ISSN 0334-9152

ISRAEL JOURNAL OF

VETERINARY MEDICINE

Formerly: **Refuah Veterinarith**

Published Quarterly:

Volume 79 • No. 2 • June 2024

Israel Veterinary Medical Association

P.O.Box 22, Raanana 4310001, Israel
Tel: 09-7419929 • Fax: 09-7431778
E-mail: ivma@zahav.net.il

M. Etinger – President
S. Yair – Secretary

Website: **www.ijvm.org.il**

Printed in Israel by E. Oren Publisher Ltd.
Email: oren.print@gmail.com

Contents

Front Cover: RETZ'S HELMETSHRIKE *(Prionops retzii)*

Retz's helmetshrike (***Prionops retzii***) is a species of bird in the helmetshrike family Vangidae, formerly usually included in the Malaconotidae. Four subspecies are recognized:

- *P. r. nigricans,* Neumann, 1899 – South Central Africa;
- *P. r. graculinus,* Cabanis, 1868 – East Africa;
- ***P. r. retzii*, Wahlberg, 1856 – Northern parts of Southern Africa**;
- *P. r. tricolor,* G.R. Gray, 1864 – Eastern and Southeastern Africa.

The bird is found in *Angola, Botswana, DRC, Eswatini, Kenya, Malawi, Mozambique, Namibia, Somalia, South Africa, Tanzania, Zambia*, and *Zimbabwe*. Its natural habitats are subtropical or tropical dry forests, subtropical or tropical mangrove forests, and subtropical or tropical moist shrub-land.

The helmetshrike is mostly black-and-brown with a piercing orange eye, red legs and eye-wattles, and an orange-tipped red bill. Calls are complex and include grating, whistling and a nasal note.

The picture of the cover were generously provided by **Mathew Axelrod** from South Africa.

Editorial

Dear Readers,

We are certainly going through turbulent times in Israel, and in fact throughout the world. Everything seems to be changing at rapid rate and hopefully we can look forward to a better future.

Still there are many issues that need to be attend to both now and in the future: One of the most important is "Climate change" which besides affecting our day to day coming and goings is and will have negative consequences.

The WHO recognizes this and have predicted that: "Climate change" is impacting our health in a myriad of ways, including by leading to death and illness from increasingly frequent extreme weather events, such as heatwaves, storms and floods, the disruption of food systems, increases in zoonoses and food-, water- and vector-borne diseases, and mental health issues (https://www.who.int/news-room/fact-sheets/detail/climate-change-and-health).

This will certainly add challenges to our work as veterinarians both in the small and large animal fields.

Best regards to all our readers.

Best wishes to all,

Sincerely,

Dr. Trevor (Tuvia) Waner
Editor-in-Chief, Israel Journal of Veterinary Medicine

Review Article

Animal Hoarding in Israel: Description and Implications of Animal Welfare and Suggested Resolutions

Shmueli Goldin, A.[1]* and Weyl Feinstein, S.[2]

[1] Animal Welfare Department, Veterinary Services, Ministry of Agriculture and Rural Development, P.O.B. 12, Bet Dagan, Hamakabim St., Rishon Letzion 7519701, Israel.

[2] Western Galilee District, Veterinary Services, Ministry of Agriculture and Rural Development, Manof 5, Akko, Israel.

* **Correspondence:** Dr. Ayelet Shmueli Goldin, DVM, Animal Welfare Department, Veterinary Services, Ministry of Agriculture and Rural Development, P.O.B. 12, Bet Dagan, Hamakabim Str., Rishon Letzion 7519701, Israel. Email: Ayelets@moag.gov.il.

ABSTRACT

Animal hoarding, a complex and often-misunderstood phenomenon, presents significant challenges to both individuals and communities. This paper offers a comprehensive review of the literature on animal hoarding, synthesizing key findings from various disciplines including psychology, sociology, veterinary medicine, animal welfare and public health. The review identifies common characteristics of animal hoarder's, such as a deep emotional attachment to animals, social isolation, and a lack of insight into the harm caused by their behavior. Furthermore, the paper explores the multifaceted nature of animal hoarding, examining its psychological, social, and ethical dimensions. Three hoarding cases that were treated by the Animal Welfare Department of Veterinary Services and districts' referents are presented, demonstrating the complexity of the theoretical division of hoarders' types. Hereby, we further discusses the impact of hoarding on both human and animal welfare, including the risks of neglect, disease transmission and environmental deterioration. Additionally, the paper highlights the challenges in identifying and intervening in cases of animal hoarding, including legal and ethical considerations. Drawing on this review, the paper proposes a conceptual framework for understanding animal hoarding that integrates individual, interpersonal, and systemic factors. This framework emphasizes the need for a multidisciplinary approach to addressing animal hoarding, highlighting the important collaboration between mental health professionals, social services, animal welfare organizations, and law enforcement agencies. Finally, the paper discusses implications for research, practice, and policy, advocating increased awareness, prevention efforts, and support services for individuals at risk of or affected by animal hoarding. By advancing our understanding of this complex phenomenon, this paper aims to contribute to interventions that are more effective and ultimately may improve the well-being of both humans and animals impacted by hoarding behaviors.

Key Words: Animal Hoarding; Animal Welfare; Law; Public Health; Mental Health.

INTRODUCTION

Animal hoarding phenomenon

Hoarding is a personality disorder characterized by the pathological collection and hoarding of various types of objects, such as furniture, newspapers, appliances and more. There are hoarders who collect and hoard animals, in addition to hoarding objects or regardless (1). Animal hoarding, like any other hoarding, is a personality disorder that can stem from many reasons such as mental disorders, difficulty connecting with the environment, unrealistic or illogical perception of reality, anxiety and

trauma, depression, searching for meaning and self-worth, the need to help a large number of animals, and more.

One of the characteristics of hoarding is a loss of control over the situation, leading to deterioration of living conditions that may harm the holder and its environment. The hoarder fills his living area with objects, finds it difficult to arrange and clean his home, does not maintain cleanliness and hygiene, neglects his surroundings and himself, and overall does not allow himself adequate living conditions. The definition of this phenomenon is holding numerous animals in a human enclosure, and the inability to provide them with minimal living conditions, take care of their needs and take care of them, thereby harming them through no fault of their own and denying the situation to some extent (2). Hoarded animals are physically and mentally affected: they often suffer from neglect, diseases and parasites. Mentally, they might develop abnormal behaviors such as fear, sensitivity to touch, attachment and attention seeking, separation-related behavior, urination and defecation when left alone, or repetitive behaviors (3).

Although little is known about this phenomenon, animal hoarding is believed to occur in every community and remains poorly understood (4).

The typical characteristics of an animal hoarder:

1. Possession of a large number of animals objectively and in relation to the ability to care for them.
2. Possession of companion animals mainly cats, dogs, and additional types of animals (5, 6).
3. Inability to care for animals and take care of all their livelihood needs.
4. Denial of the situation and unrealistic perception of reality
5. Self-neglect and the neglect of the all animals and premises.

According to literature, there are three types of hoarders: the "overwhelmed caregiver", the "rescuer hoarder" and the "exploitative hoarder" (7, 8):

1. **The "overwhelmed caregiver":** A person who decides to take care of animals in order to save them and find them good homes, out of an understanding that there are animals that need help and with a sense of mission and self-meaning. His personal value is related to the collection and care of animals. Collecting animals is usually done passively: they arrive to his possession through private bonds, people who hear about him and make contact, or authorities who consider him an association. At first, he manages to provide the animals with their livelihood needs, but soon he loses control of the situation, both financially and therapeutically, but does not stop receiving more and more animals and does not try to improve the situation. Moreover, he usually isolates him-self from society.
2. **The "rescuer hoarder":** Similar in motives and characteristics to the "overwhelmed" caregiver, but sees rescuing animals not only as part of his self-definition, but as a life task that slowly becomes an uncontrollable need. He does not take into account his ability to care for animals in the first place as a prerequisite for the task, although at the beginning of his actions he possesses certain abilities. He is usually very afraid of death and this fear motivates him to actively and passively collect animals: both from outside sources and by self-collection. He believes that he is the only one who can take care of the animals properly and even works to put animals up for adoption, but slowly become reluctant to remove them and concentrates mainly on saving them. Hence, very quickly the number of animals exceeds his ability to care for them. He is unable to refuse accepting additional animals from the outside, and he isolates from the society. However, he still maintains a certain relationship with others due to the desire to continue receiving animals. Nevertheless, he tries to prevent intervention as much as possible and is characterized by manipulative qualities.
3. **The "exploitative hoarder":** Actively collect animals in order to satisfy his needs (such as money or prestige). He often seems social, charismatic, having personal charm and manipulative, but lacks empathy for people and animals. He is indifferent to them and even deny their condition. He usually does not cooperate with authorities or any other intervention. This type of hoarder thinks and believes that he knows best, condescends and considers himself an anti-establishment expert. He is often able to lie and cheat in order to get his own and all ways are legitimate in his eyes.

The importance of understanding the types of hoarders can influence how they are treated. The "overwhelmed caregiver" and the "rescue hoarder" are able to cooperate with the authorities' demands, but the "rescue hoarder" will not cooperate, so enforcement measures must be taken (7). In many cases, characteristics of the three types of hoarders can be found in one hoarder or the development of all three types in the same hoarder, over time.

CASES DESCRIPTIONS

Case 1:

A man in his 40's began collecting stray cats at his home, following the death of his beloved cat, and was worried about cats on the street. At the same time, he made contacts with citizens and authorities to transfer to him stray cats that needed to be cared for. Within a short time, he reached holding of 500-600 cats in a closed enclosure and found it very difficult to care for them financially and physically. Following repeated complaints about the cats' poor state of holding, representatives of the authorities arrived at the scene and began a process of arrangement with him, which often failed due to his lack of cooperation and inability to take responsibility for the situation. He used to move the cats between different enclosures, in different households, and it did not improve the situation at all. The last holding place was very dirty, it smelled strongly of ammonia, the cats looked neglected and sick and after being examined it was found that some of them were infected with leishmania, which is a zoonotic disease. The case came to the attention of the authorities responsible for enforcing the Animal Welfare Law and the municipal veterinary service. Due to the infestation of the disease, great distress was expressed by the neighbors, and in light of all these, the hoarder agreed to cooperate: he hired an attending veterinarian who examined all the cats and marked them with an electronic chip. The infected cats were euthanized and the remaining cats (30 in number) were transferred to another facility, treated with preventive treatment (vaccination, deworming and treatment against skin parasites), neutered and spayed. It was agreed that the hoarder would not continue to collect cats and would focus on the remaining cats. As long as the hoarder persisted in the agreement, no indictment will be filed against him. The case was accompanied by the authorities, together with the hoarder's father, and long talks were held with him during the visits that took place. The local authority continued to monitor the situation, but no public health authority intervened.

Case 2:

A man in his 30's who was working as a taxi driver and during his work noticed stray cats, some of which to him appeared sick, and decided to collect them, treat them, neuter and sterilize them and put them up for adoption. He soon realizes that it was difficult to find a home for all cats and found it difficult to take care of all the cats. Following the complaints that arrived to the authorities and lead to their intervention, he made sure to move with all the cats in his custody from one authority to another, in order to avoid interference. According to his perception, he is the only one who could save the stray cats from their cruel fate. While he suffers as a result in his personal life, he was still unwilling to separate from his cats and return them to the places from where he collected them. He used to move with the cats from one place to another and disperse the cats to other residences, without updating the authorities and by literally concealing the data. This behavior caused increased stress to the cats and demonstrated his lack of reliability in actually rescuing them.

Case 3:

A 60-year-old woman, who lived alone in an apartment in a urban residential building owned 43 cats. At first, the woman kept "Khao Manee" cats (Thai cats) in order to breed and sell them, and following a personal loss, the number of cats she kept grew to include stray cats collected from the street. Following complaints from neighbors about the bad smell coming from the apartment, representatives of the authorities came for an inspection but were unable to obtain the cooperation to carry out a proper inspection of the apartment. Due to real concern for the welfare of the cats, a court order was issued allowing entry into the apartment to document the findings. The inspection found 43 cats living in an apartment full of furniture and objects, a lot of dirt, cat secretions and various types of garbage. There was a very strong smell of ammonia in the apartment since the windows were closed for fear of the cats escaping. Additionally, the woman scattered odor fresheners throughout the apartment, which made the smell worse. Only one bowl of drinking water and one litter box were found, and some frozen meat in the freezer that was used as cat food. The cats were found to be neglected, thin, suffering from skin infections and skin and ear parasites. The

woman had no documentation of veterinary treatments or vaccinations for the cats. Due to the difficult state of possession, the cats were confiscated.

DISCUSSION

Animal hoarding, a distinct subset of hoarding disorder, has a significant public health impact on the humans involved, as well as animal welfare. Individuals exhibit self-neglect, apathy, social withdrawal and object hoarding; living within squalid, deteriorated, structurally unsafe and uninhabitable premises, alongside neglected animals (6). The critical issue that lies in the heart of animal hoarding from the "animal welfare" point of view is the failure to provide them with minimal care (9).

The difference between hoarding animals and keeping a large number of animals

A holder of an animal is responsible for providing it with all its needs, taking care of it, taking care of its health and preventing its suffering (10). For this purpose, a person who owns an animal must know what is required in order to keep an animal and what are its natural needs. Naturally, people keep animals that are familiar, such as dogs and cats, but some people will keep parrots, reptiles, and farm animals. The number and types of animals depend to a considerable extent on the ability of the holder to provide them with all their needs and care for them, as well as to provide them with a sufficiently large and suitable living area. The holder will take into account his financial ability, the size of the enclosure he can provide and his attitude towards the animals in his custody, as sentient being, with necessary natural needs and being part of the family and household lifestyles.

Keeping animals without taking this responsibility is considered animal hoarding. Moreover, animals are perceived by the hoarder as an object and its collection fulfills a mental need, similar to the collection of inanimate objects, and when the conditions of possession are examined, the basic holding requirement by law are not fulfilled.

Implications of hoarding:

The implications of the hoarding on the immediate environment relate to sanitary problems that can reach the point of health hazards:

- **Noise**: mainly in the possession of dogs and poultry.
- **Dirt and filth:** Animals soil their environment and the hoarder is unable to clean and maintain proper hygiene. In addition, the living area full of animals is also loaded with animal-related equipment and lack of concern for waste disposal – all of which tend to invade a neighbor's living area or yard or residential building.
- **Bad smell:** due to poor hygiene conditions and a large number of animals.
- **Poor maintenance of a residential complex:** the neglect in residential complexes of hoarders not only of the animals and their living conditions, but also of the hoarder's living conditions and place of residence.
- **Animal morbidity:** a large number of animals, neglect of living conditions and their care provokes, among other things, pathogens that can also be zoonotic (such as in Case 1 – Leishmaniosis in cats).
- **Decline in property value:** Hoarder's living area is severely damaged, as are the other properties nearby. Neighbors of hoarders suffering from the hazards find it difficult to sell their properties and the value of their property drops significantly.
- **Pests:** Sanitary hazards attract pests (such as flies, rats and mice). These pests damage the hoarder's habitat and its immediate environment.
- **Isolation from society:** In many cases, the hoarder is isolated from his environment as part of the hoarder's characteristics, and also due to the hostile attitude of the environment. This includes his family members, many of whom fail to help him, do not receive cooperation from him, surrender to the situation and cut off contact with him.
- **Possible health hazards to the hoarder** due to the sanitary conditions self-neglect and lack of medical attention.

Legislation regarding welfare and protection of animals

The Israeli Animal Welfare (Protection of Animals) Act from 1994, aimed at protecting animals and preventing them from unnecessary suffering, including prohibiting animal abuse, torture and cruelty (10). The title of the law contains two main sections: 2A: "No person shall torture, cruel or abuse an animal in any way", and 2A1(a): "The owner or occupier of an animal must provide for its livelihood, take care of its

health and prevent abuse" (10). These clauses are subjected to interpretation but also reinforce each other. An owner of an animal who does not provide living needs and does not take care of the animal's health neglects the animal he owns and chronic neglect constitutes abuse. A hoarder who neglects animals in his custody is actually abusing them passively and over time and violates the law. In addition to the main Animal Welfare Act, there are the Animal Welfare (Protection of Animals) (Possession Not for Agricultural Purposes) Regulations, from 2009 (hereinafter: the "Keeping Regulations"; 11), which set minimum conditions for the care and keeping of animals, including the provision of medical treatment and prevention of suffering.

The possession regulations specify the minimum conditions necessary for satisfying the needs of animals and constitute a necessary minimum threshold, below which is considered an offense. To the extent that many sections of the Possession Regulations are not complied with, this is considered a violation of the provisions of the Animal Welfare Act itself. In some countries, there are specific animal hoarding legislations, for example, in Hawaii, Illinois and Colorado (12, 13, 14). The regulations in Hawaii refers to a specific number of animals (it is forbidden to keep more than 15 animals), refers only to dogs and cats and describes the conditions for keeping them (12). In Illinois there is a reference to a large number of animals, but no specific number is specified. However, the quantity of animals is subject to interpretation, there is no reference to the type of animal and the holding conditions which they are examined (13). In Colorado, there are legislations in various municipalities relating to limiting the number of pets in a housing unit. In most municipalities, there is a restriction to keep 4-6 dogs over the age of 4 months (14). Other states in the United States have legislations that prohibits hoarding in general. (15).

Different types of hoarders: is the division precise?

The classical idea of dividing hoarders into three different types ("the overwhelmed caregiver", "the rescuer" and "the exploiter") is intended to ease dealing with the situation, but based on the experience of the Animal Welfare Department. In most cases, there is no clear distinction between the three types rather a fusion of the different characteristics. For example, in case be characteristics that are suitable for both the "overwhelmed caregiver" (caring for the animal, loss of control over the situation, separation from the environment) and the "rescuer hoarder" (active and passive collection, contact with the environment, fear of death). In case 2, on the other hand, we can see characteristics of a "rescuer hoarder" (rescue as part of self-definition, active collection, does not differentiate from the environment, manipulates and prevents intervention by the authorities), and of the "exploitative hoarder" (knows best, condescending, anti-establishment). Nevertheless, case 3 began with characteristics of the "exploitative type" and over time characteristics of the "saving type" were interwoven with it. Patronek *et al.* (2006) described different treatments for the different types, however, we found that there is an overlapping in the types of treatment and the division is inconclusive (8). For example, Case 1, which corresponded in its characteristics to the "the overwhelmed caregiver", did not cooperate until there was a danger to health and the health of those around it. Even Case 2, which suited the characteristics of the "rescuer type", there was no cooperation from the beginning to the end of the intervention, including cheating of the law enforcement officials, contrary to the potential for cooperation with this "type", according to Patronek (8). Case 3 is characterized by the traits of the "exploitative type", but clearly, the characteristics of the "saving type" can be seen. In this case, only enforcement methods improved the situation, and welfare agencies were ineffective until representatives of the authorities intervened.

Ways of coping with hoarding

In order to influence and deal with the phenomenon, it is first necessary to understand the existing obstacles: Since cases of animal hoarding reach the sanitation authorities, the municipal veterinary services and the enforcement authorities of the Animal Welfare Act and its regulations – the treatment begins and continue by those authorities. They operate under the authority of the Animal Welfare Act and treat neglected animals, but they are unable to treat the hoarder. The hoarder suffers from a personality disorder, has difficulty integrating into the community, harms his entourage and his environment, and is likely to repeat collecting more animals and harm them. Most of hoarding cases that reach the court are hardly handled in a practical and efficient manner. The hoarder isn't usually perceived by the court as a criminal, furthermore the punishment existing in the law is not suitable for the treatment and prevention of continued hoarding. The hoarder does not turn to the welfare authorities and

the Ministry of Health for help, since hoarders usually deny and condescend over the establishment. Unfortunately, the authorities of the Ministry of Health and Animal Welfare cannot intervene to influence and improve the hoarder's condition in the absence of powers, a psychiatric opinion or a court order.

So how can we influence and improve?

The improvement in these cases lies in the broad consensus of all stakeholders and relevant authorities, that animal hoarding cases are complex situations that require the intervention and cooperation of several authorities. The causes for animal hoarding and the specific characteristics may vary; however, the outcomes remain constant: the harm to the animals. Optimal coping with animal hoarders incorporates aspects related to the conditions of the residential complex, sanitary conditions, safety of the residential complex (whether there are minors in the residential complex), animal welfare, and concern of harm to public health.

In the hoarding assessment, cooperative terminology should be used, which applies to all relevant authorities and interested parties:

1. Enforcement under the Animal Welfare law: as it is a criminal law, it is rather challenging for law enforcement officials and the court to treat the hoarder as a criminal. More importantly, the act of abrupt removal of animals, prosecuting and effectively criminalizing individuals with potential mental health issues also poses questions of public interest. Legal action is only part of the solution with high levels of repetition (up to 100%) reported (16, 17).
2. It is possible to ask the court to issue an order requiring diagnosis and treatment of the hoarder and thus begin a process of mental health treatment and animal welfare intervention combined.
3. Turning to a district psychiatrist and submitting a request for observation out of fear of harm to himself or his environment. Harm to the environment may concern public health aspects and possible suffering of animals, which are consciously sensing living beings. The examination can raise conclusions that will require the treatment providers and the hoarder to work together, monitor his condition, and thus prevent harm to himself, to his environment and to animals. He may even receive many social rights that other mentally ill people receive.
4. Legislation of bylaws in local authorities that relate to the restriction of keeping animals in residential compounds. It is important to take into account that Israel is becoming densely populated (18), and keeping large numbers of animals in residential compounds may be problematic for both the public and animal welfare.
5. Legislative amendments related to sanitation: sanitation, noise, odor and other hazards.
6. Education and information regarding the proper keeping of animals and the responsibility of owners to them.

SUMMARY

Animal hoarding entails many challenges related to animal welfare, hoarders' health and the environment. Given that animal hoarding is linked to mental health, the treatment is particularly complex. The causes and characteristics of the disorder can be different, but the result is, most often, real harm to animal welfare. Under the "One Welfare" notion, when one comes to solve the problem, there is a need for a holistic solution that will benefit both humans and animals, and this requires sensitive cooperation of all parties, which rarely are required to work together. As in many cases, prevention may be a key factor and can be obtained by initial identification of risk factors, which will lead to early and close supervision and legal actions, especially by limiting the possession of the number of animals at the earliest possible stage.

Overall, this review demonstrates the importance of a multidisciplinary approach to manage animal hoarding, for the benefit of both animals and human subjects.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. de Moura, R.R., de Castro, W.A.C., Farinhas, J.H., da Cunha, G.R., Pegoraro, M.M., Kmetiuk, L.B., dos Santos, A.P. and Biondo, A.W.: Association of hoarding case identification and animal protection programs to socioeconomic indicators in a major

metropolitan area of Brazil. Front. Vet. Sci. 9:872777, 2022. doi: 10.3389/fvets.2022.872777.
2. Wilkinson, J., Schoultz, M., King, H.M., Neave, N. and Bailey, C.: Animal hoarding cases in England: Implications for public health services. Front. Public Health, 10:899378, 2022. doi: 10.3389/fpubh.2022.899378.
3. McMillan, F.D., Vanderstichel, R., Stryhn, H., Yu, J. and Serpell, J.A.: Behavioural characteristics of dogs removed from hoarding situations. Appl. Anim. Behav. Sci. 178:69-79, 2016.
4. Patronek, G.: Hoarding of animals: an under-recognized public health problem in a difficult to study population. Public Health Rep. 114:82-7, 1999. doi: 10.1093/phr/114.1.81.
5. Nadal, Z., Ferrari, M., Lora, J., Revollo, A., Nicolas, F., Astegiano, S. and Díaz Videla, M.: Noah's syndrome: systematic review of animal hoarding disorder. Hum. Anim. Interact Bull. 10:21, 2020.
6. Wilkinson, J., Schoultz M., King, H.M., Neave, N. and Bailey, C.: Animal hoarding cases in England: Implications for public health services. Front. Public Health. 10:899378, 2022. doi: 10.3389/fpubh.2022.899378.
7. Patronek, G.J., Loar, L. and Nathanson, N.: Animal hoarding: Structuring interdisciplinary responses to help people, animals and communities at risk. Hoarding of Animal Research Consortium, 2006.
8. Patronek, G. J.: Hoarding of Animals: An Under-recognized Public Health Problem in a Difficult-to-study Population, Public Health Reports, January/February, VOL. 1, 14, 1999.
9. Stumpf, B.P., Cala´cio, B., Branco, B.C., Wilnes, B., Soier, G., Soares, L., Diamante, L. and Cappi, C.: Animal hoarding: a systematic review. Braz, J, Psychiatry., 45: 356-365, 2023. http://doi.org/10.47626/1516- 4446-2022-3003
10. Animal Welfare Act, Israel (Protection of Animals), 1994.
11. Animal Welfare Regulations, Israel (Protection of Animals) (Possession for Non-Agricultural Purposes), 2009.
12. Hawai State Legislations, Penal Code Chapter, Animal hoarding 711-1109.6.
13. Illinois complied statutes, Section 510, ILCS 70/2.10 – Companion animal hoarder.
14. Colorado Animal Cruelty law, Sections 18-9-201.5, 18-9-202, 18-9-202.5, and 18-9-204.5.
15. U.S. State Animal Protection Laws Rankings, Animal Defense Fund, *2021*. https://aldf.org/project/us-state-rankings/.
16. Berry, C., Patronek, G. and Lockwood, R.: Long-term outcomes in animal hoarding cases. Animal Law, 11: 167-94, 2005.
17. Johnson, B.M.: Animal hoarding: beyond the crazy cat lady. J Agric Food Inf., 9: 374-81, 2008. doi: 10.1080/10496500802482959.
18. WorldBank, Population density (people per sq. km of land area) – Israel, https://data.worldbank.org. Retrieved in Nov. 2022.

Sedative and Recovery Effects of Intramuscular Alfaxalone-Butorphanol-Midazolam Compared with Medetomidine-Butorphanol-Midazolam in Cats: A Randomized, Blinded Clinical Study

Bernstain, Y., Epstein, A., Abu Ahmad, W. and Shilo-Benjamini, Y.*

Koret School of Veterinary Medicine, The Robert H Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Israel

* **Corresponding author:** Dr. Yael Shilo-Benjamini, Koret School of Veterinary Medicine, The Robert H Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem. P.O.B. 12, Rehovot 7610001, **Israel** E-mail: shilo.yael@gmail.com

ABSTRACT

The goals of this study were to evaluate the effectiveness and physiological effects of alfaxalone-butorphanol-midazolam sedation in cats compared with the common sedation protocol used at our institution; medetomidine-butorphanol-midazolam. Thirty-one cats requiring sedation for various procedures were recruited randomly to receive intramuscular butorphanol (0.4 mg/kg) and midazolam (0.3 mg/kg) combined with alfaxalone (2 mg/kg) (ABM; n=16) or medetomidine (0.02 mg/kg) (MBM; n=15). Physiological variables and sedation quality (scale 7-28; 7=awake, 28=deeply sedated) were collected every 10 minutes until recovery. For medetomidine antagonism, the MBM cats received atipamezole intramuscularly. Induction and recovery times were recorded, and recovery quality was scored (1-4 scale: 1=poor, 4=excellent). Evaluations were performed by one blinded observer. Mann-Whitney U test, Fischer's exact and repeated measures mixed-effects were used for analysis, and p<0.05 was set for significance. Six cats (ABM) and three cats (MBM) required an additional dose. At 10-40 minutes sedation scores were significantly better in the MBM (21-24) compared with ABM group (19-20). Significant lower heart rate, higher blood pressure and respiratory frequency were recorded in the MBM group. Time to recovery was significantly faster (9±7 versus 26±21 minutes) and recovery of better quality (4 [1-4] versus 3 [1-4]) in the MBM compared with the ABM group. During recovery, cats in the ABM group showed opisthotonos, twitching, and paddling, which resolved within an hour. In conclusion, at the doses used, ABM was a viable alternative to MBM with less cardiovascular effects, however, sedation plane was inferior and recovery, longer, accompanied by adverse behaviors.

Keywords: Cats; Alfaxalone; Butorphanol; Medetomidine; Midazolam; Sedation.

INTRODUCTION

Cats may require sedation to undergo minor procedures during veterinary visits, especially if they are stressed and resist restraint and handling (1, 2). Medetomidine is a commonly used α2-agonist in cats, which cause significant cardiovascular effects, including initial vasoconstriction, hypertension, reflex bradycardia and reduction in cardiac output (3-5). α2-agonists are often combined with other injectables such as ketamine, opioids and/or benzodiazepines in order to achieve a synergistic effect and thus lower its dose and enhance its sedative effect (6, 7).

Many of the cats submitted for medical treatments in Israel are fractious or difficult to handle without prior deep sedation. A combination of medetomidine-butorphanol-midazolam is commonly used to sedate cats at our Veterinary Teaching Hospital, however, the effects of medetomidine on the cardiovascular system and the resultant decrease in cardiac output may be harmful in geriatric or sick cats (5).

Alfaxalone is a neurosteroid injectable anesthetic, which produces its effects via γ-aminobutyric acid receptor A ($GABA_A$) and can be administered via intravenous or intramuscular (IM) routes (8-10). Alfaxalone has been combined with various sedatives and tranquilizers to produce sedation or anesthesia in cats (11-14). It has also been advocated for use in cats with underlying diseases (15, 16) or in animals with high anesthetic risk (17).

The objectives of this study were to evaluate the sedative and adverse effects of IM alfaxalone-butorphanol-midazolam (ABM) and compare it with medetomidine-butorphanol-midazolam (MBM) protocol in cats. Our hypotheses were that sedation would be similar while cardiovascular effects would be less marked in cats administered ABM compared with MBM, although recovery time of ABM was likely to be longer.

MATERIALS AND METHODS

Animals

The Internal Ethics Review Committee (KSVM-VTH/14_2015) approved this study and a verbal or written informed consent was obtained from the owners or legal guardians. In addition, established internationally recognized high standards ('best practice') of veterinary patient care were followed.

All recruited cats required deep sedation for short (5-30 minutes), minor procedures, such as ultrasound, radiographs, bandage change, blood sampling and physical examination in stray animals prior to neutering, etc. Cats were considered healthy based on physical examination; however, some cats were fractious and required sedation to perform the examination. Inclusion criteria included: age 5-months to 12-years-old with body weight greater than 2 kg and fasting for at least 6 hours. Exclusion criteria were cats requiring general anesthesia following sedation or cats with suspected systemic disease (heart/lung/kidney/liver).

Procedures

Prior to sedation, the cats' temperament was assessed on a 1-4 scale (1=nice, quiet, easy to handle; 2=not nice, but able to handle with restraint; 3=aggressive, struggle, require a lot of restraint or sedation; 4=fractious, cannot be restraint without deep sedation). When it was possible, heart rate (HR) was measured using a stethoscope, respiratory frequency (f_R) by watching chest movements and rectal temperature (RT) with a digital thermometer. All cats were administered 0.4 mg/kg butorphanol (Butomidor; Richter Pharma AG, Wels, Austria; 10 mg/ml) and 0.3 mg/kg midazolam (Midolam; Rafa Laboratories, Jerusalem, Israel; 5 mg/ml). These drugs were combined with alfaxalone (Alfaxan, Jurox, Rutherford NSW, Australia; 10 mg/ml; 2 mg/kg; ABM) or medetomidine (Domitor, Orion Pharma, Espoo, Finland; 1 mg/ml; 0.02 mg/kg; MBM), which were assigned via a random generated list (https://www.random.org/lists/). Drugs were administrated IM in the quadriceps muscles via a squeeze cage using a 21-gauge, 25-mm needle.

Following injection, cats were left in the cage and monitored until becoming laterally recumbent. If lateral recumbency did not occur within 15 minutes or if the cat responded to stimuli during the procedure, an additional dose was administered IM (alfaxalone 1 mg/kg [ABM] or medetomidine 0.01 mg/kg [MBM]).

Sedation quality was scored on a 7-28 scale, including seven parameters, and the total score was summarized (Table 1). Additionally, response to procedure was scored on a 1-4 scale (1=cannot be performed; 2=performed with a lot of restraint; 3=performed with a little restraint; 4=performed without restraint). Vital signs included HR, f_R, RT, indirect measurement of mean arterial blood pressure (MAP) using an oscillometric technique with the cuff (40% circumference) placed above the carpus, and pulse oximetry (SpO_2) with the probe placed on the tongue/ear/paw (Cardell 9402 Vital Signs Monitor, MIDMARK, Tampa, FL, USA). Pain level of the procedures (painful/non-painful) and the noise level at the room (noisy/quiet) were recorded. Data was collected 5 and 10 minutes after injection, and then every 10 minutes until the end of the procedure. Eye drops were instilled for corneal moisture (Hydroxyethylcellulose 0.19% LYTEERS®; Fischer Pharmaceutical, Bnei Brak, Israel).

At the end of each procedure, cats in the MBM group received 0.05 mg/kg atipamezole (Antisedan; Orion Pharma; 5 mg/ml) IM in the epaxial muscles, while cats in the ABM group did not receive any antagonist medication. The assessor was out of the room during antagonist administration (or no administration). Recovery quality was scored on a 1-4 scale (1=poor, severe muscle rigidity, severe twitching/paddling, and severe hypersensitivity to touch/noise/light; 2=fair, moderate muscle rigidity and twitching/paddling,

Table 1. Quality of sedation guidelines (scale 7-28; 7=awake, 28=deeply sedated).

Parameter	Score 1	Score 2	Score 3	Score 4
Body position	Standing or walking	Sternal	Lateral but moving head	Lateral not moving
Pupil Position	Central			Rotated
Response to noise (tested by clapping the hands loudly near the cat's ear and waiting for response)	Jumping	Moving head	Moving ears	Not responding
Palpebral reflex (tested by tapping at the medial canthus and waiting for a blink response)	Spontaneous	Strong	Reduced	Absent
Ear flick reflex (tested by placing the tip of a hemostat gently in the ear and waiting for an auricle flick response)	Spontaneous	Strong	Reduced	Absent
Withdrawal reflex (tested by pinching the middle digit of the hind limb using a hemostat for a few seconds and waiting for a withdrawal response)	Spontaneous	Strong	Reduced	Absent
Jaw tone	Cannot open the jaws	Strong	Reduced	Flaccid

and/or hypersensitivity to touch/noise/light; 3=good, mostly smooth, mild twitching and/or hypersensitivity; 4=excellent, smooth and calm). Time of injection, lateral recumbency, end of procedure, atipamezole injection, sternal recumbency and adverse effects were recorded. All data collection and scorings were performed by one investigator who was unaware of the treatment.

Statistical analysis

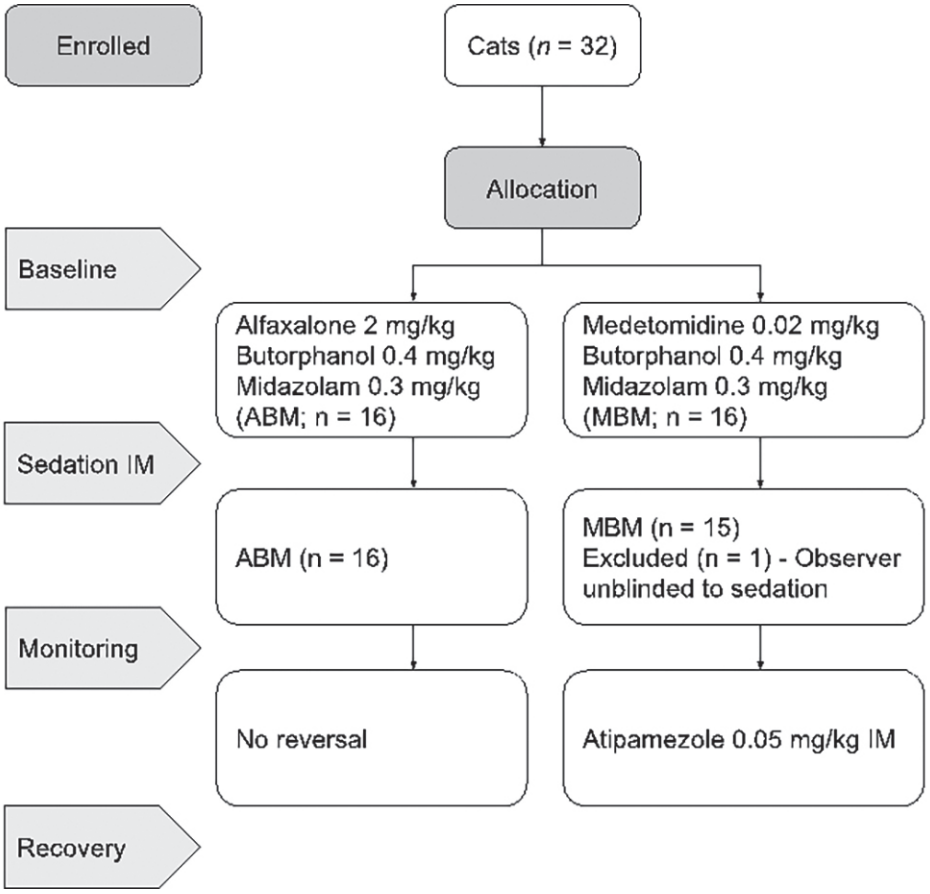
A sample size calculation determined that 12 cats per group would be required to detect a difference of 20±13 beats per minute (bpm) in HR between groups at 30 minutes from injection (yielding a power of 97% with α of 5%) (18). This time point was chosen as the average time between alfaxalone (9) *versus* medetomidine (3) maximum effect on HR (20 *versus* 40 minutes, respectively). Several more cats were recruited to account for differences in procedure length and incomplete data collection.

The Shapiro-Wilk test was used to assess normal distribution of the variables. Quantitative variables were compared by Student's *t*-test for normally distributed variables (presented as mean±standard deviation [SD]). Non-normally distributed variables were analyzed with the Mann-Whitney U-test (presented as median [range; maximum-minimum]), and qualitative variables were compared with Fisher's exact test (median [range]). For testing the relationship between ordinal variables, the Spearman's rank correlation coefficient was used. Wilcoxon signed-rank test was used to compare the HR at different times with baseline in the same group. Due to the small sample size, in both groups a correction for multiple comparison was not done. Repeated measures mixed-effects was used to determine the relationship between independent variables (age, sex, weight, temperament, location and additional drug dose) and sedation quality. Significance was set at p-value<0.05. Analyses were performed with commercial statistical software programs (SPSS Version 22, IBM, New York, NY, USA and STATA Version 14, StataCorp., College Station, TX, USA).

RESULTS

Thirty-two client-owned cats were recruited. The evaluator was accidently exposed to the injection volume of one cat in the MBM group, therefore it was omitted from the study (Figure 1). Data from 31 cats (16 females, 15 males) weighting 4.5±1.7 kg and aged 3.0±3.0 years were analyzed. Except for one Ragdoll cat in the ABM group, all cats were domestic short-haired. There was no difference in sex, temperament, pain/noise or procedure between the groups, however, cats in the ABM group were significantly older (p=0.033) and weighed more (p=0.027) (Table 2). A positive correlation between cats' age and weight (R=0.711) was found. Additionally, in the ABM group a positive correlation

Figure 1. Flow diagram of study design to compare between intramuscular (IM) alfaxalone-butorphanol-midazolam (ABM) *versus* medetomidine-butorphanol-midazolam (MBM) sedation in cats.



was found between cats' age and weight to total sedation time (R=0.737 and R=0.600, respectively). Cats who had a painful procedure were administered non-steroidal anti-inflammatory drugs and when applicable also local anesthesia. There were no differences in induction time (p=0.078), procedure duration (p=1.0) or total sedation time (p=0.131) between groups (Table 2).

Median sedation quality score was significantly higher in the MBM group compared with the ABM group at 5, 10, 20 and 40 minutes (p=0.017, 0.001, p<0.001, p=0.029, respectively; Table 3). Withdrawal reflex was decreased or lost between 5-20 minutes following injection in both groups, and no difference between groups was observed in response to the procedure (Figure 2A). Six cats (38%) in the ABM group and three cats (20%) in the MBM group required an additional dose for performing and completing the procedure (p=0.433). The procedure could not be completed in one cat from each group: ABM- a 5-month-old cat moved at 20 minutes after drugs administration, when handled for blood sampling (non-painful procedure, noisy room), and additional alfaxalone was not sufficient for resedation. MBM- a 2-year-old cat moved at 30 minutes after drugs administration, while placing sutures in a dehiscence incision, although lidocaine infiltration was performed (painful procedure, noisy room), and additional medetomidine was not sufficient for procedure completion.

Mean HR was significantly higher in the ABM group at all time points following injection (Table 4). In the ABM group HR did not change from baseline, while in the MBM group, it decreased significantly at 10-40 minutes following injection. An arrhythmia was heard in two cats in the MBM group (6-months and 1-year old); in one of them atrial premature complexes were suspected. Spontaneous breathing was preserved in all cats throughout sedation. f_R was significantly higher in the MBM group at 5-20 minutes (p=0.016, 0.001, 0.024, respectively; Table 4).

Table 2. Median (range) of demographic data, temperament scores, noise and pain levels, and description of minor procedures performed in cats sedated with an intramuscular combination of alfaxalone-butorphanol-midazolam (ABM; *n*=16) or medetomidine-butorphanol-midazolam (MBM; *n*=15). And mean±standard deviation (SD) induction time (from injection to lateral recumbency), procedure time (from injection to the end of procedure), total sedation time (from injection back to sternal recumbency) and recovery time (from atipamezole administration [MBM] or no antagonist [ABM] to sternal recumbency).

Parameter	ABM	MBM
Female/male	8/8	8/7
Age (years)	4.2 (1-7)*	1 (0.5-1.9)
Weight (kg)	4.5 (3.7-7.1)*	3.5 (3-4.7)
Temperament score	2 (1-4)	2 (1-3)
Noise level	2 (1-2)	2 (1-2)
Pain level	2 (1-2)	2 (1-2)
Procedures`	Blood sampling (5), bandage change (4), oral exam (4), ultrasound & skin biopsy (1), wound debridement (1), haircut (1)	Blood sampling (7), bandage change (4), incision dehiscence suturing (1), castration (1; under local anesthesia), ultrasound (1), radiographs & arthrocentesis (1)
Period (minutes)		
Induction time	10.4±9.1	5.9±2.8
Procedure time	49.6±19.2	49.3±17.7
Total sedation time	75.6±26.6	61.3±24.3
Recovery time	25.9±20.9	12.0±15.6*

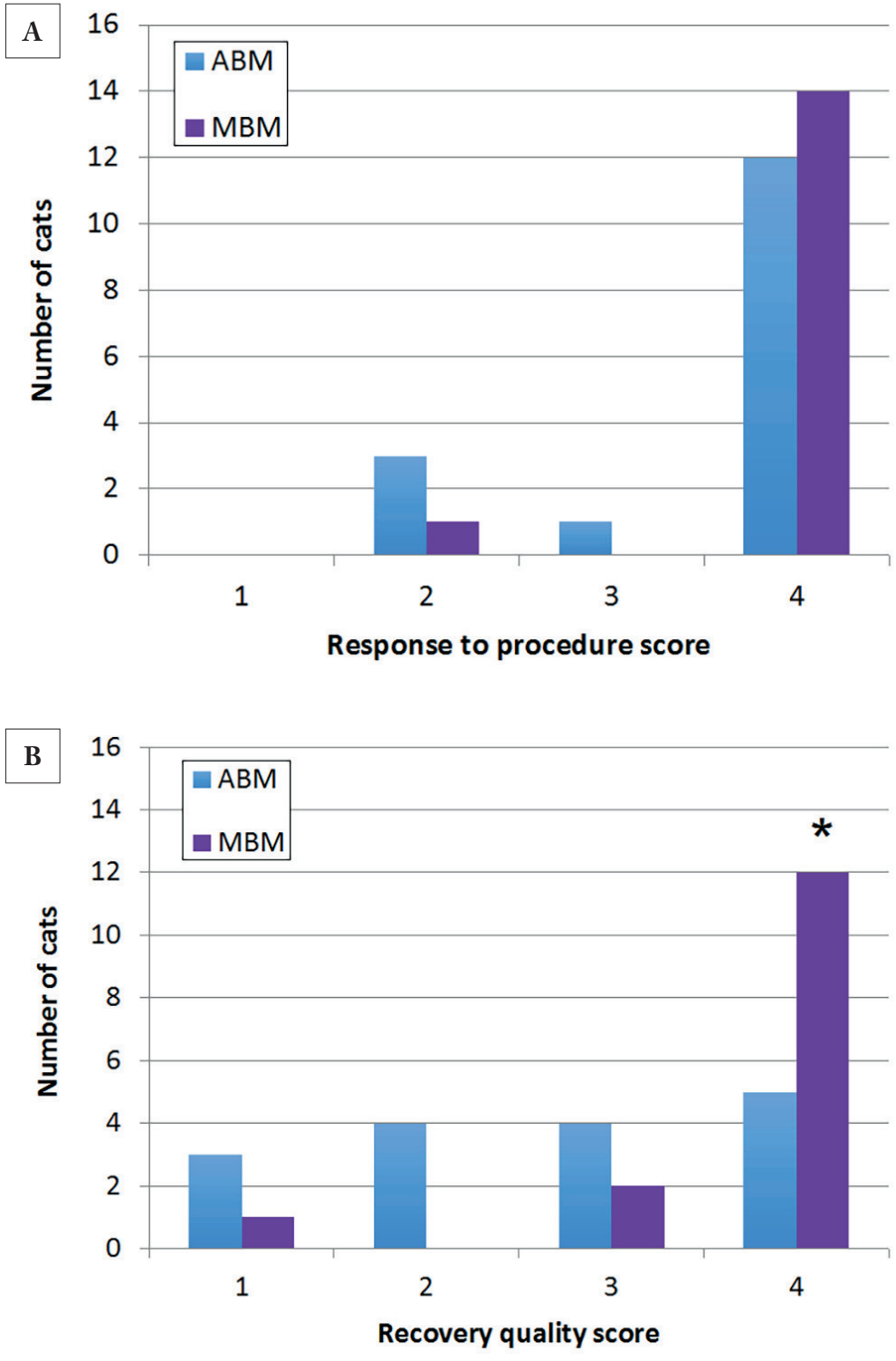
Temperament score used a 1-4 scale (1=easy to handle, 4=cannot be restraint without sedation).
Noise level scoring: 1=noisy, 2=quiet.
Pain level scoring: 1=painful, 2=non-painful.
* Significantly different between groups (p<0.05).

Table 3. Sedation quality scores (scale 7-28; 7=wide awake, 28=deeply sedated; Table 1) in cats at times 5 to 60 minutes following intramuscular administration of alfaxalone-butorphanol-midazolam (ABM; *n*=16) or medetomidine-butorphanol-midazolam (MBM; *n*=15). The number of cats (*n*) is added in parenthesis when not all the cats in the group were assessed at that time point.

Time following injection (minutes)	ABM	MBM
Baseline	7 (7)	7 (7)
5	21 (20-22) (*n*=6)	22.5 (22-23.5) (*n*=8)*
10	20 (18-21.5) (*n*=12)	23 (22-24)*
20	20 (16-21.5)	24 (23-24)*
30	20 (17-21) (*n*=13)	21 (21-24)
40	19 (15-20) (*n*=10)	23 (20-24) (*n*=9)*
50	19 (16.5-19.5) (*n*=7)	21.5 (19-22) (*n*=6)
60	15 (14-23) (*n*=5)	21 (19.5-21.5) (*n*=3)

Data is presented as median (range).
* Significantly different between groups (p<0.05).

Figure 2. Number of cats at each score category of (A) response to procedure (scale 1-4; 1=cannot be performed, 4=performed without restraint) and (B) recovery quality (scale 1-4; 1=poor, 4=excellent) of cats sedated with intramuscular alfaxalone-butorphanol-midazolam (ABM; *n*=16) or medetomidine-butorphanol-midazolam (MBM; *n*=15).



* Significantly different between groups (p<0.05).

Table 4. Physiological parameters collected from baseline to 50 minutes after administration of intramuscular alfaxalone-butorphanol-midazolam (ABM; *n*=16) or medetomidine-butorphanol-midazolam (MBM; *n*=15). The number of cats (*n*) is added in parenthesis when not all the cats in the group were assessed at that time point.

Variable	Group	Time (minutes from injection)						
		Baseline	5	10	20	30	40	50
HR (bpm)	ABM	186±43	182±20*	162±25*	165±20*	157±24*	161±27*	139±34*
		(*n*=9)	(*n*=8)	(*n*=12)			(*n*=10)	(*n*=7)
	MBM	188±22	107±19	100±18	98±17	99±17	101±19	98±2
		(*n*=8)	(*n*=7)				(*n*=10)	(*n*=5)
f_R (rpm)	ABM	53±30	31±9	29±7	31±10	30±7	31±10	32±8
		(*n*=11)	(*n*=6)	(*n*=11)	(*n*=14)	(*n*=11)	(*n*=10)	(*n*=6)
	MBM	49±18	45±12*	46±15*	40±11*	38±15	37±9	35±12
		(*n*=12)	(*n*=8)	(*n*=13)			(*n*=10)	(*n*=5)
RT (°C)	ABM	38.1±0.1	37.9±0.9	37.9±0.9	37.7±0.7	37.3±1.0	37.4±0.9	36.7±0.9
		(*n*=2)	(*n*=5)	(*n*=11)		(*n*=10)	(*n*=8)	(*n*=5)
	MBM	38.6±0.5	38.0±0.6	38.1±0.6	38.1±0.6	37.7±0.5	37.4±0.6	36.9±0.9
		(*n*=2)	(*n*=8)	(*n*=14)	(*n*=14)	(*n*=13)	(*n*=6)	(*n*=5)
MAP (mmHg)	ABM	NR	91±28	98±26	98±24	108±32	117±29	100±32
			(*n*=5)	(*n*=11)	(*n*=15)	(*n*=12)	(*n*=8)	(*n*=6)
	MBM	NR	143±22*	142±20*	125±26*	124±21	114±23	97±17
			(*n*=8)	(*n*=14)	(*n*=14)	(*n*=14)	(*n*=9)	(*n*=5)
MAP (number of cats with values <60 mmHg)	ABM	NR	1	1	0	0	0	1
	MBM	NR	0	0	0	0	0	0
SpO2 (%)	ABM	NR	94±2	94±3	94±3	95±3	95±3	97±2
			(*n*=4)	(*n*=9)	(*n*=13)	(*n*=11)	(*n*=7)	(*n*=5)
	MBM	NR	92±4	94±3	93±4	93±6	95±3	96±2
			(*n*=6)	(*n*=12)			(*n*=8)	(*n*=3)
SpO2 (number of cats with values <90%)	ABM	NR	0	1	1	0	0	0
	MBM	NR	1	1	3	2	0	0

HR, heart rate; bpm, beats per minute; f_R, respiratory frequency; rpm, respirations per minute; RT, rectal temperature; MAP, mean arterial pressure; SpO2, hemoglobin oxygen saturation; NR, not recorded.
Data is presented as mean±standard deviation, unless stated otherwise.
* Significantly greater than the other group (p<0.05).

RT decreased over time in both groups with no difference at any time point. At 50 minutes mean RT was lower than 37.0°C in both groups. Mean MAP was significantly higher in the MBM group at 5-20 minutes (p=0.007, p<0.001, p=0.010, respectively; Table 4). MAP below 60 mmHg was recorded in three different cats from the ABM group, once for each cat, although, these cats were moving during the measurement (paddling and opisthotonos). There was no difference between groups in SpO_2 levels at any time point (Table 4). One cat from the ABM group which experienced SpO_2 lower that 90% also experienced opisthotonos at these time points.

Mean recovery time was more rapid (p=0.046; Table 2), median recovery score better (4 versus 3; p=0.007), and significantly more cats were scored as "excellent" (p=0.017) in the MBM group (Figure 2B). Cats which were administered additional alfaxalone dose (ABM group) had significantly poorer recovery scores (p=0.021). No correlation was found between recovery score and age, weight, or temperament.

Table 5. Adverse effects during recovery in cats following sedation with intramuscular alfaxalone-butorphanol-midazolam (ABM; *n*=16) or medetomidine-butorphanol-midazolam (MBM; *n*=15). Data is presented as the number of cats that showed the adverse effect.

Adverse effect	ABM	MBM
Opisthotonos	10*	0
Twitching	9*	1
Paddling	6*	0
Obsessive licking	3	0

*Significantly different between groups (p<0.05)

Adverse effects were observed mostly in the ABM group during recovery (Table 5). These behaviors ceased without any treatment within an hour following recovery. Correlation was found between opisthotonos and additional alfaxalone dose (p=0.037). Two cats, one from each group, demonstrated aggressive behavior during recovery, both were defined as pleasant before sedation. All cats were discharged home following recovery. Abnormal vocalization was reported for one ABM cat on the day following sedation. That cat was administered a second alfaxalone dose. No other long-term effects were reported.

DISCUSSION

According to the results of the present study, ABM via IM injection route provided sufficient sedation for minor, short, non-painful procedures in most cats. However, sedation quality was better in the MBM group, although no difference was found in the withdrawal reflex or in response to the procedure between groups. One study demonstrated the efficacy of 2.5 mg/kg alfaxalone IM as a sole agent, although, 5-10 mg/kg produced better sedation for longer duration (9). Administration of 2 mg/kg alfaxalone and 0.2 mg/kg butorphanol IM in cats was reported to provide good sedation in one study (16), while in another study sedation was not sufficient and additional alfaxalone dose was required to produce immobility in 11/19 cats (14). Administration of butorphanol (0.2 mg/kg) combined with alfaxalone (2 mg/kg) or dexmedetomidine (0.007 mg/kg) in cats provided sufficient sedation for abdominal ultrasound or CT with no differences in sedation scores between protocols, although these cats were considered nice and habituated to handling (19). In the present study 6/15 cats required an additional alfaxalone dose in order to perform or complete the procedure. It is possible that a dose of 2 mg/kg is too low for young or fractious cats and a higher dose may be necessary (e.g., 2.5-3 mg/kg). In a study comparing butorphanol (0.2 mg/kg) combined with alfaxalone 2 *versus* 5 mg/kg, it was reported that sedation was significantly better for 30 minutes with the higher alfaxalone dose (14). However, it should be taken into account that higher dose will require higher injection volume, which may result in more discomfort compared with a lower volume when administered IM. Additionally, in the present study older cats took longer time to recover, therefore, geriatric cats or cats with comorbidities may require a lower dose than 2 mg/kg.

The median (reference range) for HR in cats was reported to be 190 (128-256) bpm in the hospital setting and 153 (110-250) bpm in the home environment (2). In the ABM group HR values were within the reference range, while in the MBM group they were lower. HR values of the ABM cats are similar to other studies administering up to 5 mg/kg alfaxalone to cats (8, 9, 13, 20, 21). The decrease in HR in the MBM group was anticipated because of medetomidine-induced reflex bradycardia and was reported previously (3-5). Arrhythmias, such as atrioventricular block were reported following medetomidine in cats and dogs (22, 23). Therefore, it is less likely that the arrhythmias heard in the two young cats from the MBM group were present before the sedation but were caused due to medetomidine administration.

All cats in both groups were breathing spontaneously throughout sedation. In the ABM group f_R decreased from baseline values, but remained in the reference range for cats (2). These findings are consistent with studies reporting alfaxalone administration up to 5 mg/kg IM in cats (9, 10). In a study in cats sedated with IM alfaxalone-butorphanol, f_R was also maintained in the reference range (14). In a study investigating target alfaxalone plasma concentrations in cats, only supraclinical plasma concentrations produced hypoventilation ($PaCO_2$>45 mmHg; f_R was not reported) (20). Administration of IM medetomidine (0.05-0.08 mg/kg) (3, 4) or dexmedetomidine (0.01 mg/kg) and butorphanol (0.2 mg/kg) (24) in cats resulted in a significant decreased f_R. At the present study the f_R was higher in the MBM group at the first 20 minutes of sedation. High f_R may be caused by hypoxemia and/or hypercarbia (25). SpO_2 lower than 90% was observed only in 3 cats in the MBM group, however PaO_2 nor $PaCO_2$ were measured. Pulse oximetry have ac-

curacy limitations, such as movement, skin pigmentation, and vasoconstriction (25). The SpO_2 measurements in the MBM group could have been affected by vasoconstriction in the first 20 minutes following drug administration.

In both groups RT decreased over time. Hypothermia following medetomidine was related to muscle relaxation or to α_2-receptors type C2, which are present in the spinal cord and are thought to be involved in thermoregulation (26). In the ABM group the decrease in RT was consistent with other studies in cats sedated with alfaxalone and can be explained by muscle relaxation and vasodilation (9, 13, 21). Therefore, during both sedation protocols it is recommended to monitor RT and provide external heat when needed.

The reference range of MAP in adult awake companion animals is 80-120 mmHg, and under anesthesia MAP should be kept above 60 mmHg (25). However, it was reported in cats that kidney autoregulation is lost below 70 mmHg (27). In the ABM group MAP was generally kept in the acceptable range, except for three readings (three different cats) in which MAP was below 60 mmHg. However, in all three low MAP events the cats were moving during measurement, which may suggest that measurements were less accurate (25). Other studies reporting hypotension in cats following alfaxalone used higher alfaxalone doses (5-15 mg/kg) (9, 10, 21). In a study characterizing hemodynamic effects of subclinical, clinical and supraclinical plasma alfaxalone concentration in cats, MAP decreased with increasing plasma target concentration, although MAP values were higher than 87 mm Hg at all plasma concentrations (20). In the MBM group a biphasic blood pressure pattern was demonstrated, starting with high MAP during the first 20 minutes, followed by a gradual decrease. This biphasic pattern was reported previously (5, 24, 28), and is attributed to the initial peripheral vasoconstriction followed by a secondary central vasodilation (23).

The youngest cat in the ABM group woke up 20 minutes following injection, which could be related to the report that $GABA_A$ receptors subunits β_2 and β_3 have different number and distribution in younger animals, and may have an impact on binding properties of drugs to $GABA_A$ (29, 30). Since alfaxalone functions as a positive modulator of $GABA_A$, an age-related difference may influence alfaxalone affinity to its receptor. Another explanation for the difference could be related to metabolism, which may be quicker in younger animals (31). This can also explain the observation that older cats in the ABM group took longer to recover.

The advantage of adding butorphanol and midazolam to the sedation protocol is their sedative effect and synergism, making the sedation more reliable, while minimally affecting cardiopulmonary function (26, 32, 33). Butorphanol also has analgesic property, which is important when painful procedures are planned (32). Alfaxalone 2.5 mg/kg IM resulted in 60 minutes of anesthesia (from recumbency until the cat was standing), while 5-10 mg/kg provided longer anesthetic duration (9). Combination of 2 mg/kg alfaxalone and 0.2 mg/kg butorphanol IM in cats resulted in 32.1-44.1 minutes sedation until seating/sternal recumbency (14, 16, 34). Sedation duration in the present study was longer probably due to the addition of midazolam.

Recovery time and recovery quality were significantly better in the MBM group, because the ability to antagonize medetomidine, which were expected. Adverse effects observed in the ABM group were reported previously in cats following alfaxalone sedation (9, 14, 15), although a study administering alfaxalone-butorphanol reported smooth recovery (16). A different study, reported poor and prolonged recovery following 5 mg/kg alfaxalone, 0.01 mg/kg dexmedetomidine with/without 0.1 mg/kg hydromorphone, however atipamezole was not administered (12). In the present study an additional alfaxalone dose resulted in decreased recovery score, which was reported previously (35, 36). These findings suggest that lower alfaxalone doses should be used in cats, although it is important to note that the adverse effects were minor and resolved without any treatment within an hour following the procedure.

Limitations to this study include (i) suboptimal comparison between an anesthetic and a sedative drug, which were not administered at equipotent doses, although, to the authors' knowledge such doses were not reported in the literature. (ii) Due to the clinical nature of the study, there was no uniformity as to the procedure, procedure length, pain or noise levels, which could have affected sedation and recovery qualities. (iii) Baseline data was lacking because of the cats' temperament, and because procedural limitations, some data was not collected. (iv) Most cats were young and healthy, and it is unknown whether old and/or sick cats would respond differently to these sedation protocols. Additionally, cats' age and weight were statistically different between groups, which could have potentially affected physiological values and sedation time. (v) Sedation quality scoring was developed by the observer instead of using previously reported scoring

systems. (vi) Direct measurements of MAP and SpO2 are more accurate than indirect techniques, but these could not be established in this clinical study due to the more invasive nature of such techniques.

CONCLUSIONS

ABM administered IM produced short sedation that allowed minor procedures in healthy cats and provided cardiopulmonary stability in comparison to MBM. As a third of the cats required an additional dose, it is suggested that in young/fractious cats a higher alfaxalone dose (2.5-3 mg/kg) may be required. In contrast, our clinical experience suggests that in geriatric/sick cats alfaxalone dose of 1-1.5 mg/kg is sufficient with this combination. During recovery some cats may experience some short-lived opisthotonos and twitching.

ACKNOWLEDGMENTS

The authors thank the staff of the Veterinary Teaching Hospital for assistance with patient recruitment, and especially grateful to Mrs. Lilith Mayron.

REFERENCES

1. Lloyd, J.K.F.: Minimising Stress for Patients in the Veterinary Hospital: Why It Is Important and What Can Be Done about It. Vet Sci. 4(2):22, 2017, doi: 10.3390/vetsci4020022.
2. Quimby, J.M., Smith, M.L. and Lunn, K.F.: Evaluation of the effects of hospital visit stress on physiologic parameters in the cat. J. Feline Med. Surg. 13(10):733-737, 2011.
3. Ansah, O.B., Raekallio, M. and Vainio, O.: Comparison of three doses of dexmedetomidine with medetomidine in cats following intramuscular administration. J. Vet. Pharmacol. Therapeutics. 21(5):380-387, 1998.
4. Granholm, M., McKusick, B.C., Westerholm, F.C. and Aspegrén, J.C.: Evaluation of the clinical efficacy and safety of dexmedetomidine or medetomidine in cats and their reversal with atipamezole. Vet. Anaesth. Analges. 33(4):214-23, 2006.
5. Lamont, L.A., Bulmer, B.J., Grimm, K.A., Tranquilli, W.J. and Sisson, D.D.: Cardiopulmonary evaluation of the use of medetomidine hydrochloride in cats. Amer. J. Vet. Res. 62(11):1745-1762, 2001.
6. Kanda, T. and Hikasa, Y.: Effects of medetomidine and midazolam alone or in combination on the metabolic and neurohormonal responses in healthy cats. Can. J. Vet. Res. 72(4):332-339, 2008.
7. Wiese, A.J. and Muir, W.W.: Anaesthetic and cardiopulmonary effects of intramuscular morphine, medetomidine and ketamine administered to telemetered cats. J. Feline Med. Surg. 9(2):150-156, 2007.
8. Whittem, T., Pasloske, K.S., Heit, M.C. and Ranasinghe, M.G.: The pharmacokinetics and pharmacodynamics of alfaxalone in cats after single and multiple intravenous administration of Alfaxan® at clinical and supraclinical doses. J. Vet. Pharm. Therap. 31(6):571-579, 2008.
9. Tamura, J., Ishizuka, T., Fukui, S. and Oyama, N.: Sedative effects of intramuscular alfaxalone administered to cats. J. Vet. Med. Sci. 77(8):897-904, 2015.
10. Rodrigo-Mocholí, D., Escudero, E., Belda, E., Laredo, F.G., Hernandis, V. and Marín, P.: Pharmacokinetics and effects of alfaxalone after intravenous and intramuscular administration to cats. New Zealand Vet. J. 66(4):172-177 2018.
11. Lazzarini, E., Martinelli, E., Brioschi, F.A., Gioeni, D., Corneliani, R.T. and Carotenuto, A.M.: Intramuscular alfaxalone and methadone with or without ketamine in healthy cats: effects on sedation and echocardiographic measurements. Vet. Anaesth. Analg. 47(5):621-630, 2020.
12. Grubb, T.L., Greene, S.A. and Perez, T.E.: Cardiovascular and respiratory effects, and quality of anesthesia produced by alfaxalone administered intramuscularly to cats sedated with dexmedetomidine and hydromorphone. J. Fel. Med. Surg. 15(10):858-865, 2013.
13. Rodrigo-Mocholí, D., Belda, E., Bosmans, T. and Laredo, F.G.: Clinical efficacy and cardiorespiratory effects of intramuscular administration of alfaxalone alone or in combination with dexmedetomidine in cats. Vet. Anaesth. Analg. 43(3):291-300, 2016.
14. Deutsch, J., Jolliffe, C., Archer, E. and Leece, E.A.: Intramuscular injection of alfaxalone in combination with butorphanol for sedation in cats. Vet. Anaesth. Analg.44(4):794-802, 2017.
15. Ramoo, S., Bradbury, L.A., Anderson, G.A. and Abraham, L.A.: Sedation of hyperthyroid cats with subcutaneous administration of a combination of alfaxalone and butorphanol. Australian Vet. J. 91(4):131-136, 2013.
16. Ribas, T., Bublot, I., Junot, S., Beaufrère, H., Rannou, B., Gagnière, P., Cadoré, J.L. and Pariaut, R.: Effects of intramuscular sedation with alfaxalone and butorphanol on echocardiographic measurements in healthy cats. J. Feline Med. Surg. 17(6):530-536, 2015.
17. Psatha, E., Alibhai, H.I., Jimenez-Lozano, A., Armitage-Chan, E. and Brodbelt, D.C.: Clinical efficacy and cardiorespiratory effects of alfaxalone, or diazepam/fentanyl for induction of anaesthesia in dogs that are a poor anaesthetic risk. Vet. Anaesth. Analg. 38(1):24-36, 2011.
18. Romagnoli, N., Zambelli, D., Cunto, M., Lambertini, C., Ventrella, D. and Baron Toaldo, M.: Non-invasive evaluation of the haemodynamic effects of high-dose medetomidine in healthy cats for semen collection. J. Feline Med. Surg.18(4):337-743, 2016.
19. Finck, C., Steagall, P. and Beauchamp, G.: Effects of Butorphanol With Alfaxalone or Dexmedetomidine on Feline Splenic Size and Appearance on Ultrasound and Computed Tomography. Front. Vet. Sci. 8:572146, 2021.
20. Pypendop, B.H., Barter, L.S., Pascoe, P.J., Ranasinghe, M.G. and Pasloske, K.: Hemodynamic effects of subclinical, clinical and supraclinical plasma alfaxalone concentrations in cats. Vet. Anaesth. Analg. 46(5):597-604, 2019.
21. Muir, W., Lerche, P., Wiese, A., Nelson, L., Pasloske, K. and Whittem, T.: The cardiorespiratory and anesthetic effects of clinical

and supraclinical doses of alfaxalone in cats. Vet. Anaesth. Analg. 36(1):42-54, 2009.
22. Cardoso, C.S., Jorge, E.B. and Santos, A.B.: Comparative study of the effects of medetomidine and xylazine in cats and reversal with atipamezole. Colloquium.;7(Suppl1):52-60, 2011.
23. Sinclair, M.D.: A review of the physiological effects of alpha2-agonists related to the clinical use of medetomidine in small animal practice. Can. Vet. J. 44(11):885-897, 2003.
24. Selmi, A.L., Mendes, G.M., Lins, B.T., Figueiredo, J.P. and Barbudo-Selmi, G.R.: Evaluation of the sedative and cardiorespiratory effects of dexmedetomidine, dexmedetomidine-butorphanol, and dexmedetomidine-ketamine in cats. JAVMA. 222(1):37-41, 2003.
25. Haskins, S.C.: Monitoring Anesthetized Patients. In: Grimm, K.A., Lamont, L.A., Tranquilli, W.J., Greene, S.A., Robertson, S.A. (eds). 5th Edition ed. Ames, Iowa: John Wiley & Sons, Inc. pp. 86-113, 2015.
26. Rankin, D.C.: Sedatives and tranquilizers. In: Grimm, K.A., Lamont, L.A., Tranquilli, W.J., Greene, S.A., Robertson, S.A. (eds). 5th Edit ed. Ames, Iowa: John Wiley & Sons, Inc. pp. 196-206, 2015.
27. Sundeman, H., Biber, B., Raner, C. and Winsö, O.: Autoregulation and vasodilator responses by isoflurane and desflurane in the feline renal vascular bed. Acta Physiol. Scand. 41(9):1180-1186. 1997.
28. Golden, A.L., Bright, J.M., Daniel, G.B. and Fefee, D.: Cardiovascular effects of the a2-adrenergic receptor agonist medetomidine in clinically normal cats anesthetized with isoflurane. Am. J. Vet. Res. 59:509-513, 1998.
29. Clark, S.E., Garret, M. and Platt, B.: Postnatal alterations of GABA receptor profiles in the rat superior colliculus. Neuroscience.104(2):441-454, 2001.
30. Rissman, R.A., Nocera, R., Fuller, L.M., Kordower, J.H. and Armstrong, D.M.: Age-related alterations in GABAA receptor subunits in the nonhuman primate hippocampus. Brain Research. 1073:120-130, 2006.
31. Seguin, M.A., Papich, M.G., Sigle, K.J., Gibson, N.M. and Levy, J.K.: Pharmacokinetics of enrofloxacin in neonatal kittens. Am. J. Vet. Res. 65(3):350-356, 2004.
32. Kukanich, B. and Wiese, Aj.: Opiods. In: Grimm, K.A., Lamont, L.A., Tranquilli, W.J., Greene, S.A., Robertson, S.A. (eds). 5th. Edit ed. Ames, Iowa: John Wiley & Sons, Inc. pp. 207-226, 2015.
33. Ilkiw, J.E., Pascoe, P.J. and Tripp, L.D.: Effects of morphine, butorphanol, buprenorphine, and U50488H on the minimum alveolar concentration of isoflurane in cats. Am. J. Vet. Res. 63(8):1198-1202, 2002.
34. Reader, R.C., Barton, B.A. and Abelson, A.L.: Comparison of two intramuscular sedation protocols on sedation, recovery and ease of venipuncture for cats undergoing blood donation. J. Feline Med. Surg.21(2):95-102, 2019.
35. Mathis, A., Pinelas, R., Brodbelt, D.C. and Alibhai, H.I.K.: Comparison of quality of recovery from anaesthesia in cats induced with propofol or alfaxalone. Vet. Anaesth. Analg. 39(3):282-290, 2012.
36. Pypendop, B.H., Siao, K.T., Ranasinghe, M.G. and Pasloske, K.: Effective plasma alfaxalone concentration to produce immobility in male neutered cats. Vet. Anaesth. Analg. 45(3):269-277, 2018.

Examination of Pathotypes, Phylogroups, and Antibiotic Resistance of *Escherichia coli* Isolates Obtained from Diarrheic Pet Dogs

Serbest, A.,[1,a] Turkyilmaz, M.K.[2,b] and S. Turkyilmaz.[3,c,*]

[1]Health Sciences Institute, Aydin Adnan Menderes University, Aydin, Türkiye.
[2]Departmant of Animal Science, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Aydin, Türkiye.
[3]Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, Türkiye.
[a]: 0009-0007-5516-6576, [b]: 0000-0002-7600-2390, [c]: 0000-0002-1363-4534

* **Corresponding author:** Prof. Dr. S. Turkyilmaz, Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Isikli, Aydin-Türkiye. E-mail: sturkyilmaz@adu.edu.tr

ABSTRACT

Intestinal pathogenic *Escherichia coli* (DEC) remains a significant zoonotic etiological agent causing diarrhea leading to fatalities in both humans and animals. This study aimed to assess the pathotypes, phylogroups, and antimicrobial resistance profiles of DEC isolates obtained from pet dogs and analyze their interrelationships. Two hundred *E. coli* isolates were collected from rectal swab samples of 40 diarrheic and 40 apparently clinically healthy (non-diarrheic) pet dogs puppies aged between 1-6 months, between January and June 2023. After *E. coli* isolation using classical conventional methods, their identification was performed phenotypically using the BD Phoenix 100 automatic microbiology system and confirmed genotypically using PCR. Pathotypes (EHEC, EPEC, ETEC, EIEC) and phylogenetic groups (A, B1, B2, C, D, E, F, Clade 1) of isolates were investigated via multiplex PCR. Resistance profiles to 19 antibiotics belonging to ten antimicrobial families were determined using the BD Phoenix 100 automated microbiology system with NMIC/ID 400 Gram negative identification cards. The relationship between the clinical status of the sampled dogs (diarrheic and healthy) and the antibiotic resistance profiles, multi-drug resistance (MDR), pathotypes, and phylogroups of *E. coli* isolates was analyzed using the Chi-square (χ^2) test. Pathotyping revealed that all *E. coli* isolates belonged to EHEC (47.2%), EPEC (34.5%), ETEC (12.8%), and EIEC (5.5%) pathotypes, while phylogenetic analysis indicated that the isolates were distributed among filogroups B2 and C (23.7%), D (20.0%), B1 (12.7%), E (9.0%), F (3.6%), and A (1.8%). Antibiotic susceptibility test results revealed that 78.2% of isolates exhibited MDR. Significant statistical associations were observed between the clinical status of dogs and resistance to ampicillin, amoxicillin-clavulanic acid, ciprofloxacin and tigecycline. However, no significant statistical relationship was found between multi-drug resistance profiles, pathotypes and phylogroups of the isolates. The presence of virulence genes specific to DEC pathotypes in canine isolates indicated that apparently healthy pet dogs could serve as a potential source of human infections, similar to diarrheic dogs. The presence of diverse phylogroups highlights the population diversity of *E. coli*, while the high prevalence of multi-drug resistant isolates underscores the necessity for careful antibiotic selection in the treatment and control of infections.

Keywords: Antibiogram; *Escherichia coli*; Phylogroup; Diarrhea; Dog; Pathotype.

INTRODUCTION

Escherichia coli, as a member of the *Enterobacteriaceae* family, is a significant part of the normal commensal biota of both humans and animals (1). However, certain *E. coli* strains are associated with numerous clinical diarrhea cases in both humans and animals (2). It has been documented that these

strains carry a wide range of virulence genes responsible for pathogenicity. In this context, seven different *E. coli* pathotypes have been identified: enteropathogenic (EPEC), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC), adherent invasive (AIEC) (1).

The EPEC, which commonly causes diarrhea in both humans and animals, contains the intimin (*eae*) gene and is classified into typical and atypical strains based on the presence of the *bfp*A gene located on a plasmid. Typical EPEC (tEPEC) strains are *eae*+ and *bfp*A+, while atypical EPEC (aEPEC) strains carry only the *eae*+ gene as they lack the plasmid (3). The EHEC pathotype, responsible for symptoms such as abdominal pain, bloody diarrhea and mild fever, produces two types of Shiga toxins, *stx*1 and *stx*2 (4). The pathogenicity of ETEC is determined by the production of heat-stable (*st*) and heat-labile (*lt*) enterotoxins (1). EIEC carries the invasive plasmid antigen H (*ipa*H) gene (5).

E. coli strains can be classified into different categories based on their phylogenetic characteristics and genetic backgrounds. It has been determined that *E. coli* strains belonging to different phylogenetic groups exhibit distinct phenotypic and genotypic characteristics (6). Clermont and colleagues (2013) divided *E. coli* isolates into eight phylogroups (A, B1, B2, C, D, E, F, and Clade I) by analyzing four genes (*arp*A, *chu*A, *yja*A, and *TspE4*.C2) (6). In recent years, a new phylogroup (G) has been reported between B2 and F phylogroups (7). Phylogroup A, which includes isolates with low virulence, is considered commensal, while phylogroup B1, commonly found in the intestinal flora, represents environmental strains (8). The more pathogenic phylogroups include B2 and D (9). The B2 phylogroup is highly virulent and poses a significant risk to human health. Phylogroup F, which typically contains enterohemorrhagic isolates, is the sister group of phylogroup B2. Phylogroup C, encompassing commensal groups with low virulence, is closely related to phylogroup B1 (6). Very little is known about the virulence of phylogroups C and F (6).

E. coli excreted in feces by domestic animals constitutes a significant source for the zoonotic transmission of pathogenic agents (10). Diarrheic animals, due to their frequent and uncontrolled defecation, contribute to a higher dissemination of *E. coli* compared to non-diarrheic animals. Pathogenic and non-pathogenic *E. coli* strains are considered potential reservoirs for antimicrobial resistance genes, and their presence in dog feces poses a serious threat to public health (2). This situation places individuals in direct contact with these animals, such as dog owners, caregivers, children and veterinarians, at a higher risk.

Pets hold a significant place in people's lives, and the number of individuals living with pets is steadily increasing. Therefore, the potential for pets to carry zoonotic diseases is an important public health concern. Phenotypic and genotypic markers of aEPEC isolated from diarrheic and non-diarrheic dogs have been reported to be similar to those found in isolates from human disease (11). Similarly, some dog aEPEC strains have been shown to share virulence genes commonly found in human pathogenic strains. Strains of the same serotype isolated from dogs and children share virulence genes and are phylogenetically closely related, indicating a potential zoonotic risk (12). In general, studies suggest that enteropathogenic *E. coli* isolates in dogs may have the potential to transmit to humans, and further research is needed to fully understand the potential risks (10,11,12).

In countries that do not have good regulations regarding antibiotic use, the arbitrary use of antimicrobial drugs in pets is a common problem. This practice leads to an increase in antimicrobial-resistant *E. coli* strains. Especially in dogs, treatment with antimicrobial agents such as β-lactams, fluoroquinolones and sulfonamides is becoming a significant public health problem (13).

As antimicrobial resistance continuously evolves, regular monitoring studies of resistance are crucial to guide treatment decisions and develop up-to-date control strategies. Overall, the variability in antibiotic resistance complicates the selection of antimicrobial agents and increases the need for culture and sensitivity testing. Additionally, while disease-causing microorganisms in pets tend to differ from those in humans, there is always potential for antibiotic resistance genes to transfer between humans and pets (14). It has been documented that multi-resistant *E. coli* strains are shared between dogs and their owners (15).

In Türkiye, there is no available information about the antimicrobial resistance profiles, pathotypes, and phylogroups of *E. coli* isolates obtained from clinical samples of pet animals. For this reason, this study aimed to evaluate the pathotypes, phylogroups, and antimicrobial resistance profiles of intestinal pathogenic *E. coli* isolates obtained from pet dogs, and analyze their interrelationships.

MATERIAL AND METHODS

Ethical Approval

This study was conducted with the approval of the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee, dated 18.05.2023, and numbered 64583101/2023/76.

Animal Material

The rectal swab samples required for *E. coli* isolation were obtained between January and June 2023 from materials brought to the Department of Microbiology at the Faculty of Veterinary Medicine, Aydın Adnan Menderes University, by clinics and from a private clinic. All the dogs included in the study were either sheltered or home-raised. Many of them were considered mixed bred and all dogs were puppies aged between 1-6 months. None of the dogs had received antibiotic treatment in the last two weeks, with 40 exhibiting diarrhea symptoms and another 40 serving as the control group, showing no signs of diarrhea. During the study, the medical history of each dog was documented and recorded. In addition, informed consent was obtained from the owners, indicating their permission for their pets to participate in the study.

Fecal samples were collected directly from the rectum by a veterinarian using a rectal swab. For this purpose, swabs were inserted approximately one centimeter byond the anal sphincter and rotated to obtain visible fecal material. Once it was ensured that a fecal sample had been collected, it was immediately placed in a semi-solid transport medium (Carry-Blair medium, Micropoint Diagnostics, USA) to prevent moisture loss. Information such as the animal's identification details, sampling date, etc., was recorded on the sample. The samples were stored in a refrigerator (4-8°C) until they were cultured, ensuring that the cultures were performed as soon as possible (within a maximum of 72 hours).

Bacterial Isolation

For the isolation of *E. coli*, differential and selective agar media including EMB and MacConkey agar were used. In aseptic conditions, rectal swab samples were streaked onto EMB agar. After incubation under aerobic conditions at 37°C for 18-24 hours, five colonies with a greenish metallic sheen on the EMB agar were selected and subcultured onto MacConkey agar. Subsequently, in order to increase the likelihood of detecting virulence genes, at least three colonies from each petri dish were taken from the lactose-fermenting pink colonies after incubation under aerobic conditions at 37°C for 18-24 hours. Colonies were passaged onto blood agar for purification purposes. Following that, Gram staining and standard biochemical tests (oxidase, catalase, indole) were performed. Isolates that exhibited Gram-negative rod morphology, lactose fermentation within one day, negative oxidase, and positive catalase and indole tests were considered as suspected *E. coli* isolates (16). Isolates were stored in BHIB containing 15% glycerol at -20°C until bacterial identification, antibiotic susceptibility testing, and molecular tests were conducted. Bacterial identification and antibiotic susceptibility testing of isolates were carried out using the BD Phoenix 100 automated microbiology system.

Reference Strains

For molecular studies, *E. coli* ATCC 35150 (EHEC; *stx*1, *stx*2, *eae*A), ATCC 35401 (ETEC; *lt*, *st*), ATCC 43893 (EIEC; *ial*) were used as positive controls, and *E. coli* ATCC 25922 strain was used as a negative control; for antibiotic susceptibility testing, *E. coli* ATCC 25922 strain was used as a quality control strain.

Antibiotic Susceptibility Tests

Antibiotic susceptibility tests of *E. coli* isolates were performed using the BD Phoenix 100 automated microbiology system (Becton-Dickinson, USA) with the NMIC/ID 400 Gram-negative identification card. The NMIC/ID 400 panel evaluates 19 antibiotics from ten antimicrobial families (Aminoglycosides: amikacin (AN), gentamicin (GM), netilmicin (NET); Carbapenems: ertapenem (ETP), imipenem (IMP), meropenem (MEM); Cephalosporins: cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP); Monobactam: aztreonam (ATM); Penicillin: ampicillin (AMP), piperacillin (PIP); β-Lactam: amoxicillin clavulanate (AXC), piperacillin tazobactam (TZP); Lipopeptide: colistin (COL); Folates: trimethoprim sulfamethoxazole (SXT); Quinolone: ciprofloxacin (CIP); Tetracycline: tigecycline (TGC)).

Multi-drug resistance (MDR) was defined as the condition where bacteria were resistant to at least one antibiotic in three or more antibiotic classes (17).

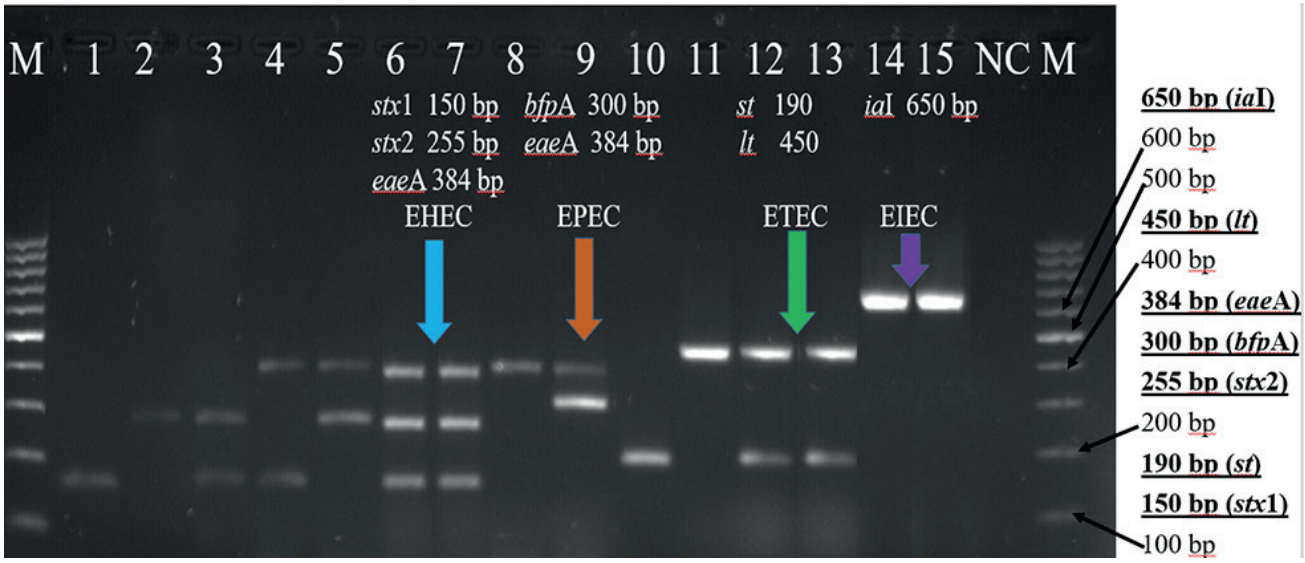


Figure 1. Agarose gel electrophoresis of virulence gene PCR products associated with the pathotype. **1.** *stx*1 (150 bp), **2.** *stx*2 (255 bp) **3.** *stx*1+ *stx*2 (150 bp+255 bp) **4.** *stx*1+*eae*A (150 bp+384 bp) **5.** *stx*2+ *eae*A 2 (255 bp+384 bp) **6**. *stx*1+*stx*2+*eae*A (150 bp+255 bp+384 bp) 7. Positive Control EHEC (*E. coli* ATCC 35150) 8. *eae*A (384 bp) 9. *bfp*A+*eae*A (300 bp+384 bp) **10.** *st* (190 bp) **11.** *lt* (450 bp) **12**. *st*+*lt* (190 bp+450 bp) **13.** Positive Control ETEC (*E. coli* ATCC 35401) **14**. *iaI* (650 bp) **15.** Positive Control EIEC (*E. coli* ATCC 43890) (*ial*) **NC:** Negative Control (master mix without DNA) **M:** 100 bp DNA Ladder (Fermentas).

Polymerase Chain Reaction (PCR)

DNA Extraction, Purity and Quantity Control: In this study, DNA extraction was performed using the sonication method (18). After DNA extractions were completed, their concentrations were checked for purity and quantity using a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). DNA with OD260/280 values between 1.6 and 2.0 were considered to have sufficient purity (19) and used at a volume of 3 µl as template DNA in each PCR reaction.

Primers: To identify the *E. coli* pathotypes causing diarrhea, the target genes *stx*1, *stx*2, *eae*A (EHEC) (20); *lt* and *st* (ETEC) (21); *eae*A and *bfp* (EPEC) (21); and ial (EIEC) (22) were examined. The phylogenetic distribution of *E. coli* isolates was determined using the quadruplex PCR method (6). This method targets the *chu*A, *yja*A, *TspE4*.C2, *arp*A, and *trp*A genes and classifies *E. coli* into eight phylogroups (A, B1, B2, C, D, E, F, clade I). The quadruplex PCR was performed using the primer sequences shown in Table 1 (6, 23, 24, 25).

PCRs were conducted in a volume of 25 µl. The final concentrations were adjusted as follows: 1x Taq enzyme buffer solution 1x, 25 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol of each primer 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, Massachusetts, USA), and 3µl of each DNA sample. After preparing the tubes, they were loaded into a thermal cycling device (Boeco, Hamburg, Germany).

Once the master mixes were prepared, the PCR tubes were labeled with the corresponding sample numbers, and 22 µl of master mix was added for each sample. Subsequently, 3 µl of the extracted DNA was added, the tube mouths were tightly closed, and then they were loaded into the thermal cycling devices and programmed. For DNA amplification, the device was set to perform initial denaturation at 95°C for 5 min: 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C (*stx*1, *stx*2, *eae*A, *bfp*A, *lt*, *st*, *iaI*) and 56°C (*chu*A, *yja*A, *TspE4*C2, *arp*A *trp*A) for 30 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 10 min.

Statistical Analysis

The statistical analysis of the obtained data was performed using SPSS (Statistical Package for Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) software package. The Pearson Chi-square (χ^2) test (Fisher's Exact χ^2 Test) was used to compare frequency data. The χ^2 test was used to examine the relationship between the clinical status of sampled dogs

Table 1. Primers used in the study.

	Primer	Target Gene	Sequence (5'-3')	Amplicon size (bp)	Tm
	EHEC	*stx*1	CTGGATTTAATGTCGCATAGTG AGAACGCCCACTGAGATCATC	150	58.0 61.0
	EHEC	*stx*2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	63.0 60.0
	ETEC	*lt*	GGCGACAGATTATACCGTG CGGTCTCTATATTCCCTGTT	450	60.0 56.0
	ETEC	*st*	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	190	51.0 60.0
	EPEC	*eae*	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	63.0 63.0
	EPEC	*bfp*A	GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTA GT	300	61.0 65.0
	EIEC	*ial*	GGTATGATGATGATGAGTCCA GGAGGCCAACAATTATTTCC	650	57.0 56.0
Quadruplex PCR	*chu*A.1b *chu*A.2	*chu*A	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	288	60.5 60.5
Quadruplex PCR	*yja*A.1b *yja*A.2b	*yja*A	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	211	58.4 58.4
Quadruplex PCR	*TspE*4C2.1b *TspE*4C2.2b	*TspE*4.C2	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	152	56.4 62.5
Quadruplex PCR	*Ace*K F *Arp*A1 R	*arp*A	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	400	60.5 60.5
Group E	*Arp*AgpE F *Arp*AgpE R	*arp*A	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	301	60.1 58.4
Group C	*trp*AgpC.1 *trp*AgpC.2	*trp*A	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCCC	219	58.4 68.1
Internal Control	*trp*BA.F *trp*BA.R	*trp*A	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	489	59.4 68.7

(diarrhea or healthy) and the resistance of *E. coli* isolates to antibiotics, multidrug resistance status, pathotypes, and phylogroups. Results were considered statistically significant when the difference between means was $p<0.05$, with a 95% confidence interval.

RESULTS

Bacterial Isolation and Identification

A total of 80 rectal swab samples were obtained from dogs in this study, comprising 40 clinical cases (with diarrhea) and 40 control samples (without diarrhea). Suspected *E. coli* colonies were obtained from 55 (68.7%; 55/80) dogs, showing metallic green sheen on EMB agar and lactose fermentation on MacConkey agar (29 diarrhea cases (72.5%; 29/40); 26 controls (65.0%; 26/40)). A total of 200 isolates were passaged, with 100 colonies from diarrhea and control group samples each. The identification of isolates was performed both phenotypically using the BD Phoenix 100 automated microbiology system and genotypically using PCR.

Isolates identified as *E. coli* were first determined for their pathotypes using PCR. The phylogroups of isolates with identified pathotypes were examined, and antibiotic susceptibility tests were conducted.

PCR

Pathotyping

To determine the presence of target virulence genes, all *E. coli* isolates were examined using PCR for pathotyping purposes. The results showed that 47.0% (47/100) of isolates from

Table 2. Pathotypes of *E. coli* isolates obtained from diarrheic and healthy dogs.

Virulence Gene/Pathotype	Clinical Status		Total (n=55) (%)
	Diarrhea (n=47) (%)	Healthy (n=8) (%)	
EHEC	24 (51.0)	2 (25.0)	26 (47.2)
***stx*1**	3 (6.4)	0 (0.0)	3 (5.5)
***stx*2**	5 (10.6)	1 (12.5)	6 (10.9)
***stx*1+*stx*2**	3 (6.4)	0 (0.0)	3 (5.5)
***stx*1+*eae*A**	5 (10.6)	1 (12.5)	6 (10.9)
***stx*2+*eae*A**	7 (14.5)	0 (0.0)	7 (12.7)
***stx*1+*stx*2+*eae*A**	1 (2.1)	0 (0.0)	1 (1.8)
EPEC	16 (34.0)	3 (37.5)	19 (34.5)
Atipik EPEC (*eae*A)	12 (25.5)	3 (37.5)	15 (27.3)
Tipik EPEC (*eae*A+*bfp*A)	4 (8.5)	0 (0.0)	4 (7.2)
ETEC	5 (10.6)	2 (25.0)	7 (12.8)
st	1 (2.1)	2 (25.0)	3 (5.5)
lt	3 (6.4)	0 (0.0)	3 (5.5)
st+lt	1 (2.1)	0 (0.0)	1 (1.8)
EIEC	2 (4.3)	1 (12.5)	3 (5.5)
iaI	2 (4.3)	1 (12.5)	3 (5.5)

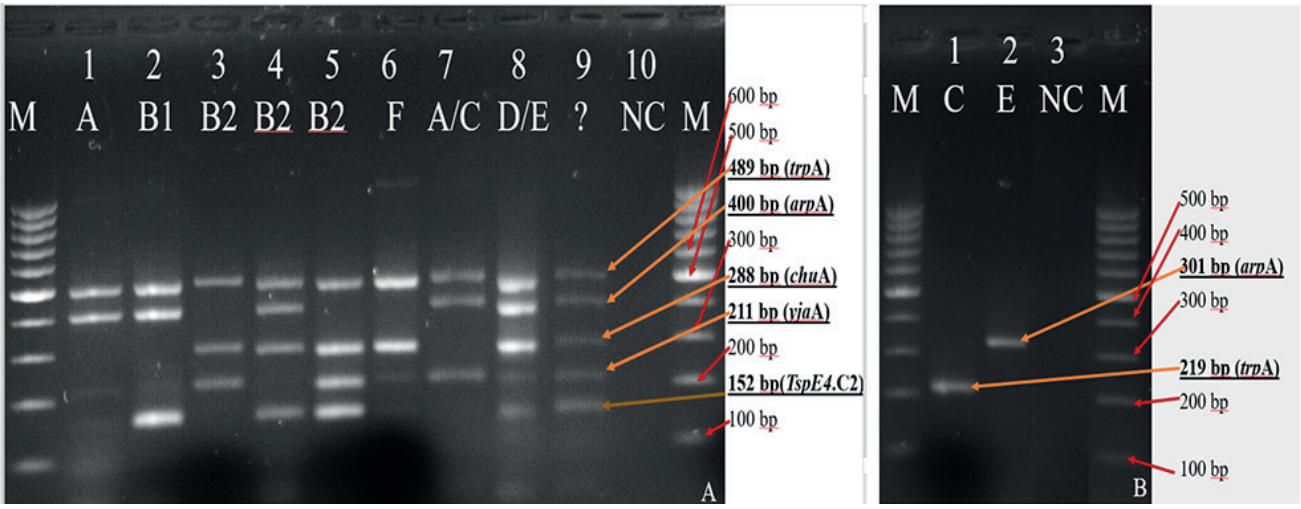


Figure 2. A. Quadruplex PCR profiles using the new Clermont phylogenetic method. **1.** Group A (– – – + +), **2.** Group B1 (+ – – + +), **3.** Group B2 (– + + – +), **4.** Group B2 (+ – + + +), **5.** Group B2 (+ + + – +), **6.** Group F (– – + – +), **7.** Group A/C (– + – + +), **8.** Group D/E (– – + + +), **9.** Unknown Group (+ + + + +) (152 bp, 211 bp, 288 bp, 400 bp, 489 bp), **NC:** DNA-free master mix, **M:** Marker (100 bp, Fermentas). **B. 1.** Group C (219 bp), **2.** Group E (301 bp), **NC:** DNA-free master mix, **M:** Marker (100 bp, Fermentas).

diarrheic dogs and 8.0% (8/100) of isolates from healthy dogs carried at least one gene associated with diarrhea. Out of 55 isolates, 60.0% (33/55) amplified only one gene (*stx*1, *stx*2, *eae*A, *st*, *lt*, *iaI*), while 40.0% (22/55) had combinations of virulence genes (*stx*1+*stx*2, *stx*1+*eae*A, *stx*2+*eae*A, *eae*A+*bfp*A, *st+lt*) (Table 2, Figure 2).

Among diarrheic dogs, 51.0% (24/47) were identified as EHEC, 34.0% (16/47) as EPEC, 10.6% (5/47) as ETEC, and 4.3% (2/47) as EIEC. In contrast, healthy dogs were identified as 25.0% (2/8) EHEC, 37.5% (3/8) EPEC, 25.0% (2/8) ETEC, and 12.5% (1/8) EIEC. Among all isolates, 47.2% (26/55) were classified as EHEC, 34.5%

Table 3. Phylogroups of *E. coli* isolates obtained from diarrheic and healthy dogs.

Phylogroup	Clinical Status		Total (n=55) (%)
	Diarrhea (n=47) (%)	Healthy (n=8) (%)	
A	0 (0.0)	1 (12.5)	1 (1.8)
B1	5 (10.6)	2 (25.0)	7 (12.7)
B2	13 (27.6)	0 (0.0)	13 (23.7)
C	10 (21.3)	3 (37.5)	13 (23.7)
D	10 (21.3)	1 (12.5)	11 (20.0)
E	4 (8.5)	1 (12.5)	5 (9.0)
F	2 (4.3)	0 (0.0)	2 (3.6)
?	3 (6.4)	0 (0.0)	3 (5.5)

?: The number of isolates for which the phylogroup could not be determined and required MLST.

Table 4. Virulence genes and phylotypes of *E. coli* isolates.

Virulence Gene/Genes	Clinical Status																Total (n=55)
	Diarrhea (n=47)								Healthy (n=8)								
	A	B1	B2	C	D	E	F	?	A	B1	B2	C	D	E	F	?	
*stx*1		1			2												3
*stx*2			2		3					1							6
*stx*1+*stx*2			3														3
*stx*1+*eae*A			2		3								1				6
*stx*2+*eae*A			5		2												7
*stx*1+*stx*2+*eae*A			1														1
Atipik EPEC (*eae*A)				6		2	2	2				3					15
Tipik EPEC (*eae*A+*bfp*A)				4													4
st		1							1	1							3
lt		2						1									3
st+*lt*		1															1
iaI						2								1			3
Total	0	5	13	10	10	4	2	3	1	2	0	3	1	1	0	0	55

(19/55) as EPEC, 12.8% (7/55) as ETEC, and 5.5% (3/55) as EIEC.

Phylotyping

For phylotyping purposes, the presence of target genes in *E. coli* isolates with known pathotypes were examined using PCR. Among isolates obtained from diarrheic dogs, 10.6% (5/47) were classified as phylogroup B1, 27.8% (13/47) as phylogroup B2, 21.3% (10/47) as phylogroup C and phylogroup D, 8.5% (4/47) as phylogroup E, and 4.3% (2/47) as phylogroup F. In contrast, isolates from healthy dogs were found to be 12.5% (1/8) phylogroup A, phylogroup D, and phylogroup E, 25.0% (2/8) phylogroup B, and 37.5% (3/8) phylogroup C. The phylogroup of 6.4% (3/47) of isolates obtained from diarrheic dogs could not be determined. Among all isolates, 1.8% (1/55) were phylogroup A, 12.7% (7/55) phylogroup B1, 23.7% (13/55) phylogroup B2 and phylogroup C, 20.0% (11/55) phylogroup D, 9.0% (5/55) phylogroup E, 3.6% (2/55) phylogroup F, while the phylogroup of 5.5% (3/55) of isolates could not be determined using the available primers (Table 3, Figure 2).

Table 5. Antimicrobial resistance of *E. coli* isolates.

Antimicrobial Family-Antibiotic Name	Clinical Status		Total (n=55) (%)
	Diarrhea (n=47) (%)	Healthy (n=8) (%)	
Aminoglycoside			
Amikacin	1 (2.1)	0 (0.0)	1 (1.8)
Gentamicin	25 (53.2)	5 (62.5)	30 (54.5)
Netilmicin	0 (0.0)	0 (0.0)	0 (0.0)
Carbapenem			
Ertapenem	6 (12.8)	0 (0.0)	6 (10.9)
Imipenem	7 (14.9)	0 (0.0)	7 (12.7)
Meropenem	1 (2.1)	0 (0.0)	1 (1.8)
Cephem			
Cefuroxime	30 (63.8)	3 (37.5)	33 (60.0)
Ceftazidime	26 (55.3)	2 (25.0)	28 (50.9)
Ceftriaxone	28 (59.6)	2 (25.0)	30 (54.5)
Cefepime	27 (57.4)	2 (25.0)	29 (52.7)
Monobactam			
Aztreonam	27 (57.4)	2 (25.0)	29 (52.7)
Penicillin			
Ampicillin	42 (89.4)	4 (50.0)	46 (83.6)
Piperacillin	36 (76.6)	4 (50.0)	40 (72.7)
Beta Lactam			
Amoxicillin Clavulanate	41 (87.2)	4 (50.0)	45 (81.8)
Piperacillin Tazobactam	20 (42.6)	1 (12.5)	21 (38.2)
Lipopeptide			
Colistin	4 (8.5)	0 (0.0)	4 (7.3)
Folate			
Trimethoprim Sulfamethoxazole	27 (57.4)	3 (37.5)	30 (54.4)
Quinolone			
Ciprofloxacin	25 (53.2)	1 (12.5)	26 (47.3)
Tetracycline			
Tigecycline	41 (87.2)	4 (50.0)	45 (81.8)

According to Clermont's phylogenetic method, the most prevalent phylogroup among diarrheic dogs was B2 (27.6%; 13/47), whereas in healthy dogs, the dominant phylogenetic group was C (37.5%; 3/8). In all isolates, phylogroups B2 and C (23.7%; 13/55) were identified (Table 3).

Virulence genes and phylotypes of *E. coli* isolates obtained from diarrheal and healthy dogs are shown in Table 4.

Antimicrobial Resistance

In this study, the antibiotic resistance profiles of a total of 55 *E. coli* isolates, comprising 47 obtained from 40 dogs with diarrhea and 8 from 40 healthy dogs, were investigated for 19 antibiotics belonging to ten antimicrobial families using an automated system (Table 5).

E. coli isolates were resistant to some antibiotics at low levels (1.8%-12.7%) (amikacin, imipenem, ertapenem, meropenem, colistin), at moderate levels to some antibiotics (38.2%, 47.3%) (pipercillin-tazobactam, ciprofloxacin), and at high levels to some antibiotics (50.9%-83.6%) (gentamicin, cefuroxime, ceftazidime, ceftriaxone, cefepime, aztreonam, ampicillin, pipercillin, amoxicillin-clavulanate, trimethoprim-

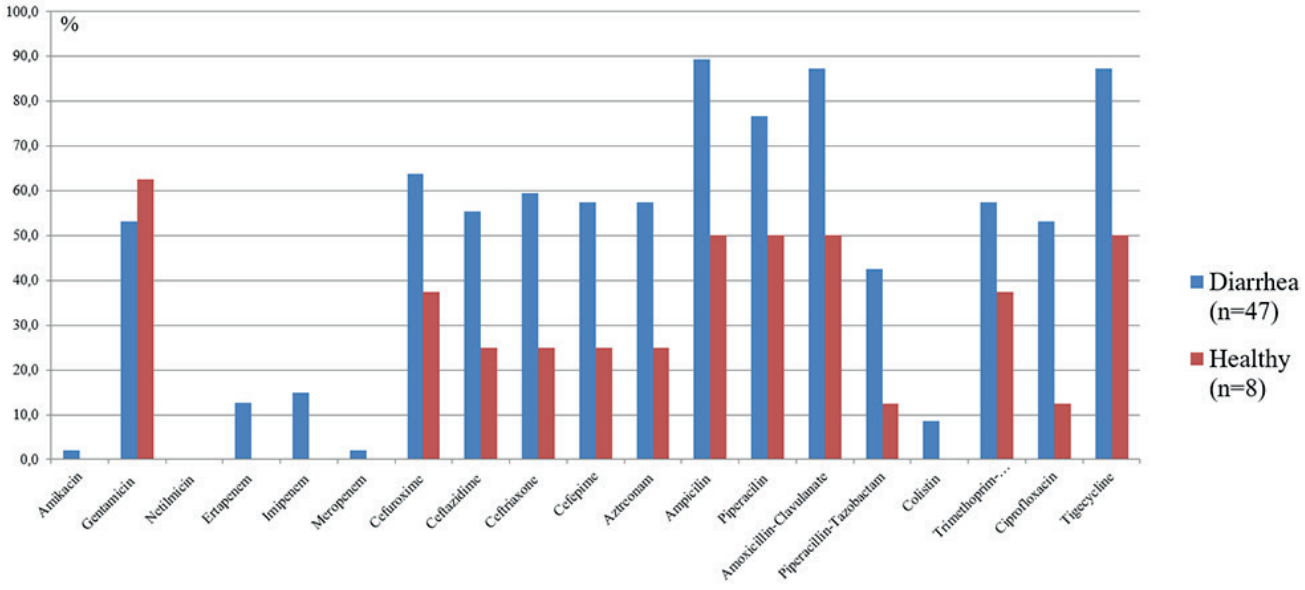


Figure 3. Antibiotic resistance profiles of *E. coli* isolates obtained from diarrheic and healthy dogs.

sulfamethoxazole, tigecycline). All isolates were susceptible to netilmicin (Table 5, Figure 3).

Multiple Antibiotic Resistance

Among the *E. coli* isolates obtained from diarrheic dogs, 83.0% (39/47), 50.0% (4/8) from apparently healthy dogs, and 78.2% (43/55) of all isolates were found to be MDR. The resistance rates of all isolates to 10, 9, 8, 7, 6, 5, 4, 3, 2, and 1 antimicrobial families were determined as follows: 5.4% (3/55), 12.7% (7/55), 21.8% (12/55), 3.6% (2/55), 5.4% (3/55), 7.3% (4/55), 1.8% (1/55), 20.0% (11/55), 14.5% (9/55), and 5.4% (3/55), respectively (Table 6, Figure 4).

Statistical Analysis

The relationship between the clinical condition of the sampled dogs and the resistance status of *E. coli* isolates to antibiotics is presented in Table 7.

The significant statistical relationship was detected between the clinical status of the dogs and resistance to ampicillin (p=0.019), amoxicillin clavulanate (p=0.029), ciprofloxacin (p=0.054), and tigecycline (p=0.019) (Table 7) ($p<0.05$).

The relationship between the clinical status of the dogs and the MDR status of *E. coli* isolates is shown in Table 8.

A significant statistical relationship between the clinical status of the dogs and their multi-antibiotic resistance status could not be detected (Table 8).

The relationship between the clinical status of the dogs and the pathotypes and phylotypes of *E. coli* isolates is shown in Table 9 and Table 10.

A significant statistical relationship between the clinical status of the dogs and the phylotypes and pathotypes of *E. coli* isolates could not be detected.

DISCUSSION

Pets are an important part of our lives and frequently come into close contact with humans. Through this close contact, pet animals can transmit *E. coli* pathotypes that can cause diarrhea in humans (26). Therefore, the health of our pets is of critical importance not only for their own well-being but also for public health. In this study, we determined the frequency of virulence factors associated with DEC pathotypes in *E. coli* isolates obtained from diarrheic and apparently healthy dogs. Subsequently, we conducted phylotyping studies to better understand the genetic origins and characteristics of the isolates. Finally, we attempted to determine the resistance status of our isolates to commonly used antibiotics in both human and veterinary medicine after identifying their pathotypes and phylotypes. In this context, by examining DEC pathotypes, phylogroups, and antibiotic resistance profiles originating

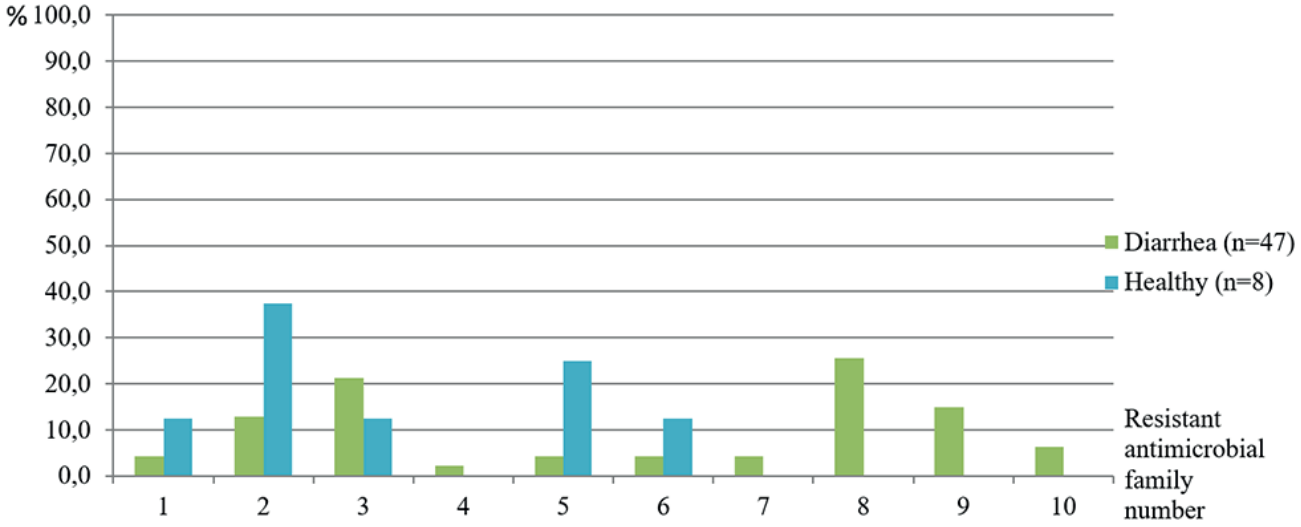


Figure 4. Distribution of MDR status of *E. coli* isolates obtained from diarrheic and healthy dogs.

Table 6. Multiple antibiotic resistance profiles of *E. coli* isolates obtained from diarrheic and healthy dogs.

Number of Resistant Antimicrobial Families	Clinical Status		Total (n=55) (%)	MDR
	Diarrhea (n=47) (%)	Healthy (n=8) (%)		
1	2 (4.3)	1 (12.5)	3 (5.4)	12 (21.8)
2	6 (12.8)	3 (37.5)	9 (14.5)	
3	10 (21.3)	1 (20.0)	11 (20.0)	43 (78.2)
4	1 (2.1)	0 (0.0)	1 (1.8)	
5	2 (4.3)	2 (20.0)	4 (7.3)	
6	2 (4.3)	1 (12.5)	3 (5.4)	
7	2 (4.3)	0 (0.0)	2 (3.6)	
8	12 (25.5)	0 (0.0)	12 (21.8)	
9	7 (14.9)	0 (0.0)	7 (12.7)	
10	3 (6.4)	0 (0.0)	3 (5.4)	

from dogs, we aimed to better understand potential risks for both our dogs and human health.

The prevalence of *E. coli* in dogs with diarrhea varies according to studies. In recent studies, the rate of *E. coli* isolation from dogs showing diarrhea symptoms was reported as 67.7% in Egypt (27) and 73.5% in Nigeria (28). In this study, the isolation rate of *E. coli* was similarly high (72.5%) as in other studies. The sampled dogs were puppies aged 1 to 6 months. This is consistent with studies that have reported a high incidence of *E. coli* isolation in young diarrheic dogs (27). Differences in isolation rates in various studies could be attributed to a combination of factors such as sample selection, isolation methods used, the ages of sampled animals, geographical factors and population variations.

Antimicrobial resistance in bacteria has been continuously evolving since the discovery of antibiotics. There are several factors contributing to bacterial resistance, including the prophylactic or incorrect use of antibiotics and the transmission of resistant bacteria from animals to humans (or vice versa) (29). However, taking into account local antibiotic usage, animal populations, and environmental factors, different resistance patterns can be observed in various regions and at different times. This variability in resistance patterns is one of the primary reasons for the emergence of differing antibiotic resistance profiles, particularly in studies conducted in different countries, and even within different regions of the same country. Therefore, our results are in line with findings from studies conducted in Egypt regard-

Table 7. Relationship between clinical status and resistance status of isolates to antibiotics.

Antibiotic	Clinical Status		χ2	P
	Diarrhea (n=47) (%)	Healthy (n=47) (%)		
Amikacin, Meropenem R+	1	0	0.170	1
Amikasin. Meropenem R-	46	8		
Gentamicin R+	25	5	0.235	0.715
Gentamicin R-	22	3		
Ertapenem R+	6	0	1.125	0.577
Ertapenem R-	41	8		
Imipenem R+	7	0	1.340	0.577
Imipenem R-	40	8		
Cefuroxime R+	30	3	1.939	0.244
Cefuroxime R-	17	5		
Ceftazidime R+	26	2	2.469	0.143
Ceftazidime R-	21	6		
Ceftriaxone R+	28	2	3.236	0.123
Ceftriaxone R-	19	6		
Cefepim. Aztreonam R+	27	2	2.835	0.131
Cefepim. Aztreonam R-	20	6		
Ampicillin R+	42	4	7.599	0.019*
Ampicillin R-	5	4		
Piperacillin R+	36	4	2.394	0.193
Piperacillin R-	11	4		
Amoxicillin Clavulanate, Tigecycline R+	41	4	6.255	0.029*
Amoxicillin Clavulanate, Tigecycline R-	6	4		
Piperacillin Tazobactam R+	20	1	2.568	0.136
Piperacillin Tazobactam R-	27	7		
Colistin R+	4	0	0.721	1
Colistin R-	43	8		
Trimethoprim Sulfamethoxazole R+	27	3	1.077	0.446
Trimethoprim Sulfamethoxazole R-	20	5		
Ciprofloxacin R+	25	1	4.459	0.054*
Ciprofloxacin R-	22	7		

* Degree of statistical significance.

ing high susceptibility to amikacin (26,27). However, there are proportional variations in resistance patterns to other antibiotics compared to the results of studies conducted in Brazil and Egypt (13,26,27).

In our study, antimicrobial susceptibility test results have shown that isolates obtained from both healthy and diarrheic dogs were resistant to β-lactams, aminoglycosides, tetracyclines and folate inhibitors. This finding is consistent with the results of many studies worldwide on antimicrobial resistance in bacteria isolated from dogs (26,27,28,30).

E. coli isolates obtained from dogs exhibited high levels of resistance to gentamicin, cefuroxime, ceftazidime, ceftriaxone, cefepime, aztreonam, ampicillin, piperacillin, amoxicillin-clavulanate, trimethoprim-sulfamethoxazole and tigecycline. Ceftazidime, ceftriaxone, aztreonam, tigecycline, and piperacillin are important drugs used in the treat-

ment of various infections in humans. The development of resistance to these antibiotics in dogs is significant in terms of the risk of spreading infections between dogs and humans.

Similarly, *E. coli* isolates exhibited low resistance (1.8%-12.0%) to imipenem, ertapenem, meropenem and colistin, which are used in human medicine, while they showed intermediate resistance (38.2%) to piperacillin-tazobactam. The fact that these antibiotics are not used in veterinary medicine may have reduced the likelihood of resistance developing in bacteria treated with these drugs in veterinary practice. However, resistance to these drugs can limit important treatment options for serious infections in humans. Especially carbapenem antibiotics like meropenem are used as a last resort in humans against highly resistant bacteria. The presence of bacteria resistant to these antibiotics in pet dogs can have implications for public health, as pet owners may come into contact with these bacteria, which have the potential to be transmitted to humans.

In the study, despite tigecycline not having been used in the sampled dogs, a high level of resistance (81.8%) was observed. There could be two possible reasons for this phenomenon. Firstly, these bacteria may have acquired the tigecycline resistance gene from the environment they came into contact with or from another source. Secondly, changes in ribosomal targets or transport systems in tetracycline-resistant bacteria may trigger tigecycline resistance, even though tigecycline has never been used. This is because tetracycline was commonly used where the samples were collected. Tetracycline and tigecycline share the same mechanism of action, binding to the same ribosomal targets to inhibit bacterial protein synthesis. Therefore, the use of tetracycline antibiotics may have contributed to the development of tigecycline resistance (31). Resistance genes can be transferred through plasmids or other genetic elements. The presence and transfer of resistance genes can contribute to the spread of resistance to different antibiotics (31).

One of the noteworthy findings of our study was that the isolates developed resistance to colistin, albeit at low levels (7.3%). Colistin resistance in *E. coli* is reported to result from mutations in chromosomal genes or the presence of plasmid-borne *mcr* genes (32). To the best of our knowledge, colistin resistance in *E. coli* isolates from diarrheic dogs in Türkiye has not been reported. Colistin is considered a last-resort antibiotic for the treatment of severe infections. Therefore, colistin resistance should be a serious concern for both veterinary and human health.

Similarly, we observed resistance to carbapenem group antibiotics, which are important for the treatment of infections in human medicine, albeit at low levels (1.8%-12.7%). The development of low-level resistance to carbapenems may be attributed to horizontal gene transfer between bacteria. This occurrence could be a result of bacteria adapting to environmental conditions. Additionally, in areas where antibiotic usage is prevalent, the likelihood of bacteria developing carbapenem resistance in the environment may be higher.

In the study, a significant statistical relationship was identified between the clinical condition of dogs and resistance to ampicillin, amoxicillin-clavulanate, ciprofloxacin, and tigecycline (Table 7). This result suggests that the likelihood of developing resistance to antibiotics is associated with the diarrheal condition of dogs. This finding may have several important clinical implications, such as limitations in the treatment options for cases of diarrhea in dogs that involve the use of certain antibiotics. Over time, antibiotics like ampicillin, amoxicillin-clavulanate, and ciprofloxacin may become ineffective. This could mean that veterinarians may need to reevaluate their treatment choices. When selecting appropriate antibiotics for the treatment of dogs, veterinarians should consider these resistance profiles. Moreover, the antibiotic resistance acquired by *E. coli* isolates is a concern not only for animal health but also for human health. Antibiotic resistance observed in dogs can be transmitted to humans through contact, consumption of contaminated food, and other means. Therefore, the proper disposal of dog feces in a hygienic manner and the elimination of the risk of contamination in the home environment are important for individuals in contact with dogs.

In the study, while the MDR rates of our isolates were not as high as those reported in studies conducted in Egypt (26) and Nigeria (28), they were still at a considerable level (78.2%). The high prevalence of multi-drug resistance in diarrheic dogs can complicate their treatment because the options for effective antibiotics against bacteria with multiple resistance are limited. In addition, no significant statistical relationship was found between the clinical condition of dogs and their multi-drug resistance profiles (Table 8). The causes of diarrhea can be quite diverse and may not only be related

Table 8. The relationship between the clinical status and the multi-antibiotic resistance.

Antibiotic Resistance Phenotype	Clinical Status		χ2	P
	Diarrhea (n=47) (%)	Healthy (n=8) (%)		
MDR+	39	4	4.280	0.059
MDR-	8	4		

Table 9. Relationship between the clinical status and the pathotypes of isolates.

Pathotypes	Clinical Status		χ2	P
	Diarrhea (n=47) (%)	Healthy (n=47) (%)		
EHEC+	24	2	1.829	0.257
EHEC-	23	6		
EPEC+	16	3	0.035	1
EPEC-	31	5		
ETEC+	5	2	1.246	0.267
ETEC-	42	6		
EIEC+	2	1	0.885	0.382
EIEC-	45	7		

to bacterial infections but also to viruses, parasites, dietary changes and other factors. These factors can influence the formation and spread of resistant strains. The antibiotic usage habits in the environments where both diarrheic and healthy-looking dogs live may be similar. Therefore, there may not have been a significant difference observed in the likelihood of acquiring multi-drug resistance between diarrheic and healthy dogs.

Enteric *E. coli* pathotypes, which are a significant concern for public health and food safety, exhibit a high diversity of pathogenic mechanisms and virulence factors. In this study, EHEC was the most frequently detected pathovar, comprising 47.2% of the isolates. This pathovar was isolated from 51.0% of diarrheic dogs and 25.0% of healthy dogs. The prevalence of EHEC seemed to be lower in samples obtained from apparently healthy animals. In studies conducted on healthy pet dogs, the prevalence of EHEC was reported to be 4.08% in Iran (33) and 5.9% in Turkey (34); whereas in diarrheic dogs, it was reported to be 2.7% in Brazil (35). Overall, these studies indicate that the prevalence of EHEC in diarrheic dogs can vary by region and may also be present in some healthy dogs. The higher prevalence of EHEC found in our study could be attributed to regional differences. The region where our study was conducted may have a different pattern of EHEC spread compared to other studies. Additionally, the laboratory methods used, population of dogs examined may differ from other studies.

In the study, EPEC was the second most frequently detected pathovar. EPEC is characterized by the production of intimin and can be classified as typical or atypical based on its presence or absence (1). Similar to studies conducted in Brazil (10,35), we found that atypical EPEC had a higher distribution than typical EPEC in our study. Our results are consistent with other studies, demonstrating that EPEC is an important diarrheagenic pathotype in dogs (10,35).

In the study, ETEC was the third most commonly detected pathovar, with a prevalence of 10.6% in clinical cases and 25.0% in dogs without diarrhea. The prevalence of ETEC in diarrheic dogs can vary according to different studies. Sancak *et al.* (2004) found that ETEC was more common in dogs with acute and chronic diarrhea compared to healthy dogs in households and kennels (34). In a study conducted in Iran, the prevalence of ETEC in healthy dogs was reported to be 2.1% (33).

Among our isolates, the lowest detected pathovar was EIEC, which was found in both diarrheic (4.3%) and non-diarrheic dogs (12.5%). In previous studies, EIEC was reported to be absent in both healthy and diarrheic dogs in

Table 10. Relationship between the clinical status and the phylotypes of istolates.

Phylotypes	Clinical Status		χ^2	P
	Diarrhea (n=47)(%)	Healthy (n=8)(%)		
A+	0	1	5.875	0.145
A-	47	7		
B1+	5	2	1.246	0.267
B1-	42	6		
B2+	13	0	2.845	0.176
B2-	34	8		
C+	10	3	0.979	0.376
C-	37	5		
D+	10	1	0.323	1
D-	37	7		
E+	4	1	0.129	0.559
E-	43	7		
F+	2	0	0.347	1
F-	45	8		

Mexico (13), in diarrheic dogs in Brazil (10), while it was detected in healthy dogs in Iran (6.1%) (33).

In this study, the prevalence of EPEC, ETEC, and EIEC was higher in healthy dogs (37.5%, 25.0%, 12.5%) compared to diarrheic dogs (37.5%, 10.6%, 4.3%). This suggests that healthy dogs may harbor virulence genes as carriers but may not exhibit symptoms. The EPEC/ETEC/EIEC strains present in healthy dogs may be less pathogenic than those in diarrheic dogs, which could explain the absence of symptoms. Alternatively, the healthy dogs sampled in this study may have had stronger immune systems, which could have prevented the development of diarrhea symptoms. Considering that these differences may be influenced by multiple factors, further research with a larger sample size is needed to elucidate the exact reasons for these variations.

In this study, no significant statistical relationship was found between the clinical status of dogs and the *E. coli* pathotypes isolated (Table 9). *E. coli* can be categorized into different pathotypes, each having the potential to cause different types of infections. However, while these pathotypes can sometimes be associated with different clinical conditions, there may also be instances where different pathotypes are found in the same clinical condition (1). Therefore, the relationship between clinical status and pathotype can be complex. This complexity can make it challenging to detect a statistical relationship.

The Clermont phylotyping method was developed to classify *E. coli* isolates into phylogroups based on their genetic backgrounds (6). The original method assigned isolates to one of four phylogroups (A, B1, B2, or D) (36), but an updated method now recognizes eight phylogroups (A, B1, B2, C, D, E, F, and clade I). The updated method has been shown to correctly assign over 95% of *E. coli* isolates to a phylogroup (6). In the *E. coli* phylogroup analysis developed by Clermont and colleagues, commensal groups are typically referred to as A and B1 phylogroups. These phylogroups encompass strains of *E. coli* that are commensal or naturally found in the intestines of humans and animals. Virulent groups, on the other hand, include B2 and D phylogroups. There is not much information available about the pathogenicity of other phylogroups. Phylogroups are used to classify *E. coli* strains based on their genetic similarities and can help interpret different characteristics and infection potential of the bacterium. However, these phylogroups are just a classification method and do not alone provide a complete picture of pathogenicity (6).

It has been documented that the distribution of phylogenetic groups among dog isolates varies concerning extraintestinal, commensal and clinical samples. When it comes to extraintestinal isolates, B2 and D groups would be expected to dominate, although Gibson *et al.* (2010) reported no isolates belonging to the B2 group (37). However, Maynard *et al.*

(2004) reported an 88.0% frequency of the B2 phylogenetic group in extraintestinal samples (38). In a study by Harada *et al.* (2012), a high prevalence of the B2 group was observed in fecal strains of dogs (39). Davis *et al.* (2011) also observed the dominance of the B2 and D groups in strains isolated from various anatomical regions, including the rectal area, in healthy dogs (40).

In this study, phylogenetic group analysis revealed that commensal phylotypes A and B1 comprised 37.5% (3/8) of the *E. coli* isolates obtained from healthy dogs and 10.6% (5/47) of isolates from diarrheic dogs, while virulent B2 and D phylogroups constituted 12.5% (1/8) of isolates from healthy dogs and 48.9% (23/47) of isolates from diarrheic dogs. In a study conducted in Mexico, when *E. coli* isolates were analyzed based on phylogenetic characterization, commensal phylogroups A and B1 were found to account for 57.0% in healthy dogs and virulent phylogroups B2 and D for 43.0%, while in diarrheic dogs, commensal phylogroups were 31.0%, and virulent phylogroups were 69.0% (13). These findings seemingly indicate that healthy dogs can be colonized by both commensal and virulent strains. Similar to the study conducted in Mexico, this study also suggests that domestic dogs could serve as a reservoir for virulent phylogroups and potentially transmit these pathogens to their owners and individuals with indirect contact (12).

There were no significant statistical differences observed between the clinical status of the sampled dogs and the *E. coli* isolates' phylotypes (Table 10). *E. coli* can be divided into eight phylogroups, each with distinct characteristics. While these phylogroups can sometimes be associated with different clinical conditions, there can also be different phylogroups within the same clinical condition. Therefore, the relationship between clinical status and phylogroup can be complex. Diarrhea can have various causes, including bacterial, viral, parasitic, dietary changes, or other factors. These factors can influence clinical status, making it challenging to establish a clear relationship with phylogroups. Similarly, the sample sizes can affect the results of statistical analysis. The much larger size of the diarrhea group may have led to an imbalance in the analysis, making it more challenging to establish a statistically significant relationship. The results of this study indicate that not only diarrheal dogs but also seemingly healthy pet dogs can carry multidrug-resistant, pathogenic *E. coli* pathotypes and phylogroups that can potentially be transmitted to humans. Additionally, the presence of various phylogroups obtained from both diarrheal and healthy dogs may demonstrate the diversity within the *E. coli* population. This suggests that *E. coli* can naturally possess different phylogroups or adapt to different environmental conditions. The resistance of *E. coli* isolates obtained from dogs to antibiotics commonly used in human medicine (imipenem, ertapenem, meropenem, colistin, piperacillin-tazobactam, tigecycline) can pose a significant public health concern by limiting treatment options. It is crucial for dog owners and veterinarians to adhere to hygiene measures and use antibiotics based on antibiotic susceptibility test results. This is necessary to protect both the health of dogs and to avoid endangering human health.

ACKNOWLEDGEMENTS

This article is an abstract of the first author's master's thesis and was supported by Aydın Adnan Menderes University Scientific Research Projects Unit (ADU BAP Project Number: VTF-23037).

CONFLICT OF INTERESTS STATEMENT

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript. This original work and is not under review at any other publication.

REFERENCES

1. Nataro, J. P. and Kaper, J.B.: Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11:142-201, 1998.
2. Majowicz, S.E., Scallan, E., Jones-Bitton, A., Sargeant, J.M., Stapleton, J., Angulo, F.J., Yeung, D.H. and Kirk, M.D.: Global incidence of human shiga toxin–producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. Foodborne Pathog. Dis. 11:447-455, 2014.
3. Trabulsi, L.R., Keller, R. and Tardelli, T.A.: Typical and atypical enteropathogenic *Escherichia coli*. Emerg. Infect. Dis. 8:508-13, 2002.
4. Singh, P., Sha, Q., Lacher, D.W., Del Valle, J., Mosci, R.E., Moore, J.A., Scribner, K.T. and Manning, S.D.: Characterization of enteropathogenic and shiga toxin-producing *Escherichia coli* in cattle and deer in a shared agroecosystem. Front. Cell. Infect. Microbiol. 5:1-13, 2015.
5. Sunabe, T. and Honma, Y.: Relationship between O-serogroup and presence of pathogenic factor genes in *Escherichia coli*. Microbiol. Immunol. 42:845-49, 1998.
6. Clermont, O., Christenson, J.K., Denamur, E. and Gordon, D.M.: The Clermont *Escherichia coli* phylo-typing method revisited:

improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5:58-65, 2013.
7. Clermont, O, Dixit, O.V.A., Vangchhia, B., Condamine, B., Dion, S., Bridier-Nahmias, A., Denamur, E. and Gordon, D.: Characterization and rapid identification of phylogroup G in *Escherichia coli*, a lineage with high virulence and antibiotic resistance potential. Environ. Microbiol. 21:3107-3117, 2019.
8. Walk, S.T., Alm, E.W., Calhoun, L.M., Mladonicky, J.M. and Whittam, T.S.: Genetic Diversity and population structure of *Escherichia coli* isolated from freshwater beaches. Environ. Microbiol. 9:2274-2288, 2007.
9. Carlos, C., Pires, M.M., Stoppe, N.C., Hachich, E.M., Sato, M.I.Z., Gomes, T.A.T., Amaral, L.A. and Ottoboni, L.M.M.: *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. BMC Microbiol. 10:161, 2010.
10. Puño-Sarmiento, J., Medeiros, L., Chiconi, C., Martins, F., Pelayo, J., Rocha, S., Blanco, J., Blanco, M., Zanutto, M., Kobayashi, R. and Nakazato, G.: Detection of diarrheagenic *Escherichia coli* strains isolated from dogs and cats in Brazil. Vet. Microbiol. 166:676-680, 2013.
11. de Almeida, P.M., Arais, L.R., Andrade, J.R.C., Prado, E.H.R.B., Irino, K. and Cerqueira, A.D.: Characterization of atypical enteropathogenic *Escherichia coli* (aEPEC) isolated from dogs. Vet. Microbiol. 158:420-424. 2012.
12. Arais, L.R., Barbosa, A.V., Andrade, J.R.C., Gomes, T.A.T., Asensi, M.D., Aires, C.A.M. and Cerqueira, A.M.F.: Zoonotic potential of atypical enteropathogenic *Escherichia coli* (aEPEC) isolated from puppies with diarrhoea in Brazil. Vet. Microbiol. 227:45-51, 2018.
13. Vega-Manriquez, X. D., Ubiarco-Lopez, A., Verdugo-Rodriguez, A., Hernandez-Chinas, U., Navarro-Ocana, A., Ahumada-Cota, R. E., Ramirez-Badillo, D., de Leon, H.D. and Eslava, C.A.: Pet dogs potential transmitters of pathogenic *Escherichia coli* with resistance to antimicrobials. Arch. Microbiol. 202:1173-1179, 2020.
14. Cao, H., Bougouffa, S., Park, T.-J., Lau, A., Tong, M.-K., Chow, K.-H. and Ho, P.L.: Sharing of antimicrobial resistance genes between humans and food animals. MSystems. 20;7(6):e0077522, 2022.
15. Carvalho, A.C., Barbosab, A.V., Arais, L.R., Ribeiro, P.F., Carneiro, V.C. and Cerqueira, A.M.F.: Resistance patterns, ESBL genes, and genetic relatedness of *Escherichia coli* from dogs and owners. Braz. J. Microbiol. 47: 150-158, 2016.
16. Koneman, E.W., Allen, S.D., Janda, W.M. and Schreckenberger, P.C.: Color: Atlas and Textbook of Diagnostic Microbiology. 5th ed. Philadelphia, Lippincott, USA, 1997.
17. Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y, Falagas, M.E., Giske, C.G., Harbart, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T. and Monnet, D.L.: Multidrug-resistant, extensively drug resistant and pandrug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18:268-281, 2012.
18. Maniatis, T. and Sambrook, J.: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, USA. 1989.
19. Turner, P.C., Mclennan, A.G., Bates, A.D. and White, M.R.H.: Molecular Biology Important Notes. Nobel Publications. 2004.
20. Paton, A.W. and Paton, J.C.: Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx*1, *stx*2, *eae*A, enterohemorrhagic *E. coli hly*A, *rfb*0111, and *rfb*0157. J. Clin. Microbiol. 36:598-602, 1998.
21. Stacy-Phipps, S., Mecca, J.J. and Weiss, J.B.: Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during the course of infection. J. Clin. Microbiol. 33:1054-1059, 1995.
22. Lopez-Saucedo, C., Cema, J.F., Villegas-Sepulveda, N., Thompson, R., Velazquez, F.R., Torres, J., Tarr, P. and Estrada-Garcia, T.: Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. Emerg. Infect. Dis. 9:127-131, 2003.
23. Clermont, O., Bonacorsi, S. and Bingen, E.: Characterization of an anonymous molecular marker strongly linked to *Escherichia coli* strains causing neonatal meningitis. *J. Clin. Microbiol.* 42:1770-1772, 2004.
24. Clermont, O., Lescat, M., O'Brien, C.L., Gordon, D.M., Tenaillon, O. and Denamur, E.: Evidence for a human specific *Escherichia coli* clone. Environ. Microbiol. 10:1000-10006, 2008.
25. Lescat, M., Clermont, O., Woerther, P.L., Glodt, J., Dion, S., Skurnik, D., Djossou, F., Dupont, C., Perroz, G., Picard, B., Catzeflis, F., Andremont, A. and Denamur, E.: Commensal *Escherichia coli* strains in Guiana reveal a high genetic diversity with host dependant population structure. Environ. Microbiol. Rep. 5:49-57, 2013.
26. Younis, K., Baddour, M. and Ibrahim, M.S.: Detection of diarrheagenic *Escherichia coli* in pet animals and its antibiotic resistance in Alexandria Governorate. A.J.V.S. 45:113-118, 2015.
27. Zarea, Z. Z., EL-Demerdash, G.O., El Shafei, A.A. and Elkader, S.A.: Occurrence of *Escherichia coli* as a causative agent of enteritis in dogs with special reference to their multidrug resistance and virulence genes. J. Anim. Health Prod. 9:7-13, 2020.
28. Mustapha, M., Audu, Y., Ezema, K.U., Abdulkadir, J.U., Lawal1, R.R., Balami, A.G., Adamu, L. and Bukar-Kolo, Y.M.: Antımıcrobıal susceptibility profiles of *Escherichia coli* isolates from diarrheic dogs in Maiduguri, Borno State, Nigeria. Maced. Vet. Rev. 44: 47-53, 2021.
29. Grave, K. and Tanem, H.: Compliance with short-termoral antibacterial drug treatment in dogs. J. Small Anim. Pract. 40:158-162, 2006.
30. Karahutová, L., Mandelík, R. and Bujnáková, D.: Antibiotic resistant and biofilm-associated *Escherichia coli* isolates from diarrheic and healthy dogs. Microorganisms. 9: 1334, 2021.
31. Yaghoubi, S., Zekiy, A.O., Krutova, M., Gholami, M., Kouhsari, E., Sholeh, M., Ghafouri, Z. and Maleki, F.: Tigecycline antibacterial activity, clinical effectiveness, and mechanisms and epidemiology of resistance: narrative review. Eur. J. Clin. Microbiol. Infect. Dis. 41: 1003-1022, 2022.
32. Li, F., Cheng, P., Li, X., Liu, R., Liu, H. and Zhang, X.: Molecular epidemiology and colistin-resistant mechanism of *mcr*-positive

and *mcr*-negative *Escherichia coli* isolated from animal in Sichuan Province, China. Front. Microbiol. 13:818548, 2022.
33. Askari, A., Ghanbarpour, R., Akhtardanesh, B., Aflatoonian, M.R., Sharifi, H., Jajarmi, M. and Molaei, R.: Detection of zoonotic diarrheagenic pathotypes of *Escherichia coli* in healthy household dogs. Iran. J. Microbiol. 12: 522-530, 2020.
34. Sancak, A., Rutgers, H., Hart, C. and Batt, R.: Prevalence of enteropathic *Escherichia coli* in dogs with acute and chronic diarrhoea. Vet. Rec. 24;154:101-106, 2004.
35. Coura, F.M., Diniz, A.N., Junior, C.A.O., Lage, A.P., Lobato, F.C.F., Heinemann, M.B. and Silva, R.O.S.: Detection of virulence genes and the phylogenetic groups of *Escherichia coli* isolated from dogs in Brazil. Ciênc. Rural. 48 (02): e20170478, 2018.
36. Clermont, O., Bonacorsi, S. and Bingen, E.: Rapid and simple determination of *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. 66: 4555-4558, 2000.
37. Gibson, J.S., Cobbold, R.N. and Trott, D.J.: Characterization of multidrug-resistant *Escherichia coli* isolated from extraintestinal clinical infections in animals. J. Med. Microbiol. 59 (Pt 5):592-598, 2010.
38. Maynard, C., Bekal, S., Sanschagrin, F., Levesque, R.C., Brousseau, R., Masson, L., Larivière, S. and Harel, J.: Heterogeneity among virulence and antimicrobial profiles of extraintestinal *E. coli* isolates of animal and human origin. J. Clin. Microbiol. 42:5444-5452, 2004.
39. Harada, K., Okada, E., Shimizu, T., Kataoka, Y., Sawada, T. and Takahashi, T.: Antimicrobial resistance, virulence profiles, and phylogenetic groups of fecal *Escherichia coli* isolates: acomparative analysis between dogs and their owners in Japan. Comp. Immunol. Microbiol. Infect. Dis. 35:139-144, 2012.
40. Davis, J.A., Jackson, C.R., Fedorka-Cray, P.J., Barrlett, J.B., Brousse, J.H., Gustafson, J. and Kucher, M.: Anatomical distribution and genetic relatedness of antimicrobial-resistant *Escherichia coli* from healthy companion animals. J. Appl. Microbiol. 110:597-604.26, 2011.

Investigation of Biofilm Formation, Virulence Genes and Antibiotic Resistance of *Acinetobacter baumannii* Isolates Obtained from Clinical Samples

Cihan, E.[1,a] **and Turkyilmaz, S.**[2,b,*]

[1] Health Sciences Institute, Aydin Adnan Menderes University, Aydin, Türkiye.
[2] Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, Türkiye.
ORCID[a]: 0000-0002-6216-3177, ORCID[b]: 0000-0002-1363-4534

* **Corresponding author:** Prof. Dr. S. Turkyilmaz, Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Isikli, Aydin-Türkiye. E-mail: sturkyilmaz@adu.edu.tr

ABSTRACT

The ability of *Acinetobacter baumannii* to form biofilms and its multiple antibiotic resistance may be responsible for the bacterium's survival in various environments. This study was aimed to investigate the biofilm-forming ability, antibiotic resistance phenotypes, frequency of genes associated with biofilm genes (*bap*, *omp*A, *aba*I, *csu*E, *bfm*S), and integron genes (*int*1, *int*2) in *A. baumannii* isolates obtained from human and bovine clinical samples. A total of 30 *A. baumannii* isolates were used, including 25 from human blood samples and 5 from mastitis-infected bovine milk samples. After conventional isolation methods, identification and antibiotic susceptibility tests were performed using an automated microbiology system (BD Phoenix™ 100, USA). Phenotypic biofilm formation was quantitatively assessed using the microplate test, and virulence genes associated with biofilm and integron genes were examined using polymerase chain reaction. Pearson's Chi-square ($\chi 2$) test was used to compare the study data. The highest resistance rate among all isolates was observed against ampicillin, followed by ertapenem and cefepime. Ninety three percent of the isolates exhibited multidrug resistance (MDR), and all could form biofilms (60% strong, 27% moderate, 13% weak). All *A. baumannii* isolates carried at least one gene associated with biofilm formation. The most commonly observed virulence gene associated with biofilm was *bfm*S, followed by *csu*E, *bap*, *omp*A and *aba*I. Integron genes were detected in 90% of the isolates (23 from humans, 4 from bovines). Statistical analysis revealed no significant relationship between the origin of isolates and the severity of biofilm formation. However, significant associations were found between the origin of isolates and the presence of *aba*I and *bfm*S virulence genes, as well as between strong biofilm formation and carrying all virulence genes or integron genes. Analyses of isolates obtained from human and bovine clinical samples indicate that *A. baumannii*'s biofilm formation capacity and resistance to antibiotics pose significant health threats. Particularly, the significant associations between strong biofilm formation and carrying all virulence genes or integron genes highlight the bacterium's complex adaptation strategies. In conclusion, this study demonstrates the comparative analysis of antimicrobial resistance profiles, biofilm formation abilities, and virulence gene carriage of *A. baumannii* isolates, emphasizing its importance as a threat to both human and animal health.

Key words: *Acinetobacter baumannii*; Biofilm; Multiple Antibiotic Resistance.

INTRODUCTION

Acinetobacter baumannii is a member of the *Moraxellaceae* family and is described as a nonmotile, catalase-positive, oxidase-negative, aerobic Gram-negative coccobacil, which is an opportunistic nosocomial pathogen (1). *A. baumannii* can lead to meningitis, urinary tract, skin, and soft tissue

infections in humans (2), with the highest mortality rates observed in ventilator-associated pneumonia and bloodstream infections (3). In the veterinary medicine, there is limited information about *A. baumannii* compared to human medicine (4, 5). *A. baumannii* has been isolated from mastitis, pneumonia, and sepsis in cattle; wound infections, sepsis, bronchopneumonia, neonatal encephalopathy, and eye infections in horses; wound, bloodstream, and urinary tract infections in dogs and cats (4, 5).

In recent years, *A. baumannii* has become an increasingly significant pathogen in both humans and animals due to its escalating antibiotic resistance and ability to form biofilms, making it a common cause of healthcare-associated infections due to its capability to persist and survive on surfaces (6). The virulence factors of *A. baumannii* consist of a series of genes that contribute to its significance as a pathogen in both humans and animals. Some of these genes play crucial roles in functions such as biofilm formation and antimicrobial resistance (7).

Biofilm is a structure where a microbial community attaches to a relevant surface via an extracellular matrix (8). Biofilm formation entails a complex regulatory system that involves bacterial adhesion ability, development of the biofilm, attachment of matrix components, mobility, synthesis, and coordination of relevant gene expression (9). The *bap* (biofilm-associated protein), *ompA* (outer membrane protein A), *csu*E (chaperon-usher pilus), *aba*I (gene for quorum sensing components), and *bfm*RS (two component system) genes have been evaluated as the most significant virulence genes associated with biofilm formation in *Acinetobacter* spp. (9,10). The *bap* protein, associated with biofilm, is a large protein found on the cell surface. Encoded by the *bap* gene, the *bap* protein serves as a surface adhesin involved in intracellular adhesion in mature biofilm and biofilm biomass volume and is homologous to *Staphylococcus* protein. Additionally, it has been found in other bacterial genera typically associated with hospital-acquired infections such as *Enterococcus* spp. and *Pseudomonas* spp. The importance of the bap gene in forming mature biofilm on both biotic and abiotic surfaces has been established (9). *A. baumannii* produces autoinducing signal molecules via the *abaI* gene, which is involved in quorum sensing. These molecules contribute to the bacterium's perception of environmental conditions and particularly to the regulation of biofilm formation. Moreover, they can regulate interactions between *A. baumannii* and other pathogens, potentially increasing the severity of infections (9). *csu*E is part of the CsuA/BABCD chaperone complex critical for *A. baumannii*'s ability to form biofilm. The *csu*E gene encodes part of the csu pilus assembly system and acts as an adhesin attaching to the surface at the onset of biofilm formation. The absence of *csu*E can reduce the bacterium's ability to form biofilm, potentially increasing the risk of infection (9, 11). *omp*A, an outer membrane porin, plays various roles in *A. baumannii*, including antimicrobial resistance, adherence to epithelial cells, and biofilm formation. The *omp*A gene strengthens the bacterial cell membrane, enhancing its resistance to the host and potentially increasing its ability to cause infection (12). The *bfm*S gene aids the bacterium in forming biofilm on inanimate surfaces such as polystyrene. Activation of *bfm*S allows the bacterium to adapt to environmental conditions and enhance its ability to cause infection (9).

In the last decade, *A. baumannii* isolates have become a significant clinical concern due to their ability to develop resistance to all known antibiotics (6). Particularly, the association of isolates showing multidrug resistance (MDR) with nosocomial and community-acquired infections has led the Infectious Diseases Society of America to include *A. baumannii* in the ESKAPE pathogens list alongside *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Enterobacter* spp. (13,14). In recent years, MDR *A. baumannii* has been frequently isolated in infections with high morbidity and mortality rates, often failing to respond to conventional treatments (e.g., penicillins, cephalosporins, carbapenems, and monobactams), primarily due to the acquisition of various resistance genes, especially against β-lactam antibiotics (15).

Integrons are mobile DNA elements that can disseminate multidrug resistance, particularly in Gram-negative pathogens (16). The basic structure of integrons consists of conserved segments containing antibiotic resistance gene cassettes that can be added to or excised from the site-specific recombination catalyzed by integrase (17). To date, five classes of integrons have been identified based on the sequence of the *int* gene; class 1 and class 2 integrons are the most commonly identified integron classes in clinical isolates of *A. baumannii* (16, 17).

Acinetobacter spp. are biofilm producers capable of acquiring and transferring resistance genes, thereby enhancing their antibiotic resistance abilities (18). Studies have shown that

clinical isolates may form more effective biofilms compared to environmental isolates, and there is a significant correlation between biofilm production and multidrug resistance (19,20). To the best of our knowledge, there is currently no study in Türkiye that evaluates both biofilm formation and antibiotic resistance profiles of human and animal-derived *A. baumannii* isolates together. This study aimed to investigate the biofilm-forming ability, antibiotic resistance phenotypes, and the frequency of the most important genes associated with biofilm (*aba*I, *csu*E, *bap*, *omp*A, *bfm*S) and integron genes (*int*1, *int*2) in *A. baumannii* isolates obtained from human (blood culture) and animal (mastitic bovine milk) clinical samples.

MATERIAL AND METHODS

Ethical Approval

The study was conducted with the ethical approval obtained from the Non-Interventional Research Ethics Committee of Aydın Adnan Menderes University, Health Sciences Institute, dated February 12, 2021 (Protocol No: 2021/005).

Material

In the scope of this study, human blood samples were collected from 105 hospitalized patients over a three-month period (May 2021 – July 2021) at Aydın Adnan Menderes University Faculty of Medicine Hospital. Additionally, as animal material, 225 subclinical mastitis milk samples, routinely submitted to the diagnostic laboratory of Aydın Adnan Menderes University Faculty of Veterinary Medicine for various studies, were examined.

Isotion and identification

After homogenizing the samples, one milliliter of Brain Heart Infusion Broth (Merc 110493, Germany) was inoculated. The BHIB cultures were then incubated at 37°C for 18-24 hours. From these cultures, a loopful of broth culture was streaked onto 5% sheep blood agar (Merc 1.10886, Germany) and MacConkey agar (Merck 100205, Germany). The streaked plates were incubated aerobically at 37°C for 18-24 hours. Colonies showing Gram-negative coccobacilli appearance underwent biochemical tests (catalase, citrate, motility, oxidation-fermentation test, hemolysis, gas production, oxidase, H_2S, indole, urea, glucose, and sucrose fermentation) (21). For the identification of suspected *Acinetobacter* spp. colonies, an automated microbiology system (BD Phoenix 100™, USA) was used.

Antibiotic susceptibility test

Antibiotic susceptibility test of the isolates was performed using the NMIC/ID 433 panel on the automated microbiology system (BD Phoenix 100™). A comprehensive panel consisting of 20 different antibiotics from nine antimicrobial classes was utilized for the test, including aminoglycosides such as amikacin, gentamicin; carbapenems such as ertapenem, imipenem, meropenem; cephalosporins such as cefazolin, cefuroxime, ceftazidime, ceftriaxone, cefepime; beta-lactams such as ampicillin, ceftolozane-tazobactam, amoxicillin-clavulanate; lipopeptides such as colistin; folates such as trimethoprim-sulfamethoxazole; quinolones such as ciprofloxacin, levofloxacin; tetracyclines such as tigecycline. The resistance profiles of the isolates were determined, and interpretation was performed according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (22). *E. coli* ATCC 25922 was used as a quality control strain. Additionally, *A. baumannii* isolates were classified as MDR when resistant to at least three different antibiotic classes from distinct categories (23).

Biofilm formation

The microplate method was used to quantitatively determine the amount of biofilm production (24). *A. baumannii* isolates were incubated overnight at 37°C in tryptic soy broth (TSB) (Merc 10459, Germany) containing 0.25% glucose. After removal of free cells, the biofilm was washed three times with sterile phosphate-buffered saline (PBS) and then fixed with 99% (v/v) methanol. The biofilm in the wells was stained with 1% (w/v) crystal violet at room temperature for 20 minutes. Subsequently, the crystal violet solutions were dissolved in 33% (v/v) ethanol/acetone (80, 20, v/v), and absorbance values were measured at 595 ηm. The optical density cutoff (ODc) was calculated as three times the sum of the average optical density and standard deviation of the negative control.

Classification of strains was performed according to the following criteria: non-biofilm producer (NB) (OD≤ODc), weak biofilm producer (W) (ODc<OD≤2xODc), moderate biofilm producer (M) (2xODc<OD≤4xODc), and strong biofilm producer (S) (4xODc<OD). The test was repeated three times for each sample, and the average optical density was calculated. *Staphylococcus aureus* 25923 was used as the

Table 1. Primers used in this study.

	Target gene	Primer Sequences (5'-3')	T_m	Amplicon size	Reference
Virulence genes associated with biofilm	*abaI*	CGCTACAGGGTATTTGTTGA TCGTAATGAGTTGTTTTGCG	56.4 54.3	370	11
	csuE	AGACATGAGTAGCTTTACG CTTCCCCATCGGTCATTC	53.0 56.1	516	
	bap	ATAACTCGGCTGTTTACGG ACTGATGGTGTTGGAAGTG	55.2 55.2	358	
	ompA	CTGGTGTTGGTGCTTTCTGG GTGTGACCTTCGATACGTGC	60.5 60.5	352	
	*bfm*S	TTGCTCGAACTTCCAATTTATTATAC TTATGCAGGTGCTTTTTTATTGGTC	60.0 60.9	1428	26
Integron gens	*Int*1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	57.2 57.2	280	27
	*Int*2	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	51.6 57.3	233	28

T_m: Melting temperature.

positive control, and Müller-Hinton broth (MHB) (Merc 110293, Germany) was used as the negative control.

DNA extraction

Genomic DNA was obtained using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific™ Biotechnology, Seongnam-Si, Korea, K0721). The purity and quantity of DNA were assessed using nanodrop device (MaestroNano Micro-Volume Spectrophotometer, Malaysia MN-913). DNA purity was determined to be within the range of OD260/280 values of 1.6 to 2.0 (25). For each PCR reaction, 3 µl of template DNA was used.

Polymerase Chain Reaction (PCR)

The presence of genes associated with biofilm (*aba*I, *csu*E, *bap*, *omp*A, *bfm*S) and integron genes (*int*1, *int*2) was examined using PCR as previously described (Table 1). PCR reactions were performed in a volume of 25 µl. The final concentrations for each PCR reaction were adjusted as follows: 1x for 10x Taq enzyme buffer solution, 2 mM for 25 mM $MgCl_2$, 0.2 mM for 10 mM dNTP, 0.4 µmol for 100 ρmol of each primer, 1.5 U for 5 U of Taq DNA polymerase (Fermentas, Massachusetts, USA), and 3 µl of DNA from each sample. Prepared reaction mixtures were loaded into a thermal cycling device (Boeco, Hamburg, Germany).

For DNA amplification, the device used a program consisting of an initial denaturation at 95˚C for 5 minutes; followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 53˚C (*aba*I, *csu*E, *bap*), 56˚C (*int*1, *int*2), and 58˚C (*omp*A, *bfm*S) for 30 seconds, extension at 72˚C for 60 seconds, and a final extension at 72˚C for 10 minutes.

A 2% agarose gel was used for electrophoresis, and the gel was subjected to an electric current of 100 volts for 60 minutes. After electrophoresis, the gel was placed in a transilluminator device (Vilbert Lourmat, Collegien, France) under UV light and photographs were taken. Amplification products were considered to contain the respective gene when they produced a band of the expected size (Table 1).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the obtained data. Pearson's Chi-square ($\chi 2$) test (Fisher's Exact $\chi 2$ Test) was used to compare frequency data. The following relationships were examined using the $\chi 2$ test: **a.** The relationship between the origin of isolates and the severity of biofilm formation **b.** The relationship between the origin of isolates and the frequency of virulence genes associated with biofilm **c.** The relationship between the strength of biofilm formation and the frequency of virulence genes associated with biofilm **d.** The relationship between the strength of biofilm formation and the frequency of integron genes. The results were evaluated at a 95% confidence

Table 2. Antimicrobial resistance rates of *A. baumannii* isolates.

Antimicrobial Family-Antibiotic Name	Human Blood (n=25), R (%)	Mastitic Bovine Milk (n=5), R (%)	All Isolates (n=30), R (%)
Aminoglycoside			
Amikasin	13 (52)	1 (20)	14 (47)
Gentamicin	17 (68)	2 (40)	19 (63)
Carbapenem			
Ertapenem	25 (100)	0 (0)	25 (83)
Imipenem	16 (64)	0 (0)	16 (53)
Meropenem	17 (68)	0 (0)	17 (57)
Cephem			
Cefazolin	15 (60)	0 (0)	15 (50)
Cefuroxime	14 (56)	0 (0)	14 (47)
Ceftazidime	22 (88)	0 (0)	22 (73)
Ceftriaxone	19 (76)	0 (0)	19 (63)
Cefepim	23 (92)	0 (0)	23 (77)
Penicillin			
Ampicillin	25 (100)	3 (60)	28 (93)
β-Lactam			
CeftolozaneTazobactam	14 (56)	0 (0)	14 (47)
Amoxicillin Clavulanate	19 (76)	4 (80)	23 (77)
Ampicillin Sulbactam	16 (64)	2 (40)	18 (60)
Piperacillin Tazobactam	11 (44)	0 (0)	11 (37)
Lipopeptide			
Colistin	3 (12)	0 (0)	3 (10)
Folate			
Trimethoprim Sulfamethoxazole	16 (64)	3 (60)	19 (63)
Quinolone			
Ciprofloxacin	17 (68)	3 (60)	20 (67)
Levofloxacin	18 (72)	0 (0)	18 (60)
Tetracycline			
Tigecycline	3 (12)	0 (0)	3 (10)

interval, and differences with $p<0.05$ between means were considered statistically significant.

RESULTS

Isolation and identification

In this study, a total of 37 *Acinetobacter* spp. suspected isolates were obtained, including 25 (24%) from 105 human blood samples and 12 (5%) from 225 subclinical mastitis bovine milk samples. On blood agar, the colonies were non-hemolytic, while on MacConkey agar, they typically appeared as light lavender color and displayed a Gram-negative coccobacilli morphology. They were catalase, citrate, glucose fermentation positive; negative for oxidase, indole, urease, hydrogen sulfide production; non-motile, non-hemolytic, and gas non-producing, thus considered as suspected *Acinetobacter* spp. isolates.

Using the automated microbiology system (BD Phoenix 100™), all 25 (100%, 25/25) isolates from human clinical blood samples and 5 (42%, 5/12) out of 12 mastitis bovine milk isolates were identified as *A. baumannii*. Thus, a total of 30 *A. baumannii* identifications were made, consisting of 25 (24%, 25/105) from human clinical samples and 5 (2%, 5/225) from bovine clinical samples.

Antimicrobial susceptibility test

Antimicrobial susceptibility test revealed that among all isolates, the highest resistance rate was observed against ampicillin (93%, 28/30), followed by ertapenem (83%, 25/30), and cefepime (77%, 23/30). All human isolates (100%, 25/25)

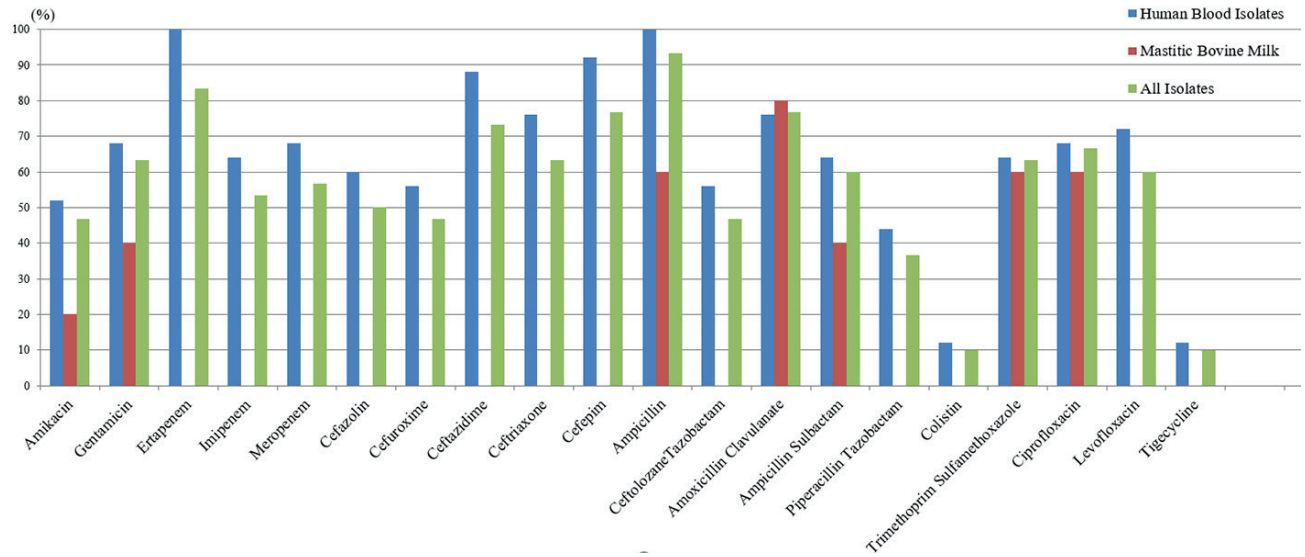


Figure 1. Antimicrobial resistance situations in *A. baumanii*.

Table 3. Antimicrobial resistance phenotypes of the isolates.

Antimicrobial Family	Antibiotic Resistance Fenotypes	Ratio (%), Isolate (n=30)
2*	β-laktam, Folat	3 (1)
2*	β-laktam, Quinolone	3 (1)
3*	Penisilin, β-laktam, Folat	3 (1)
3	Carbapenem, Penisilin, β-laktam	3 (1)
4*	Aminoglikozid, Penisilin, β-laktam, Quinolone	3 (1)
5*	Aminoglikozid, Penisilin, β-laktam, Quinolone, Folat	3 (1)
5	Carbapenem, Cephem, Penisilin, β-laktam, Folat	3 (1)
5	Aminoglikozid, Carbapenem, Cephem, Penisilin, Quinolone	3 (1)
6	Carbapenem, Cephem, Penisilin, β-laktam, Folat, Quinolone	7 (2)
6	Carbapenem, Cephem, Penisilin, β-laktam, Lipopeptid, Quinolone	7 (2)
6	Aminoglikozid, Carbapenem, Cephem, Penisilin, β-laktam, Quinolone	13 (4)
6	Aminoglikozid, Carbapenem, Cephem, Penisilin, β-laktam, Folat	7 (2)
7	Aminoglikozid, Carbapenem, Cephem, Penisilin, β-laktam, Folat, Quinolone	27 (8)
7	Aminoglikozid, Carbapenem, Cephem, Penisilin, β-laktam, Lipopeptid, Folat	3 (1)
7	Carbapenem, Cephem, Penisilin, β-laktam, Folat, Quinolone, Tetrasiklin	3 (1)
8	Aminoglikozid, Carbapenem, Cephem, Penisilin, β-laktam, Folat, Quinolone, Tetrasiklin	3 (1)
8	Aminoglikozid, Carbapenem, Cephem, Penisilin, β-laktam, Lipopeptid, Quinolone, Tetrasiklin	3 (1)

*: Resistance patterns of bovine isolates.

were resistant to ampicillin and ertapenem, while all bovine isolates were susceptible to carbapenems, cephalosporins, lipopeptides, and tetracyclines (Table 2). High (50%-100%) resistance was detected against 70% of the tested antibiotics, moderate (49%-20%) resistance against 20%, and low (19%-1%) resistance against 10% (Table 2, Figure 1).

A total of 17 different antibiotic resistance patterns were identified, with the most common pattern observed in humans being "aminoglycoside, carbapenem, cephalosporin, penicillin, β-lactam, folate, quinolone" resistance pattern, while in bovines, five different resistance patterns were observed for five isolates (β-lactam), Folate), (β-lactam,

Table 4. Number of antimicrobial families to which isolates were resistant.

Isolate origin	Isolate (%)	Number of Antimicrobial Families to Resistance	NMDR/MDR status of isolates
Human (n=25)	1 (4)	3	100% MDR
	2 (8)	5	
	10 (40)	6	
	10 (40)	7	
	2 (8)	8	
Bovine (n=5)	2 (40)	2	40% NMDR
	1 (20)	3	60% MDR
	1 (20)	4	
	1 (20)	5	
Total (n=30)	2 (7)	2	7% NMDR
	2 (7)	3	93% MDR
	1 (3)	4	
	3 (10)	5	
	10 (33)	6	
	10 (33)	7	
	2 (7)	8	

Table 5. Phenotypic capacities of isolates for biofilm formation.

	Weak (%)	Moderate (%)	Strong (%)
Human (n=25)	3 (12)	5 (20)	17 (68)
Bovine (n=5)	1 (20)	3 (60)	1 (20)
Total (n=30)	4 (13)	8 (27)	18 (60)

quinolone), (penicillin, β-lactam), Folate, (aminoglycoside, penicillin, β-lactam, quinolone), (aminoglycoside, penicillin, β-lactam, quinolone, folate)) (Table 3).

Multiple antibiotic resistance

Ninety-three percent (28/30) of all *A. baumannii* isolates were resistant to three or more antimicrobial agents and were considered MDR. Two bovine isolates (40%, 2/5) were resistant to two antimicrobial families (non-MDR, (NMDR)), while all human isolates (100%, 25/25) were MDR (Table 4).

Biofilm formation

All *A. baumannii* isolates have the ability to produce biofilm. Sixty percent of these isolates (18 isolates: 17 human, 1 bovine) demonstrated a strong biofilm-forming ability, while 27% (8 isolates: 5 human, 3 bovine) formed biofilms at a moderate level, and 13% (4 isolates: 3 human, 1 bovine) exhibited a weak biofilm-forming capability (Table 5, Figure 2). These findings indicate that *A. baumannii* possesses varying levels of biofilm-forming ability, observed in both human and bovine isolates. However, there is a higher tendency for strong biofilm formation in human isolates and moderate biofilm formation in bovine isolates.

Biofilm-related virulence genes

All *A. baumannii* isolates harbored at least one virulence gene associated with biofilm formation. The most commonly observed virulence gene related to biofilm formation was *bfm*S (87%), followed by *csu*E (83%), *bap* (73%), *omp*A (70%), and *aba*I (30%). The *aba*I gene was detected at the lowest rate (20%) in human isolates, while its presence in bovine isolates (80%) was higher. Interestingly, while the bap gene was found

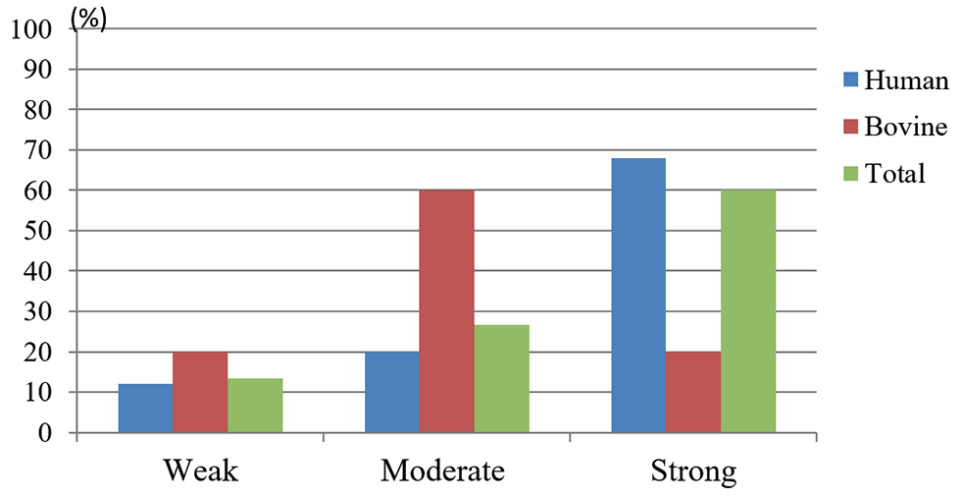


Figure 2. Biofilm formation status of isolates.

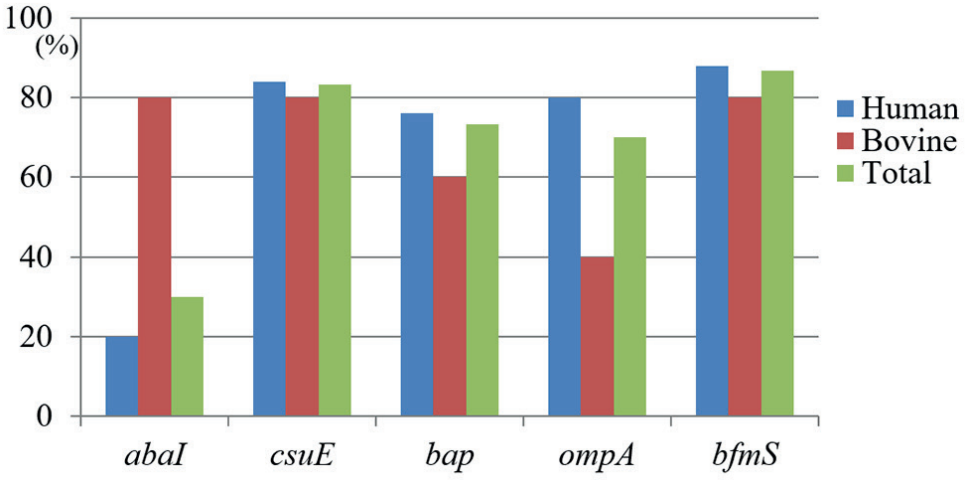


Figure 3. Distribution of biofilm-related virulence genes.

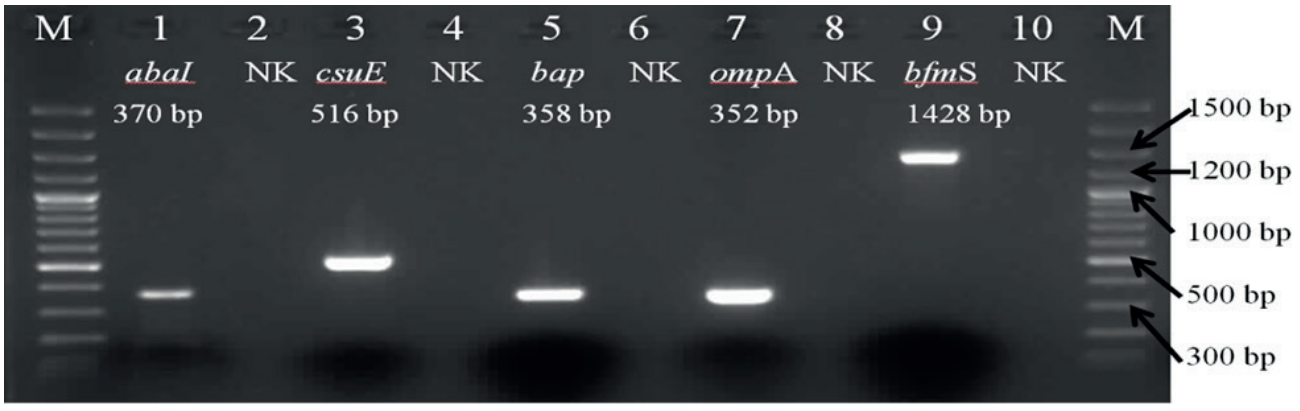


Figure 4. Gel electrophoresis of *A. baumannii* virulence genes **1.** *aba*I (370 bp) **3.** *csu*E (516 bp) **5.** *bap* (358 bp) **7.** *omp*A (352 bp) **9.** *bmf*S (1428 bp **2,4,6,8,10.** Negative Control (NK) (master mix without DNA) **M:** 100 bp DNA ladder (Vivantis).

Table 6. Frequency of biofilm-related virulence genes.

	*aba*I (%)	*csu*E (%)	*bap* (%)	*omp*A (%)	*bfm*S (%)
Human (n=25)	5 (20)	21 (84)	19 (76)	20 (80)	22 (88)
Bovine (n=5)	4 (80)	4 (80)	3 (16)	2 (60)	4 (80)
Total (n=30)	9 (30)	25 (83)	22 (73)	21 (70)	26 (87)

Table 7. Virulence gene phenotypes of isolates.

Number of virulence genes	Virulence gene phenotypes	Ratio (%), Isolate number (n=30)
2	*bfm*S, *omp*A	3 (1)
2	*csu*E, *aba*I	10 (3) (One bovine isolate)
2	*csu*E, *bap*	3 (1)
3	*bfm*S, *csu*E, *omp*A	7 (2)
3	*bfm*S, *bap*, *omp*A	13 (4) (One bovine isolate)
3	*bfm*S, *csu*E, *aba*I	3 (1)
4	*bfm*S, *csu*E, *bap*, *omp*A	43 (13)
4	*bfm*S, *csu*E, *bap*, *aba*I	10 (3) (Two bovine isolates)
4	*bfm*S, *csu*E, *omp*A, *aba*I	3 (1) (One bovine isolate)
5	*bfm*S, *csu*E, *bap*, *omp*A, *aba*I	3 (1)

at a low level in bovine isolates (16%), it was present at a high level (76%) in human isolates (Table 6, Figure 3, Figure 4).

Five isolates carried 2 virulence genes, six isolates (human) carried 2, seven isolates carried 3, fourteen isolates (human) carried 4 genes, seventeen isolates carried 4, and one isolate carried 5 virulence genes. In total, ten different virulence gene patterns were identified, with the most common pattern being the "*bfm*S, *csu*E, *bap*, *omp*A" resistance pattern (Table 7).

Integron genes

In this study, integron genes were detected in 90% of all isolates (23 human isolates and 4 bovine isolates out of a total of 27 isolates). It was determined that the *int*1 gene was carried at a higher rate (92%) compared to the *int*2 gene (52%) in human isolates, while in animal isolates, the presence of both genes was at an equal level (60%). According to the findings, only the class 1 integron gene was present in 37% of the isolates, while only the class 2 integron gene was found in 3% of the isolates. Notably, both classes of integron genes were detected together in 50% of the isolates. However, 10% of the isolates did not contain any class of integron gene (Table 8, Figure 5, Figure 6).

Statistical analysis

In our study, no statistically significant relationship was detected between the origin of the isolates and the severity of biofilm formation (Table 9).

When examining the relationship between the origin of isolates and the frequency of virulence gene presence, a significant association was found between the carriage of *aba*I and *bfm*S virulence genes. However, no significant relationship was detected between the origin of the isolate and the presence of virulence genes for the other three virulence genes (*bap*, *csu*E, *omp*A).

A significant relationship was found between strong biofilm formation and carrying all virulence genes (*aba*I, *csu*E, *bap*, *omp*A, *bfm*S), while similarly, a significant relationship was observed between moderate biofilm formation and carrying *aba*I and *bap* virulence genes, and between weak biofilm formation and carrying *omp*A and *bfm*S virulence genes (Table 11).

A significant relationship was found between strong biofilm formation and carrying integron genes in the isolates, whereas no significant relationship was identified between weak and moderate biofilm formation and carrying integron genes (Table 12).

Table 8. Rates of isolates carrying integron genes.

	*int*1 (%)	Only *int*1 (%)	*int*2 (%)	Only *int*2 (%)	*int*1+*int*2 (%)
Human (n=25)	23 (92)	10 (40)	13 (52)	0 (0)	13 (52)
Bovine (n=5)	3 (60)	1 (20)	3 (60)	1 (20)	2 (40)
Total (n=30)	26 (87)	11 (37)	16 (53)	1 (3)	15 (50)

Table 9. The relationship between the origin of the isolates and the severity of biofilm formation.

Biofilm formation	Origin of isolate		P	χ^2
	Human	Bovine		
Weak (+)	3	1	0.538	0.223
Weak (-)	22	4		
Moderate (+)	5	3	0.102	3.29
Moderate (-)	20	2		
Strong (+)	17	1	0.128	3.87
Strong (-)	8	4		

Table 10. Relationship between the origin of isolates and the frequency of virulence genes related to biofilm formation.

Virulance genes	Origin of isolate		P	χ^2
	Human	Bovine		
***aba*I (+)**	5	4	0.019*	6.91
***aba*I (-)**	20	1		
***csu*E (+)**	21	4	0.046	1
***csu*E (-)**	4	1		
***bap* (+)**	19	3	0.589	0.527
***bap* (-)**	6	2		
***omp*A (+)**	20	2	0.102	3.30
***omp*A (-)**	5	3		
***bfm*S (+)**	22	4	0.017*	6.77
***bfm*S (-)**	3	5		

*: Degree of statistical significance.

DISCUSSION

Acinetobacter species are known for their rapid development of antimicrobial resistance and their ability to persist in the environment for extended periods, which makes them a major concern (9). Moreover, it has been previously reported that *Acinetobacter* spp. can be found in soil, water, and sewage, as well as in the flora of both animal and human skin (29). One of the objectives of this study was to determine the prevalence of *A. baumannii* in clinical samples from humans and animals. In our study, we detected *A. baumannii* in 24% of human clinical samples and 2% of animal clinical samples. These findings indicate that *A. baumannii* is more frequently associated with human-derived isolates, but can also be found in isolates from animals. This highlights the potential significance of infections related to animal health as a source of concern for human health. For example, the prevalence of A. baumannii obtained from milk and dairy products in Egypt was found to be 3% (30). Similarly, a study conducted in Korea reported an incidence rate of 8% for *Acinetobacter*

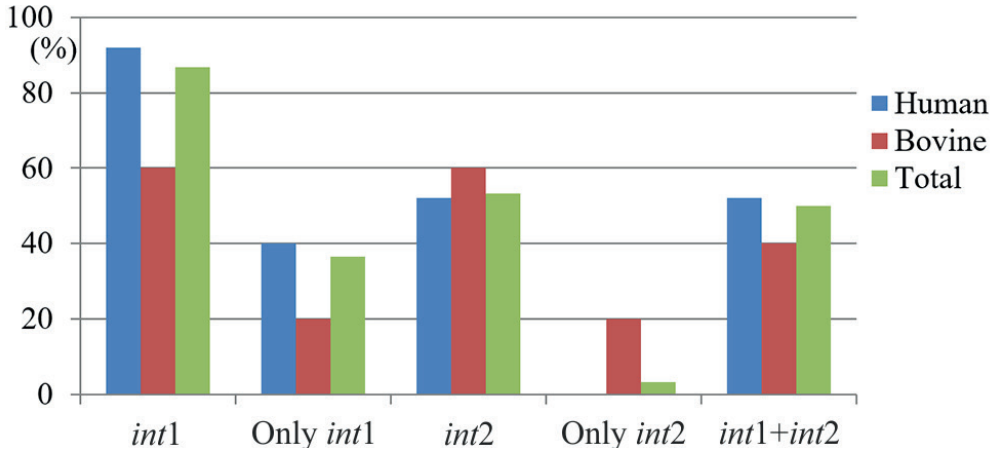


Figure 5. Integron gene carrying status of isolates.

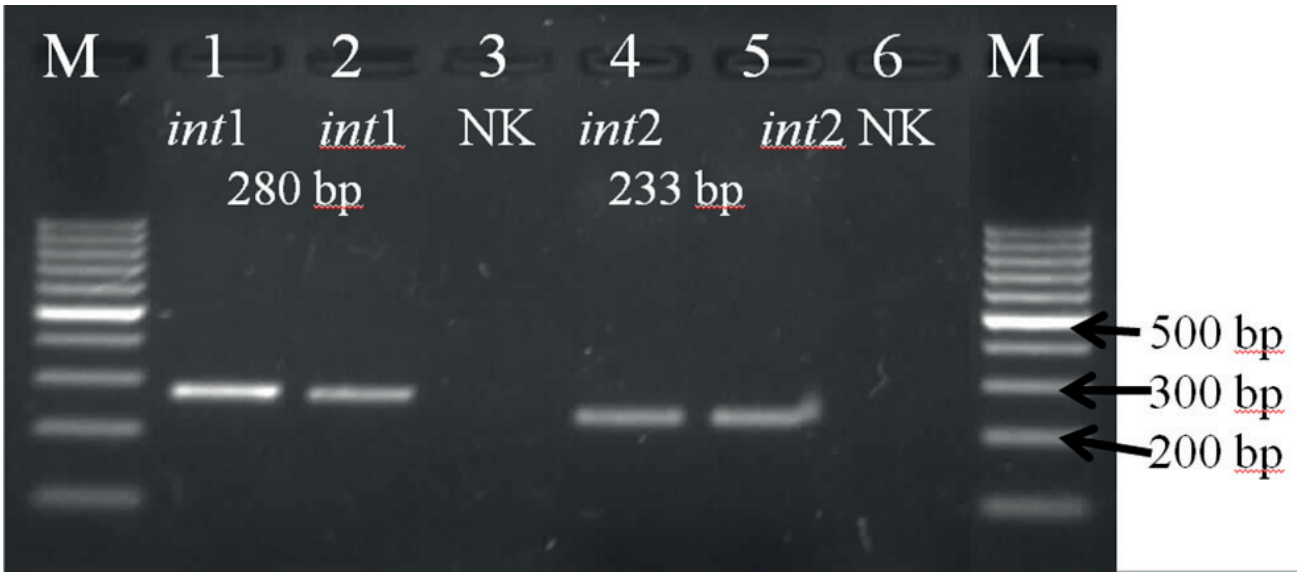


Figure 6. Integron classes of *A. baumannii* isolates. **1,2:** *int*1 gene positive *A. baumannii* isolates **4,5**: *int*2 gene positive *A. baumannii* isolates **3,6:** Negative Control (NK) (DNA-free master mix) **M:** 100 bp molecular marker (Fermentas).

spp. (31). Additionally, *Acinetobacter* spp. were isolated from 23% of raw milk samples from farmers' homes, with *A. baumannii* detected in 20% of these samples. These findings indicate that these microorganisms pose a serious threat to both animal and human health.

Bacterial infections resistant to antibiotics, especially those involving multidrug-resistant bacteria, can pose challenging treatment scenarios, leading to serious health complications and even death due to prolonged hospital stays and unsuccessful treatment attempts (32). In this study, similiar to previous research (33), it was found that all human isolates exhibited multidrug resistance, showing moderate to high levels of resistance to all antimicrobial agents except for tetracycline and lipopeptides. Although resistance rates were lower in animal isolates, resistance to aminoglycosides, penicillin, β-lactams, folate, and quinolone antibiotics were observed. These findings are consistent with prior studies (13). Overall, the high rates of antimicrobial resistance can be associated with the assumption that the samples were largely collected from individuals treated with antimicrobial agents. These results particularly highlight the broad spectrum of antibiotic resistance among human-derived *A. baumannii* isolates. They underscore the need for more effective and personalized treatment strategies to successfully manage *A.*

Table 11. Relationship between the biofilm-forming ability of isolates and the presence of virulence genes associated with biofilm formation.

Genes	Biofilm formation		P	χ2	Biofilm formation		P	χ2	Biofilm formation		P	χ2
	S (+)	S (-)			M (+)	M (-)			W (+)	W (-)		
*aba*I (+)	1	8	0.001***	12.377	5	3	0.016*	6.924	3	6	0.069	4.302
*aba*I (-)	17	4			3	19			1	20		
*csu*E (+)	13	12	0.004**	9.50	8	17	0.287	2.109	4	21	1	0.892
*csu*E (-)	5	0			0	5			0	5		
bap (+)	17	5	0.003**	9.91	3	19	0.016*	6.92	2	20	0.284	1.242
bap (-)	1	7			5	3			2	6		
*omp*A (+)	18	4	0.001***	15.818	4	18	0.158	2.936	0	22	0.003***	12.269
*omp*A (-)	0	8			4	4			4	4		
*bfm*S (+)	18	8	0.018*	6.692	7	19	1	0.006	1	25	0.004***	14.682
*bfm*S (-)	0	4			1	3			3	1		

*: Degree of statistical significance; **???????????; ***p<0.05.

Table 12. Relationship between the biofilm-forming ability of isolates and the presence of integron genes.

Biofilm formation	Integron genes		P	χ2
	Positive	Negetaive		
Weak (+)	3	1	0.360	1.115
Weak (-)	24	2		
Moderate (+)	6	2	0.166	2.636
Moderate (-)	21	1		
Strong (+)	18	0	0.050*	4.833
Strong (-)	9	3		

baumannii infections. Additionally, they demonstrate that *A. baumannii* presents a widespread and serious antimicrobial resistance issue in both humans and animals.

The ability of *A. baumannii* to colonize surfaces and form biofilms is known to be a significant factor contributing to chronic and persistent infections (34). Our findings indicate that all *A. baumannii* isolates possess the capacity to form biofilms; however, notably, there is a significantly higher tendency for strong biofilm formation in human isolates (68%) compared to animal isolates (20%). Our results align with previous reports (10,34), demonstrating that over 58% of *A. baumannii* isolates form strong biofilms. This suggests that human-derived isolates may be better adapted to biofilm formation, potentially increasing their infection potential. Conversely, a moderate tendency for biofilm formation appears to be more common in animal-derived isolates. This indicates that the biofilm-forming ability of *A. baumannii* isolates obtained from animals is milder compared to human isolates and may vary depending on environmental factors.

Studies have shown that *A. baumannii* possesses biofilm-associated genes such as *aba*I, *csu*E, *bap*, *omp*A, and *bfm*S, which influence biofilm formation (10, 11). According to the findings of this study, the presence of the *bfm*S gene was the highest, followed by *csu*E, *bap*, *omp*A, and *aba*I genes, which is consistent with other studies conducted in Iran, Thailand, and Korea (10,11,34,35). The presence of various virulence genes detected in *A. baumannii* isolates indicates various pathogenic mechanisms that enhance the pathogen's disease-causing potential. Specifically, the widespread presence of virulence genes such as *bfm*S, *csu*E, *bap*, *omp*A, and *aba*I is regarded as significant factors that may affect *A. baumannii*'s biofilm formation ability, cell adhesion, antibiotic resistance, and other infection-related properties (10, 11). The presence of different combinations of these virulence genes may reflect the complexity and diversity of the pathogen's infection dynamics.

Differences in the distribution of biofilm-associated virulence genes in *A. baumannii* isolates from human and animal sources highlight the diversity in the pathogen's infection dynamics. Particularly, the higher prevalence of the *aba*I gene in animal-derived isolates suggests the need for a different adaptation strategy, possibly due to the natural microbiome of animals and environmental conditions. During transitions between animals, *A. baumannii* may activate different virulence genes to adapt to envi-

ronmental conditions and initiate infections. However, the higher expression of the *bap* gene in human-derived isolates may suggest a more prominent role of biofilm formation in hospital environments and in the pathogenesis of infections associated with this gene. This finding suggests that the virulence mechanisms of the bacterium may vary depending on environmental factors and infection sources, indicating that different types of infections may exhibit varying pathogenicity characteristics.

The presence of integrons, known as the main reservoirs of antibiotic resistance genes in microbial populations, plays a significant role in the emergence of multidrug-resistant isolates (36). In our study, we found that class I integrons were more prevalent compared to class II integrons, which is consistent with other research findings (10,16). Our findings indicate the widespread presence of integron genes in *A. baumannii* isolates. The notably higher frequency of the *int*1 gene compared to the *int*2 gene in human-derived isolates resembles findings from other studies (16), suggesting a more common occurrence of this gene in human isolates. Conversely, the equal prevalence of both integron genes in animal-derived isolates indicates a different distribution in isolates obtained from animals. Additionally, the low occurrence of isolates carrying only class 1 or class 2 integron genes, and the high occurrence of isolates with both classes together, suggest that combinations of different integron classes may be common in the *A. baumannii* population. These findings suggest that integrons may play a significant role in the spread of antibiotic resistance in *A. baumannii.* However, the concurrent increase in multidrug-resistant isolates and integron resistance genes will lead to the failure of antibiotic treatment strategies. The absence of any class integron gene in some isolates suggests the presence of different resistance mechanisms, which may not be dependent on integrons.

The statistically non-significant relationship between the origin of isolates and the severity of biofilm formation is a noteworthy finding of our study. These results indicate that the biofilm-forming abilities of *A. baumannii* isolates from human and animal origins do not vary depending on their sources. This suggests that the potential for biofilm formation in *A. baumannii* is similarly distributed among isolates from different sources. This finding may imply that the biofilm-forming ability of *A. baumannii* is more dependent on genetic characteristics rather than environmental factors. However, further research with a larger sample size is needed to investigate this aspect more thoroughly.

The investigation of the relationship between the origin of isolates and the frequency of virulence genes reveals an important finding of our study. Specifically, the association of *aba*I and *bfm*S virulence genes with the origin of isolates suggests that certain virulence genes may exhibit different distributions among isolates from human or animal sources. This finding implies that *A. baumannii* isolates from different sources may show variations in virulence gene profiles, indicating that the pathogen employs different genetic strategies to adapt to environmental conditions.

The significant association between strong biofilm formation and the carriage of integron genes highlights the role of integrons in bacterial biology as a critical component in the spread of biofilm formation and antimicrobial resistance. However, the lack of a significant relationship between weak and moderate biofilm formation and the presence of integron genes may suggest that biofilm formation alone may not be sufficient as a determinant factor in resistance development. This finding could contribute to a better understanding of the role of biofilm formation in antimicrobial resistance mechanisms and lay a foundation for future research endeavors.

This study aimed to determine the frequency of *A. baumannii* in human and animal clinical samples, analyze antimicrobial resistance profiles, investigate biofilm formation abilities, and examine the presence of *int*1 and *int*2 genes. Our findings indicate that *A. baumannii* isolates from both human and animal sources have the potential to form biofilms and pose a significant antimicrobial resistance problem. Specifically, human-derived isolates show higher levels of antibiotic resistance compared to those from animals, although isolates from both sources are concerning in terms of antimicrobial resistance. Additionally, our data revealed the more widespread presence of *int*1 compared to *int*2 suggesting the significant role of integrons in the spread of antimicrobial resistance. The diversity of virulence genes and biofilm formation ability are believed to have a decisive impact on the pathogenic potential of *A. baumannii*. In conclusion, the widespread antimicrobial resistance and biofilm formation ability observed in human and animal-derived *A. baumannii* isolates represent a significant public health issue. These findings underscore the need for the development of more effective strategies for the treatment and control of

A. baumannii infections, as well as the necessity for further research in this field.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support from the Scientific Research Projects Unit of Aydın Adnan Menderes University (Project Number: ADU-BAP, VTF-21011) for funding this study, which was summarized from the first author's doctoral thesis.

REFERENCES

1. Peleg, A.Y., Seifert, H. and Paterson, D.L.: *Acinetobacter baumannii*: emergence of a successful pathogen. Clin. Microbiol. Rev. 21:538-582, 2008.
2. Dijkshoorn, L., Nemec, A. and Seifert, H.: An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat. Rev. Microbiol. 5:939-951, 2007.
3. Alrahmany, D., Omar, A.F., Alreesi, A., Harb, G. and Ghazi, I.M.: *Acinetobacter baumannii* infection-related mortality in hospitalized patients: risk factors and potential targets for clinical and antimicrobial stewardship interventions. Antibiotics. 11(8): 1086, 2022.
4. van der Kolk, J.H., Endimiani, A., Graubner, C., Gerber, V. and Perreten, V.: *Acinetobacter* in veterinary medicine with emphasis on *A. baumannii*. J. Glob. Antimicrob Resist. 16:59-71, 2019.
5. Müller, S., Janssen, T. and Wieler, L.H.: Multidrug resistant *Acinetobacter baumannii* in veterinary medicine – emergence of an underestimated pathogen? Berl. Munch. Tierarztl. Wochenschr. 127(11-12):435-446, 2014.
6. Cavallo, I., Oliva, A., Pages, R., Sivori, F., Truglio, M., Fabrizio, G., Pasqua, M., Pimpinelli, F. and Di Domenico, E.G.: *Acinetobacter baumannii* in the critically ill: complex infections get complicated. Front. Microbiol. 14:1196774, 2023.
7. Nocera, F.P., Attili, A.R. and De Martino, L.: *Acinetobacter baumannii*: its clinical significance in human and veterinary medicine. Pathogens. 10(2):127, 2021.
8. Flemming, H.C., Wıngender, J., Szewzyk, U., Steınberg, P., Rıce, S.A. and Kjelleberg, S.: Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14(9): 563-575, 2016.
9. Gedefie, A., Demsis, W., Ashagrie, M., Kassa, Y., Tesfaye, M., Tilahun, M., Bisetegn, H. and Sahle, Z.: *Acinetobacter baumannii* biofilm formation and its role in disease pathogenesis: a review. Infect. Drug Resist. 14: 3711-3719, 2021.
10. Zeighami, H., Valadkhanı, F., Shapouri, R., Samadi, E. and Haghi, F.: Virulence characteristics of multidrug resistant biofilm forming *Acinetobacter baumannii* isolated from intensive care unit patients. BMC Infect. Dis. 19(1): 629, 2019.
11. Ghasemi, E., Ghalavand, Z. Goudarzi, H., Yeganeh, F., Hashemi, A., Dabiri, H., Mirsamadi, E.S. and Foroumand, M.: Phenotypic and genotypic investigation of biofilm formation in clinical and environmental isolates of *Acinetobacter baumannii*. Arch. Clin. Infect. Dis. 13(4):e12914, 2018.
12. Weng, Z., Yang, N., Shi, S., Xu, Z., Chen, Z., Liang, C., Zhang, X. and Du, X.: Outer membrane vesicles from *Acinetobacter baumannii*: biogenesis, functions, and vaccine application. Vaccines. 12: 49, 2024.
13. Mohamed, H.M.A., Abd-Elhafeez, H.H., Al-Jabr, O.A. and El-Zamkan, M.A.: Characterization of *Acinetobacter baumannii* isolated from raw milk. Biology.11: 1845, 2022.
14. Tiku, V.: *Acinetobacter baumannii*: virulence strategies and host defense mechanisms. DNA Cell Biol. 41(1):43-48, 2022.
15. Logan, L.K., Gandra, S., Trett, A., Weinstein, R.A. and Laxminarayan, R.: *Acinetobacter baumannii* resistance trends in children in the United States, 1999-2012. J. Pediatr. Infect. Dis. Soc. 8:136-142, 2019.
16. Goudarzi, H., Azad, M., Seyedjavadi, S.S., Azimi, H., Salimi Chirani, A. and Fallah Omrani, V.: Characterization of integrons and associated gene cassettes in *Acinetobacter baumanni*i strains isolated from intensive care unit in Tehran, Iran. J. Acute Dis. 5(5):386-92, 2016.
17. Koczura, R., Przyszlakowska, B., Mokracka, J. and Kaznowski A.: Class 1 integrons and antibiotic resistance of clinical *Acinetobacter calcoaceticus-baumannii* complex in Poznan, Poland. Curr. Microbiol. 69(3):258-62, 2014.
18. Eze, E.C., Chenia, H.Y. and El Zowalaty, M.E.: *Acinetobacter baumannii* biofilms: effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. Infect. Drug Resist. 11, 2277: 2018.
19. Babapour, E., Haddadi, A., Mirnejad, R., Angaji, S.A. and Amirmozafari, N.: Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pac. J. Trop. Biomed. 6:528-533, 2016.
20. Bardbari, A.M., Arabestani, M.R., Karami, M., Keramat, F., Alikhani, M.Y. and Bagheri, K.P.: Correlation between ability of biofilm formation with their responsible genes and MDR patterns in clinical and environmental *Acinetobacter baumannii* isolates. Microb. Pathog. 108: 122-128, 2017.
21. Constantiniu, S., Romaniuc, A., Iancu, L.S., Filimon, R. and Tarasi, I.: Cultural and biochemical characteristics of *Acinetobacter* spp. strains isolated from hospital units. J. Prev. Med. 12:35-42, 2004.
22. EUCAST. Breakpoint tables for interpretation of MICs and zone diameters, version 12.0; 2022. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_12.0_Breakpoint_Tables.pdf. Accessed August 18, 2022.
23. Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbart, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T. and Monnet, D.L.: Multidrug-resistant, extensively drug resistant and pandrug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18: 268-281, 2012.
24. Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penadés, J.R. and Lasa, I.: The enterococcal surface protein, *esp*, is involved in enterococcus faecalis biofilm formation. Appl. Environ. Microbiol. 67(10):4538-45, 2001.
25. Turner, P., Mclennan, A., Bates, A. and White, M.: BIOS Instant Notes in Molecular Biology. 4th Edition, Taylor & Francis, England, 2012.

26. Lee, H.W., Koh, Y.M., Kim, J., Lee, J.C., Lee, Y.C., Seol, S.Y., Cho, D.T. and Kim, J.: Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin. Microbiol. Infect. 14(1):49-54, 2008.
27. Bass, L., Liebert, C.A., Lee, M.D., Summers, A.O., White, D.G., Thayer, S.G. and Maurer, J.J.: Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. Antimicrob. Agents and Chemother. 43:2925-2929, 1999.
28. Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A.O., White, D.G. and Maurer, J.J.: Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents and Chemother. 45:723-726, 2001.
29. Almasaudi, S.: *Acinetobacter* spp. as nosocomial pathogens: epidemiology and resistance features. Saudi J. Biol. Sci. 25: 586-596, 2018.
30. Saad, N.M., Amin, W.F. and Mostafa, S.M.: Detection of *Acinetobacter* species in milk and some dairy products. Assiut Vet. Med. J. 64(156):34-40, 2018.
31. Nam, H., Lim, S., Kang, H., Kim, J., Moon, J., Jang, K., Joo, Y. and Jung, S.: Prevalence and antimicrobial susceptibility of gram-negative bacteria isolated from bovine mastitis between 2003 and 2008 in Korea. J. Dairy Sci. 92(5):2020-2026, 2009.
32. Salam, M.A., Al-Amin, M.Y., Salam, M.T., Pawar, J.S., Akhter, N., Rabaan, A.A. and Alqumber, M.A.A.: Antimicrobial resistance: a growing serious threat for global public health. Healthcare. 11:1946, 2023.
33. Albayrak, H., Bayraktar, M.T. and Zeyrek, F.Y.: Antibiotic resistance profile of *Acinetobacter* species isolated from blood cultures of inpatients in Harran University Hospital. J. Harran Univ. Med. Fac. 18(2):165-169, 2021.
34. Thummeepak, R., Kongthai, P., Leungtongkam, U. and Sitthisak, S.: Distribution of virulence genes involved in biofilm formation in multi-drug resistant *Acinetobacter baumannii* clinical isolates. Int. Microbiol. 19(2):121-9, 2016.
35. Youn Sung, J.: Molecular characterization and antimicrobial susceptibility of biofilm-forming *Acinetobacter baumannii* clinical isolates from Daejeon, Korea. Korean J. Clin. Lab. Sci. 50:100-109, 2018.
36. Bhat, B.A., Mir, R.A., Qadri, H., Dhiman, R., Almilaibary, A., Alkhanani, M. and Mir, M.A.: Integrons in the development of antimicrobial resistance: critical review and perspectives. Front. Microbiol. 31(14):1231938, 2023.