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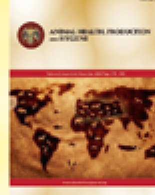

Research Article

Investigation of Mobilized Colistin Resistance Gene-1 in Poultry Pathogenic *Escherichia coli*

Ecenur YILMAZ[1], Süheyla TÜRKYILMAZ[2*]

[1]*Aydın Adnan Menderes University, Health Sciences Institute, Aydın, TÜRKİYE*

[2]*Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydın, TÜRKİYE*

ABSTRACT

This study aimed to determine the presence of the mobilized colistin resistance gene 1 (*mcr*-1), which provides plasmid-mediated colistin resistance in avian pathogenic *Escherichia coli* (APEC) isolates, and to examine the antibiotic resistance profiles of colistin resistant isolates. In this study, isolates from 200 broilers with suspicion of colibacillosis from previous studies were used as material. Following the isolation of *E. coli* through classical conventional methods, identification and antibiotic susceptibility tests were conducted using an automated microbiology system (BD Phoenix 100TM, USA). The presence of the *mcr*-1 gene in isolates phenotypically determined as colistin resistant was investigated using polymerase chain reaction (PCR). Ten (6.4%) out of one hundred fifty-six *E. coli* isolates were found to be phenotypically resistant to colistin. It was found that 80% of colistin resistant *E. coli* isolates were resistant to l evofloxacin, 70% to cefazolin and cefuroxime, 60% to ceftazidime, 50% to gentamicin and ceftriaxone, 40% to cefepime, 30% to ceftolozane-tazobactam, and 20% to piperacillin-tazobactam. All isolates were sensitive to amikacin, ertapenem, imipenem, meropenem, and resistant to ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, trimethoprim-sulfamethoxazole, and tigecycline, exhibiting multidrug resistance (MDR). All isolates that were found to be phenotypically resistant to colistin also carried the *mcr*-1 gene. These findings indicate that *mcr* in APEC contributes to the rapid spread of plasmid-mediated resistance genes and the escalation of broad-spectrum antibiotic resistance. The detection of resistance to antibiotics used in human medicine may pose a potential threat to public health. Future studies should be conducted with samples from different regions and include a diverse sample group to better understand this risk.

Keywords: *Antibiotic resistance, avian pathogenic Escherichia coli, mobile colistin resistance gene.*

Kanatlı Patojenik *Escherichia coli*'lerde Mobilize Kolistin Direnç Geni-1'in Araştırılması

ÖZET

Bu çalışma, kanatlı patojenik *Escherichia coli* (APEC) izolatlarında plazmit aracılı kolistin direnci sağlayan mobilize kolistin direnç geni 1 (*mcr*-1) varlığının belirlenmesi ve kolistin dirençli izolatların antibiyotik direnç profillerinin incelenmesi amaçlandı. Çalışmada, kolibasillozis şüpheli 200 broylerden daha önceki çalışmalar kapsamında elde edilen izolatlar materyal olarak kullanıldı. *E. coli* izolasyonları klasik konvansiyonel yöntemler ile gerçekleştirildikten sonra identifikasyonlar ve antibiyotik duyarlılık testleri otomatize mikrobiyoloji sistemi (BD Phoenix 100™, ABD) sistemi yardımı ile yapıldı. Fenotipik olarak kolistin dirençli olduğu tespit edilen izolatlarda *mcr*-1 gen varlığı polimeraz zincir reaksiyonu (PZR) ile incelendi. Yüz elli altı *E. coli*'nin 10 (%6,4)'unun fenotipik olarak kolistin dirençli olduğu saptandı. Kolistin dirençli *E. coli* izolatların %80'inin levofloksasine, %70'inin sefazolin ve sefuroksime, %60'ının sefotazidime, %50'sinin gentamisin ve seftriaksona, %40'ının sefepime, %30'unun seftolozan tazobaktama, %20'sinin piperasilin tazobaktama dirençli oldukları belirlendi. Tüm izolatlar amikasin, ertapenem, imipenem, meropenem duyarlı; ampisilin, amoksisilin klavulanat, ampisilin sulbaktam, trimethoprim sulfamethoksazol ve tigesiklin dirençli olup; çoklu antibiyotik direncine (MDR) sahip idi. Fenotipik olarak kolistin dirençli oldukları tespit edilen tüm izolatların genotipik olarak da *mcr*-1 genini taşıdıkları saptandı. Bulgular, APEC izolatlarında kolistin direncinin mobilize olmasının, plazmidik direnç genlerinin hızlı yayılmasına ve antibiyotik direncinin artmasına katkı sağladığını göstermektedir. Beşeri hekimlikte de kullanılan antibiyotiklere direnç saptanması halk sağlığı için tehdit oluşturabilir. Gelecekteki çalışmalar, farklı bölgelerden alınan örneklerle yapılmalı ve bu riskin daha iyi anlaşılması için geniş bir örneklem grubunu içermelidir.

Anahtar kelimeler: *Antibiyotik direnci, kanatlı patojenik Escherichia coli, mobilize kolistin direnç geni.*

****Corresponding author:*** *Süheyla TÜRKYILMAZ, Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydın, TÜRKİYE. sturkyilmaz@adu.edu.tr*

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Introduction

In recent years, antimicrobial resistance against bacteria poses a serious threat to global public health. The increasing use of antibiotics in both animals and humans, particularly in the case of animals, leads to the spread of antimicrobial resistance in society (Salam et al., 2023). The use of antimicrobials in animals for disease control or growth promotion results in commensal microflora acquiring resistance genes from resistant strains, with horizontal gene transfer being the underlying mechanism of this process (Salinas et al., 2019). Studies indicate that antimicrobial resistance in humans can arise through horizontal transfer of antibiotic resistance genes from foodborne sources or direct transfer of resistant bacteria (Marshall et al., 2011).

Escherichia coli, a microorganism belonging to the *Enterobacteriaceae* family, is found in the intestines of both animals and humans. However, it can cause life threatening infections in animals and humans, especially in poultry. Antimicrobial resistant *E. coli* strains pose a potential risk to public health and can function as carriers capable of transferring antimicrobial resistance determinants to their own strains or other bacterial species (Rasheed et al., 2014).

Colistin, previously avoided in human medicine due to systemic toxicity, has been reintroduced for the treatment of Gram-negative bacteria showing multidrug resistance (MDR). Colistin is critically important as a last-resort antibiotic in human medicine (Poirel et al., 2017).

Colistin is also used in veterinary medicine for disease control and growth promotion in food-producing animals (Kempf et al., 2016; Apostolakos & Piccirillo, 2018). The widespread use of colistin in animals can lead to the emergence of colistin resistance in animal-origin bacteria and its transmission to humans (Poirel and Nordmann, 2016). The origin of resistant microorganisms in humans is thought to be cattle and food-producing animals as chickens. Until the discovery of the transferable plasmid-mediated *mcr*-1 gene in 2015, colistin resistance in bacteria was considered to result from chromosomal mutations. The emergence of mcr-mediated colistin resistance poses a significant threat to the treatment of infections (Liu et al., 2016).

Since the initial report of *mcr*-1, many studies have continuously reported new *mcr* genes in *E. coli* worldwide (Lemlem et al., 2023). Following the discovery of *mcr*-1 in China, numerous studies on colistin resistance, especially in food animals including poultry, have been conducted globally, particularly in Asia (Kempf et al., 2016). Many countries have banned the use of colistin as a growth promoter in food additives due to the increased in colistin resistance in animals (Wang et al., 2020). Although colistin resistance has decreased after the complete ban on colistin use in animal production, significant levels of colistin resistance are still reported worldwide, especially in pigs and poultry (Wang et al., 2020). Commonly reported genes encoding colistin resistance include those from *mcr*-1 to *mcr*-10 (Valiakos et al., 2021).

While studies on colistin resistance in Türkiye are limited (Kurekci et al., 2018; Adıgüzel et al., 2021; Erzaim and İkiz, 2021; Aslantaş and Küçükaltay, 2023; Seferoglu et al., 2024). An *mcr*-1 positive *E. coli* isolate reported in Hatay stands out as the first case in the country (Kurekci et al., 2018). Studies conducted in Erzurum and İstanbul also indicate the persistence of colistin resistance in isolates obtained from chicken meat, highlighting it as a significant problem across a wide geography (Adıgüzel et al., 2021; Erzaim and İkiz, 2021).

As of our current knowledge, there is no information regarding the presence of *mcr*-1 mediating colistin resistance in APEC isolates in Aydın province in western Türkiye, and there is no information about the antibiotic resistance profiles of colistin-resistant isolates. This study was conducted to determine the presence of the *mcr*-1 gene in APEC isolates and to examine the antibiotic resistance profiles of colistin-resistant isolates. This study focuses on an important issue for both veterinary medicine and public health, serving as a fundamental in the development of broader and more effective strategies to combat colistin resistance. Additionally, the results of the antibiotic resistance testing will guide veterinarians in directing treatment strategies and monitoring antimicrobial resistance.

Materials and Methods

Ethical Approval

Isolates obtained from previous studies, brought to Aydın Adnan Menderes University Faculty of Veterinary Medicine, Department of Microbiology, Routine Diagnostic Laboratory for routine diagnosis purposes, were used in the study.

Animal Material

In this study, isolates obtained from 200 chickens brought to the Laboratory of Microbiology Department, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, for disease diagnosis throughout the year 2023 (January-December) were used, based on previous studies.

Bacterial Isolation and Identification

Isolates obtained in previous studies were revitalized and purity checks were performed. Isolates were plated on MacConkey agar (Merck 105465, Germany) and incubated aerobically at 37 °C for 24 hours. The following day, a single lactose-positive colony on MacConkey agar was subcultured onto EMB agar (Merck 101347, Germany). After another 24 hours of incubation at 37 °C, *E. coli* colonies exhibiting a characteristic green metallic sheen were selected. These colonies underwent biochemical tests (motility, oxidase, catalase, indole, etc.) (Koneman et al., 1997). Bacterial identification was confirmed using an automated system (BD Phoenix, Becton-Dickinson, USA) following the manufacturer's instructions. Isolates were stored in Brain Heart Infusion Broth (BHIB) supplemented with 20% glycerol (Merck 110493, Germany) at

-20°C.

Antibiotic Susceptibility Test

Antibiotic susceptibility testing (AST) of the *E. coli* isolates was conducted utilizing the BD Phoenix 100™ automated system (Becton-Dickinson, USA) with NMIC/ID 433 panels. A comprehensive panel of 20 antibiotics, spanning nine antimicrobial families, was employed for testing, including aminoglycosides (amikacin, gentamicin), carbapenems (ertapenem, imipenem, meropenem), cephems (cefazolin, cefuroxime, ceftazidime, ceftriaxone, cefepime), penicillin (ampicillin), beta-lactams (ceftolozane-tazobactam, amoxicillin clavulanate, ampicillin sulbactam, piperacillin-tazobactam), lipopeptide (colistin), folate (trimethoprim-sulfamethoxazole), quinolones (ciprofloxacin, levofloxacin), and tetracycline (tigecycline). The resistance profiles of the isolates to these antibiotics were determined, with interpretation based on the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2022). *E. coli* ATCC 25922 strains were used for quality control. Additionally, colistin resistance was specifically assessed in all isolates using the automated system, and the minimum inhibitory concentration (MIC) results were interpreted according to EUCAST clinical breakpoints (susceptible ≤ 2 mg/l; resistant > 2 mg/l) (EUCAST, 2022).

Multidrug Resistance (MDR)

MDR was characterised as resistance to three or more classes of antimicrobial agents (Magiorakos et al., 2012).

Polymerase Chain Reaction

DNA Extraction, Purity, and Quantification Controls

In this study, DNA was extracted using the sonication method (Maniatis & Sambrook, 1989). *E. coli* stock cultures were plated on EMB agar and incubated at 37 °C for 24 hours. A single colony was selected and transferred to 5 ml of nutrient BHIB broth and incubated at 37 °C for 18-24 hours. After centrifugation at 13,500 rpm for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 200 µl of PBS (~10^8/ml). The suspension was sonicated at 40 Hz for 10 minutes and then centrifuged at 13,500 rpm for 5 minutes. The resulting supernatant was collected, and DNA concentration was assessed using a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) to ensure purity and quantify DNA (Turner et al., 2012). Samples with OD260/280 values between 1.6 and 2.0 were considered sufficiently pure. Finally, 3 µl of template DNA was used in each PCR reaction.

Genotypic Detection of Colistin Resistance

Primers previously designed by Lui et al. were used to amplify the *mcr*-1 gene (Liu et al., 2016). The *mcr*-1 gene was detected by conventional PCR in ten isolates that were phenotypically resistant to colistin. The product size of the *mcr*-1-positive amplicon was 309 bp. Bands of the expected size were visualised on a 2% agarose gel after electrophoresis at 100 V for 45 min. The genomic DNA of the *E. coli* NCTC 13846 strain was used as a positive control and the *E. coli* ATCC 25922 strain was used as a negative control in the polymerase chain reactions.

Results

Bacterial Isolation and Identification

In this study, 156 (78.8%) *E. coli* isolates were obtained from 200 broilers suspected of colibacillosis. Gram-negative rod-shaped isolates with a metallic green sheen on EMB agar and forming pink colonies on MacConkey agar, along with negative oxidase and positive catalase and indole tests, were considered suspicious for *E. coli*.

All 156 suspected *E. coli* isolates were identified as *E. coli* using NMIC/ID 433 panels in the BD Phoenix 100™ automated microbiology system. The biochemical test results of the isolates as a result of the identification process carried out with the help of the automated testing system are shown in Table 1.

Antibiotic Susceptibility Testing

An automated microbiology system was used to determine the resistance status of 156 *E. coli* isolates to 20 antimicrobial drugs belonging to nine antimicrobial families. As a result of the antibiotic susceptibility test, 10 (6.4%) of the 156 *E. coli* isolates were found to be phenotypically resistant to colistin. The results of the antibiotic susceptibility testing of ten colistin-resistant isolates are shown in Table 2, Table 3 and Figure 1.

Eight (80%) of the colistin-resistant *E. coli* isolates were found to be resistant to levofloxacin; 7 (70%) to cefazolin and cefuroxime; 6 (60%) to ceftazidime; 5 (50%) to gentamicin and ceftriaxone; 4 (40%) to cefepime; 3 (30%) to ceftolozane tazobactam; 2 (20%) to piperacillin tazobactam. While all colistin-resistant isolates were sensitive to amikacin, ertapenem, imipenem, and meropenem; it was resistant to ampicillin, amoxicillin clavulanate, ampicillin sulbactam, trimethoprim sulfamethoxazole and tigecycline.

While it was determined that *E. coli* isolates showed low rates (10%-30%) of resistance to some antibiotics (ceftolozane-tazobactam, piperacillin-tazobactam), moderate rates (31%-75%) of resistance to many antimicrobials (gentamicin, cefazolin, cefuroxime, ceftazidime, ceftriaxone, cefepime); and high levels of resistance (80%-100%) to others (ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, colistin, trimethoprim-sulfamethoxazole, ciprofloxacin, levofloxacin, tigecycline). While the most effective antibiotics against isolates were amikacin, ertapenem, imipenem, meropenem; ampicillin, amoxicillin clavulanate, ampicillin-sulbactam, trimethoprim-sulfamethoxazole and tigecycline were ineffective antibiotics.

The number of antibiotics to which colistin-resistant *E. coli* isolates are resistant, moderately sensitive and sensitive according to the antibiotic susceptibility test is shown in Table 4.

While one isolate 7, one isolate 8, two isolates 9, two isolates 11, two isolates 12, two isolates 13 were resistant to antibiotics; three isolates were resistant to 6, two isolates 7, one isolate 8, two isolates 9, one isolate 10, and one isolate was susceptible to 12 antibiotics.

Multiple Antibiotic Resistance

All ten (100%) colistin-resistant *E. coli* isolates obtained from broilers with colibacillosis had multiple antibiotic resistance.

Of the ten colistin-resistant *E. coli* isolates, 7 (70%) were resistant to 6 and 3 (30%) were resistant to 7 antimicrobial families.

Table 1. Biochemical test results of *E. coli* isolates

Test	Result	Test	Result	Test	Result
A-ARARR	V	A-GLPRB	-	A-GLYB	-
A-GUGAH	-	A-LARGH	-	A-LGTA	V
A-LEUH	V	A-LPHET	-	A-LPROB	-
A-LPYR	-	A-LTRY	-	A-LYALD	V
A-ACT	-	C-ADO	-	C-CIT	-
C-CLST	V	C-DMNT	V	C-KGA	V
C-MLO	-	C-PXB	V	C-TIG	-
M-NAG	-	N-LGGH	-	N-LPROT	V
P-BDGLU	-	P-BPHO	V	R-BALL	V
R-BGEN	-	R-DEX	+	R-DFRU	+
R-DGAL	V	R-DGUA	+	R-DMLB	V
R-DSBT	V	R-DSUC	-	R-GRA	+
R-LARA	+	R-LRHA	V	R-MBGU	-
R-MTU	-	R-NGA	V	R-NGU	+
S-ORN	V	S-URE	-	t-ESC	-

V: Variable

Table 2. Antibiotic susceptibility test results of colistin-resistant isolates

Antimicrobial Family	Antibiotic Name	1	2	3	4	5	6	7	8	9	10
Aminoglycoside	Amikacin	S	S	S	S	S	S	S	S	S	S
	Gentamicin	S	R	S	R	R	S	S	S	R	R
Carbapenem	Ertapenem	S	S	S	S	S	S	S	S	S	S
	Imipenem	S	S	S	S	S	S	S	S	S	S
	Meropenem	S	S	S	S	S	S	S	S	S	S
Cephem	Cefazolin	R	R	R	R	I	R	R	I	R	S
	Cefuroxime	R	R	I	R	S	R	R	R	R	S
	Ceftazidime	R	R	S	S	S	R	R	R	R	S
	Ceftriaxone	R	R	S	S	S	S	R	R	R	S
	Cefepime	R	R	S	S	S	S	S	R	R	S
Penicillin	Ampicillin	R	R	R	R	R	R	R	R	R	R
Beta lactam	Ceftolozane tazobactam	S	S	S	S	S	R	R	R	S	S
	Amoxicillin clavulanate	R	R	R	R	R	R	R	R	R	R
	Ampicillin sulbactam	R	R	R	R	R	R	R	R	R	R
	Piperacillin tazobactam	S	S	R	S	S	S	S	S	S	S
Folate	Trimethoprim sulfamethoxazole	R	R	R	R	R	R	R	R	R	R
Quinolone	Ciprofloxacin	R	R	R	R	R	R	R	R	R	R
	Levofloxacin	R	R	R	R	R	R	I	R	R	S
Tetracycline	Tigecycline	R	R	R	R	R	R	R	R	R	R

S: Susceptible, I: Intermediate, R: Resistant

Polymerase Chain Reaction

DNAs obtained from all isolates were examined molecularly by polymerase chain reaction for the presence of the *mcr*-1 gene. All ten isolates that were found to be phenotypically resistant to colistin were also found to carry the *mcr*-1 gene genotypically (Figure 2).

All phenotypically colistin-resistant isolates had the plasmid-mediated *mcr*-1 gene.

Discussion

Colistin, also known as polymyxin E, is a decapeptide antimicrobial compound discovered shortly after World War II. However, its use has been restricted due to concerns about systemic toxicity which poses a serious risk to public health. In recent years, there has been an increased reutilization of colistin as a last-resort antibiotic against infections caused by multidrug-resistant Gram-negative

Table 3. Susceptibility and resistance status of colistin-resistant *E. coli* isolates to antibiotics

Antibiotic Name	Total (n=10)		
	Resistant (%)	Intermediate (%)	Susceptible (%)
Amikacin	0 (0)	0 (0)	10 (100)
Gentamicin	5 (50)	0 (0)	5 (50)
Ertapenem	0 (0)	0 (0)	10 (100)
Imipenem	0 (0)	0 (0)	10 (100)
Meropenem	0 (0)	0 (0)	10 (100)
Cefazolin	7 (70)	2 (20)	1 (10)
Cefuroxime	7 (70)	1 (10)	2 (20)
Ceftazidime	6 (60)	0 (0)	4 (40)
Ceftriaxone	5 (50)	0 (0)	5 (50)
Cefepime	4 (40)	0 (0)	6 (60)
Ampicillin	10 (100)	0 (0)	0 (0)
Ceftolozane tazobactam	3 (30)	0 (0)	7 (70)
Amoxicillin clavulanate	10 (100)	0 (0)	0 (0)
Ampicillin sulbactam	10 (100)	0 (0)	0 (0)
Piperacillin tazobactam	2 (20)	0 (0)	8 (80)
Trimethoprim sulfamethoxazole	10 (100)	0 (0)	0 (0)
Ciprofloxacin	10 (100)	0 (0)	0 (0)
Levofloxacin	8 (80)	1 (10)	1 (10)
Tigecycline	10 (100)	0 (0)	0 (0)

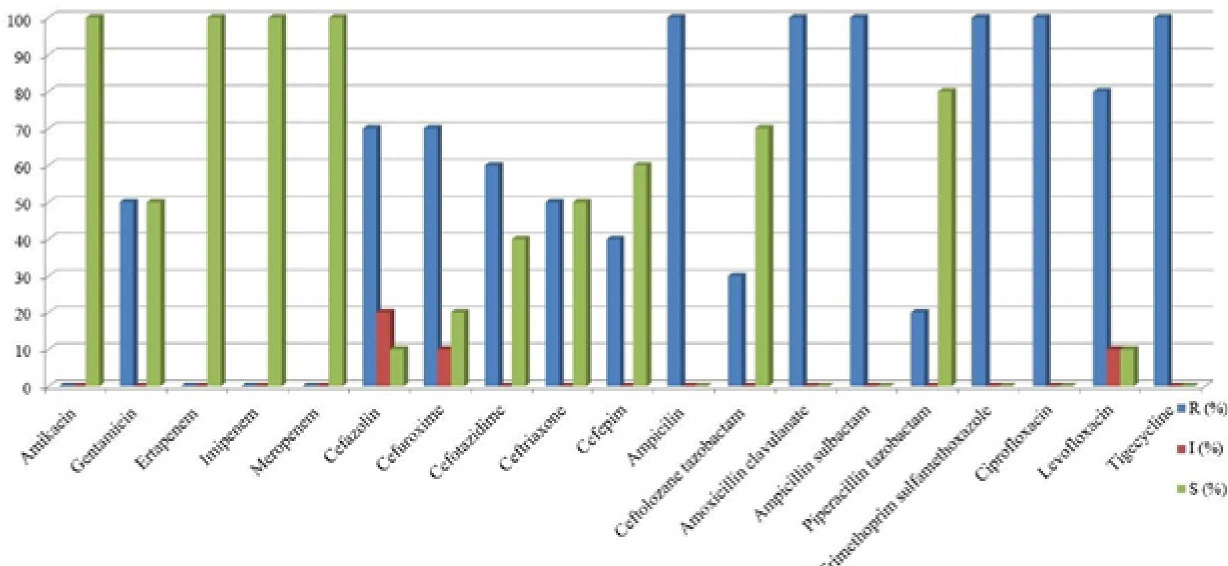

Figure 1. Resistance and sensitivity of colistin-resistant *E. coli* isolates to antibiotics

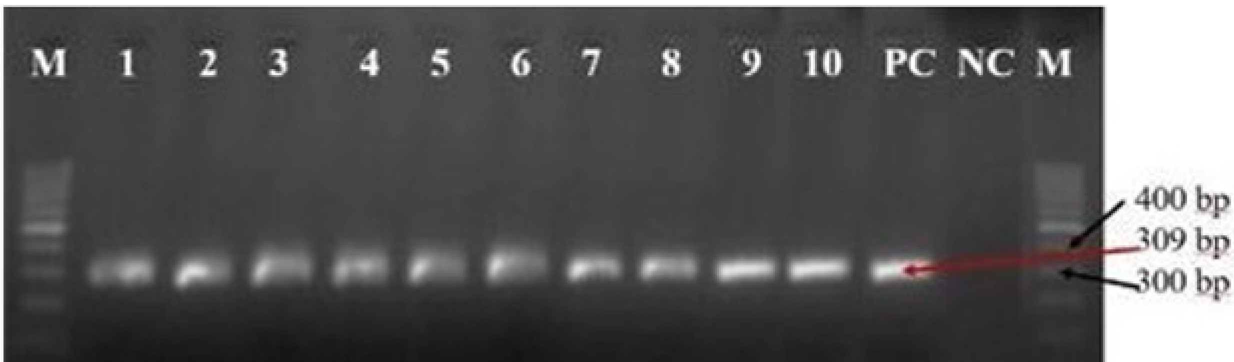

Figure 2. Gel electrophoresis for colistin resistance encoded by the *mcr*-1 gene. M: 100 bp DNA Ladder, 1-10: field isolates with *mcr*-1 gene (309 bp) 11: Positive Control (*E. coli* NCTC 13846), NC: Negative Control (*E. coli* 25922)

bacteria, particularly *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Klebsiella pneumoniae* (Poirel et al., 2017). The discovery of mobile colistin resistance determinants in humans and animals has raised concerns about the future of antimicrobials (Apostolakos and Piccirillo, 2018). Therefore, this study aimed to determine the presence of *mcr*-1, which provides plasmid-mediated colistin resistance in APEC isolates, and to examine the antibiotic resistance profiles of colistin-resistant isolates.

The dissemination of the *mcr*-1 gene to many countries and its presence in bacteria isolated from various environmental sources have been reported (Skov and Monnet, 2016). This situation could increase the transmission of colistin resistance from animal husbandry and agricultural areas to the environment and humans. Therefore, the spread of the *mcr*-1 gene could lead to a serious public health issue.

Studies conducted in food-producing animals have demonstrated an increase in colistin resistance, especially among poultry in Asia (Kempf et al., 2016; Webb et al., 2017). The cheap source of protein provided by poultry and the increasing trend of colistin resistance necessitate a thorough evaluation of colistin use in chickens (Apostolakos and Piccirillo, 2018).

In Türkiye, colistin sulphate is applied to treat gastrointestinal and respiratory system infections caused by *E. coli* and *Salmonella* species in poultry. This study confirms the use of colistin in broiler chickens for therapeutic purposes on the farms where the samples were collected. While studies on colistin resistance in Türkiye are limited (Kurekci et al., 2018; Adıgüzel et al., 2021; Erzaim and İkiz, 2021; Aslantaş and Küçükaltay, 2023; Seferoglu et al, 2024) and there is increasing awareness of this issue. The first reported case of *mcr*-1 positive *E. coli* isolate in the country emerged in Hatay (Kurekci et al., 2018). In a study conducted in Istanbul, phenotypic colistin resistance was detected in 7.5% of 200 *E. coli* isolates obtained from broiler intestinal samples. However, none of the examined samples, including isolates with phenotypic colistin resistance, were found to carry the *mcr*-1 gene. Although colistin resistance was identified phenotypically, the absence of the plasmid-mediated *mcr*-1 gene suggested a chromosomal origin of resistance or the involvement of other resistance genes (Erzaim and İkiz, 2021). Furthermore, a study of colistin resistance in commensal *E. coli* strains isolated from chicken flocks in Hatay revealed that out of 454 isolates examined from cloacal swabs, five isolates carried the *mcr*-1 gene as determined by PCR. Phylogenetic analysis based on whole-genome and multi-locus sequence typing showed that these strains were closely related to *mcr*-1 carrying isolates previously reported from chicken and human clinical isolates in different regions of the world (Aslantaş and Küçükaltay, 2023). In this study, all isolates identified as phenotypically resistant to colistin were found to carry the *mcr*-1 gene, indicating that resistance was plasmid-mediated and mobile. Of the via plasmid transfer of the *mcr*-1 gene can confer colistin resistance to other bacteria, increasing the risk of disease spread. Additionally, the *mcr*-1 gene can be transferred from animal sources to humans, increasing the risk of zoonotic infections. Considering other studies conducted in our country (Adıgüzel et al., 2021; Erzaim and İkiz, 2021), it is evident that colistin resistance persists in isolates obtained from chicken meat in Türkiye, highlighting it as a significant problem over a wide geographical area.

Table 4. The number of antibiotics to which colistin-resistant *E. coli* isolates are resistant, moderately sensitive and sensitive according to the antibiotic susceptibility test

	Number of antibiotics									
Antibiotic sensitivity test result	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Resistance	12	13	9	9	8	11	11	12	13	7
Intermediate	0	0	1	1	1	0	1	1	0	0
Susceptible	7	6	9	9	10	8	7	6	6	12

In veterinary medicine, colistin is commonly used for disease prevention, treatment, or growth promotion (Rhouma et al., 2016). However, the discovery of a plasmid-mediated gene transferable between bacterial species in 2016 raised global concerns. The *mcr*-1 gene, one of the mobile colistin resistance genes, was first discovered in 2016 (Liu et al., 2016), and its resistance development mechanism has not been fully elucidated in many cases (Fukuda et al., 2018). Epidemiological data indicate evidence for the emergence of transferable colistin resistance due to the widespread of colistin use in livestock, with evidence of transfer from animals to humans (Poirel and Nordmann, 2016).

The *mcr*-1 gene is located on a plasmid, a small fragment of DNA that can be transferred from one bacterium to another. The rapid spread potential of the gene to other bacteria increases the likelihood that bacteria resistant to multiple antibiotics will also becoming resistant to colistin as well, underscoring its critical importance in the fight against antibiotic resistance (Tenover, 2006).

Determination of colistin resistance in countries and establishment of gene pools are crucial to identify the prevalence of colistin resistance genes (Etebu and Ukpong, 2016). Phenotypic methods, such as broth and agar microdilution, and disc diffusion methods are commonly used to determine colistin resistance in *E. coli*. However, these methods are time-consuming, impractical, and require laboratory skills. The disc diffusion method provides faster results but lacks standardised disc diffusion zone diameters, making interpretation difficult due to the large molecular structure of colistin (EUCAST, 2022).

Recent studies have reported that automated systems for determining colistin resistance in *E. coli* isolates do not lead to significant errors and demonstrate have acceptable performance (Yiş, 2022; Zhang et al., 2023). The BD Phoenix 100^{TM} automated microbiology system is one of the automated systems used for antimicrobial susceptibility testing. This system uses the broth microdilution method to determine minimum inhibitory concentrations. Due to its automated nature, the BD Phoenix 100^{TM} system can speed up testing processes and reduce operator intervention. Automated systems provide standardised test conditions, this leads to more consistent results. The BD Phoenix 100^{TM} can perform susceptibility testing for many different antibiotics simultaneously (BD Phoenix, 2023). In this study, the BD Phoenix 100^{TM} automated microbiology system was used due to its advantages, such as rapid results for determining phenotypic colistin resistance, standardised test conditions, and multiple antibiotic testing.

The widespread use of antibiotics in poultry farming and the emergence of antibiotic-resistant bacteria pose a significant challenges to both animal and human health. In our study, all isolates were resistance to multiple antibiotics, indicating the limited treatment options available. Similar findings have been reported in previous studies of poultry isolates carrying the *mcr*-1 gene, highlighting the global concern of surrounding antibiotic resistance in poultry production (Apostolakos and Piccirillo, 2018; Aslantas and Kucukaltay, 2023).

Conclusion

In this study, the presence of the *mcr*-1 gene in APEC isolates was confirmed, highlighting the widespread prevalence of colistin resistance and its impact on other antibiotics. All phenotypically colistin-resistant isolates were found to carry the *mcr*-1 gene, indicating plasmid-mediated mobilisation of colistin resistance. These findings highlight the urgent need for surveillance and control measures to address antibiotic resistance in animal pathogens, and emphasise the importance of revising veterinary health policies and regulating the use of colistin. Monitoring the use of antibiotics in animal husbandry and promoting sustainable practices are crucial steps in the fight against antibiotic resistance and the protection of animal and human health. In addition, further research on colistin resistance and genetic spread is essential, highlighting the importance of implementing appropriate measures to prevent the spread of resistance genes.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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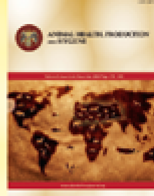

Research Article

Molecular Typing of Vibrio Species Isolated from Sea Bass (*Dicentrarchus labrax*) and Detection of Antibiotic Resistance

Oğuzhan DOLGUN[1], Şükrü KIRKAN[2], Hafize Tuğba YÜKSEL DOLGUN[2*]

[1]Aydın Adnan Menderes University, Health Sciences Institute, Department of Microbiology, Aydın, TÜRKİYE
[2]Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydın, TÜRKİYE

ABSTRACT

This study was conducted to isolate *Vibrio* species from sea bass, identify them using molecular methods, and determine their antibiotic resistance. In the research, 100 sea bass samples taken from fish farms in the Aegean region between May and September 2021 were examined. After the phenotypic and genotypic identification of the isolates obtained from the samples, antibiotic resistances were determined by the disk diffusion method, and antibiotic resistance genes were determined by multiplex PCR. In this study, 46 (46%) *Vibrio* spp. isolates were obtained from 100 sea bass samples by conventional and biochemical methods. The obtained 46 isolates were confirmed to be *Vibrio* spp. by 16S rRNA PCR. From 46 isolates, 22 (47.8%) isolates were identified as *V. alginolyticus*, 13 (28.2%) isolates as *V. harveyi*, 3 (6.5%) isolates as *V. parahaemolyticus*, 1 (2%) isolate as *V. vulnificus* and 7 (15%) isolates as *Vibrio* spp. by multiplex PCR. The highest resistance was found to ampicillin (84.8%) in the isolates in the antibiogram. All isolates were found to be susceptible to enrofloxacin and sulfamethoxazole-trimethoprim (100%). In isolates, the highest resistance gene was found to be trimethoprim resistance gene (63%), and the lowest resistance gene was found to be fluoroquinolone resistance gene (6.5%). In this study, it was determined that *Vibrio* species have an important role as a primary agent in fish diseases, molecular methods give more reliable results in identification, and there is single and multiple antibiotic resistance among isolates.
Keywords: *Antibiotic resistance, identification, PCR, Vibrio spp.*

Levreklerden (*Dicentrarchus labrax*) İzole Edilen Vibrio Türlerinin Moleküler Tiplendirilmesi ve Antibiyotik Dirençliliklerinin Belirlenmesi

ÖZET

Bu çalışma, *Vibrio* türlerinin levreklerden izolasyonu, moleküler yöntemlerle tanımlanması ve antibiyotik dirençlerinin belirlenmesi amacıyla yapılmıştır. Araştırmada Mayıs-Eylül 2021 tarihleri arasında Ege bölgesindeki balık çiftliklerinden alınan 100 levrek örneği incelendi. Örneklerden elde edilen izolatların fenotipik ve genotipik olarak tanımlanmasının ardından disk difüzyon yöntemiyle antibiyotik dirençleri ve multipleks PCR ile antibiyotik direnç genleri belirlendi. Araştırmada 100 levrek örneğinden konvansiyonel ve biyokimyasal yöntemle 46 (%46) *Vibrio* spp. izolatı elde edilmiştir. Elde edilen 46 izolatın16S rRNA PCR ile *Vibrio* spp. olduğu doğrulanmıştır. Multipleks PCR ile 22 (%47,8) izolatın *V. alginolyticus*, 13 (%28,2) izolatın *V. harveyi*, 3 (%6,5) izolatın *V. parahaemolyticus*, 1 (%2) izolatın *V. vulnificus*, 7 (%15) izolatın *Vibrio* spp. olduğu belirlenmiştir. Antibiyogramda en yüksek direncin ampisiline (%84,8) karşı geliştiği belirlenirken tüm izolatların enrofloksasin ve sulfametoksazol-trimetoprime (%100) duyarlı olduğu tespit edilmiştir. İzolatlarda en yüksek direnç geninin trimetoprim direnç geni (%63), en düşük direnç geninin ise florokinolon direnç geni (%6,5) bulunmuştur. Bu çalışmada *Vibrio* türlerinin balık hastalıklarda primer erken olarak önemli bir rolü olduğu ve identifikasyonda moleküler yöntemlerin daha güvenilir sonuç verdiği, izolatlar arasında tekli ve çoklu antibiyotik direnci bulunduğu tespit edilmiştir.
Anahtar kelimeler: *Antibiyotik dirençliliği, identifikasyon, PCR, Vibrio spp.*

****Corresponding author:*** *Hafize Tuğba YÜKSEL DOLGUN, Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydın, TÜRKİYE. tugba.yuksel@adu.edu.tr*

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Introduction

Aquaculture has emerged as a critical sector in meeting the escalating global demand for affordable, high-quality protein, driven by rapid population growth. Technological advancements have further elevated the significance and expansion of aquaculture (Done et al., 2015). According to the United Nations Food and Agriculture Organization (FAO), global aquaculture production reached 114.5 million tons, valued at 263.6 billion US dollars in 2018. Of this production, 54.3 million tons comprised fish, with 47.0 million tons from freshwater and 7.3 million tons from marine fish farming (FAO, 2020).

In Türkiye, aquaculture began in the 1960s with *Cyprinus carpio* and rainbow trout (*Oncorhynchus mykiss*), gaining momentum with the introduction of sea bream and sea bass farming in the 1980s (Arslan & Yıldız, 2021). Currently, sea bream represents 35% and sea bass 26% of marine aquaculture in Türkiye, while rainbow trout is the dominant species in freshwater aquaculture (TÜİK, 2021).

The major bacterial diseases affecting cultured fish in Türkiye include Vibriosis (Candan, 1991; Onuk et al., 2018; Duman et al., 2023), Photobacteriosis (Çağırgan, 1993), motile Aeromonas septicaemia (Baran, 1980; Duman et al., 2018), Pseudomonas infection (Matyar et al., 2010; Duman et al., 2021), Mycobacteriosis (Korun et al., 2005; Urku et al., 2018), Streptococcosis (Akaylı et al., 2008), Rickettsia infection (Timur et al., 2005), and Flavobacteriosis (Yardımcı, 2011; Saticioglu et al., 2018; Saticioglu et al., 2019).

Vibriosis, caused by *Vibrio* species, is particularly problematic in aquaculture, affecting various aquatic organisms including fish, molluscs, crustaceans, rotifers, and corals (Chong et al., 2011; Gomez-Gil et al., 2014). *Vibrio* species are Gram-negative, motile, rod-shaped bacteria, forming smooth, cream-colored colonies, and are oxidase-positive, fermentative, and susceptible to many Vibriostatic agent O/129 (Toranzo and Barja, 1990; Alsina and Blanch, 1994; Yaman et al., 2003; Noga, 2010; Actis et al., 2011).

The challenge of vibriosis disease to sustainable aquaculture is significant, and is further compounded by interactions between host, pathogen and environmental stressors (Toranzo et al., 2005; Noga, 2010). The ability of *Vibrio* species to thrive independently in aquatic environments underscores the urgency of addressing bacterial diseases in aquaculture (Pridgeon and Klesius, 2012).

In Türkiye, *V. harveyi* has been isolated from sea bream and sea bass, *V. anguillarum* from rainbow trout, sea bass, cultured sea bream, and red coral fish, and *V. ordalii* from sea bream and sea bass. Additionally, *V. alginolyticus*, *V. scophthalmi*, and *V. logei* have been isolated from cultured sea bass, alongside other pathogenic bacteria (Timur et al., 2005; Demircan and Candan, 2006; Korun, 2006; Tanrıkul, 2007; Akaylı et al., 2008; Tanrıkul and Gültepe, 2011).

Globally, antibiotics (such as oxytetracycline, enrofloxacin, florfenicol, and sulfonamides) are extensively used in Türkiye to treat fish bacterial diseases. However, the misuse and overuse of antibiotics contribute to the emergence of antibiotic-resistant bacteria and diminishing antibiotic efficacy over time (Colquhoun et al., 2007; Rodgers, 2009). Hence, accurate and prompt diagnosis of fish diseases and appropriate treatment selection are crucial (Kırkan et al., 2006; Akşit and Kum, 2008; Boran et al., 2013).

The aim of this study is to identify *Vibrio* species isolated from sea bream using traditional and molecular methods and to determine their antibiotic resistance and antibiotic resistance genes.

Materials and Methods

Samples

The research material consists of specimens of sea bass (*Dicentrarchus labrax*) raised in cage systems at aquaculture facilities along the Aegean Region coast. Sampling was conducted between May and September 2021, when water temperatures increase and fish losses due to diseases increase. A total of 100 samples were collected from suspected sea bass with lesions found dead in the cage systems of these farms and transported under a cold chain to the diagnosis laboratory of the Department of Microbiology.

Phenotypic Isolation and Identification of Vibrio Isolates

Samples were taken from the internal organs (liver, spleen, kidney) of naturally infected and dead sea bass. Initially, blood agar with 1.5% NaCl (Merck, Germany) and MacConkey agar (Merck, Germany) were inoculated with the samples and incubated at 25°C for 24 hours. Then, round, smooth, semi-transparent, or cream-colored colonies with haemolysis were detected on blood agar, and transparent colonies on MacConkey agar in equivalent petri dishes were selected for Gram staining. The selected colonies were stained with Gram staining (Merck, Germany), and Gram-negative comma-shaped bacteria were used for further analysis. Catalase tests were initially performed on colonies determined to be Gram-negative. Oxidase tests (Merck, Germany) were performed on colonies that tested positive for catalase. Colonies showing a positive reaction in the oxidase test were subjected to the Vibriostatic agent O129 (Bio-Rad, USA) resistance test and incubated at 30 °C for 24 hours. Strains showing inhibition zones around the Vibriostatic agent O129 disks were considered sensitive. Colonies identified as sensitive were passaged onto TCBS agar (Merck, Germany) and incubated at 25 °C for 24 hours. Colonies observed in yellow and green colours were recorded as *Vibrio* spp. and stored at -20 °C in Brain Heart Broth (Merck, Germany) supplemented with 20% glycerol (Merck, Germany) and 1.5% NaCl (Thompson et al., 2004).

Table 1. Primer sequences and targeted amplicon sizes used in PCR analysis

Target *Vibrio* Species	wPrimer Name	Primer Sequence (5'-3')[a]	Primer conc. (µM)	Amplicon size (bp)
Vibrio spp.	VG C2694352F46 VGC2694352R734	GTCARATTGAAAARCARTTYGGTAAA-GG ACYTTRATRCGNGTTTCRTTRCC	1	689
V. parahaemolyticus	VP 1155272F VP 1155272R	AGCTTATTGGCGGTTTCTGTCGG CKCAAGACCAAGAAAAGCCGTC	0.24	297
V. cholerae	VC C634002F VC C634002R	CAAGCTCCGCATGTCCAGAAGC GGGGCGTGACGCGAATGATT	0.24	154
V. vulnificus	VV2055918F79 VV 2055918R	CAGCCGGACGTCGTCCATTTTG ATGAGTAAGCGTCCGACGCGT	0.4	484
V. alginolyticus	VA 1198230F VA 1198230R	ACGGCATTGGAAATTGCGACTG TACCCGTCTCACGAGCCCAAG	0.1	199
V. mimicus	VMC727581F VMC727581R	ATAAAGCGGGCTTGCGTGCA GATTTGGRAAAATCCKTCGTGC	0.8	249
V. harveyi	VH-4F VH-7R	GTGATGAAGAAGCTTATCGCGATT CGCCTTCTTCAGTTAACGCAGGA	0,5	601

[a] Mixed base: K = G + T; R = A + G; Y = C + T; N = A + C + G + T

Phenotypic Identification of Vibrio Isolates with BD Phoenix™

In this study, *Vibrio* spp. suspected isolates were identified using the BD Phoenix (Becton Dickinson, USA) device. Fresh cultures of 24 h pure *Vibrio* spp. isolates on tryptic soy agar (Merck, Germany) were prepared in suspension with ID broth available in glass tubes, adjusted to McFarland 0.5 colony density. The BD Phoenix NMIC/ID-433 panel kit was used for Gram-negative bacterial isolates. The diagnosis was made using separate panels for each sample. ID Broth suspension tubes prepared separately for each sample were placed in the device for bacterial identification. Biochemical identification data obtained from the device were evaluated.

Genotypic Identification

DNA isolation of Vibrio isolates

Vibrio spp. colonies were passaged onto TSA agar (Merck, Germany) supplemented with 1.5% NaCl and incubated at 25 °C for 24 h. DNA was extracted from the fresh cultures with a DNA extraction kit (MagAttract® HMW DNA Kit, Qiagen, Germany).

Primer sequences used for identification of Vibrio species

The primer sequences used for the PCR analysis to detect *Vibrio* genus and *Vibrio* species in the study were designed by the manufacturer as specified in the studies by Kim et al. (2014, 2015) (Table 1). In the PCR analysis, the following standard strains were used as positive controls: *Vibrio alginolyticus* ATCC 17749, *Vibrio cholerae* ATCC 39050, *Vibrio harveyi* ATCC 33842, *Vibrio mimicus* ATCC 33653, *Vibrio parahaemolyticus* ATCC 17802, *Vibrio vulnificus* ATCC 27562.

16S rRNA PCR analysis

The DNA obtained from *Vibrio* spp. isolates were subjected to 16S rRNA analysis. For this purpose, a PCR mixture was prepared with a total volume of 25 µl containing 25 ng of sample DNA, 200 µM of each dNTP, 0.5 U of Ex Taq DNA polymerase, 1X Ex Taq Buffer, and final concentrations of each primer at 0.24 µM. PCR conditions consisted of an initial denaturation at 94 °C for 5 minutes followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes (Kim et al., 2015). The isolates identified in the 689 bp were identified as *Vibrio* spp.

Species-specific multiplex PCR analysis

Multiplex PCR was performed for species identification of the strains identified as *Vibrio* spp. For the multiplex PCR analysis used for the detection of *Vibrio* species, a total volume of 25 µl was prepared containing 25 ng of sample DNA, 200 µM of each dNTP, 0.5 U of Ex Taq DNA polymerase, 1X Ex Taq Buffer, and primer concentrations as specified in Table 1. PCR conditions consisted of an initial denaturation at 94°C for 5 minutes followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes (Kim et al., 2014; 2015). Following the 2% agarose gel electrophoresis, the agarose gel was analysed using a gel documentation system (Vilber Lourmat®, France) and the presence of bands at 297 bp for *Vibrio parahaemolyticus*, 154 bp for *Vibrio cholerae*, 484 bp for *Vibrio vulnificus*, 199 bp for *Vibrio alginolyticus*, 249 bp for *Vibrio mimicus*, and 601 bp for *Vibrio harveyi* were identified (Kim et al., 2014; 2015).

Antibiotic Susceptibility Tests of Vibrio Isolates

The antibiotic susceptibility of *Vibrio* strains was examined using the Kirby Bauer Disk diffusion method. For this purpose, antibiotic disks containing tetracycline (30 µg/disk), streptomycin (10 µg/disk), sulfamethoxazole-trimethoprim (25 µg/disk), gentamicin (10 µg/disk), enrof-

Table 2. List of primers used to identify antibiotic resistance genes

Primer name	Primer Sequence (5'-3')	Amplicon size (bp)	Target Gene
aadA1-F *aadA1-R*	TATCCAGCTAAGCGCGAACT ATTTGCCGACTACCTTGGTC	447	Streptomycin resistance
tetA-F *tetA-R*	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	577	Tetracycline resistance
tetB-F *tetB-R*	CCTCAGCTTCTCAACGCGTG GCACCTTGCTGATGACTCTT	634	Tetracycline resistance
dfrA1-F *dfrA1-R*	GGAGTGCCAAAGGTGAACAGC GAGGCGAAGTCTTGGGTAAAAAC	367	Trimethoprim resistance
Qnr-F *Qnr-R*	GGGTATGGATATTATTGATAAAG CTAATCCGGCAGCACTATTTA	670	Fluoroquinolone resistance
aac[3]-IV-F *aac[3]-IV-R*	CTTCAGGATGGCAAGTTGGT TCATCTCGTTCTCCGCTCAT	286	Gentamicin resistance
Sul1-F *Sul1-R*	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	822	Sullphonamide resistance
blaSHV-F *blaSHV-R*	TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG	768	Cephalothin resistance
CITM-F *CITM-R*	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	Ampicillin resistance
ereA-F *ereA-R*	GCCGGTGCTCATGAACTTGAG CGACTCTATTCGATCAGAGGC	419	Erythromycin resistance

loxacin (5 µg/disk), cefalothin (30 µg/disk), ampicillin (10 µg/disk), and florfenicol (30 µg/disk) (Oxoid, UK) were used. After incubation, the inhibition zone diameters around the disks were measured, and the susceptibility or resistance of the respective isolate to antibiotics was evaluated according to CLSI standards (CLSI, 2018).

Multiplex PCR for Determining Antibiotic Resistance Genes

To determine the antibiotic resistance genes of molecularly typed *Vibrio* strains, multiplex PCR was performed using the primers specified in Table 2 (Shahrani et al., 2014). PCR amplification was carried out in a 25 µl total volume, containing 5 µl of 10X Taq enzyme buffer solution, 25 mM magnesium chloride ($MgCl_2$), 200 µmol of each dNTP, 2 U Taq DNA polymerase, and 3 µl template DNA. The concentrations of each primer in the prepared master mixes were adjusted to 0.5 µM. PCR conditions included an initial denaturation at 94°C for 8 minutes, followed by 32 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 70 seconds, extension at 72°C for 2 minutes, and a final extension at 7 °C for 8 minutes. PCR products were run on a 1.5% agarose gel to visualize bands of the sizes specified in Table 2 using a gel documentation system.

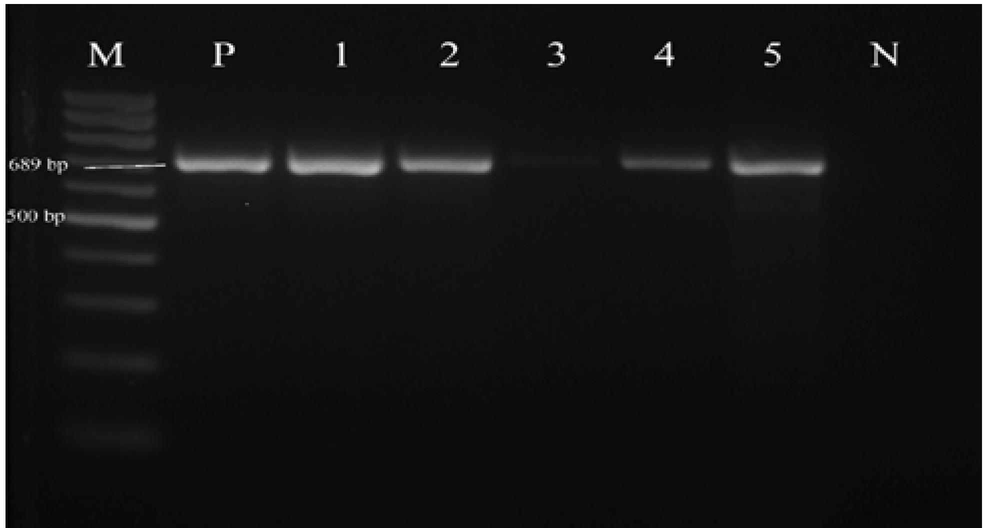

Figure 1. 16S rRNA PCR analysis electrophoresis image of *Vibrio* species. M: Molecular marker 100 bp; P: *Vibrio alginolyticus* ATCC 17749 positive control; N: Negative control (sterilised ddH_2O); 1-5: *Vibrio* spp. positive samples

Results

Phenotypic Isolation and Identification Results for Vibrio spp.

In this study, bacterial growth was observed in 46 out of 100 samples (46%). The suspected colonies were determined to be round, smooth, translucent, and cream-colored and were subjected to Gram staining. The Gram staining results showed that the 46 *Vibrio*-suspected colonies were Gram-negative and comma-shaped bacteria and were positive for both catalase and oxidase tests. These colonies were sensitive to the Vibriostatic agent O129. The 46 suspected *Vibrio* colonies were then subcultured onto TCBS agar, where 25 (54.3%) of the colonies formed yellow colonies and 21 (45.6%) formed green colonies. The isolates confirmed by growth on the Vibrio selective medium TCBS were identified as *Vibrio* spp.

Phenotypic Identification Results of Vibrio spp. Using BD Phoenix™

Among the 46 isolates identified as *Vibrio* spp. based on their growth on *Vibrio* selective TCBS agar, identification using the BD Phoenix™ M50 system revealed that 43 (93.5%) of the isolates were *Vibrio alginolyticus*, while 3 (6.5%) were *Vibrio parahaemolyticus*.

Genotypic Identification Results

16S rRNA PCR results

16S rRNA PCR analysis was conducted on the 46 *Vibrio* spp. isolates identified by phenotypic methods. During electrophoresis, samples within the range of 689 bp were examined, and all 46 strains (100%) were identified as *Vibrio* spp. (Figure 1).

Multiplex PCR analysis results

Multiplex PCR was performed to identify the species of the 46 isolates determined to be *Vibrio* spp. The multiplex PCR results showed bands in the 199 bp range in 22 isolates (48%), identifying them as *V. alginolyticus*; bands in the 601 bp range in 13 isolates (28%), identifying them as *V. harveyi*; bands in the 297 bp range in 3 isolates (7%), identifying them as *V. parahaemolyticus*; and a band at 484 bp in 1 isolate (2%), identifying it as *V. vulnificus*. The analysis did not show band formation at 249 bp and 154 bp, indicating that there were no isolates of *V. mimicus* and *V. cholerae* (Figure 2). In the remaining 7 isolates (15%), no bands were observed from the multiplex PCR, and these isolates were determined to be *Vibrio* spp. (Figure 3).

Antibiogram Results

An antibiogram was performed on 46 identified *Vibrio* isolates using the Kirby-Bauer disk diffusion method. The results of the antibiogram are shown in Table 3. The *Vibrio* isolates (n=46) were resistant to 84.8% of the ampicillin and 54.4% of the streptomycin.

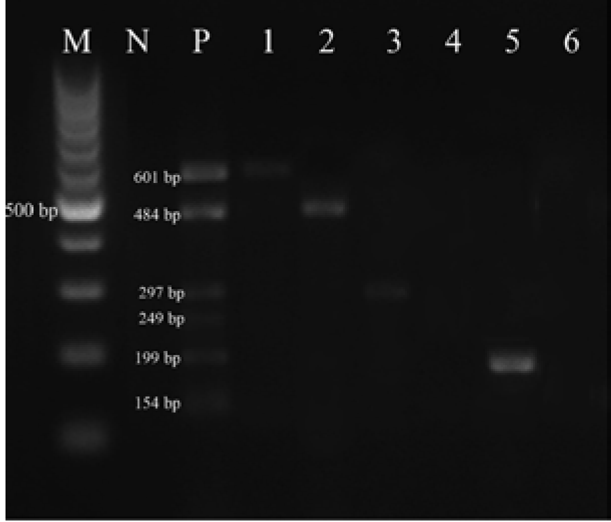

Figure 2. Multiplex PCR analysis electrophoresis image of *Vibrio* species. M: Molecular marker 100 bp; P: *Vibrio* positive control- *Vibrio alginolyticus* ATCC 17749 (199 bp), *Vibrio cholerae* ATCC 39050 (154 bp), *Vibrio harveyi* ATCC 33842 (601 bp), *Vibrio mimicus* ATCC 33653 (249 bp), *Vibrio parahaemolyticus* ATCC 17802 (297 bp), *Vibrio vulnificus* ATCC 27562 (484 bp); N: Negative control; 1: *V. harveyi* positive sample; 2: *V. vulnificus* positive sample; 3: *V. parahaemolyticus* positive sample; 4: *Vibrio* negatives ample; 5: *V. alginolyticus* positive sample; 6: *Vibrio* negative sample.

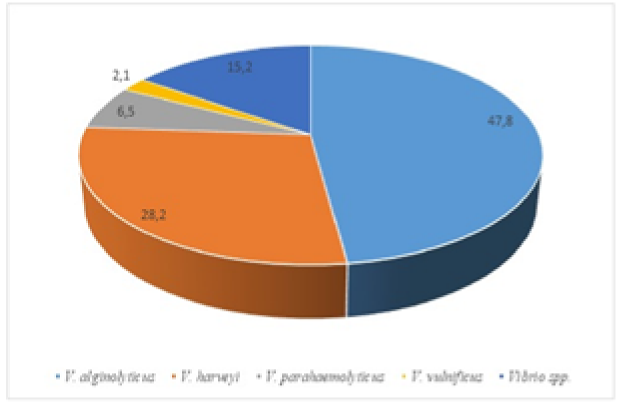

Figure 3. Identification percentages of *Vibrio* species

The isolates were found to be 100% susceptible to sulfamethoxazole-trimethoprim and enrofloxacin, 97.8% to florfenicol, 93.5% to tetracycline, 82.6% to cephalothin, and 80.5% to gentamicin. The susceptibility/resistance profiles of the *Vibrio* isolates (n=46) to different antibiotic types are shown in Figure 4.

Antibiotic Resistance Genes Results

The results of multiplex PCR analysis performed to detect antibiotic resistance genes of 46 *Vibrio* isolates are shown in Table 4.

Multiplex PCR analysis revealed that trimethoprim resistance gene (*dfrA1*) was detected in 29 (63%) isolates, erythromycin resistance gene (*ereA*) in 27 (58.7%) isolates, gentamicin resistance gene (*aac3-IV*) in 24 (52.2%) isolates, cephalothin resistance gene (*blaSHV*) in 20 (43.5%) isolates, tetracycline resistance genes (8 isolates *tetA*, 3 isolates *tetB*) in 11 (24%) isolates, ampicillin resistance

Table 3. Antibiogram results of *Vibrio* isolates

Sample No	Multiplex PCR	SXT	AMP	KF	ENR	FFC	S*	CN	T
1	*V. harveyi*	S	R	S	S	S	R	R	S
2	*V. alginolyticus*	S	R	S	S	S	R	I	S
3	*V. harveyi*	S	R	I	S	S	R	I	S
4	*V. parahaemolyticus*	S	R	S	S	S	R	S	S
5	*V. alginolyticus*	S	R	S	S	S	R	S	S
6	*V. alginolyticus*	S	R	S	S	S	I	S	S
7	*V. alginolyticus*	S	R	S	S	S	R	S	S
8	*V. alginolyticus*	S	R	S	S	S	I	S	S
9	*V. alginolyticus*	S	R	S	S	S	I	S	S
10	*V. alginolyticus*	S	R	S	S	S	R	S	I
11	*V. alginolyticus*	S	R	S	S	S	R	S	S
12	*V. alginolyticus*	S	R	S	S	S	I	S	S
13	*V. alginolyticus*	S	R	S	S	S	R	S	S
14	*V. harveyi*	S	R	S	S	S	I	S	S
15	*V. alginolyticus*	S	I	S	S	S	R	S	S
16	*V. harveyi*	S	R	S	S	S	I	S	S
17	*V. alginolyticus*	S	R	S	S	S	R	S	S
18	*V. vulnificus*	S	R	I	S	S	I	I	S
19	*V. alginolyticus*	S	I	S	S	S	I	R	S
20	*V. harveyi*	S	R	S	S	S	I	S	S
21	*V. alginolyticus*	S	R	S	S	S	I	S	S
22	*V. harveyi*	S	R	I	S	S	R	S	S
23	*V. alginolyticus*	S	R	S	S	S	I	S	S
24	*V. harveyi*	S	R	R	S	S	I	S	S
25	*V. harveyi*	S	R	I	S	S	I	S	S
26	*Vibrio* spp.	S	R	S	S	S	R	S	S
27	*V. alginolyticus*	S	R	S	S	S	R	S	S
28	*V. alginolyticus*	S	R	S	S	S	R	S	S
29	*V. alginolyticus*	S	I	S	S	S	R	S	R
30	*V. alginolyticus*	S	I	S	S	S	I	S	S
31	*Vibrio* spp.	S	R	S	S	I	R	S	S
32	*V. alginolyticus*	S	R	S	S	S	I	S	S
33	*V. alginolyticus*	S	R	S	S	S	R	I	S
34	*Vibrio* spp.	S	S	S	S	S	R	S	S
35	*Vibrio* spp.	S	R	S	S	S	I	S	S
36	*V. parahaemolyticus*	S	S	S	S	S	S	S	S
37	*V. harveyi*	S	R	S	S	S	I	S	S
38	*V. parahaemolyticus*	S	S	S	S	S	R	S	S
39	*V. alginolyticus*	S	R	S	S	S	I	S	S
40	*Vibrio* spp.	S	R	S	S	S	S	S	S
41	*V. harveyi*	S	R	S	S	S	R	S	S
42	*V. harveyi*	S	R	I	S	S	I	I	S
43	*Vibrio* spp.	S	R	S	S	S	R	I	S
44	*V. harveyi*	S	R	S	S	S	R	S	S
45	*V. harveyi*	S	R	R	S	S	R	R	S
46	*Vibrio* spp.	S	R	R	S	S	R	S	R

SXT: Sulfamethoxazole-trimethoprim; AMP: Ampicillin; KF: Cephalothin; ENR: Enrofloxacin; FFC: Florfenicol; S*: Streptomycin; CN: Gentamicin; T: Tetracycline; S: Susceptible; I: Intermediate; R: Resistance

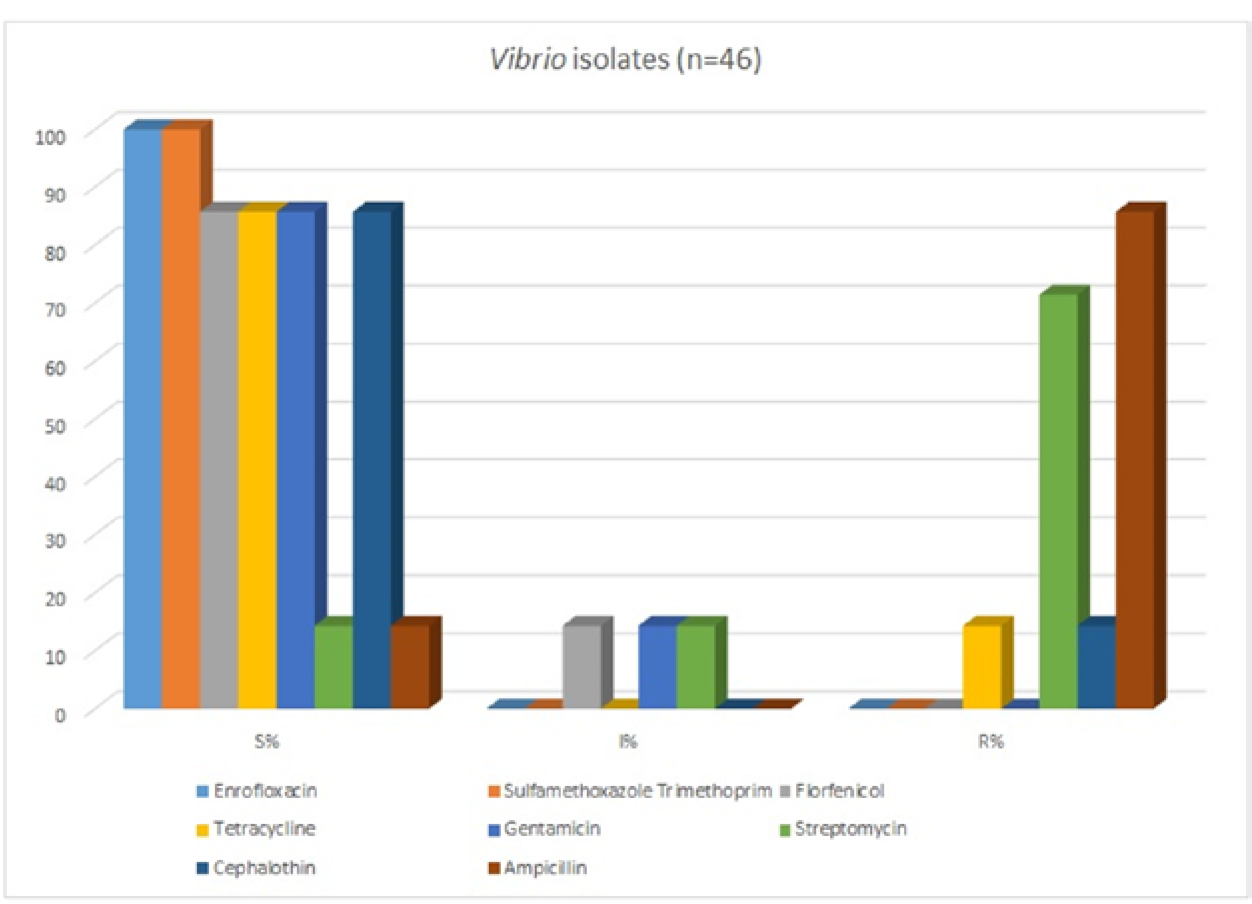

Figure 4. Antibiotic sensitivity/resistance percentages of *Vibrio* isolates

gene (*CITM*) in 6 (13%) isolates, sulfonamide resistance gene (*sul1*) in 5 (10.9%) isolates, streptomycin resistance gene (*aadA1*) in 5 (10.9%) isolates, and fluoroquinolone resistance gene (*Qnr*) in 3 (6.5%) isolates, either individually or in multiple combinations. The number of resistance genes present in the *Vibrio* isolates (n=46) was examined, revealing that 2 isolates (4.3%) had 1 resistance gene, 15 isolates (32.6%) had 2 resistance genes, 16 isolates (34.7%) had 3 resistance genes, 6 isolates (13%) had 4 resistance genes, 4 isolates (8.6%) had 5 resistance genes, and 1 isolate (2%) had 7 resistance genes, while 2 isolates (4.3%) did not have any resistance genes.

Discussion

The aquaculture industry is currently the fastest-growing sector in meeting nutritional standards and contributing to global economic development by providing high protein sources. Bacterial diseases in aquaculture affect both economical and social development in many countries. Vibriosis is one of the most common bacterial diseases affecting various marine fish. The most common species infecting animals in aquaculture farms are *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. owensii*, and *V. campbellii* (Ina-Salwany et al., 2019).

Our study comprised 100 suspected sea bass with vibriosis. Through bacteriological culturing, biochemical analyses, and TCBS agar incubation, 46 isolates (46%) were isolated as *Vibrio* spp. It was determined that 25 colonies (54.4%) on TCBS agar were yellow, while 21 colonies (45.6%) were green. For species-level identification, the BD Phoenix automated identification system was utilized, identifying 43 isolates (93.5%) as *V. alginolyticus* and 3 isolates (6.5%) as *V. parahaemolyticus*. All isolates (n=46) were confirmed as *Vibrio* spp. using 16S rRNA PCR analysis. Subsequent multiplex PCR analysis for species identification revealed that 22 isolates (48%) were identified as *V. alginolyticus*, 13 isolates (28%) as *V. harveyi*, 3 isolates (7%) as *V. parahaemolyticus*, and 1 isolate (2%) as *V. vulnificus*. Seven isolates (15%) showed no bands in multiplex PCR and were identified as *Vibrio* spp. It was determined in our study that *V. alginolyticus* was the predominant species, as *V. cholerae* and *V. mimicus* species were not isolated and identified from the analysed samples.

In our study, isolation, and identification were initially conducted using conventional methods and the Vibrio selective medium TCBS agar, followed by PCR analyses for species identification. Similar to our research, studies by Raissy et al. (2012), Uzun and Ogut (2015), Suresh et al. (2018), Abd El Tawab et al. (2018), Deng et al. (2020), El-Gamal and El Bahi (2020), Gxalo et al. (2021), Sadat et al. (2021), and Zin et al. (2021) performed *Vibrio* spp. identification primarily using conventional methods and culture on TCBS agar, followed by PCR analyses for species identification.

Our findings from both biochemical analysis-based identification using the BD Phoenix automated identification

Table 4. Antibiotic resistance genes detected in *Vibrio* isolates

Sample No	Multiplex PCR	Antibiotic Resistance Genes					
1	*V. harveyi*	*ereA*	*CITM*	*tetB*	*blaSHV*	*sul1*	
2	*V. alginolyticus*	*aac 3-IV*	*dfrA1*				
3	*V. harveyi*	*ereA*	*dfrA1*	*tetA*			
4	*V. parahaemolyticus*	*aac 3-IV*	*ereA*	*dfrA1*	*aadA1*	*CITM*	*blaSHV*
5	*V. alginolyticus*	*aac 3-IV*	*ereA*	*blaSHV*	*sul1*		
6	*V. alginolyticus*	*ereA*	*dfrA1*				
7	*V. alginolyticus*	*ereA*	*dfrA1*				
8	*V. alginolyticus*	*aac 3-IV*	*blaSHV*	*sul1*			
9	*V. alginolyticus*	*aac 3-IV*	*dfrA1*				
10	*V. alginolyticus*	*aac 3-IV*	*ereA*	*dfrA1*	*blaSHV*		
11	*V. alginolyticus*	*aac 3-IV*	*ereA*	*dfrA1*	*blaSHV*		
12	*V. alginolyticus*	*ereA*	*dfrA1*				
13	*V. alginolyticus*	*aac 3-IV*	*dfrA1*	*ereA*	*blaSHV*		
14	*V. harveyi*	*ereA*	*dfrA1*	*blaSHV*			
15	*V. alginolyticus*	*aac 3-IV*	*ereA*				
16	*V. harveyi*	*ereA*	*dfrA1*				
17	*V. alginolyticus*	*aac 3-IV*	*blaSHV*				
18	*V. vulnificus*	*dfrA1*	*CITM*	*tetA*	*Qnr*	*sul1*	
19	*V. alginolyticus*						
20	*V. harveyi*	*aadA1*	*Qnr*				
21	*V. alginolyticus*	*aac 3-IV*	*ereA*	*blaSHV*			
22	*V. harveyi*	*ereA*	*aadA1*	*tetA*	*blaSHV*		
23	*V. alginolyticus*	*dfrA1*	*aadA1*				
24	*V. harveyi*	*ereA*	*dfrA1*	*tetA*			
25	*V. harveyi*	*aac 3-IV*	*dfrA1*	*tetA*			
26	*Vibrio* spp.	*aac 3-IV*	*ereA*	*dfrA1*	*aadA1*	*blaSHV*	
27	*V. alginolyticus*	*aac 3-IV*	*ereA*	*blaSHV*			
28	*V. alginolyticus*	*aac 3-IV*	*ereA*	*blaSHV*			
29	*V. alginolyticus*	*aac 3-IV*	*blaSHV*				
30	*V. alginolyticus*	*aac 3-IV*					
31	*Vibrio* spp.	*ereA*	*dfrA1*	*sul1*			
32	*V. alginolyticus*	*aac 3-IV*	*ereA*	*blaSHV*			
33	*V. alginolyticus*	*aac 3-IV*	*ereA*	*blaSHV*			
34	*Vibrio* spp.	*aac 3-IV*	*ereA*	*CITM*			
35	*Vibrio* spp.	*aac 3-IV*	*ereA*	*dfrA1*	*tetA*	*blaSHV*	
36	*V. parahaemolyticus*	*aac 3-IV*	*dfrA1*	*CITM*	*blaSHV*		
37	*V. harveyi*	*ereA*	*dfrA1*	*blaSHV*			
38	*V. parahaemolyticus*	*ereA*	*dfrA1*	*tetB*			
39	*V. alginolyticus*						
40	*Vibrio* spp.	*aac 3-IV*	*dfrA1*				
41	*V. harveyi*	*ereA*	*dfrA1*	*Qnr*			
42	*V. harveyi*	*dfrA1*	*tetB*				
43	*Vibrio* spp.	*dfrA1*					
44	*V. harveyi*	*aac 3-IV*	*dfrA1*	*tetA*			
45	*V. harveyi*	*dfrA1*	*tetA*				
46	*Vibrio* spp.	*dfrA1*	*CITM*				

52 colonies were obtained on TCBS media, 34 of which were yellow, and 18 were green. The 18 (34.6%) green colonies were identified as *V. parahaemolyticus*. API 20E tests identified 15 isolates as *V. parahaemolyticus*, and sequencing confirmed 18 isolates as *V. parahaemolyticus*. Comparing our study with these studies reveals differences in identification methods, species diversity investigated, and water temperature parameters, which could explain the discrepancies in our findings.

Duman et al. (2023) investigated the genetic relationship of Vibrio species isolated from fish farms in Türkiye. For this purpose, a total of 256 Vibrio isolates were typed using multilocus sequence analysis (MLSA) and typing (MLST) methods. For all isolates studied (n = 256), diversity analysis, population structure, determination of recombination, demographic history and gene flow were performed according to the MLST scheme. As a result of the study, Vibrio isolates showed high diversity within the Vibrio population and also genetic interactions within the genus. 17 new sequence types were identified by MLST analysis isolated from rainbow trout, sea bream and sea bass in fish farms in Türkiye, and it was emphasized by the researchers that this situation clearly showed that genes frequently undergo recombination. As a result of the research, it was concluded that *V. anguillarum* and *V. alginolyticus* are the dominant species fish farms in Türkiye and have biochemically heterogeneous properties, but it is argued that genotype differences should be evaluated in case of a disease or to prevent measurements.

In our study, *Vibrio* isolates were resistant to ampicillin at a rate of 84.8%, streptomycin at 54.4%, cephalothin at 6.5%, gentamicin at 6.5%, and tetracycline at 4.3%. The isolates were found to be 100% sensitive to sulfamethoxazole-trimethoprim and enrofloxacin, 97.8% sensitive to florfenicol, 93.5% sensitive to tetracycline, 82.6% sensitive to cephalothin, and 80.5% sensitive to gentamicin. The highest resistance was observed against ampicillin. Raissy et al. (2012) performed a disk diffusion analysis to determine the antibiotic resistance profiles of *Vibrio* spp. isolates (n=72) and found that 70 (97.2%) isolates were resistant to ampicillin. Similarly, Suresh et al. (2018) reported that 93.38% of their 15 *Vibrio* spp. isolates were resistant to ampicillin, with the highest resistance observed against this antibiotic. Our study also found that the highest resistance among all *Vibrio* isolates was to ampicillin (84.8%), consistent with these studies.

In contrast to our findings, Raissy et al. (2012) reported that of the 72 *Vibrio* spp. isolates, 60 (83.3%) were resistant to gentamicin, 18 (25%) to streptomycin, and 13 (18.1%) to tetracycline. Suresh et al. (2018) observed that their Vibrio spp. isolates exhibited high levels of resistance to gentamicin (80%), tetracycline (33.33%), and streptomycin (6.66%). Deng et al. (2020) used the disk diffusion method to for the antibiogram of 70 *Vibrio* spp. isolates and found resistance rates of 12.8% to gentamicin, 10% to tetracycline, and 7.1% to sulfamethoxazole-trimethoprim. The differences in findings between our study and these studies are thought to be due to variations in the antibiotics used for treatment.

In our antibiogram results, it was determined that 21 isolates (45.6%) were resistant to 1 antibiotic type, 19 isolates (41.3%) to 2 antibiotic types, 1 isolate (2%) to 3 antibiotic types, and 2 isolates (4%) to 4 antibiotic types. Evaluation by antibiotic groups revealed that 44 isolates (95%) were resistant to at least 1 antibiotic group, 20 isolates (43.4%) to 2 antibiotic groups, and 2 isolates (4.3%) to 3 antibiotic groups. Two isolates (4.6%) did not develop resistance to any antibiotic group. Shahimi et al. (2021) found that 45.8% of *V. alginolyticus* isolates were resistant to one or more antibiotics; Raissy et al. (2012) found that 11% of *Vibrio* isolates were resistant to 4 antibiotics, 26.4% to 5 antibiotics, 13.8% to 6 antibiotics, 47.2% to 7 antibiotics, and 1.3% to 8 antibiotics; Yang et al. (2017) reported that 68.38% of *Vibrio* isolates were resistant to more than 3 antibiotics. It is noteworthy that the resistance rates and resistance diversity obtained in these studies are higher than the research findings we obtained. This difference may be attributed to variations in treatment protocols and the diversity of antibiotics used in the regions where the studies were conducted, in comparison to the findings of our study.

When we examined the number of resistance genes in our study, 1 resistance gene in 2 (4.3%) isolates, 2 resistance genes in 15 (32.6%) isolates, 3 resistance genes in 16 (34.7%) isolates, 4 resistance genes in 6 (13%) isolates, 5 resistance genes in 4 (8.6%) isolates, 7 resistance genes in 1 (2%) isolate were determined. It was observed that no resistance gene was present in two isolates (4.3%). Trimethoprim resistance gene in 29 (63%) isolates, erythromycin resistance gene in 27 (58%) isolates, gentamicin resistance gene in 24 (52%) isolates, cephalothin resistance gene in 20 (43%) isolates, tetracycline gene in 11 (24%) isolates, ampicillin resistance gene in 6 (13%) isolates, sulphonamide resistance gene in 5 (11%) isolates, streptomycin resistance gene in 5 (11%) isolates, and fluoroquinolone resistance gene in 3 (7%) isolates were detected with single or multiple combinations. Raissy et al. (2012) reported that streptomycin resistance gene was detected in 18 (25%) of *Vibrio* isolates (n = 72), tetracycline resistance gene was detected in 6 (8.3%), and erythromycin resistance gene was detected in 5 (7%). Gxalo et al. (2021) stated that in the PCR analysis performed for the presence of the resistance gene, the ampicillin resistance gene was detected in all (100%) *V. vulnificus* isolates. Faja et al. (2019) stated that the highest resistance gene (80.64%) belongs to beta-lactamases, and the lowest resistance gene (16.12%) belongs to florfenicol. When these studies are examined, antibiotic resistance genes also show differences in isolates with multiple antibiotic resistance. In our research, the highest antibiotic resistance gene detected in *Vibrio* isolates was trimethoprim, and the lowest resistance gene was the fluoroquinolone resistance gene.

Conclusion

Aquaculture has an important place in the supply of animal protein in the world and in our country. In addition to its contributions to the food chain, it also contributes to the country's economy by providing employment and exports. The healthy maintenance of the created system is possible with fish health. Diseases damage the economy of businesses by increasing workload, increasing antibiotic costs, and failing to achieve anticipated sales. It is known that *Vibrio* species are frequently encountered in aquaculture and are one of the primary factors that cause deaths.

Appropriate determination of the technique chosen for the diagnosis of diseases and the chosen treatment method is essential to preventing losses. As a result of incorrect identification and antibiotic sensitivity tests, microorganisms gain resistance, and the use of antibiotics to which microorganisms are not sensitive causes both the prolongation of the treatment process, unnecessary antibiotic costs, and antibiotic residues in the seas and aquaculture systems.

In our research, *Vibrio* species in dead sea bass fish with suspicion of vibriosis were investigated. As a result of the analyses, the presence of *Vibrio* species in sea bass fish was detected molecularly. As a result of PCR analyses of antibiogram and antibiotic resistance genes, it was revealed that multiple antibiotic resistances developed in the *Vibrio* species obtained. It is recommended that the identification of *Vibrio* species be done with molecular methods instead of conventional methods, and that antibiotic species that are sensitive as a result of antibiotic sensitivity or resistance tests be used in antibiotic selection.

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Author contribution statement

Concept: O.D., S.K., H.T.Y.D.; Design: O.D., S.K., H.T.Y.D.; Data Collection or Processing: O.D., S.K., H.T.Y.D.; Analysis or Interpretation: O.D., H.T.Y.D.; Literature Search: O.D., S.K., H.T.Y.D.; Writing: O.D., S.K., H.T.Y.D.

Conflict of interest

The authors declare that they have no conflict of interest in this study.

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Research Article

Determination of Thiol/Disulfide Homeostasis and Oxidative Stress Index in Sheeppox Virus

Sinan ADIGÜZEL[1], Oğuz MERHAN[2*]

[1]Kafkas University, Institute of Health Sciences, Kars, TÜRKİYE.
[2]Kafkas University, Faculty of Veterinary, Department of Biochemistry, Kars, TÜRKİYE.

ABSTRACT

The study aimed to evaluate the balance of thiol/disulfide homeostasis and oxidative stress markers in sheep infected with pox virus and to explore their potential diagnostic value. 1-2 years old Morkaraman breed sheep (20 infected with pox virus, 10 healthy) were used in the study. Analyses of total thiol, native thiol, total antioxidant capacity, and total oxidant capacity were conducted on serum samples collected from the sheep's jugular vein using tubes without anticoagulant. Comparison between the pox virus-infected group and healthy sheep revealed a significant decrease in total thiol and native thiol levels, while disulfide, disulfide/native thiol, and disulfide/total thiol levels increased, though these changes were not statistically significant. In addition, it was determined that serum total oxidant capacity and oxidative stress index levels increased, while total antioxidant capacity level decreased. In conclusion, the study's findings indicate that the disease induces oxidative stress. The use of oxidative stress markers, particularly those related to thiol/disulfide homeostasis, may provide insights into the pathogenesis of sheeppox and serve as an additional diagnostic tool.

Keywords: *Oxidative stress index, sheeppox, thiol/disulfide homeostasis.*

Koyun Çiçek Hastalığında Tiyol/Disülfid Homeostazı ve Oksitadif Stres İndeksinin Belirlenmesi

ÖZET

Çalışmadaki amacımız çiçek virüsü ile enfekte koyunlarda tiyol/disülfid homeostazisi ve oksidatif stres parametreleri düzeylerinin belirlenmesi ve bunların diagnostik önemlerinin araştırılmasıdır. Çalışmada 1-2 yaşlı Morkaraman ırkı koyunlar (20 adet çiçek virüsü ile enfekte, 10 adet sağlıklı) kullanıldı. Koyunların *Vena jugularis*'inden antikoagulansız tüplere alınan kan örneklerinden elde edilen serumlarda total tiyol, natif tiyol, total antioksidan kapasite ve total oksidan kapasite analizleri yapıldı. Çiçek virüsü ile enfekte grup ile sağlıklı koyunlar karşılaştırıldığında total tiyol ve natif tiyol düzeylerinin anlamlı olarak azaldığı; disülfid, disülfid/natif tiyol ve disülfid/total tiyol düzeyleri ise artmakla beraber istatistiksel olarak anlamsız olduğu belirlendi. Bunun yanı sıra serum total oksidan kapasite ve oksidatif stres indeksi düzeylerinin arttığı, total antioksidan kapasite düzeyinin ise azaldığı saptandı. Sonuç olarak, çalışmada elde edilen bulgular hastalığın oksidatif strese neden olduğu, oksidatif stres belirteçlerinden özellikle de tiyol/disülfid homeostazis parametrelerinin kullanımı çiçek hastalığının patogenezine katkı sağlayacağı ve teşhisi için yardımcı parametre olabileceği düşünülmektedir.

Anahtar kelimeler: *Koyun çiçeği, oksidatif stres indeksi, tiyol/disülfid homeostazı.*

***Corresponding author:** *Oğuz MERHAN, Kafkas University, Faculty of Veterinary, Department of Biochemistry, Kars, TÜRKİYE.*
oguzmerhan@hotmail.com
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Introduction

Sheeppox virus is a zoonotic disease that affects sheep, goats and humans. The disease still maintains its importance as it is commonly seen in sheep and goats in many parts of the world. In addition to abortion, death, loss of productivity and treatment costs resulting from the disease, the implementation of import bans on live animals and animal products in countries where it is seen negatively affects the economy of small livestock farming (Bowden, 2008; Plowright, 2012). The disease, caused by a virus belonging to the Capripox genus in the Poxvirus family. While the severity of the disease can be influenced by factors such as the animal's age, sex, breed, and physiological condition, it tends to be particularly severe in young lambs (Bhanuprakash et al., 2006).

Thiols are a very important antioxidant in preventing damage caused by oxidative stress and protects the cell against oxidative stress. It appears that the thiol status changes in various diseases and that thiol/disulfide homeostasis is very important in the pathogenesis of diseases. Consequently, assessing thiol/disulfide homeostasis can offer valuable insights into a range of physiological and pathological processes. It is known that oxidative stress occurs in situations such as infection and stress, and as a result, the total oxidant/antioxidant capacity may change and can be used as a marker. In light of this information, researching early markers of inflammation in human and veterinary medicine has gained importance in recent years (Erel and Neselioglu, 2014; Kükürt et al., 2021). When the balance between antioxidants and oxidants in the organism is disrupted for various reasons, free reactive radicals appear (Pizzino et al., 2017). Free radicals can cause damage by affecting the basic building blocks of the body such as lipids, carbohydrates and proteins. If the organism's defence mechanism cannot repair the damage, the cells cannot perform their functions, and the immune system weakens and the severity of the infection increases. As a result, apoptosis may occur in cells (Özcan et al., 2015).

It has been reported that free radicals play a role in the formation of diseases (Akaike, 2001), and that the sheeppox virus affects membrane phospholipids by causing an increase in free radicals and, accordingly, causes damage to cells and tissues (İssi et al., 2008). Therefore, our aim in the study is to determine the levels of thiol/disulfide homeostasis and oxidative stress parameters in sheep infected with pox virus and to investigate their diagnostic importance.

Material and Methods

This study was started after receiving the ethics committee approval of Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK) dated 21.11.2022 and coded 2022/178. In the study, 20 sheep naturally infected with smallpox virus (showing clinical symptoms such as fever, loss of appetite, rhinitis, respiratory problems, smallpox lesions in different parts of the mucosa and skin such as under the tail, eyelids, armpits, between the hind legs, etc.) and 10 healthy sheep (1-2 year old Morkaraman breeds) were used. Before starting the study, the infection was confirmed by taking samples from the lesions in these sheep and isolating and identifying the poxvirus in the research laboratory (Erzurum Veterinary Control Institute-Turkey). The samples taken into the 10 mL tubes without anticoagulant were centrifuged at 3000 rpm for 15 minutes. The serum samples were stored at -20 °C until the analysis would be made.

Total thiol, native thiol, total antioxidant capacity (TAC) and total oxidant capacity (TOC) (Rel Assay Diagnostics, Turkey) in the obtained serum samples were measured colorimetrically (Epoch, Biotek, USA) using commercial test kit. A novel automatic and spectrophotometric technique established by Erel and Neselioglu (2014) was used to determine the thiol/disulphide concentrations. The principle of this method is based on the degradation of dynamic disulphide bonds (-S-S-) to functional thiol groups (-SH) with a sodium borohydride ($NaBH_4$) solution. The remaining $NaBH_4$ residue was totally removed by formaldehyde. Thus, this inhibited extra reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) along with any disulphide bonds resulting from the reaction with DTNB. The following reaction with the DTNB-modified Ellman reagent was used to detect the amount of total thiol. Disulfide = (Total thiol-Native thiol)/2, Disulfide/Native Thiol (%) = (Disulfide x 100)/Native thiol, Disulfide/Total Thiol (%) = (Disulfide x 100)/Total thiol and Native Thiol/Total Thiol (%) = (Native thiol x 100)/Total thiol (Erel and Neselioglu, 2014).

TAC measurement is based on the principle that antioxidant substances present in the sample react with the blue-green ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] radical, causing a decrease or loss of color in the color of the compound. TOC measurement is based on the principle that oxidants present in the sample oxidize the Fe^{+2}-o-dianisidine complex to ferric (Fe^{+3}) ion. Fe^{+3} forms a colored complex with xylenol orange in acidic medium. It was calculated with the formula oxidative stress index (OSI) = (TOC/10xTAC) (Karababa et al., 2013).

Statistical Analysis

The study data were analyzed using the SPSS software package (SPSS 20.0, IBM SPSS Statistics®, Chicago, IL, USA). The distribution of the data obtained from the groups were shown as normal distribution according to the Kolmogorov-Smirnov test. An Independent Samples T-test was employed to compare the groups. $P \leq 0.05$ value was statistically considered significant.

Results

When the sheeppox virus-infected group was compared with healthy sheep, it was determined that total thiol and native thiol ($P<0.01$) levels decreased statistically significantly, while disulfide, disulfide/native thiol and disulfide/total thiol levels increased insignificantly. In

addition, although the native thiol/total thiol level decreased, it was determined to be statistically insignificant (P>0.05) (Table 1). It was determined that serum TOC and OSI levels increased, while TAC (P<0.01) levels decreased statistically significantly (Table 2).

Table 1. Thiol/disulfide homeostasis parameters in clinically healthy and sheeppox virus (X±SEM)

Parameters	Control	Infected	P
Total Thiol (µmol/L)	317.31±20.66	243.09±9.13	0.006
Native Thiol (µmol/L)	267.90±19.30	187.21±9.22	0.002
Disulfide (µmol/L)	24.71±3.05	27.93±3.76	0.509
Disulfide/Native Thiol (%)	9.60±1.27	17.17±3.39	0.135
Disulfide/Total Thiol (%)	7.87±0.93	11.37±1.49	0.127
Native Thiol/Total Thiol (%)	84.25±1.87	77.25±2.99	0.126

Table 2. TAC, TOC and OSI parameters in clinically healthy and sheeppox virüs (X±SEM)

Parameters	Control	Infected	P
TAC (mmol Trolox Equiv/L)	1.42±0.07	1.15±0.06	0.007
TOC (µmol H_2O_2 Equiv/L)	5.61±0.26	7.78±0.52	0.008
OSI (Arbitrary Unit)	0.40±0.02	0.72±0.07	0.004

TAC: Total Antioxidant Capacity, TOC: Total Oxidant Capacity, OSI: Oxidative Stress Index

Discussion

Albumin contains thiol sulfhydryl groups, which are proteins and low molecular weight thiols. This sulfhydryl group plays a crucial role in mitigating oxidative stress. Thiol groups found in sulfur-containing amino acids, such as methionine and cysteine, are primary targets for reactive oxygen species (Erel and Neselioglu, 2014). Thiol groups oxidized by the effect of reactive oxygen species form disulfide bonds (Ates et al., 2016). Determination of thiol/disulfide homeostasis is an indicator of free radical formation (Aksoy and Kirmit, 2020). Free radicals cause deterioration in the functions of thiol-related enzymes and thiol/disulfide homeostasis in the cellular environment (Erel and Neselioglu, 2014).

Thiol/disulfide homeostasis, a new biomarker for oxidative stress, was determined in veterinary medicine; toxoplasmosis (Aydın et al., 2023), gastrointestinal nematodes (Schmidt et al., 2021), foot diseases (Deveci and Erdal, 2022), babesiosis (Tarhan et al., 2023), canine distemper (Değirmençay et al., 2021), sarcoptic mange in sheep (Çamkerten et al., 2019) in calves with neonatal diarrhea (Terzi et al., 2023), dehorning in cattle (Erdoğan et al., 2019), and young cattle with pneumonia (Ertaş et al., 2023). It has been reported that total thiol and native thiol significantly decreased, while disulfide, disulfide/native thiol and disulfide/total thiol increased in sheep with toxoplasmosis (Aydın et al., 2023). In calves with neonatal diarrhea, total thiol and native thiol levels were lower, and disulfide and disulfide/native thiol levels were higher in the diarrheal group (Terzi et al., 2023). Additionally, Emre et al. (2021) reported in a study conducted in cattle with endometritis that native thiol and total thiol levels decreased statistically significantly compared to the control group, while disulfide, disulfide/native thiol, disulfide/total thiol and native thiol/total thiol levels were statistically insignificant. In this study, when the sheeppox virus infected group and healthy sheep were compared, native thiol and total thiol levels decreased statistically significantly, disulfide, disulfide/native thiol and disulfide/total thiol levels increased but were statistically insignificant, as well as native thiol levels. Although the native thiol/total thiol level decreased, it was determined to be statistically insignificant. The reason for the change in homeostasis can be explained by the severity of thiol oxidation and oxidative stress.

Under physiological conditions, oxidants and antioxidants are in balance. However, in case of disease, this balance is eliminated, and oxidative stress occurs. Antioxidants prevent the formation of free radicals and eliminate the oxidants that form. Although oxidative stress level is determined by many methods, it is reported that measuring total oxidant or antioxidant capacity is more useful in studies since the methods that measure molecules one by one are both expensive and time-consuming (Erel, 2004; Erel, 2005). Oxidative stress is important in the formation of inflammatory conditions, and studies have reported that oxidative stress occurs in infectious diseases (Bozukluhan et al., 2017; Merhan et al., 2017; Merhan et al., 2020). In their study on sheep infected with ecthyma, Deveci et al. (2017) reported that TOC and OSI levels increased, indicating oxidative stress. This stress resulted from an imbalance between pro-oxidant and antioxidant molecules, driven by rising oxidant levels. It was reported that oxidative stress occurred, and the level of antioxidants decreased in sheeppox-infected sheep (Kirmizigül et al., 2016). Additionally, in cattle infected with lumpy skin, El Mandrawy and Alam (2018)

reported that the MDA level, increased and the GSH level decreased. In the study, it was determined that TOC and OSI levels increased, and TAC levels decreased in sheep infected with sheeppox. It is thought that the change in oxidative stress index values may be due to the increase in free radicals formed against the virus in host cells.

Conclusion

As a result, it is thought that the findings obtained in the study cause oxidative stress in the disease and the use of thiol/disulfide homeostasis markers will contribute to the pathogenesis of sheeppox disease and may be useful in studies to be conducted for its diagnosis.

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This research was summarized from the Master's thesis of the first named researcher.

Conflict of interest

The authors declare that there is no conflict of interest between them.

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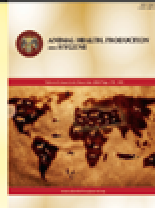

Research Article

Utilization of Computed Tomography at the Aydın Adnan Menderes University Faculty of Veterinary Medicine Research and Application Hospital: An Analysis of Preliminary Results on Patient Demographics and Case Distribution

Murat SARIERLER[1*], Cavit KUM[2], Yalçın Alper ÖZTURAN[1], Bülent ULUTAŞ[3], İbrahim AKIN[1]

[1]*Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Surgery, Aydın, TÜRKİYE ,*
[2]*Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Aydın, TÜRKİYE,*
[3]*Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Internal Medicine, Aydın, TÜRKİYE,*

ABSTRACT

This study examines the distribution of computed tomography (CT) usage at Aydın Adnan Menderes University Faculty of Veterinary Medicine Research and Application Hospital between 2023 and 2024. CT, a widely adopted diagnostic tool in veterinary medicine, allows for detailed imaging of internal organs and anatomical structures, enhancing the diagnosis of complex cases. The present study analyzed data from 143 animals, including dogs, cats, and other species, to assess the frequency of CT scans based on species, age, sex, breed, and imaged anatomical regions. A total of 350 CT scans were reviewed, with dogs (n=87) undergoing the majority of scans (200), followed by cats (n=50) with 175 scans, and other species accounting for 26 scans. Cranium, thorax, and abdomen were the most frequently imaged regions across all species. The study found that older animals, particularly large-breed dogs and male cats, required more imaging, while younger animals (0-6 months) underwent fewer CT scans. The findings emphasize the need for tailored diagnostic approaches based on species, age, and sex to improve early detection and treatment outcomes in veterinary practice. Furthermore, the study highlights the potential for expanding CT use in non-traditional veterinary species, with the goal of improving diagnostic precision. The results provide important insights for enhancing veterinary services through the strategic application of CT imaging.

Keywords: *Computed tomography, diagnostic imaging, veterinary medicine*

Aydın Adnan Menderes Üniversitesi Veteriner Fakültesi Araştırma ve Uygulama Hastanesinde Bilgisayarlı Tomografi Kullanımı: Hasta Demografisi ve Vaka Dağılımı Üzerine Ön Sonuçların Analizi

ÖZET

Bu çalışma, 2023 ve 2024 yılları arasında Aydın Adnan Menderes Üniversitesi Veteriner Fakültesi Araştırma ve Uygulama Hastanesi'nde bilgisayarlı tomografi (BT) kullanımının dağılımını incelemektedir. Veteriner hekimlikte yaygın olarak benimsenen bir tanı aracı olan BT, iç organların ve anatomik yapıların detaylı görüntülenmesine olanak tanıyarak karmaşık vakaların tanısını iyileştirmektedir. Sunulan çalışma, tür, yaş, cinsiyet, ırk ve görüntülenen anatomik bölgeler temelinde BT taramalarının sıklığını değerlendirmek için köpekler, kediler ve diğer türleri de içeren 143 hayvanın verilerini analiz etmiştir. Toplamda 350 BT taraması incelenmiş olup, köpekler (n=87) en fazla tarama (200) gerçekleştirirken, kediler (n=50) 175 tarama ile ikinci sırada yer almakta ve diğer türler 26 tarama ile devam etmektedir. Kafa, toraks ve abdomen, tüm türler arasında en sık görüntülenen bölgeler olmuştur. Çalışma, daha yaşlı hayvanların, özellikle büyük ırk köpekler ve erkek kedilerin daha fazla görüntülemeye ihtiyaç duyduğunu, genç hayvanların (0-6 ay) ise daha az BT taraması geçirdiğini bulmuştur. Bulgular, veteriner pratiğinde erken teşhis ve tedavi sonuçlarını iyileştirmek için tür, yaş ve cinsiyete dayalı özelleştirilmiş tanı yaklaşımlarına ihtiyaç duyulduğunu vurgulamaktadır. Ayrıca, çalışma, tanısal hassasiyeti artırma amacıyla geleneksel olmayan veteriner türlerinde BT kullanımının genişletilmesi potansiyelini de öne çıkarmaktadır. Sonuçlar, BT görüntülemenin stratejik uygulanması yoluyla veteriner hizmetlerin geliştirilmesi için önemli bilgiler sunmaktadır.

Anahtar kelimeler: *Bilgisayarlı tomografi, tanısal görüntüleme, veteriner hekimlik*

***Corresponding author:** *Murat SARIERLER, Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Surgery, Aydın, TÜRKİYE. e-mail: msarierler@adu.edu.tr.*

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Introduction

Advances in veterinary medicine technologies allow for more precise and detailed assessments of animal health. Among these technologies, computed tomography (CT) has emerged as a critical diagnostic tool, particularly in complex cases. While CT devices are widely used in human medicine, they have also become invaluable in veterinary settings, especially for small animals (cats, dogs) and large animals (horses, cattle). CT provides detailed imaging of internal organs, bony structures, and soft tissues, playing a pivotal role in various areas such as pre-surgical planning, tumor diagnosis, trauma, and fracture evaluations.

The application of computed tomography in veterinary medicine varies depending on animal species, age, breed, and the specific reasons for clinical presentation, as well as the distribution of diseases. Neurological disorders, orthopedic issues, and oncological cases are among the primary areas where CT is most frequently utilized (Gielen et al., 2012; Puchalski, 2012; Ballegeer, 2016; Keane et al., 2017). The distribution of patients undergoing CT scans in veterinary clinics offers insight into the prevalence of different conditions across species. For instance, trauma cases may dominate in certain animal groups (Dozeman et al., 2020; Sepuya et al., 2022), while tumor diagnoses could be more frequent in others (Keane et al., 2017; Greco et al., 2023). The use of CT also plays an important role in treatment planning and prognosis assessment.

This study aims to analyze the distribution of CT usage at Aydın Adnan Menderes University Faculty of Veterinary Medicine Research and Application Hospital across different animal species, focusing on the frequency of scans based on breed (in dogs), gender, age groups, and specific body regions imaged. The analysis will categorize data into three main groups: dogs, cats, and other animal species, providing insights into the clinical applications of CT in veterinary medicine. By identifying frequently imaged anatomical regions, the study seeks to enhance early diagnosis and preventive treatment strategies, raising awareness of health issues and guiding veterinary services toward priority species.

Table 1. Descriptive statistics of animals undergoing computed tomography (CT) scan at the Aydın Adnan Menderes University Faculty of Veterinary Medicine Research and Application Hospital between 2023 and 2024 (n)

	Sex			Breed (kg)			Age (month)			
Species	**Male**	**Female**	**Unknown**	**Small (0-10)**	**Medium (11-25)**	**Large (26+)**	**0-9**	**10-18**	**19-108**	**109+**
Dog	46	41	0	20	34	33	1	10	49	27
							0-6	**7-35**	**36-83**	**83+**
Cat	25	25	0				3	16	17	14
Other animals	2 (rabbit)	0	4*							

* The sex records of these animals (two reptile, one parrot and one falcon) were missed.

Table 2. The number of tomographic imaging regions in dogs according to sex, breed, and age groups (n, %)

Sex	Breed (kg)	Age (Month)	Region, n (%)						
			Cranium	Cervical	Thoracal	Abdominal	Lumbar	Extremity	Total
Male	**Small (0-10kg)**	**0-9**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		10-18	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		19-108	9 (2.57)	8 (2.29)	8 (2.29)	9 (2.57)	8 (2.29)	8 (2.29)	50 (14.29)
		109+	1 (0.29)	1 (0.29)	2 (0.57)	2 (0.57)	1 (0.29)	1 (0.29)	8 (2.29)
	Middle (11-25kg)	**0-9**	1 (0.29)	1 (0.29)	1 (0.29)	1 (0.29)	1 (0.29)	1 (0.29)	6 (1.71)
		10-18	3 (0.86)	3 (0.86)	4 (1.14)	3 (0.86)	3 (0.86)	3 (0.86)	19 (5.43)
		19-108	10 (2.86)	7 (2.00)	7 (2.00)	7 (2.00)	7 (2.00)	7 (2.00)	45 (12.86)
		109+	3 (0.86)	3 (0.86)	3 (0.86)	3 (0.86)	3 (0.86)	3 (0.86)	18 (5.14)
	Large (26+ kg)	**0-9**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		10-18	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.29)	0 (0.00)	0 (0.00)	1 (0.29)
		19-108	3 (0.86)	3 (0.86)	6 (1.71)	4 (1.14)	6 (1.71)	4 (1.14)	26 (7.43)
		109+	5 (1.43)	4 (1.14)	4 (1.14)	5 (1.43)	5 (1.43)	4 (1.14)	27 (7.71)
Subtotal			35 (10.00)	30 (8.57)	35 (10.00)	35 (10.00)	34 (9.71)	31 (8.86)	200 (57.14)
Female	**Small (0-10kg)**	**0-9**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		10-18	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.29)	1 (0.29)
		19-108	3 (0.86)	2 (0.57)	2 (0.57)	4 (1.14)	2 (0.57)	2 (0.57)	15 (4.29)
		109+	1 (0.29)	1 (0.29)	2 (0.57)	1 (0.29)	1 (0.29)	1 (0.29)	7 (2.00)
	Middle (11-25kg)	**0-9**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		10-18	2 (0.57)	2 (0.57)	2 (0.57)	2 (0.57)	2 (0.57)	4 (1.14)	14 (4.00)
		19-108	7 (2.00)	6 (1.71)	8 (2.29)	6 (1.71)	6 (1.71)	7 (2.00)	40 (11.43)
		109+	4 (1.14)	2 (0.57)	2 (0.57)	2 (0.57)	2 (0.57)	2 (0.57)	14 (4.00)
	Large (26+ kg)	**0-9**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		10-18	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		19-108	5 (1.43)	3 (0.86)	3 (0.86)	4 (1.14)	3 (0.86)	2 (0.57)	20 (5.71)
		109+	8 (2.29)	7 (2.00)	6 (1.71)	6 (1.71)	6 (1.71)	6 (1.71)	39 (11.14)
Subtotal			30 (8.57)	23 (6.57)	25 (7.14)	25 (7.14)	22 (6.29)	25 (7.14)	150 (42.86)
Total			65 (18.57)	53 (15.14)	60 (17.14)	60 (17.14)	56 (16.00)	56 (16.00)	350 (100.00)

Materials and Methods

This study analyzed the records of patients who underwent CT scanning at Aydın Adnan Menderes University Faculty of Veterinary Medicine Research and Application Hospital between 2023 and 2024. The collected data included demographic informations such as species, sex, age, and breed, as well as the anatomical regions (cranium, cervical, thoracal, abdominal, lumbar, and extremity) imaged during the CT procedures. In this study, age determination for dogs was based on tooth wear time (Sutton et al., 2018). For cats, age determination was based on tooth eruption times and dietary changes (Little, 2011). Dog breeds were categorized into small (0-10 kg), medium (11-25 kg), and large (26 kg and above) groups according to their weight classification (Butković et al. 2001). The obtained data were presented using standard descriptive statistics to summarize the distribution and frequency of imaging across different animal species.

Results

The descriptive statistics of the animals in the study, classified by species, sex, weight, and age, are summarized in Table 1. A total of 87 dogs were identified, with 46 males and 41 females. Based on body weight, 20 dogs were classified as small (0-10 kg), 34 as medium (11-25

Table 3. The number of tomographic imaging regions in cats according to sex and age groups (n, %)

Sex	Age (Month)	Region, n (%)						
		Cranium	Cervical	Thoracal	Abdominal	Lumbar	Extremity	Total
Male	0-6	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
	7-35	5 (2.86)	6 (3.43)	5 (2.86)	6 (3.43)	6 (3.43)	5 (2.86)	33 (18.86)
	36-83	7 (4.00)	5 (2.86)	6 (3.43)	6 (3.43)	5 (2.86)	5 (2.86)	34 (19.43)
	84+	4 (2.29)	4 (2.29)	5 (2.86)	7 (4.00)	4 (2.29)	4 (2.29)	28 (16.00)
Subtotal		16 (9.14)	15 (8.57)	16 (9.14)	19 (10.86)	15 (8.57)	14 (8.00)	95 (54.29)
Female	0-6	2 (1.14)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	2 (1.14)
	7-35	7 (4.00)	3 (1.71)	5 (2.86)	3 (1.71)	3 (1.71)	3 (1.71)	24 (13.71)
	36-83	6 (3.43)	4 (2.29)	4 (2.29)	6 (3.43)	4 (2.29)	4 (2.29)	28 (16.00)
	84+	4 (2.29)	4 (2.29)	5 (2.86)	5 (2.86)	4 (2.29)	4 (2.29)	26 (14.86)
Subtotal		19 (10.86)	11 (6.29)	14 (8.00)	14 (8.00)	11 (6.29)	11 (6.29)	80 (45.71)
Total		35 (20.00)	26 (14.86)	30 (17.14)	33 (18.86)	26 (14.86)	25 (14.29)	175 (100.00)

kg), and 33 as large (26 kg and above). The age distribution revealed that the majority of dogs (n=49) were between 19-108 months, followed by 27 dogs older than 109 months, 10 dogs aged 10-18 months, and one dog in the 0-9 month category. For the cats, there were 25 males and 25 females. Age data revealed that 17 cats were aged 36-83 months old, 16 were 7-35 months old, 14 were older than 83 months, and 3 were in the 0-6 months old. The group of other animals consisted of 2 male rabbits and 4 additional animals (two reptiles, one parrot, and one falcon), though sex records for these four were missing.

The distribution of tomographic imaging regions in dogs based on sex, breed (based on weight), and age is shown in Table 2. A total of 350 scans were conducted across different anatomical regions, with the cranial region having the highest number of scans (n=65, 18.57%), followed by the thoracic and abdominal regions, each accounting for 60 scans (17.14%). The lumbar and extremity regions contributed equally with 56 scans (16%), and cervical scans represented 15.14% of the total. The majority of scans were performed on medium (11-25 kg) and large (26 kg and above) breed dogs, particularly in the 19-108 months age group. Notably, large dogs aged 109 months and older accounted for a significant number of scans, especially in the cranial (n=5, 1.43%), lumbar (n=5, 1.43%), and extremity regions (n=4, 1.14%). Small dogs (0-10 kg) underwent fewer scans overall, with the majority concentrated in the 19-108 months age range (n=50, 14.29%). In terms of sex, both male and female dogs exhibited a similar distribution of scans across all regions. However, slightly more scans were performed on male dogs (n=200, 57.14%) compared to females (n=150, 42.86%). A notable finding is the higher number of cranial and lumbar scans in large females aged 109 months and older (n=8, 2.29%), suggesting an increased need for imaging in this demographic.

The distribution of tomographic imaging regions in cats, based on sex and age were presented in Table 3. A total of 175 scans were performed across different regions, with cranial scans accounting for the highest percentage (n=35, 20%), followed by abdominal scans (n=33, 18.86%) and thoracic scans (n=30, 17.14%). Cervical, lumbar, and extremity regions contributed similarly, with each accounting for 14.29% to 14.86% of the scans. Male cats underwent more scans overall, particularly in the cranial (9.14%), cervical (8.57%), and abdominal (10.86%) regions. In contrast, female cats had a similar distribution across regions, with the highest number of scans occurring in the cranial (10.86%) and thoracic (8%) regions. Cats aged 7-35 months accounted for the highest number of scans, with 33 (18.86%) across various regions, while older cats (aged 84+ months) represented a significant portion of cranial, lumbar, and abdominal scans.

The distribution of tomographic imaging regions in other

Table 4. The number of tomographic imaging regions in other animal species (n, %)

Species	Region, n (%)						
	Cranial	Cervical	Thoracal	Abdominal	Lumbar	Extremity	Total
Avian	1 (3.85)	1 (3.85)	2 (7.69)	1 (3.85)	1 (3.85)	1 (3.85)	7 (26.92)
Rabbit	2 (7.69)	2 (7.69)	2 (7.69)	2 (7.69)	2 (7.69)	2 (7.69)	12 (46.15)
Reptile	1 (3.85)	1 (3.85)	2 (7.69)	1 (3.85)	1 (3.85)	1 (3.85)	7 (26.92)
Total	4 (15.38)	4 (15.38)	6 (23.08)	4 (15.38)	4 (15.38)	4 (15.38)	26 (100.00)

animal species is presented in Table 4. A total of 26 scans were performed across avian, rabbit, and reptile species. The thoracic region accounted for the highest number of scans (n=6, 23.08%), while cranial, cervical, abdominal, lumbar, and extremity regions each contributed equally, with 4 scans each (15.38%). Rabbits underwent the most tomographic scans (n=12, 46.15%), with equal distribution across all anatomical regions. Avian and reptile species each accounted for 7 scans (26.92%), with thoracic scans being the most common in both species (7.69%).

Discussion

This study evaluated the demographic distribution and tomographic imaging practices among animals at Aydın Adnan Menderes University Faculty of Veterinary Medicine Research and Application Hospital from 2023 to 2024, comprising a total of 143 animals and 350 tomographic images. In a previous study by Caspanello et al. (2023) reported five hundred and sixty-one CT exams on 512 dogs and 49 cats were reported in six years. Similarly, the majority of subject animal in the present study were dogs (n=87) with 200 scan, followed by cats (n=50) with 175 scan, and a minor representation from other species, including rabbits (n=4) and reptiles (n=2), which collectively accounted for 26 scan. The present findings indicate that the utilization of the CT scan is in alignment with the previous reports in aid of diagnostic validations, aside from diversity in animal species such as rabbits and reptiles.

The use of CT in veterinary medicine is becoming increasingly prevalent. This auxiliary diagnostic method offers numerous advantages in patient treatment by providing valuable information to practitioners. Additionally, it enables surgeons to visualize detailed anatomical structures in patients requiring surgical intervention, facilitating more precise and less invasive procedures. In the postoperative phase, CT allows for the assessment of complications and the evaluation of surgical success (Ando et al., 2012; Iwanaga et al., 2016; Del Busto et al., 2020; Griffeuille et al., 2021; Cohen et al., 2023). However, the data presented in the current study do not describe the specific advantages of CT for patients. Future research should investigate the benefits of CT across various animal species and diseases, potentially enhancing this diagnostic modality through advancements in software, specialization, and apparatus development.

The CT using in horses (Zimmerman et al., 2022) and cattle (Nuss et al., 2011) have been stated in the literature. The records from our hospital reveal that computed tomography (CT) scans have been performed on various animal species, including avian, reptiles, and rabbits; however, CT scans for large animals have not yet been performed. This limitation may arise from the current CT device and the area designated for its operation is not suitable for larger species, such as cattle and horses. Acquiring a compatible tomography device for large animals, which possess significant economic value, and conducting proper area planning during installation will facilitate the performance of CT scans on these animals and increase the number of large animals undergoing such procedures. By considering the aforementioned recommendations, the prevalence of CT scans for large animals can be enhanced in our country, thereby contributing positively to the economy.

The data analyzed in this study indicates a lack of tomographic imaging for dogs younger than nine months (Table 2). In small breed dogs (0-10 kg) aged 19-108 months, the cranial and abdominal regions were the most frequently imaged, possibly reflecting a higher incidence of conditions in these areas. Middle-aged male dogs (11-25 kg) exhibited a considerable number of scans, particularly in the thoracic region, which might suggest a greater occurrence of respiratory or cardiovascular issues. For large breed dogs (26kg and above), the scans were distributed across multiple regions, especially the abdominal and lumbar areas, potentially highlighting the complexity of health issues that arise with aging in these breeds. The relatively high number of scans in older dogs (109+ months) underscores the importance of routine imaging to monitor age-related health conditions (Table 2). Overall, these findings suggest that tomographic imaging in dogs could benefit from a tailored approach based on breed, age, and sex to enhance early detection and treatment outcomes. Special attention may be needed for the cranial, thoracic, and lumbar regions, particularly in older and larger dogs.

In the present study, similar to dogs, there was a lack of tomographic imaging in cats aged between 0-6 months, and none was performed in males (Table 3). Among male cats aged 7-35 months, the cranial and abdominal regions were most imaged, possibly reflecting a greater prevalence of health problems in these regions and the need for more focused diagnostic approaches for cats in this age group. Female cats aged 36-83 months had a significant number of scans, particularly in the abdominal region, and this finding may emphasize the importance of regular health monitoring in this age group of cats. Older cats (84+ months) had a moderate imaging frequency, especially in the abdominal and thoracic regions (Table 3). The findings of the present study highlight the role of age and sex in influencing veterinary imaging practices in cats and support the need for specific diagnostic strategies to improve feline health outcomes. Miniter et al. (2019) reported 130 abdominal CT scans (75 for canines and 22 for felines) over a two-year period. In this study, 93 abdominal CT scans were identified within one year (60 dogs and 33 cats). The number of CT scans performed within one year in this study suggests a need for CT imaging in the patients presenting to our hospital.

The findings also suggest the potential necessity of tomographic imaging for animals besides companion animal species (Table 4). The variability in imaging requirements among avian and reptilian species may indicate that imaging protocols could benefit from customization to address the specific diagnostic needs of each species.

Conclusion

The present study demonstrates a lack of tomographic imaging in very young animals, particularly in male dogs

and cats aged between 0-6 months. In contrast, older animals exhibit increased imaging frequencies across various regions, suggesting a potential need for diagnostic approaches for certain diseases. The findings highlight the importance of considering species, age, and sex to optimize health outcomes in different animal populations. This study aims to raise awareness of the increasing use of CT in veterinary medicine and to assess the frequency of CT usage between 2023 and 2024. Summarizing the trends in CT scans for patients admitted to our hospital during this period may provide valuable insights for veterinarians, animals, and their owners.

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Conflict of interest

The authors declared that there is no conflict of interest.

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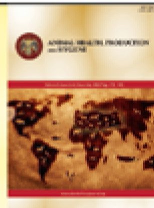

Research Article

Knowledge and Perception of Antimicrobial Resistance in Aquaculture in the Nairobi River Basin, Kenya

Mary A. OPIYO[1*], Fonda Jane AWUOR[2], Hezron AWANDU[2], Kevin ONSONGO[2], Dennis OTIENO[2], Lorna BENTON[3], Claire HEFFERNAN[4]

[1]Kenya Marine and Fisheries Research Institute, National Aquaculture Research Development and Training Center, Sagana, KENYA
[2]Kenya Marine and Fisheries Research Institute, Kisumu Centre, KENYA
[3]The London School of Hygiene & Tropical Medicine, Keppel Street, London, UNITED KINGDOM
[4]The Royal Veterinary College, Royal College Street, London, UNITED KINGDOM

ABSTRACT

Increased food demand in low and middle-income countries (LMICs) has led to the intensification of production, underpinning environmental and health hazards such as increased water needs or misuse of antimicrobials. Epidemics of diseases still emerge often, necessitating the routine administration of antimicrobials to curb their spread. Sub-therapeutic concentrations of these medications persist in water and sediments for extended periods, creating favourable circumstances for developing and selecting resistant microorganisms and stimulating horizontal gene transfer. This study aimed to understand the knowledge and perception of fish farmers towards antimicrobial resistance (AMR) to further responsible usage of antimicrobials and promote antimicrobial stewardship programs. A study involving 34 farmers was undertaken using structured questionnaire interviews and face-to-face workshops to determine the Source, Exposure pathway and Main receptors (S-P-R) of antimicrobials along the Nairobi River basin. Most respondents (59%; n=20) were familiar with "antimicrobial resistance." However, in the last five years, fish diseases and infections on the farm were recognized as a concern, although not a major one (50%; n=17). This is supported by the fact that the majority of respondents (65%; n=22) reported having no specific training in fish health management. Untreated or partially treated wastewater and solid waste disposal/scavenging are the major hotspots for human exposure to AMR. The results of this study provide a baseline understanding of potential risk factors for AMR in aquaculture and can be used in the formulation of appropriate risk-management measures to prevent AMR in cultures fish.

Keywords: *Antimicrobial resistance, aquaculture, farming, Nairobi River Basin.*

Kenya'daki Nairobi Nehri Havzası'nda Su Ürünleri Yetiştiriciliğinde Antimikrobiyal Direnç Hakkında Bilgi ve Algı

ÖZET

Düşük ve orta gelirli ülkelerde (LMICs) artan gıda talebi, üretimin yoğunlaşmasına yol açarak artan su ihtiyacı veya antimikrobiyallerin yanlış kullanımı gibi çevresel ve sağlık riskleri gibi sorunları beraberinde getirmiştir. Hastalık salgınları hala sıkça ortaya çıkmakta, bu durum da antimikrobiyallerin rutin olarak kullanılmasını ve yayılmalarının önlenmesine engel olmaktadır. Bu ilaçların su ve sedimentlerde uzun süreler boyunca sub-terapötik konsantrasyonlarda kalması, dirençli mikroorganizmaların gelişimini ve seçilimini teşvik eden ve gen transferini tetikleyen uygun koşullar oluşturmaktadır. Bu çalışma, balık çiftlikçilerinin antimikrobiyal direnç (AMR) konusundaki bilgi ve algılarını anlamayı ve antimikrobiyallerin bilinçli kullanımını teşvik etmeyi amaçlamaktadır. Nairobi Nehri havzası boyunca antimikrobiyallerin Kaynak, Maruziyet yolu ve Ana alıcılar (S-P-R)ını belirlemek için 34 çiftçi ile planlanmış anket görüşmeleri ve yüz yüze atölye çalışmaları kullanılarak çalışma yürütülmüştür. Katılımcıların çoğu (%59; n=20) "antimikrobiyal direnç" terimi ile tanıdık olmakla birlikte, son beş yılda çiftliklerdeki balık hastalıkları ve enfeksiyonları önemli bir sorun olarak görmemektedir (%50; n=17). Bu durum, katılımcıların çoğunun (%65; n=22) özgül balık sağlığı yönetimi eğitimi almamış olması gerçeği ile desteklenmektedir. Arıtılmamış veya kısmen arıtılmış atık sular ve katı atık bertarafı/taraması, insanların AMR'ye maruz kalmasının başlıca kaynaklarıdır. Bu çalışmanın sonuçları, akuakültürde AMR'yi önlemek için uygun risk yönetimi önlemlerinin formülasyonunda kullanılabilecek akuakültürdeki AMR için potansiyel risk faktörlerine dair temel bir anlayış sağlamaktadır.

Anahtar kelimeler: *Antimikrobiyal direnç, çiftçilik, Nairobi Nehri Havzası, su ürünleri yetiştiriciliği.*

***Corresponding author:** *Mary A. OPIYO, Kenya Marine and Fisheries Research Institute, National Aquaculture Research Development and Training Center, Sagana, KENYA. mopiyo@kmfri.go.ke*

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Introduction

Global fish production is experiencing rapid growth and increased from 21.8 to 156.4 million tonnes between 1960 and 2018 (Little et al., 2016; FAO, 2020). Due to declining capture fisheries, aquaculture accounts for the majority (52%) of aquatic food production, with expansion expected through 2030 (FAO, 2022). The increasing demand for animal protein has led to a transition from extensive to intensive farming, which in terrestrial food animal sectors has historically been accompanied by the increasing use of antimicrobials (Van Boeckel et al., 2015; 2017). The terrestrial and aquatic food production industries have emerged as the largest consumers by volume (73.7% and 5.7%, respectively) of antimicrobials globally (Laxminarayan et al., 2013; Schar et al., 2020).

Antimicrobial resistance (AMR) has been described as one of the global serious public health threats especially for developing countries (Dadgostar, 2019). Antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi, and parasites adapt and grow in the presence of an antimicrobial drug at a concentration that typically kills or inhibits their growth (Founou et al., 2017). This occurs due to natural processes and widespread anthropogenic activity or through mutation or, more likely, horizontal gene transfer (HGT) in the environment via natural transformation, transduction, or conjugation (Watts et al., 2017; Sun et al., 2020). The rise in intensification of aquaculture and farming in small areas has led to increased disease occurrence and a rise in the use of antimicrobials to treat diseases and also as growth promoters (Watts et al., 2017; Limbu et al., 2018). Though there is a national policy on prevention and containment of antimicrobial resistance using the one health approach in Kenya, specific regulations when it comes to the use of antimicrobials in aquaculture do not exist, and there is a reliance on the Animal Disease Act CAP 364 of 2012, which provides guidelines on the treatment of animals.

Most aquaculture and livestock production in the city occurs in small spaces along the riparian land of the Nairobi River (CoG, 2021), mainly in the informal settlements of Mukuru kwa Ruben, Mathare, Kawangware, Kayole, and Ruai (Madara et al., 2022). Farming in these places is unregulated and is characterized by the use of antimicrobials, which are purchased over the counter without any prescription and administered to the animals without the supervision of a veterinarian increasing the risk of antimicrobial resistance (Muloi et al., 2019).

Source, exposure pathway, and main receptors (S-P-R) mapping of antimicrobials in the Nairobi river basin is vital in indicating the possible hotspots of antimicrobial resistance and the various linkages with the food web and the environment, thus enabling the identification of targeted areas to address the challenges associated with antimicrobial resistance. It is therefore important to have an understanding of the knowledge and perception of the fish farmers towards antimicrobial resistance for successful antimicrobial stewardship programs. This study was carried out to assess the level of awareness, attitudes, usage of antimicrobials, and behaviours related to antimicrobial resistance among farmer communities in the Nairobi River basin.

Materials and Methods

Ethical Statement

The study was conducted following the standard operating procedures (SOPs) of the Kenya Marine and Fisheries Research Institute (KMFRI) guidelines for research registered with the National Commission for Science, Technology, and Innovation (NACOSTI) registration number NACOSTI/2016/05/001. The SOPs comply with the Prevention of Cruelty to Animals Act 1962, CAP 360 (Revised 2012) of the laws of Kenya, and the EU regulation (EC Directive 86/609/EEC). Informed consent for this study was obtained from all individual participants included in the study. The study did not involve animals as subjects; hence, ethical committee approval was not required.

Study Area

The study was carried out in Nairobi County at 1.2921° S, 36.8219° E. The target population was fish farmers along the Nairobi River basin in Nairobi County. The locations of the surveyed farms are shown in Figure 1.

Instrument and Procedure

A questionnaire was developed into four sections. The sections were: preliminary information describing the location of the farm; socio-demographics; farm profile, including the species of farmed fish; experience in farming; culture systems; management practices and antimicrobial use; feeds and feeding and the additives used in the feed, including the antimicrobials; risk for diseases and biosecurity measures in the farm; and awareness of antimicrobial resistance in aquaculture. Respondents were required to provide only simple responses (e.g., yes or no) or to select from a list of options. A pilot study was conducted on ten farmers who attended the workshop to improve the comprehension and consistency of the items in the questionnaire.

Questionnaire Administration

The contacts and locations of the farms were obtained from the County Director of Fisheries Office in Nairobi County. The farmers were visited for self-administration of the questionnaire using a mobile application tool (Kobo Collect), which was also used to take the GPS position of the farm. The questionnaire was administered to fish farmers along the Nairobi River basin in Nairobi County between July and August 2021. The study involved 34 fish farmers who were located in the Riverine sub-counties in the Nairobi river basin. Data were submitted to a central database and collated for analysis.

Workshop to Map Aquaculture Systems

A workshop was held with seasoned aquaculture and

AMR experts. The workshop had four aims: 1) to develop systems thinking and experience in mapping systems; 2) to build collaborations and understanding of different expertise; 3) to create maps of the aquaculture sector and the drivers of AMR; 4) to identify the most likely routes of exposure to AMR for humans. The workshop had a combination of presentations, focus group discussions, and plenary discussions.

Statistical Analysis

Data analysis was done using Stata (Version 14.0) statistical software. Data analysis entailed descriptive analysis presented in tables and graphs. We followed a pathway analysis to develop the source, exposure pathway, and main receptors (S-P-R) map.

Results

A total of 34 participants were surveyed (Table 1). The majority were male (79%, n=27) between the ages of 40 and 49. More than 35% of the farmers' level of education was below secondary school level. The commonly used culture system was liner ponds, while other culture systems reported to be common were tanks and aquaponics.

The farms sampled were conducting integrated farming (57.58%), whereby both animal and crop production were connected, as indicated in Figure 2. Animal manure from livestock was being used in crop production and pond fertilization. The pond water was also used to water crops on the farms.

In these integrated systems, chicken dominated other poultry being kept by farmers along the Nairobi River basin (Figure 3).

The majority of the farmers, 66%, did not use any additives in the feeds, and more than 80% of respondents agreed to procure fish feeds from commercial companies rather than on-farm formulated feeds (Table 2). The minority who mixed additives into the feeds, such as vitamins, antibiotics, and probiotics, may reflect farm-lev-

Table 1. Socio-demographic characteristics of fish farmers in NairXobi County

Variable	Indicator	Frequency
Age (Years)	18-29	3(9)
	30-39	7(21)
	40-49	13(38)
	50-59	6(18)
	60 – 69	3(9)
	70 +	2(5)
Gender	Female	7(21)
	Male	27(79)
Education Level	Postgraduate	3(9)
	Diploma	5(15)
	Certificate	8(24)
	Secondary	9(26)
	Primary	3(9)
Responsibility Role	Farm manager	9(26)
	Farm manager & Farm owner	9(26)
	Farm owner	9(26)
	Farm worker	7(22)
Culture system	Earthen ponds	5(15)
	Earthen ponds & Liner ponds	1(3)
	Earthen ponds & Tanks	1(3)
	Liner ponds	14(41)
	Liner ponds & Tanks	2(6)
	Other	1(3)
	Tanks	9(26)
	Tanks & Other	1(3)

* Values in brackets indicate percentages

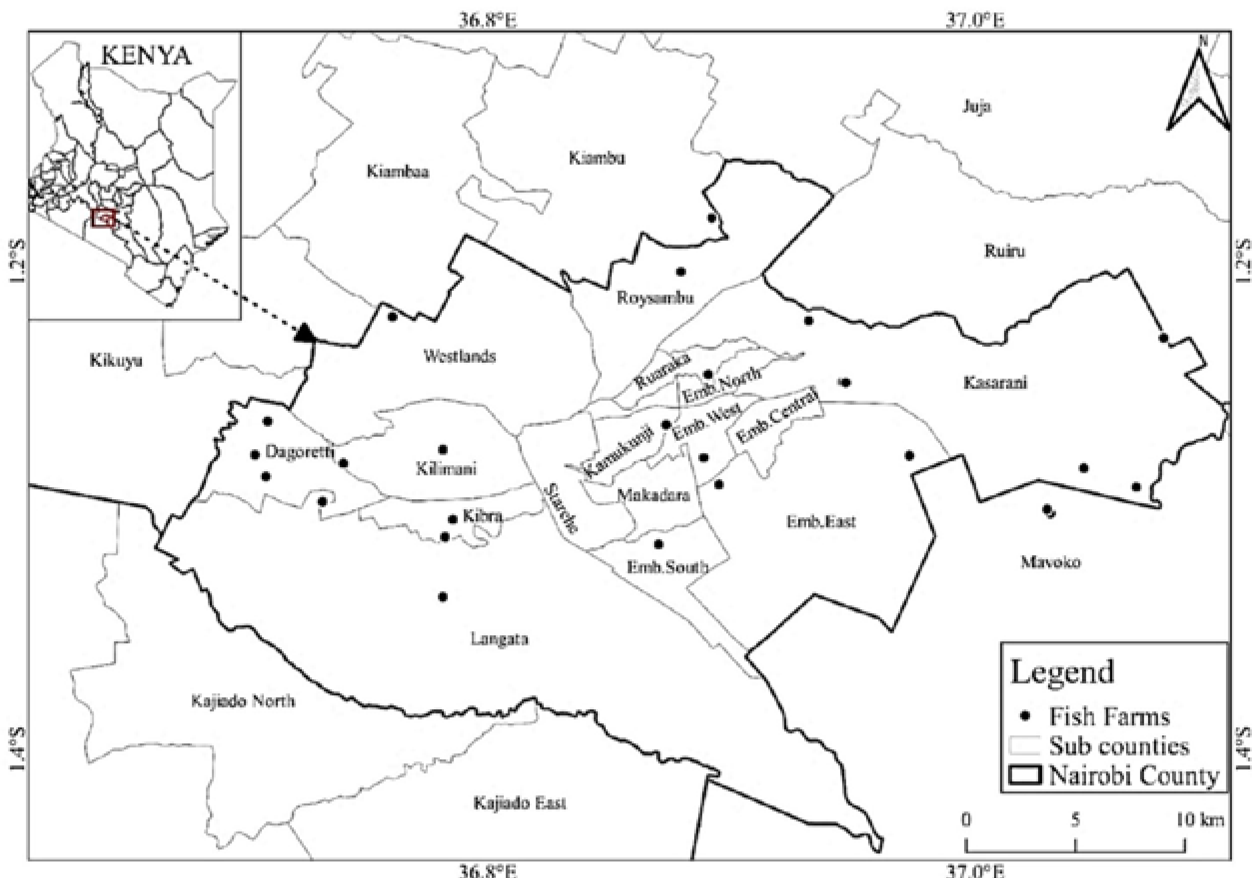

Figure 1. Map of Nairobi County showing the surveyed aquaculture farms

el formulated feeds since the commercial feeds did not indicate the additives in the labels as indicated during the workshop.

Most of the fish farmers did not report mortality of the fish in the last 12 months (56%). The mortality observed was linked to the lack of knowledge of husbandry practices (Table 3).

The respondents' awareness and lack of awareness of antimicrobial resistance were split at 59% and 41%, respectively (Table 4). This is also related to the level of education, whereby about 35% of the respondents had secondary education and below, while the rest had post-secondary education, of which only 35% had received training in fish health management.

More than half of the respondents (59%; n=20) were aware of the term "antimicrobial resistance." In contrast, fewer than half (41%; n=14) were aware of the use of probiotics. These findings are validated by the fact that most respondents reported that they had no specific training in fish health management (65%; n=22) (Table 4). Fish disease and infections on the farm in the last five years were reported as a problem but not major (50%; n=17).

Source, Exposure pathway and Main receptors (S-P-R) of Antimicrobials

The SPR model, informed by the workshop and the survey, shown in Figure 4, is supported by a summary of current evidence on the SPR of AMR in aquaculture. Mapping the SRP of antimicrobials identifies practices and operations that warrant prioritization by future surveillance efforts for antimicrobial resistance. The potential routes of exposure were occupational (different handling points of antimicrobials), food consumption, water contaminated with antimicrobial residues and bacteria, and the environment where the antimicrobials are disposed of. Due to the interconnection of the production systems, the cultured animals (fish and livestock) and the cultivated crops become the receptors of the antimicrobials in the environment.

The potential interventions to reduce antimicrobial resistance identified during the workshop are presented in Table 5 with the responsible stakeholders and actors in the respective areas. The intervention ranges from the farm level to the market level and the consumers of the farmed products. Implementation of best management practices at the farm level and leadership from the government and the industry are required.

Discussion

Antimicrobial resistance is one of the global challenges, and awareness about it is important among Kenyans. The use of antimicrobials must be accepted as a responsibility rather than a right for improved fish health to minimize the potential risk of poor antimicrobial usage in animal production. The results of this study show a low level of women participating in aquaculture activities. This is in

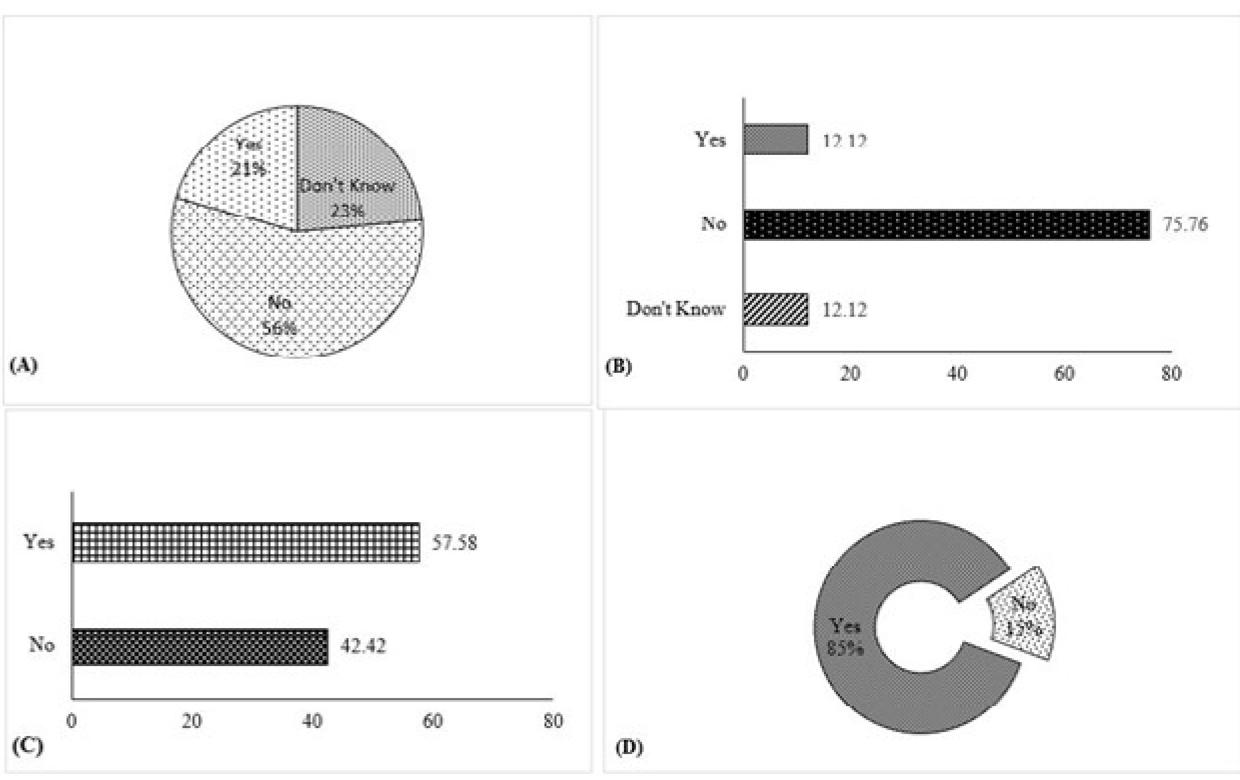

Figure 2. (A) Connection of farm water source with Nairobi River, (B) Sharing of water with nearest farms, (C) Integration with crops, and (D) Presence of livestock in farm site

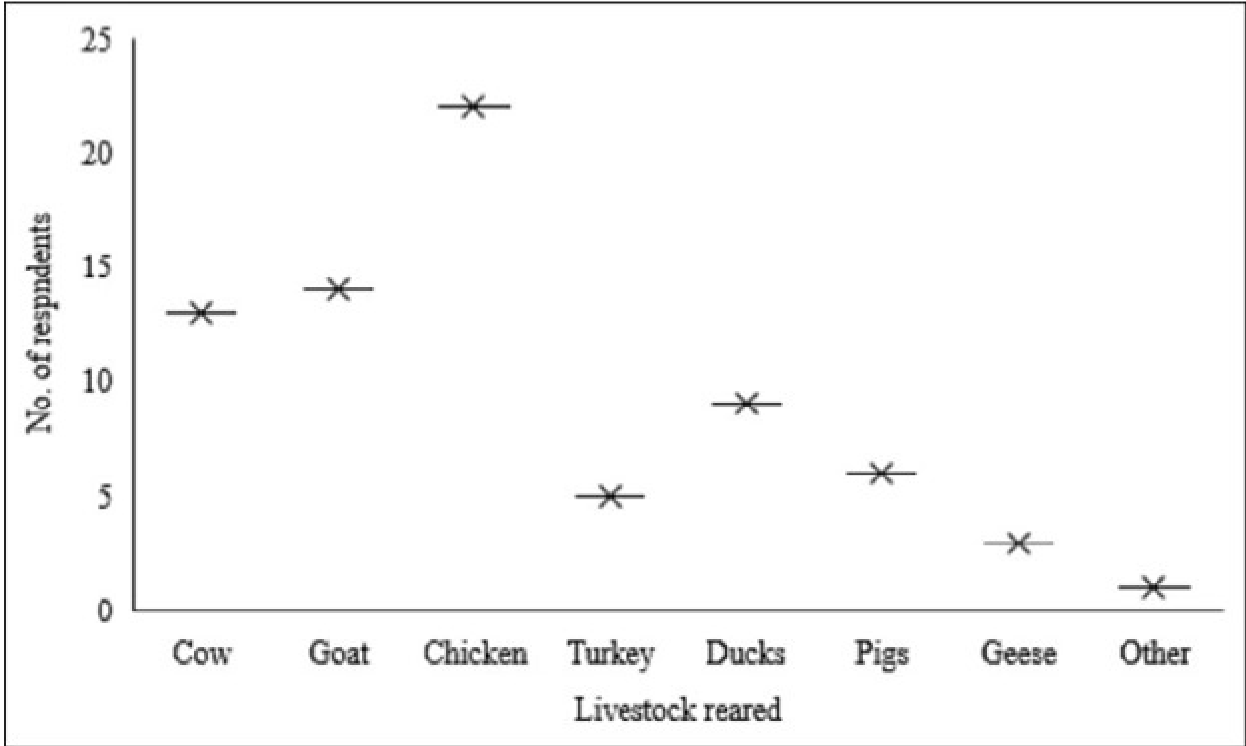

Figure 3. Livestock reared in surveyed farms in Nairobi County, Kenya

line with previous studies, which reported less engagement among women in aquaculture activities (Abwao & Fonda, 2019). Aquaculture is labour-intensive in pond construction; thus, more men are involved, while women sometimes participate in feeding and post-harvest fish processing (Kipkemboi et al., 2007; Awuor, 2021).

The most commonly used culture systems were liner ponds, followed by aquaponics. The dominance of liner ponds could be attributed to the soil type. At the same time, other culture units, such as tanks, are suitable in areas with limited land, such as urban and peri-urban areas, which was the case in the Nairobi river basin. The

Table 2. Fish feeds source, feeding and feed additives in surveyed farms in Nairobi County, Kenya

Variable	Indicator	%
Additives to feed in the last production cycle	Antibiotics	16
	Nothing	66
	Other	6
	Probiotics	3
	Vitamins	9
Ever fed your fish on chicken feed/ dairy meal	No	61
	Yes	39
Farm's main source of fish feeds	Commercial animal feeds	3
	Commercial fish feeds	80
	Own formulation	17

Table 3. Fish mortality, related signs and treatment of surveyed farms in Nairobi County, Kenya

Variable	Indicator	Frequency
Fish mortalities in the last 12 months	No	19(56)
	Yes	15(44)
No. of dead fish per pond (300m^2)	0-100	12(80)
	201-300	2(13)
	1001-1500	1(7)
Presence Clinical signs before fish mortality	Don't Know	3(20)
	No	5(33)
	Yes	7(47)
Actual signs identified	Bloated belly, Feacal dragging	1(14)
	Eroded fins	1(14)
	Gaping gills	1(14)
	White spots & cloudy eyes	1(14)
	Yellow fluid from vent	1(14)
	Red spots, Bloated belly	2(30)
Attempt to treat fish	Don't Know	2(6)
	No	27(79)
	Yes	5(15)
Treatment/chemical Used	Antibiotic	2(40)
	Potassium permanganate	1(20)
	Salt	1(20)
	Smash over-ripe avocado and fish feed	1(20)
Mode of administration/Treatment	In Feed	2(40)
	In water	2(40)
	Other	1(20)

* Values in brackets indicate percentages

Table 4. Disease infections in farms, training in fish health management, awareness of AMR and probiotics in surveyed farms in Nairobi, Kenya

Variable	Indicator	Frequency
Fish disease/infections in the farm in the last 5 years	A major problem	6(18)
	A problem but not major	17(50)
	Don't Know	4(12)
	Not a problem	7(20)
Specific training in fish diseases/fish health management	No	22(65)
	Yes	12(35)
Awareness of AMR	No	14(41)
	Yes	20(59)
Awareness of Probiotics	No	20(59)
	Yes	14(41)

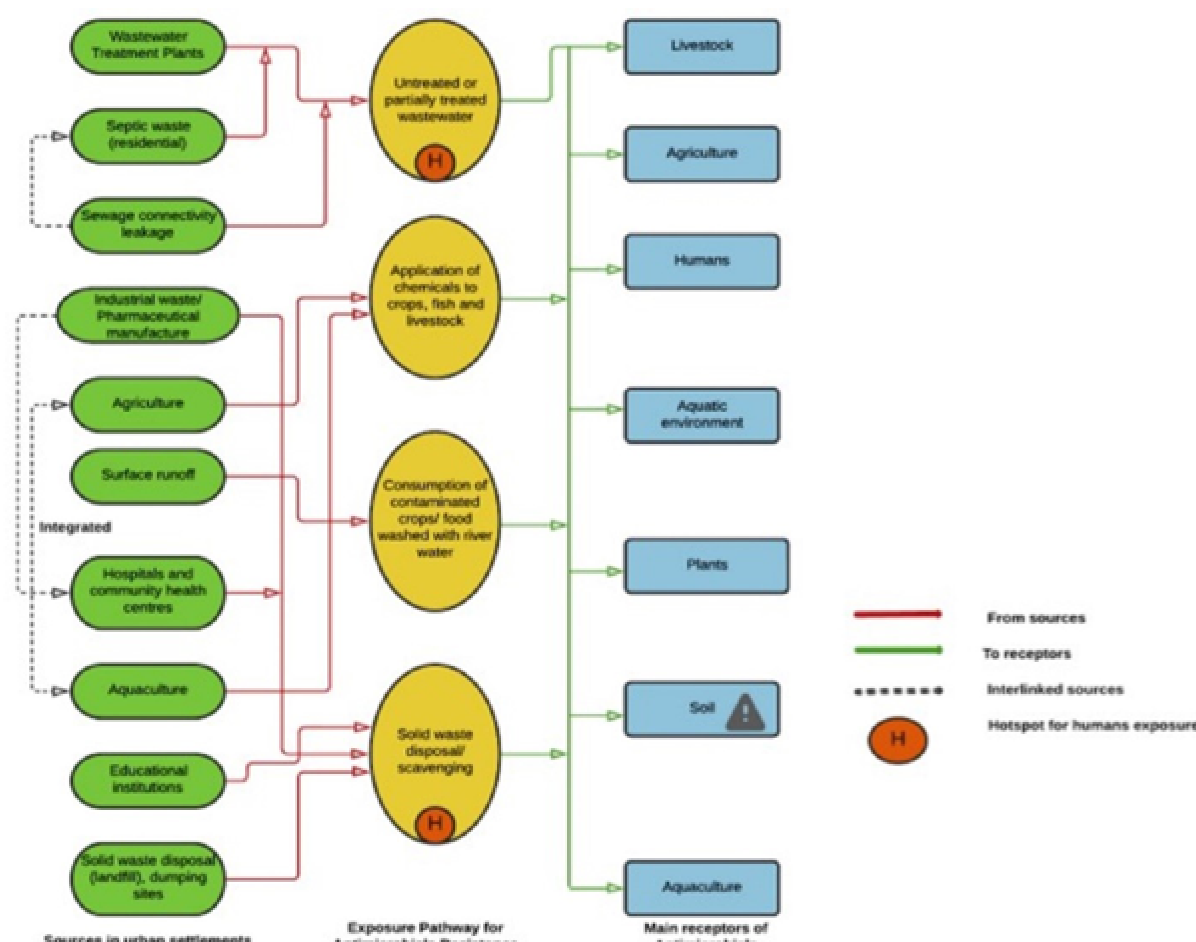

Figure 4. A schematic diagram showing the source, exposure pathway and main receptors (S-P-R) of antimicrobials

use of aquaponics, which are integrated systems used in vertical gardening while farming in small spaces, was also common in the study area. Aquaponics are known to maximize space, water utilization and efficiency, especially in water-deficit areas (Ani et al., 2022).

Integrated aquaculture provides a proven method to increase production efficiency. It also involved the use of crops, grass, and manure as feed and fertilizer, all in a complex, mutually beneficial system (Kaleem & Sabi, 2021; Awuor et al., 2023). The design allows waste from one system to be used as input in another system, conserving resources and boosting returns (Shoko et al., 2019). For instance, chicken droppings or cow manure

Table 5. Potential interventions to reduce antimicrobial resistance

Intervention	Proposed Stakeholder/Actor
Implementation of effective hygiene and biosecurity measures in the farm	Fish farmer
Promotion of better management practices (Good aquaculture practices)	Fish farmer / Industry
Strengthen Farmer Association and Cooperatives for knowledge sharing	Fish farmer / Industry
Avail alternatives to antibiotics (Vaccines, Prebiotics, Probiotics, Immunostimulants)	Industry / Research
Provision of Incentives to produce antibiotic free products	Government/Industry
Improve enforcement (in veterinary sector) on the sale of antibiotics	Government/Industry
Creation and implementation of AMR awareness campaigns	Government / Industry
Development and application of certification systems for antibiotic-free products	Government / Industry
Development of rapid diagnostic tools and increased diagnostic capacity in the field	Government / Research / Industry
Consumer awareness to encourage smart choices	Government / Industry
Adherence to standards to reduce AM residues	Certification bodies/ Government

are used to fertilize fish ponds, while the pond water is used to water crops or vegetables. In integrated farming involving livestock and crops, chicken was the poultry most kept among the farmers in the Nairobi river basin. This could be attributed to the fact that chicken is the most popular poultry reared in Kenya's households and in many countries in sub-Saharan Africa (Shoko et al., 2019). Its manure is also used for crops and pond fertilization.

Feeds and feed management practices are critical to the growth of the aquaculture sector. Fish farmers require nutritionally adequate and cost-effective feeds, as well as good feeding practices and feed management practices, to achieve high levels of aquaculture production (Munguti et al., 2021). Fish farmers need nutritionally adequate and cost-effective feeds, which are coupled with good feed management practices. Access to high quality and cost-effective feeds is one of the prerequisites to successful fish farming. This paper reviews the current status of the Kenyan fish feed industry and feed management practices. The review includes constraints and opportunities in fish feeds from a farmer's perspective. The review shows that the fish feed industry has been boosted by the development of fish feed standards, which has ensured access to high-quality fish feeds by all farmers. Feed management practices considerably impact on the economic performance in fish production. Thus, adopting appropriate feed management technologies and feeding strategies is instrumental in maximizing aquaculture productivity. Some of the major challenges faced by fish farmers in the feed sector including limited access to finance, lack of appropriate technical innovations, limited knowledge in feed formulation and processing and poor feed handling and storage are discussed. These challenges pose limitation in investment opportunities for a viable and sustainable fish feed processing and manufacturing to meet the rising demand occasioned by increased demand for fish food in Kenya There is a huge potential to develop public-private partnerships with farmer groups to improve access to training and information dissemination on feeds availability and quality. Training fish farmers on feed formulation using locally available feed ingredients provide an opportunity to reduce feed costs, increase feeding efficiency and improve profitability. This paper reviews the current status of the Kenyan fish feed industry and feed management practices including constraints and opportunities from a farmer's perspective. Farmers in the study area largely sourced their feeds from commercial manufacturers; farm-made feeds also existed where individual farmers made their feed at the farm level (Opiyo et al., 2014; Ragasa et al., 2022). Twice after 1 day, and twice after 2 days for a period of 7 months. Three hundred and seventy five fish were stocked into each of the nine, 150 m2 ponds with three replicates for each treatment. The fish were fed with a commercial diet (26% crude protein. In this study, the farmers were incentivized to buy and use packaged feeds, thus ensuring the source and quality of feeds.

Fish mortality observed in this study could be attributed to diminishing dissolved oxygen in the culture water, less digestible feed, and parasitic and bacterial infections (Opiyo et al., 2018; Alfred et al., 2020) Bacterial infections can be treated with proper diagnosis, however, misdiagnosis and treatment with the wrong drugs or dosage are more lethal since the bacteria can develop resistance to drugs (Leonard et al., 2022). The lack of diagnosis of the diseases can result in the misuse of antimicrobials since the farmers reported that they could not identify sick fish. This can be linked to inadequate training of fish farmers to recognize fish diseases and could explain the reason some farmers were unable to identify clinical symptoms of fish diseases. A study by Nzeve et al. (2024) indicated that 10.9% of pond-based fish farmers

can observe and document clinical signs in diseased fish. This lack of knowledge can lead to poor documentation of diseases among fish farmers.

The Nairobi River, as an important water source for aquaculture, may pose a challenge in terms of antimicrobial resistance if antibiotics find their way into the river through point or non-point sources (Moldovan, 2006). Antibiotics have been found in a variety of ecosystems, causing widespread concerns globally (Kümmerer et al., 2004; Ben et al., 2019). They are continuously discarded in natural ecosystems through human and animal excretion, individual drug abuse, and hospital effluents. These drugs' residues can persist in aquatic environments, causing severe changes in the composition and structure of the bacterial community, leading to the development of drug-resistant genes (Leonard et al., 2022). The lack of awareness of antimicrobial resistance can be linked to limited training in fish diseases and health management and education levels. Since the farmers had limited knowledge of fish health management, their knowledge of probiotics used as prophylactics was also low. A similar scenario was reported by Nzeve et al. (2024) among pond fish farmers in Kenya.

Mapping and surveillance of antimicrobial resistance using the SPR model is critical to guiding AMR interventions. AMR hotspots are reported in untreated or partially treated wastewater and solid waste disposal as sources. Enhanced documentation of the SPR for AMR has the potential to inform targeted surveillance programmes and interventions in aquaculture (Brunton et al., 2019). A rise in AMR rates is expected to affect low and middle-income countries disproportionately, jeopardizing the development gains in vulnerable communities, widening economic inequality, and contributing to a rise in extreme poverty by 2030 (World Bank, 2017). Furthermore, resistant pathogens significant to aquaculture may reduce the efficacy of treatment options in commercial aquaculture, with potential implications for food security and nutrition (Watts et al., 2017; Henriksson et al., 2018). The SPR mapping undertaken during this study indicates that aquaculture is connected with other agricultural food production systems, and addressing antimicrobial resistance needs to be done in a one-health approach rather than focusing on human health while ignoring the environment and animals for human consumption and animals being handled by the various users. A similar scenario was reported in a study carried out in Vietnam targeting small-scale farmers (Brunton et al., 2019). The interventions require leadership from the government and the industry with clearly defined roles, and this will depend on the production systems at the farmer level. The implementation of biosecurity measures, best management practices, record-keeping for traceability, and farmer training are required in the management of antimicrobial resistance.

Conclusion

The study also indicated that the interconnectivity of the various farm practices led to the transfer of antimicrobial residues from one unit to another, which led to antimicrobial resistance in animals that were not exposed to antimicrobials. The results of this study indicate that more awareness of antimicrobial resistance and training on best management practices need to be given to fish farmers in Nairobi Country for the sustainable development of the aquaculture sector. Monitoring and surveillance will be required in the identified hotspots, and the various stakeholders need to work together to address the challenges of antimicrobial resistance in aquaculture.

Limitations of this study

Previous studies carried out in Kenya in aquaculture production indicated a large number of active fish farming activities. This study was limited to the Nairobi River basin which had a limited number of fish farmers hence the small sample size.

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Conflict of interest

The authors declare that they have no conflict of interest in this study.

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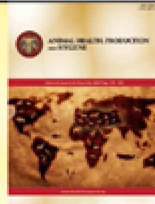

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Research Article

Comparative Macroscopic and Morphometric Analysis of Mandible in Different Mouse (Balb-c; Cd-1; C57bl/6) and Rat Strains (Wistar Albino; Sprague Dawley; Wag/Rij)

Duygu KÜÇÜK AĞAÇ[1*], Burcu ONUK[2]

[1]*Gümüşhane University, Şiran Mustafa Beyaz Vocational School, Department of Veterinary Medicine, Gümüşhane, TÜRKİYE*

[2]*Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Anatomy, Samsun, TÜRKİYE*

ABSTRACT

Since the morphology of the mandible varies among animal species, it is the subject of many studies. The aim of this study was to investigate the macroanatomical and morphometric aspects of the mandible in three different rat (Spague Dawley, Wistar Albino, Wag/Rij) and mouse strains (Balb-c, C57bl/6, CD-1). In this study, which was conducted using a total of 96 mandibles, 8 females and 8 males from each strain, measurements were taken from 6 parameters. Similarities and differences between strains and sexes were revealed by statistical analyses. The mandibles were examined under a stereomicroscope. For Balb-c, the termination of *proc. angularis* at the level of *proc. condylaris* and the shallow *tuberculum masseterica* were identified as distinctive anatomical structures; for Wag/Rij, the termination of *proc. angularis* at the level of *proc. condylaris*, the prominent *tuberculum masseterica*, and the localization of the molar teeth were macroscopically revealed as distinctive features. Specific measurement parameters were *proc. coronoideus* height for Balb-c; mandible length and lower M3 crown length for CD-1, while specific measurement point was no available for C57bl/6. In rats, *proc. coronoideus* height was the specific measurement point for Wag/Rij; lower M3 crown length was decisive for Sprague Dawley. The mandible length parameter was specific for all rat strains. It was concluded that strain determination could be made by looking at these distinctive parameters.

Keywords: *Anatomy, laboratory animals, mandible, morphometry, statistics.*

Farklı Fare (Balb-c; Cd-1; C57bl/6) ve Sıçan Soylarında (Wistar Albino; Sprague Dawley; Wag/Rij) Mandibula'nın Karşılaştırmalı Makroskobik ve Morfometrik Analizi

ÖZET

Mandibula'nın morfolojisi hayvan türleri arasında farklılıklar gösterdiği için çok sayıda araştırmaya konu olmaktadır. Yapılan bu çalışma ile üç farklı rat (Spague Dawley, Wistar Albino, Wag/Rij) ve fare soyunda (Balb-c, C57bl/6, CD-1) mandibula'nın makroanatomik ve morfometrik açıdan incelenmesi amaçlandı. Her soydan 8'er dişi ve erkek olmak üzere toplam 96 mandibula kullanılarak yapılan bu çalışmada, 6 parametreden ölçümler alındı. Soylar ve cinsiyetler arasındaki benzerlik ve farklılıklar istatistiksel analizlerle ortaya kondu. Mandibula'lar steromikroskop altında incelendi. Balb-c için, *proc. angularis*'in *proc. condylaris* hizasında sonlanması ve *tuberculum masseterica*'nın sığ oluşu; Wag/Rij soyu için ise *proc. angularis*'in, *proc. condylaris* hizasında sonlanması, *tuberculum masseterica*'nın belirgin oluşu ve molar dişlerin lokalizasyonu makroskobik olarak ayırt edici anatomik yapılar olarak ortaya kondu. Spesifik ölçüm parametreleri Balb-c için *processus coronoideus* yüksekliği; CD-1 için mandibula uzunluğu ve alt M3 taç uzunluğu iken C57bl/6 için herhangi bir spesifik ölçüm noktası mevcut değildi. Ratlarda ise Wag/Rij rat için *proc. coronoideus* yüksekliği spesifik ölçüm noktası iken; Sprague Dawley için alt M3 taç uzunluğu belirleyici oldu. Mandibula uzunluğu parametresi ise tüm rat soyları için spesifikti. Bu ayırt edici parametrelere bakılarak soy tayini yapılabileceği sonucuna varıldı.

Anahtar Kelimeler: *Anatomi, istatistik, laboratuvar hayvanları, mandibula, morfometri.*

***Corresponding author:** *Duygu KÜÇÜK AĞAÇ, Gumushane University, Şiran Mustafa Beyaz Vocational School, Department of Veterinary Medicine, Gümüşhane, Türkiye. duygu.kucuk@gumushane.edu.tr*

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Introduction

Knowledge of morphology and morphometry is very important in the evaluation of variation between populations and closely related species (Bookstein, 1991). In studies on the quantitative genetics of shape, the mouse mandible stands out as a highly suitable morphological structure as a model (Boell and Tautz, 2011). There are 400 different mouse strains, including inbred and outbred, which are bred for research purposes in the world (Dikmen, 2011). In addition, rats are another species widely used in experimental research and originated from *Rattus norvegicus*. Today, most of the inbred strains used in research are Wistar Albino. There are approximately 400 inbred and 50 outbred rat strains genetically defined (Dikmen, 2011). Since studies that will be beneficial to humans are carried out on experimental animals, it is important to know the anatomical differences in the bones that make up the cranium in terms of clinical and surgical intervention (Treuting et al., 2017).

The mandible is a flat pair of bones that form the lower part of the facial skeleton, where the teeth in the lower jaw are located, and which forms the jaw joint with the *os temporale* (Dursun, 2008; Bahadır and Yıldız, 2018; Demiraslan and Dayan, 2021). It has two parts, the *corpus mandibulae* and the *ramus mandibulae*, which is curved upwards (Aspinall and O'Reilly, 2004; Dursun, 2008; Ketani et al., 2017; Bahadır and Yıldız, 2018; Dyce et al., 2018; König and Liebich, 2018), and the right and left parts of this structure unite at the front at the *synchondrosis intermandibularis* (Aspinall and O'Reilly, 2004; Dursun, 2008; Bahadır and Yıldız, 2018).

In the literature reviews, there are studies on the mandible in different animals (İnce and Pazvant, 2010; Gürbüz et al., 2016; İlgün and Özüdoğru, 2020; Yılmaz, 2020; Demirtaş et al., 2023). The geometric morphometry of the mandible in the laboratory animals Wistar albino (İnce and Pazvant, 2010), brush-tailed mouse (*Calomyscus*) (Zarei et al., 2013) and *Mus domesticus* and *Mus macedonicus* (Demirtaş et al., 2023) and the morphology and geometric morphometry of the mandible of the Brazilian shrew (Missagia and Perini, 2018) were studied. In addition, although Ağaç et al. (2024a; b) revealed the shape differences of the mandible in different mouse and rat strains using geometric morphometry, these studies did not address the macroscopic structural differences and morphometric data of the strains. In this study, it was aimed to reveal the anatomical structures and parameters that can be used to distinguish strains from each other by performing macroscopic and morphometric analysis of the mandible in different rat and mouse strains.

Materials and Methods

In this study, 3 different mouse strains (Balb-c; CD-1; C57bl/6) and 3 different rat strains (Wistar Albino; Sprague Dawley; Wag/Rij) and 8 female and 8 male adults (8 weeks old) experimental animal mandibles from each strain were used. The study was carried out with the permission of the Ondokuz Mayıs University Animal Experiments Local Ethics Committee (2021/32).

The mandibles of the animals euthanized under deep anaesthesia "50 mg Ketasol (Interhas-Turkey)-5 mg Xylazinebio (Bioveta-Czech Republic)" were separated from their bodies. The maceration processes of the separated bones were performed using *Dermestes maculatus* larvae. Thus, all soft tissues were removed from the skull and the bone tissue was exposed. The skulls were bleached in 3% hydrogen peroxide (Oktet-Türkiye) to obtain more cleaning. Nomina Anatomica Veterinaria (2017) was used for naming. Morphometric data were taken. For each mandible, measurements were taken with digital caliper (Mitutoyo, Japan) in accordance with the literature (Şeker, 2009) for the length of the mandible, the height of the *proc. coronoideus*, the length of the lower molar alveoli, and the crown lengths of the lower M1, M2 and M3 teeth. The measurement points are indicated below and shown in Figure 1 and Figure 2.

1. Mandibular length: The length of the distance between the anterior edge of the lower incisor alveolus and the most posterior point of the *proc. angularis* (Figure 1-b).

2. *Proc. coronoideus* height: The length of the shortest distance between the most anterior point of the coronoideus and the most inferior point of the mandible (Figure 1-a).

3. Mandibular alveolus length: The length of the most anterior point and the most posterior point of the right mandibular alveolus (Figure 2-a).

4. M1 crown length: The length of the chewing surface of the 1st molar of the mandibular (Figure 2-d).

5. M2 crown length: The length of the chewing surface of the 2nd molar of the mandibular (Figure 2-c).

6. M3 crown length: The length of the chewing surface of the 3rd molar of the mandibular (Figure 2-b).

Statistical analysis

SPSS package program (SPSS 21.0, IBM SPSS Statistics®, Chicago, IL, USA) was used for all calculations and analyses. Normality of variables in the study was checked with Shapiro-Wilk test and homogeneity was checked with Levene's test. Anova analysis was used to evaluate variables showing normal distribution. Posthoc test Duncan was used to detect differences in Anova analysis.

Results

In all rat and mouse strains, the mandible, which forms the lower part of the facial skeleton, was a flat bone with strong protrusions and formed the jaw joint with the *os temporale*, which carries the lower jaw teeth. The right and left two bones were united with *synchondrosis intermandibularis* in all rats and mice. The *corpus mandibulae*, which is the front and lower part, and the *ramus mandibulae*, which continues upwards from here, were

present in all animals in the study (Figures 3-4). In all rat and mouse strains, one long incisive tooth and three molar teeth were observed in each half of the corpus part of the mandible, which is located in the lower part of the cranium and carries the teeth (Figures 3-5). Canine and premolar teeth were not found in the strains in the study (Figures 3-4). The tooth arrangement was 1-0-0-3 as incisive, canine, premolar and molar, respectively (Figures 3-5). Again, *dentes canini* was absent in all strains and the distance between the incisive tooth and molar tooth was long (Figures 3-5). While the molar teeth were arranged in a straight position in all mice, they were located with a significant inclination towards the caudoventral in Wag/Rij rats. This inclination in the arrangement of the teeth was slight in Sprague Dawley, but not evident in Wistar Albino. It was present as *margo interalveolaris* in all rats and mice. This part was 7.65±0.4 mm on average in Sprague Dawley male, 7.11±0.3 mm in female rats, 6.32±0.3 mm in Wistar Albino male and 5.89±0.3 mm in females. In the Wag/Rij male, the *margo interalveolaris* was determined as 5.40±0.2 and in the female as 5.21±0.2 mm. The same margin was measured as 2.85±0.1 mm in the Balb-c and 2.13±0.3 mm in the female; 3.01±0.3 mm in the C57bl/6 male and 52.76±0.2 mm in the female; and 2.60±0.1 mm in the CD-1 male and 2.18±0.2 mm in the female.

A *foramen mentale* was seen on the lateral surface of the mandible, close to the molar teeth, in all strains of rats and mice (Figures 3-4). A sharp line starting at the level of the *dentes molares* on the lateral side and continuing along the ventral edge to the *proc. angularis* was also seen in all strains. It was seen that the ventral edge of the corpus indented dorsally at the level of the last molar tooth in all animals.

In the *ramus mandibulae*, a wide and large ramus was observed to be quite high and long from the tooth level and the presence of the *proc. angularis* was observed in all rats and mice (Figures 3-5). While this protrusion was approximately at the level of the *proc. condylaris* in Wag/Rij rats, it exceeded the *proc. condylaris* caudally in Wistar Albino and Sprague Dawley rats (Figure 3). In mice, the *proc. angularis* ended in front of the *proc. condylar*is in CD-1 and C57bL/6 strains, while the *proc. angularis* extended to the level of the *proc. condylaris* in Balb-c strain (Figure 4). The direction of this protrusion was observed to be caudal in rat and mouse strains and its lower edge was convex. There was a very deep concave in the medial part of the *proc. angularis*. This depth was seen as deepest in Wistar Albino rats, then Sprague Dawley and shallower in Wag/Rij rats. In mice, the same depression was observed deeper in the C57bL/6 strain than in the CD-1 and Balb-c strains. The articular surface, the *proc. condylaris*, was present in all strains as a large and strong structure and connected to the rest of the skull by forming the *os temporale* and temporomandibular articulation. The *proc. condylaris* ended with a protuberance in all three strains of mice. In rats, however, there was not such a prominent protuberance as in mice. There was a quite prominent protuberance at the level of the middle of the outer surface of the *ramus mandibulae*. This protuberance, the *tuberculum masseterica*, was less prominent in Wag/Rij rats, while it was a prominent elevation in the others (Figure 3). In mice, it was less prominent in CD-1 and C57bL/6 mice, while it was prominent in Balb-c mice (Figure 4). The *proc. coronoideus* of the *ramus mandibulae* was present in all strains of rats and mice (Figures 3-4). The tip of the *proc. coronoideus* was directed caudodorsally in Sprague Dawley and Wistar albino rats and Balb-c and C57bL/6 strains, while it was directed caudal in Wag/Rij rat and CD-1 strains. The tip of the *proc. coronoideus* was clearly pointed in the Balb-c strain, while it was blunt in C57bL/6 and CD-1 strains. A clearly visible *inc. mandibulae* between the *proc. coronoideus* and *proc. condylaris* was observed in all rats and mice (Figures 3-5). It was seen that the *proc. coronoideus* was higher than the *proc. condylaris* in all animals. The presence of a hole called *foramen mandibulae* at the medial and middle level of the *ramus mandibulae* was present in all rats and mice (Figures 3- 5). There was another hole observed in all rats and mice, in the medial of the *corpus mandibulae* and *ramus mandibulae* border, caudal to the molar teeth (Figure 5).

Whether the mouse and rat species and gender used in the study had a statistically significant effect on the length of the mandible, the height of the *proc. coronoideus*, the length of the lower molar alveoli, and the crown lengths of the lower M1, M2 and M3 teeth were evaluated with a two-way ANOVA test and the results are presented in Tables 1 for mice and 2 for rats.

Table 1 shows the results of the partial eta squared test showing the mean, standard deviation, f value, probability value and effect value of change regarding the effect of the species, sex and species-sex interaction of mice on the dependent variables. When Table 1 is examined, it is seen that there are statistically significant differences between mouse strains in the dependent variables of *proc. coronoideus* height, M2 and M3 crown length ($P<0.0001$, $P<0.0001$, $P=0.045$, respectively).

When the differences in terms of gender were examined, it was seen that gender had a statistically significant effect on these variables since the probability (sig.) value of the variables belonging to the length of the mandible and the length of the lower molar alveoli was less than the threshold value of 0.05. When the species-gender interaction results were examined; the P value for the sub-variables of the mandible length, M2 crown length and M3 crown length was found to be below the significance level of 0.05. These results showed that the species and gender interaction had a statistically significant effect on the means of these dependent variables. Since the P values belonging to the species and gender interactions for the other variables were above the significance

level of 0.05, it was concluded that these interactions did not create a statistically significant difference on the dependent variables.

Since significant differences were detected between mouse strains according to ANOVA results, Duncan test was performed to examine in more detail which groups these differences were between and to separate the groups into homogeneous subsets. As a result of Duncan test, significant differences were detected between the groups and it was determined which groups were similar to each other and which were different. When Table 1 is examined, it was determined that there was no statistically significant difference between CD-1, C57bl/6 and Balb-c species in terms of the length of the lower molar alveoli, M1 crown length subvariables and M2 crown length subvariables. It was determined that the CD-1 lineage was statistically significant compared to C57bl/6 and Balb-c lines in terms of the mandible length and M3 crown length subvariables; and the Balb-c lineage was statistically significant compared to CD-1 and C57bl/6 lines in terms of the *proc. coronoideus* height variable. Based on all these results of the mice, the parameters that differed between the lines were summarized in Figure 6.

When Table 2 was examined, it was concluded that there was a statistically significant ($P<0.0001$, $P<0.0001$, $P=0.006$, respectively) difference between rat strains for all variables except for the length of the lower molar alveoli, M1 crown length lower and M2 crown length lower. Again, since the probability (sig.) value of all variables except for the length of the lower molar alveoli, M1 crown length lower, M2 crown length lower and M3 crown length sub-variables was less than the 0.05 threshold value ($P<0.0001$), it was determined that there was a significant difference between the rat genders and the means of the dependent variables. When the species-gender interaction results were examined, it was concluded that there were significant differences between the means of this interaction result and the means of the dependent variables, since the probability (sig.) value of all variables except for the M1 crown length lower, M2 crown length lower and M3 crown length sub-variables was less than the 0.05 threshold value. M1 crown length sub-variables and M2 crown length sub-variables were variables that had no significant difference between them in terms of species, sex and species-sex interaction.

Since significant differences were detected between rat strains according to ANOVA results, Duncan test was performed to examine in more detail which groups these differences were between and to separate the groups into homogeneous subsets. As a result of Duncan test, significant differences were detected between the groups and it was determined which groups were similar to each other and which were different. In this direction, homogeneous subgroups in terms of dependent variables according to rat strain variable are presented in Table 2.

When Table 2 was examined, it was seen that there was a statistically significant difference between the Wag/

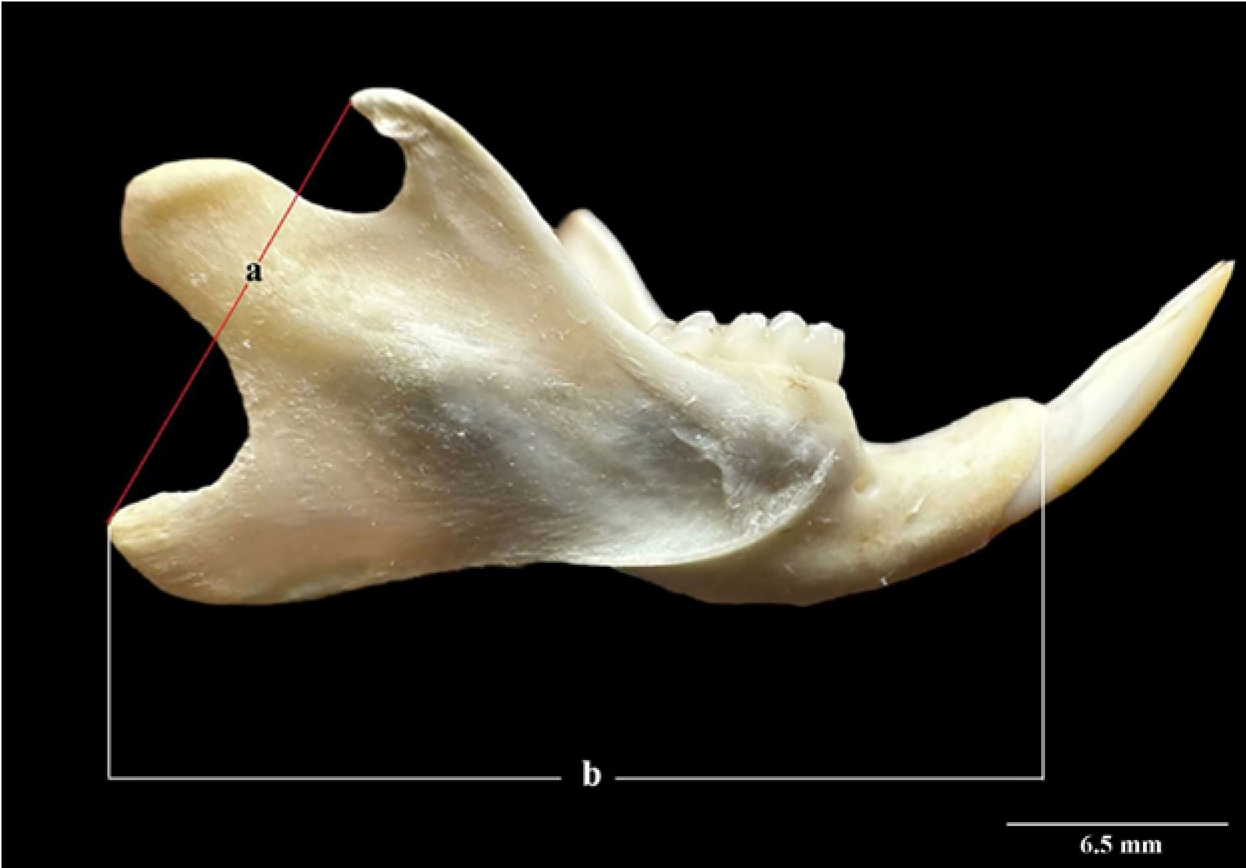

Figure 1. Measurement locations taken from the lateral view of the Wag/Rij mandible. a: *Proc. coronoideus* height, b: Mandibular length

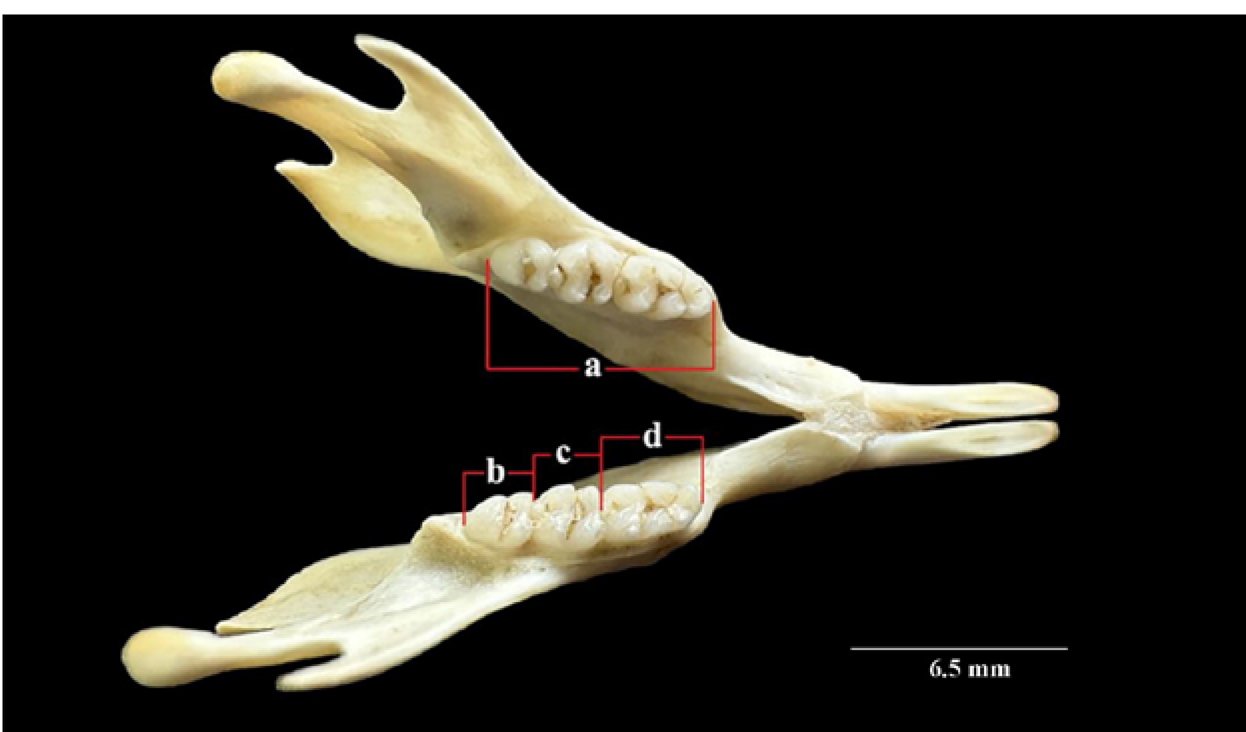

Figure 2. Measurement locations taken from the dorsal view of the Wag/Rij male mandible. a: Lower molar alveolus length, b: M3 crown length, c: M2 crown length, d: M1 crown length

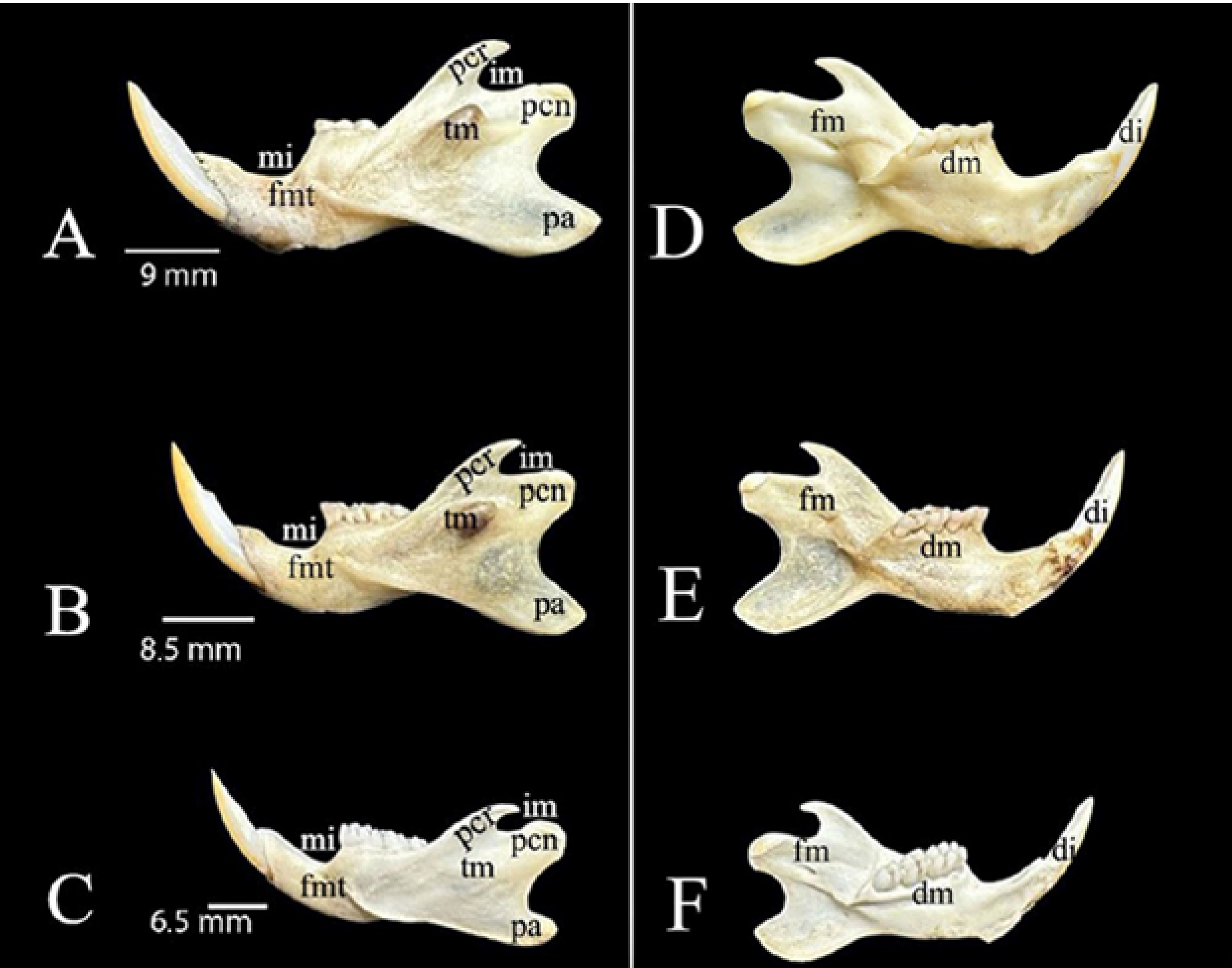

Figure 3. Lateral (A, B, C) and Medial (D, E, F) View of Male Rat Mandibles A-D: Sprague Dawley Rat, B-E: Wistar Albino Rat, C-F: Wag/Rij Rat. fmt: *For. mentale*, tm: *Tuberculum masseterica*, pcr: *Proc. coronoideus*, pcn: *Proc. condylaris*, pa: *Proc. angularis*, fm: *For. mandibulae*, dm: *Dentes molares*, di: *Dentes incisivi*, mi: *Margo interalveolaris*, im: *Inc. mandibulae*

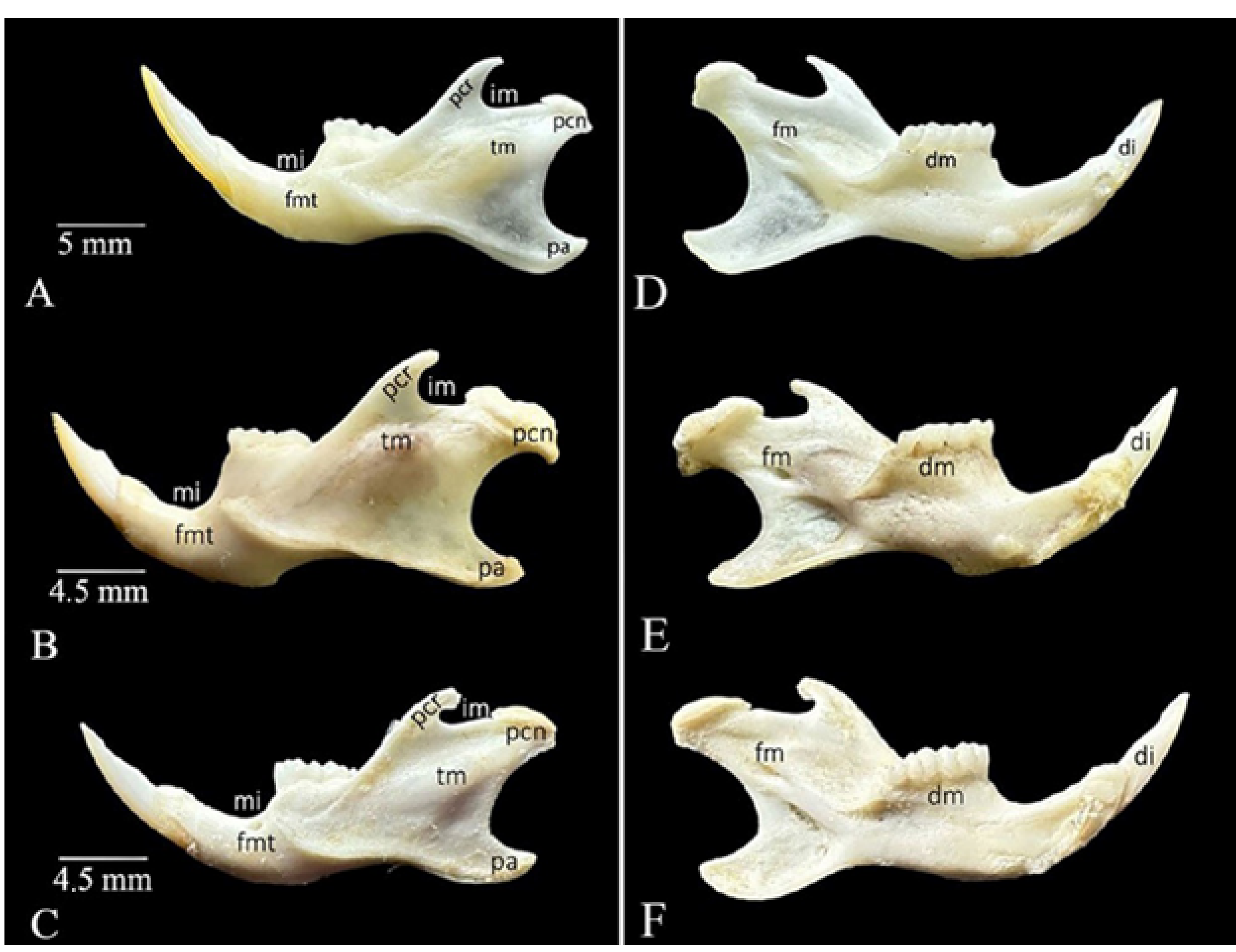

Figure 4. Lateral (A, B, C) and Medial (D, E, F) View of Female Mouse Mandibles, A-D: Balb-c, B-E: C57bL/6, C-F: CD-1. fmt: *For. mentale*, tm: *Tuberculum masseterica*, pcr: *Proc. coronoideus*, pcn: *Proc. condylaris*, pa: *Proc. angularis*, fm: *For. mandibulae*, dm: *Dentes molares*, di: *Dentes incisivi*, mi: *Margo interalveolaris*, im: *Inc. mandibulae*

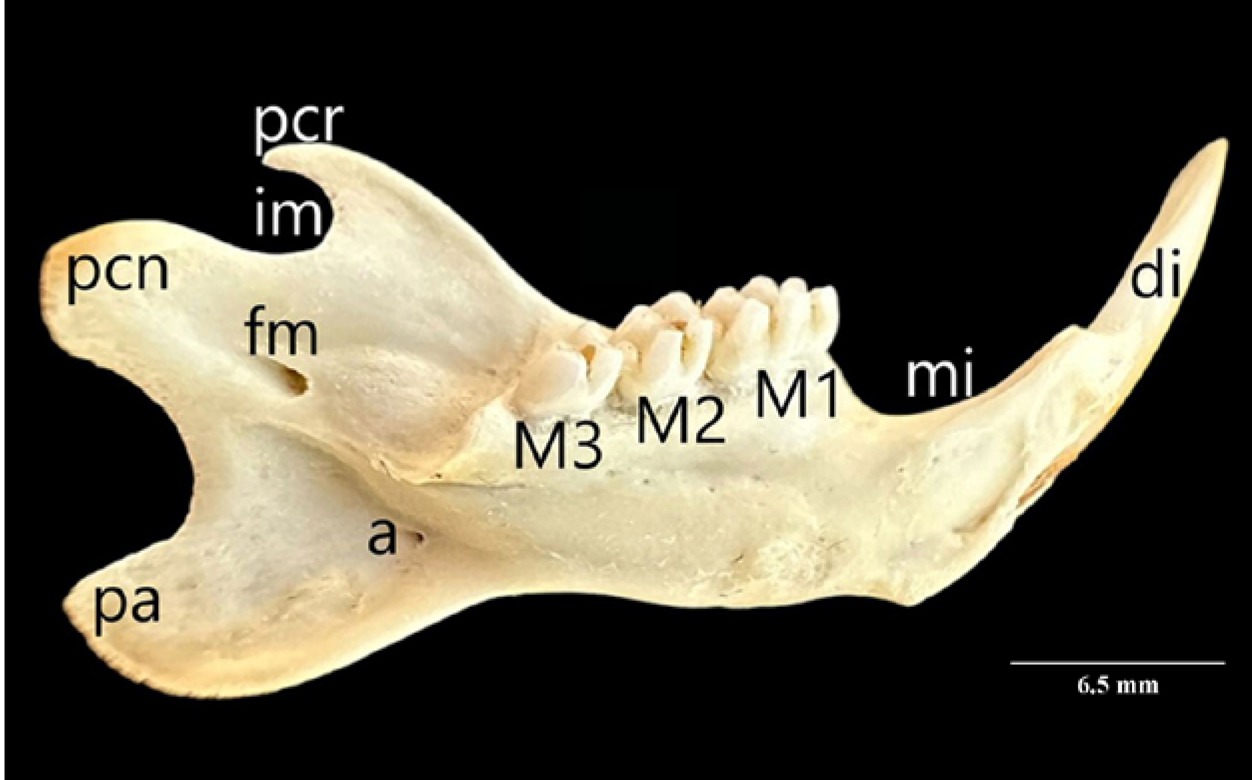

Figure 5. Medial view of the mandible of the Wag/Rij male rat, a: Mentioned hole, pa: *Proc. angularis*, pcn: *Proc. condylaris*, pcr: *Proc. coronoideus*, fm: *For. mandibulae*, im: *Inc. mandibulae*, mi: *Margo interalveolaris*, di: *Dentes incisivi*, M1: First molar, M2: Second molar, M3: Third molar

Table 1. Two-way analysis of variance (ANOVA) test results for mice

Dependent Variable	Species/Gender/ Species*Gender	N	Mean	Standard Deviation	F Value	P Value	Partial Eta Squared
Mandible Length	Balb-c	16	12.43	0.54	3.04	0.058	0.13
	C57bL/6	16	12.53	0.72			
	CD-1	16	12.16	0.32			
	Female	24	12.17	0.47	9.87	0.003	0.19
	Male	24	12.58	0.58			
	Species*Gender	48	12.37	0.56	8.24	0.001	0.28
Proc. coronoideus Height	Balb-c	16	6.89[a]	0.29	20.05	<0.0001	0.49
	C57bL/6	16	6.39[b]	0.28			
	CD-1	16	6.22[b]	0.34			
	Female	24	6.45	0.41	1.15	0.290	0.03
	Male	24	6.55	0.42			
	Species*Gender	48	6.50	0.41	0.65	0.527	0.03
Molar Alveolus Length	Balb-c	16	3.14	0.20	2.32	0.111	0.10
	C57bL/6	16	3.24	0.18			
	CD-1	16	3.12	0.18			
	Female	24	3.10	0.15	8.84	0.005	0.17
	Male	24	3.24	0.20			
	Species*Gender	48	3.17	0.19	1.79	0.180	0.08
M1 Crown Length	Balb-c	16	1.47	0.15	2.33	0.110	0.10
	C57bL/6	16	1.48	0.08			
	CD-1	16	1.55	0.10			
	Female	24	1.51	0.09	0.25	0.619	0.006
	Male	24	1.50	0.14			
	Species*Gender	48	1.50	0.12	4.93	0.012	0.19
M2 Crown Length	Balb-c	16	0.95[a]	0.10	9.47	<0.0001	0.31
	C57bL/6	16	0.91a	0.08			
	CD-1	16	1.03a	0.08			
	Female	24	0.96	0.08	0.20	0.654	0.005
	Male	24	0.97	0.12			
	Species*Gender	48	0.96	0.10	7.72	0.001	0.27
M3 Crown Length	Balb-c	16	0.63[b]	0.11	3.33	0.045	0.14
	C57bL/6	16	0.63[b]	0.06			
	CD-1	16	0.68[a]	0.09			
	Female	24	0.66	0.08	2.11	0.154	0.05
	Male	24	0.63	0.09			
	Species*Gender	48	0.65	0.09	13.52	<0.0001	0.39

[a,b,c] The difference between means in the same column is statistically significant (P<0.05).

Rij, Sprague Dawley and Wistar Albino strains in terms of mandible length. This difference was Sprague Dawley> Wistar Albino> Wag/Rij. No statistically significant difference was observed between the strains in terms of M1 crown length sub-variables and M2 crown length sub-variables. While the *proc. coronoideus* height variable was a statistically significant variable in the Wag/Rij strain compared to other strains, the M3 crown length sub-variable showed a statistically significant difference for the Sprague Dawley strain. Based on all these results, the parameters that differ between the strains are shown in Figure 7.

Figure 6. Mandibular parameters distinguishing mouse strains from each other

Table 2. Two-way analysis of variance (ANOVA) test results for rats

Dependent Variable	Species/Gender/ Species*Gender	N	Mean	Standard Deviation	F Value	P Value	Partial Eta Squared
Mandible Length	Sprague Dawley	16	26.47[a]	1.80	202.19	<0.0001	0.91
	Wag/Rij	16	21.75[c]	0.77			
	Wistar Albino	16	24.50[b]	0.82			
	Female	24	24.49	2.92	6.88	0.012	0.14
	Male	24	23.99	1.46			
	Species*Gender	48	24.24	2.30	51.49	<0.0001	0.71
Proc. coronoideus Height	Sprague Dawley	16	13.05[a]	1.03	95.04	<0.0001	0.82
	Wag/Rij	16	10.56[b]	0.63			
	Wistar Albino	16	12.81[a]	0.54			
	Female	24	12.43	1.62	12.44	0.001	0.23
	Male	24	11.86	0.98			
	Species*Gender	48	12.14	1.36	14.21	<0.0001	0.40
Lower Molar Alveolus Length	Sprague Dawley	16	6.93	0.23	3.04	0.059	0.13
	Wag/Rij	16	7.00	0.00			
	Wistar Albino	16	7.13	0.34			
	Female	24	7.00	0.14	0.17	0.682	0.004
	Male	24	7.03	0.32			
	Species*Gender	48	7.02	0.25	3.44	0.042	0.14
M1 Crown Length Lower	Sprague Dawley	16	2.94	0.14	2.25	0.118	0.10
	Wag/Rij	16	3.00	0.00			
	Wistar Albino	16	3.06	0.25			
	Female	24	2.99	0.10	0.24	0.630	0.006
	Male	24	3.01	0.22			
	Species*Gender	48	3.00	0.17	1.22	0.304	0.06
M2 Crown Length Lower	Sprague Dawley	16	1.99	0.15	0.80	0.455	0.04
	Wag/Rij	16	2.06	0.25			
	Wistar Albino	16	2.00	0.00			
	Female	24	1.99	0.08	1.30	0.260	0.03
	Male	24	2.05	0.23			
	Species*Gender	48	2.02	0.17	0.55	0.580	0.03
M3 Crown Length Lower	Sprague Dawley	16	1.74[b]	0.14	5.79	0.006	0.22
	Wag/Rij	16	2.00[a]	0.00			
	Wistar Albino	16	1.88[a]	0.34			
	Female	24	1.85	0.25	0.26	0.611	0.006
	Male	24	1.88	0.22			
	Species*Gender	48	1.87	0.24	0.26	0.770	0.01

[a,b,c] The difference between means in the same column is statistically significant (P<0.05).

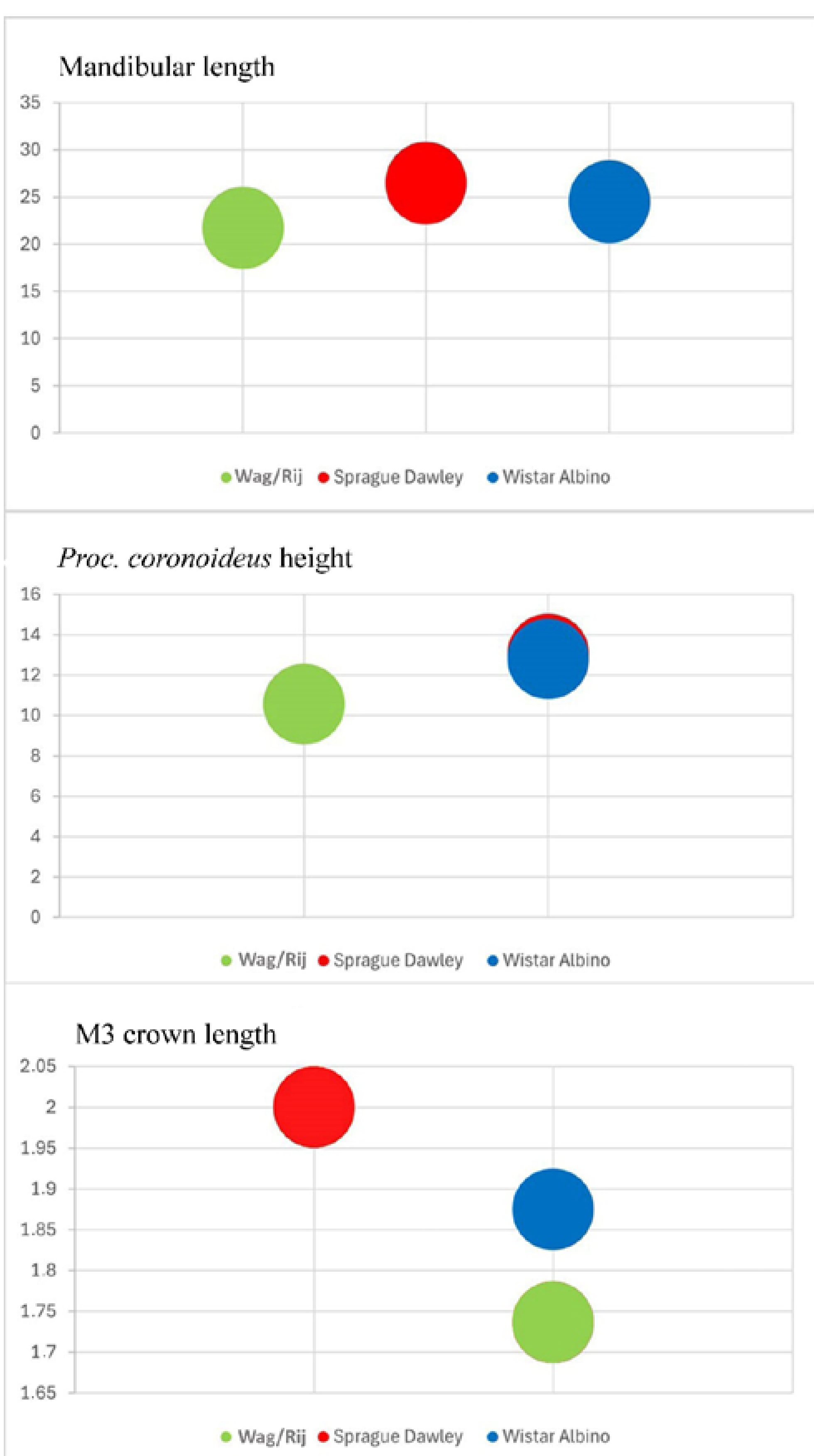

Figure 7. Parameters distinguishing rat strains from each other.

Discussion

Studies have reported that the differences observed in the mandible between animal species develop depending on various factors such as nutrition, stress, breed, hormones, masticatory joint and genetic factors (Rohlf and Marcus 1993; Bodner et al., 1998; Luca et al., 2003; Fujita, 2004). Luca et al. (2003) and McFadden et al. (1986) evaluated the development and morphometry of the mandible by applying different diet types to rats and determined that more growth was observed in the dimensions of the mandible of those fed a solid diet compared to liquid and soft food. Odman et al. (2008) similarly reported differences in the condylar base inclination in animals fed a solid diet. Enomoto et al. (2010) mentioned that diet affects the growth and development of the mandible and chewing function in mice. Akbulut et al. (2014) reported that there was a slight difference in the length and width of the mandible in males and females in rabbits with the same feeding and housing conditions. Ince and Pazvant (2010) concluded in their study on rats that the total mandibular length and mandibular incisive corona length of males were greater than those of females. In the study, the parameters of the mandible length and lower molar alveolus were statistically greater in males than in females in mouse strains; and the parameters of the *proc. coronoideus* and mandible length were statistically greater in rat strains.

Similar to what was reported for rabbits in the literature (Çalışlar, 1978), the *ramus mandibulae* was large and wide in rat and mouse strains. In addition, it was reported that the *ramus mandibulae* was short and barely exceeded the molar tooth level in guinea pigs (Çalışlar, 1978). In all rat and mouse strains in the study, unlike guinea pigs, the *ramus mandibulae* considerably exceeded the molar tooth level. In the literature (Çalışlar, 1978), it was stated that the *proc. angularis* was short and blunt in rabbits, and extended caudally in guinea pigs, and had a more or less rounded, distinct structure. It was also reported that the *proc. angularis* had a distinct protrusion and extended caudolaterally in the mole rat (Özkan, 2007). In the examined rat and mouse strains, there was a distinct *proc. angularis* and its direction extended caudally, similar to the literature. The depression located medially to this structure was seen the deepest in Wistar Albino rats, moderately deep in Sprague Dawley rats, and shallower in Wag/Rij rats. In addition, it was observed that the *proc. angularis* was outside the *proc. condylaris* alignment in Sprague Dawley and Wistar Albino rats, while it was in line with the *proc. condylaris* in Wag/Rij rats. It has been reported that the *foramen mandibulae* is located medially to the last molar tooth in guinea pigs and medially to the *ramus mandibulae* in rats (Çalışlar, 1978). In the mole rat, the *foramen mandibulae* has been reported to be at the base of the *proc. condylaris* and on the medial side (Özkan, 2007). In the study, the location of this hole was consistent with that reported in the literature for rats (Çalışlar, 1978) and mole rats (Özkan, 2007).

In a study conducted on Brazilian shrews and Akodontini rodents (Missagia and Perini, 2018), it was reported that variations in the skull were related to nesting habits, soil digging, and diet differences. In addition, the prominence and position of the *tuberculum masseterica*, which is the origin of the superficial masseter muscle, has been associated with diet (Ağaç et al., 2024). In the study conducted by Ağaç et al. (2024), it was shown that the *tuberculum masseterica* differed in terms of position among Balb-c, C57bl/6, and CD-1 strains. While the *tuberculum masseterica* was a prominent elevation in Sprague Dawley and Wistar Albino rats and Balb-c mice, it was shallow in Wag/Rij rats and CD-1 and C57bL/6 mice. Based on this, it can be said that the muscle attached here is shaped more strongly in Sprague Dawley and Wistar Albino rats and Balb-c mice compared to other strains.

It has been reported that the *proc. coronoideus* is short and tapered in guinea pigs and is located just behind the last molar tooth (Çalışlar, 1978). In the study, the tip of the *proc. coronoideus* was clearly pointed only in the Balb-c strain, while it was blunt in the C57bL/6 and CD-1 strains. In the study, the tip of the *proc. coronoideus* was observed to be oriented caudodorsally in Sprague Dawley and Wistar albino and in the Balb-c and C57bL/6 strains, similar to that reported in the mole rat (Özkan, 2007). In addition, the prominence of the *inc. mandibulae* and the level of the *proc. coronoideus* being higher than the *proc. condylaris* in all species examined were similar to those in the mole rat (Özkan, 2007). Although it has been reported in the literature (Ellerman, 1948; Ellerman & Morrison-Scoot, 1951; Ketani, 2017) that there is an extra protrusion called *proc. alveolaris* on the *ramus mandibulae* in rodents of the *Nannospalax* genus, such a protrusion was not present in the strains examined in the study. It has been reported that there are two wedge-shaped incisors in the mandible in guinea pigs and a pair of long incisors in rats (Çalışlar, 1978). In the study, there was a long pair of incisors in all strains of rats and mice, similar to what has been reported in the literature (Çalışlar, 1978).

There are differences in the number of teeth in the mandible and the number of these teeth among species. In each half of the mandible, incisor, canine, premolar and molar are reported as 1-0-1-3 in tree and ground squirrels (Yalçın and Arslan, 2009); 1-0-0-3 in Sprague Dawley rats (Yalçın et al., 2007); and 1-0-0-3 in blind mole rats (Ketani et al., 2017). The dental formula of the otter was reported as 3-1-3-2 (Yılmaz et al., 2000), while it was expressed as 3-1-4-3 in the raccoon (Hidaka et al., 1998), 1-0-1-3 in the hedgehog (Yılmaz, 1998), chinchillas (Brenner et al., 2005), and 3-1-3-2 in the badger (Dinç, 2001). In our study, the 1-0-0-3 dental arrangement reported in the literature in the blind mole rat (Ketani et al., 2017) and the Sprague Dawley rat (Yalçın et al., 2007) was present in both rat and mouse strains. In addition, in the study conducted, it was striking that there was a significant inclination towards the caudoventral in the arrangement of the molar teeth in Wag-Rij. This feature

was evaluated as an important parameter that can be used in strain determination.

Conclusion

In conclusion, this study found that there were significant differences in parameters such as height of the *proc. coronoideus*, crown lengths of lower M2 and M3 teeth, and length of the mandibular and length of the mandibular molar alveolus between strains in mice, but no differences in other variables. It was concluded that there were significant differences in parameters such as length of the *proc. coronoideus*, crown lengths of lower M3 teeth, and length of the mandibular and height of the *proc. coronoideus* in rats, and there were significant differences in parameters such as length of the *proc. coronoideus*, crown lengths of lower M3 teeth, and length of the mandibular and height of the *proc. coronoideus* in macroscopic examination. For Wag/Rij, termination of the *proc. angularis* at the level of the *proc. condylaris*, shallowness of the *tuberculum masseterica*, and localization of the molar teeth were found to be distinguishing parameters for the Balb-c strain.

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Author contribution statement

BO designed and planned the experiments. DKA conducted the experiments, performed the analyses, interpreted the results, and took the lead in writing the manuscript. BO contributed to the interpretation of the results. All authors provided critical feedback and contributed to shaping the investigation, analysis, and manuscript.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the paper's content.

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Review

The Past, Present and Future of Three-Day Sickness with Epidemiological Data

Yavuz KAYA[1*], Oya BULUT[2,3]

[1]Yalıhüyük Directorate of District Agriculture and Forestry, Konya, TÜRKİYE
[2]Selçuk University, Faculty of Veterinary Medicine, Department of Virology, Konya, TÜRKİYE
[3]Dokuz Eylül University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology İzmir, TÜRKİYE

ABSTRACT

Three-day sickness, also known as Bovine Ephemeral Fever (BEF), is an arboviral-derived disease of cattle and water buffaloes progressing with acute, rapidly developing polyphasic fever and showing symptoms such as stiffness of muscle tissue and excessive salivation. While clinical findings generally continue between 1-3 days, it has importance due to a sudden and high decrease in milk yield in cows during lactation, infertility, loss of condition, treatment costs and sometimes serious economic losses due to death of sick animals. The possible spread mechanism is reported to be related to the inter-regional wind movement of *Culicoides*-type sandflies and various insect vectors and host feeding of these blood-fed vectors. The objective of this review is to provide information about the current situation of the three-day sickness in the light of current epidemiological data and to draw a perspective for the future.

Keywords: *Arbovirus, Bovine Ephemeral Fever, Culicoides.*

Epidemiyolojik Verilerle Üç Gün Hastalığının Dünü, Bugünü ve Geleceği

ÖZET

Üç gün hastalığı diğer adıyla Bovine Ephemeral Fever (BEF) hastalığı sığır ve mandaların akut, hızlı gelişen polifazik ateşle ilerleyen, kas dokusunun sertliği, aşırı salivasyon gibi bulgular gösteren arboviral kaynaklı bir hastalığıdır. Klinik bulgular genellikle 1-3 gün arasında sürerken, laktasyon döneminde bulunan ineklerde süt veriminin aniden ve yüksek oranda azalması, infertilizasyon, kondüsyon kaybı, tedavi masrafları ve bazen hastalanan hayvanların ölümü nedeniyle ciddi ekonomik kayıplara neden olması sebebiyle önem teşkil etmektedir. Muhtemel yayılım mekanizması *Culicoides* türü tatarcık ve çeşitli insekt vektörlerin bölgeler arası rüzgarla hareketi ve kan ile beslenen bu vektörlerin konakçı beslenmesi ile ilgili olduğu bildirilmektedir. Bu derlemenin amacı güncel epidemiyolojik veriler ışığında üç gün hastalığının günümüzdeki durumu hakkında bilgi vermek ve geleceğe dair perspektif çizmektir.

Anahtar kelimeler: *Arbovirus, Culicoides, Üç gün hastalığı.*

***Corresponding author:** Yavuz KAYA, Yalıhüyük Directorate of District Agriculture and Forestry, Konya, TÜRKİYE. vetyavuzkaya@gmail.com*
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Introduction

Three-day sickness is an acute, rapidly progressing arboviral-derived disease of cattle and buffalo characterized by polyphasic fever, excessive salivation, nasal and ocular discharge, and reluctance to move (St George 1988; Walker and Klement, 2015). While the clinical signs persist for a few days, the disease causes significant economic losses due to a sudden drop in milk production in lactating animals, infertility, loss of condition, treatment expenses, trade restrictions, and sometimes the death of affected animals (Tonbak et al., 2013; Trinidad et al., 2014; Oguzoglu et al., 2015).

The first detailed description of the disease was reported in Egypt in 1895. It was first described as "Bovine Dengue Fever" whose clinical findings were closer to the modern definition of BEF (St George, 1988). In 1907, an epidemic began in Zimbabwe. In this epidemic, the first experimental data regarding the transmission of the disease from the blood of cattle were reported (Bevan, 1907).

In 1967, the virus was isolated by Bianca Van Der Westhuizen through intracerebral inoculation in mice by obtaining the leukocyte-platelet fraction. The citrated blood samples taken by Bianca Van Der Westhuizen in 1958 were stored in dry ice, and in 1967, the leukocyte-platelet fraction was obtained and inoculated intracerebrally into mice, and the first isolation of the BEF virus was achieved. Virus isolation was also performed from cattle that were experimentally infected with these isolated viruses (Van der Westhuizen, 1967).

Etiology

Bovine fever ephemerovirus is a virus of the genus *Ephemerovirus*, which belongs to the subfamily *Alpharhabdovirinae* of the family *Rhabdoviridae* (Walker et al., 2022). *Bovine fever ephemerovirus*, which causes three-day sickness, is a single-stranded, enveloped and non-segmented negative-polarity RNA virus. *Ephemerovirus* genomes contain single-stranded and negative-polarity, non-segmented RNA ranging between 14.5 kb-16.1 kb. Virions are bullet shaped and the genome contains 5 structural and 6 non-structural proteins. There are 5 structural proteins as nucleoprotein (N), surface glycoprotein (G), RNA polymerase (L), Phosphoprotein (P) and Matrix protein (M) (Walker et al., 1991).

Epidemiology Transmission and Economics

Epidemiology

Bovine Ephemeral Fever emerges seasonally in tropical, subtropical and temperate regions of countries geographically located in Africa, Asia, Australia and the Middle East. To date, the disease has been reported in 46 countries (St George 1986; Walker and Klement, 2015). The disease has economic importance due to yield losses and deaths in fattening and dairy enterprises. Contrary to general information, the rate reported as 100% for morbidity differs. Although a low rate has been reported for mortality (1%), there are also data suggesting a higher rate (Walker et al., 2012; Pyasi et al., 2020). For instance, the mortality rate was reported to be lower than 1% in the first epidemic in Türkiye in 1986, and 15-20% in the epidemic in 2012 (Girgin et al., 1986; Tonbak et al., 2013). An average of 17% mortality was reported in Henan province of China in 2004, 2005 and 2011 (Zheng and Qiu, 2012).

In BEF infection, infected animals, asymptomatic carrier animals and insect vectors are the most important sources of infection (Akakpo, 2015). The geographical distribution and spread of BEF disease indicate vector-related diseases. The disease has been reported to be limited to the regions where insect vectors of the genus *Culicoides* are intense in farms in the countries where it is observed. It has also been reported that the disease usually starts after an intense rainy period and continues until summer or autumn months (Murray, 1970; George et al., 1977; Uren et al., 1987).

The analysis of the glycoprotein (G) gene, one of the structural proteins of the virus, led to the classification of the virus into South Africa, East Asia, Australia and the Middle East according to the isolation locations (Aziz-Boaron et al., 2012; Trinidad et al., 2014; Omar et al., 2020).

In 2020, when the last epidemic was seen in Türkiye, the provinces in the south of the country were affected. A study conducted on the envelope glycoprotein of the isolate obtained from this pandemic revealed that the isolate clustered phylogenetically in the Middle East region. It has been reported that this isolate represents a separate branch from isolates obtained during the outbreaks in 1986, 2008, and 2012. In the 2012 epidemic, it was reported that while East Asian and Middle Eastern strains were circulating in Türkiye, the 2020 isolate belonged only to the Middle East (Abayli et al., 2017; Alkan et al.,2017; Karayel-Hacioglu et al., 2021). Another finding regarding this isolate is its close similarities with the isolate obtained in India in 2018. The exact reason for this similarity cannot be explained; however, it is suggested that the geographical proximity of Middle Eastern countries to Türkiye enabled the spread of the disease between continents through vector translocation (Pyasi et al., 2020; Karayel-Hacioglu et al., 2021).

In the 2020 epidemic in Türkiye, 168 serum samples taken from BEFV suspected animals, 79 EDTA-treated blood and 2 spleen samples (Tokgoz et al., 2023) were examined in a study. While the positivity rate was determined as 69.04% in serologically examined serum, 64.20% positivity was reported in 81 samples examined molecularly. In this study conducted by (Tokgoz et al., 2023), it was determined that the phylogenetic analyses of the 2 isolates were consistent with the results of the study conducted by (Karayel-Hacioglu et al., 2021), and both isolates were in the same cluster (Tokgoz et al., 2023).

Transmission

It has been reported that direct or indirect contact has no

role in the transmission of the disease. As a result of the data obtained from experimental infections in animals, it can be stated that the need for intravenous transmission of the virus in order for the infection to start, witnessing the infection from the beginning of spring until the autumn months and its geographical distribution (tropical, subtropical and temperate regions) prove the claim that the infection is transmitted through blood-fed insects (Mackerras et al., 1940; Coetzer and Tustin, 2004; Walker, 2005).

The virus has been isolated from many species of *Culicoides* and insects. These include *Culiocides imicola* in Zimbabwe and *C. coarctus*, C. *oxystoma* in Taiwan, *C. arakawae, Anopheles sinensis, Cx. tritaeniorhynchus*, *C. kingi* and *C. nivosis*, *C. bedfordi* and *C. pallidipennis* in Kenya, *C. punkticollis* in Turkmenistan, *C. brevitarsis*, *An. bancroftii, Culex, Uranotenia and Aedes* species of insects and sandflies in Australia (Davies and Walker, 1974; St George et al., 1976; Blackburn et al, 1985; Muller and Standfast, 1986; Cybinski and Muller, 1990; Lvov et al., 2015; Tzeng et al., 2023).

Simultaneous or recent disease notifications in distant regions are thought to be resulting from the transportation of vectors to the region with strong winds (Murray, 1970; Kirkland, 2002). A study conducted in Australia based on the meteorological data suggests that the winds cause BEF cases to spread in waves along a 800 km front and move 3000 km away from the region. This spreading model is supported by the fact that most animal movements are in the opposite direction to the movement in the relevant country (Murray, 1970; Newton and Wheatley, 1970). It is also argued that heavy precipitation, which promotes the emergence of large vector populations after a prolonged drought, precedes these epidemics (Walker and Klement, 2015).

Findings regarding the spread of vectors with wind are also present in BEF epidemics in Asia and the Middle East. The relationship of the BEF epidemic that started in Japan and Korea with the wind direction of a low-level airflow from China and the genetic similarity among the strains isolated from these countries were supported with evidence (Kato et al., 2009).

The fact that the isolates are clustered according to geographical regions suggests that there is another reason for the spread of the disease over long distances through animal transportation. In addition, several studies suggest that some isolates obtained from Egypt and Türkiye are phylogenetically clustered with isolates from the East Asian branch, not from the geographical regions of these countries (Aziz-Boaron et al., 2012; Trinidad et al., 2014; Oguzoglu et al., 2015). Although cattle were exported from China to the Middle East 1 year before the 2005 epidemic in Egypt, and cattle were not exported from Asia before the epidemic in Türkiye in 2012, transmission is thought to be due to neighbouring countries. Moreover, this isolate in Türkiye is thought to come through Africa by cattle transportation (Walker and Klement, 2015).

Economics

Although the low mortality of the disease and its improvement in terms of clinical findings within three days are underestimated by breeders, its economic effect is quite high. It has highly important economic effects for the industry due to serious losses in milk and meat production, its rapidly spreading character in herd, and the sequelae it causes in high yielding breeds, even if it shows improvement in terms of clinical findings. In this context, there is a study conducted with veterinarians offering service in the regions where the epidemic emerged in Türkiye in 2020 through a questionnaire on the economic effects of the disease. According to the results of this study, the cost of vaccination against infection in a herd of 50 animals was determined to be $8,10 per animal, and the cost of treatment for each unvaccinated animal was $20,2. In addition, it is stated that the cost increases to $85 depending on the milk yield lost in a dairy cow in addition to the treatment cost, and the unit cost per animal increases to $148 with the lost yield in addition to the treatment cost in livestock. It is stated that the loss is $5,381 in a dairy farm with 50 animals and $7,902 in a fattening farm. In addition, when the disease is seen as an epidemic, businesses need to spend an additional $138 in addition to all costs for fighting against insects (Ayvazoğlu and Demir, 2022).

A retrospective statistical study was conducted on the economic effects of the disease by examining the data reported by farms in the 2021 epidemic in Israel. In a study conducted on 30 dairy farms, culling rates and milk yield losses were examined. Considering the sequelae caused during infection and after healing of the loss per infected cow, the total milk yield loss was found to be $315, and it was estimated that it caused a loss of $123,000 in a herd of 300 animals due to milk yield lost during the first 40 days after infection. Depending on the culling chosen as one of the disease control methods, it was estimated that the economic loss would be $33,120 in a herd of 300 cows. When examined in terms of herd, it was estimated that the total loss of the same herd was $253,000 due to the loss of 110 cows in a herd of 700 animals, and the total loss of milk yield due to culling and death was estimated to be $275,000 (Lavon et al., 2023).

Clinical Findings

BEF disease is characterized by a sudden onset of fever in cattle and a tendency to recover quickly in uncomplicated cases. It is called “three-day sickness” because it usually shows significant improvement in animals within 3 days from the onset of the disease. The course of the disease usually begins with a sudden fever up to 41°C. Monophasic, biphasic or polyphasic fever can be seen in the disease (Kirkland, 2002).

The fever seen in the disease is usually biphasic and returns to normal levels within 1-1.5 days. Clinical findings of the disease in the first fever stage are milder than subsequent findings and may often go unnoticed. Loss of ap-

petite, depression and behavioural abnormalities are observed 12 hours after the onset of the fever (St George, 1988). The clinical symptoms of the disease are mostly associated with secondary fever (Uren, 1989). The body temperature in the first phase is always lower than the next phase (Davis et al., 1984).

On the 2nd day of the disease, a high amount of salivation, ocular and nasal flows are observed in sick animals. Tremor may be seen in some animals, muscles may become stiff with an altered lameness, and unwillingness to move is observed in sick animals. Rumen function may stop, and constipation may develop accordingly. Animals showing symptom of paresis may be affected by dehydration due to high fever and excessive fluid loss if they remain shadowless and dehydrated in very hot weather (St George, 1988).

On the 3rd day of the disease, many animals start to stand up and feed. However, symptoms such as lameness and weakness may last for a few more days. Affected animals lose their strength rapidly, and it may take time to recover the lost weight. Abortion can be seen in approximately 5% of pregnant animals, especially in the 2-3 months of pregnancy (Uren et al., 1987). There is no evidence that disease-related venereal transmission or infection during pregnancy adversely affects the development of the foetus (Parsonson and Snowdon, 1974).

Immunity

Neutralizing antibodies that develop in infected animals were thought to be permanent (Mackerras et al., 1940). However, it has been reported that specific neutralizing antibodies can be detected in the blood for at least 422 days following infection in animals infected with the disease, and infected animals have been reported to be protected against the disease for at least 2 years (Spradbrow, 1975). In fact, the results of serological studies conducted in Taiwan between 2001 and 2013 and obtained from three major outbreaks in the country between these dates show that neutralizing antibody titers were at very low levels during the dates of BEF disease and that there is a significant relationship between outbreaks and herd immunity (Ting et al., 2014). Although it has been reported that antibodies obtained through colostrum protect the offspring, the fact that the disease is seen at certain intervals makes the protection with passive immunity insignificant (St George, 1986).

Vaccines

Vaccination has been reported to be the only effective method in the control of the BEF disease (Radostits et al., 2006). There is evidence that vaccines developed by using isolated BEF virus isolates from about half a century ago continue to be effective against strains still in circulation. The neutralization and sequence similarities between among strains isolated in different geographical regions also support this argument (Hsieh et al., 2005; Gao et al., 2017).

There are live, inactive, subunit and recombinant vaccines developed against the disease. Among all these vaccines, live attenuated vaccines have generally been reported to provide longer-lasting immunity (Radostits et al., 2006). Live, inactive and subunit vaccines are used in the field. Current vaccines are used in countries such as Australia, Japan, South Korea, China, Taiwan, Türkiye, Saudi Arabia, Israel and the Philippines. In Türkiye, there is a commercially available live attenuated vaccine and one experimental recombinant DNA vaccine developed in 2018 (Abaylı, 2018; Vetal, 2024). Although experimental and commercial vaccines have been developed in various formulations, there are few reports on their effectiveness in the field. For most of the vaccines, protective immunity seems to be a limited period of time, and due to this period, additional applications are recommended at 6-month or 1-year intervals (Walker and Klement, 2015).

Diagnosis

If the first cases of the disease are seen in regions where the disease has not been seen before, it is difficult to diagnose from the clinical findings. Furthermore, diagnosis may be difficult due to clinical findings and histories due to the presence of other diseases similar to the disease as well as its rapid onset and recovery. Symptoms such as sudden onset of fever, lameness and rapid recovery in fattening and dairy enterprises can help to diagnose the disease. (Akakpo, 2015).

However, the diagnosis should be confirmed by laboratory method due to insufficient data to make an overall estimate at times such as the presence of sporadic cases or the onset of the epidemic. For this purpose, blood and blood serum should be obtained. However, successful results are not always achieved due to difficulties such as the short viremia phase in sick animals and rapid transfer of blood samples to laboratories (Bakhshesh and Abdollahi, 2015).

Laboratory Diagnosis

Laboratory diagnosis is necessary to confirm the pre-diagnosis of the disease. For this purpose, the disease can be diagnosed virologically, molecularly and serologically from blood samples taken during the hyperthermic phase to detect the virus and specific antibodies. During the convalescent period, diagnosis can be made serologically from samples taken at that time (Akakpo, 2015).

Various molecular techniques have been developed to detect BEFV infections. These include Dot Blot, Traditional RT-PCR, RT-qPCR, RT-LAMP and LFD-RPA (Hsieh et al., 2005; Zheng et al., 2011; Zaghloul et al., 2012; Blasdell et al., 2013; Hou et al., 2018; Gao et al., 2020). Conventional RT-PCR is routinely used in real-time quantitative PCR BEFV detection (Hou et al., 2018).

Serological methods include the most commonly used methods in the diagnosis of BEF disease. Serological diagnosis aims to detect neutralizing antibodies by using viral neutralization tests, enzyme-linked immunosorbent

assay (ELISA), immunoperoxidase (IP), immunofluorescence assay (IFA), dot blot hybridization and similar tests (Kirkland, 1992; Johal et al., 2008; Vorster and Mapham, 2012; Tonbak and Abaylı, 2016).

Treatment-Prevention-Control

Treatment

There is no effective treatment for the disease as in other viral diseases. However, by treating its symptoms, the disease is tried to be relieved. Resting, providing adequate shelter, feed and water to sick animals help the treatment since dehydration will be exacerbated in animals exposed to hot air. Moreover, animals raised with the pasture system move to meet their feed and water needs. This may adversely affect the course of the disease. Animals leaning on their sides should be rotated several times a day to prevent circulatory system disorders and muscle damage (Kirkland, 2002; Akakpo, 2015). When given daily during the incubation period, nonsteroidal anti-inflammatory drugs prevent the onset of clinical findings and can induce rapid recovery by relieving the symptoms after the onset of clinical disease (Uren et al., 1989).

The hypocalcaemia seen in the disease is thought to be due to hypersecretion of immunoreactive calcitonin or decreased circulating levels of vitamin D metabolites (Uren and Murphy, 1985).

Digestive disorders and increased salivation due to loss of the swallowing reflex prevent calcium absorption from the rumen (St George, 1992). Calcium borogluconate can be used in the treatment of hypocalcaemia, which is considered to be responsible for symptoms such as ruminal stasis, paresis and reflex loss. It is clear that further studies are required on the low milk production, which is presumably due to the high inflammatory response that occurs in the disease, and on the relationship between milk production and the hypocalcaemia presentation (St. George et al., 1986). In the post-infection period, animals should not be stressed for several days after clinical symptoms have subsided to ensure that biochemical functions return to normal (Coetzer and Tustin, 2004).

Prevention and Control

The use of disinfection methods in preventing the spread of the disease is relatively insignificant. The agent is rapidly inactivated in the secretions and carcass after the death of the animal. Since the disease is transmitted with vectors rather than direct or indirect contact, it is stated that the struggle should be against insects (Lvov et al., 2015). For this purpose, insecticides used in the struggle against insects are frequently preferred (Weetman et al., 2018). However, it is an important challenge to limit its effect due to the resistance to insecticides (Thomas, 2018). Quarantine measures may be beneficial in countries where the risk of spreading the bovine ephemeral fever virus from neighbouring countries is not high (Kirkland, 2002).

Future Perspective

It is clear that it is an arboviral disease due to the role of blood-fed insects and sandflies in the spread of the three-day sickness. Arboviral diseases are highly affected by ecological changes. Climate changes allow insects related to these diseases to expand and adapt to their habitats by causing them to multiply in areas where they have not been seen before. Due to deforestation caused by intense and unplanned urbanization, the nature of these insects is highly affected by reasons such as the intense contact among the habitats of different species, increasing trade between countries and socioeconomic factors that damage infrastructures (Mellor et al., 2000; Gould et al., 2006; Purse et al., 2015). Ecological changes and increasing average temperatures not only have a negative effect on insects, but also cause animals to become more susceptible to diseases due to weakening of the immune system (Lavon et al., 2023).

Although the disease has not been seen in Europe and the American continent, the risk of a sudden appearance of the disease is quite high, as in the case of the Bluetongue virus (BTV) and the Schmallenberg virus (SBV). The strong warming tendency of the climate in Europe and the change in this climate have caused the prolongation of the active periods of sandflies and increased their contact with the host by shortening their feeding and reproduction cycles. Accordingly, the transmission duration also increases. Virus detection made in common *Culicoides* species in diseases developing due to BTV and SBV is actually a striking example of how serious the risk is (Purse et al., 2005; Carpenter et al., 2009; De Regge et al., 2012; Hoffmann et al., 2012; Elbers et al., 2013). Since the animals in this region have not encountered the disease immunologically before and do not contain antibodies against the disease serologically, the result will be important for the industry if they infected with this disease (Lavon et al., 2023).

Considering the small body sizes of the *Culicoides*, which are associated with the disease, infective insects can be easily transported to overseas countries even with the effect of the wind, apart from the insect movement (Gale et al., 2015; Aguilar-Vega et al., 2019). Simultaneous or recent epidemics in distant regions in three-day sickness reports and isolate similarities in these epidemics also support this claim (Murray, 1970; Kirkland, 2002).

From an epidemiological perspective, surveillance, early warning systems and on-call animal practices will also be able to provide important information about arboviral infections for authorities, local officials and researchers. For this purpose, some practices applied in countries, where the disease is seen, may help in the struggle against the disease. In order to create a vector detection and early warning system in Türkiye, an early warning system is used by periodically scanning for many diseases, including BEFV, in insects with the light trap application applied in regions where the risk is high within the scope of the “Animal Disease Control Program”. In addi-

tion, there are antigen and antibody screening programs performed at regular intervals in sensitive areas (GKGM, 2023). Seronegative cattle, which are designated as sentinels under a program called "National Arbovirus Monitoring Program" in Australia, are periodically screened for various arboviral diseases, including BEFV. It provides importing countries with documentation of freedom from certain diseases and offers early warning capabilities to local producers regarding the onset and spread of the disease (Kirkland et al., 2016). The mentioned systems will be able to provide important information to local authorities regarding the examination of the movements of insects, the presence of existing pathogens and identification of threatened areas (ECDC, 2021).

Vaccines are an important part of the struggle against viral diseases. Vaccines have been developed in various formulations for use in the struggle against BEF (Walker, 2005). However, the low protection period of vaccines, the fact that the disease is seen every few years and requires repetition of the application, and the short-term immunity developed in the animals infected with the disease make the struggle difficult. Therefore, vaccines that provide long-term immunity are needed to obtain the desired results from vaccination practices which are indisputable in the struggle against the disease (Walker and Klement, 2015; Zheng et al., 2016).

As a result**,** vector identification in three-day sickness is an important stage of the struggle against the disease. It is important to perform this with more modern methods in order to eliminate the concerns caused by the insect struggle in terms of the environment and human health and to prevent the resistance to insecticides. There are many vaccines in various technologies and formulations developed to be used in the struggle against the disease. However, the protection duration of these vaccines is quite low considering that the disease is seen at intervals of several years due to the fact that the immunity that develops in infected and recovering animals does not protect animals for life. Accordingly, further researches should be conducted for vaccines that provide effective and long-term immunity to be used against the disease. Establishing effective surveillance and early warning systems will pave the way for more accurate and healthy information to be conveyed to local authorities and policy makers about the disease. Furthermore, the selection of these systems among the methods that can be applied in neighbouring countries, not in a single country, will allow regional struggle for diseases transmitted by insects.

Conflict of Interest

The authors declare no conflict of interests.

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Faculty of Veterinary Medicine, Aydın Adnan Menderes University,
West Campus, 09020, Efeler, Aydın, Türkiye
Tel : Tel:+90 256 220 60 00 Fax :+90 256 220 62 99
E-mail : animalhealth@adu.edu.tr
Dergipark: https://dergipark.org.tr/tr/pub/aduveterinary