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RESEARCH ARTICLE

The Quinolone Resistance Genes in the Bacteriophage DNA Fractions in the Healthy Calf Stool Samples Via qPCR

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Abstract: The One Health approach shows that people, animals, plants, and environmental factors can affect each other. Phages are one of the mobile genetic elements. Quinolones are a critical group of antibiotics for both human and animal health and monitoring their antimicrobial resistance is very important. The aim of the study is to determine the frequency of the quinolone resistance gene in bacteriophage DNA fractions obtained from healthy calf stool samples. In our study, 50 samples from 6-9 months old calves, which were found to be healthy and not treated with any group of antibiotics in Sanlıurfa province, were included. DNA isolation was made from phage lysates of stool samples and specific primers were used *qnrA*, *qnrB* and *qnrS* genes. qPCR was performed on LightCycler480. Despite not receiving any antibiotic treatment, *qnrB* was the most detected gene among the phage DNA fractions detected in 11 calves. While *qnrA*, *qnrB* and *qnrS* quinolone resistance genes were detected together in one sample, *qnrB* and *qnrS* resistance genes were found together in two samples. Our data, obtained from the study in Türkiye to search for antimicrobial resistance genes in phage fractions, showed the importance of the One Health approach and determined that it was highly effective in quinolone resistance gene shedding in healthy calves that had never been treated with antibiotics. It has been concluded that in empirical treatment with quinolone, attention should be paid to all living things and unconscious antibiotic use may cause the spread of resistance genes more than expected.

Keywords: *Antibiotic resistance genes, Bacteriophage, qPCR, Quinolone*

Sağlıklı Buzağı Dışkı Örneklerindeki Bakteriyofaj DNA Fraksiyonlarındaki Kinolon Direnç Genlerinin qPCR ile Belirlenmesi

Öz: "Tek Sağlık" yaklaşımı, insanların, hayvanların, bitkilerin ve çevresel faktörlerin birbirini etkileyebileceğini gösterir. Fajlar, hareketli genetik elemanlardan biridir. Kinolonlar hem insan hem de hayvan sağlığı için kritik bir antibiyotik grubudur ve antimikrobiyal dirençlerinin izlenmesi çok önemlidir. Bu nedenle çalışmamızın amacı, sağlıklı buzağı dışkı örneklerinden elde edilen bakteriyofaj DNA fraksiyonlarında kinolon direnç gen belirteçlerinin *(qnrA*, *qnrB* ve *qnrS* genleri) sıklığını belirlemektir. Çalışmamıza Şanlıurfa ilinde bulunan mandıralardan alınan 6-9 aylık buzağılardan sağlıklı olduğu tespit edilen ve herhangi bir grup antibiyotik ile tedavi edilmeyen 50 dışkı örneği dahil edildi. Dışkı numunelerinin faj lizatlarından DNA izolasyonu yapılmış ve *qnrA*, *qnrB* ve *qnrS* genleri için spesifik primerler kullanılmıştır. qPCR, LightCycler480'de gerçekleştirilmiştir. Hiçbir antibiyotik tedavisi görmemesine rağmen 11 buzağı dışkısında tespit edilen faj DNA fraksiyonları arasında en çok tespit edilen gen *qnrB* idi. Bir örnekte *qnrA*, *qnrB* ve *qnrS* kinolon direnç gen belirteçleri birlikte saptanırken, iki örnekte *qnrB* ve *qnrS* direnç gen belirteçleri birlikte bulundu. Türkiye'de faj fraksiyonlarında antimikrobiyal direnç geni araştırması yapan çalışma ile elde edilen verilerimiz, "Tek Sağlık" yaklaşımının önemini göstermiş, ayrıca sağlıklı, antibiyotikle hiç tedavi edilmemiş buzağılarında kinolon direnç geni saçılımında oldukça etkili olduğu belirlenmiştir Kinolon ile ampirik tedavide tüm canlılara dikkat edilmesi gerektiği ve bilinçsiz antibiyotik kullanımın tahmin edilenden fazla direnç genlerinin yayılmasına neden olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Antibiyotik direnç genleri, Bakteriyofaj, qPCR, Kinolon*

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Introduction

Bacterial antimicrobial resistance (AMR), which reduces the effect of drugs used to treat bacterial infections, threatens the whole world as one of the leading public health problems of the 21st century. According to the reports of the World Health Organization (WHO), it is estimated that 10 million people will die in 2050 due to antimicrobial resistance [1]. The One Health approach indicates that factors originating from humans, animals, plants and the environment can have effects on each other. Therefore, it is thought that the development of antimicrobial resistance may be related to the misuse of antimicrobials in these resources and contribute to the spread of antimicrobial resistant bacteria and antimicrobial resistance markers throughout the world within or between these sectors. It is a well-known fact that many similar classes of antimicrobials used to treat bacterial infections in humans are also frequently used in animals [2]. Transduction by bacteriophages (phages) is one of the many horizontal gene transfer mechanisms and it has been demonstrated that phage-mediated transduction makes an important contribution to the spread of antimicrobial resistance genes [3,4].

Bacteriophages are known as bacterial viruses that invade the cells of Gram-positive and Gram-negative bacteria [5]. According to their genomic and morphological structures, there is a wide variety among phages. The size of phage genomes can range from a number of to 100 kb [6]. It is known that ARGs are transferred to the environment from antibiotic-resistant bacteria when bacteriophages invade these bacterial cells [7,8]. Phages are one of the mobile genetic elements (MGEs), and antimicrobial resistance genes (ARGs) can be acquired and transferred between bacteria via these MGEs such as phages, conjugative plasmids, insertion sequences, integrons, and transposons [9]. Metagenomic studies have shown that due to the bacterial diversity in the gut microbiota, bacteriophages can also be found extensively in the human and animal gut microbiota, and that the gut is an excellent ecological environment for these phages, which can probably proliferate by infecting bacterial communities of the gut. In addition to this, ARGs of Gram-negative and Gram-positive bacteria can be carried and transferred within these phage DNA fractions [10,11]. Quinolones and fluoroquinolones have been classified as critical antibiotics for human health by the WHO. Resistance to these compounds is widespread in Europe, and due to this rapid spread, monitoring of antimicrobial resistance to quinolones is crucial for both human and animal health [12]. The most common disease treated in cattle is the neonatal calf diarrhea. According to the recommendations and depending on the results of antimicrobial susceptibility test, the use of quinolones in the treatment of this disease should be in a limited amount and should be used as a last choice in the treatment in cases of diarrhea due to *E. coli* and *Salmonella* spp. infections [13]. However, some 3rd generation fluoroquinolones, such as enrofloxacin, can be used empirically for the treatment of several diseases of animals, which may lead to the development of resistance genes against quinolones [14].

In our study, we aimed to determine the frequency of quinolone resistance gene markers (*qnrA*, *qnrB* and *qnrS* genes) by qPCR, which are critical for human and animal health, in bacteriophage DNA fractions obtained from healthy calf stool samples.

Material and Methods

Ethical Statement

This study was approved by the Veterinary Control Central Research Institute Local Ethics Committee (Approval no: 2022/24).

Sampling and DNA Isolation

In our study, random sampling was used to select stool samples. 50 stool samples taken from 6-9 months old Holstein calves (25 male and 25 female) from the dairy farms in Sanliurfa, that were found to be healthy and not treated with any kind of antibiotics, were included. Stool samples were taken into sterile containers and delivered to the laboratory under appropriate conditions. All stool samples were checked for the *Bovine Coronavirus* (BcoV), *Bovine Rotavirus* (BRV) *group A*, *Escherichia coli* K99+, *Cryptosporidium parvum* and *Giardia* by using the Anigen Rapid BoviD-5 Ag rapid test kit (Bionote, Inc. Korea) according to the instructions of manufacturer. No pathogen was detected in these 50 stool samples which were included in the study.

Standard PCR Procedures

Stool samples were diluted with a 1:5 (weight/volume) ratio in PBS solution and homogenized with magnetic stirrer for 15 min (2 g stool sample were homogenized in 10 mL of PBS). The homogenate was centrifuged at 3.000 x g and the phage lysate or the homogenate was concentrated by purification. DNase (100 U/mL) was added in order to eliminate free DNA outside the phage particles in the suspension [11]. The phage DNA fraction was extracted from the 200 µL homogenate suspension by using the QIAamp DNA stool minikit (Qiagen GmbH, Hilden, Germany) according to the instructions of manufacturer [15].

qPCR Procedures

Quinolone resistance gene markers (*qnrA, qnrB* and *qnrS* genes) were analyzed by using the qPCR method in the LightCycler 480 system according to the instructions of manufacturer. Specific primers for *qnrA, qnrB* and

qnrS genes and the qPCR procedure were used for the detection of antimicrobial resistance gene markers [16]. qPCR reactions were performed in accordance with the instructions of manufacturer by using the specific primers, LightCycler 480 Sybr Green I Master kit. 5 µL template DNA and 15 µL PCR master mix (3 µL sterile water, 1 µL forward primer [10 mmol/L], 1 µL reverse primers [10 mmol/L] and 10 µL master mix) were added in 96-wells. qPCR melting analysis was performed for both internal control of DNA presence and specific *qnr* determination.

Results

Antimicrobial resistance gene markers of *qnrA, qnrB* and *qnrS* genes detected in phage DNA fractions obtained from 50 stool samples which were included in the study are shown in *Table 1*. Quinolone resistance gene markers were detected in the phage DNA fractions which were detected in a total of 11 samples (22%), whereas quinolone resistance gene markers were not detected in remaining 39 samples (78%). Among the quinolone resistance genes, the most commonly detected one was *qnrB* gene which was found in 7 (14%) samples.

Table 1. *Distribution of quinolone resistance gene markers in phage DNA fractions*

Result	Positive	
	n	%
Total qnrA	4	8
Total qnrB	7	14
Total qnrS	6	12

While *qnrA, qnrB* and *qnrS* quinolone resistance gene markers were detected together in one sample (6%), *qnrB* and *qnrS* gene resistance gene markers were detected together in two samples (4%) and shown in *Table 2*.

Table 2. *Distribution of quinolone resistance gene markers in phage DNA fractions*

Result	Positive	
	n	%
qnrA only	1	2
qnrB only	3	6
qnrS only	2	4
qnrA + qnrB	1	2
qnrA + qnrS	1	2
qnrB + qnrS	2	4
qnrA + qnrB + qnrS	1	2

Discussion

Antibiotic resistance is an important and expanding public health problem. For this reason, many studies are currently being conducted about the mechanisms and spread of antibiotic resistance. The contribution of bacteriophages to the mobilization of ARGs in the environment is known but this issue has not been extensively studied. However, recent studies suggest that phages play an important role in animal and human diseases [17-20]. Therefore, in our study, we focused on the detection of quinolone resistance gene markers in phage lysates in healthy calf stool samples.

It has been determined as a result of many studies that, phages have the potential to be a reservoir and vector for the acquisition of ARGs [20,21]. Furthermore, it has been shown in several studies that transfer of ARGs is done by phages through transduction in natural environments such as mud, wastewater, sediment, soil, animal and human stool [22-28].

In our study, ARG scanning was performed on healthy calf stool samples and the rate was determined as 22%. Although the mechanism of AMR spread is not known exactly, it is generally thought that it occurs as a result of unconscious antibiotic use in both humans and animals. It is known that there is a continuous flow of ARGs among humans, animals and the environment in which they form a triad on the ecosystem, and it is planned to carry out the necessary controls and applications at these 3 key points in order to prevent the flow of ARGs.

The contribution of phages to the spread of antibiotic resistance is not fully known. Some recent researches suggest that the role of ARG-bearing phages in the environment is much more important than previously thought [29]. For this reason, many studies have investigated the transport of ARGs in bacteriophage DNA fractions in samples of sludge, wastewater, sediment, soil, water, and animal and human stool. The key role played by phages in the construction of the bacterial microbiota of the human gut flora has been extensively investigated by Mills et al.[30], Scanlan [31], and Guerin and Hill [32]. Camarillo-Guerrero et al.[33] showed in their study that the gene flow produced by phages is not limited to a single bacterial species or genus, but they form gene flow networks among phylogenetically different bacteria. Phages dominate the viral fraction of the human gut microbiota [34,35]. Up to 1012 virus-like particles (VLP) per mL in human stool have been reported by Hoyles et al.[36]. Camarillo-Guerrero et al.[33] have identified more than 142,000 redundant viral genomes in the human gut, mostly belonging to phages. Dutilh et al.[37] and Edwards et al.[38] determined in their studies that crAssphage and crAss-like phages are quite common worldwide. CrAss-like phages are associated with Bacteroidetes, which is the most abundant bacteria phylum in the human gut microbiota [39].

In the study of Quirós et al.[11], within the stool of 80 healthy human, ARGs were detected in 70% of the samples. The

most detected genes in bacteriophage DNA fragments isolated in the study were *blaTEM, qnrA* and *blaCTX-M-1* genes. Brown-Jaque et al.[39], in their study, determined the rates of 9 ARGs *(blaTEM, blaCTX-M, blaCTX-M-9, blaOXA-48, qnrA, qnrS, mecA, sul1,* and *armA)* found in the bacteriophage DNA fragments obtained from 150 healthy human stool. 72% of the samples included in the study were positive for at least one ARG.

In several studies, fluoroquinolone resistance genes *(qnrA* and *qnrS)* were frequently detected in environmental samples [25-27]. Similarly, in our study, *qnrA* and *qnrS* genes were detected in DNA fractions of phages found in the healthy calf stool. Colomer-Llunch et al.[15] detected *qnrA* and *qnrS* in bacteriophage DNA fragments obtained from samples of urban wastewater, river water and animal stool and they suggested that *qnr*-encoding phages might be generalized transforming particles. It is thought that the presence of qnr-encoding phages is an important factor for the formation of quinolone resistant strains and the spread of ARGs [40].

To investigate the contribution of bacteriophages to the spread of resistance genes, in China, a large-scale screening for 32 ARGs was performed in pig stool from three different commercial farms. The most common gene detected as a result of this scan was the *qnrA* gene [41]. Transfer of ARGs to the environment is a critical issue for both human and animal health. In several other studies conducted about the bacteriophage DNA fractions, a large number of ARGs have been similarly detected in waters contaminated with human and animal stool [42,43]. Although the fact that our study is single-centred and performed with a low number of samples in which these seem to be our limitations, it is still valuable in terms of presenting a preliminary data on this situation in our country.

As a result, our data, which is the study on the antimicrobial resistance genes in phage fractions in our country, showed that a One Health approach is very important, because it has been found that bacteriophage fractions can be detected in the stool of healthy calves and quinolone resistance genes can be carried in these fractions. It has been concluded that antibiotics, quinolone groups particularly, which are frequently used in different areas, can be transported through these phages and so, the antibiotic applications should be done carefully regardless of the type of living organism, whether it is animal or human.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (S. Ekici).

Financial Support

There is no funding source

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

The study was conducted with the permission of the Veterinary Control Central Research Institute Local Ethics Committee (Approval no: 2022/24).

Author Contributions

SE, MD, DD and AY conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. SE, AY, MD collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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