinical isolates exhibited antibacterial activity against *S. aureus*.

Discussion and Conclusion

Bacteriocins were produced from different microorganisms such as *Lactobacillus* sp., *Leuconostoc* sp., *Lactococcus* sp., *Pediococcus* sp., *Streptococcus* sp., *Enterococcus* spp. and different origin such as food, clinical substances, and environmental etc. (23). *E. faecalis* and *E. faecium* are the main species of enterococci, the most commonly found both in food and in clinical samples (3). On this sense, *E. faecalis* and *E. faecium* were selected as bacteriocinogenic isolates because previous researchers mostly reported *E. faecium* and *E. faecalis* as bacteriocin producer strains (5, 7, 16, 19, 25, 28, 29).

Table 1. Inhibition zone diameter from foodborne enterocins against pathogens (mm).

Code	Food samples	Source of enterocins	*B. cereus*	*S.* Enteritidis	*E. coli*	*S. aureus*
L13	*Hatay cow cheese*	*E. faecium*	6.00	9.50	6.50	9.00
P18	*Kasseri*	*E. faecium*	-	8.50	5.50	-
H8	*Erzincan Tulum cheese*	*E. faecium*	-	-	-	-
E5	*Antep cheese*	*E. faecium*	10.00	7.00	-	-
YS1	*Kangal sucuk*	*E. faecium*	-	-	-	-
JS1	*Chicken meat*	*E. faecalis*	7.00	10.00	-	-
NS1	*Homemade sucuk*	*E. faecalis*	-	7.00	-	-
A1	*Urfa cheese*	*E. faecalis*	13.00	12.00	-	-
LS1	*Chicken meat*	*E. faecalis*	11.00	-	-	-
AS1	*Homemade cheese*	*E. faecalis*	-	-	-	-

Table 2. Inhibition zone diameter from clinical enterocins against pathogens (mm).

Code	Source of enterocins	*B. cereus*	*S.* Enteritidis	*E. coli*	*S. aureus*
V150	*E. faecium*	-	9.00	-	-
V105	*E. faecium*	-	12.00	-	-
V98	*E. faecium*	-	-	8.00	-
V146	*E. faecium*	-	-	-	-
V198	*E. faecium*	7.00	9.00	-	-
225	*E. faecalis*	-	-	-	-
226	*E. faecalis*	10.00	14.00	-	-
227	*E. faecalis*	8.00	9.00	8.00	-
228	*E. faecalis*	-	11.00	-	-
V188	*E. faecalis*	-	11.00	-	-

In this study, diameters of inhibition zone caused by enterocins show their effectiveness of the antimicrobial activity. According to this, the presence and absence of antimicrobial activity of enterocins were evaluated as "Bac +" and "Bac -", respectively for the strains from which they were obtained. The present research clearly demonstrated the importance of enterocins from *E. faecium* and *E. faecalis* in both food and clinical sources with regard to their inhibitory activity against major foodborne pathogens including *E. coli* O157:H7, *B. cereus, S.* Enteritidis and *S. aureus*. Khalkhali and Mojgani (14) stated that enterocin-like substances produced by *E. faecalis* and *E. faecium* caused strong antibacterial activity (zone diameter ≥ 20 mm) against *E. coli, Salmonella typhi* and *S. aureus* but caused weak (zone diameter ≤ 15 mm) or no (absence of a zone of inhibition) antibacterial activity against *B. cereus.* Enterocins obtained from the present study have weak antibacterial activity. Similar to our data based on inhibition zone diameter, Savadago et al. (21) reported that bacteriocins produced by lactic acid bacteria gave zones of inhibition (between 9 and 10 mm) onto *B. cereus, E. coli,* and *S. aureus.*

Nowadays, there is a trend to detect novel enterocins from different enterococcal sources (16). Especially, the importance of enterocins from *E. faecium* and *E. faecalis* was emphasized as regards antibacterial spectrum (12, 22, 26). For example, Javed et al. (12) isolated and identified *E. faecium* and *E. faecalis* as bacteriocin producing enterococcal strains from indigenous fermented dairy products of Pakistan. Similarly, the present study detected that some of *E. faecium* and *E. faecalis* strains were Bac (+). Isleroglu et al. (11) confirmed that several bacteriocinogenic enterococcal strains isolated from various food products exhibited an antibacterial effect on foodborne pathogens and food spoilage bacteria. Franz et al. (8) found that approximately 3 % of lactic acid bacteria isolates were bacteriocinogenic activity against one or more of the indicator strains. Similarly, in our study, not all isolates had "Bac +", some of them were found as Bac (-). Both clinical and foodborne enterococci used in this paper may be candidate strains for practical use. However, there is a need for information in order to distinguish enterocins (16). Therefore, researchers should focus on the risk factors associated with virulence trait of bacteriocinogenic enterococcal strains and their technological properties. The presence of virulence traits in these enterococcal strains should be carefully monitored for safety parameters of their enterocins (14).

Previous researchers reported that majority of enterocins displayed antibacterial activity against Gram-positive bacteria as well as few effective against Gram-negative bacteria (1, 9, 20, 26). Isleroglu et al. (11) reported that enterocins had little or no activity against *Bacillus* and *Staphylococcus*, *Salmonella* and *E. coli*. Similarly, in our study, inhibitory effect of enterocin against *S. aureus* was found less than other strains. *S. aureus* is generally more resistant to enterocins because *Staphylococcus* possesses the ability to form a viscous or gelatinous polysaccharide capsule that prevents the penetration of antimicrobial compounds into the bacterial cells (17). On the other hand, the present paper concluded that enterocins showed inhibition effect against gram-positive and negative bacteria. In accordance with our results, Sparo et al. (24), stated that enterocins from some enterococcal strains displayed the ability to inactivate the growth of both gram positive and negative bacteria. As a result, it was considered that the inhibition effect is strain specific. Bacteriocins have different antimicrobial spectra due to their different modes of action (27). The mode of antibacterial activity of bacteriocins depends on various factors such as the available concentration, characterizations of target or indicator strains and media. For example, antimicrobial resistance of target strains is the main factor related with the effectiveness of bacteriocins (16, 22).

The present study emphasized the importance of *E. faecalis* as a source of enterocin with broad antimicrobial spectrum similar to the work of Belguesmia et al. (5). The differentiation related to antimicrobial spectrum of enterocins may explain resistance mechanisms of microorganisms (5). Additionally, enterocin produced by *E. faecium* isolates from food had inhibition effect on all pathogenic bacteria used in this study. This situation considered that enterocin from *E. faecium* in food isolates may provide protection against pathogen. Additionally, enterocin from *E. faecalis* in clinical sources exhibited the highest antibacterial activity against *S.* Enteritidis. As seen our results, there is variability in antimicrobial activity of enterocins from different strains or sources. The variation of inhibitory spectrum among enterococcus isolates results from different enterocin genotypes among species (14, 15, 16).

The present results revealed that there are huge differences among the *E. faecium* and *E. faecalis* strains in terms of inactivation potential. On the other hand, any correlation could not be established between the origin of strains and inhibition efficacy. Generally, antimicrobial potential of enterococci was heterogeneous and strain-specific because of its ubiquitous nature and persistence (4, 10, 17). Furthermore, inhibitory spectrum of *E. faecium* and *E. faecalis* may change according to pathogenic bacteria strains (7).

In conclusion, the present study revealed that enterococcal isolates from food and clinical isolates have the ability to produce bacteriocinogenic substances against pathogenic bacteria. Enterocins from food and clinical sources has potential to use in food industry as

biopreservatives against pathogens. However, the effectiveness of enterocins should be tested in the food systems and stability of enterocins should be investigated at different conditions such as pH and temperature. Additionally, the relationship between bacteriocin production, hemolysis, antibiotic resistance and the presence of virulence factors should be individually evaluated to determine the safety and risk factors of bacteriocins from food and clinical sources.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Comparison of periapical radiography, panoramic, and cone-beam CT in the detection of dental caries in dog teeth

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Abstract: The aim of this study was to compare the effectiveness of panoramic, periapical and two different Cone Beam Computed Tomography (CBCT) devices in the detection of dental caries of dog teeth *ex vivo*. A total of 880 teeth were investigated, 33 of which were with caries, whereas; 33 healthy teeth were the controls. Periapical, panoramic and CBCT scans were made for the assessment of the teeth. All images were evaluated separately by two observers experienced in image interpretation. The presence or absence of occlusal caries was scored using a 5-point scale. Kappa values were calculated to assess intra and interobserver agreement. Receiver Operating Characteristic (ROC) analysis was performed to compare the effectiveness of different imaging methods in the detection of dental caries. For both observers, the order of success of the image sets in the estimation of the caries tooth was CBCT Morita, CBCT Iluma, periapical and panoramic radiograph (Area Under Curve (AUC): 0.929, 0.882, 0.861, and 0.704 for observer 1, AUC: 0.927, 0.896, 0.875, and 0.693 for observer 2, respectively). CBCT was found to be the best imaging method for the *ex vivo* detection of caries in dog teeth. In addition, panoramic images performed worse than all other modalities.
Keywords: CBCT, dental caries, dogs, panoramic radiography, periapical radiography.

Köpek dişlerindeki çürüklerin tespitinde periapikal, panoramik ve konik ışınlı BT görüntülerinin karşılaştırılması

Özet: Bu çalışmanın amacı, *ex vivo* olarak köpeklerde diş çürüğü tespitinde panoramik, periapikal ve iki farklı Konik Işınlı Bilgisayarlı Tomografi (KIBT) cihazının etkinliğini karşılaştırmaktır. Toplam 880 diş değerlendirilmiş olup 33 çürük diş tespit edilmiştir. Sağlıklı dişlerden rastgele 33'ü seçilerek kontrol grubu oluşturulmuştur. Çürüklerin radyografik olarak değerlendirilmesi için periapikal, panoramik ve KIBT yöntemleri kullanılarak görüntüler elde edildi. Tüm görüntüler, görüntü yorumlamada deneyimli iki gözlemci tarafından ayrı ayrı değerlendirildi. Okluzal çürüğün varlığı veya yokluğu, 5 puanlık bir ölçek kullanılarak puanlandı. Kappa değerleri, gözlemci içi ve gözlemciler arası uyumu değerlendirmek için hesaplandı. Diş çürüğü tespitinde farklı görüntüleme yöntemlerinin karşılaştırılması için ROC analizi yapıldı. Her iki gözlemci için, çürük dişin tespitinde görüntüleme yöntemlerinin başarı sırası KIBT Morita, KIBT Iluma, periapikal ve panoramik radyograf olarak bulunmuştur. (1. gözlemci için Area Under Curve (AUC): 0.929, 0.882, 0.861 ve 0.704, 2. gözlemci için AUC: 0.927, 0.896, 0.875 ve 0.693). KIBT'ın köpek dişlerinde çürüklerin *ex vivo* tespiti için en iyi görüntüleme yöntemi olduğu belirlenmiştir. Ayrıca, panoramik görüntüler diğer tüm yöntemlerden daha kötü performans göstermiştir.
Anahtar sözcükler: Diş çürüğü, köpek, KIBT, panoramik radyografi, periapikal radyografi.

Introduction

Dental caries is plaque-induced demineralization of the teeth formed by the effect of bacteria that ferment carbohydrates. This fermentation leads to the production of acids, which demineralize enamel and dentin. As a result of this, bacteria spread into the dentin, undermining the enamel, leading to a collapse of the enamel and cavitation of the tooth (9). Dental caries can affect any tooth. Teeth of dogs with deeper pits and fissures may be more susceptible to caries. In the dogs, dental caries most commonly occurs on the occlusal surface of the distal aspect of the mandibular first molar tooth, the remaining

mandibular molar teeth, maxillary molar teeth, and teeth with prominent developmental grooves. The maxillary first molar tooth is particularly prone to caries (2). In the literature, the incidence of dental caries is between 3.1-5.3% of dogs (2). Dental caries is less common in dogs than in humans (2). This is because dogs do not have too many fermented carbohydrates in their diet and have an increased salivary pH (9).

Imaging modalities utilized for veterinary dentistry showed an enormous development during the last decade. Consequently, veterinarians' knowledge regarding complex diagnostic imaging methods and treatments, and the importance of orofacial health for domestic animals has increased progressively (12). In consideration of several pathological conditions, radiographic imaging of domestic animal teeth plays a critical role in clinical diagnosis (6). Radiological examination in veterinary dentistry is necessary for certain conditions, such as; caries diagnosis, periodontal assessment, endodontics, restorative procedures and maxillofacial surgery etc. (10). Yet, adequacy of radiographic information seen on conventional two dimensional radiographs, such as; periapical and panoramic radiography is usually limited by the superimposition of anatomical structures. Generally, several radiographs of the suspected teeth are needed during the initial examination. A full-mouth examination is regarded as a series of radiographs describing not only existing teeth but also the toothless segment of the jaw (15). Pathologic radiographic changes are often challenging to diagnose, and for this reason, clarity and detail of radiographic images are important (6). Cone beam computed tomography (CBCT) which offered high resolution three dimensional images of dentomaxillofacial structures with relatively lower radiation doses and costs than medical computed tomography was introduced in 1999 (14). CBCT also enabled three dimensional imaging of rabbits, pigs, cats, and dogs (8).

The aim of this study was to compare the effectiveness of periapical, panoramic, and two different CBCT devices in the detection of dental caries of dog teeth *ex vivo*. If the diagnostic capability of conventional radiographs is found better than CBCT there would be no need to three-dimensional (3D) images.

Materials and Methods

Sample: This study was performed with local ethical committee approval (Baskent University, D-KA 18/21). The sample consisted of randomly selected 40 fresh cadaver mandibles frozen within the post mortem 24th hour. The sample was defrosted 24-hours prior to making the scans. (Figure 1) Firstly, according to tooth size and position, three groups of teeth were formed. The first group included lower incisors, the second group consisted of lower canines, and the third group comprised lower premolars and molars (6). However, there is no caries found on incisors, canines and premolars. All the caries lesions were on molar teeth. A total of 880 teeth were investigated, 33 of with dentine caries (on occlusal surfaces) and 33 healthy teeth were selected randomly for the control group. We selected the same type of teeth (molar) with the caries and compared them with the healthy ones. A 1.5 cm red wax material was used as a soft tissue equivalent.

Figure 1. One of the fresh cadaver mandible used in the study.

All caries lesions were diagnosed with visual inspection and the use of fine dental explorer for the gold standard.

Periapical, panoramic and CBCT assessments: A full-mouth radiographic survey was made for the assessment of teeth. A standard wall-mounted dental radiography unit (Sirona Dental, Salzburg, Austria), along with photostimulable phosphor (PSP) digital intraoral imaging system (Digora, Optime, Soredex, Finland) was used. Intraoral radiographs of the mandibular premolars and molars were obtained by using paralleling technique. Bisecting angle technique was used to evaluate mandibular incisors and canine teeth because of the anatomy of the dogs' mandible.

All digital panoramic images were acquired using the same machine (Veraviewpocs 2D, Morita, Japan), with the following exposure parameters: 64–66 kVp; 6–9 mA; and 10 s. The isolated mandibles were positioned with the occlusal plane perpendicular to the floor.

Two CBCT systems (3D Accuitomo 170, Morita, Japan and Iluma, OrthoCATTM, IMTEC Imaging, Ardmore, OK, US) were used to scan the sample. CBCT Iluma and Morita are two different systems that each of them has its own technical parameters and software program. Technical parameters for 3D Accuitomo 170 and Iluma were; 90 kV, 5 mA, 17.5 s, 10x5 cm FOV, 0.25 mm voxel size and 120 kV, 3.8 mA, 40 s, 18x14 cm FOV, 0.09

mm voxel size, respectively. The isolated mandibles were positioned with the occlusal plane perpendicular to the floor. Axial scans and multiplanar reconstructions were obtained from CBCT scans.

Two experienced observers assessed all the images, separately. Image evaluation was done in a dimly lit room without time constraints. (FUJITSU L20T1 20'' 1600x900 resolution LCD monitor, Kawasaki, Japan). The presence or absence of occlusal/incisal caries was scored using the following 5-point scale: 1= caries definitely present; 2= caries probably present; 3= uncertain-unable to tell; 4= caries probably not present; 5= caries definitely not present. Each observer evaluated the images twice in four weeks to analyze the intra- and interobserver agreement.

Statistical analysis: Statistical analysis was performed with the SPSS (Version 22.0, SPSS Inc., Chicago, IL, USA) package program. Weighted kappa statistics with confidence intervals were calculated to determine the level of agreement between the imaging methods and the gold standard. Kappa values were calculated to assess intra-and inter-observer agreement. The kappa values were interpreted as follows: (< 0.20: Poor, 0.21 - 0.40: Fair, 0.41 - 0.60: Moderate, 0.61 - 0.80: Good, 0.81 - 1.00: Very good). Receiver Operating Characteristic (ROC) analysis was carried out to compare different imaging methods for caries detection. The areas under the curve (AUC), with 95% confidence intervals (CI) were determined. The categories used to classify the accuracy of the imaging methods in ROC analysis were as follows: excellent (0.9–1), good (0.8–0.9), fair (0.7–0.8), poor (0.6–0.7) and fail (0.5–0.6). Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood value, and accuracy were also calculated to compare classification success of dental caries (> 3 without caries, <3 caries).

Results

Fourteen of the 40 mandibles (35%) had bilaterally symmetrical lesions. Caries lesions were most common on mandibular first molar teeth, with 25 on mandibular first molar teeth and eight on mandibular second molar teeth. The incidence of caries lesions was found 4.1% and 35% of 40 mandibles had bilaterally symmetrical lesions in our study.

Intraobserver agreement was 0.932 (very good), 0.924 (very good), 0.835 (moderate) and 0.820 (very good) for Oberver 1, 0.940 (very good), 0.938 (very good), 0.842(very good) and 0.826 (very good) for Observer 2 for CBCT Morita, CBCT Iluma, periapical and panoramic radiography, respectively. Interobserver agreement was 0.896 (very good), 0.833 (very good), 0.601(moderate) and 0.437 (moderate) for CBCT Morita, CBCT Iluma, periapical and panoramic radiography, respectively (Table 1).

Table 1. Interobserver weighted kappa coefficients by image sets.

Image sets		Observers 1 and 2		
		Weighted kappa values		
		κ	**LB**	**UB**
CBCT	Morita	0.896	0.812	0.980
	Iluma	0.833	0.759	0.907
Periapical radiograph		0.601	0.483	0.719
Panoramic radiograph		0.437	0.308	0.566

κ: kappa value, LB: Lower Bound, UB: Upper Bound, CBCT: Cone beam computed tomography

The agreement results between the gold standard test and the image sets were calculated with weighted kappa statistics. Kappa values are presented in Table 2. According to Table 2, the highest agreement with the gold standard test for both observers was found for CBCT Morita (observer 1: $\kappa = 0.586$: moderate agreement, observer 2: $\kappa = 0.616$: high agreement). For observer 1, the agreement value of the other image sets was $\kappa = 0.525$: moderate agreement, $\kappa = 0.515$: moderate agreement, $\kappa = 0.303$: poor agreement, for periapical radiography, CBCT Iluma, and panoramic radiography, respectively. For observer 2, the agreement value of the other image sets was $\kappa = 0.525$: moderate agreement, $\kappa = 0.485$: moderate agreement, $\kappa = 0.192$: poor agreement, for CBCT Iluma, periapical and panoramic radiography, respectively. The scores obtained from panoramic radiographs for both observers showed poor agreement with the gold standard test. Images obtained from a carious tooth by using each imaging technique were shown in Figure 2 (A-C).

In order to compare prediction successes of different imaging techniques according to the gold standard the AUCs obtained by Binormal ROC analysis and post-hoc comparisons with the gold standard were presented in Table 3. ROC curves were also shown in Figure 3 (A) and (B) in consideration to observers. There was a statistically significant difference between the success of correct diagnosis of the four different devices used by the first and second observers ($p = 0.001$, $p = 0.002$, respectively; Table 3). This difference was due to the panoramic radiographic scores obtained from both observers, according to post-hoc ROC comparisons. For both observers, success order of the image sets in the estimation of the caries tooth was CBCT Morita, CBCT Iluma, periapical and panoramic radiography (AUC: 0.929, 0.882, 0.861, and 0.704 for observer 1, AUC: 0.927, 0.896, 0.875, and 0.693 for observer 2, respectively; Table 3).

Table 2. Observers' results in image sets for area under the ROC curve, sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and accuracy (n=66).

Observer	Image set	AUC (95 % CI)	P for ROC comparison	Pairwise comparison	Se	Sp	PPV	NPV	LR+	Ac (%)
1	A	0.929 (0.867-0.992)	0.001	1-2: 0.345 **1-3: <0.001** 1-4: 0.249	0.879	0.727	0.763	0.857	3.22	80.3
	B	0.882 (0.797-0.967)		**2-3: 0.004** 2-4: 0.706	0.788	0.788	0.788	0.788	3.71	78.8
	C	0.704 (0.563-0.845)		**3-4: 0.014**	0.697	0.636	0.657	0.677	1.92	66.7
	D	0.861 (0.766-0.956)			0.818	0.727	0.750	0.800	3.00	77.3
2	A	0.927 (0.862-0.992)	0.002	1-2: 0.540 **1-3: <0.001** 1-4: 0.304	0.848	0.758	0.778	0.833	3.50	80.3
	B	0.896 (0.818-0.974)		**2-3: 0.001** 2-4: 0.700	0.848	0.788	0.800	0.839	4.00	81.8
	C	0.693 (0.540-0.846)		**3-4: 0.015**	0.879	0.455	0.617	0.789	1.61	66.7
	D	0.875 (0.782-0.967)			0.879	0.667	0.725	0.846	2.64	77.3

A: CBCT Morita; B: CBCT Iluma; C: Panoramic radiograph; D: Periapical radiograph; AUC: Area under the ROC curve, CI: Confidence interval, Se: Sensitivity, Sp: Specificity, PPV: Positive predictive value, NPV: Negative predictive value, LR+: Positive likelihood ratio, Ac: Accuracy.

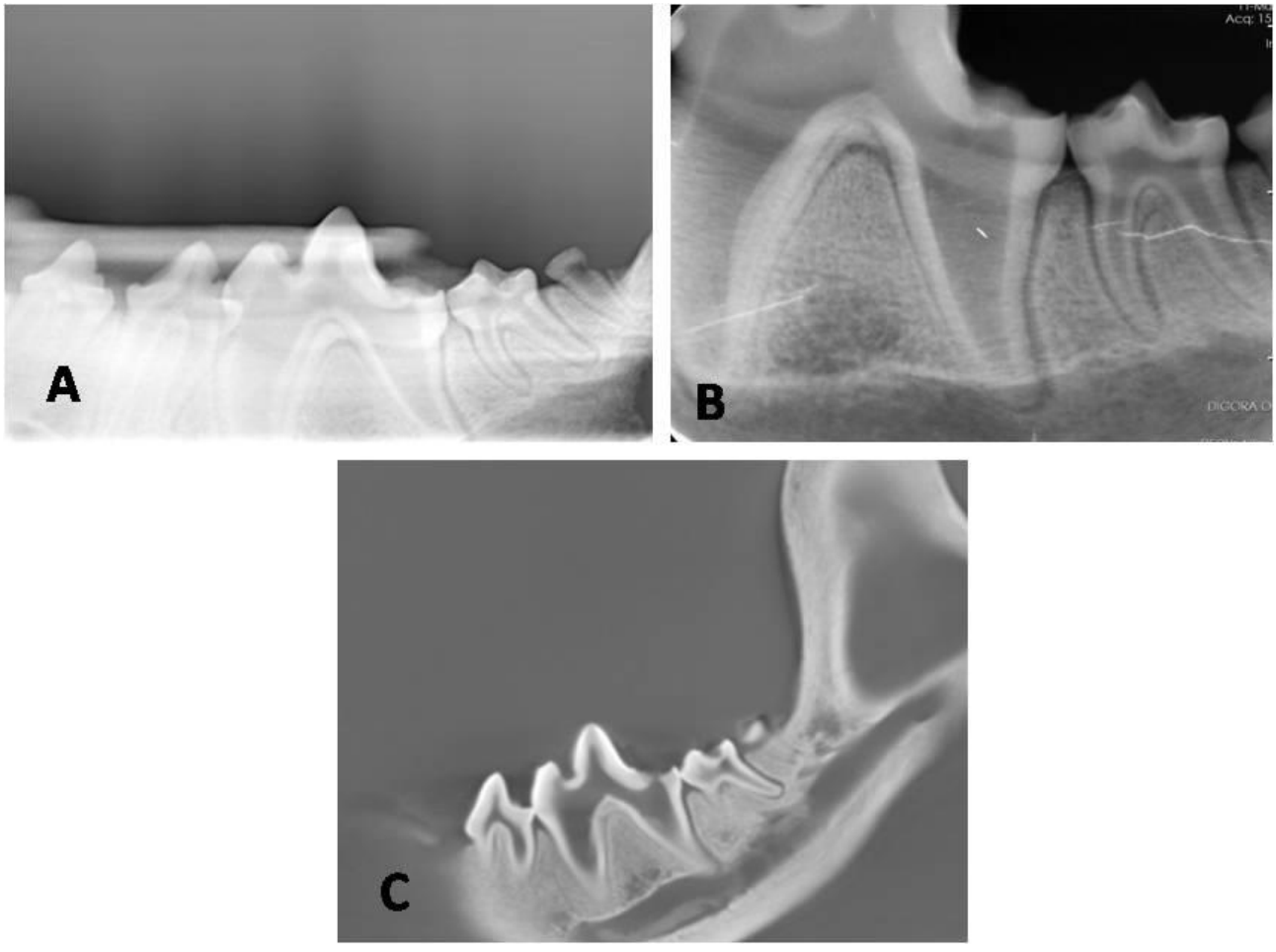

Figure 2. The images of left lower first molar tooth with occlusal caries were shown A: Panoramic radiograph, B: Periapical radiograph, C: CBCT Morita.

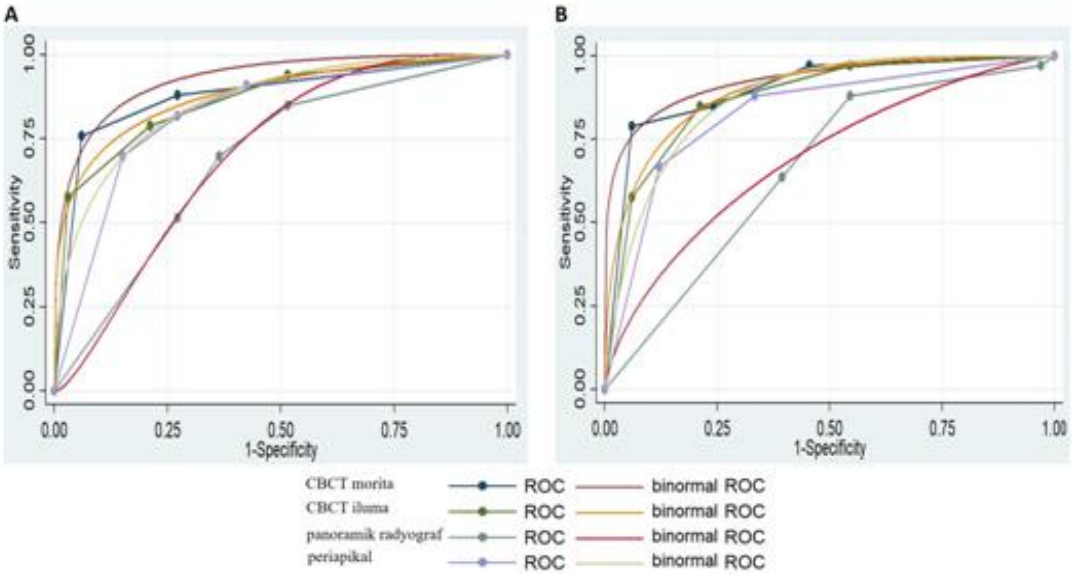

Figure 3. ROC curves were shown according to observers (A, B).

Table 3. Weighted kappa coefficients to assess agreement between image sets and gold standard.

Observer	Image sets	P	Kappa values		
			κ	LB	UB
1	A	<0.001	0.586	0.451	0.721
	B	<0.001	0.515	0.370	0.660
	C	0.008	0.303	0.109	0.497
	D	<0.001	0.525	0.354	0.696
2	A	<0.001	0.616	0.473	0.759
	B	<0.001	0.525	0.386	0.664
	C	0.024	0.192	0.049	0.335
	D	<0.001	0.485	0.342	0.628

CI: Confidence interval, κ: kappa value, LB: Lower Bound, UB: Upper Bound
A: CBCT Morita; B: CBCT Iluma; C: Panoramic radiograph; D: Periapical radiograph.

Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood and accuracy values calculated to compare correct classification success (>3 without caries, and <3 caries) were given in Table 2. For both observers, the highest correct classification was achieved with CBCT Morita (80.3-81.8% Accuracy, 84.8-87.9% sensitivity and 72.7-78.8% specificity).

Discussion and Conclusion

Veterinarians should monitor the risky or already decaying areas and recommended reasonable preventative or restorative treatments even though the incidence of dental caries is lower than humans.

To the authors' knowledge, no previous veterinary dentistry study compared two CBCT units with periapical and panoramic radiography techniques in the detection of dental caries in dog teeth. We compared images obtained from intraoral PSP, panoramic and two CBCT units in the detection of dental caries. We found that both CBCT systems performed similarly and better than two-dimensional (2D) systems suggesting the use of 3D imaging for better caries diagnosis. Panoramic imaging showed the worst diagnostic performance in detecting of dental caries. In recent years, to evaluate the accuracy of CBCT in detecting proximal and occlusal caries lesions, several studied reported varying results (1, 11, 16). In 2007, the study comparing a Sirona CBCT unit and conventional radiography in the detection of proximal caries (3). They did not find any differences between the two imaging methods (3). Tsuchida et al. noticed that the accuracy of the 3D Accuitomo in assessing early proximal caries was not superior to conventional radiography (13). Similarly, Haiter-Neto et al. demonstrated no differences between NewTom 3G, 3DX Accuitomo, DIGORA PSP and Kodak conventional film system in detecting occlusal caries (1). We found that the highest agreement with the gold standard test for both observers was with CBCT Morita. This result might be attributable to CBCT detector and software capabilities, observer performance or

imaging settings used. Voxel size which defines the smallest component of a three dimensional image may be detrimental in diagnostic quality and patient dose. We used voxel sizes smaller than 0.2 mm and this could positively have affected observer performance. In addition, unless the patient is anesthetized, patient motion which may be an important clinical drawback was not an issue in this ex vivo study.

On the contrary, Young et al. reported that Accuitomo 3DX CBCT images were better than Charge Coupled Device (CCD) images in detecting dentin caries lesions (16). However, the authors showed that the differences between CBCT and CCD images did not statistically significant in detecting enamel caries lesions. The reason for these differences in the studies is that both of the studies (1, 13) used a population of teeth in which the most of the proximal surface lesions were limited to the enamel, whereas Young et al. (16) evaluated both enamel and dentinal lesions equally. Another study by Rathore et al. showed no statistically significant differences between Sirona CBCT unit and conventional radiography in detecting occlusal caries (7). In our study, all caries lesions extended into the dentin and similar to Young et al. (16) we found CBCT Morita images to be superior to the periapical radiographs in detecting caries lesions. In our study, a comparison between enamel and dentin caries lesions could not be performed since all caries lesions extended into the dentin.

The concern about radiation exposure is one of the most important factors when choosing between imaging modalities. CBCT systems deliver far greater effective doses when compared to intraoral imaging in general (4, 5). Future improvements in CBCT imaging can be expected to result in innovative systems with better diagnostic abilities and lower effective doses.

Limitations of this study; all caries lesions was on occlusal surfaces of teeth, histopathologic analysis of caries lesions was not performed so that the diagnosis was made with visual inspection and dental explorer.

In conclusion, conventional 2D radiographical techniques have some advantages such as low cost, shorter irradiation time, however, we found that panoramic images performed worse than all other modalities. In our study, CBCT was found to be the best imaging method for the *ex vivo* detection of caries in dog teeth. So that it would be recommended to increase the use of CBCT in veterinary dentistry. In addition, further studies about comparing the different imaging modalities in the detection of occlusal and proximal caries lesions in dog teeth were encouraged.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Clinical significance of pretreatment Ca-P solubility product in 47 cats with chronic kidney disease

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Abstract: Chronic kidney disease (CKD) and mineral disorders are one of the most common reasons of cats. Alterations in mineral metabolism occur in early stage of CKD and increasing the severity in advanced stages. In Turkey, although some clinical studies on CKD in cats are available, no data concerning the clinical significance of pretreatment Ca-P solubility product is present. The purpose of the current study was to determine of Ca-P solubility product and its association with the life quality of cats with CKD. Staging system for classifying cats with chronic kidney disease was based on IRIS guideline. The following groups were occurred based on serum creatinine (SCr) and urine specific gravity < 1035: Stage 2= SCr 1.6 - 2.8 mg/dl; Stage 3= SCr 2.9 - 5.0 mg/dl; Stage 4= SCr > 5.0 mg/dl. Solubility product (calcium x phosphorus) was also defined. Although calcium levels were within reference ranges in groups, Ca-P product were above 72 mg^2/dl^2 in stage 3 and 4 cats. In conclusion, determination of pretreatment Ca-P solubility product in cats with different stages of CKD could be useful to modify and manage the life quality of cats with CKD.

Keywords: Calcium, cat, kidney disease, phosphorus.

Kronik böbrek yetmezliği bulunan 47 kedide tedavi öncesi belirlenen Ca - P çarpımının klinik önemi

Özet: Kronik böbrek yetmezliği (KBY) ve mineral metabolizması sorunları kedilerin yaygın klinik problemlerinden biridir. Mineral metabolizmasındaki değişimler KBY'nin erken aşamalarında başlamakta ve ileri aşamalarda ise şiddeti artmaktadır. Her ne kadar Ülkemizde KBY'li kedilerde yapılan bazı klinik çalışmalar mevcutsa da, bu çalışmaların hiç birinde tedavi öncesi belirlenen Ca-P çarpımının klinik önemine dair veri bulunmamaktadır. Çalışmanın amacı; KBY'li kedilerde Ca-P çarpımı ve yaşam kalitesiyle olan ilişkisini ortaya koymaktır. Çalışmada kronik böbrek yetmezliği olan kediler IRIS klasifikasyonuna göre sınıflandırıldı. Serum kreatinin (SCr) ve 1035'in altındaki idrar dansitesi baz alınarak böbrek yetmezliğinin şiddetine göre gruplar; 2. aşama= SCr 1.6 - 2.8 mg/dl; 3. aşama SCr = 2.9 - 5.0 mg/dl ve 4. aşama= SCr > 5.0 mg/dl olarak oluşturuldu. Kalsiyum fosfor çarpımı ayrıca tespit edildi. Her ne kadar gruplarda kalsiyum düzeyleri referans değerler arasındaysa da, kalsiyum fosfor çarpımı 3 ve 4. aşama kedilerde 72 mg^2/dl^2'nin üzerinde belirlendi. Sonuç olarak KBY'nin çeşitli aşamalarındaki kedilerde Ca-P çarpımının tedavi öncesi belirlenmesi KBY'li kedilerde yaşam kalitesinin yönetilmesinde faydalı olabilir.

Anahtar sözcükler: Böbrek hastalığı, fosfor, kalsiyum, kedi.

Introduction

Chronic kidney disease (CKD) and mineral disorders are a common clinical problem in aging cats. Structural or functional renal abnormalities develop in 1.6-20 % of all cats during their lifetime (24, 26). Alterations in mineral metabolism occur in early stage of CKD and increase the severity in advanced stage (26). Because of the progression of the disease, literature have focused the survival in cats with CKD (7, 17). In Turkey, although some clinical studies including heamatological and biochemical changes have been published in cats with CKD (2, 8), these studies have consisted of less case series and none of them had an explanation concerning the clinical significance of Ca-P solubility product. Human studies revealed the association between increased levels of serum phosphorus and increased mortality (18, 20). It has also been reported that human patients with a Ca-P product > 72 mg^2/dl^2 have higher mortality risk compared to those with a Ca-P product between 42-52 mg^2/dl^2 (5). The purpose of the current study was that although it is

known the progression of CKD in cats, determination of Ca-P solubility product in different stages of CKD may be used to manage the life quality of cats with CKD.

Material and Methods

This study was a prospective clinical study in 69 clint-owned cats with the signs of polyuria, polydipsia, vomiting, anorexia, halitosis, lethargy, depression or small kidneys on palpation. Staging system for classifying cats with chronic kidney disease based on IRIS guidelines was used (16). The following groups were occurred based on serum creatinine (SCr) and urine specific gravity < 1035: Stage 2= SCr 1.6 – 2.8 mg/dl; Stage 3= SCr 2.9 - 5.0 mg/dl; Stage 4 = SCr > 5.0 mg/dl. Cats with proteinuria, high blood pressure and CKD Stage 1 (SCr < 1.6 mg/dl) were not included the IRIS staging. Cats with systemic disease such as diabetes mellitus, hyperthyroidism, neoplasia, postrenal obstruction and hepatic pathology were excluded from the study. Cats with feline infectious peritonitis, feline immunodeficiency virus or feline leukemia virus performed with speed tests were also excluded from the study. Cats were treated with appropriate medications. Clinical procedures including physical examination, complete blood count (Exigo® cbc analyzer), serum profiles (Erba xl 600®), urinalysis (urine test strip) and abdominal ultrasonography (Shimadzu ® - Sdu 450) were performed in all cats. Solubility product (calcium x phosphorus-mg^2/dl^2) was also defined (4). Cats were fasted for at least 12 hour prior to blood sampling. Written owner consents were also obtained from the owners.

Statistical analysis: Data regarding characteristics of the cats were subjected to the Mean Procedure (SPSS, Version 16, Chicago, ILL). Clinical data were analyzed using cross-tabulation in chi-square test. Finally hematological parameters and serum biochemistry parameters were analyzed using the NPAR1WAY procedure. Differences by the stage were attained using Kruskal-Wallis test, employing chi square value and degree of freedom. Statistical significance was declared at $P<0.05$.

Results

Data were collected from 69 clint-owned cats referred to hospital with the signs of kidney disease. Stage 1 cats (n:15) with renal insufficiency but not renal failure were excluded from the study. 7 cats with postrenal obstruction, hyperthyroidism, renal mass or hepatic pathology were not also included the study. Study were performed with 47 cats with chronic kidney disease. The mean age, weight, breed and sex distribution of cats in groups were shown in Table 1. Historical and physical examination findings in each group were also presented in Table 2. Table 3 and Table 4 revealed complete blood counts, serum profiles and urinalysis in groups.

Table 1. Characteristics of cats in groups.

Staging*	**Age (years)**	**Weight (kg)**	**Breeds (n)**	**Sex (n♂, n♀)**
Stage 2, n:7	10.85 ± 3.48	3.42 ± 0.51	DSH (n:3), Persian (n:2), Siamese (n:2)	4♂, 3♀
Stage 3, n:11	11.59 ± 4.87	2.96 ± 0.64	DSH (n:8), Siamese (n:2), Van cat (n:1)	3♂, 8♀
Stage 4, n:29	10.41 ± 4.72	3.09 ± 0.80	DSH (n:15), Persian (n:4), Siamese (n:4), BSH (n:3), Chinchilla (n:1), Ankara cat (n:1) Van cat (n:1)	14♂, 15♀
P	> 0.05	> 0.05		

DSH: Domestic short hair; BSH: British short hair; *Based on IRIS guidelines.

Table 2. Historical and physical examination findings of cats in groups.

Historical exam findings	**Stage 2, n: 7 (%)**	**Stage 3, n: 11 (%)**	**Stage 4, n: 29 (%)**
Polyuria/Polydipsia	2 (28)	4 (36)	17 (58)
Vomiting	3 (42)	8 (72)	19 (65)
Anorexia	1 (14)	5 (45)	22 (75)
Halitosis	-	3 (27)	6 (20)
Lethargy	4 (57)	9 (81)	12 (41)
Depression	-	3 (27)	26 (89)
Physical exam findings	**Stage 2, n: 7 (%)**	**Stage 3, n: 11 (%)**	**Stage 4, n: 29 (%)**
Dehydration	3 (42)	9 (81)	26 (89)
Pale mucous membranes	1 (14)	2 (18)	8 (27)
Murmur	1 (14)	1 (9)	13 (44)
Poor oral health	-	3 (27)	18 (62)
Poor hair health	-	6 (54)	22 (75)
Small kidneys on palpation* (Uni- or Bilateral)	1 (14%)	5 (45)	24 (82

*confirmed by abdominal ultrasonography

Table 3. Heamatology profiles in cats with chronic kidney disease.

	Stage 2, n:7 (Mean ± SD)	**Stage 2, n:7 (Median)**	**Stage 3, n:11 (Mean ± SD)**	**Stage 3, n:11 (Median)**	**Stage 4, n:29 (Mean ± SD)**	**Stage 4, n:29 (Median)**	X^2 ; P
WBC, 10^9 / l (5.5-19.5)	8.40 ± 4.45	7,84	9.52 ± 3.78	7,25	12.58 ± 9.65	9,07	2.3 ; 0.3
LYM, 10^9 / l (1-7)	1.84 ± 0.40	2,01	1.36 ± 0.84	1,54	1.94 ± 1.55	1,3	1.9 ; 0.45
MONO, 10^9 / l (0.2-1)	0.38 ± 0.21	0,38	1.14 ± 1.64	0,6	0.97 ± 0.78	0,66	4.9 ; 0.28
NEUT, 10^9 / l (2.8-13)	4.70 ± 5.32	1,96	6.80 ± 4.14	6,3	9.20 ± 9.15	6	4.3 ; 0.34
EOS, 10^9 / l	0.21 ± 0.10	0,28	0.23 ± 0.14	0,2	0.45 ± 0.51	0,29	4.4 ; 0.20
LYM, %	35.65 ± 17.6[a]	42,9	18.95 ± 15.6[b]	11,4	18.9 ± 15.5[b]	14	**1.9 ; 0.04**
MONO, %	5.90 ± 2.24	6,1	7.30 ± 5.00	6,1	7.62 ± 4.18	6,9	4.9 ; 0.62
NEUT, %	54.02 ± 21.3	41,8	71.78 ± 17.5	78,7	63.83 ± 22.79	69,7	3.6 ; 0.24
EOS, %	4.51 ± 3.30	6,9	2.18 ± 1.21	1,9	3.64 ± 3.82	3	1.6 ; 0.31
RBC, 10^{12} / l (5-11)	6.61 ± 1.50	6,29	6.41 ± 2.12	5,89	5.87 ± 1.92	5,72	1.8 ; 0.55
HGB, g/dl (8-15)	10.34 ± 3.20	10	11.14 ± 2.94	10,6	9.83 ± 2.56	9	1.8 ; 0.40
HCT, % (25-45)	27.88 ± 6.65	24,9	31.05 ±9.50	32,23	26.52 ± 9.75	26,6	2.4 ; 0.39
MCV, fl (39-50)	42.28 ± 3.88	42,8	48.68 ± 3.88	48	46.68 ± 6.13	45	4.9; 0.08
MCH, pg (12.5-17.5)	15.47 ± 2.75	14	18.9 ± 6.90	17,4	17.57 ± 4.99	16,9	2.9 ; 0.40
MCHC, g/dl (31-38.5)	34.4 ± 6.17	32,2	34.02 ± 4.09	34,6	33.43 ± 3.41	33,1	0.07 ; 0.81
RDW, % (14-18.5)	16.37 ± 3.24	15	15.42 ± 2.94	14,8	14.40 ± 2.86	13,7	3.67 ; 0.24
PLT, 10^9 / l (200-500)	313.2 ± 242.8	245	289.6 ± 194. 1	254	370.53 ± 225.5	354	1.67 ; 0.54
MPV, fl (8-12)	11.68 ± 1.53	12	11.11 ± 3.36	10,5	11.07 ± 3.49	11,6	0.33 ; 0.90

[a,b] different letters are significantly different

Table 4. Serum profiles in cats with chronic kidney disease.

	Stage 2, n:7 (Mean ± SD)	Stage 2, n:7 (Median)	Stage 3, n:11 (Mean ± SD)	Stage 3, n:11 (Median)	Stage 4, n:29 (Mean ± SD)	Stage 4, n:29 (Median)	X^2 ; P
Urea, mg/dl (15-64.2)	93.3 ± 42.4	84,8	126.05 ± 59.46	116,6	162.69 ± 100.2	130	3.54 ; 0.13
Creatinin, mg/dl (0.8-1.8)	2.30 ± 0.39[a]	2,4	3.96 ± 0.59[a]	3,93	7.90 ± 2.42[b]	7,7	**34.4 ; < 0.0001**
T. Protein, g/dl (5.4-7.8)	6.65 ± 1.57	7,5	7.01 ± 0.90	6,71	7.50 ± 1.34	7,4	1.23 ; 0.23
Glucose, mg/dl (70-110)	97.7 ± 15.1	95	120.46 ± 44.11	97	126.56 ± 33.23	124	5.3 ; 0.14
Albumin, g/dl (3.5-4.5)	2.58 ± 1.22	2,5	3.26 ± 0.71	3,1	2.92 ± 0.40	2,9	2.6 ; 0.10
T. Bilirubin, mg/dl (0.1-0.2)	0.25 ± 0.15	0,24	0.26 ± 0.16	0,2	0.35 ± 0.38	0,21	0.7 ; 0.65
ALP, IU/l (25-93)	29.6 ± 11.4	30	59.92 ± 70. 9	36,9	36.17 ± 26.43	28	1.77 ; 0.19
AST, IU/l (26-43)	18.3 ± 7.82[a]	15	43.55 ± 36.40[b]	34	56.18 ± 35.13[b]	59	**9.92 ; 0.03**
ALT, IU/l (6-83)	36.2 ± 31.7	24	65.02 ± 59. 17	25	64.74 ± 44.70	59	2.75 ; 0.33
GGT, IU/l (6-28)	2.00 ± 1.82	1	5.41 ± 8.30	2,9	2.68 ± 4.17	1	1.97 ; 0.27
Ca, mg/dl (7.5-10.8)	9.80 ± 1.11	9,5	9.59 ± 0.95	9,6	9.77 ± 1.07	9,7	0.08 ; 0.86
I. Phosphorus, mg/dl	5.34 ± 2.27[a]	4,3	7.66 ± 3.61[a]	5,6	13.05 ± 3.04[b]	13,1	**21.5 ; < 0.0001**
Na, mmol/l (146-159)	151.5 ± 7.5	155	148.45 ± 5.98	149	147.117 ± 26.04	151	2.29 ; 0.88
K, mmol/l (3.8-5.3)	3.91 ± 0.59	3,9	4.07 ± 0.76	3,9	4.53 ± 1.10	4,3	3.74 ; 0.20
Ca x P Product, mg^2/dl^2	52.4 ± 23.2[a]	41,65	73.12 ± 33.95[a]	58,3	127.427 ± 31,52[b]	136,4	**22.21 ; <0.0001**
Urine Specific Gravity	1032.5 ± 6.3	1025	1030.8 ± 6.4	1024	1025.8 ± 5.3	1021	7.5 ; 0.04
Urine Ph	6.25 ± 0.41	6	6.32 ± 0.52	6.1	6.81 ± 0.43	6.5	8.8 ; 0.04

[a,b] different letters are significantly different

Discussion and Conclusion

CKD is the most common metabolic disease of aging cats. Age-related increase in the prevalence of CKD in cats has been reported (23). Renal disorders in cats above 13 years of age have been identified as the most common cause of death (14). Mortality in cats with CKD has defined as 4% between 1 to 5 years of age and 17% in cats dying at ≥ 11 years of age (19). Although survival was not the aim of this study and younger cats included in each group, the mean age of all cats in groups were about 11 year old.

Severity of the CKD associated complications such as hyperphosphatemia, secondary renal hyperparathyroidism, hypokalemia, anemia, proteinuria, hypertension and uremia as well as the process of treatment and prognosis vary in advancing IRIS stage (7, 16). In 30% to 65% of cats with CKD, anemia could develop attributable to decreased erythropoietin production (10). In a study previously defined, no significant correlations were found between anemia and survival in cats with CKD (15). Although anemia is a common finding in cats with end stage CKD (11), in this study it was not a remarkable finding. Dehydration was thought as the possible reason of masking the anemia in cats with CKD.

Some studies performed in cats with CKD based on elevated blood creatinine concentrations to diagnose the kidney disease in cats with or without clinical signs (4, 17). IRIS guidelines also confirmed creatinine measurements for staging the renal failure (16). Creatinine levels obtained from this study were statistically significant in each IRIS stages as consistent with the reports previously described.

Hyperphosphatemia is a clinicopathologic marker of renal fibrosis, mortality and progression of CKD in cats (22). Phosphorus level in early stages of CKD is kept within reference ranges by parathyroid hormone (PTH). However, phosphorus level can no longer maintain in cats with IRIS stage 2, 3 and 4 because of decreasing the glomerular filtration rate (1, 13). Data obtained from the current study supported this finding. Although we do not know the PTH levels in cats with different IRIS stages of CKD, inorganic phosphorus levels in IRIS stages gradually increased in this study.

CKD is a concurrent disease in cats with hypercalcemia (9). Several mechanism including decreased glomerular filtration and bone storage of calcium and, increased tubular reabsorption have supported the hypercalcemia risk (25). Whether CKD is a risk factor for hypercalcemia is still unclear (9). In some studies 11.5-21% of cats with CKD had concurrent hypercalcemia (3, 12, 27). In this study presented here mean calcium concentrations in groups were within reference ranges and no significant differences were found among groups.

In a study obtained from 50 cats, higher phosphorus level has been associated with a higher risk of death within 1 month. In the same study, higher urea and serum creatinine concentrations were also related to increased risk of death (21). In another study (7) 11.8 % increase in the risk of death was defined for each 1 unit increase in

phosphorus level. In Turkey, although some studies including clinical, heamatological and biochemical changes have been published in cats with CKD (2, 8), none of them had an explanation concerning the clinical significance of Ca-P solubility product. Point out the determination of Ca-P solubility product in different stages of CKD to modify the prognosis and manage the life quality of cats with CKD is the main purpose of the current study. In a human study (5), patients with a Ca-P solubility product above 72 mg^2/dl^2 had a 34% higher risk of death compared with those with a Ca-P product between 42-52 mg^2/dl^2. It has been also reported that cats with soft tissue calcifications had elevated levels of Ca-P product (above 70 mg^2/dl^2) with or without hypercalcemia (4, 6). In the current study although calcium levels were within reference ranges in each group, Ca-P product were above 72 mg^2/dl^2 in stage 3 and 4 cats as consistent with the reports previously described (4, 6).

In conclusion, determination of pretreatment Ca-P solubility product in cats with different stages of CKD could be useful to modify and manage the life quality of cats with CKD.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Estimation of genetic parameters for some performance traits in a selected Barred Rock line

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Abstract: This study was conducted to determine the genetic correlation and heritability for age at sexual maturity, body weight at sexual maturity, egg yield, egg weight, egg shape index and L*, a*, b* values of egg shell in a Barred Rock line was applied selection. For this aim, a total of 1622 pedigreed Barred Rocks were selected. The present estimated heritability values for investigated traits were found between moderate-to-high. However, the heritability of egg yield, eggshell color and body weight at sexual maturity were found lower than those of other traits. It was calculated positive correlations among monthly egg yields, especially between egg yield at the second month with total egg yield. As a conclusion, selection studies did not cause much decrease in genetic variation of studied traits, except for egg yield and body weight at sexual maturity. Monthly egg yield data can be used selection studies.
Keywords: Genetic correlation, heritability, layer breeder, performance traits.

Seleksiyon uygulanan Barred Rock hattında bazı performans özellikleri için genetik parametrelerin tahmini

Özet: Bu çalışma, seleksiyon uygulanan Barred Rock hattında yumurta verimi, cinsel olgunluk yaşı, cinsel olgunluk ağırlığı, yumurta verimi, yumurta ağırlığı, yumurta şekil indeksi ve yumurta kabuk L*, a*, b* değerlerinde kalıtım derecesi ve genetik korelasyonları belirlemek için yürütülmüştür. Bu amaçla, toplam 1622 pedigrili Barred Rock seçilmiştir. İncelenen özelliklerin hesaplanan kalıtım dereceleri orta ile yüksek değerler arasında olduğu görülmüştür. Bununla birlikte, yumurta verimi, yumurta kabuk rengi ve cinsel olgunluk ağırlığı özelliklerinin kalıtım dereceleri diğerlerine göre daha düşük bulunmuştur. Özellikle ikinci aydaki yumurta verimi ile toplam yumurta verimi arasında olmak üzere, aylık yumurta verimleri arasında pozitif korelasyonlar hesaplanmıştır. Sonuç olarak seleksiyon çalışmaları, incelenen özelliklerden cinsel olgunluk ağırlığı ve yumurta verimi dışındaki özelliklerin genetik varyasyonunda fazla bir azalmaya neden olmamıştır. Aylık yumurta verimi seleksiyon çalışmalarında kullanılabilir.
Anahtar sözcükler: Genetik korelasyon, kalıtım derecesi, performans özellikleri, yumurtacı damızlık.

Introduction

Poultry meat and egg have been the most important contributors to animal protein requirement of increasing world population. Turkey has been one of the important leading countries in the poultry production and relevant technologies (18). North American and European breeding companies have dominated for hybrid animal materials more than 2/3 of global market (11, 32, 33). The Poultry Research Institute in Turkey has been continuing its mission of poultry breeding based on layers for many years. In 1995, the selection studies were conducted on 10 pure lines taken from abroad by taking into consideration egg weight, egg yield, age and weight at sexual maturity. In these studies, one white and two brown native hybrids were developed. The performance of hybrids and comparison studies with foreign hybrids have been continued while selection studies have been conducted on pure lines to be used to produce hybrid combinations.

Aims of the poultry breeding programs are to improve the genetic potential of birds through crossbreeding and selection schedules. Poultry breeding studies include, mainly, two stages. The first one covers selection studies to improve performance at pure line level. All genetic parameter estimations, breeding value estimations and molecular genetic methods are performed at this stage. This provides the highest homozigotization in terms of additive gene effects with all the processes (19, 25). Almost all current studies have been related to this stage. The breeding companies have worked on selection studies to improve their layer lines for some performance

traits such as age at sexual maturity, body weight at sexual maturity, feed conversion ratio, livability, egg yield, egg weight, shell strength, shell color, blood and meat spots and albumen height (33). The second stage of poultry breeding covers hybridization processes of lines developed by selection and comparison of hybrid combinations. At this stage, the special combination capabilities of hybrid lines with general combination capabilities are determined, and thus, non-additive (dominance and epistatic) gene effects are utilized. Highly selected elite sire and dam pure lines construct the basis of today's layer breeding programs. Commercial layers are obtained from three-way or four-way crosses of specific closed pure breeding lines. Barred Rock is one of the lines developed in the Poultry Research Institute. In this breed, egg yield parameters were studied. The aim of this study was to estimate heritability and genetic correlations for some performance traits (age at sexual maturity, body weight at sexual maturity, egg yield, egg weight, egg shape index and L*, a*, b* values of egg shell) in a Barred Rock line was applied selection.

Material and Methods

Animals: The Barred Rock line used in this study was selected for generations using multi-trait selection methodology considering four traits (egg yield, egg weight, age and body weight at sexual maturity) from 1996 up to now. With regards to the selection criteria, superior birds (450 hens and 50 cocks) were selected from the base populations; 50 families were formed (9 hens were artificially inseminated with the semen from 1 male). Eggs were obtained from these full-pedigree families and they were incubated. Chicks were hatched and vaccinated against diseases according to the institute vaccination program. Chicks were reared until 16 weeks of age under standard management conditions. When the pullets reached to 16 weeks of age, they were randomly distributed into individual cages with conventional cages. All birds had *ad libitum* access to water and feed. During rearing and growing periods, the birds were fed commercial starter and grower diets for breeder flock. Layers were fed according to the feeding strategy of the Turkish selection and crossbreeding experiment (10, 18). Pullets were kept under a constant artificial lighting for 18 h/day. Records of 1622 pedigreed Barred Rocks were taken. The data were collected between 20 to 43 weeks of age. Monthly egg records were generated by summing each 4 continuous weekly eggs records from 20 to 24, 25 to 29, 30 to 34, and 35 to 39 weeks of age. The mean sexual maturity age was measured as the number of days until the first egg was laid. The hen was weighed on the day it laid its first egg and this weight was recorded as sexual maturity weight. Average egg weight of each hen's eggs was recorded by weighing three sequential eggs at every 4 weeks starting from the 24th week to end of the experiment. Egg shape and L*, a* and b* values of egg shell were determined at the same time. In this study, L*, a* and b* values were measured (19) with Minolta Chroma Meter CR 400 (Minolta Co., Osaka, Japan).

Statistical analyses: The descriptive statistics, normality (Kolmogorov-Smirnov) and homogeneity (Levene) tests of the traits were performed by using UNIVARIATE procedure of SAS 9.3 software (Statistical Analysis Systems Institute Inc., Cary, NC). The non-parametric rank transformation was applied in R package for color parameters and shape index which parametric Box-Cox transformation did not Gaussian distributed traits (6, 19).

The restricted maximum likelihood (REML) estimator was used to estimate the variance-covariance components for following multi-trait model;

$$\mathbf{y=X\beta+Zu+e}$$

Where **y**, vector of observations for the trait; **β**, vector of fixed effects for the trait; **u**, vector of random animal effects for the trait; **e**, vector of random residual effects for the trait; and **X** and **Z** are incidence matrices relating records of the trait to fixed and random animal effects, respectively (20, 30). The sire, dam and residual variance components and additive genetic and environmental covariance matrices for multivariate analysis were estimated from the mixed-model equations by SAS PROC MIXED. Heritability (h_i^2) and genetic correlations ($r_{g(ii')}$), were calculated from the variance and covariance parameters as follows:

$$h_i^2 = \frac{\sigma_{ia}^2}{\sigma_{ia}^2 + \sigma_{ie}^2} \qquad r_{g(ii')} = \frac{\sigma_{ii'a}}{\sigma_{ia}^2 + \sigma_{i'a}^2}$$

Where *i* and i' represents the trait(s) of interest and σ_{ia}^2 and σ_{ie}^2 are the diagonal elements of $\mathbf{G_0}$ and $\mathbf{R_0}$ matrices, respectively. Also, $\sigma_{ii'a}$ stands for the additive genetic covariance between the traits *I* and i'. The estimations of genetic correlation and heritability and their standard errors for subjected traits were obtained by SAS interactive matrix language (IML) procedure.

Results

The mean values of the age and body weight at sexual maturity, egg weight, egg shape index, and L*, a*, b* values of eggshell are shown in Table 1. The means of age and body weight at sexual maturity, egg weight, shape index, L*, a* and b* values of eggshell, monthly egg yield from 20 to 24, 25 to 29, 30 to 34, and 35 to 39 weeks of age and total egg yield from 20 to 43 weeks of age were found as 136.14, 1538.87, 51.31, 76.44, 63.58, 7.95,

20.51, 14.25, 26.88, 28.22, 28.39 and 143.93, respectively. The heritability estimations of age and body weight at sexual maturity, egg weight, L*, a* and b* values of eggshell, egg shape index and total egg yield from 20 to 43 weeks of age were determined as 0.41, 0.20, 0.48, 0.20, 0.21, 0.22, 0.33 and 0.23, respectively (Table 2). The heritability estimations of monthly records from 20 to 24, 25 to 29, 30 to 34, and 35 to 39 weeks of age, and the total egg yield were found as 0.33, 0.24, 0.04, 0.01 and 0.23, respectively (Table 3).

Table 1. The descriptive statistics of some performance traits

Variable	N	Mean	Min.	Max.	CV	SD	SEM
ASM	1176	136.14	119	164	3.97	5.4	0.16
BWSM	1176	1538.87	960	2080	8.41	129.44	3.77
EW	1176	51.31	42	63	5.33	2.73	0.08
L*	1078	63.58	51.1	76.7	9.22	5.86	0.18
a*	1078	7.95	1.2	17.8	74.97	5.96	0.18
b*	1078	20.51	4.0	45.1	14.67	3.01	0.09
SI	1077	76.44	71.93	80.39	2.65	2.02	0.06
EP_1	1176	14.25	0	31	35.88	5.11	0.15
EP_2	1176	26.88	0	32	18.36	4.94	0.14
EP_3	1176	28.22	10	32	8.57	2.42	0.07
EP_4	1176	28.39	2.0	32	9.58	2.72	0.08
EP_T	1176	143.93	61	173	8.66	12.46	0.36

ASM: age at sexual maturity, BWSM: body weight at sexual maturity, EW: egg weight, L*: lightness, a*:redness, b*:yellowness, SI: egg shape index, EP_1: 20 to 24 weeks of age, EP_2: 25 to 29 weeks of age, EP_3:30 to 34 weeks of age, EP_4: 35 to 39 weeks of age, EP_T: from 20 to 43 weeks of age.

Table 2. The estimates of heritability (on diagonal) and genetic correlation (below diagonal) for some performance traits and phenotypic correlations (above diagonal) between traits

	ASM	BWSM	EW	L*	a*	b*	SI	EP_T
ASM	**0.41** (0.01)	0.00 (0.04)	0.05 (0.03)	-0.07 (0.02)	0.13 (0.02)	0.04 (0.02)	0.07 (0.02)	-0.44 (0.02)
BWSM	0.04 (0.04)	**0.20** (0.02)	0.51 (0.02)	-0.01 (0.02)	0.01 (0.02)	0.04 (0.02)	-0.01 (0.02)	0.06 (0.02)
EW	0.23 (0.03)	0.58 (0.02)	**0.48** (0.02)	-0.10 (0.02)	0.12 (0.02)	0.10 (0.02)	-0.04 (0.02)	-0.51 (0.02)
L*	-0.50 (0.01)	-0.02 (0.04)	-0.14 (0.04)	**0.20** (0.03)	-0.68 (0.02)	-0.30 (0.02)	0.04 (0.02)	0.00 (0.02)
a*	0.82 (0.01)	-0.14 (0.04)	0.62 (0.01)	-0.33 (0.02)	**0.21** (0.02)	0.43 (0.02)	-0.09 (0.02)	-0.07 (0.02)
b*	-0.19 (0.04)	-0.01 (0.05)	0.27 (0.02)	-0.68 (0.01)	0.44 (0.01)	**0.22** (0.02)	-0.10 (0.02)	0.06 (0.02)
SI	0.63 (0.01)	-0.79 (0.01)	0.16 (0.03)	-0.23 (0.03)	-0.02 (0.04)	-0.13 (0.02)	**0.33** (0.01)	-0.09 (0.02)
EP_T	-0.82 (0.01)	0.03 (0.05)	-0.68 (0.01)	-0.16 (0.03)	-0.21 (0.04)	0.06 (0.05)	-0.66 (0.01)	**0.23** (0.01)

ASM: age at sexual maturity, BWSM: body weight at sexual maturity, EW: egg weight, L*: lightness, a*:redness, b*:yellowness, SI: egg shape index, EP_T: from 20 to 43 weeks of age.

Table 3. The estimates of heritability (on diagonal) and genetic correlation (below diagonal) for some egg yield traits and phenotypic correlations (above diagonal) between traits

	EP_1	**EP_2**	**EP_3**	**EP_4**	**EP_T**
EP_1	**0.33** (0.02)	0.27 (0.02)	0.08 (0.02)	0.03 (0.02)	0.66 (0.02)
EP_2	0.81 (0.01)	**0.24** (0.02)	0.34 (0.02)	0.15 (0.02)	0.75 (0.02)
EP_3	0.40 (0.02)	0.84 (0.02)	**0.04** (0.03)	0.27 (0.02)	0.53 (0.02)
EP_4	0.52 (0.01)	0.73 (0.01)	0.86 (0.01)	**0.01** (0.03)	0.47 (0.02)
EP_T	0.79 (0.01)	0.87 (0.01)	0.79 (0.01)	0.63 (0.01)	**0.23** (0.02)

EP_1: 20 to 24 weeks of age, EP_2: 25 to 29 weeks of age, EP_3:30 to 34 weeks of age, EP_4: 35 to 39 weeks of age, EP_T: from 20 to 43 weeks of age.

Discussion and Conclusion

The principal traits in layer stocks are weight and age at sexual maturity of hen, egg weight and egg yield. In order to achieve early maturity and egg yield, it is crucial to have correct body weight and uniformity in growing period. The mean of sexual maturity age in this study was found as 136.14 days. This value was lower than those reported by Kumar et al. (14) and Sing et al. (31). The earlier sexual maturity in the current study was the results of selections for sexual maturity and egg yield. The average sexual maturity age of the flock was 162.31 days in 1996 (18) and it has reached 136.14 days at the present. A 3.97% coefficient of variation for sexual maturity age shows a close uniformity within flock. Selection studies from 1996 to 2019, the sexual maturity weight was reduced from 1996 g to 1538 g. However, this weight was higher than that of commercial market counterparts, which were 1350-1400 g (4). Sexual maturity weight is a very important parameter associated with egg weight. The average egg weight in this study was 51.31 g, which was lower than the reported values 55.46 to 62.74 g in Lohmann and Hyline commercial breeders, respectively (3, 5). And also this values was found lower than results of Peebles et al. (23) and Rayan et al. (26). The heavier eggs in laying hens not only decreases the total egg yield but also causes excessive feed consumption. So, the present mean egg weight would not be an obstacle for either higher egg yield or feed efficiency.

Production of uniform dark-brown colored egg shells through laying period is the goal of brown layer breeders (28). In this study, the mean values of L*, a* and b* were found as 63.58, 7.95, 20.51, respectively. It is an important component for measuring shell color (27). The eggshell color of a commercial flock is influenced by many genetic factors, where it is important to ensure uniformity. The coefficients of variation of L*, a* and b* traits were quite high as 9.22%, 74.97% and 14.67%, respectively. Eggs can be classified with respect to shape index, namely as the sharp egg (<72), the standard egg (72–76) or the round egg (>76) (31). The shape index value was found in this study in the upper limit of standard type. The average egg yield from 20 to 24, 25 to 29, 30 to 34, and 35 to 39 weeks of age and total egg yield from 20 to 43 weeks of age were found as 14.25, 26.88, 28.22, 28.39 and 143.93, respectively. These findings are similar with reported by other researchers (36, 38, 37).

It is known that age at sexual maturity was a moderately heritable trait, and there were various heritability estimations between 0.28 to 0.36 in the literature (1, 21, 35). High heritability estimation (0.41) of the age at sexual maturity in this study was in agreement with that of Lillpers and Wilhelmson (16). The heritability estimation (0.20) for body weight at sexual maturity was lower than those reported in the previous studies (1, 21, 22, 35). The low heritability value for body weight at sexual maturity might be indicated that there was little scope for improving this trait through selection. Although the main effort in layer breeding strategy is focused on egg yield, egg weight is, also, a factor in selection schemes. The objective of commercial layer breeding strategies is to obtain lines characterized by moderate egg weight (34). Franches et al. (9) reported that the heritability values for egg weight ranged between 0.20 and 0.33 in different breeds. However, Zhang et al. (39) reported a high heritability value of 0.63 for egg weight. In the current study, the heritability estimation of egg weight trait is similar with those found in the literature (7, 13, 17).

Estimated heritability (0.20 to 0.22) for eggshell color traits in the current study were lower than those (0.27 to 0.53) reported by Franches et al. (9) and Zhang et al.

(39) in the different breeds. Johansson et al. (12) and Besbes and Gibson (8) estimated heritability as moderate to high values (0.23 to 0.41) for egg shape index. A moderate heritability estimation (0.33) for shape index was consistent with the estimates reported by Johansson et al. (12) and Besbes and Gibson (8).

The heritability estimates of egg yield in the first, second, third, and fourth months of laying hens were determined as 0.65, 0.36, 0.36, and 0.38, respectively. A high heritability (0.43) was estimated for total egg yield. In the literature, the heritability for egg yield in layer breeder were estimated moderate to high (0.21 to 0.48) in previous studies (2, 7, 24, 29). Present findings support these estimations.

There was a quite high genetic correlation (0.58) between body weight at sexual maturity and egg weight (Table 2). Egg weight is strongly correlated with layer age and, consequently, with body weight. Similarly, genetic correlations between age at sexual maturity with brightness and yellowness egg shell were calculated as -0.50 and 0.82, respectively. In spite of different estimations of genetic correlations between egg yield and other performance traits in the literature, it is well known that egg yield is, negatively, correlated with body weight. The negative relation between sexual maturity age with egg yield implies that the improvement in egg yield will be maintained by earlier sexual maturity. In the present study, the monthly egg yields had a positive correlation among them, indicating that short term of egg production can be used selection studies. Genetic correlations among all traits were fairly high, ranging from 0.40 (between first and third month) to 0.86 (between third and fourth month). Kumari et al. (15) estimated similar genetic correlations for cumulative egg yield up to 12, 16, and 20 weeks of age in two quail lines. In the present study, the genetic correlation between second month and total egg yield was fairly high (0.87), in particular when compared to correlation coefficient (0.79) between both first month and total egg yield, third month and total egg yield.

In conclusion, it was seen a low level of genetic correlation between the traits in this study. The heritabilities of sexual maturity age and egg weight were high, which indicates that it is possible to improve Barred Rock pure line rapidly with respect to egg yield and egg weight. There was a wide variation in the Barred Rock line in terms of egg shape index. In the study, the estimated heritability for shape index showed that the uniform line can be obtained by selection of this trait.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Rapid identification of *Klebsiella pneumoniae* isolates from various samples with biosensor and genotyping

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Abstract: The most important cause of *Klebsiella* spp. contamination of drinking water is the leakage of animal faeces into drinking water sources. Recently, the biosensor technology has quickly begun to replace other methods with its faster finding and reliability. The aim of this study was to investigate the reliability of the biosensor technology in the rapid detection of *Klebsiella pneumoniae (K. pneumoniae)* and to determine the presence of the relationship between *K. pneumoniae* isolates isolated from the drinking water thought to be contaminated by animal faeces and the clinical isolates. For this purpose, portable, microfluidic electrochemical sensor device version 2 (V2) was used for the detection of *K. pneumoniae* and results were confirmed with VITEK MALDI-TOF Mass Spectrometry (VITEK MS) automated system. For the molecular typing of *K. pneumoniae* isolates, pulsed-field gel electrophoresis (PFGE) and multiple locus variable-number tandem repeat analysis (MLVA) methods were employed and the results were compared. For these bacteria, the most appropriate typing method was tried to be determined comparatively. PFGE analysis indicated the presence of six different strains, while MLVA divided them into 23 clusters. Clonal relationships were viewed between environmental and clinical isolates. The main goal of this paper is to present, the detailed report of the comparison of the samples isolated from drinking water, animal and human faeces for *K. pneumoniae*. To accomplish of this goal we introduced that MLVA and PFGE methods. Also, gold nanoparticies enhanced electrochemical biosensor device is used for the determination of *K. pneumoniae* for the first time.

Keywords: Biosensor, *K. pneumoniae*, MLVA, PFGE, VITEK MS.

Çeşitli örneklerden elde edilen *Klebsiella pneumoniae* izolatlarının biyosensör ile hızlı teşhisi ve genotiplendirilmesi

Özet: İçme sularında *Klebsiella* türlerine rastlanmasının en önemli sebebi, hayvan dışkılarının içme suyu kaynaklarına sızmasıdır. Son yıllarda, biyosensör teknolojisi hızlı tespit ve güvenilirliği sayesinde diğer yöntemlerin yerini almaya başladı. Bu çalışmada biyosensör teknolojisinin *Klebsiella pneumoniae* (*K. pneumoniae)'* nin hızlı tespitindeki güvenilirliğinin belirlenmesi ve hayvan dışkısı ile kirlenmiş olduğu düşünülen içme suyundan izole edilen *K. pneumoniae* izolatları arasındaki ilişkinin varlığının tespit edilmesi amaçlandı. Bu amaçla *K. pneumoniae* izolatlarının tespiti için, portatif, mikroakışkan, elektrokimyasal biyosensör cihazı sürüm 2 (V2) kullanıldı ve sonuçlar VITEK Matriks aracılı lazer desorpsiyon/iyonizasyon-uçuş zamanlı kütle spektrometresi (VITEK MS) otomatik tanımlama sistemi ile doğrulandı. *K. pneumoniae* izolatlarının genotiplendirilmesinde değişken alanlı jel elektroforezi (PFGE) ve çok lokuslu değişken sayıda ardışık tekrar analizi (MLVA) yöntemleri kullanılarak sonuçlar karşılaştırıldı. İzolatlar PFGE analizi ile 6, MLVA ile ise 23 farklı genotipe ayrıldı. Çevresel ve klinik izolatlar arasında benzer genotiplerin görüldüğü belirlendi. Bu çalışmanın temel amacı; içme suyundan, hayvan ve insan dışkısından izole edilen *K. pneumoniae* örneklerinin MLVA ve PFGE yöntemi ile karşılaştırılmasıdır. Bunun ile birlikte ilk kez *K. pneumoniae* tayini için sinyalleri altın nanopartiküler ile güçlendirilmiş elektrokimyasal biyosensör cihazı kullanılmıştır.

Anahtar sözcükler: Biyosensör, *K. pneumoniae*, MLVA, PFGE, VITEK MS.

Introduction

Strains of *Klebsiella pneumoniae* (*K. pneumoniae)* have been known to be one of the most common Gram-negative pathogens causing pneumonia, septicemia, and urinary tract and soft tissue infections (4).

The detection of bacteria from drinking water is possible with several methods. From past to future with order, cultural methods, enzyme-dependent ELISA method, molecular tests such as reverse transcription-polymerase chain reaction, DNA hybridization and

microarray (28). In recent years, matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF MS) has emerged as a powerful technique for identification. Cultural methods have lengthy and time consuming process (27). Immunological-based methods take 3-4 h to complete (29). These test quantities of 0.1-1 ng of antigen/ml can be detected. Molecular based methods need trained laboratory personnel required for the test performing VITEK MS is faster and less expensive than molecular tests, but the huge size and the high initial cost of the VITEK MS equipment are big disadvantages (27). The biosensor technology is widely used because of the advantages such as the low amount of measurable microliter range, the short time analysis (maximum 10-15 minutes), portable, rapid, no need for a trained personal, user friendly, and most importantly the low detection limit (1, 25).

The most important cause of bacterial contamination of drinking water is the leakage of animal feces into drinking water sources (13). For this reason, the detection of the source of contamination is very significant in terms of taking measures in a short time and preventing the disease. This is also possible with the choice of the most appropriate molecular typing method as much as fast detection of bacteria causing outbreaks. However, the specificity of the used method varies from microorganism to microorganism (11, 31).

Various molecular methods have been used in the molecular typing of *K. pneumoniae* strains (3, 8, 17). Multilocus sequence typing (MLST) method is based on DNA sequencing method and it has been found that the discriminant power and solubility are lower compared to the PFGE method (16, 19, 21, 22). Therefore, fast, easy, relatively inexpensive PCR-based Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) method has been used in molecular typing.

In this study, fast, electrochemical rapid detection device V2 developed by TUBITAK/Bilgem Biosensor and Bioelectronic Group was used to determine the presence of *K. pneumoniae* in drinking water human and animal feces after cultured. The same samples were also studied with the VITEK MS automated system and the results were compared. Molecular typing methods were evaluated to determine their ability to detect clonal relationships among the isolated *K. penumoniae* strains. Until now, MLVA method was used for only several times and results are different from each other. Thus, this study is important in terms of revealing the first detailed report molecular characteristics of *K. pneumoniae* strains from environmental and clinical samples were studied with this article.

Material and Methods

Bacterial strains: Forty-nine isolates were used in the study and the distribution of them according to their origin was as follows: drinking water 19; animal stool 10; human faeces 20 (Table 1).

Detection of bacteria: Suspicious drinking water samples were passed through filter paper and then inoculated to human blood agar. After 24 hours of incubation, VITEK MS method was used to detect colonies. For electrochemical measurement, the liquid suspension formed by phosphate-buffered saline (PBS) medium was taken from the colonies grown in blood agar and adjusted to 100 cfu mL^{-1} concentration. The animal and human stool samples were stored in enrichment medium (GN broth) for 7 hours and subsequently cultivated to sheep blood agar. The VITEK MS (bioMérieux, France) measurement was performed directly from the resulting colonies while the colonies were suspended in PBS medium for electrochemical measurement at 100 cfu mL^{-1}.

***Development of antibody biosensor and biosensor-based electrochemical detection*:** Sensor chip cleaning and self-assembled monolayer (SAM) deposition were optimized in previous studies and standardized for all studies (2, 25). Sensor chips were prepared, vacuum-packed, and stored at +4°C till use.

Table 1. General information for *Klebsiella pneumoniae* isolates.

Source	Location	Strain Numbers
Faeces	IDS	ST 124, 135, 73, 96, 119, 130, 26, 85, 141, 88, 23
Faeces	PS	ST 30, 11, 97, 13, 139, 129, 99
Faeces	EC	ST 128, 36
Water	DHM	ST 112, 21, 81, 83, 80, 50
Water	PHC	ST 7, 16, 5, 136, 19, 1 113, 20, 115, 9, 102, 134, 15
Cattle farm	C1-C10	ST 90, 118, 140, 8, 138, 17, 6, 144, 75, 77

IDS: Infectious Diseases Service, PS: Pediatric Service, EC: Emergency Clinic, DHM: District Health Management, PHC: Public Health Centre.

The 200 nm gold-plated sensor chip surface, which was produced in accordance with the electrochemical sensor device, was immobilized with the *K. pneumoniae* specific monoclonal capture antibody (BioRad, USA). In previous studies, the optimized immobilization protocol was modified using *K. pneumoniae* specific antibody (25). First, the sensor surface was activated with EDS/NHS. The surface was then coated with *K. pneumoniae* specific monoclonal primary antibody. The sensor chip was treated with 10 ug mL^{-1} bovine serum albumin (BSA) and 1M ethanol was used to block the non-binding regions. The gold (Au) chip surface was purged with PBS buffer (pH 7.5) between each step. The immobilized sensor chip was inserted into the electrochemical measuring device. Firstly, the water sample and then the horseradish peroxidase (HRP)-labeled secondary antibody conjugated Au nanoparticle sent to the surface of the sensor chip. Amperometric measurement was then carried out at -0.4V using 3,3', 5,5'-tetramethylbenzidine (TMB) reagents which are specific substrate for HRP enzyme. Afterwards, the *K. pneumoniae* antibody immobilized chip was measured with *Salmonella* spp. *Shigella* spp. *Escherichia coli (E. coli)* for the detection of cross reaction. The measurements were taken with electrochemical sensor device V2. PsTrace program was used as software for measurements (Palmsens, Netherlands).

To prepare of Au nanoparticles, commercially purchased 40 nm diameter liquid gold nanoparticles were mixed in a tube with 2.5 µl HRP enzyme (1 mg mL^{-1}) and 1.5 µl *K. pneumoniae* specific detection antibody (1 mg mL^{-1}) and incubated for 15 minutes at room temperature in a shaker. The supernatant portion was discarded and the pellet was dissolved with 30 µl BSA (10 mg mL^{-1}) and 70 µl Tris buffer (20mM). The prepared mixture was kept at +4 ° C until use.

***VITEK MS*:** Bacteria identification was performed using the VITEK MS (bioMérieux, France). The card AST-N261 was used for identification of isolates. Strains direct transfer method was used for the preparation of the samples. At the end of 24-hours incubation a small amount of bacteria spread onto a single-use target slides and spread over a thin layer 1 µl matrix solution (α-cyano4-hydroxy cinnamic acid (CHCA) matrix solution (bioMérieux, France) was added. The prepared slides were processed in the device and automatically analyzed via the MYLA software (bioMérieux, France). At the ending of the analysis, spectra were obtained (12).

Molecular typing methods: The DNA extraction method was modified (6). The isolates were grown on blood agar. Then, a single colony was taken and added in 5 ml tryptic soy broth. After incubation at 37 ºC for 20 hours, 1.5 ml of this was taken and centrifuged at 15,000 rpm for 10 minutes. The upper liquid was discarded and 500 µl of sterile distilled water was added. The boiling method was used to break down the cells and reveal the DNA. After boiling at 95 °C for 10 minutes, it was centrifuged at 15,000 rpm for 10 minutes to remove cellular debris. After centrifugation, the upper liquid was removed and stored at -20 °C for DNA amplification.

Eight variable number tandem repeat (VNTR) loci were amplified by multiplex polymerase chain reaction (PCR) using all of the oligo primer DNA pairs (Table 2) for MLVA (Multiple - Locus Variable Tandem Repeat) Analysis. Amplifications were performed in 50 µl final reaction volume containing 2 mM $MgCl_2$ (Fermantas, Germany). 0.2 mM each of dNTPs, 10 pmol forward

Table 2. Oligoprimers used for the amplification of VNTRs.

Number of loci	**Target range (Indicesin CP000647) (NC_009648)**	Sequence (Primers (5′–3′)	**Reference**
L1	153111-153861	GGTGGCTCATTTTTTGGC GATAAACACGCGCTAATGACC	Turton et al. (32)
L2	287217-287244	GCAGGTCTCGTCTTCATTCC TGACCATCGAAGAGGCG	Turton et al. (32)
L3	323741-324007	GAGCTGGCGGCTGGAATA GCAATCTGCCCGGAAATA	Turton et al. (32)
L4	557947-558627	AGCGTATCTGCCATTGCC CAGCATGGCCAGTTTGTC	Turton et al. (32)
L5	2077669-2077992	CCAAATCCGGGTATTTATCG TTCGATACCCATCCGGAAG	Turton et al. (32)
L6	4176549-4177448	ATGACCAAGGAAGAACCCG CTTTACCTGGCATGCGAACG	Turton et al. (32)
L7	4323141-4323168	CAGGATCACCGATATTTTGCG GATCGGCGGCAGGTTGAG	Turton et al. (32)
L8	4934720-4935748	ACCGGATTAAGCGCTATTCC TTCCTCGCCCACGGATAG	Turton et al. (32)

primer, 10 pmol reverse primer, 5 µl template DNA, and 0.2 µl Taq DNA polymerase (Fermantas, Germany). An initial denaturation cycle, for 5 min 96 °C, was followed by 30 reaction cycles, each comprising 30s denaturation at 96 °C, 30s annealing at 60°C, and 30s extension at 70 °C. The reaction program ended with a final 10 min extension step at 72 °C. The amplification products were run in a 2% agarose gel and their size was estimated by comparison with a DNA ladder marker (Fermantas, Germany). Amplicons were also analyzed by capillary gel electrophoresis (fragment analysis) in a Beckman-Coulter CEQ8000 sequencer. Fragment size analysis was performed using the PeakScanner software (Beckman-Coulter CEQ8000). Allele description was noted into the MLVA plug-in of the BioNumerics software (version 6. 01, Applied Maths, Belgium).

Chromosomal DNA of the isolates was analyzed by PFGE as described (9). Chromosomes were digested in agar plugs with *Xba*I (Fermentas, Germany), and resulting DNA fragments were resolved in a PFGE CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA) for 20 h at 6V/cm2 in 0.5 × Tris-borate-EDTA buffer at 14 ºC. The initial and final switch times were 5s and 30s. The banding patterns were then analyzed with BioNumerics software (Version 6.01, Applied Math, Sint-Maten-Latem, Belgium). Cluster analysis was performed by the Dice similarity coefficient and unweighted pair group method using arithmetic average (UPGMA) dendrogram (optimization 1.0 %, position tolerance 1.5%). Strains were categorized as different, closely related, or indistinguishable, based on the criteria of Tenover et al. (30).

Results

Gold nanoparticle enhanced antibody sensor for K. pneumoniae detection: The optimum HRP concentration in the electrochemical sensor was investigated in previous studies and 12 ng mL^{-1} HRP concentration was decided optimum for enhanced gold nanoparticle antibody sensor for *K. pneumoniae* detection (25). A new immobilized chip was used for each measurement with each sample. In Figure 1, the electrochemical measurements of 8 samples were overlapped and the signals between 77-155 nA were considered as indicators of the presence of *K. pneumoniae*. The colony suspension prepared in 100 cfu mL^{-1} in PBS was tested individually with chips coated with *Salmonella enterica*, *Shigella* spp, *E. coli* and *K. pneumoniae* specific antibody. The same samples showed a signal between 1-5 nA in *Salmonella enterica*, *Shigella* spp and *E.coli* specific antibody coated chips. This value shows that it is not one of these microorganisms (Figure 2). The results were also studied and validated using the VITEK MS method.

Identification with VITEK MS: Strains were analyzed by the VITEK MS system. As a result, ionizable cell surface components' spectra were compared with VITEK MS biotyping software's spectra. Peaks of several samples that were selected randomly were shown in Figure 3. *K. pneumoniae* were identified in all samples isolated from water, human and animal by the VITEK MS automated identification system with a similarity rate of 99.9%.

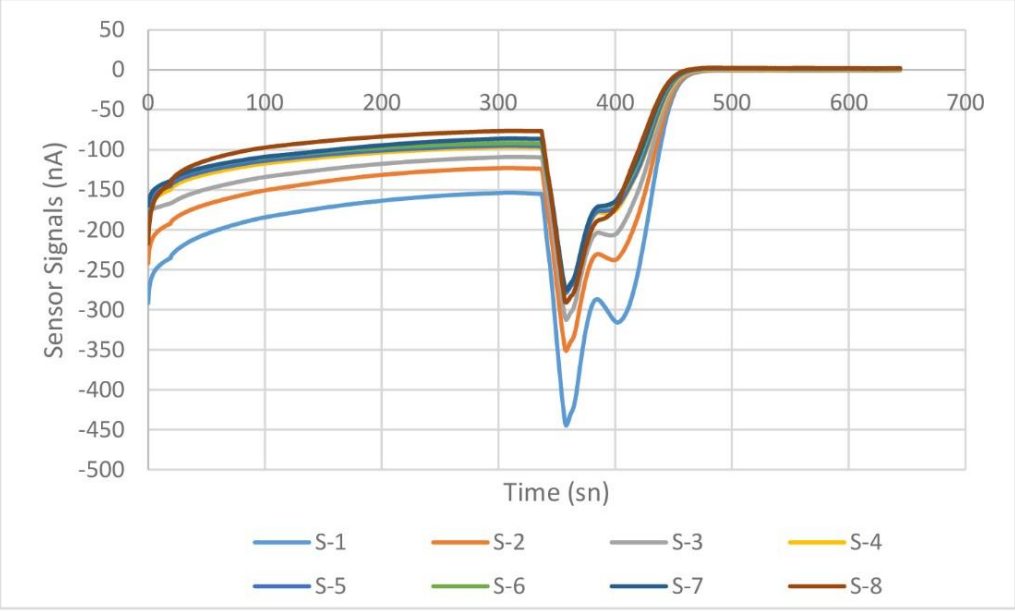

Figure 1. The electrochemical measurements of random 8 samples were overlapped and the signals were between 77-155 nA.

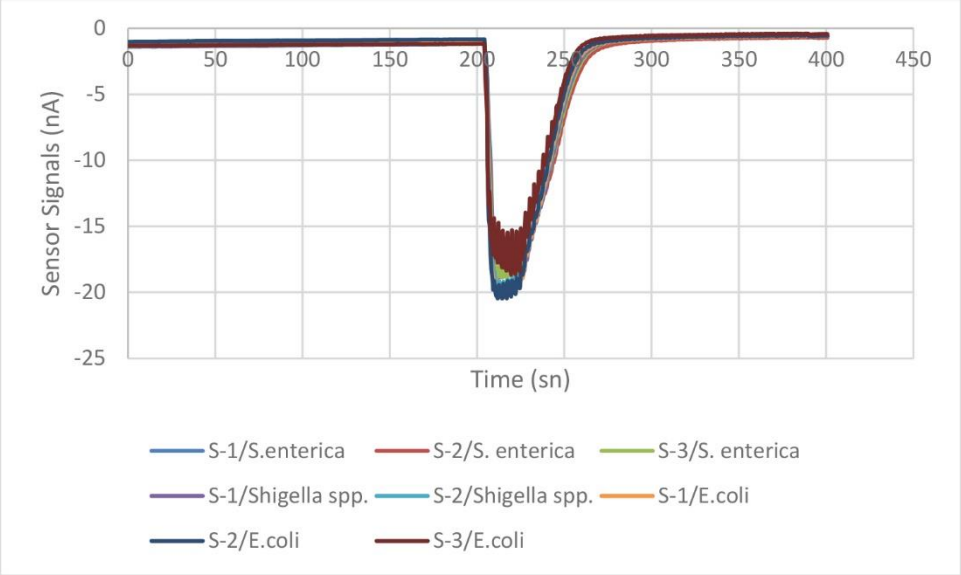

Figure 2. The same samples showed a signal between 1-5 nA in cross reaction with *Salmonella enterica*, *Shigella* spp and *E.coli*.

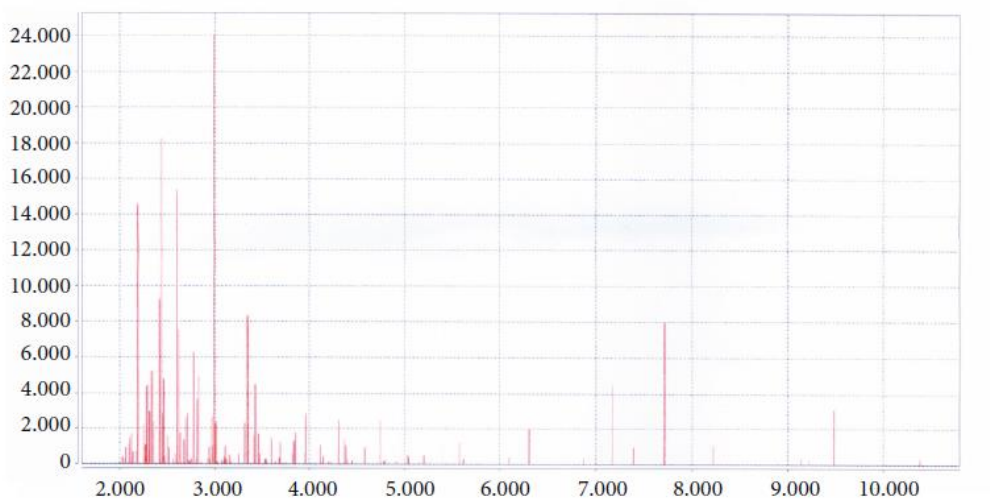

Figure 3. VITEK MS spectra of 99.9% *K. pneumoniae.*

MLVA typing: Eight variable number tandem repeats distributed the 49 isolates into 23 MLVA patterns (Figure 4a). Sixteen of these patterns were represented only by a single isolate while the remaining contained between 2 and 5 members. The number of repeats found within each of the loci was also determined from the amplicon lengths and by sequencing. The number of repeats was 4.8, 2.8, 7.3, 8.3, 2.3, 2.0, 2.0, and 4.5 for the eight loci, respectively.

PFGE analysis: *K. pneumoniae* strains produced six different PFGE pulsotypes (A-F), containing 23-25 bands following the digestion *Xba*I restriction enzyme. These were obtained using "dice" similarity coefficient and UPGMA program (Figure 4b). The six major PFGE pulsotypes (A-F) contained 2.04%, 36.7%, 4.0 %, 30.61%, 2.04%, and 24.4% of all the isolates, respectively. The similarity rate (A, B, C, D) between for pulsotype was over 95% and they were identic with each other. Pulsotype E had only one member. Similarity within each of these five pulsotypes was higher than 90%. The PFGE pattern of E had three fewer bands, and displayed a varying similarity to the others (A, B, C, D), ranging from 95% to 85%. Pulsotype F was found to be unrelated to all other clusters. *K. pneumoniae* strains isolated from the same cattle farm at different times were grouped into the same pulsotype with the water sample isolates and some human source isolates. PFGE patterns obtained from different sources of drinking water at different time points enabled the identification of a single patient as the source of wide-spread infection in outpatient clinics and services. A single animal isolate (ST128) had specific MLVA and PFGE profiles and was found to be an independent subtype. In summary, the cause of *K. pneumoniae* found in the drinking water of the city was found to be a similar genotype pattern between animal and human feces and drinking water.

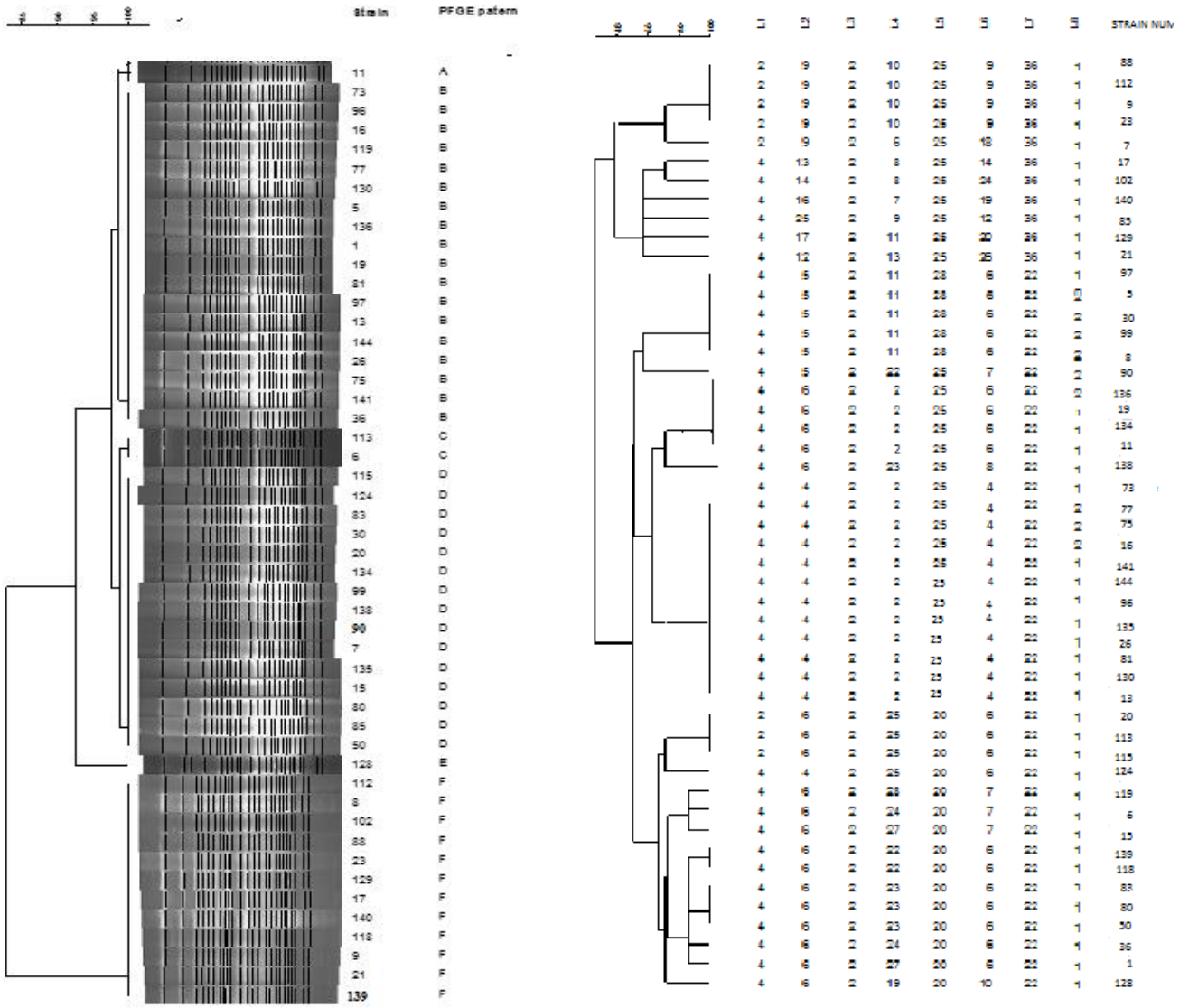

Figure 4. a) Dendogram of *K. pneumoniae* isolates showing percent similarity calculated by the Dice similarity of PFGE (XbaI) restriction endonuclease digestion, constructed using UPGMA algorithm (BioNumerics version 6.01), b) Dendrogram of MLVA results

Discussion and Conclusion

The general habitat of *K. pneumoniae* is people, animals, sewage, polluted waters and soils. Fecal contamination from human or animal is known to be the most important route to its transmission.

In the last decade, VITEK MS was developed for the identification of microorganisms (18). In the past, conventional methods and VITEK MS were compared and their results were verified by 16S sequencing in the detection of isolated strains of *K. pneumoniae* from different sources. VITEK MS results were consistent with sequence results (12). Based on this study, the results obtained with VITEK MS method were compared with the results biosensors based on electrochemical sensor device V2 (TUBITAK/Bilgem) which has more reliability, more reproducibility and takes a shorter amount of time compared to other identification methods. The fact that the results are completely matched to the VITEK MS method. Therefore, it can be assumed that this device may be appropriate in cases requiring rapid detection in the field of microbiology. Sub-versions of the "electrochemical sensor machine" were used before for the detection of *E. coli*. The quantification of *E. coli* in water samples was investigated with standard and nanomaterial amplified immunoassay which resulted in LODs of 1.99×10^4 cfu mL^{-1} and 50 cfu mL^{-1}, respectively (2). The last V2 was used respectively for the detection of bacteria (25). *Salmonella Typhimurium* (*S. typhimurium*) was analyzed in a concentration range of $1–2.48\times10^6$ cfu mL^{-1} that resulted in a LOD of 12 cfu mL^{-1} using the antibody biosensor. The specificity of immunoassay was tested by studying with non-specific bacteria including *E. coli* and

Staphylococcus aureus (*S. aureus)* that revealed only 2.01% and 2.66% cross-reaction. The quantification of *Salmonella* DNA was investigated in a concentration range of 0.002-200 μM that demonstrated very high specificity and sensitivity with a LOD of 0.94 nM. Also Zhang et al. (2017) was developed a label-free DNA hybridization electrochemical sensor for the detection of *K. pneumoniae* and could detect target DNA down to 3 × 10-11 M . Kumar et al., (2015) have focused on the fabricated of electrochemical biosensor for the detection of *K. pneumoniae* and detected 3.0×10^7 cfu/50 ml in PBS. When we compare our study with these studies, it was thought that the measured limit value was lower and the applied process was easier.

Biosensor technology was used in the detection of pathogens and converted into various devices (23, 24, 26).

There have been several studies that have employed different methods for the identification of various pathogenic bacteria. Discrimination power of these methods, at strain level, plays a crucial role in understanding the pathogen diversity and identifying the source of infection. For example, it has been reported that the discriminatory power of the PFGE method was insufficient because of the high homogeneity of the genomic structure of *Bacillus anthracis* strains and that MLVA differentiated the same strains with much better efficiency (34). In another study in which *Acinetobacter baumannii* strains have been identified by both MLVA and PFGE, comparison of the results has argued that MLVA had better discriminating power for these bacteria (11). Similar data have been produced for the typing of *Staphylococcus aureus* strains (16). On the other hand, MLVA and PFGE displayed similar efficiencies in the discrimination of *Listeria monocytogenes* strains. (31).

So far, there have been many studies on the use of MLST and PFGE molecular typing methods for *K. pneumoniae* (15, 20). However, the MLST method requires intensive laboratory work and it can be practiced in reference and research laboratories. The PFGE method, on the other hand, maintains its most reliable feature as gold standard method. However, the PFGE method is also time consuming and expensive to install in a laboratory. The MLVA method can be completed within 6 hours, although a standard protocol with PFGE method for typing *K. pneumoniae* takes approximately 3 days. The MLVA method, which is an established method of amplification, can be successfully applied in epidemics that develop in a short time. If infrastructure exists, confirmation of the results can be done by PFGE method. In the molecular typing of *K. pneumoniae* strains, the number of articles which compared with the PFGE method and the MLVA method was inadequate and different results were obtained. Researchers investigated the source of KPC-2-producing *K. pneumoniae* infections and inter-hospital spread with MLVA, MLST and PFGE methods and achieved the same results with 3 methods (20). *K. pneumoniae* isolates with MLVA and MLST methods and indicated that both methods have equal resolution but MLVA is more suitable for laboratory use of in terms of its low cost and labor power (5, 10). These studies support our work but MLST is known stronger than PFGE typing method. Despite this, The discriminant power of the PFGE method, which was studied by the MLVA and PFGE method and PFGE was much higher in the MLVA method in a study (7). However, contrary to our study, the results of both methods overlap with each other, but MLVA method has been shown to have a greater proportion of the discrimination power for *K. pneumoniae.*

When water and animal source *K. pneumoniae* samples are examined by molecular typing methods, similar types of *Klebsiella* isolates isolated from different sources suggests that microorganism spreading from animal source enters drinking water. There is a need for frequent control of the water network of the provincial network and the microorganisms that are rapidly spreading in the hospital and which have acquired antibiotic resistance should be controlled.

To our knowledge, this study represents the detailed report of the comparison of the samples isolated from drinking water, animal and human faeces for *K. pneumoniae*. Also, gold nanoparticle enhanced electrochemical biosensor device is used for the determination of *K. pnemoniae* for the first time and the results were compared with VITEK MS automated system. Because of its advantages such as fast detection, portable, ease of use and lack of need for experienced personnel, it is thought that this application with biosensor based electrochemical sensor device V2 may be important for microbiology laboratory and field work. In addition, genetic relationship among the strains was determined by PFGE and MLVA. This data provides better understanding of the molecular characteristics and their transmission dynamics in the city, emphasizing the need for implementation of efficient control measures for disease prevention.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Determination of antibiotic susceptibility, ESBL genes and pulsed-field gel electrophoresis profiles of extended-spectrum β-lactamase-containing *Escherichia coli* isolates

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Abstract: The purpose of this study was to determine the phenotypic antibiotic susceptibility patterns, extended-spectrum β-lactamase (ESBL) genes and genotypic profiles of ESBL-positive *Escherichia coli* strains isolated from urine samples obtained from outpatients with urinary tract infection in Turkey. A total of 120 *E. coli* strains during 2017, 2018, and 2019 (40 patients per year) were examined for antibiotic susceptibility patterns by disc diffusion method, for ESBL genes using PCR and sequencing and for molecular typing by pulsed-field gel electrophoresis (PFGE) method. The isolates were evaluated for their sensitivity to 21 different antibiotics. Four different antimicrobial resistance patterns were determined according to antibiotic susceptibility status of the isolates. The β-lactamase genes detected in the isolates were CTX-M-15 + OXA-1 (n= 14), CTX-M-15 (n= 24), TEM-1 + CTX-M-15 (n= 52), TEM-1 + SHV-12 (n=6), SHV-12 1 (n= 6), TEM-1 + CTX-M-1 (n= 6), TEM-1 + CTX-M-16 (n= 6) and TEM-1 + CTX-M-9 (n= 6). The CTX-M-15 was the most prevalent ESBL enzyme in the isolates. As a result of PFGE analysis performed by XbaI enzyme restriction process, one major PFGE profile and three main groups (Group I-II-III) were observed. While antibiotic resistance profiles of the strains showed four groups (RI-RII-RIII-RIV), PFGE band profiles showed a major group (90% similarity ratio). High ESBL production and decreased susceptibility to broad-spectrum cephalosporins were observed in *E. coli* strains. In addition, PFGE analysis showed high clonal similarity among *E. coli* isolates.

Keywords: Antimicrobial drug resistance, ESBL genes, *Escherichia coli*, molecular subtyping, pulsed-field gel electrophoresis.

Genişlemiş spektrumlu β-laktamaz üreten *Escherichia coli* izolatlarının antibiyotik duyarlılıklarının, GSBL genlerinin ve pulsed-field jel elektroforez yöntemiyle genotipik profillerinin belirlenmesi

Özet: Bu çalışmada Türkiye'de idrar yolu enfeksiyonu bulunan hastalardan alınan idrar örneklerinden izole edilmiş olan genişlemiş spektrumlu β-laktamaz (GSBL) pozitif *E. coli* suşlarının antibiyotik duyarlılık paternlerinin (fenotipik), GSBL genlerinin ve genotipik profillerinin belirlenmesi amaçlandı. Çalışmada 2017, 2018 ve 2019 yıllarında ve her yıl 40 hastadan olmak üzere, toplam 120 adet *E. coli* suşunun disk difüzyon yöntemi ile antibiyotik duyarlılıkları, PCR ve sekanslama ile GSBL genleri, pulsed-field jel elektroforez (PFGE) yöntemi ile moleküler tipleri belirlendi. Numunelerden elde edilen izolatların 21 farklı antibiyotiğe karşı duyarlılığı değerlendirildi. İzolatların antibiyotik duyarlılık durumlarına göre, değerlendirmede dört farklı antimikrobiyal direnç paterni tespit edildi. İzolatlarda β-laktamaz genleri olarak CTX-M-15 + OXA-1 (n= 14), CTX-M-15 (n= 24), TEM-1 + CTX-M-15 (n= 52), TEM-1 + SHV-12 (n= 6), SHV-12 1 (n= 6), TEM-1 + CTX-M-1 (n= 6), TEM-1 + CTX-M-16 (n= 6) ve TEM-1 + CTX-M-9 (n= 6) tespit edildi. CTX-M-15 izolatlarda en yaygın görülen GSBL enzim tipi olarak belirlendi. PFGE analizi sonucunda, bir majör PFGE profili ve üç ana grup (Grup I-II-III) gözlendi. Suşların antibiyotik direnç profilleri, dört grupta (RI-RII-RIII-RIV) bulunurken, PFGE bant profilleri ise bir majör grup içinde bulunduğu (% 90 benzerlik oranı) belirlendi. Numunelerde *E. coli* yüksek GSBL üretimi ve geniş spektrumlu sefalosporinlere karşı azalan bir duyarlılık gözlendi. Ayrıca, PFGE analizi ile bu izolatların yüksek klonal benzerliğe sahip olduğu da gösterildi.

Anahtar sözcükler: Antimikrobiyal direnç, GSBL genleri, *Escherichia coli*, moleküler alt tiplendirme, pulsed-field jel elektroforez.

Introduction

Escherichia coli is a commensal bacteria of the digestive tract microflora of humans and animals, some of them cause intestinal and extraintestinal pathologies (25). Animals are defined as important zoonotic reservoirs for human intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) causing diseases in farm and pet animals (4, 53). Extraintestinal pathogenic *E. coli* is an important cause of diverse infections, which includes urinary tract infections (UTI) in human and animals (26, 32, 53). UTI is a significant bacterial infection that causes serious complications including emphysematous cystitis and pyelonephritis when infection is inadequately managed. Although most of the patients face a single or rare episode of the disease, many patients experience recurrent UTIs (19, 50). Approximately 80 % of patients who suffer from UTI is caused by *E. coli* and *Staphylococcus saprophyticus* (15).

Antibiotic resistance leads to failure in the treatment of both community- and hospital-acquired infections and appears to be a growing problem worldwide. The application of antibacterial drugs in clinical therapy results in the emergence of microorganisms that are resistant to these drugs. One of the most common bacterial-resistance mechanism against antimicrobial drugs is the inactivation of the drug by the enzymes they synthesize (8). β-lactamases are enzymes that are produced by bacteria providing multiple resistance to β-lactam compounds by hydrolyzing the β-lactam ring in these antibiotic groups. The prolonged exposure of bacterial strains to a large number of β-lactam antibiotics has increased their activities by inducing mutation of the β-lactamases, which are known as extended-spectrum β-lactamases (ESBLs), against the third-generation cephalosporins (42). Types of ESBL generated by mutations in genes coding the narrow-spectrum β-lactamases (TEM-1, TEM-2, or SHV-1) are TEM, SHV, CTX-M, OXA and Amp C. The most frequently identified ESBL genes produced by *E. coli* and *Klebsiella* spp. are TEM and SHV (34). The emergence of ESBL-producing bacteria has been commonly reported in veterinary medicine since β-lactam antibiotics have been used mostly for therapeutic and prophylactic reasons in livestock (35, 47). Several studies showed that similar ESBL isolates were found in human and livestock, suggesting a zoonotic transfer (11, 22, 28).

The emergence of antibiotic resistance is accelerated by the overuse and misuse of antibiotics and the lack of development of new antimicrobial drugs (48). Antimicrobial agents are widely used for therapeutic or nontherapeutic purposes in animal husbandry. Use of these drugs results in selection for antimicrobial resistant *E. coli* in the microflora of these animals. Subsequently, antimicrobial-resistant *E. coli* can be transferred from animals to humans through cross-contamination or consumption of raw or insufficiently cooked meat contaminated with antimicrobial resistant bacteria (4, 38).

Pulsed-field gel electrophoresis is a molecular fingerprinting method considered the "gold standard" among molecular typing methods to classify bacteria. The method is based on the determination and interpretation of the profiles formed by the appropriate restriction endonuclease enzyme of genomic DNA isolated from the bacterial cell embedded in low melting agarose without deterioration of the structural integrity. PFGE technique has been used safely in the typing of many bacteria such as *Salmonella typhimurium*, *Neisseria gonorrhoeae*, methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, and *E. coli* (12, 14, 16, 20, 49).

The purpose of the present study was to determine the antibiotic susceptibility patterns and genotypic profiles of ESBL positive *E. coli* strains isolated from urine samples collected between 2017 and 2019 and also determine the prevalence of ESBL genes among the isolates. The most effective antibiotic selection was provided for the empirical treatment of *E. coli*-induced UTI by forming an antibiotic susceptibility pattern of *E. coli*. In addition, by determining the possible clonal relationship between isolates via PFGE analysis, the similarity of antibiotic susceptibilities of strains with common band profiles was investigated.

Material and Methods

***Sample collection*:** A total of 120 *E. coli* isolates obtained from UTI patient's urine samples collected from outpatients (n= 120) in Public hospital in Konya province of Turkey between 2017-2019 were evaluated in the present study. Patient data anonymized in this study. These samples were collected with collection containers and transported to the microbiology laboratory.

***Isolation and identification of Escherichia coli*:** *E. coli* isolates were incubated in Eosin Methylene-blue (EMB) and Nutrient agar (NA) media overnight at 37ºC by a single colony incubation technique. Colonies which appeared metallic sheen were again fished out into nutrient broths and subcultures were maintained on nutrient agar and used for further identification and antimicrobial sensitivity testing. A single colony picked up and identified as *E. coli* using IMVIC test (citrate, methyl red, Voges-Proskauer, citrate, ornithine, urea, indole, kligler iron agar media). The strains were confirmed as *E. coli* using with gram-negative crystal identification kit (BBL Crystal ID System, Becton Dickinson, Cockeysville). ESBL production was confirmed if the presence of a β-lactamase inhibitor enlarged, the zone size of inhibition by ≥5 mm for all 120 isolates (10).

***Antimicrobial susceptibility testing*:** Disc diffusion method according to Clinical and Laboratory Standards Institute (10) was used for antibiotic susceptibility test.

Tested antibiotics (BBL, Becton Dickinson) were cefoxitin (FOX, 30 µg), cefotaxime (CTX, 30 µg), cefepime (FEP, 30 µg), ceftazidime (CAZ, 30 µg), cefazolin (CFZ, 30 µg), cephalothin (CEF, 30 µg), cefuroxime (CXM, 5 µg), ampicillin (AMP, 10 µg), ampicillin-sulbactam (SAM, 10 µg), amoxicillin-clavulanate (AMC, 30 µg), imipenem (IPM, 10 µg), piperacillin (PIP, 100 µg), trimethoprim-sulfamethoxazole (SXT, 23.75 µg/1.25 µg), ofloxacin (OFX, 5 µg), amikacin (AMK, 30 µg), gentamicin (GEN, 10 µg), sulfisoxazole (SXZ, 0.25 µg), nitrofurantoin (NIT, 100 µg), piperacillin-tazobactam (TZP, 110 µg), ticarcillin-clavulanate (TIM, 85 µg), carbenicillin (CAR, 100 µg). Quality control was performed with *E. coli* ATCC 25922 strain. Isolates were grouped as resistant (R), intermediate-resistant (I), or susceptible (S) according to the CLSI (10).

***Characterization of ESBL genes*:** The presence of genes encoding TEM, SHV, OXA and CTX-M type β-lactamases was examined in this study. PCR screening for the presence of different β-lactamase genes, *bla*TEM-type, *bla*CTX-M-type, *bla*SHV-type and *bla*OXA-1-type, were performed as described previously (6, 7, 24). Amplicons obtained from PCR were sequenced on both strands and sequences were compared to those reported in the Database of the GenBank and on the Lahey Clinic beta-lactamase website (http://www.lahey.org/Studies/) to identify the β-lactamase genes.

***PFGE analysis*:** The isolates, which were identified as *E. coli* by biochemical and molecular methods and confirmed as ESBL-producing *E. coli* by antibiotic susceptibility test, were subjected to PFGE to analyze the genetic diversity of them in order to investigate genetic similarities. A single colony of each isolate was suspended with CSB buffer (cell suspension buffer, 10 mM Tris-HCl, 50 mM EDTA, 20 mM NaCl, pH 7.2). 2 % low-melting agarose (LMA, Sigma-Aldrich) was prepared in the CSB buffer supplemented with Sodium dodecyl sulfate (a final concentration of 1 %) (SDS, Sigma-Aldrich). The agarose-buffer mixture was melted by heating in magnetic stirrer to 45-50°C. Bacteria suspension was added to the agarose tubes by means of a pipette, and the pipette was used for mixing the suspension. This mixture was transferred to plug molds (10mm x 5mm x 1.5mm, Bio-Rad) and after solidification of agarose for the preparation of high-quality DNA, the plugs were incubated overnight at 55°C in lysis buffer (50 mmol Tris-HCI, pH 8.0; 50 mmol EDTA, pH 8.0; 1 % sarcosine; 1 mg of proteinase K/ml). The agarose was washed three times with sterile distilled water, followed by three washes with TE buffer (10 mmol Tris, pH 8.0; 1 mmol EDTA, pH 8.0) Agarose-embedded DNA was transferred to the mixture containing XbaI restriction enzyme (Thermo Scientific), and incubated to digest at 37°C for 2 hours. PFGE was carried out with the CHEF-DR® II system (Bio-Rad Laboratories, Nazareth, Belgium) using a 1 % of pulsed-field certified agarose prepared in standard 0.5xTris-boric acid-EDTA (TBE buffer). The electrophoresis condition was set as follows: Initial switch time, 2 s; final switch time, 35 s, run time, 20 h; gradient, 6V/cm; angle 120°; temperature, 14°C. After electrophoresis, the gel was stained by putting into 400 ml ultrapure water solution containing 5 µg/ml ethidium bromide for 20 minutes and the fingerprinting profile was photographed under ultraviolet light using a Gel Logic 220 imaging system (Kodak Company, USA). The band profiles were analyzed using the Gel Compar II software system (version 3.0, Applied Maths, Sint-Martens-Latem). First of all, three external standard strains (1, 7, 15, carried out in wells, *E. coli* ATCC 25922 strain) in each gel were used to normalize the images. Dendrograms and clustering analysis of PFGE profiles were performed using "the unweighted-pair group method with mathematical averaging" (UPGMA), the Dice coefficient with a 1-1.5 % band position tolerance and optimization. The interpretation of PFGE patterns was categorized as follows: indistinguishable, closely related, possibly related, or different according to the criteria of Tenover et al. (44).

Results

All isolates were tested for antibiotic resistance using Disc diffusion method. Table 1 shows the resistant

Table 1. Antibiotic resistance profiles for *E. coli* isolates.

Resistance phenotype	Resistance pattern	Isolate number	%
RI	SXT, OFX, AMP	1,2,3,5,6,7,8,9,10,13,14,15,17,18,19,20,21,22,25,26,28,34,35,38,39,43,47,51,56,59,63, 69,73,74,78,81,84,88,92,96,97,99,105,106,107,108,109,110,111,112,113,114	43
RII	AMC, TZP, TIM	42,46,50,54,57,60,64,66,67,71,72,77,80,85,87,91,93,94,95,98 100,101,102,103,104,115,116,117,118,119,120	26
RIII	GEN, AMK, CAR, PIP	4,12,16,23,27,29,33,37,41,45,49,52,61,62,65,68,70,75,76,79,82,83,86,89,90	21
RIV	NIT, G25, IMP	11,24,30,31,32,36,40,44,48,53,55,58	10

SXT: Trimethoprim-sulfamethoxazole; OFX: Ofloxacin; AMP: Ampicilin; AMC: Amoxicillin-clavulanate; TZP: Piperacillin-tazobactam; TIM: Ticarcillin-clavulanate; GEN: Gentamicin; AMK: Amicasin; CAR: Carbenicillin; PIP: Piperacillin; NIT: Nitrofurantoin; G25: Gentamicin high resistance; IMP: Imipenem.

phenotypes of *E. coli* isolates. Among the isolates, the predominant resistance profile was RI (SXT-OFX-AMP). Approximately 43 % of all isolates were RI phenotype, whereas isolates with RII phenotype (Combinations of β-lactam-β-lactam inhibitors) accounted for 26 %. 21 % of all isolates were resistant to 4 antibiotics belonging to RIII phenotype (GEN, AMK, CAR, and PIP). Aminoglycoside antibiotics (GEN, AMK) were considered as the most important ones in this group. Isolates (10 %) which are resistant to other antibiotics were classified as RIV phenotype (NIT, G25, and IMP).

In the PFGE study for genotypic typing, after the *E. coli* DNAs were cut with FastDigest XbaI enzyme with restriction endonuclease activity, PFGE gel images in which various band patterns were formed were determined. Dendrogram analysis was performed in the next stage of gel images of *E. coli* strains in which PFGE band profiles were observed. After the band profile analysis using Gel-Compare-II, PFGE profile dendrograms were formed and the relationships between the strains were determined according to the Dice similarity coefficient. When the dendrogram of 120 *E. coli* strains of 2017, 2018 and 2019 were examined; based on Tenover criteria (44), 120 strains were found to be related to each other according to 85% and higher similarity rates. Although there is only one major clone, the strains are divided into three pulsotypes. Group I (90-3/113 strains), Group II (51-81/2 strains), Group III (120-61-67-64-66/5 strains) (Figure 1).

After all the studies, epidemiological data were obtained from the clinical files of the patients in order to correlate all the data with significant results. In the present study that examined patients admitted to the hospital at different times, a data (Table 2) with information about epidemiological information, antimicrobial susceptibility patterns and PFGE types of 120 isolates collected from 2017 to 2019 were obtained.

Group I (113 strains) and Group III (5 strains) were the most common PFGE profile groups in the genotypic investigation of *E. coli* strains. Among the 113 strains in Group I, 50 had RI resistance and had SXT, OFX and AMP resistance. In addition, 27, 24, and 12 strains had RII, RIII and RIV resistances, respectively. The two identical strains in Group II showed the same antibiotic resistance pattern (RI). The 4 strains in Group III show the same antibiotic resistance pattern (RII). Furthermore, there is also a strain with RIII resistance (Table 2).

All the phenotypic ESBL-producing *E. coli* isolates were confirmed by PCR and sequencing for detection of genes encoding TEM, SHV, OXA and CTX-M type β-lactamases. The β-lactamase genes detected in the isolates were CTX-M-15 + OXA-1 (n= 14), CTX-M-15 (n= 24), TEM-1 + CTX-M-15 (n= 52) , TEM-1 + SHV-12 (n= 6),

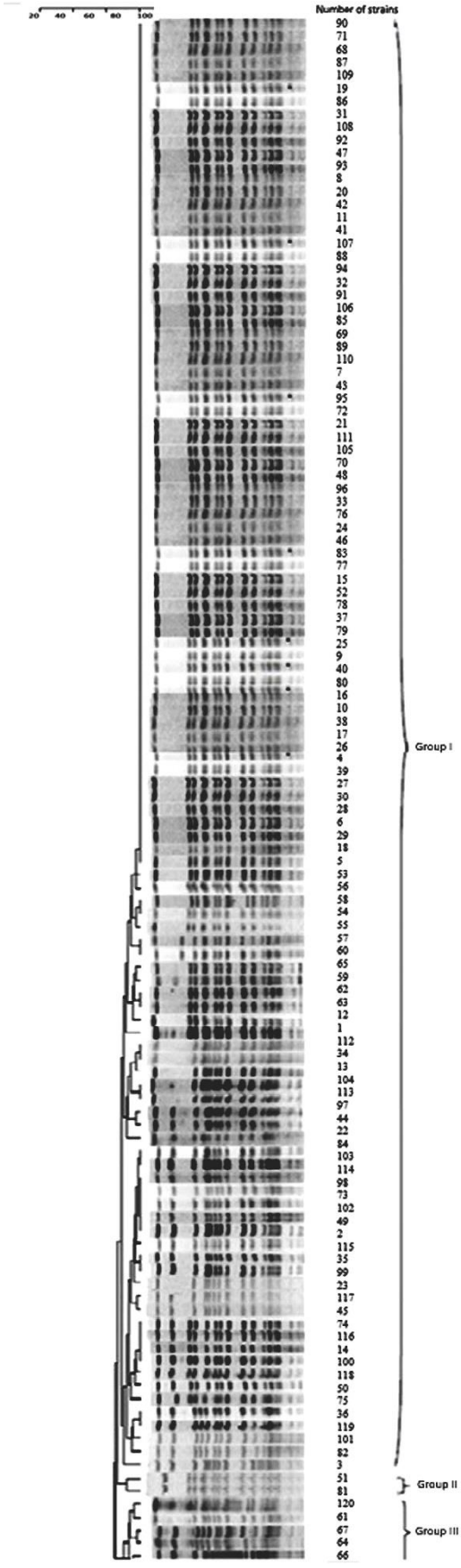

Figure 1. PGFE XbaI digestion patterns and clonal analysis of 120 ESBL-producing *E. coli* isolates (2017-2019) (1-120). The dendrogram using the Dice coefficient and UPGMA clustering methods showing the relationships between *E. coli* strains.

Table 2. Comparison of PFGE and antibiotic resistance profiles of *E. coli* strains.

Isolation year	Isolates number	*bla* gene(s)	Antibiotic resistance phenotype	PFGE profile
2017	1	CTX-M-15, OXA-1	RI	Group I
2017	2	CTX-M-15, OXA-1	RI	Group I
2017	3	CTX-M-15, OXA-1	RI	Group I
2017	4	CTX-M-15	RIII	Group I
2017	5	TEM-1, SHV-12	RI	Group I
2017	6	CTX-M-15, OXA-1	RI	Group I
2017	7	CTX-M-15, OXA-1	RI	Group I
2017	8	CTX-M-15, OXA-1	RI	Group I
2017	9	CTX-M-15, OXA-1	RI	Group I
2017	10	SHV-12 1 (4)	RI	Group I
2017	11	TEM-1, CTX-M-1	RIV	Group I
2017	12	CTX-M-15	RIII	Group I
2017	13	TEM-1, SHV-12	RI	Group I
2017	14	TEM-1, CTX-M-1	RI	Group I
2017	15	TEM-1, CTX-M-15	RI	Group I
2017	16	CTX-M-15	RIII	Group I
2017	17	CTX-M-15, OXA-1	RI	Group I
2017	18	CTX-M-15, OXA-1	RI	Group I
2017	19	CTX-M-15, OXA-1	RI	Group I
2017	20	SHV-12 1 (4)	RI	Group I
2017	21	TEM-1, CTX-M-15	RI	Group I
2017	22	TEM-1, CTX-M-16	RI	Group I
2017	23	CTX-M-15	RIII	Group I
2017	24	TEM-1, CTX-M-15	RIV	Group I
2017	25	CTX-M-15, OXA-1	RI	Group I
2017	26	CTX-M-15, OXA-1	RI	Group I
2017	27	CTX-M-15	RIII	Group I
2017	28	TEM-1, SHV-12	RI	Group I
2017	29	CTX-M-15	RIII	Group I
2017	30	SHV-12 1 (4)	RIV	Group I
2017	31	TEM-1, CTX-M-1	RIV	Group I
2017	32	TEM-1, CTX-M-15	RIV	Group I
2017	33	CTX-M-15	RIII	Group I
2017	34	TEM-1, SHV-12	RI	Group I
2017	35	TEM-1, CTX-M-15	RI	Group I
2017	36	TEM-1, CTX-M-15	RIV	Group I
2017	37	CTX-M-15	RIII	Group I
2017	38	CTX-M-15, OXA-1	RI	Group I
2017	39	CTX-M-15, OXA-1	RI	Group I
2017	40	SHV-12 1 (4)	RIV	Group I
2018	41	CTX-M-15	RIII	Group I
2018	42	TEM-1, CTX-M-15	RII	Group I
2018	43	TEM-1, SHV-12	RI	Group I
2018	44	TEM-1, CTX-M-15	RIV	Group I
2018	45	CTX-M-15	RIII	Group I
2018	46	TEM-1, CTX-M-15	RII	Group I
2018	47	TEM-1, CTX-M-9	RI	Group I
2018	48	TEM-1, CTX-M-15	RIV	Group I
2018	49	CTX-M-15	RIII	Group I
2018	50	TEM-1, SHV-12	RII	Group I
2018	51	SHV-12 1 (4)	RI	Group II
2018	52	TEM-1, CTX-M-15	RIII	Group I
2018	53	CTX-M-15	RIV	Group I
2018	54	TEM-1, CTX-M-15	RII	Group I
2018	55	TEM-1, CTX-M-1	RIV	Group I
2018	56	TEM-1, CTX-M-15	RI	Group I
2018	57	SHV-12 1 (4)	RII	Group I
2018	58	TEM-1, CTX-M-15	RIV	Group I
2018	59	TEM-1, CTX-M-16	RI	Group I
2018	60	TEM-1, CTX-M-15	RII	Group I

2018	61	CTX-M-15	RIII	Group III
2018	62	CTX-M-15	RIII	Group I
2018	63	TEM-1, CTX-M-15	RI	Group I
2018	64	TEM-1, CTX-M-15	RII	Group III
2018	65	CTX-M-15	RIII	Group I
2018	66	TEM-1, CTX-M-9	RII	Group III
2018	67	TEM-1, CTX-M-15	RII	Group III
2018	68	TEM-1, CTX-M-15	RIII	Group I
2018	69	TEM-1, CTX-M-15	RI	Group I
2018	70	CTX-M-15	RIII	Group I
2018	71	TEM-1, CTX-M-15	RII	Group I
2018	72	TEM-1, CTX-M-16	RII	Group I
2018	73	TEM-1, CTX-M-15	RI	Group I
2018	74	TEM-1, CTX-M-15	RI	Group I
2018	75	CTX-M-15	RIII	Group I
2018	76	CTX-M-15	RIII	Group I
2018	77	TEM-1, CTX-M-15	RII	Group I
2018	78	TEM-1, CTX-M-15	RI	Group I
2018	79	TEM-1, CTX-M-1	RIII	Group I
2018	80	TEM-1, CTX-M-15	RII	Group I
2019	81	TEM-1, CTX-M-9	RI	Group II
2019	82	TEM-1, CTX-M-15	RIII	Group I
2019	83	CTX-M-15	RIII	Group I
2019	84	TEM-1, CTX-M-15	RI	Group I
2019	85	TEM-1, CTX-M-16	RII	Group I
2019	86	TEM-1, CTX-M-15	RIII	Group I
2019	87	TEM-1, CTX-M-15	RII	Group I
2019	88	CTX-M-15	RI	Group I
2019	89	TEM-1, CTX-M-15	RIII	Group I
2019	90	TEM-1, CTX-M-15	RIII	Group I
2019	91	TEM-1, CTX-M-15	RII	Group I
2019	92	TEM-1, CTX-M-16	RI	Group I
2019	93	TEM-1, CTX-M-15	RII	Group I
2019	94	TEM-1, CTX-M-9	RII	Group I
2019	95	TEM-1, CTX-M-15	RII	Group I
2019	96	CTX-M-15	RI	Group I
2019	97	TEM-1, CTX-M-15	RI	Group I
2019	98	TEM-1, CTX-M-15	RII	Group I
2019	99	TEM-1, CTX-M-15	RI	Group I
2019	100	TEM-1, CTX-M-15	RII	Group I
2019	101	TEM-1, CTX-M-1	RII	Group I
2019	102	TEM-1, CTX-M-15	RII	Group I
2019	103	TEM-1, CTX-M-15	RII	Group I
2019	104	TEM-1, CTX-M-15	RII	Group I
2019	105	CTX-M-15	RI	Group I
2019	106	TEM-1, CTX-M-15	RI	Group I
2019	107	TEM-1, CTX-M-9	RI	Group I
2019	108	TEM-1, CTX-M-15	RI	Group I
2019	109	TEM-1, CTX-M-15	RI	Group I
2019	110	TEM-1, CTX-M-9	RI	Group I
2019	111	TEM-1, CTX-M-15	RI	Group I
2019	112	CTX-M-15	RI	Group I
2019	113	TEM-1, CTX-M-15	RI	Group I
2019	114	TEM-1, CTX-M-15	RI	Group I
2019	115	TEM-1, CTX-M-16	RII	Group I
2019	116	TEM-1, CTX-M-15	RII	Group I
2019	117	TEM-1, CTX-M-15	RII	Group I
2019	118	TEM-1, CTX-M-15	RII	Group I
2019	119	TEM-1, CTX-M-15	RII	Group I
2019	120	CTX-M-15	RII	Group III

SHV-12 1 (4) (n= 6), TEM-1 + CTX-M-1 (n= 6), TEM-1 + CTX-M-16 (n= 6) and TEM-1 + CTX-M-9 (n= 6) (Table 3). One hundred and eight out of 120 isolates were found to harbor a *bla*CTX-M gene, with the *bla*CTX-M-15 group being the most common type. Most of the *bla*CTX-M-15–containing *E. coli* isolates also harbored different β-lactamase genes, including especially *bla*TEM-1 and *bla*OXA-1. The *bla*TEM-1 was found in 76 isolates, alone and in combination with other genes. Sixteen out of all isolates harbored the *bla*CXT-M-9 gene, 14 isolates harbored a *bla*OXA-1 gene and other genes found in the isolates were *bla*CXT-M-1 (n= 6), *bla*CXT-M-9 (n= 6), *bla*SHV-12 (n= 6) and *bla*SHV-12 1 (4) (n= 6).

Table 3. Detected ESBL genes of *E. coli* isolates.

***bla* gene(s)**	**Number of *E. coli* isolates (%)**
CTX-M-15, OXA-1	14 (12)
CTX-M-15	24 (20)
TEM-1, CTX-M-15	52 (43)
TEM-1, SHV-12	6 (5)
SHV-12 1 (4)	6 (5)
TEM-1, CTX-M-1	6 (5)
TEM-1, CTX-M-16	6 (5)
TEM-1, CTX-M-9	6 (5)

Discussion and Conclusion

In Turkey, as well as in the world, especially outbreaks of infections with ESBL-producing *Enterobacteriaceae* has an increasing frequency. Although ESBLs have been described in almost all enteric bacteria, they are frequently found in *E. coli* and *K. pneumoniae*. The high prevalence of ESBL-positive *E. coli* isolates reported for farm animals, especially poultry, due to misuse and overuse of antimicrobial agents is a zoonotic risk factor for human (5, 35, 47). A study on examining the ESBL prevalence of *E. coli* isolated from urine samples of patients. The results of the study demonstrated that the prevalence of ESBL producers was a significant increase among isolates from inpatients (12.5 % to 44.7 %) and from outpatients (9.6 % to 22.8 %) (41). In 2012, 66 (37.1 %) of a total of 178 patients were ESBL positive-*E. coli* isolated from urine samples (n= 322) collected from Ankara Training and Research Hospital in Ankara province of Turkey (27). In a study conducted in Turkey, Akçam et al. (1) reported that the production of ESBL was found in 7.2 % of *E. coli* and 35 % of *Klebsiella* spp. In another study, although ESBL positivity was observed in 52.2 % of 52 *E. coli* strains and in 58.2 % of 12 *K. pneumoniae* strains, it was not observed in 6 *Proteus* spp. (2). In a similar study, Sahin et al. (40) reported that positivity rates of ESBL for *Enterobacteriaceae* were detected 19.4 % for *E. coli* (n= 108), 15.9 % for *Klebsiella* spp. (n= 44) and 13.6 % for *Proteus* spp. (n= 22). In studies conducted in other countries, the frequency of ESBLs were reported to be 11.0 % to 63.6 % in *E. coli* and 13.0 % to 86.6 % in *Klebsiella* spp. (21, 23, 46).

The differences in ESBL production rates in both Turkey and other countries are related to the fact that the production in bacteria changes with certain conditions. It is known that the increase in the production is closely related to the use of broad-spectrum β-lactam antibiotics and in parallel with the increase in β-lactam resistance. Ozkan et al. (33) found that *E. coli* and *K. pneumoniae* strains were sensitive to 80-85 % and 60-63 % of third-generation cephalosporins including cefinaxone, ceftazidime, and cefotaxime, respectively. The third-generation cephalosporin resistance of *E. coli* strains in the present study is also proportional to the ESBL production in these bacteria.

In Turkey, SHV-2, SHV-5, SHV-12, OXA-1, CTX-M-2, CTX-M-15, CTX-M-16, and TEM-1 type ESBLs were reported in *E. coli* isolates (17, 18, 47). Sequencing of β-lactamase genes revealed that *bla*CTX-M-15 was the most prevalent (90/120) in the ESBL-producing *E. coli* isolates, followed by *bla*TEM-1(76/120), *bla*CTX-M-9 (16/120), *bla*OXA-1 (14/120), *bla*CXT-M-1 (6/120), *bla*CXT-M-9 (6/120), *bla*SHV-12 (6/120) and *bla*SHV-12 1 (4) (6/120) in this study. A study conducted in Izmir province of Turkey between 2004 and 2005 showed that *E. coli* isolated from patients with UTI produced an ESBL, of which CTX-M-15 was predominant (53 %) (51). Similarly, CTX-M-15 group has been reported to be found in 86.8 % of *E. coli* isolated from inpatients and outpatients at the hospital of İstanbul Faculty of Medicine between 2002 and 2004 (17). Among ESBLs, CTX-M-15 was found as the most prevalent type enzymes as reported in different studies from Turkey and several other countries (9, 17, 18, 29, 30, 31, 51). In several studies conducted in different province of Turkey, it has been reported that ESBL-positive *E. coli* isolates harbored *bla*TEM gene (3, 7). TEM-1 was found to be harbored in 63 % of ESBL-positive *E. coli* isolates in combination with other genes in this study. In animal studies performed in Turkey, it has been reported that CTX-M-15 was the most frequent ESBL enzyme type in ESBL-positive *E. coli* isolates obtained from both healthy broilers (47) and laying hens (35). The results of our study are similar to the results of these studies. CTX-M-15 is the predominant ESBL in both human and poultry. This may be caused by the transmission of ESBL-producing bacteria between humans and animals.

In the present study, the standard criteria of Tenover (13, 43-45) were used for the analysis of *E. coli* strains using PFGE system. If the restriction patterns of isolates according to the Tenover criteria have the same number of

bands and the reciprocal bands are the same size, these isolates are the same. In the present study, 120 strains which constitute the main cluster and evaluated as 3 groups in this cluster may be epidemiologically identical strains. When these strains were examined epidemiologically, it was determined that patients admitted to the hospital from the same region. However, four different groups were observed when resistance profiles were compared. According to XbaI digestion profiles, 120 strains were found to be related to each other epidemiologically.

Studies in different countries show that resistance to β-lactam antibiotics is increasing and this is an emerging threat in today's world. In the last decade, plasmid-encoded ESBL-producing organisms have increased rapidly (13, 36, 37). Currently, most of these strains can be treated with combinations of β-lactam and β-lactamase inhibitor. However, the number of strains that are not treated with these combinations is also increasing (52). In addition, ESBL-producing strains are becoming more and more resistant to other antimicrobial drugs such as aminoglycosides, sulphonamides, and tetracyclines through various mechanisms (39), and it is foreseen that our antibiotic options will be severely restricted if no precautions are taken.

CTX-M-15-positive *E. coli* is more widespread in the isolates and the presence of different types of enzymes in each isolates shows that the epidemiology of ESBLs in the hospital is complex. High ESBL production and decreased susceptibility to broad-spectrum cephalosporins are present in *E. coli* strains in our hospital. If empirically treated with these broad-spectrum antimicrobial agents, treatment of ESBL-producing *E. coli*-associated infections with these agents may result in failure. The resistance problem is still observed for infections with *E. coli*. However, it is known that resistance properties can be transferred to different types of bacteria. In the coming years, resistance can be encountered in different bacteria in our hospital.

Consequently, in order to help slow the increasing antibiotic resistance, it is necessary to develop activities and policies to promote more rational use of antibiotics. The local prevalence and antibiotic susceptibility of the bacterial organism should be considered during the selection of empirical antibiotic therapy. High levels of ofloxacin, trimethoprim-sulfamethoxazole, and ampicillin resistance were found in the *E. coli* strains isolated from the patients who admitted to our hospital. Overuse of these antibiotics may have led to the development of resistance to bacteria.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Clinical approaches for genital and extragenital metastasis of transmissible venereal tumor in a bitch with ovarian remnant syndrome

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Abstract: A 25 kg, 4 years old, Boxer breed bitch which had ovariohysterectomy section 2 years ago, was presented with weakness, loss of appetite, masses on abdominal and inguinal mammary glands and vaginal bleeding. Transmissible venereal tumor (TVT) was diagnosed with vaginal cytology. Intra-abdominal mass around the iliac artery and remnant ovary behind the right kidney were determined on ultrasonographic examination. The remnant ovary and the masses on mammary glands were removed by surgical approach. However, intra-abdominal mass could not be extirpated because of its localization during the same laparotomy. A biopsy was taken from the vaginal mass. According to histopathology, TVT and collagenous hamartoma were detected on mammary glands. Also, TVT was observed in vaginal and intra-abdominal masses. Chemotherapy protocol was arranged for intra-abdominal mass and vaginal TVT which occupied for ten weeks. Hemogram, vaginal smear and transabdominal ultrasonography were repeated before each chemotherapy course. Size of the intra-abdominal mass, pulsatility index (PI) and relative index (RI) values of iliac artery were evaluated with B-mode ultrasonography and doppler ultrasonography during the treatment, respectively. As a result, positive correlation between PI and RI values were determined. In the presented case, TVT and its metastases were successfully treated with both surgery and chemotherapy. It was concluded that evaluation of the metastases with doppler ultrasonography will be favorable to reveal the hemodynamic alterations of related organs or tissues.

Keywords: Canine, metastasis, transmissible venereal tumor.

Ovaryan remnant sendromlu bir köpekte bulaşıcı veneral tümörün genital ve ekstragenital metastazlarına klinik yaklaşımlar

Özet: Dört yaşında, 25 kg ağırlığında, 2 yıl önce ovariohisterektomi operasyonu uygulanmış, Boxer ırkı dişi bir köpek; halsizlik, iştahsızlık, vajinal kanama, abdominal ve inguinal meme bezlerinde kitle şikayetiyle getirildi. Vajinal sitolojik muayene ile bulaşıcı veneral tümör (TVT) teşhis edildi. Ultrasonografik muayenede, arteria iliaka etrafında yer alan intra-abdominal bir kitle ve sağ böbreğin gerisinde kalıntı ovaryum varlığı tespit edildi. Kalıntı ovaryum ve meme bezlerindeki kitleler cerrahi yaklaşım ile uzaklaştırıldı. Laparotomi sırasında intra-abdominal kitle lokalizasyonu nedeniyle esktirpe edilemedi. Vajinal kitleden biyopsi örneği alındı. Histopatolojik inceleme neticesinde, meme bezlerinde TVT ve kollajenöz hamartom, vajinal ve intrabominal kitlelerde de TVT varlığı saptandı. Vaginal ve intra-abdominal TVT kitleleri için 10 haftalık kemoterapi protokolü oluşturuldu. Her kemoterapi öncesi hemogram, vajinal smear ve transabdominal ultrasonografi tekrarlandı. Tedavi süresince intra-abdominal kitlenin boyutu ve arteria illiaka'ya ait pulsatil indeks (PI) ve relative indeks (RI) değerleri sırasıyla B-mod ultrasonografi ve doppler ultrasonografi ile değerlendirildi. Bunun sonucunda, PI ve RI değerleri arasında pozitif korelasyon tespit edildi. Sunulan bu vakada, TVT ve metastazları hem cerrahi yaklaşımla hem de kemoterapi ile başarılı bir şekilde tedavi edildi. Metastazların doppler ultrasonografi ile değerlendirilmesinin, ilgili organ veya dokuların hemodinamik değişikliklerini ortaya koymak için faydalı olacağı sonucuna varıldı.

Anahtar sözcükler: Köpek, metastaz, bulaşıcı veneral tümör.

Canine transmissible venereal tumor (CTVT) is a contagious venereal tumor of dogs which are infected with close contact and mating without noticing any breed, age and sex (5, 11). However, CTVTs are commonly seen between 2 and 8 years old (6). The clinico-pathological feature of CTVT is cauliflower-like, pedunculated, lobular, papillar or multilobular. The surface of the tumor is usually ulcerated, hemorrhagic and friable (13). CTVTs are easily diagnosed with anamnesis, clinical and cytological examinations. Even if exfoliated cells of the tumors can be definitely obtained by swabs or fine needle aspiration, cytogenetic and molecular techniques are also applied for diagnosis (7, 17, 18). Although TVT is usually a benign neoplasm, it starts to transform into an invasive and malignant form that cause metastases at the range of 5-17% (23). The occurrence of the tumor and its metastases depends on the immune system of the dog (26, 27). Metastases commonly occur in males (16%) rather than in females (2%) (6). Nasal mucosa, gluteal region, maxillar gingiva, subcutaneous tissue, skin, lymph node, uterus, ovarium, brain and ocular metastases of CTVTs are reported (3, 12, 14, 25). Regional lymph node metastases are frequently seen in males with large tumors (18). Several treatments can be applied for CTVT such as radiotherapy, chemotherapy, immunotherapy, biotherapy and excisional surgery (22). But the autogenous vaccine was not suitable in the treatment of the bitches with TVT (9). Chemotherapy is the most effective and common method for treatment that provides good prognosis in CTVT cases (1). However, it was reported that CTVT could be successfully treated by doxorubicin (10).

Figure 1. A. Hemorrhagic vaginal discharge and intra-vaginal mass on the presentation day.
B. Vulvar condition at the end of the chemotherapy.

A 25 kg, 4 years old, Boxer breed bitch was presented with weakness, inappetence, masses on abdominal and inguinal mammary glands and vaginal bleeding. Although ovariohysterectomy was performed two years ago, the bitch continued to come into heat regularly. Upon clinical examination, vulvar swelling, bloody vaginal discharge, round-shape and edematous intra-vaginal mass (Figure 1) were detected in addition to masses on mammary glands. Vaginal smear revealed the existence of abundant transmissible venereal tumor cells, erythrocytes and intermediate cells (Figure 2). Ovarian remnant tissue behind the right kidney and a mass (4.14 x 6.24 cm) around iliac artery were diagnosed by transabdominal ultrasonography. The complete blood count and biochemistry revealed normal reference ranges, only mild anemia was detected. Surgical intervention was decided to perform for removal of the remnant ovary and mammary glands. After the bitch was premedicated with atropine sulfate (0.03 mg/kg, sc) (Atropin®, Teknovet, Turkey), general anesthesia was induced with propofol 1% (4 mg/kg, iv) (Lipuro®, Braun, England) and maintained with isoflurane (3%) (Forane likid®, Abbott Laboratories, England) and oxygen (0.5-1%). Though remnant ovary was removed after laparotomy, localization of the intra-abdominal mass prevented to extirpate it from iliac artery with surgical intervention. Total mastectomy was performed and biopsy was taken from the vaginal mass in the same anesthesia. Chemotherapy was decided to perform for treatment of intra-abdominal mass and CTVT. For the postoperative care, ranitidin (2 mg/kg, im, SID, Ulcuran®, Yavuz, Turkey), enrofloxacin (5 mg/kg, sc, SID, Baytril-K® 5%, Bayer, Turkey), cyanocobalamin (50 mcg/kg, SID, Dodex®, Deva, Turkey) and Iron III Hydroxide Polimalose (20 mg/kg, im, SID, Ferrum Hausmann®, Abdi İbrahim, Turkey) was applied to the bitch until leucocytosis and anemia had disappeared. Meloxicam (0.04 mg/kg, im, Melox®, Nobel, Turkey) was prescribed for management of the postoperative analgesia. Mammary and vagina tissues were previously fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4 µm to examined by light microscopy. The dimensions of the tumoral mass that was extirpated from the inguinal mammary lobe and the vagina were 10 cm x 6 cm x 5 cm and 2 cm x 2 cm x 1 cm, respectively. The histological examination revealed round to polyhedral cells arranged in densely packed masses separated by fine strands of connective tissue both in the inguinal mammary lobe and vagina that were exhibited the existence of TVT (Figure 3A,3B,3C). Hamartoma was observed in all the other mammary lobes (Figure 3D).

Due to hematologic examination revealed anemia (erythrocytes: 2.82 M/µL, haematocrit: 18.2%, haemoglobin: 5.3 g/dL, MCH: 18.8 pg, MCHC: 29.1 g/dL), blood

transfusion was applied one week before the first chemotherapy cycle. After hemagramme parameters were suitable for chemotherapy treatment, vincristine sulfate (0.025 mg/kg, Vincristine®, Kocak Farma, Turkey) was administered with 0.9% NaCl isotonic serum (10 ml/kg, Deva®,Turkey) intravenously to the bitch once a week for ten weeks. Chemotherapy section had been paused for a week at the end of the sixth cure but immediately after TVT cells were cytologically detected, the cure had continued four more weeks. Hemogram and vaginal cytology were repeated before each course of chemotherapy. Cyanocobalamin (Dodex®, Deva, Turkey) was prescribed to avoid the anemia during the cure. However, doppler ultrasonography was performed to evaluate the mass around iliac artery in every week before chemotherapy treatment (Figure 4). The mass exhibited a decrease in size during chemotherapy protocol was applied and its size stayed at 1.97 x 3.84 cm which is anatomically compatible with median iliac lymph node (Figure 4). At the end of the chemotherapy, hemogram parameters were measured in the reference ranges.

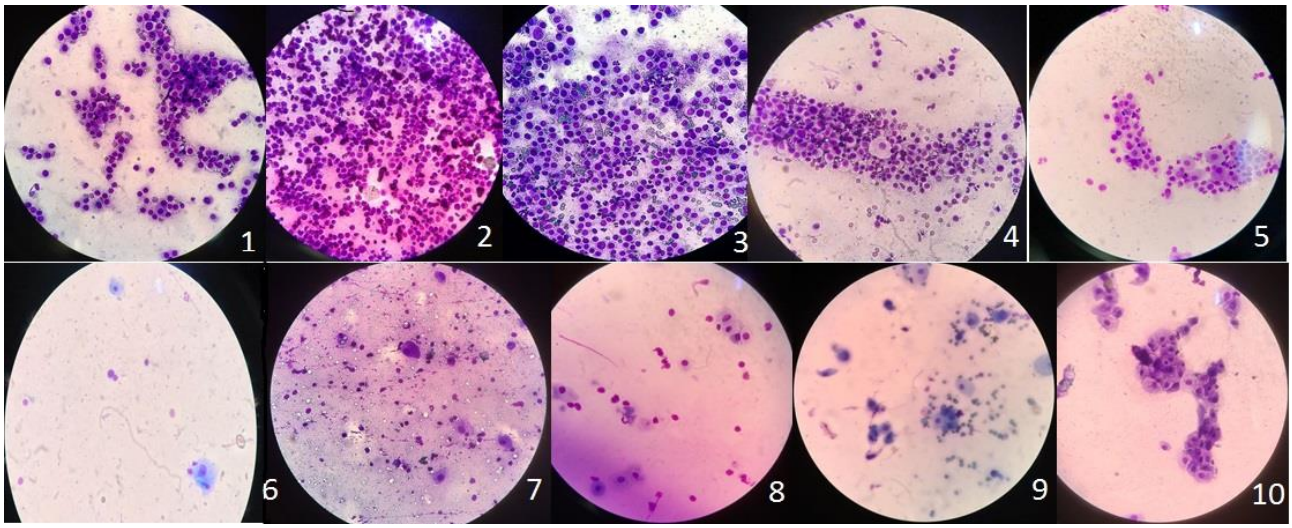

Figure 2. Vaginal cytology images during chemotherapy (1st-10th week).

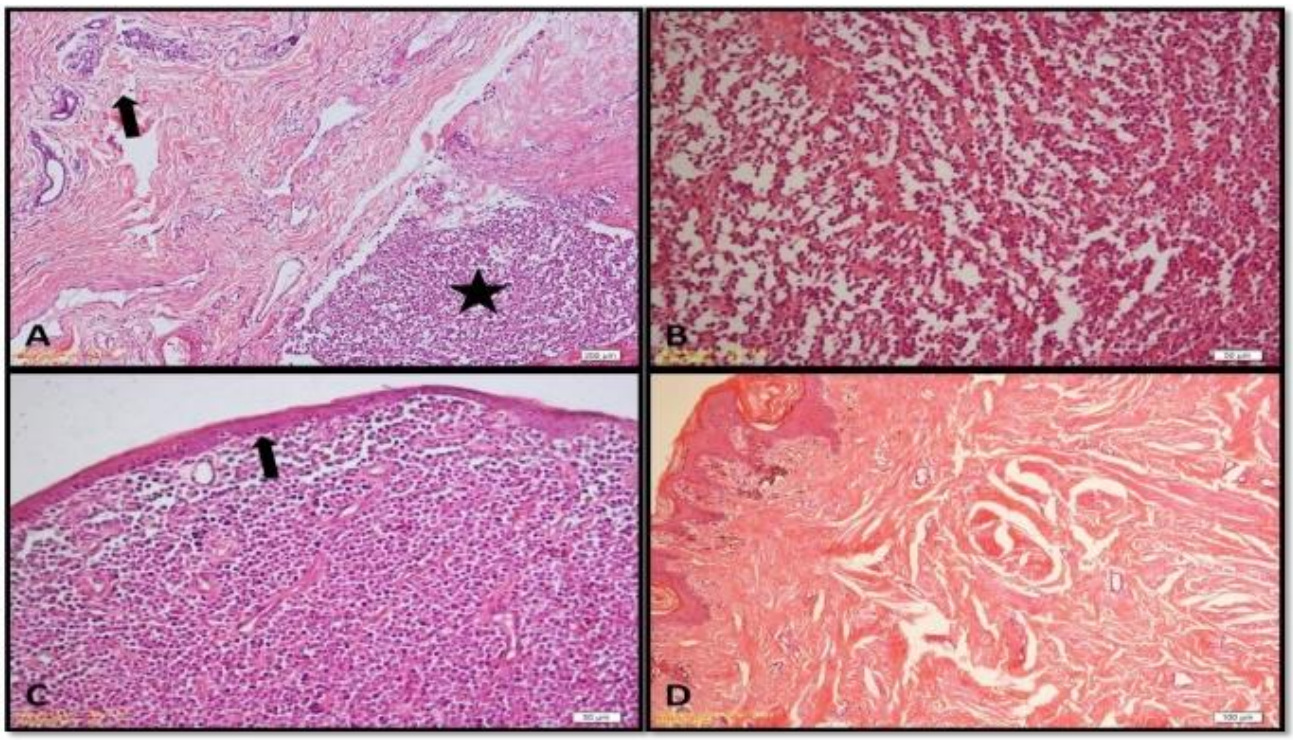

Figure 3. A. Inguinal mammary lobe. Mammary glands (arrow). Area of transmissible venereal tumor (star). **B.** Closer appearance of the transmissible venereal tumor. The tumor cells are arranged in densely packed masses separated by fine strands of connective tissue. **C.** Vagina. Transmissible venereal tumor. Round to polyhedral cells are arranged in sheets beneath the mucosa (arrow). **D.** Mammary tissue. Hamartoma.

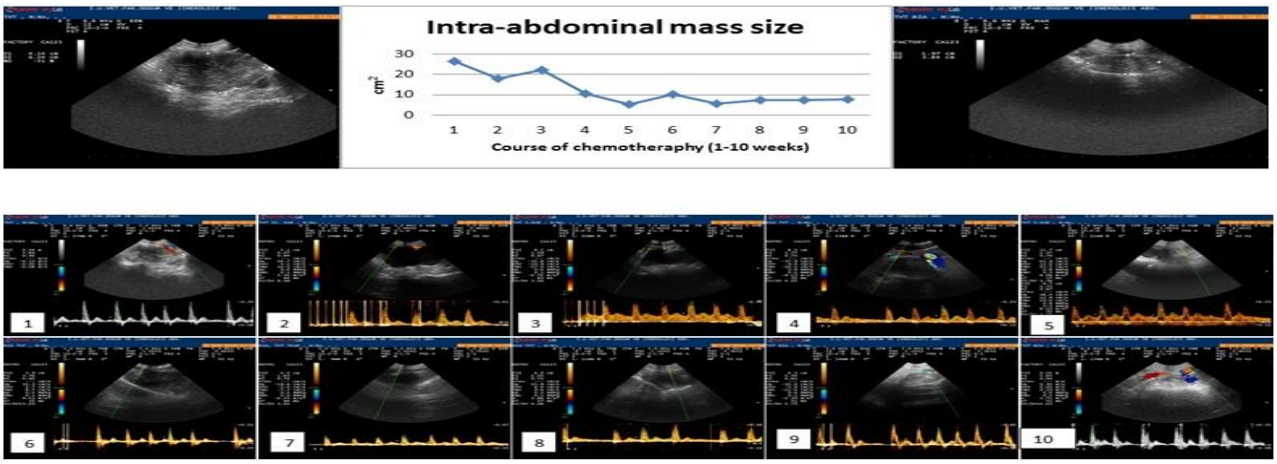

Figure 4. Size of the mass around median iliac lymph node by B-mode USG: Before and after the chemotherapy, respectively. Doppler USG findings during chemotherapy for ten weeks.

The ovariohysterectomized bitch exhibited estrus signs because of ORS and TVT contaminated during mating in the presented case, in line with Turna Yilmaz et al (24) reported that CTVT can occur in bitches with ORS. Also, the shape of the intravaginal TVT mass was round and lobular in this case unlike with Champour et al. (8) reported as cauliflower-like feature in their report. In contrast with Kevin et al. (16) reported that CTVTs were rarely metastasized, genital and extragenital metastasis of CTVT was evaluated in the presented case. The occurrence of the metastases was thought to be due to the suppression of the immune system as Behera et al. (4) reported. Transabdominal B-mode ultrasonography and doppler ultrasonography were preferred to evaluate the metastatic mass around the iliac artery, contrarily with the researchers' (21, 25) imaging techniques (radiography and computed tomography). Pulsatility index is the most sensitive index for differentiating abnormal waveforms which are used in obstetric and abdominal evaluations (20). In line with the researchers' report (20), PI and RI values are measured to evaluate the hemodynamics of the metastasis in a gynecologic pathology that was correlated statistically significant ($P<0.001$) according to Spearmann correlation test. This results indicate that metastatic mass on the iliac lymph node affects the blood flow in iliac artery negatively. It was concluded that doppler ultrasonography should be performed to evaluate the blood flow where the metastasis occurs in TVT cases and it will be a useful tool for examination of blood circulation in the affected tissues.

Two different treatment techniques were applied for the management of TVT in the presented case. In line with Nak et al. (19), metastasis of TVT on inguinal mammary glands was treated by surgery. But chemotherapy protocol was performed for intravaginal mass and its metastasis on median iliac lymph node due to the inoperable location. Vincristine sulfate administrated intravenously in the same dose that Jacob et al. (15) reported. As Kevin et al. (16) notified that TVT and its metastasis were very invasive in the presented case. Because of that reason, the chemotherapy treatment for them took ten weeks.

Hamartoma is a lesion between developmental disorder and benign tumor of various body parts and organs which occurs frequently in human but rare in domestic animals (2). Although the researchers (2) stated that hamartoma occurs rarely in domestic animals, it had seen together with TVT in mammary glands in the presented report. Also, they were successfully treated by surgical intervention without recurrence in postoperative two years.

It was concluded that TVT can complicate with different pathologies such as hamartoma in genital organs, and it can take form in extra-genital tissues. Evaluation of intra-abdominal metastasis of TVT with doppler ultrasonography will be favorable to reveal the hemodynamic alterations of related organs or tissues. Both surgery and chemotherapy are performed as suitable treatment options for CTVT. For the prophylactic purpose, ovariohysterectomy or ovariectomy should be performed to avoid the spread of TVT.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Review / *Derleme*

Behavioral and health problems of poultry related to rearing systems

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Abstract: This review paper aims to give an overview of the literature data on common behavioral and health problems in broiler chickens and laying hens and to explain their interrelation and relation to the rearing system. In the initial part, the mechanism of arising of the most common forms of abnormal behavior and the way they affect the poultry are briefly described. Furthermore, the possibility of poultry to fulfill behavioral needs in different rearing systems and some of the consequences to the birds in the case of their inability to meet their needs are displayed. The influence of the main microclimate and space factors is also discussed, as well as the occurrence of some infectious, parasitic and production diseases in poultry rearing systems. The welfare problems of poultry are complexes and most often caused by the system design and its improper use. Continuous work on improving rearing systems is as important as better informing the producers and the public on poultry welfare problems.

Keywords: Behavior, health, broilers, hens, rearing.

Introduction

Poultry production for decades has had the status of the fast-growing agricultural sector, and it is expected to continue to grow along with demand for poultry meat and eggs. The progress has been made by changes in technology which increased the number of birds per farmer and labor productivity, and also by selective breeding directed to improving the size, fecundity and growth rate of birds (13, 50). However, intensification of the production has also resulted in many welfare problems manifested as damaging behavior, injuries of specific body parts, various kinds of diseases, and the increased mortality rate of poultry. Welfare problems are usually multifactorial but basically, they arise when animals cannot realize their natural behavioral needs, and that is the case in most of the existing poultry rearing systems (23, 24). Certain species-specific behavior patterns in poultry species are very strongly motivated, such as nesting, perching, dust bathing, and scratching; if the birds are not allowed their expression that leads to frustration, abnormal behavior manifesting and/or to injury (17, 22). The wounds cause physical pain to the birds and may also be a source of infection and disease (22).

Abnormal behavior may cause damage to the animal itself or its conspecifics. Behaviors harmful for the animal are feather-plucking (self-removal of feathers), hysteria or "fright disease", and "excessive gregariousness" - which may lead to suffocation (1). Behaviors that harm other animals appear more frequent than previous and include injurious pecking (gentle and severe feather pecking, cloaca i.e. vent pecking, and cannibalistic pecking), toe pecking, aggressiveness, and bullying. Gentle feather pecking is a light, repeated pecks on the tail, wings, back, and neck of the other hen whilst the severe form is a hard, fast, and singular pecks on the tail, back, vent, and neck of the bird. The injury after removal a feather could trigger cannibalism i.e. continued pecking on the skin leading to serious bleeding and wounds which may cause the victim's death (1, 23, 24, 27, 40). There are also some altered behaviors usually not directly harmful to the bird, such as stereotypic pacing, vacuum nesting, and vacuum dust bathing (1). This review considers data from the literature on common behavioral and health problems of chickens and their interrelation, as well as their linkage to the characteristics of rearing systems.

Rearing systems and the possibility of expressing the natural behaviors

Poultry rearing systems may be classified as the cage and alternative systems and both groups comprise a wide variety of different models. The use of cages has the same limitations in different countries. The conventional barren cages have been banned for laying hens in the EU since 2012 by Directive 1999/74/EC but enriched i.e. furnished cages are available (21, 22, 27, 36). Cage systems for broilers are in use in some parts of the world (44). However, considering welfare aspects and consumers' demands, the development of alternative (non-cage) systems for both, broilers and laying hens is encouraged (23, 28, 45): floor, aviary and free-range systems. These systems for broilers have no nests and commonly have no perches. In free-range system birds may use outdoor areas i.e. verandas and areas covered with vegetation (22, 47, 48).

Domestic fowls always tend to exhibit behavior very similar to their progenitor - the Jungle fowl (17). Alternative systems are superior regarding freedom of movement and fulfillment of behavioral needs, which is limited or completely disabled in conventional cages. The use of furnished cages allows for the expression of behaviors such as perching, nesting, and dust bathing, whilst loose housing systems additionally allow for activities such as walking, wing flapping, and foraging. On the other hand, alternative systems may have side-effects that cause other welfare problems (22). Many problems arise directly or indirectly due to agonistic interactions in the production group which may consist of several thousand to a few tens of thousands of birds in one facility whilst chickens normally live in a small, mixed-sex and -age family groups of 5 to 30 birds (24). Both in nature and farm conditions ranking begins from the moment when unfamiliar birds are put together. Method of establishing and maintaining a hierarchy is by fighting and pecking the head of other bird, what is called "a pecking order." In commercial conditions, this behavior can turn into aggressiveness and may lead to serious injuries in the area of the head and neck, including comb, wattles, and eyes (24). Increased aggression as well as and stereotyped pacing behavior may be a form of response to severe or long-term frustration and suffering in adverse conditions (18, 20).

Chickens also peck to escape from the eggshell, to feed, to drink, to explore, and to obtain and keep personal space. Searching for food is performed by ground pecking and scratching with their feet, and in nature, it takes the most time of the day (24). Beak trimming, a practice still performed in many countries for preventing injurious pecking, makes difficult for birds to perform the above-mentioned activities but also to orient themselves in free-range rearing (26). In systems without litter, some of the elements of foraging may be directed at other birds' feathers rather than the feed (3). Injurious pecking may be a serious problem in all systems and is especially difficult to control in the large-group furnished cages and the non-cage systems (22).

Dust bathing, together with preening, is a common strategy for feather and skincare (24). Birds that cannot perform either of these two behaviors (e.g. due to the lack of a dustbathing substrate or shortened beak) may have a severe problem with ectoparasites (7) such as red mite (*Dermanyssus gallinae*) (22). A form of preening behavior which domestic fowl may show after mild or short-term frustration is "displacement preening" (19). Displacement behavior usually occurs when an animal is torn between two conflicting drives, such as fear and aggression (4).

In the case of high air temperature thermoregulation is mainly conveyed by emitting evaporated water via respiratory organs, and a certain amount of warmth loss also occurs via the skin. When the air temperature is low, birds lift the feather and let the air go in making a layer of warm air around the body. Therefore the full feather cover is very important for cold protection, and also for protection against skin abrasions. Losing and re-growing feathers ("molting") normally occurs every year when the days get shorter. Feather loss is also possible due to stress, sudden weather changes, dehydration, overcrowding, contact with equipment, feather pecking, nutrient dysbalance, etc. (24).

Sleeping behavior is performed by roosting high off the ground. In reaching a branch or a perch chickens move in a specific way: when they go up, they jump on the branch and gradually jump to a higher one, but when they go back, they fly down directly from the branch to the ground using their wings. Damage or loss of feathers on the wings makes birds less efficient in maintaining balance (31), which can be a problem in using perches and flight control during landings. Long-term sitting on perch are common in the systems where the movements are restricted as in furnished cages. This may lead to developing deformation of the keel bone in laying hens which is very rarely observed in systems where perches are absent (16, 22, 39, 41). Deformations are also noticed in systems where birds roost on objects such as the edges of feed troughs, water pipes, wires or litter boxes, thereby applying pressure to the keel (22). In the collision with housing structures and other birds, especially during failed landings, keel bone fractures in layers may occur (29). Fractures have been noticed mainly in aviaries and other non-cage litter systems (6, 38). Both deformations and fractures of the keel bone (commonly named "Keel Bone Damage" or KBD) have been reported as highly frequent, multifactorial welfare problems of commercially raised laying hens (16, 29).

Environmental factors triggering behavioral and health problems in chickens

The environment in a poultry house is a combination of different factors that interact. One of the main tasks in indoor housing systems is maintaining a good aerial environment (i.e. air quality), which considers that the temperature, humidity, dust level and concentrations of certain gasses should be controlled and kept within recommended limits (11, 13).

Air temperature and humidity are affected by stocking density, season and ventilation rate. Air humidity also depends on indoor temperature, type of drinkers, water spillage, litter type, and other factors (14). Mutual action of both parameters influences the thermal comfort of the birds (42). Elevated values of these two parameters may cause thermal stress and death.

Factors affecting airborne dust and ammonia content include litter type and quality, birds' activity, stocking density, manure handling, ventilation rate, and indoor temperature and air humidity. Dust and ammonia levels are commonly high in aviaries and floor housing systems, whereas they are usually lower in furnished cages (9, 10). Dust particles are usually a carrier of bacteria, fungi, bacterial toxins, and mycotoxins. In laying hens exposed to respirable dust for a longer period, a hypersensitivity reaction and respiratory diseases may occur (9, 10). Similarly, ammonia exposure results in the formation of lesions in the respiratory tract, higher predisposition to respiratory disease and secondary infections, as well as keratoconjunctivitis (5, 10). Elevated ammonia levels are correlated with an increased level of stress hormones, and potentially to the behaviors indicative of stress as well (15) such as damaging behaviors. The harmful effect can be intensified by the simultaneous occurrence of other stressors, such as heat and humidity (42).

Litter quality affects the occurrence of respiratory diseases and has a direct influence on the skin. Wet litter is a major risk factor for contact dermatitis lesions i.e. footpad dermatitis, hock burns and breast dermatitis in broiler chickens (13). The lesions are common in heavy birds that spend most of the day time sitting, e.g. due to leg weakness (49). Moisture content in the litter is associated with litter material, high stocking density, diarrhea in birds, water consumption and diet composition which affects the amount, water contents and viscosity of feces (2, 13, 14, 43).

Light management (which includes photoperiod, light intensity, source and wavelength of light) is an important tool in regulating broiler and laying hens' production and behavior. Broilers provided with sufficient dark periods have fewer health-related problems, including sudden death syndrome ("flip-over disease"), ascites syndrome associated with pulmonary hypertension, spiking mortality syndrome, eye enlargement, tibial dyschondroplasia, and other skeletal disorders (13, 34, 35). Dimming the light is one of the effective measures in the case of problems with feather pecking during the laying period (12, 27).

Some systems provide lower density (i.e. organic and free-range systems) and the cage density is higher than in most floor systems. High density reduces locomotion and ground pecking, final body weight, feed intake, and feathering (33, 13, 25). It increases the time that birds spend sitting and behavioral disturbances in the last week of broilers rearing, and there are more scratches and bruising on the body surface. The effects may be magnified or caused by increased temperature, humidity or litter moisture (13, 25).

Both restricted movement and disturbed rest may impact birds' physical development and the occurrence of leg deformations which predispose to further behavioral restriction (13). Restricted movement is considered as the main factor for progressive osteoporosis ("cage layer fatigue") development in hens in conventional cages during the laying period. It is manifested as skeletal weakness and bone fragility which may lead to bone fractures and sudden death. Keel bone damage also reduces birds' mobility, increase time spent in the nest, and it is linked with bumble foot and poor feather cover (30, 38) as well as with the consumption of more feed and water (32). Bone strength has been found better in systems where pullets and hens can exercise, including furnished cages, comparing to conventional cages. Factor related to "fatty liver hemorrhagic syndrome" is also restricted locomotion (22, 37), in combination with high environmental temperature and a high level of stress (22).

Other diseases related to rearing system

Diseases related to the production system are multifactorial and involve genotype, high production performances, rearing system and technology, diet composition, pathogen exposure, etc. (22). In such a way, housing systems in which stressors are present e.g. crowding, social stress and lack of general stimulation may increase the risk of infection and clinical disease of the reproductive tract. Salpingitis and peritonitis in laying hens may be caused by pecking around the cloacal region which is more common in non-cage systems and aviaries when birds were not beak trimmed than in conventional cages (22).

Taking into account infectious and parasitic diseases, in non-cage systems has been recorded the higher incidence of bacterial/protozoa infections like erysipelas, *E. coli*, pasteurellosis and histomoniasis, and *Ascaridia* compared with both, furnished and conventional cages. Systems rich in fittings such as roosts, nests, and slatted floors are more demanding in terms of implementing thorough cleaning and disinfection, and there are areas

where parasites, mainly red mites, may live and survive. The risk of coccidiosis can be increased e.g. by cumulating the feces under perches (22). Pathogens and parasites that could be directly transmitted from other domesticated or wild (migratory) birds are more prevalent in outdoor i.e. free-range systems (47).

Discussion and Conclusion

This paper presents a brief overview of the most significant behavioral and health i.e. welfare problems in one poultry species, but some of the problems are noticed in other domestic fowls reared in similar conditions, e.g. contact dermatitis in turkey (43). Each of the existing systems and the technological solutions for laying hens or broiler chicken is associated with certain welfare problems. The ideal system has been not created yet and in all of them some of the behavioral needs are deprived; the natural behavior and the needs of animals are principally incompatible with the production goals.

The welfare problems are caused both by the system design and by its improper use. For instance, to achieve high production, contemporary systems for laying hens are based on a high stocking density, with an automated process of feeding, manure removal, microclimate control and control of the animals. Generally, it is easier to carry out all of that in a cage system, which is also more convenient in terms of controlling infectious and parasitic diseases and achieving cleanliness of eggs, and producers usually prefer it. In a desire for higher profits, sometimes they keep more birds per square meter than recommended, and in this way, they reduce already limited space. Consequently, the birds come more into collision with equipment and to each other, and thus damage and lose the feathers. The increase in the number of birds is an additional burden for the ventilation system, which is further contributed by the increased quantity of feces. If the manure removal system is not regularly in operation and/or if the collected manure is kept in the facility for days or even weeks, all parameters of the microclimate may be above the recommended values and the conditions in the facility may be very harmful to the hens but also the workers. Avian behavior is largely dependent on the housing system and microclimate (46), and providing, among other, environmentally stable temperature, lighting, and air quality conditions may safeguard against the development of feather pecking (8). Therefore, in the above-described conditions, the appearance of harmful behaviors is quite expected.

Another example is related to the maintenance of equipment. If the water is constantly dripping from some of the nipple drinkers, the litter underneath becomes wet; warm and moist bedding together with the presence of organic matter from the feces is an ideal medium for the survival of microorganisms and parasites from feces as well as harmful gases production, which increases risk of the occurrence of dermatitis and other diseases.

There are many more examples of how producers may contribute to the poultry welfare problems occurrence. Poor welfare is usually associated with a decrease in productivity (30, 32) and may also impact animal products' quality (23, 32) which posing financial concerns for producers. Wherefore, poor welfare is also opposite to production goals. In contrast to the period when the development of industrial poultry rearing systems began, nowadays, consumers can quickly obtain through the media various information on how poultry is grown and the quality of poultry products they use in nutrition. Based on the way how and which information has been presented, it may contribute to showing poultry production in a poor context and make harm the producers and the entire poultry industry. The goal is something else, to find the best solution that will strike a balance between producer goals, the right of consumers to get a quality product they can afford and the right of production animals to live according to their needs and to end their lives humanely. Regarding that, continuous work on improving existing rearing systems is as important as the work on better informing the producers and the public on poultry welfare problems.

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Conflict of Interest

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Yayım Koşulları

1. Dergi, Ankara Üniversitesi Veteriner Fakültesi'nin hakemli bilimsel yayın organı olup, üç ayda bir yayımlanır. Derginin kısaltılmış adı "Ankara Üniv Vet Fak Derg" dir.
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   Anahtar sözcükler, alfabetik sıralanmış olarak yazılmalı ve 5 sözcükten fazla olmamalıdır.
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   Lamont LA, Bulmer BJ, Sisson DD, et al. (2002): *Doppler echocardiographic effects of medetomidine on dynamic left ventricular outflow tract obstruction in cats.* J Am Vet Med Assoc, **221**, 1276-1281.
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