IJVST

Number 1
Volume 14
Year 2022
ijvst.um.ac.ir

Iranian Journal of Veterinary Science and Technology

Ferdowsi Univerity of Mashhad

ISSN (Print): 2008-465X
ISSN (Online): 2423-6306
Serial number: 26

In the Name of God, the Compassionate, the Merciful

Iranian Journal *of* Veterinary Science and Technology

Editorial Office:
Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

FERDOWSI UNIVERSITY OF MASHHAD PRESS

GENERAL INFORMATION

ISSN Print Edition: 2008-465X
ISSN Online Edition: 2423-6306

Journal Homepage:
ijvst.um.ac.ir

Abstracting and Indexing:
Scopus, ISI Master Journal List, Zoological Record; EMBASE, EBSCO, MIAR, Scientific Information Database (SID); Islamic World Science Citation Database (ISC); Magiran; Google Scholar; Centre for Agriculture and Biosciences International (CABI), DOAJ.

This journal has achieved the rating of:
- "Scientific-Research", by Commission of Evaluation of Iranian Scientific Journals, the Ministry of Science, Research and Technology, from Vol.7, No. 1, July 2015 onward.
- "International", by Commission of Evaluation of Iranian Scientific Journals, the Ministry of Science, Research and Technology, from Vol.13, No. 2, 2021 onward.

Publication Date:
Iranian Journal of Veterinary Science and Technology (IJVST) is published 4 times a year. Volume 14 with 4 issues appear in 2022.

Managing Director:
Abolghassem Naghibi, DVM, PhD

Editorial Officer:
Monir Taheri

Logo Design and Illustration:
Dr. Behrooz Fathi, Taraneh Ebnalnassir

SCOPE

Iranian Journal of Veterinary Science and Technology (IJVST) publishes important research advances in veterinary medicine and subject areas relevant to veterinary medicine including anatomy, physiology, pharmacology, bacteriology, biochemistry, biotechnology, food hygiene, public health, immunology, molecular biology, parasitology, pathology, virology, large and small animal medicine, poultry diseases, diseases of equine species, and aquaculture. Articles can comprise research findings in basic sciences, as well as applied veterinary findings and experimental studies and their impact on diagnosis, treatment, and prevention of diseases. IJVST publishes four kinds of manuscripts: Research Article, Review Article, Short Communication, and Case Report.

ON THE COVER

The scanning electron photomicrograph of Methicillin-resistant Staphylococcus aureus treated with Fractional inhibitory concentration of Vancomycin- cLFchimera.

Editorial Office:
Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

FERDOWSI UNIVERSITY OF MASHHAD PRESS

TABLE OF CONTENTS

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Editorial Office:
Faculty of Veterinary Medicine, Ferdowsi University of Mashhad,
Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: +98 51 3880 3742 ***Fax:*** +98 51 3876 3852
Web: ijvst.um.ac.ir ***Email:*** ijvst@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Sep- 21
Accepted after revision: 2021- Oct- 30
Published online: 2022- Mar- 01

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2021.72596.1084

Evaluation of the antibacterial activity of cLFchimera and its synergistic potential with vancomycin against methicillin-resistant *Staphylococcus aureus*

Sahar Roshanak[a], Fakhri Shahidi[a], Atefeh Soradeghi Toopkanloo[a], Zana Pirkhezranian[b], Mohammad Hadi Sekhavati[b]

[a] Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

Frequent and unlimited use of antibiotics caused the development of antibiotic resistance by microorganisms. Therefore, there is an urgent need to discover novel antibacterial agents or a combination of agents as a safe treatment strategy for various infections. In the present study, the synergistic effects of cLFchimera, an antimicrobial peptide, and the vancomycin antibiotic were evaluated using the checkerboard method against methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteria strain. cLFchimera had antimicrobial activity against MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) (MIC: 256 and 512 µg/mL, respectively). A synergistic effect was observed in the combination of cLFchimera with vancomycin (FIC: 0.375). The results showed that at FIC concentrations, the release of cytoplasmic materials from bacterial cells and the number of surviving cells were significantly ($p \leq 0.05$) higher and lower, respectively, than when peptides or antibiotics were used alone. SEM electron microscopic analysis at FIC concentration showed severe membrane damage of bacterial cells. In conclusion, the use of cLFchimera and vancomycin at FIC concentration reduces the consumption of both substances.

Keywords

Synergetic effect, Antimicrobial peptides, Antibiotics, Resistant bacteria

Number of Figures: 3
Number of Tables: 1
Number of References:: 41
Number of Pages: 8

Abbreviations

MRSA: Methicillin-resistant *Staphylococcus aureus*
MSSA: Methicillin-sensitive *Staphylococcus aureus*
Antimicrobial peptides: AMPs
MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration
FIC: Fractional inhibitory concentration

Corresponding author: *Fakhri Shahidi*
Email: *fshahidi@um.ac.ir*
Tel: *+98 (51)3880-6056*

Introduction

Staphylococcus aureus is a dominant pathogen both in the community and within hospitals. *S. aureus* is a Gram-positive bacteria which classified as a member of the family Micrococcaceae [1]. In humans, these bacteria commonly colonize on surfaces of the skin and the upper respiratory tract. Inflammation of lungs (pneumonia), infection of the mammary glands (mastitis), infections of the skin (impetigo), infection of the bone (osteomyelitis), infection of the endothelial lining of the heart and valves (endocarditis), and infection in the blood (bacteremia) are some disorders caused by *S. aureus*. *S. aureus* can also cause food poisoning, the result of enterotoxin production [1].

Administration of benzylpenicillin, β-lactam antibiotic, was the first treatment of *S. aureus* infections before the 1950s [2, 3], but producing a β-lactamase which inactivated the β-lactam, by resistant strains were causing increasing concern in the late 1950s [4]. Efforts were made to synthesize penicillin derivatives, methicillin, that were resistant to β-lactamase hydrolysis. Unfortunately, as soon as methicillin was used clinically, methicillin-resistant *S. aureus* (MRSA) strains were isolated [4]. We are currently in a situation where, in some cases, the glycopeptide antibiotic vancomycin, is the only choice for antimicrobial therapy. However, some reports demonstrated that vancomycin resistance-conferring genes from other bacterial groups can be expressed in *S. aureus* [5]. Therefore, an imperative need to propose new anti-staphylococcal agents to reduce or moderate methicillin resistance in *S. aureus* to an existing antibiotic.

Antimicrobial peptides (AMPs), which since the 1980s have been considered a possible alternative to existing antibiotics [6]. AMPs play an important role in the natural defense mechanism for destroying microbial infections [6]. AMPs have a net positive charge and an amphipathic structure and usually contain 12–50 amino acid residues [7, 8]. As is the case with AMPs, bacterial cells are less likely to develop resistance to AMPs because they disrupt the structure and function of cell membranes [9].

More recently, a chimeric form of peptide named cLFchimera has been expressed and purified in *E. coli* [10] in our lab. The results of in vitro studies showed that this peptide has antibacterial [10-12], antiviral [13], and anticancer [14] properties. Furthermore, the results of an in vivo experiment showed that supplementing *E. coli* challenged broilers with cLFchimera improved villi morphology in the jejunum, restored microbial balance in the ileum, and improved gene expression of cytokines and tight junctions in the jejunum of challenged birds [15]. These results revealed that this peptide could be nominated as an alternative for growth promoter antibiotics.

Enhancing the efficacy, restoring the sensitivity, and reducing the minimum effective dose of antibiotics, combined with other antimicrobial agents is one of the promising strategies[16, 17]. It has also been demonstrated that the combined use of APMs and antibiotics increase the bacterial killing of antibiotics regardless of the antibiotics' mode of action [18-20]. Membrane perturbation in or pore formation on the bacterial cell wall may enhance the uptake of antibiotics and increase their antibacterial effect [21].

The present study aimed to evaluate the combined effects of cLFchimera and vancomycin antibiotic compared with peptide alone, against methicillin-resistant *S. aureus* bacteria. The final goal of the present study was to determine the synergistic effects of these compounds to decrease the effective dose of antibiotics, thereby minimizing the potentially toxic side effects of these drugs and reducing the chance of antibiotic resistance.

Results

MIC determination and Check board assay

The MIC and MBC of vancomycin and cLFchimera were determined using the broth microdilution method before the synergy examination, and the highest concentration obtained was 512 µg/mL (Table 1). cLFchimera showed weak activity against *S. aureus* and MRSA in the present study (256 and 512 µg/mL, respectively). We chose commercially available vancomycin to investigate whether the combination of the cLFchimera with this antibiotic provided a synergistic effect. In combination with a low peptide concentration equivalent to 8-16 of its MIC (Fig. 1, A), vancomycin had improved antimicrobial activity, with 2–8-fold reduced MIC values (Figure 1B).

The microdilution checkboard method was carried out to evaluate the synergistic effects of the antibiotics combined with the cLFchimera, which were determined as FICIs (Table 1). The combination of vancomycin and cLFchimera had a synergic effect against both *S. aureus* and MRSA giving total synergism (FIC= 0.375), (Table 1).

Release of cytoplasmic material absorbing at 260 nm

Figure 2 shows that, for both bacteria, the release of cellular content, absorbing at 260 nm, is higher in vancomycin-treated bacteria than in cLFchimera -treated bacteria ($p \leq 0.05$) (Figure 2A). This release of cytoplasmic material was associated with total cellular mortality (Figure 2B). As shown in Figures 2A and 2B, the release of cytoplasmic materials and sur-

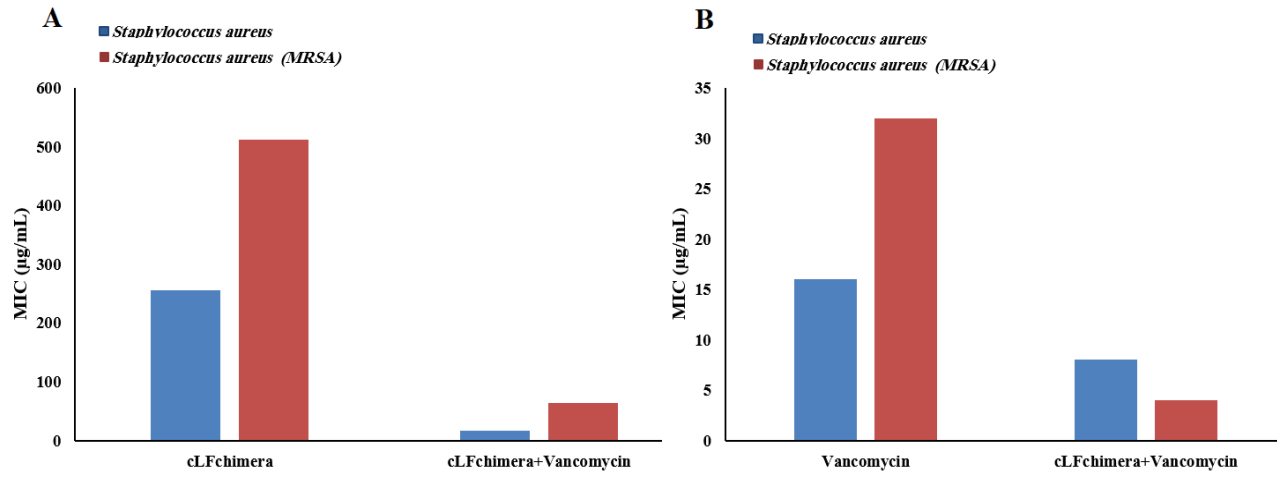


Figure 1.
Antibacterial activity of vancomycin in combination with cLFchimera. Bacterial cultures were treated with a series of concentrations of antibiotics in the presence of a low peptide concentration equivalent to 8-16 of its MIC at 37° C overnight (A). Vancomycin had improved antimicrobial activity, with 2–8-fold reduced MIC values (B). The OD600 was recorded using a microtiter plate reader. The MIC was defined as the lowest antibiotics concentration that inhibited the bacterial growth.

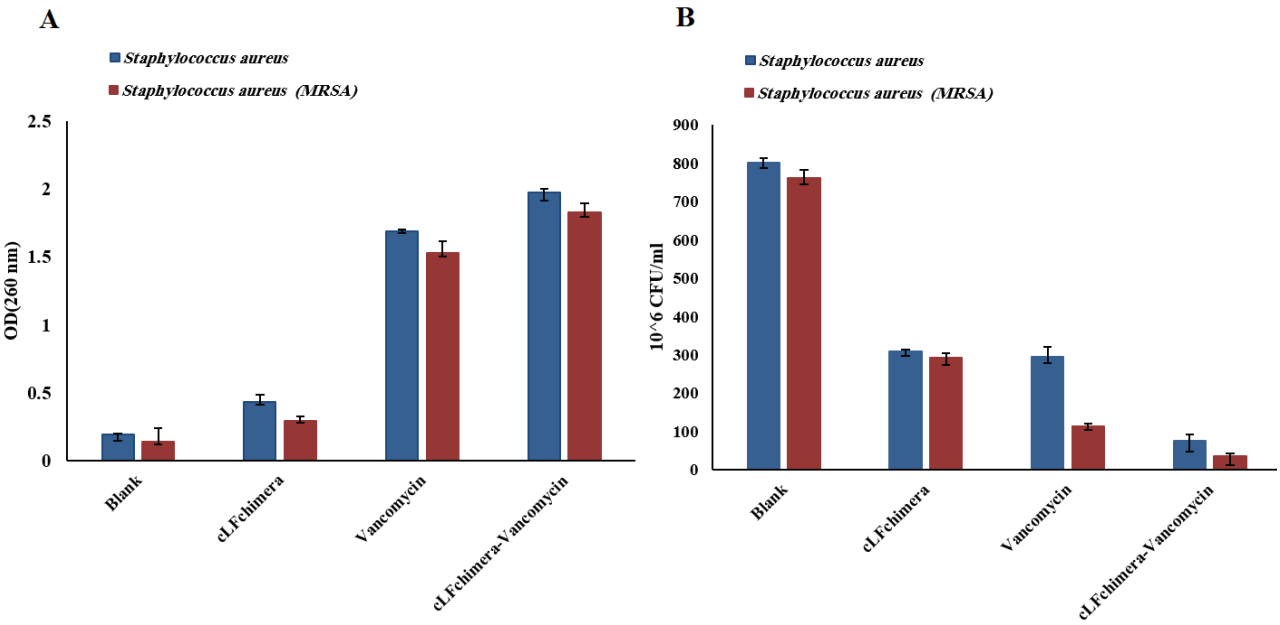


Figure 2.
Leakage of cytoplasmic material (OD at 260 nm) (A) and viable cell concentration in CFU/ml after incubation (B). Blank: without any treatment.

Table 1.
The MIC/MBC, FIC, and FIC Index of peptide and vancomycin against bacterial strains.

Microorganism	cLFchimera (µg/ml)		vancomycin (µg/ml)		FIC (µg/ml)		FIC index cLFchimera + Vancomycin
	MIC	MBC	MIC	MBC	cLFchimera	Vancomycin	
S. aureus (MSSA)	256	> 512	16	32	16	8	0.375[a]
S. aureus (MRSA)	512	> 512	32	64	64	4	0.375[a]

MSSA: Methicillin-sensitive *Staphylococcus aureus;* MRSA: Methicillin-resistant *Staphylococcus aureus*; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; FIC: Fractional inhibitory concentration ; [a]: Total synergism

vival of bacterial cells at MIC concentration of vancomycin and/or cLFchimera in comparison with FIC concentration of the combination of vancomycin and cLFchimera were higher and lower, respectively ($p \leq 0.05$).

Morphological study of S. aureus treated with peptide and vancomycin

For a better understanding of the effect of cLFchimera, vancomycin, and their combination (cLFchimera + vancomycin) on the bacterial morphology, the treated bacteria were observed using SEM (Figure 3). *S. aureus* was treated with cLFchimera (256 µg /mL), vancomycin (32 µg/mL), and their combination (4 and 64 µg/mL for antibiotic and peptide, respectively), and any damaging effects were observed with SEM (Figure 3). The shape of the bacteria treated with vancomycin, cLFchimera, and their combination did not differ greatly from the control group (blank, Figure 2A), suggesting that perhaps some molecular-level mechanisms that interact with peptidoglycan precursors, may disrupt efflux pumps, and cause irreversible changes in the flip-flop of membrane phospholipids [22] and may play important roles in the synergistic action of cLFchimera-vancomycin.

Discussion

Increasing the treatment difficulty and complexity regarding MRSE strains due to incorrect antibiotic use is currently a global issue in human health. AMPs are one of the newest and most promising classes of potent antibacterial drugs that can be considered as an alternative to antibiotics because they have useful features such as broad-spectrum antimicrobial activity and distinct membrane action mechanisms and are less likely to induce drug resistance in comparison to current antibiotics [23-25]. Furthermore, the effects of AMPs combined with antibiotics as a combination therapy often exceed those of the individual drugs, reduce the dose of drugs to minimize adverse effects, and thus be a way to overcome problems with toxicity and the development of resistance [26]. AMPs facilitate the passage of conventional small-molecule antibiotics through the membrane by disrupting the cell membrane, increasing access to these components to the cell, and exerting synergistic effects [27, 28].

The present study confirmed the synergistic effect between cLFchimera and vancomycin as the currently used antibiotic, against *S. aureus* and MRSA strains. When used as monotherapy, vancomycin showed moderate antibacterial activity against *S. aureus* and MRSA strain, with MIC values of 16 and 32 µg/mL, respectively. However, these antibacterial activities were significantly improved (2–8-fold reduced MIC values) in combination with the cLFchimera at a low concentration equivalent to 8-16 of the MICs. In addition, a total synergistic effect was also observed for the combination of the peptides and vancomycin against both strains in this study by using checkerboard assays. Similar to our results, Wu et al., (2017) showed that vancomycin had a high synergistic activity with a combination of DP7 peptides against *S. aureus* isolates [29].

The release of cellular content in treated samples with cLFchimera was significantly lower than in the antibiotic-treated groups. These results led us to hypothesize that cLFchimera exerts its antibacterial activity from other pathways instead of membrane disruption. Moreover, the results of SEM analysis showed that cLFchimera had no visible damaging effect on the outer layer of *S. aureus* as a model of Gram-positive bacteria, suggesting that maybe a molecular-level mechanism plays an important role in the synergistic action of cLFchimera. In this regard, Reyes-Cortes et al. (2016) showed that this chimeric peptide mediates its antibacterial activity by entering the cytoplasm through translocation across the bacterial membrane and possibly by interacting with internal organelles [30]. Consistent with these results, Pirkhezranian et al., using molecular simulation analysis showed that cLFchimera and its derivatives had a higher affinity for DNA interaction and hypothesized this chimeric peptide mediates its activity by intramolecular mechanisms which is the interference of DNA related pathways such as DNA replication [31, 32]. Moreover, treatment of both strains with vancomycin and its combination (vancomycin + peptide) resulted in significantly increased release of cellular contents. Vancomycin inhibits cell wall biosynthesis in Gram-positive bacteria by binding to D-alanine residues in the glycopeptide chain and thereby inhibiting cell wall synthesis [33]. Therefore, our results regarding the release of cellular content fit well with the mode of action of vancomycin.

Our results suggest that the cLfchimera may be used as promising synergistic agents to improve the antibacterial effectiveness of vancomycin against *S. aureus* and MRSA strains and to reduce the therapeutic dose of antibiotics, thus minimizing their toxic side effects. Overall, our results may suggest that cLFchimera mediates its synergistic activity independent of antibiotics by disrupting the cell membrane and intramolecular mechanisms which requires more investigation in future studies.

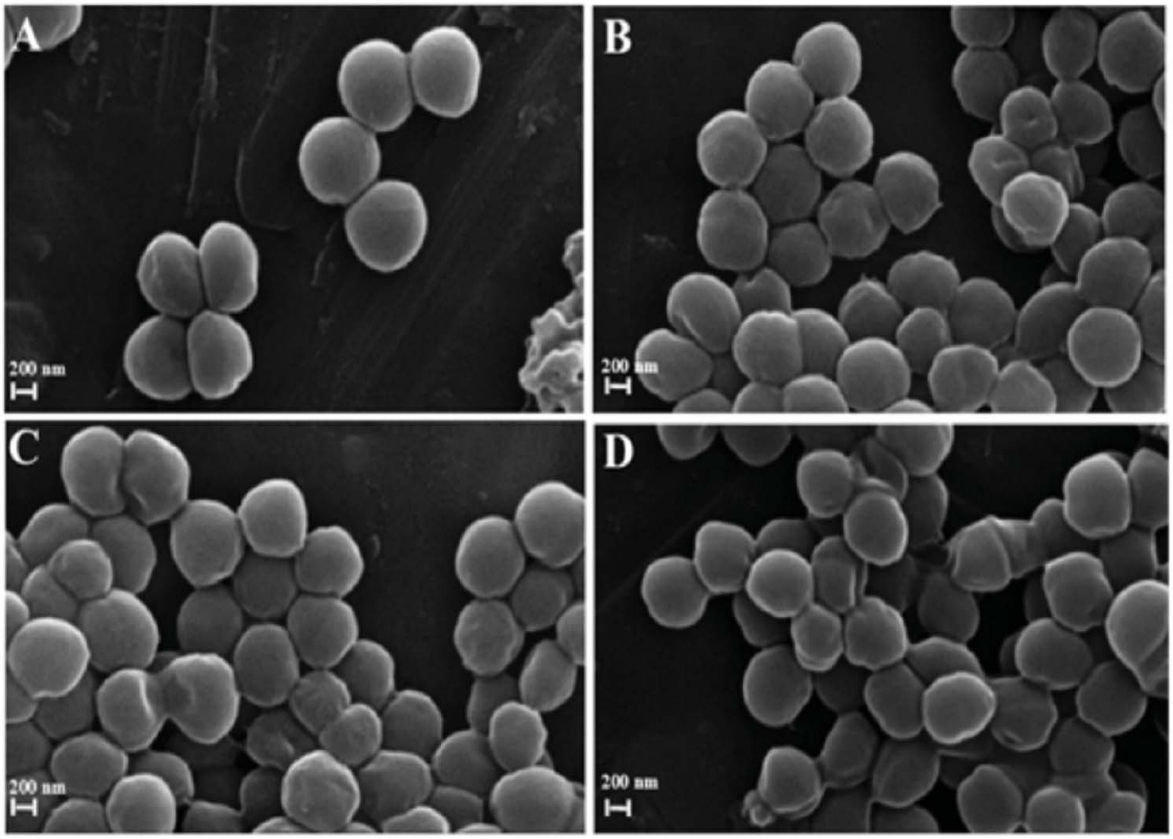


Figure 3.
SEM of *S. aureus* without vancomycin (A) and with 32 µg/mL vancomycin (B), with 256 µg /mL cLFchimera (C), and with the combination of 4 µg/mL vancomycin and 64 µg/mL cLFchimera (D).

Materials & Methods

Preparation of cLFchimera and antibiotic concentrations

The cLFchimera recombinant peptide developed in our previous study [10], was prepared n a sterile culture medium at 1000 mg/ml concentration and was filtered with a 0.22µm filter. This stock solution was used to prepare other dilutions [34]. Vancomycin was purchased from Jaber Ebne Hayyan Pharmaceutical Company, Tehran, Iran, and prepared according to CLSI [35].

Preparation of inoculum

The bacterial strains were obtained from the microbial collection of the Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad. Strains were *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* (MRSA) ATCC 33591. The microbial strains were cultured in Muller Hinton Broth (MHB) (Sigma-Aldrich) for 24 hours at 37 °C, standardized with the 0.5 McFarland standard, and antimicrobial tests were performed according to the instructions [36], equivalent to 1.5×10^8 CFU/mL of microorganism.

Determination of minimum inhibitory concentration

MIC was performed using the micro broth dilution as suggested by the Clinical and Laboratory Standards Institution [34]. The cLFchimera dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, 512 µg/mL) were prepared in sterile MHB. 20 µL of microbial suspensions with an optical density of 630 nm (OD630) equal to 0.08-0.13, was added to 190 µL of each dilution in 96-well plates. The microwell plates were incubated at 37 °C for 24 h. ELISA reader model BioTek ELx808 was used to consider absorbance at 630 nm to determine the MICs. The protocol was repeated at the same concentrations for each microorganism using vancomycin. Growth medium without inoculum was used as negative control and MIC was defined as the lowest concentration with no growth of microorganism [37]. The experiments were repeated three times to confirm the results.

Determination of minimum bactericidal concentration

100 µl of each well, in which microbial growth was not observed according to the previous section, was cultured on Müller Hinton agar (Sigma-Aldrich). Then, the plates were incubated at 37 °C for 24 h and MBC was defined as the lowest concentration with no observable colony of microorganism [38]. The experiments were repeated three times to confirm the results.

Checkerboard assay to analyze the synergistic interaction between cLFchimera and antibiotic

The synergistic interactions between cLFchimera peptide and vancomycin were measured by the checkerboard method [17, 39]. Seven numerals of two-fold serial dilutions (from 2MIC to MIC/32) of the cLFchimera and vancomycin according to obtained MIC in the previous section for each microorganism were prepared. An equal amount (25 µl) of each dilution was poured into 96-well microplates to obtain a fixed amount of both antimi-

crobial peptides. Therefore, each row (and column) contained a fixed amount of the first agent and increased amounts of the second one. A total of 50 µl of fresh bacteria suspension (108 CFU/ml) were added to each well and cultured at 37˚C. The Fraction Inhibitory Concentration Index (FICI) was calculated using the following formula:

$FIC_I = MIC_{A/B} / MIC_A + MIC_{B/A} / MIC_B$

In the above formula, MIC_A and MIC_B belong to compounds A and B, respectively. $MIC_{A/B}$ belongs to the MIC of compound A in combination with B. Total synergism ($FIC_I \leq 0.5$), partial synergism ($0.5 < FIC_I \leq 0.75$), Indifference ($0.75 < FIC_I \leq 2$) or antagonism ($FIC_I > 2$) between the two compounds were obtained using FIC_I [40].

Survival Curve

The effect of cLFchimera and Vancomycin were evaluated and combination on the growth of microbial strains through the construction of a survival curve was illustrated [41]. The final concentration of suspension of the strains (adjusted to 10^6-10^8 CFU/ml) was added to the wells of 96-well microplates, and 50µl of the antimicrobial agent (at MICs or FICs concentrations), was added to each well. The bacterial strains were cultured at 37˚C for 24 h. After incubating, a 50 µl liquid from each dilution was spread on the surface of the agar plates and were incubated at 37 °C for 24 h, eventually, the number of CFU/ml was counted. It is worth noting that 50µl of the microbial suspensions without antimicrobial agents were used as a control group. Finally, survival curves were constructed using plotting the log number of CFU/ml against time (h).

Release of cytoplasmic material absorbing at 260 nm

The suggested method by Fadli et al. (2012) was used to measure the release of cytoplasmic material at 260 nm. Viable cells in their exponential phase were collected using centrifugation (4000 rpm for 15 min.), washed three times, and resuspended in a saline buffer solution. Three milliliters of cell suspension of approximately 108 UFC/ml were incubated, under agitation, for 1 h at 37 °C in the presence of the antimicrobial agent (at MICs or FICs concentrations). After incubation, cells were centrifuged at 4000 rpm for 20 min, and the absorbance (260 nm) of the supernatant was determined using a WPA Lightwave S2000 UV/Vis Spectrophotometer (Richmond Scientific Ltd, England). The untreated cells (control) were corrected with buffer saline [16].

Scanning electron microscopy

The bacterial strains were cultured to the logarithmic phase in 100 ml of MHB at 37 °C. The suspension was divided into four portions. Antimicrobials were added to three of the portions at MICs or FICs concentrations. The remaining portion was left untreated as a control. The resuspension was incubated at 37 °C for 3 h, and subsequently, the cells from all four tubes were harvested through centrifugation and fixed with 2.5% glutaraldehyde overnight at 4 °C. Subsequently, the cells were dehydrated using sequential ethanol concentrations ranging from 30 to 100%. The samples were gold-covered through cathodic spraying. The morphology of the bacterial cells was observed through scanning electron microscopy (SEM, LAO-1450VP, Germany) [17] .

Authors' Contributions

Conceptualization, SR and FS; methodology, SR and AS; software, ZP; validation, FS and MHS; investigation, MHS; original draft preparation, SR; review and editing, SR and ZP; supervision, FS; funding acquisition, FS. All authors have read and confirmed the final version of the manuscript.

Acknowledgements

This study was supported by the Ferdowsi University of Mashhad, Grant number 2/50305.

Competing Interests

The authors declare that they have no competing interests.

References

1. Stapleton PD, Taylor PW. Methicillin resistance in Staphylococcus aureus: mechanisms and modulation. Science Progress. 2002;85(1):57-72.

2. Lowy FD. Antimicrobial resistance: the example of Staphylococcus aureus. The Journal of Clinical Investigation. 2003;111(9):1265-73.

3. Peacock SJ, Paterson GK. Mechanisms of methicillin resistance in Staphylococcus aureus. Annual Review of Biochemistry. 2015;84: 577-601.

4. Walsh C, Wencewicz T. Antibiotics: challenges, mechanisms, opportunities: John Wiley & Sons; 2016.

5. Zeng D, Debabov D, Hartsell TL, Cano RJ, Adams S, Schuyler JA, et al. Approved glycopeptide antibacterial drugs: mechanism of action and resistance. Cold Spring Harbor Perspectives in Medicine. 2016;6(12):1-16.

6. Wang S, Zeng X, Yang Q, Qiao S. Antimicrobial peptides as potential alternatives to antibiotics in food animal industry. International Journal of Molecular Sciences. 2016;17(5):603-615.

7. Linde A, Ross C, Davis E, Dib L, Blecha F, Melgarejo T. Innate immunity and host defense peptides in veterinary medicine. Journal of Veterinary Internal Medicine. 2008;22(2):247-265.

8. Pirkhezranian Z, Tanhaeian A, Mirzaii M, Sekhavati MH. Expression of Enterocin-P in HEK platform: evaluation of its cytotoxic effects on cancer cell lines and its potency to interact with cell-surface glycosaminoglycan by molecular modeling. International Journal of Peptide Research and Therapeutics. 2019:1-10.

9. Rodríguez-Rojas A, Moreno-Morales J, Mason AJ, Rolff J. Cationic antimicrobial peptides do not change recombination frequency in Escherichia coli. Biology Letters. 2018;14(3):777-782.

10. Tanhaiean A, Azghandi M, Razmyar J, Mohammadi E, Sekhavati MH. Recombinant production of a chimeric antimicrobial peptide in E. coli and assessment of its activity against some avian clinically isolated pathogens. Microbial Pathogenesis. 2018;122:73-85.

11. Tanhaieian A, Sekhavati MH, Ahmadi FS, Mamarabadi M. Heterologous expression of a broad-spectrum chimeric antimicrobial peptide in Lactococcus lactis: Its safety and molecular modeling evaluation. Microbial Pathogenesis. 2018;125:51-69.

12. Tanhaeian A, Ahmadi FS, Sekhavati MH, Mamarabadi M. Expression and purification of the main component contained in camel milk and its antimicrobial activities against bacterial plant pathogens. Probiotics and Antimicrobial Proteins. 2018;10(4):787-93.

13. Tahmoorespur M, Azghandi M, Javadmanesh A, Meshkat Z, Sekhavati MH. A Novel Chimeric Anti-HCV Peptide Derived from Camel Lactoferrin and Molecular Level Insight on Its Interaction with E2. International Journal of Peptide Research and Therapeutics. 2019:1-13.

14. Tanhaeian A, Jaafari MR, Ahmadi FS, Vakili-Ghartavol R, Sekhavati MH. Secretory expression of a chimeric peptide in Lactococcus lactis: assessment of its cytotoxic activity and a deep view on its interaction with cell-surface glycosaminoglycans by molecular modeling. Probiotics and Antimicrobial Proteins. 2019;11(3):1034-1041.

15. Daneshmand A, Kermanshahi H, Sekhavati MH, Javadmanesh A, Ahmadian M. Antimicrobial peptide, cLF36, affects performance and intestinal morphology, microflora, junctional proteins, and immune cells in broilers challenged with E. coli. Scientific Reports. 2019;9(1):1-9.

16. Fadli M, Saad A, Sayadi S, Chevalier J, Mezrioui N-E, Pagès J-M, et al. Antibacterial activity of Thymus maroccanus and Thymus broussonetii essential oils against nosocomial infection–bacteria and their synergistic potential with antibiotics. Phytomedicine. 2012;19(5):464-471.

17. Liu H, Pei H, Han Z, Feng G, Li D. The antimicrobial effects and synergistic antibacterial mechanism of the combination of ε-Polylysine and nisin against Bacillus subtilis. Food Control. 2015;47:444-450.

18. Maisetta G, Mangoni ML, Esin S, Pichierri G, Capria AL, Brancatisano FL, et al. In vitro bactericidal activity of the N-terminal fragment of the frog peptide esculentin-1b (Esc 1–18) in combination with conventional antibiotics against Stenotrophomonas maltophilia. Peptides. 2009;30(9):16-22.

19. Gupta K, Singh S, Van Hoek ML. Short, synthetic cationic peptides have antibacterial activity against Mycobacterium smegmatis by forming pores in membrane and synergizing with antibiotics. Antibiotics. 2015;4(3):358-378.

20. Regmi S, Choi YH, Choi YS, Kim MR, Yoo JC. Antimicrobial peptide isolated from Bacillus amyloliquefaciens K14 revitalizes its use in combinatorial drug therapy. Folia Microbiologica. 2017;62(2):127-138.

21. Nuding S, Frasch T, Schaller M, Stange EF, Zabel LT. Synergistic effects of antimicrobial peptides and antibiotics against Clostridium difficile. Antimicrobial Agents and Chemotherapy. 2014;58(10):5719-5725.

22. Zasloff M. Antimicrobial peptides in health and disease. New England Journal of Medicine. 2002;347(15):1199-1215.

23. Michael N, Stuart B. Molecular mechanisms of antibacterial multidrug resistance.Cells. 2000;128(3): 1037-1050.

24. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nature Reviews Microbiology. 2005;3(3):238-250.

25. Niyonsaba F, Ushio H, Hara M, Yokoi H, Tominaga M, Takamori K, et al. Antimicrobial peptides human β-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. The Journal of Immunology. 2010;184(7):3526-3534.

26. Soren O, Brinch KS, Patel D, Liu Y, Liu A, Coates A, et al. Antimicrobial peptide novicidin synergizes with rifampin, ceftriaxone, and ceftazidime against antibiotic-resistant Enterobacteriaceae in vitro. Antimicrobial Agents and Chemotherapy. 2015;59(10):6233-6240.

27. Zhou Y, Peng Y. Synergistic effect of clinically used antibiotics and peptide antibiotics against Gram-positive and Gram-negative bacteria. Experimental and Therapeutic Medicine. 2013;6(4):1000-1004.

28. Shang D, Liu Y, Jiang F, Han X. Synergistic antibacterial activity of Trp-containing antibacterial peptides in combination with antibiotics against multidrug-resistant Staphylococcus epidermidis. Frontiers in Microbiology. 2019;10:27-39.

29. Wu X, Li Z, Li X, Tian Y, Fan Y, Yu C, et al. Synergistic effects of antimicrobial peptide DP7 combined with antibiotics against multidrug-resistant bacteria. Drug Design, Development and Therapy. 2017;11:939-1012.

30. Reyes-Cortes R, Acosta-Smith E, Mondragón-Flores R, Nazmi K, Bolscher JG, Canizalez-Roman A, et al. Antibacterial and cell penetrating effects of LFcin17–30, LFampin265–284, and LF chimera on enteroaggregative Escherichia coli. Biochemistry and Cell Biology. 2017;95(1):76-81.

31. Pirkhezranian Z, Tahmoorespur M, Daura X, Monhemi H, Sekhavati MH. Interaction of camel Lactoferrin derived peptides with DNA: a molecular dynamics study. BMC Genomics. 2020;21(1):60-72.

32. Pirkhezranian Z, Tahmoorespur M, Monhemi H, Sekhavati MH. Computational Peptide Engineering Approach for Selection the Best Engendered Camel Lactoferrin-Derive Peptide with Potency to Interact with DNA. International Journal of Peptide Research and Therapeutics. 2020:1-10.

33. Kapoor G, Saigal S, Elongavan A. Action and resistance mechanisms of antibiotics: A guide for clinicians. Journal of Anaesthesiology, Clinical Pharmacology. 2017;33(3):300-305.

34. van der Kraan MI, van Marle J, Nazmi K, Groenink J, van't Hof W, Veerman EC, et al. Ultrastructural effects of antimicrobial peptides from bovine lactoferrin on the membranes of Can-

dida albicans and Escherichia coli. Peptides. 2005;26(9):1537-1542.

35. CLSI. Performance Standards for Antimicrobial Susceptibility Testing 28th ed CLSI supplement M100 Wayne, PA: Clinical and Laboratory Standards Institute. 2018.

36. Amin Mir M, Sawhney S, Manmohan SJ. Antimicrobial Activity of Various Extracts of Taraxacum officinale. Journal of Microbial and Biochemical Technology. 2016;8(3):210-215.

37. Habibipour R, Moradi Haghgou L. Study on hydro-alcoholic extract effect of pomegranate peel on Pseudomonas aeruginosa biofilm formation. Scientific Journal of Hamadan University of Medical Sciences. 2015;22(3):195-202.

38. Wendakoon C, Calderon P, Gagnon D. Evaluation of selected medicinal plants extracted in different ethanol concentrations for antibacterial activity against human pathogens. Journal of Medicinally Active Plants. 2012;1(2):60-68.

39. Roshanak S, Shahidi F, Tabatabaei F, Javadmanesh A. Evaluation of Antimicrobial Activity of Buforin I and Nisin and Synergistic Effect of the Combination of them as a Novel Antimicrobial Preservative. The Journal of Food Protection. 2020;43(1):140-153.

40. Mackay M, Milne K, Gould I. Comparison of methods for assessing synergic antibiotic interactions. International Journal of Antimicrobial Agents. 2000;15(2):125-134.

41. Magalhães L, Nitschke M. Antimicrobial activity of rhamnolipids against Listeria monocytogenes and their synergistic interaction with nisin. Food Control. 2013;29(1):138-142.

How to cite this article
Roshanak S, Shahidi F, Soradeghi Toopkanloo A, Pirkhezranian Z, Sekhavatib MH. Evaluation of antibacterial activity of cLFchimera and its synergistic potential with vancomycin against methicillin-resistant *Staphylococcus* aureus. Iran J Vet Sci Technol. 2022; 14(1): 1-8.
DOI: https://doi.org/10.22067/ijvst.2021.72596.1084
URL:https://ijvst.um.ac.ir/article_41006.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Dec- 23
Accepted after revision: 2021- Mar- 05
Published online: 2022- Apr- 01

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2022.72134.1071

Role of central opioid receptors on serotonin-Induced hypophagia in the neonatal broilers

Behrouz Rahmani[a], Kimia Mahdavi[a], Morteza Zendehdel[a], Mina Khodadadi[a], Maryam Keshavarz[a], Ali Baghbanzadeh[a], Majid Shahabi[b]

[a] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
[b] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

ABSTRACT

Serotonin (5-HT) plays an underpinning role in appetite regulation and the opioid system has a role in the modulation of the ingestion behavior in birds. The current survey was aimed to evaluate the effect of opioid receptors on serotonin-induced hypophagia in neonatal broilers. During experiments, food-deprived chickens received intracerebroventricular (ICV) injection and thereafter, the cumulative food intake was measured after 30, 60, and 120 minutes. In experiment 1, to determine the effective dose of serotonin, the control solution and the various doses of serotonin (2.5, 5, and 10 µg) were administered to birds. In the second experiment, groups received not only the control solution, but also an effective dose of serotonin (10 µg), µ-opioid receptor antagonist (β_FNA, 5 µg), and a co-injection of β_FNA (5 µg) and serotonin (10 µg), respectively. The next experiments were similar to the second experiment, however, in place of β_FNA, the antagonist of κ- opioid receptor (nor_BNI, 5 µg), the δ- opioid receptor antagonist (NTI, 5 µg), and the agonist of µ opioid receptor (DAMGO, 62.25 pmol) were used in experiments 3, 4, and 5, respectively. The results showed a dose-dependent hypophagic impact of serotonin. This effect was attenuated by β_FNA; however, nor_BNI and NTI had no effect. Furthermore, the diminishing effect of serotonin on food consumption in chickens was strengthened following DAMGO administration ($p < 0.05$). According to the results, the hypophagic effect of serotonin is possibly mediated through µ opioid receptors in neonatal broilers.

Keywords

Serotonin; central opioid receptors; food intake; anorexigenic effects; broilers

Number of Figures: 5
Number of Tables: 2
Number of References:: 57
Number of Pages: 11

Abbreviations

5-HT: 5-hydroxytryptamine
ICV: intracerebroventricular
β_FNA: beta-funaltrexamine
NTI: naltrindole
nor_BNI: norbinaltorphimine
DAMGO: [D-Ala, N-MePhe, Gly-ol]-enkephalin

Corresponding author: *Mina Khodadadi*
Email: : *mina.khodadadi@alumni.ut.ac.ir*
Tel: *+98 (21)6111-7081*

Introduction

The appetite regulation is one of the interesting topics for research in physiology and nutrition sciences nowadays. Ingestion habits are modulated via external factors such as environmental and dietary alterations as well as the internal ones, those are in relevance with the gastrointestinal, hormonal, and brain elements [1]. In this respect, central control of appetite is related to the function of various neurotransmitters and related neuronal pathways in the central nervous system (CNS) [2]. Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter with some roles in physiologic functions such as sleep, circadian rhythm, motor control, pain perception, and behaviors such as mood, anxiety, aggressiveness, depression, and so on [3-5]. The serotonergic neurons are primarily located in the raphe nuclei, central grey and reticular formation in the CNS, and seven main classes of 5-HT receptors called 5-HT1–5-HT7, have been discovered and categorized as G protein-coupled receptors (GPCRs) [6]. It has been revealed that the central serotonergic system has a key role in the modulation of the ingestion behavior in different species. Based on the former studies, the ICV injection of 5-HT decreased food intake in chickens [7-10].

It has been demonstrated that opioids are a kind of inhibitory neurotransmitters in the brain. The opioid receptors are categorized into three main types containing mu (μ), delta (δ), and kappa (κ) [11]. Opioid receptors exist within the vast regions of the CNS especially septo-hypothalamic one [12]. A myriad of studies has illustrated the effect of the central opioidergic system on pain perception, respiration control, and immune system response [13]. Recently, the interest to study the effect of central opiates in the regulation of food intake has increased [14]. But it seems that the involvement of endogenous opiates in food intake has been assigned controversial results. For example, in mammals, the ICV injection of μ- and δ-, receptors' agonist increased food intake while κ-opioid one had no same effect [15,16]. However, in avian species, the ICV injection of μ-opioid receptors agonist could decrease food consumption, and the administration of δ- and κ-opioid receptors agonists enhanced it [17]. Based on the literature, similar to mammals, 6 opioid peptides encrypted by proenkephalin (PENK), pro-opiomelanocortin (POMC), prodynorphin (PDYN), and 4 opioid receptors were considered as highly-preserved ones in chickens. Also, it has been suggested that the ligand-receptor pairs of the chicken opioidergic system are similar to those of mammals, while it is not identical [18]. By taking into consideration of revealed differences among species, several studies showed that the feeding behavior was stimulated by μ-opioid receptor activation in broilers [19,20]. Presumably, food intake behavior is controlled via neuroendocrine and the balance of energy is a complex process in which a whole host of overlapping integrated pathways have potential roles. In this view, the possibility of the interaction between endogenous opioids and other neurotransmitters has been suggested by different research studies [21-23]. To exemplify, in some previous studies, the interaction of the central opioidergic system with oxytocin, histaminergic, and dopaminergic systems have been demonstrated [24-26].

In terms of the interplay between the opioidergic and serotonergic systems at the level of the CNS, several studies, such as those on nociception, have detected an interaction between these two systems [27]. However, there is no report available concerning the evaluation of possible interconnection between these two systems in food intake behavior, especially in avian species. Since different opioid receptor subtypes were found in raphe nuclei in which 5-HT are the major neurotransmitter [28], and in consideration of the effects of both opioidergic and serotonergic systems on appetite, we designed and performed this study to investigate the possible effect of the central opioid system on feeding behavior related to serotonin in broilers.

Results

In the first experiment, the ICV injection of 2.5 μg serotonin had no important effect on food consumption in comparison with the control in none of the time points ($p \geq 0.05$), while the ICV administration of 5 and 10 μg of serotonin noticeably and dose-dependently declined the consumption of food compared with the control group at all the time-points ($p < 0.05$). The results suggested a dose-dependent hypophagic impact of serotonin on the eating habit of neonatal meat-type chicken (Fig. 1).

Regarding experiment 2, the hypophagic effect of serotonin was remarkably attenuated by β_FNA pretreatment in chickens compared to the control group at all the time points ($p < 0.05$). In addition, β_FNA alone did not affect food intake. The data suggested that the hypophagic effect of serotonin was mediated through the central receptors of μ opioid in broilers (Fig. 2).

In the next experiment neither ICV administration of 5 μg nor_BNI nor ICV co-injection of 5 μg nor_BNI plus 10 μg serotonin altered the hypophagic impact of serotonin at different time points compared with the control group ($p \geq 0.05$). These results suggested that the hypophagic effect of serotonin was not

mediated via the central kappa opioid receptors in chickens (Fig. 3).

In the fourth experiment, administration of 5 µg NTI made no major change in cumulative food consumption compared to the control group at all the time points. The hypophagic effect of serotonin was not altered by the addition of 5 µg NTI and 10 µg serotonin rather than the control group at all times ($p \geq 0.05$). This information suggested that, regarding the hypophagic effect of serotonin, delta-opioid receptors do not have a mediatory role (Fig. 4).

In experiment 5, the hypophagic effect of serotonin was noticeably increased by administration of 62.25 pmol DAMGO in FD3 chicks than the control group at all the time points after injection ($p < 0.05$), while 62.25 pmol DAMGO alone had no impact on food intake in comparison with the control group. This suggests the hypophagic effect of serotonin in broilers is possibly mediated via µ opioid receptors (Fig. 5).

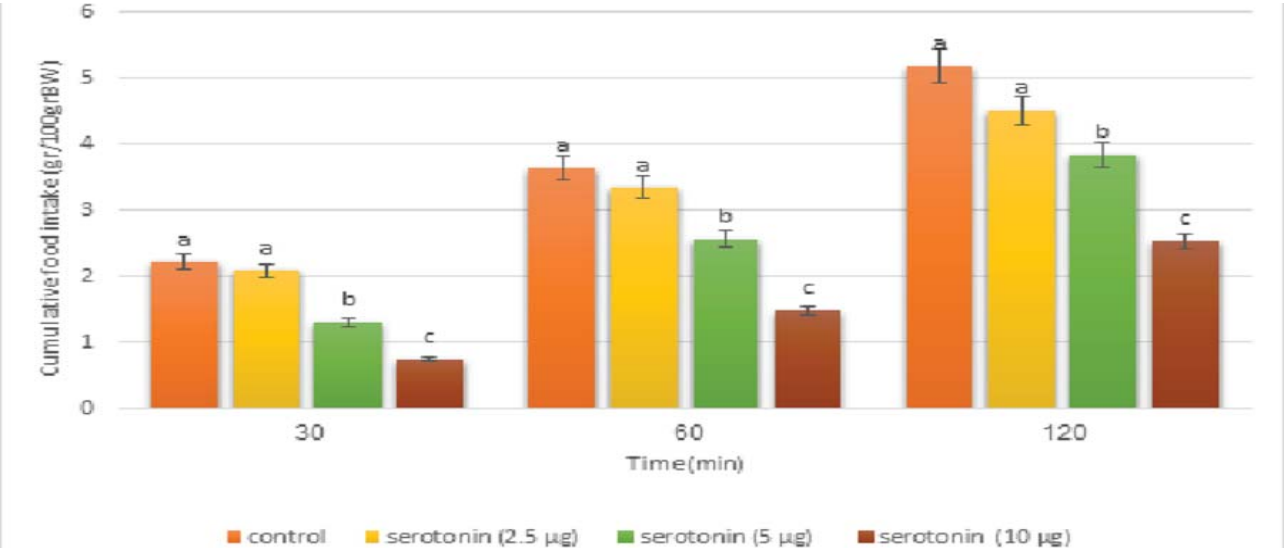


Figure 1.
Effects of ICV injection of different doses of serotonin (2.5, 5 and 10 µg) on cumulative food intake (gr/100gr BW) in neonatal chicks (n=44). Data are expressed as mean ± SEM. Different letters (a, b and c) indicate significant differences between treatments at each time ($P < 0.05$).

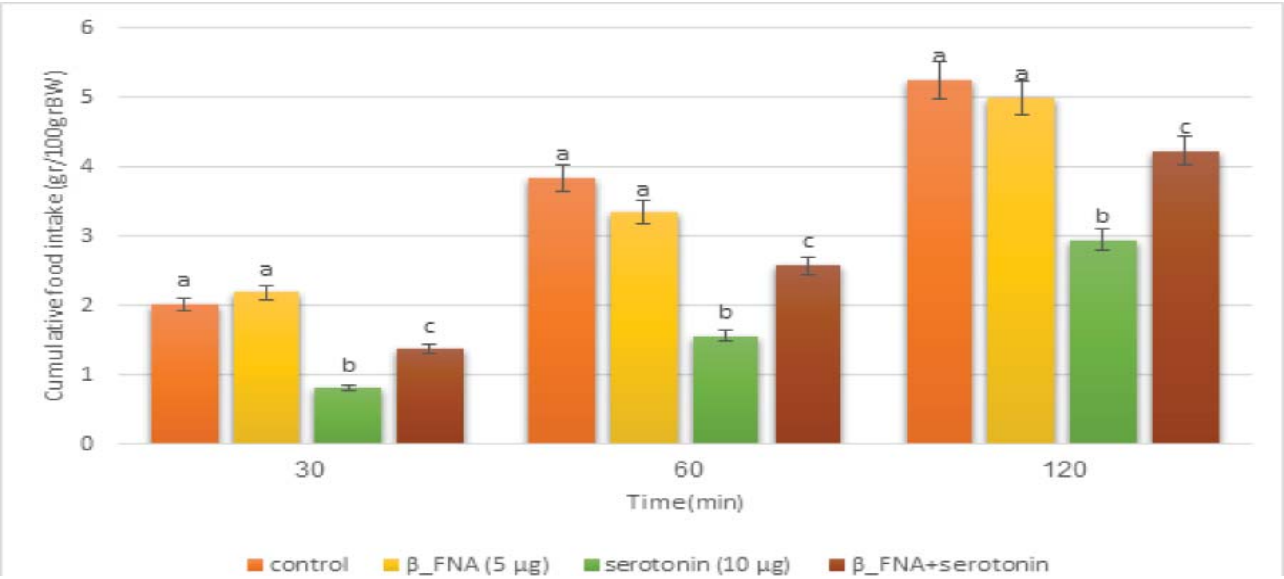


Figure 2.
Effects of intracerebroventricular injection of control solution, serotonin (10 µg), β–FNA (5 µg) and a combination of serotonin plus β–FNA on cumulative food intake (gr/100gr BW) in neonatal chicks (n=44). β–FNA: µ receptor antagonist. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time ($p < 0.05$).

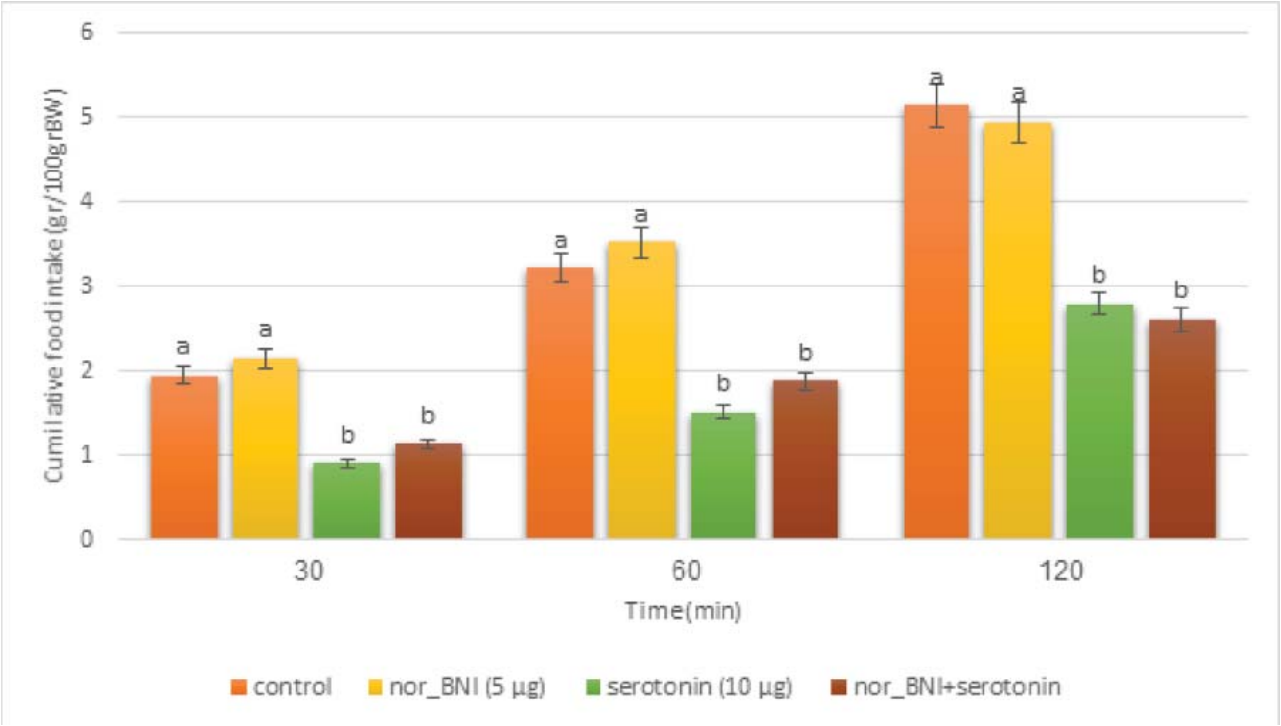


Figure 3.
Effects of intracerebroventricular injection of control solution, serotonin (10 µg), nor-BNI (5 µg) and a combination of serotonin plus nor-BNI on cumulative food intake (gr/100gr BW) in neonatal chicks (n=44). nor-BNI: Kappa receptor antagonist. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time ($p < 0.05$).

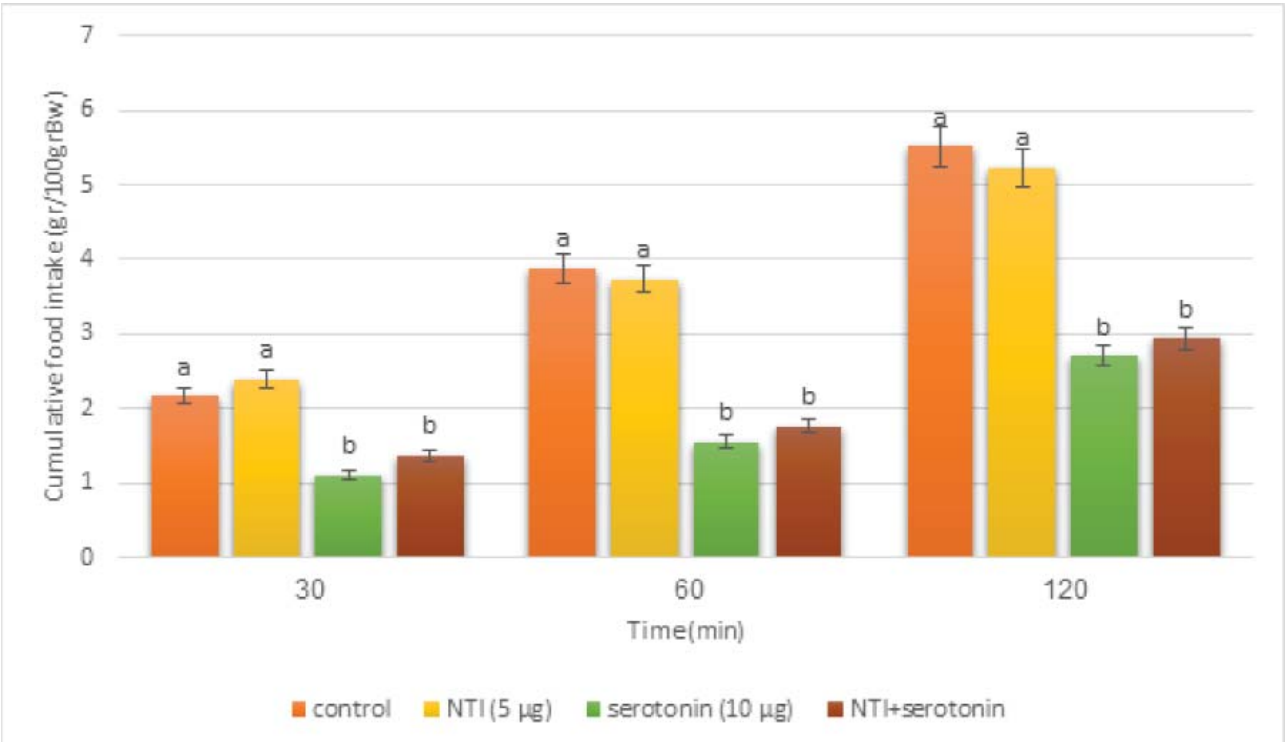


Figure 4.
Effects of intracerebroventricular injection of control solution, serotonin (10 µg), NTI (5 µg) and a combination of serotonin plus NTI on cumulative food intake (gr/100gr BW) in neonatal chicks (n=44). NTI: Delta receptor antagonist. Data are expressed as mean ± SEM. Different letters (a and b) indicate significant differences between treatments at each time ($p < 0.05$).

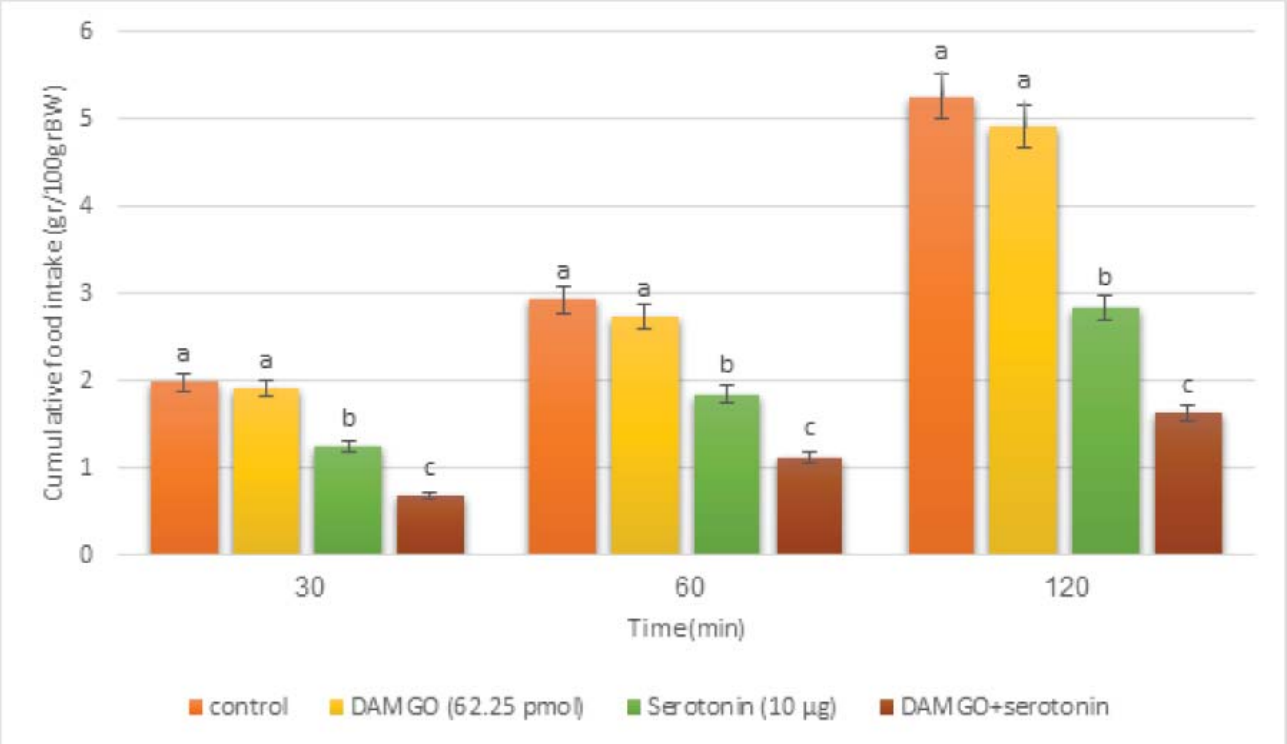


Figure 5.
Effects of intracerebroventricular injection of control solution, serotonin (10 µg), DAMGO (62.25 pmol) and a combination of serotonin plus DAMGO on cumulative food intake (gr/100gr BW) in neonatal chicks (n=44). DAMGO: µ receptor agonist. Data are expressed as mean ± SEM. Different letters (a, b, and c) indicate significant differences between treatments at each time ($p < 0.05$).

Discussion

This report is the first one concerning the interplay of opioidergic receptors in hypophagic behavior which is induced by serotonin in broilers. In the current study, serotonin decreased food intake in neonatal broilers, which agreed with former surveys in some birds' breeds besides mammals [29-32]. In consideration of the receptor's subtype, serotonergic receptors have different effects on appetite and food consumption. It seems that in mammals the appetite is attenuated via 5-HT2c receptors while 5-HT1A receptors do not affect that [9,10]. Although in one study done on adult rats, the 5-HT1A receptor showed a pivotal effect on water consumption [33]. Although the mechanism of appetite regulation varies between mammals and birds, a large amount of 5-HT receptors has been found in different regions of the CNS in both species such as the hypothalamus and the prefrontal cortex and its homologous the pallial part of the birds' brain [34]. Also, pharmacological studies in mammals showed that 5-HT receptors have a decreasing effect on food intake; in this regard, 5-HT2c receptors located in POMC neurons might have a contributing role in appetite regulation. This latter has highlighted that the serotonergic system modulates the ingestion behavior through the melanocortin pathway [3,7]. Likewise, the 5HT2c receptors can regulate appetite through several other neurotransmitters, such as dopamine and ghrelin [9,10]. Since lack of similar molecular investigation in the birds' brain, this pathway might be similar to those in mammals at least at the level of septo-hypothalamic regions, which is more conservative in avian species than the mammalian ones. Moreover, in consideration of the function of the opioid receptor in ingestion behavior, the role of various kinds of receptors has been illustrated. For instance, one research on rodents has depicted that the ICV administration of the agonists of µ and δ-, but not κ-, could induce the orexigenic behavior [15,16]. Interestingly, the research on poultry has revealed the hypophagic effect induced by µ-opioid receptor activation and the orexigenic function of δ- and κ-opioid receptors, during ICV injection of opioid agonists [17].

In terms of the interplay between two central systems in chickens' brains, it sounds like the serotonin signaling is only mediated by µ opioid receptors. In our study, the co-administration of β_FNA, the antagonist of µ opioid receptor, with serotonin significantly blocked hypophagia which is induced by serotonin in neonatal broilers. In addition, the hypophagic effect induced by serotonin was remarkably increased by administration of DAMGO, the agonist of µ opioid receptor, however nor_BNI and NTI, κ- and δ- receptors antagonists, had no impact on hypophagia induced by serotonin.

The feeding behavior can be modulated within

many regions of the brain such as the striatum, hypothalamus, amygdala, etc. While the arcuate nucleus of the hypothalamus (ARC) has a prominent role among all, it has a lot of neurotransmitters with complicated interactions with one another to regulate appetite in mammals and birds. Finally, the net output of all these interactions can regulate energy expenditure in living creatures [20]. In mammals, it has been shown that at the site of ARC appetite is mainly regulated by releasing different neuropeptides and proteins from higher-order neurons located in this nucleus [36]. In addition, it is suggested that in the ARC, the μ-opioid receptors are contributed to the hyperphagic properties of neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons [12]. The conspicuous role of ARC in food intake regulation has been demonstrated within avian species [35,36]. Although it seems that there are similar pathways in the hypothalamus, more complementary molecular investigations are needed in the future.

Furthermore, the interaction between the μ-opioid receptors and the serotonergic system has been demonstrated in the previous research on the different properties of opioids in mammals. For instance, the co-administration of the agonists of the μ-opioid receptors and the 5-HT receptor agonists has shown the postulated antinociceptive effects [27]. According to several research studies, a wide distribution of opioid receptors has been indicated in different brain regions with a regulatory function in the feeding behavior such as ARC, NAc, amygdala, and NTS [37]. The 5-HT containing neurons originated from the raphe nuclei innervate different parts of the brain such as the ARC and NAc [9, 38]. Under the administration of serotonin or its reuptake inhibitor, the *in vivo* microdialysis assessment in rats has shown the increasing levels of beta-endorphins within ARC and NAc [39]. This, in turn, has postulated an interplay between serotonin and opioid receptors in these nuclei. By taking into account the effect of different brain regions such as ARC and NAc nuclei in feeding behavior and the detected interaction between the serotonergic and opioidergic systems in the mentioned nuclei, it is proposed that this interaction may be one of the mechanisms in which the food intake can be regulated. In terms of the observation of the interplay between serotonin and opioid receptors, this finding can be in line with our obtained results, which showed a mediatory effect of opioid receptors in the serotonin-induced behavior and inverse. However, the exact mechanism remains to be experimentally determined in birds.

From a different aspect of view, anatomical studies have illustrated that μ, δ, and κ opioid receptors are expressed in raphe nuclei. Owing to the existence of various subtypes of receptors in both systems, the explanation of the opioid-5-HT interaction is to some extent difficult. In this relation, researchers have presented that the μ- and δ-opioid receptors stimulation increased the levels of serotonin in raphe nuclei, even though the activation of κ-opioidergic receptors diminished it [28]. In addition, a significant role of 5-HT in opioid biosynthesis has been mentioned [40]. Nevertheless, the exact process of the interaction between μ-opioid and 5-HT receptors needs more investigation [28]. The evidence revealed that the post-synaptic 5-HT2c receptor is located in different neuronal systems such as GABAergic, with a high level of heterogeneity in the rat and human CNS cells [10]. In this respect, previous research referred to the possibility of an indirect act of the μ-opioid receptors on the 5-HT2C receptors by inhibiting local GABAergic neurons. As we know this GABAergic neurons synapse on serotonergic neurons in the raphe nuclei [28].

In terms of the determination of the underlying mechanisms for opioid- 5-HT interaction, the involvement of other neurotransmitters and neuropeptides should be considered. For instance, it seems that the opioidergic and serotonergic systems might be regulated via other peptides in the CNS [41], the functional assessment of this neuropeptide in the modulation of the opioid- 5-HT interaction, is a notable subject that needs to be determined in the future research studies. Similar to the opioid receptors, the serotonin receptor couples to Gi/o. Consequently, extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinase (MAPK) signaling pathways will be active by activation of 5-HT1A [42]. Furthermore, different signaling pathways such as activation of p38 and ERK1/2 MAPK are indicated by opioid's effect [43]. Related to the mediatory effect of the 5-HT2C receptor on μ-opioid receptors, the major role of the dopaminergic system is already seen in mammals, which is in agreement with our findings [44]. To sum, the role of the μ-opioid receptors in terms of regulation in food intake and its direct and/or indirect interplay with the serotonin is a complicated matter which needs more investigation. The new findings showed herein could be a starting spot for more investigation in this field on broiler-type chicken. Undoubtedly, in this respect, future surveys are needed to explain the direct and/or indirect relevant neurological pathway(s).

Materials & Methods

Animals

220 male neonatal one-day hatched chicks (Ross 308) were prepared (from Mahan Company nearby Tehran, Iran). At first,

all birds were placed in a group and followed for 2 days. Then they were put in individual cages. In this study, the electrically heated cages with a stabilized temperature of 32 °C ± 1 were employed. The relative humidity of the housing room was set at 40–50%, and the 23:1 lighting/dark period was determined [45]. Chickens had access without any limitation to a diet of the commercial starter (Table 1). On the 5th day of age, the ICV injections were performed on birds. Three hours before ICV injections, all birds were deprived of food, while they freely access water. All the experimental procedures were performed based on the US Guide (publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics Committee of Faculty of Veterinary Medicine, University of Tehran.

Drugs

Drugs consisted of serotonin, β_FNA (antagonist of μ opioid receptor), nor_BNI (antagonist of the kappa-opioid receptor), NTI (antagonist of the delta-opioid receptor), DAMGO (an agonist of μ opioid receptor), and color as Evans Blue (Sigma, USA). Drugs were prepared in a solution of absolute dimethyl sulfoxide (DMSO) which were diluted with 0.85% NaCl 0.9% plus color at the proportion of 1:250. The using DMSO has no cytotoxic effects [46,47]. The mixture of DMSO/ Saline containing color was used as a control solution.

ICV

Chickens were divided into 5 experiments (n=44) in such a way that every experiment involved 4 groups (n = 11). Before performing the experiments, the birds were accurately weighed and distributed into different groups. Averagely, body weight (BW) between understudied groups was uniform. In each experiment, the ICV injection was performed by a microsyringe (Hamilton, Switzerland) without the need for anesthesia (1979) and Furse et al. (1997) methods [48,49]. In brief, by using an acrylic device consisting of a holder of a bill at 45 degrees, the calvarium of the chicken head was being placed parallel to the table surface, as was stated previously [50]. Immediately after the head positioning, a hole was made over the right lateral ventricle of the brain. Via the orifice, the tip of the needle penetrated 4 mm below the skull [51]. All drugs were administered in the volume of 10 μL via the ICV route [52] and the control group merely received the solution of control (10 μL). It should be mentioned that the procedure made no physiological stress in newly hatched birds [53]. In the end, the chicken was decapitated (according to AVMA Guidelines for the Euthanasia of Animals 'No: M3.6, cervical dislocation), and the preciseness of the injection site was evaluated based on the method published in our previous publications.

Food intake measurement

The experiment procedure and all the details are described in Supplementary file 1. Also, the design of the experiments is shown in Table 2. Following performing the ICV injections, birds were returned to their cages with available food which was pre-weighed and freshwater. 30, 60, and 120 minutes after injections, and the cumulative food intake was measured. To adjust the diversity among the weights, all measurements were calculated as %BW. The dosage of the drugs was determined based on the pilot and previous research studies [54-57].

Statistical Analysis

As mentioned before, cumulative food intake was applied to analysis as extracted data. The statistical analysis was done by using repeated measure two-way analysis of variance (ANOVA) and the outcomes were raised as mean ± SEM. The analytical procedure was the same as previous publications [54-57].

Table 1.
Ingredient and nutrient analysis of experimental diet

Ingredients	%	Nutrient analysis	
Corn	52.85	ME (kcal/g)	2850
Soybean meal, 48% CP	31.57	Crude protein (%)	21
Wheat	5	Linoleic acid (%)	1.69
Gluten meal, 61% CP	2.50	Crude fiber (%)	3.55
Wheat bran	2.47	Calcium (%)	1
Di-calcium phosphate	1.92	Available phosphorus (%)	0. 5
Oyster shell	1.23	Sodium (%)	0.15
Soybean oil	1.00	Potassium (%)	0.96
Mineral premix	0.25	Chlorine (%)	0.17
Vitamin premix	0.25	Choline (%)	1.30
Sodium bicarbonate	0.21	Arginine (%)	1.14
Sodium chloride	0.20	Isoleucine (%)	0.73
Acidifier	0.15	Lysine (%)	1.21
dl-Methionine	0.10	Methionine (%)	0.49
Toxin binder	0.10	Methionine + cystine (%)	0.83
l-Lysine HCl	0.05	Threonine (%)	0.70
Vitamin D3	0.1	Tryptophan (%)	0.20
Multi enzyme	0.05	Valine (%)	0.78

ME: metabolisable energy, CP: crude protein, per kg of diet, the mineral supplement contains 35.2 g manganese from $MnSo_4 \cdot H_2O$; 22 g iron from $FeSO_4 \cdot H_2O$; 35.2 g zinc from ZnO; 4.4 g copper from $CuSO_4 \cdot 5H_2O$; 0.68 g iodine from ethylene diamine dihydroiodide; 0.12 g seleniumfrom $Na2SeO_3$. The vitamin supplement contains 1.188 g of retinyl acetate, 0.033 g of dl-α-tocopheryl acetate, 8.84 g of tocopherol, 1.32 g of menadione, 0.88 g of thiamine, 2.64 g of riboflavin, 13.2 g of nicotinic acid, 4.4 g of pantothenic acid, 1.76 g of pyridoxin, 0.022 g of biotin, 0.36 g of folic acid, 1500 mg of choline chloride

Table 2.
Intracerebroventricular injections in experiments

Groups	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
A	CS[a]	CS	CS	CS	CS
B	Serotonin (2.5 µg)	β_FNA[b] (5 µg)	nor_BNI[c] (5 µg)	NTI[d] (5 µg)	DAMGO[e] (62.25 pmol)
C	Serotonin (5 µg)	Serotonin (10 µg)	Serotonin (10 µg)	Serotonin (10 µg)	Serotonin (10 µg)
D	Serotonin (10 µg)	β_FNA+serotonin (5 µg)+(10 µg)	nor_BNI+serotonin (5 µg)+(10 µg)	NTI+serotonin (5 µg)+(10 µg)	DAMGO+serotonin (62.25 pmol)+(10 µg)

[a] Control solution
[b] µ opioid receptor antagonist
[c] kappa opioid receptor antagonist
[d] delta opioid receptor antagonist
[e] µ opioid receptor agonist

Authors' Contributions

M.Z. conceived and planned the experiments. K.M. carried out the experiments. M.Z. and M.KH. contributed to the interpretation of the results. M.KH took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and the manuscript.

Acknowledgements

This study was assisted by the authors' self-fund.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Sharkey KA, Darmani NA, Parker LA. Regulation of nausea and vomiting by cannabinoids and the endocannabinoid system. European Journal of Pharmacology. 2014; 722:134–146.

2. Parker KE, Johns HW, Floros TG, Will MJ. Central amygdala opioid transmission is necessary for increased high-fat intake following 24-h food deprivation, but not following intra-accumbens opioid administration. Behavioural Brain Research. 2014;260: 131–138.

3. Nonogaki K, Ohba Y, Sumii M, Oka Y. Serotonin systems upregulate the expression of hypothalamic NUCB2 via 5-HT2C receptors and induce anorexia via leptin-independent pathway in mice. Biochemical and Biophysical Research Communications. 2008; 372:186–190.

4. Nonogaki K, Kaji T, Ohba Y, Sumii M, Wakameda M, Tamari T. Serotonin 5-HT2C receptor-independent expression of hypothalamic NOR1, a novel modulator of food intake and energy balance, in mice. Biochemical and Biophysical Research Communications. 2009; 386:311–315.

5. Zendehdel M, Mokhtarpouriani K, Babapour V, Baghbanzadeh A, Pourrahimi M, Hassanpour S. The effect of serotonergic system on nociceptin/orphanin FQ induced food intake in chicken. The Journal of Physiological Sciences. 2013; 63:271–277.

6. Idova GV, Alperina EL, Cheido MA. Contribution of brain dopamine, serotonin and opioid receptors in the mechanisms of neuroimmunomodulation: evidence frompharmacological analysis. International Immunopharmacology. 2012; 12:618–625.

7. Zendehdel M, Taati M, Jonaidi H, Amini E. The role of central 5-HT (2C) and NMDA receptors on LPSinduced feeding behavior in chickens. The Journal of Physiological Sciences. 2012; 62:413–419.

8. Zendehdel M, Hamidi F, Babapour V, Mokhtarpouriani K, Fard RM. The effect of melanocortin (Mc3and Mc4) antagonists on serotonin-induced food and water intake of broiler cockerels. The Journal of Veterinary Science. 2012; 13:229–234.

9. Zendehdel M, Mokhtarpouriani K, Hamidi F, Montazeri R. Intracerebroventricular injection of ghrelin produces hypophagia through central serotonergic mechanisms in chicken. Veterinary Research Communications. 2013; 37:37–41.

10. Zendehdel M, Hasani K, Babapour V, Mortezaei SS, Khoshbakht Y, Hassanpour S. Dopamine-induced hypophagia is mediated by D1 and 5HT-2c receptors in chicken. Veterinary Research Communications. 2014; 38:11–19.

11. Le Merrer J, Becker JAJ, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. Physiological Reviews. 2009; 89:1379–1412.

12. Barnes MJ, Holmes G, Primeaux SD, York DA, Bray GA. Increased expression of mu opioid receptorsin animals susceptible to diet-induced obesity. Peptides. 2016; 27:3292–3298.

13. Solati J. Dorsal hippocampal N-methyl-d-aspartate glutamatergic and δ-opioidergic systems modulate anxiety behaviors in rats in a noninteractive manner. Kaohsiung Journal of Medical Science. 2011; 27:485–493.

14. Kozlov AP, Nizhnikov M, Kramskaya TA, Varlinskaya EI, Spear NE. Mu-opioid blockade reduces ethanol effects on intake and behavior of the infant rat during short-term but not long-term social isolation. Pharmacology, Biochemistry and Behavior. 2013; 103:773–782.

15. Taha SA. Preference or fat? Revisiting opioid effection food intake. Physiology and Behavior. 2010; 100:429–437.

16. Kaneko K, Yoshikawa M, Ohinata K. Novel orexigenic pathway prostaglandin D2–NPY system – Involvement in orally active orexigenic δ opioid peptide. Neuropeptides. 2012; 46:353–357.

17. Bungo T, Kawamura K, Izumi T, Dodo K, Ueda H. Effects of various μ-, δ- and κ-opioid ligands on food intake in the meat-type chick. Physiology and Behavior. 2005; 85:519–523.

18. Bu G, Cui L, Lv C, Lin D, Huang L, Li Z, Li J, Zeng X, Wang Y. Opioid Peptides and Their Receptors in Chickens: Structure, Functionality, and Tissue Distribution. Peptides. 2020; 128, 170307.

19. Dodo K, Izumi TH, Ueda H, Bungo T. Response of neuropeptide Y-induced feeding to μ-, δ- and κ-opioid receptor antagonists in the neonatal chick. Neuroscience Letters. 2005; 373:85–88.

20. Shiraishi J, Yanagita K, Fujita M, Bungo T. μ-Opioid receptor agonist diminishes POMC gene expression and anorexia by central insulin in neonatal chicks. Neuroscience Letters. 2008; 439:227–229.

21. Bodnar RJ. Endogenous opiates and behavior. Peptides. 2012; 38:463–522.

22. Guy EG, Choi E, Pratt WE. Nucleus accumbens dopamine and mu-opioid receptors modulate the reinstatement of food-seeking behavior by food associated cues. Behavioural Brain Research. 2011; 219:265–272.

23. Echo JA, Lamonte N, Ackerman TF, Bodnar RJ. Alterations in food intake elicited by GABA and opioid agonists and antagonists administered into the ventral tegmental area region of rats. Physiology and Behavior. 2004; 76:107–116.

24. Raji-Dahmardeh F, Vazir B, Zendehdel M, Asghari A, Panahi N. Interaction Between Oxytocin and Opioidergic System on Food Intake Regulation in Neonatal Layer Type Chicken. International Journal of Peptide Research and Therapeutics. 2020; 26:1905–1912.

25. Jaefari-Anari M, Zendehdel M, Gilanpour M, Asghari A, Babapour V. Central Opioidergic System Interplay with Histamine on Food Intake in Neonatal Chicks: Role of μ-Opioid and H1/H3 Receptors. Brazilian Journal of Poultry Science. 2018; 20(3):595-604.

26. Zendehdel M, Ghashghayi E, Hassanpour S, Baghbanzadeh A, Jonaidi H. Interaction Between Opioidergic and Dopaminergic Systems on Food Intake in Neonatal Layer Type Chicken. International Journal of Peptide Research and Therapeutics. 2016; 22:83–92.

27. Li J, Koek W, Rice KC, France CP. Effects of direct- and indirect-acting serotonin receptor agonists on the antinociceptive and discriminative stimulus effects of morphine in rhesus monkeys. Neuropsychopharmacology. 2011; 36:940–949.

28. Kirby LG, Zeeb FD, Winstanley CA. Contributions of serotonin in addiction vulnerability. Neuropharmacology. 2011; 61:421–432.

29. Denbow DM. Body temperature and food intake of turkeys following ICV injections of serotonin. Nutrition and Behavior. 1983; 1:301-308.

30. Denbow DM, Van Krey HP, Lacy MP, Dietrick TJ. Feeding, drinking and body temperature of Leghorn chicks: effects of ICV injections of biogenic amines. Physiology and Behavior. 1983; 31:85–90.

31. Baranyiova Eva. Effects of serotonin on the food intake in chickens in the post hatching period. Acta Veterinaria. 1990; 23-33.

32. Gobert A, Rivet JM, Lejeune F. serotonin receptors tonically suppress the activity of mesocortical dopaminergic and adrenergic but not serotonergic pathways a combined dialysis and electrophphysiological analysis in the rat. Synapse. 2000; 36:205-201.

33. Villa SP, Menani JV, De Arruda Camargo GMP, De Arruda Camargo LA, Saad WA. Activation of the serotonergic 5-HT1A receptor in the paraventricularnucleus of the hypothalamus inhibits water intake and increases urinary excretion in water-deprived rats. Peptides. 2008; 150:14–20.

34. Martin-Ruiz R, Puig MV, Celada P, Shapiro DA, Roth BL, Mengod G, Artigas F. Control of serotonergic function in medial prefrontal cortex by serotonin-2A receptors through a glutamate-dependent mechanism. Neuroscience. 2001; 21:9856–9866.

35. Zendehdel M, Hassanpour S. Ghrelin-induced hypophagia is mediated by the β2 adrenergic receptor in chicken. The Journal of Physiological Sciences. 2014; 645:383– 391.

36. Tachibana T, Tsutsui K. Neuropeptide Control of Feeding Behavior in Birds and Its Difference with Mammals. Frontiers in Neuroscience. 2016; 10:485.

37. Shojaei M, Yousefi AR, Zendehdel M, Khodadadi M. The Roles of Neurotransmitter on Avian Food and Appetite Regulation. Iranian Journal of Veterinary Medicine. 2020; 14(1):99-115.

38. Zendehdel M, Baghbanzadeh A, Babapour V, Cheraghi J. The efects of bicuculline and muscimol onglutamate-induced feeding behavior in broiler cockerels. The Journal of Comparative Physiology. 2009; 195:715–720.

39. Zangen A, Nakash R, Yadid G. Serotonin-mediated increases in the extracellular levels of beta-endorphin in the arcuate nucleus and nucleus accumbens: a microdialysis study. Journal of Neurochemistry. 2000; 73(6):2569-2574.

40. Di Benedetto M, D'Addario C, Collins S, Lzenwasser S, Candeletti S, Romualdi P. Role of serotonin on cocaine-mediated effects on prodynorphin gene expression in the rat brain. The Journal of Molecular Neuroscience. 2004; 22:213–222.

41. Rothman RB, Vu N, Wang X, Xu H. Endogenous CART peptide regulates mu opioid and serotonin 5-HT2A receptors. Peptides. 2003; 24:413-417.

42. Polter AM, Li X. 5-HT1A receptor-regulated signal transduction pathways in brain. Cellular signalling. 2010; 22(10):1406-1412.

43. Cussac D, Rauly-Lestienne I, Heusler P, et al. μ-Opioid and 5-HT1A receptors heterodimerize and show signalling crosstalk via G protein and MAP-kinase pathways. Cellular signalling. 2012; 24(8):1648-1657.

44. Navailles S, De Deurwaerdere P, Porras G, Spampinato U. In vivo evidence that 5-HT2C receptor antagonist but not agonist modulates cocaine-induced dopamine outflow in the rat nucleus accumbens and striatum. Neuropsychopharmacology. 2004; 29:319-326.

45. Olanrewaju HA, Thaxton P, Dozier WA, Purswell J, Roush WB, Branton SL. A review of lighting programs for broiler production. International Journal of Poultry Science. 2006; 5(4):301–308.

46. Blevins JE, Stanley BG, Reidelberger RD. DMSO as a vehicle for central injections: tests with feeding elicited by norepinephrine injected into the paraventricular nucleus. Pharmacology, Biochemistry and Behavior. 2002; 71:277–282.

47. Qi W, Ding D, Salvi RJ. Cytotoxic efects of dimethyl sulphoxide (DMSO) on cochlear organotypic cultures. Hearing Research. 2008; 236:52–60.

48. Davis JL, Masuoka DT, Gerbrandt LK, Cherkin A. Autoradiographic distribution of L-proline in chicks after intracerebral injection. Physiology and Behavior. 1979; 22:693–695.

49. Furuse M, Matsumoto M, Okumura J, Sugahara K, Hasegawa S. Intracerebroventricular injection of mammalian and chicken glucagon-like peptide-1 inhibits food intake of the neonatal chick. Brain Research. 1997; 755:167–169.

50. Van Tienhoven A, Juhasz LP. The chicken telencephalon, diencephalon and mesencephalon in sterotaxic coordinates. The Journal of Comparative Neurology. 1962; 118:185–197.

51. Jonaidi H, Noori Z. Neuropeptide Y-induced feeding is dependent on GABAA receptors in neonatal chicks. The Journal of Comparative Physiology. 2012; 198:827–832.

52. Furuse M, Ando R, Bungo T, Ao R, Shimo JOM, Masuda Y. Intracerebroventricular injection of orexins does not stimulate food intake in neonatal chicks. British Poultry Science. 1999; 40:698–700.

53. Saito S, Tachibana T, Choi YH, Denbow DM, Furuse M. ICV melatonin reduces acute stress responses in neonatal chicks. Behavioural Brain Research. 2005; 165(2):197-203.

54. Ahmadi F, Zendehdel M, Babapour V, Panahi N. CRF1/CRF2 and MC3/MC4 receptors afect glutamate-induced food intake in neonatal meat-type chicken. Brazilian Journal of Poultry Science. 2019; 21(1):1–10.

55. Heidarzadeh H, Zendehdel M, Babapour V, Gilanpour H. The effect of Nesfatin-1 on food intake in neonatal chicks: role of CRF1 /CRF2 and H1/H3 receptors. Veterinary Research Communications. 2017; 42(1):39–47.

56. Newmyer BA, Cline MA. Neuropeptide SF is associated with reduced food intake in chicks. Behavioural Brain Research. 2009; 205(1):311–314.

57. Cline MA, Bowden CN, Calchary WA, Layne JE. Short-term anorexigenic effects of central neuropeptide VF are associated with hypothalamic changes in chicks. Journal of Neuroendocrinology. 2008; 20(8):971–977.

How to cite this article
Rahmani B, Mahdavi K, Zendehdel M, Khodadadi M, Keshavarz M, Baghban Zadeh A,Shahabi M. Role of central opioid receptors on serotonin-Induced hypophagia in the neonatal broilers. Iran J Vet Sci Technol. 2022;14(1): 9-19.
DOI: https://doi.org/10.22067/ijvst.2022.72134.1071
URL: https://ijvst.um.ac.ir/article_41006.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Jul- 12
Accepted after revision: 2022- Feb- 12
Published online: 2022- Mar- 01

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2022.71423.1059

Evaluation of resistance to fluoroquinolones and determination of mutations in *gyr*A and *par*C genes in *Escherichia coli* isolated from raw milk of dairy cows with coliform mastitis in Khorasan Razavi province, Iran

Mahdie Mahdavi[a], Behrooz Fathi[b], Abdollah Jamshidi[c], Babak Khoramian[d]

[a] Graduated from Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[b] Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[c] Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[d] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

The present study was performed to assess the resistance profile to fluoroquinolone and to determine mutations in gyrA and parC genes of *Escherichia coli* in bovine coliform mastitis. Fluoroquinolones (norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (NFX), levofloxacin (LEV), and ofloxacin (OFL) were tested against *E. coli* isolates, isolated from bovine mastitis (100 milk samples) by disk diffusion method. To determine the extent of gyrA and parC mutations associated with fluoroquinolone resistance in *E. coli*, two isolates with the highest resistance to each fluoroquinolone were submitted for the amplification and sequencing of the quinolone resistance-determining regions (QRDRs) of gyrA and parC genes. The disk diffusion method indicated that *E. coli* isolates had the highest intermediate resistance to OFL (16.7%), followed by NFX and NOR (15%), while they had low resistance to CIP and LEV (3.33%). A few silent mutations in gyrA (in codons 91, 100, 111, 131, 132) and in parC (in codons 91, 157, 159) were detected in QRDRs, and mutations in nucleotides 65, 80, and 83 in gyrA, and 195, 209, 212 in parC were detected in the other isolate. These results showed an intermediate rate of resistance to fluoroquinolones in *E. coli* isolates from raw milk of cows with coliform mastitis

Keywords

Escherichia coli; fluoroquinolone resistance; gyrA gene; mastitis, parC gene

Number of Figures: 7
Number of Tables: 3
Number of References:: 37
Number of Pages: 9

Abbreviations

E. coli: *Escherichia coli*
FQ: Fluoroquinolone
QRDR: Quinolone resistance determining region
MC: MacConkey
EMB: Eosin Methylene Blue

Corresponding author: *Behrooz Fathi*
Email: : *b-fathi@um.ac.ir*
Tel: *+98 (41)3880-5628*

Introduction

Mastitis is considered one of the most important diseases in dairy animals that causes severe losses to the dairy industry [1]. The economic losses due to clinical mastitis include production loss, lower milk yield and value, treatment expenses, and loss of animal value [2]. Coliforms such as *Escherichia*, *Klebsiella spp.*, and *Enterobacter spp.* are the most common etiological agents causing clinical mastitis [3]. *Escherichia coli* is the most common species isolated from coliform mastitis which is a Gram-negative, non-spore-forming rod bacterium that belongs to the family *Enterobacteriaceae* [4, 5]. Clinical signs of *E. coli* mastitis include a wide range from a mild disease with only local inflammation changes in the mammary gland to severe with systemic signs, generally including high fever, increased pulse frequency, lack of appetite, decreased milk production, dehydration, rumen stasis, shock, and death [6, 7]. In cases of mild to moderate *E. coli* mastitis, the use of anti-inflammatory drugs and supportive treatments is recommended. In peracute or acute cases of *E. coli* mastitis, due to the potential risk of bacterial growth in the mammary gland, which in turn may lead to bacteremia, administration of broad-spectrum antimicrobials is recommended to reduce the number of bacteria [8].

The fluoroquinolones are broad-spectrum and bactericidal antibiotics. They are used against gram-positive and especially gram-negative bacteria such as members of the *Enterobacteriaceae* family [9, 10]. They block DNA synthesis by targeting bacterial DNA gyrase and topoisomerase IV, both of which are essential for bacterial DNA supercoiling as the replicating strands separate [11]. DNA gyrase and topoisomerase IV are tetrameric structures composed of two pairs of subunits. The four subunits of DNA gyrase include 2 monomers of A and 2 monomers of B, with the names GyrA and GyrB, respectively. The topoisomerase IV also has ParC and ParE subunits, which are encoded by parC and parE genes, respectively [12].

The major mechanisms of resistance to quinolone antibiotics include mutations that occur at the target drug sites, mutations that reduce drug accumulation, and plasmid-mediated quinolone resistance [13]. The most common mechanism that produces significant levels of clinical resistance to fluoroquinolones is an alteration in the target enzymes. These changes are caused by self-mutations occurring within the responsible genes. Resistance to fluoroquinolones is due to the substitution of amino acids in a certain region of GyrA or ParC subunits [13, 14]. The broad-spectrum activity of quinolones against various infections and the widespread use of these antibiotics, the abuse and unnecessary use of them, especially in developing countries, has accelerated the development of resistance mechanisms [15].

Fluoroquinolones are used in the treatment of infectious diseases, including coliform mastitis caused by *E. coli*. Since the drug resistance pattern has regional distribution, determination of this pattern of *E. coli* resistance can be used to determine the appropriate treatment regimen for clinical coliform mastitis [16]. This study aimed to determine the resistance pattern of *E. coli* isolated from cows with coliform mastitis to some fluoroquinolones and also to detect the mutations in QRDR of fluoroquinolone-resistance *E. coli* isolates.

Results

Identification of E. coli

In this study, 100 milk samples were subjected to isolation of *E. coli* by selective plating followed by streaking on the Eosin Methylene Blue (EMB) agar at 37 ˚C for 24 h. Typical colonies of *E. coli* were produced from 45 samples. These 45 presumptive *E. coli* isolates on EMB agar were confirmed by biochemical tests (Figure 1).

Susceptibility testing

The results of susceptibility study showed that less than 20% of *E. coli* isolates had intermediate resistance to each antibiotic (Figure 2). Intermediate resistance was 3.33% to ciprofloxacin and levofloxacin, 15% to enrofloxacin and norfloxacin, and 16.7% to ofloxacin (Table 1).

Identification of gyrA and parC mutations in clinical isolates of E. coli

Amplification of the QRDRs of gyrA and parC genes was performed by PCR (Figure 3). The results of the DNA sequencing of gyrA and those of parC were consistent and provided information from both standards for a region between nucleotides 247 to 840 (corresponding to codons 82 to 280) of gyrA and from nucleotides 167 to 539 (corresponding to codons 55 to 180) of the parC gene, respectively (Table 2, Figures 4, 5, 6, and 7). Accession numbers of *E. coli* isolates based on QRDRs of gyrA and parC genes deposited in the GenBank are as follows: SRX5988183, SRX5982112 for sample number 2968, and SRR17711097, SRR17711096 for sample number 3077 (Accession to cite for these SRA data: PRJNA547542).

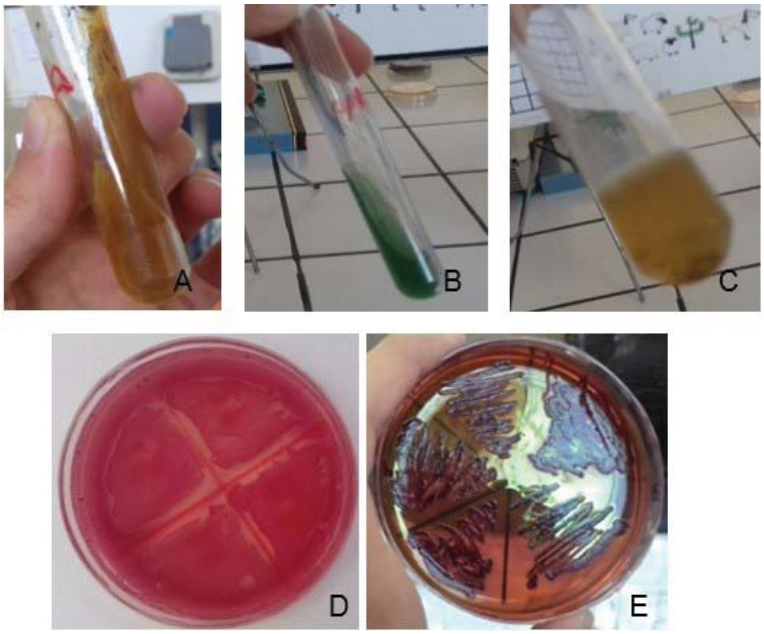


Figure 1.
Isolation and identification of *E. coli*. A) Triple Sugar Iron agar, acid/acid reaction with gas production and no H2S. B) Simmons citrate agar, the medium remained green. This is a negative result for citrate test. C) Sulfur Indole Motility (SIM) medium, *E. coli* is hydrogen sulfide negative, indole positive, and the cloudy appearance of the medium indicates that *E. coli* is motile. D) MacConkey agar, pink colonies. E) Eosin Methylene Blue agar, colonies of purple with black center and green metallic sheen.

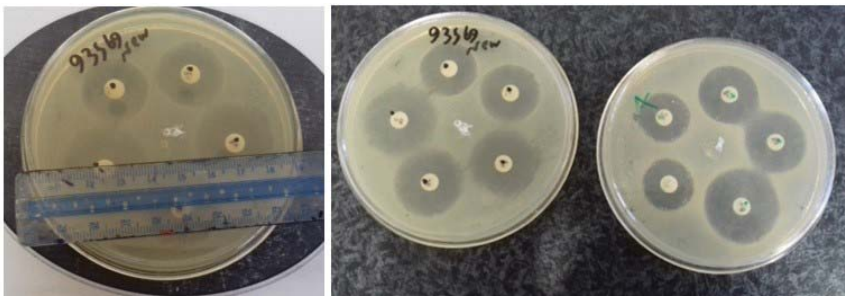


Figure 2.
The results of evaluation of resistance to fluoroquinolones with disk diffusion method. The scale is in mm.

Table 1.
Antibiotic resistance pattern of 60 *E. coli* isolates.

Antibiotic (µg)	No. of sensitive isolates	% sensitive isoates	% intermediate resistant isolates	% resistant isolates
Ofloxacin (5)	50	83.3	16.7	-
Enrofloxacin (5)	51	85	15	-
Norfloxacin (10)	51	85	15	-
Ciprofloxacin (5)	58	96.6	3.33	-
Levofloxacin (5)	58	96.6	3.33	-

Discussion

The focus of the current study was to assess the resistance of *E. coli* to some fluoroquinolones in bovine coliform mastitis and to generate the fluoroquinolones resistance profile of isolates. The level of resistance of *E. coli* isolates to enrofloxacin and norfloxacin was similar to the level of resistance to ofloxacin. This is due to the development of cross-resistance to one of the fluoroquinolones. In the present study, among all 60 *E. coli* isolates, less than 20% of isolates had intermediate resistance to fluoroquinolones. This is still a relatively low figure compared with other published studies, in which the proportion of resistant isolates has ranged from 23% to 63% [15, 17-19]. In the results from Su et al. (2016) *E. coli* isolates showed 4% resistance to ciprofloxacin and levofloxacin; similarly, *E. coli* isolates in the present study showed only 3.33% resistance to ciprofloxacin and levofloxacin whereas Metzger and Hogan (2013) found 12% of *E. coli* isolated from bovine milk samples were non-susceptible to ciprofloxacin [20, 21]. Among fluoroquinolones, enrofloxacin and norfloxacin resistance were found in nine (15%) *E. coli* isolates, and all other isolates

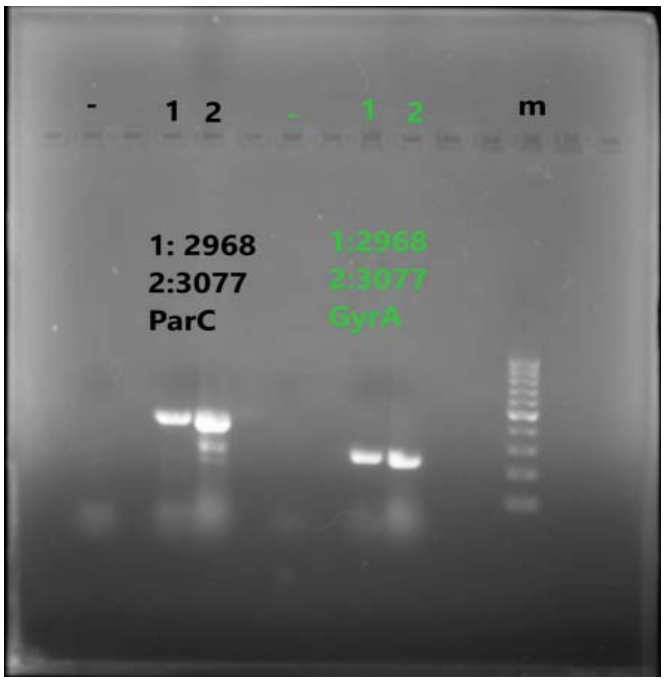


Figure 3.
PCR amplification of QRDR of gyrA and parC genes for *E. coli*.
Lane M: DNA marker; 100 bp plus. Lane 1, 2: test isolates. The expected product size of gyrA is 253 bp and the expected product size of parC is 434 bp.

Score	Expect	Identities	Gaps	Strand
261 bits(141)	2e-73	151/156(97%)	0/156(0%)	Plus/Minus

```
NC_000913.3 2227 CAGTTCATGGGCAATTTTCGCCAGACGGATTTCCGTATAACGCATTGCCGCCGCAGAGTC 2286
gyrA_2968   168  .........TT.............................................. 109

NC_000913.3 2287 GCCGTCGATAGAACCGAAGTTACCCTGACCGTCTACCAGCATATAACGCAGCGAGAATGG 2346
gyrA_2968   108  .........G................................G.............. 49

NC_000913.3 2347 CTGCGCCATGCGGACGATCGTGTCATAGACCGCCGA 2382
gyrA_2968   48   .........A........................... 13
```

Figure 4.
The result of alignment of the gyrA gene of sample number 2968. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 2227 to 2382 from NC_000913.3:2336793-2339420 Escherichia coli str. K-12 substr. MG1655, complete genome.

Score	Expect	Identities	Gaps	Strand
678 bits(367)	0.0	385/393(98%)	3/393(0%)	Plus/Plus

```
NC_000913.3 156 GGGCCTGAATGCCAGCGCCAAATTTAAAAAAT-CGGCCCGTACCGTCGGTGACGTACTGG 214
parC_2968   10  ...-...-.......................C.......................... 67

NC_000913.3 215 GTAAATACCATCCGCACGGCGATAGCGCCTGTTATGAAGCGATGGTCCTGATGGCGCAAC 274
parC_2968   68  ..............................................................G. 127

NC_000913.3 275 CGTTCTCTTACCGTTATCCGCTGGTTGATGGTCAGGGGAACTGGGGCGCGCCGGACGATC 334
parC_2968   128 ............................................................ 187

NC_000913.3 335 CGAAATCGTTCGCGGCAATGCGTTACACCGAATCCCGGTTGTCGAAATATTCCGAGCTGC 394
parC_2968   188 ............................................................ 247

NC_000913.3 395 TATTGAGCGAGCTGGGGCAGGGGACGGCTGACTGGGTGCCAAACTTCGACGGCACTTTGC 454
parC_2968   248 ............................................................ 307

NC_000913.3 455 AGGAGCCGAAAATGCTACCTGCCCGTCTGCCAAACATTTTGCTTAACGGCACCACCGGTA 514
parC_2968   308 ................G.....T..................................... 367

NC_000913.3 515 TTGCCGTCGGCATGGCGACCGATATTCCACCGC 547
parC_2968   368 ....................T.C..... 400
```

Figure 5.
The result of alignment the parC gene of sample number 2968. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 156 to 547 from NC_000913.3:c3165973_3163715 Escherichia coli str. K_12 substr. MG1655, complete genome.

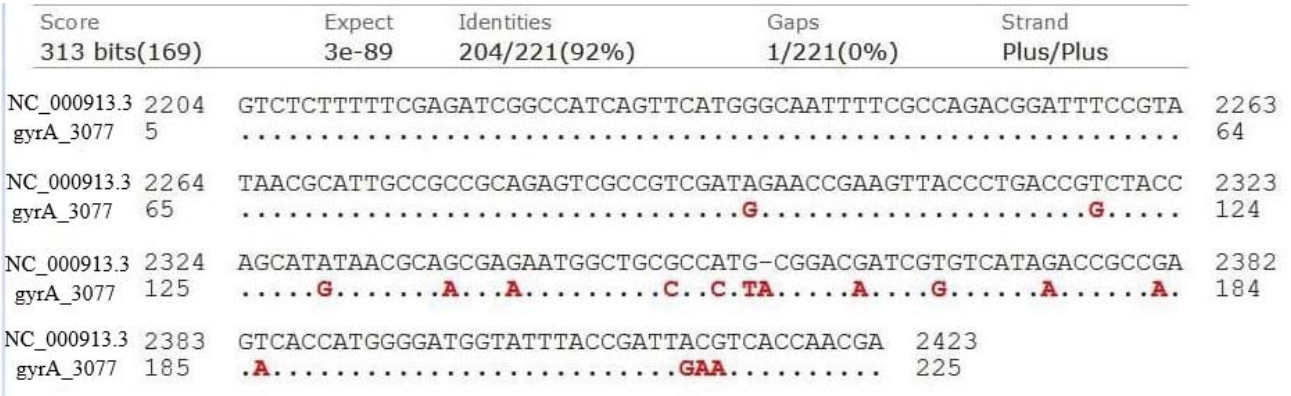


Figure 6.
The result of alignment the gyrA gene of sample number 3077. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 2204 to 2423 from NC_000913.3:2336793-2339420 Escherichia coli str. K-12 substr. MG1655, complete genome

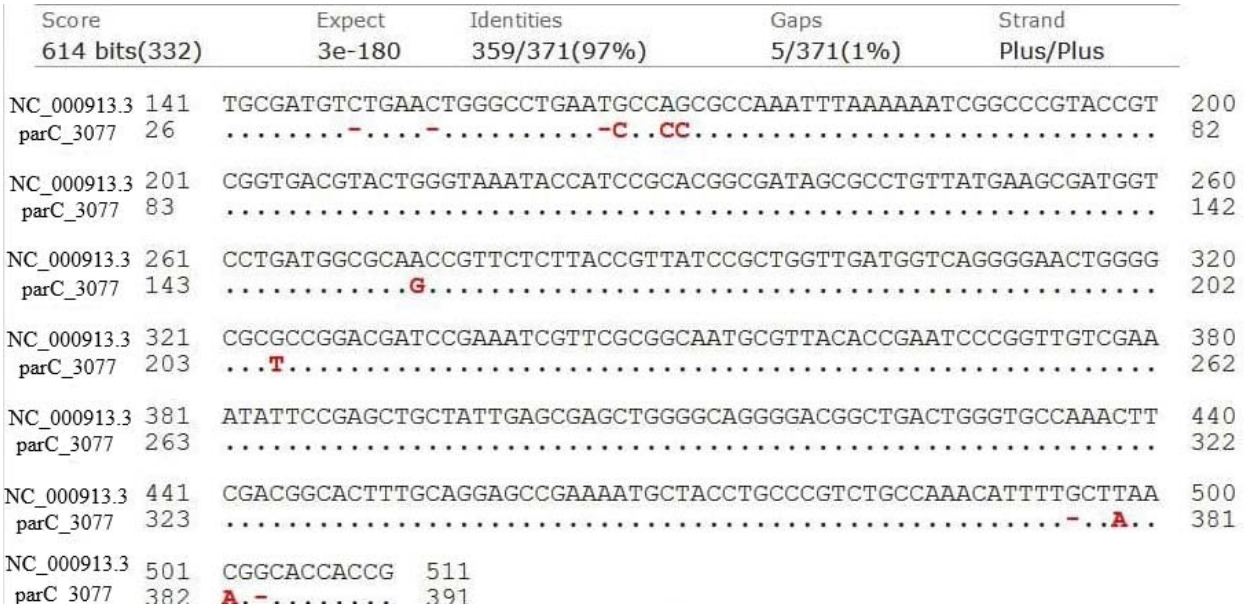


Figure 7.
The result of alignment the parC gene of sample number 3077. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 141 to 511 from NC_000913.3:c3165973_3163715 Escherichia coli str. K_12 substr. MG1655, complete genome.

were susceptible to norfloxacin and enrofloxacin.

This is in general agreement with Malinowski et al. (2008) who found that 16.1% and 14.9% mastitis *E. coli* isolates from Poland were resistant to enrofloxacin and norfloxacin, respectively [22]. However, in a study in Bangladesh, no resistance to fluoroquinolones including ofloxacin, ciprofloxacin, and levofloxacin was reported in *E. coli* isolated from milk of mastitis cattle [23]. Persson et al. (2011) reported that there was no fluoroquinolone resistance in *E. coli* isolated from milk samples of cows with mastitis [24]. In another study by Persson and her colleagues in Sweden (2015), they reported that all isolates (n=57) of *E. coli* from dairy cows with acute clinical mastitis were susceptible to enrofloxacin [25]. Armanullah et al. (2018), studied the antibiotic resistance profile of *E. coli* isolates from bovine clinical mastitis and reported resistance to ciprofloxacin (16.67%), norfloxacin (8.33%), ofloxacin (8.33%), and intermediate resistance to norfloxacin (8.33%) that was somewhat similar to the finding of the present study [26].

Fluoroquinolone resistance of *E. coli* isolates from bovine mastitis has been studied by several authors and the results have varied, which may be due to different methods and breakpoints used to determine susceptibility. Resistance to fluoroquinolones is still uncommon among *E. coli* isolated from bovine mastitis. In comparison to other studies [15, 17-19, 27], the

results of this study showed a low level of resistance to fluoroquinolones, which may be due to the controlled use of these antibiotics. However, in the present study ciprofloxacin and levofloxacin were proved to be the best antibiotics to treat *E. coli* mastitis in cattle since they were highly effective.

In the present study, the *E. coli* isolates did not have resistance to fluoroquinolones and the rate of intermediate resistance to fluoroquinolones was very low. It is generally accepted that gyrA mutations play a major role in the development of fluoroquinolone resistance in *E. coli,* while the mutations in the parC gene are additionally associated with resistance [28]. To analyze the correlation between genetic characterization and resistance phenotype, two isolates with the most resistance to each fluoroquinolone were submitted to amplification and sequencing of the QRDR in gyrA and parC genes. There were two silent mutations in the gyrA gene at wobble position in codons 91 and 100; similarly, *E. coli* isolates in the Heisig study showed silent mutations in codons 91 and 100 [28]. Mutation at codons 83 and 87 was found to be the most common gyrA mutations of *E. coli* in several studies, and in the present study, there was a mutation in codon 83 of gyrA in sample number 3077 [29-32]. In addition, we found a silent mutation in codon 91 in the parC gene. Similarly, *E. coli* isolates in the Heisig study showed silent mutation only in codon 91, whereas the most common mutations in parC were reported at codons 80, 84, and 87 [19, 28, 31, 32].

In conclusion, the current investigation showed that most *E. coli* isolates isolated from raw milk of cows with coliform mastitis in Khorasan Razavi province were sensitive to fluoroquinolones and some *E. coli* isolates had intermediate resistance to fluoroquinolones. In gyrA and parC genes of *E. coli* isolates with the most intermediate resistance to studied fluoroquinolones, there were silent mutations and mutations. There is some evidence that silent mutations can especially affect the regulation of transcription [33-35].

Materials & Methods

Sample collection

A total of one hundred (100) milk samples were examined in this study. Samples were collected from the milk of dairy cattle with clinical mastitis of three dairy farms in Mashhad (Khorasan Razavi province, Iran). Fifteen isolates of *E. coli* were obtained from "Bacterial Collection of the Mastitis Laboratory", Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Isolation of E. coli

Milk samples were cultured on MacConkey agar media (Merk, Darmstadt, Germany) and were incubated at 37 ˚C for 24 h. Suspected E. coli lactose-fermenting colonies (pink colonies) were used for culture on the Eosin methylene blue (EMB) agar (Merk, Darmstadt, Germany). The appearance of the colonies of purple with black center and green metallic sheen were considered positive for *E. coli* on EMB agar and were selected for further studies. The colonies of presumptive *E. coli* on EMB agar were confirmed by standard biochemical tests, including triple sugar iron agar, Simmons citrate agar, and motility assay.

Antibiotic susceptibility study

Antibiotic susceptibility testing was carried out with equivalence of 0.5 McFarland turbidity standard by agar disk diffusion method on Mueller-Hinton agar (Himedia, Mumbai, India) plates following the Clinical and Laboratory Standards Institute [36]. All *E. coli* isolates were subjected to an antibiotic susceptibility test. The antimicrobial disks (Padtan Teb, Tehran, Iran) used in the experiment included 5 µg ciprofloxacin, 10 µg norfloxacin, 5 µg levofloxacin, 5 µg ofloxacin, and 5 µg enrofloxacin. The antibiotic

Table 2.
Mutations in genes *gyrA* and *parC*.

E. coli isolate	gyrA mutation			parC mutation		
	Codon position	Nucleotide exchange	Amino acid exchange	Codon position	Nucleotide exchange	Amino acid exchange
2968	91	CGT → CGC	ginine [a]	91	CAG → CAA	Glutamine [a]
	100	TAC → TAT	Tyrosine [a]	157	CTG → CTA	Leucine [a]
	111	TCC → TCT	Serine [a]	159	GCT → GCC	Alanine [a]
	131	GCA → GCC	Alanine [a]			
	132	AAT → CAT	Asparagine → Histidine			
3077	65	AAT → CAT	Asparagine → Histidine	195	GGT → GTT	Glycine → Valine
	80	GCA → TCA	Alanine → Serine	209	GTG → TTA	Valine → Leucine
	83	AGG → GGG	Arginine → Glycine	212	GGC → CAC	Glycine → Histidine

[a] Silent mutation

disks were placed on Mueller-Hinton agar culture plate. The plates were incubated for 18-24 h at 37 ˚C. The size of the zone of inhibition was recorded and resistance zone diameter breakpoints adopted for these antimicrobials were the following: ≤ 15 mm for ciprofloxacin, ≤ 12 mm for norfloxacin, ≤ 13 mm for levofloxacin, ≤ 12 mm for ofloxacin, and ≤ 14 mm for enrofloxacin.

DNA extraction

E. coli isolates were grown overnight in Nutrient agar (Merk, Darmstadt, Germany) at 37 ˚C. One colony was suspended in 250 µL of sterile distilled water. After boiling the suspension for 15 min, followed by freezing and subsequent centrifugation at 14000 rpm for 15 min, the cell debris was pelleted and the supernatant was used as a template for the amplification reaction.
[37].

Amplification of quinolone resistance determining regions (QRDRs)

Polymerase chain reaction (PCR) was used to amplify QRDR of gyrA and parC for mutation detection. The list of primers that were used for amplification of gyrA and parC genes is shown in Table 3. The PCR amplification was performed in a total reaction volume of 25 µL. The reaction mixture contained 12.5 µL of 2x master mixtures (CinnaGen, Tehran, Iran), 1 µL of each forward and reverse primer (10 pmol/µL), 8.5 µL of deionized water and 2 µL of DNA template. The PCR program included initial denaturation at 94 ˚C for 5 min followed by 30 cycles of denaturation (94 ˚C for 1 min), annealing (55 ˚C for gyrA and 56 ˚C for parC for 1 min), and extension (72 ˚C for 1 min) with a final extension at 72 ˚C for 5 min. For amplification of DNA, the PCR was performed with a thermocycler (Techne, Chelmsford, UK). The PCR products were run on a 1% agarose gel in TAE buffer at 100 V for 45 min. After electrophoresis (Padideh Nojen Pars, Mashhad, Iran) in the agarose gel and staining with the green viewer (Sinaclone, Tehran, Iran), they were observed and documented under gel documentation system (Kimiagene, Mashhad, Iran). A 100 bp plus DNA ladder was used to determine the molecular size of the PCR products. Primers used in the study were custom synthesized from Macrogen Inc. (South Korea).

Sequencing and Alignment

The PCR product of gyrA and parC genes with forward and reverse primers sent for sequencing to Microsynth (Switzerland). DNA sequences were analysed using Chromas software. DNA sequence data were compared to data in the GenBank database using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

Table 3.
The primers were used for amplification of *gyrA* and *parC*

Primer name	Primer direction [a]	Sequence (5' to 3')	Product size (bp)	Annealing temperature (°C)	Reference
gyrA4	F	TCGTTGGTGACGTAATCGGT	253	55	31
gyrA5	R	TCCGTGCCGTCATAGTTATC	253	55	31
parC1	F	AACCTGTTCAGCGCCGCATT	434	56	31
parC2	R	ATGCGGTGGAATATCGGTCG	434	56	31

[a] F, forward; R, reverse

Authors' Contributions

B.F., A.J., and B.KH. conceived and planned the experiments. M.M. carried out the experiments. B.F., A.J. and B.KH. planned and carried out the simulations. M.M., K.L., and B.KH. contributed to sample preparation. B.F., A.J., and B.KH. contributed to the interpretation of the results. B.F. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and the manuscript.

Acknowledgements

We wish to thank H.K. Rahmani, A. Kargar and N. Shahbazi for their technical assistance. This research was financially supported by Ferdowsi University of Mashhad. Mashhad, Iran.

Conflict of interest

The authors declare that they have no competing interests.

References

1. Janzen J. Economic losses resulting from mastitis. A review. J Dairy Sci. 1970; 53(9):1151-60.

2. Jingar SC, Mahendra S, Roy AK. Economic losses due to clinical mastitis in cross-bred cows. Dairy Vet Sci J. 2017; 3(2):555606.

3. Hogan J, Smith KL. Coliform mastitis. Vet Res. 2003; 34(5):507-19.

4. Cullor JS, Smith WL. Endotoxin and disease in food animals. Compend. Contin. Educ. Vet. 1996;18(1): 31-38.

5. Bradely AJ, Leach KA, Breen JE, Green LE, Green MJ. Survey of the incidence and etiology of mastitis in dairy farms in En-

gland and Wales. Vet Rec. 2007; 160:253-258.

6. Wenz JR, Barrington GM, Garry FB, Dinsmore RP, Callan RJ. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. J Am Vet Med Assoc. 2001; 218(4):567-72.

7. Burvenich C, Van Merris V, Mehrzad J, Diez-Fraile A, Duchaeau L. Severity of E. coli mastitis is mainly determined by cow factors. Vet Res. 2003; 34:521-564.

8. Suojala L, Kaartinen L, Pyörälä S. Treatment for bovine Escherichia coli mastitis – an evidence based approach. J Vet Pharmacol Ther. 2013; 36(6):521-31.

9. Sharfaraj Nawaz M, Bodla R, Kant R, Pratab Singh S, Bhutani R, Kapoor G. Fluoroquinolone as antimicrobial agent: A Review. Int J Pharm Sci Res. 2017; 2(3):57-63.

10. Suh B, Lorber B. Quinolones. Med Clin North Am. 1995; 79(4):869-894.

11. Hooper DC. Mechanisms of action of antimicrobials: focus on fluoroquinolones. Clin. Infect. Dis. 2001; 32(1): 9-15.

12. Higgins PG, Fluit AC, Schmitz FJ. Fluoroquinolones: structure and target sites. Curr. Drug. Targets. 2003; 4(2): 181-190.

13. Hooper DC. Mechanisms of quinolone resistance. In: Hooper DC, Rubinstein E (Eds.), Quinolone antimicrobial agents. (3rd Edn.), Washington, D.C, USA: American Society of Microbiology Press. 2003; PP:41-67.

14. Hopkins KL, Davies RH, Threlfall EJ. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. Int J Antimicrobial Agents. 2005; 25:358–373.

15. Saranya K, Pavulraj S, Kalaiselvi L, Amsaveni S, Ramesh S. Antibacterial susceptibility profiles of coliforms isolated from bovine subclinical and clinical mastitis against fluoroquinolones.Tamilandu Journal. Vet Anim Sci. 2013; 9 (4):279-284.

16. Srinivasan V, Gillespie BE, Lewis MJ, Nguyen LT, Headrick SI, Schukken YH, Oliver SP. Phenotypic and genotypic antimicrobial resistance patterns of Escherichia coli isolated from dairy cows with mastitis. Vet. Microbiol. 2007; 124(3-4): 319-328.

17. Alekish M, Al-Qudah K, Al-Saleh A. Prevalence of antimicrobial resistance among bacterial pathogens isolated from bovine mastitis in northern Jordan. Rev Vet Med. 2013; 164:319-26.

18. Chandrasekaran D, Venkatesan P, Tirumurugaan K, Nambi A, Thirunavukkarasu P, Kumanan K, et al. Pattern of antibiotic resistant mastitis in dairy cows, Vet World. 2014; 7(6):389-394.

19. Balakrishnan S, Antony PX, Mukhopadhyay HK, Pillai RM, Thanislass J, Padmanaban V, et al. Genetic characterization of fluoroquinolone-resistant Escherichia coli associated with bovine mastitis in India. Vet World. 2016; 9(7):705-709.

20. Su Y, Yu C-Y, Tsai Y, Wang S-H, Lee C, Chu C. Fluoroquinolone-resistant and extended-spectrum β-lactamase-producing Escherichia coli from the milk of cows with clinical mastitis in Southern Taiwan. J Microbiol Immunol Infect. 2016;49(6):892-901.

21. Metzger SA, Hogan JS. Short communication: Antimicrobial susceptibility and frequency of resistance genes in Escherichia coli isolated from bovine mastitis. J Dairy Sci. 2013; 96:3044–3049.

22. Malinowski E, Lassa H, Smulski S, Kłossowska A, Kaczmarowski M. Antimicrobial susceptibility of bacteria isolated from cows with mastitis in 2006-2007. Bulletin of the Veterinary Institute in Pulawy. 2008; 52(565):72.

23. Tanzin T, Nazir KHMNH, Zahan MN, Md. Parvej S, Zesmin K, Rahman MT. Antibiotic resistance profile of bacteria isolated from raw milk samples of cattle and buffaloes. J Adv Vet Anim Res. 2016; 3 (1): 62-67.

24. Persson Y, Nyman AK, Grönlund-Andersson U. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Vet Scand. 2011; 53(1):36.

25. Persson Y, Katholm J, Landin H, Mörk Mj. Efficacy of enrofloxacin for the treatment of acute clinical mastitis caused by Escherichia coli in dairy cows. Vet Rec. 2015; 176(26):673-673.

26. Armanullah MD, Anjay Dr, Kumar P, Kumari S, Kaushik P, Archana S, Arya S. Prevalence of Multi-Drug Resistant (MDR) Escherichia coli in bovine clinical samples. Int. J. Curr. Microbiol. App. Sci. 2018; 7: 1476-1485.

27. Firouzi R, Rajaian H, Tabaee I, Saeedzadeh A. In vitro antibacterial effects of marbofloxacin on microorganisms causing mastitis in cows. J Vet Res. 2010; 65(1):51-55.

28. Heisig P. Genetic evidence for a role of parC mutations in development of high-level fluoroquinolone resistance in Escherichia coli. Antimicrob. Agents Chemother. 1996; 40(4): 879-85.

29. Chen JY, Siu LK, Chen YH, Lu PL, Ho M, Perg CF. Molecular epidemiology and mutations at gyrA and parC genes of ciprofloxacin-resistant Escherichia coli isolates from Taiwan medical center. Microb Drug Resist. 2001; 7, 47-53.

30. Ruiz J, Gomez J, Navia MM, Ribera A, Sierra JM, Marco F. High prevalence of nalidixic acid resistant, ciprofloxacin susceptible phenotype among clinical isolates of Escherichia coli and other Enterobacteriaceae. Diagn Microbiol and infect Dis. 2002; 42, 257-61.

31. Ogbolu DO, Daini O, Ogunledun A, Alli AT, Olusoga-Ogbolu F, Webber M. Effects of gyrA and parC mutations in quino-

lones resistant clinical gram negative bacteria from Nigeria. Afr J Biomed Res. 2012; 15(2):97-104.

32. Sáenz Y, Zarazaga M, Briñas L, Ruiz-Larrea F, Torres C. Mutations in gyrA and parC genes in nalidixic acid-resistant Escherichia coli strains from food products, humans and animals. J Antimicrob Chemother. 2003; 51(4):1001-5.

33. Yadegari H, Biswas A, Akhter MS, Driesen J, Ivaskevicius V, Marquardt N, Oldenburg J. Intron retention resulting from a silent mutation in the VWF gene that structurally influences the 5' splice site. Blood. 2016; 128(17):2144-2152.

34. Agashe D, Sane M, Phalnikar K, Diwan GD, Habibullah A, Martinez-Gomez NC, et al. Large-effect beneficial synonymous mutations mediate rapid and parallel adaptation in a bacterium. Mol Biol Evol. 2016; 33(6):1542-53.

35. Hauber DJ, Grogan DW, DeBry RW. Mutations to less-preferred synonymous codons in a highly expressed gene of Escherichia coli: fitness and epistatic interactions. PLoS One 2016; 11(1):e0146375.

36. CLSI. Performance standards for antimicrobial susceptibility testing. 27th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.

37. Reischl U, Youssef MT, Kilwinski J, Lehn N, Zhang WL, Karch H, et al. Real-time fluorescence PCR assay for detection and characterization of shiga toxin, intimin, and enterohemolysin genes from shiga toxin-producing Escherichia coli. J Clin Microbiol. 2007; 40(7):2555-2565.

How to cite this article
Mahdavi M, Fathi B, Jamshidi A, Khoramian B.Evaluation of fluoroquinolones resistance and determination of mutations in gyrA and parC genes in Escherichia coli isolated from raw milk of dairy cows with coliform mastitis in Khorasan Razavi province, Iran. Iran J Vet Sci Technol.2022; 14(1): 20-28.
DOI: https://doi.org/10.22067/ijvst.2022.71423.1059
URL: https://ijvst.um.ac.ir/article_42057.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Sep- 15
Accepted after revision: 2022- Mar- 25
Published online: 2022- Apr- 30

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2022.72394.1080

Antibiotic resistance patterns of bacteria isolated from *Clarias gariepinus* farms in Kaduna state, Nigeria

Deborah Arimie Adah[a], Saidu Lawal[b], Sonnie Oniye[c], Sylvanus Adakole Adah[d], Susan Mosebolatan David[e], Omodolapo Morohunranti Obisesan[f]

[a] Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ilorin, Ilorin, Nigeria.
[b] Veterinary Teaching Hospital, Ahmadu Bello University Zaria, Nigeria.
[c] Department of Zoology, Ahmadu Bello University Zaria, Nigeria.
[d] Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine University of Ilorin, Ilorin Nigeria.
[e] Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Science, University of Ilorin, Ilorin Nigeria.
[f] Department of Animal Health Technology, Oyo State College of Agriculture and Technology Igboora, Oyo State Nigeria.

ABSTRACT

Fish farming is increasing globally, with an increase in bacterial infections known to cause morbidity and varying mortality, affecting the productivity and profitability of aquaculture. The objective of this study was to determine the antibiotics susceptibility and multiple antibiotic resistance index of bacteria isolated from fish in some selected fish farms in Kaduna State to ten commonly used antibiotics using the Kirby-Bauer disc diffusion method. In total, 84 bacteria were isolated from 75 *Clarias gariepinus* in this study, belonging to 12 genera. The antibiotic profile of the bacteria isolated displayed different sensitivity and resistance to the antibiotics used. The highest numbers of the Gram-positive (59.5%) and Gram-negative (69%) bacteria, respectively, were sensitive to ciprofloxacin compared to the other antibiotics. All the bacterial isolates displayed varying diversity of multidrug-resistant patterns. A total of 38 and 41 different resistance patterns for Gram-positive and Gram-negative respectively were observed. The multiple antibiotic resistance (MAR) index analysis reveals that 97.3% of the bacteria had a high MAR index value (> 0.2). In conclusion, there is a diversity of bacteria organisms within the fish farms that are pathogenic to both fish and humans. Therefore, there is a need to implement optimal preventive management measures and control the use of antibiotics.

Keywords

Antimicrobials, aquaculture, health risk, multidrug resistance, pathogens

Number of Figures: 1
Number of Tables: 4
Number of References:: 43
Number of Pages: 10

Abbreviations

C. gariepinus: *Clarias gariepinus*
E. coli: *Escherichia coli*
MAR: Multiple antibiotic resistance
AMP: Ampicillin
CIP: Ciprofloxacin
FFC: Florfenicol

Corresponding author:
Deborah Arimie Adah
Email: : *adah.ad@unilorin.edu.ng*
Tel: *+23(480)6065-2832*

Introduction

Fish production through aquaculture provides an alternative supply of fish for human consumption [1, 2]. This has led to an increase in fish production levels to meet the protein demand of the growing population [3, 4]. In bridging the demand and supply gap of fish, *Clarias gariepinus* is a suitable choice for aquaculture in Africa, especially Nigeria, owing to its hardy nature and wide acceptability [5]. However, increasing demand for fish is associated with the intensification of fish farming activities, such as increased stocking density, and a rise in water quality challenges, which facilitate a higher incidence of disease outbreaks [6, 7]. Furthermore, the occurrence of various types of diseases, most of which are caused by bacteria, at any stage of fish culture has a significant impact on the economic viability of fish farms [8, 9]. Consequently, this has led to the use of antibiotics as a growth promoter, for prophylactic and therapeutic purposes [10, 11]. Excessive use of antibiotics in aquaculture in many countries has been attributed to the development and dissemination of antibiotic-resistant bacteria [12, 13, 14].

Assessing and monitoring antimicrobial-resistant bacteria from fish for human consumption from different parts of the world is needed regularly to evaluate and detect the emergence, trend, and changes in the resistance pattern towards antimicrobial drugs [15, 16]. Therefore, this study is aimed at isolating and identifying bacteria from *Clarias gariepinus* in some selected fish farms in Kaduna State, Nigeria, and determining their antimicrobial susceptibility and resistance pattern to 10 commonly used antibiotics.

Results

A total of 84 bacteria belonging to 12 genera were isolated from 75 *Clarias gariepinus* samples from this study. Out of 42 bacteria isolated, 16 (19.0%) were *Bacillus subtilis*, 3 were (3.6%) *Corynebacteria aquaticum*, 17 were (20.3%) *Staphylococcus aureus,* and 6 were (7.1%) *Streptococcus agalactiae*. Forty-two Gram-negative bacteria were also isolated, which consisted of *Aeromonas hydrophila* (2.4%, n = 2), *Citrobacter freundi* (4.8 %, n = 4), *Escherichia coli* (13.1%, n= 11), *Klebsiella pneumoniae* (3.6%, n = 3), *Proteus mirabilis* (11.9%, n = 10), *Pseudomonas aeruginosa* (7.1%, n = 6), and *Salmonella enterica* and *Shigella* species (3.6%, n = 3) (Figure 1). *Staphylococcus aureus* (20.3%) was the most prevalent species followed by *Bacillus subtilis* (19.0%) and *E. coli* (13.1%). *Aeromonas hydrophila* was the least prevalent (2.4%) bacteria isolated (Figure 1).

The antibiotic profile of the bacteria isolated revealed different sensitivity and resistance to the ten antibiotics used. The majority of the Gram-positive bacteria (59.5%) were sensitive to ciprofloxacin. Among the Gram-positive bacteria, no level of sensitivity was detected to Vancomycin. With a *Chi*-Square value of 80.30, the difference in the sensitivity level of antibiotic susceptibility was significant at $p \leq 0.01$. There was no significant difference ($p = 0.27$) in susceptibility to different antibiotics. There was a level of resistance to all the antibiotics used, with vancomycin causing the highest level of resistance to the Gram-positive bacteria. There was a significant statistical difference ($p \leq 0.01$) in the resistance of the Gram-positive bacteria to different antibiotics (Table 1).

The Gram-negative bacteria were mostly susceptible to Ciprofloxacin (69.0%) and showed the lowest (4.8%) level of susceptibility to penicillin. The level of sensitivity to other antibiotics ranged between gentamicin (66.7%) and ampicillin (7.1%). There was a significant difference ($p \leq 0.01$) in the sensitivity level to antibiotics in the Gram-negative bacteria. In addition, there was a significant difference ($p \leq 0.01$) between the antibiotics in terms of their intermediate and resistance profiles with the Gram-negative bacteria. (Table 2). All the bacterial isolates displayed varying diversity of multidrug-resistant patterns to more than one antibiotic. There were differences in the multidrug-resistance profiles of the bacteria within the different species of the isolates. The prevalence of multidrug resistance was 97.6% for both Gram-positive and Gram-negative bacteria respectively and was resistant to more than two antibiotics. From the Gram-positive, one of the isolates was resistant to two antibiotics 1(2.4%), 4 (9.52 %) were resistant to three antibiotics, 6 (14.29%) were resistant to four antibiotics, 13 (30.92%) were resistant to five antibiotics, 11 (26.1%) were resistant to six antibiotics, 4 (9.52 %) were resistant to seven antibiotics, and 3 (3.74%) were resistant to eight antibiotics out of the ten antibiotics used. A total of 38 different resistance patterns were observed. The multidrug resistance patterns for Gram-positive bacteria isolated from *Clarias gariepinus* showed a significant difference ($p \leq 0.01$) with a Chi-Square value of 22.56. The highest prevalence was recorded among the 5 antibiotics combinations (30.9%), with the double antibiotic combinations having the least

Abbreviations-Cont'd

CN: Gentamicin
OXE: Oxytetracycline
OX: Oxacillin
P: Penicillin
S: Streptomycin
TE: Tetracycline
VA: Vancomycin
SEM: Standard error of the mean

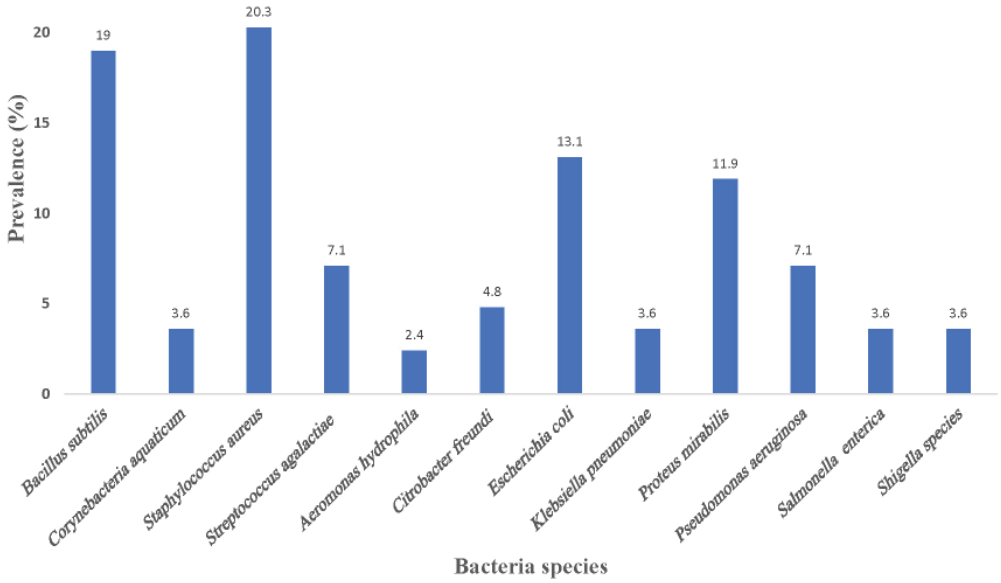


Figure 1.
Prevalence of Gram-positive and Gram-negative bacteria isolates from Gills of *Clarias gariepinus* from fish farms

(2.4%) (Table 3).

For the Gram-negative bacteria, one of the isolates was resistant to two and three antibiotics, respectively. Eight (19.04%) were resistant to four antibiotics, 18 (42.86%) were resistant to five antibiotics, 9 (21.43%) were resistant to six antibiotics, 4 (9.52%) were resistant to seven antibiotics and 1(2.4%) was resistant to eight antibiotics out of the ten antibiotics used. A total of 41 different resistance patterns were observed for Gram-negative in this study. The multidrug resistance patterns and MAR Index of Gram-negative bacteria from *Clarias gariepinus* are presented in Table 4. The highest prevalence of multidrug resistance patterns was seen in the five antibiotic combinations (42.9%) with a MAR value of 0.5. The difference between the multidrug resistance patterns was significant ($p \leq 0.01$) with a Chi-Square value of 45.89 (Table 4).

Table 1.
Percentage distribution of antibiotics susceptibility of Gram-positive bacteria isolates from Gills of *Clarias gariepinus* from some fish farms in Kaduna State, Nigeria

Antibiotic	N (µg)	Sensitive (%)	χ^2	*p*	Intermediate (%)	χ^2	*p*	Resistance (%)	χ^2	*p*
Ciprofloxacin	5	25 (59.5)			6 (14.3)			11 (26.2)		
Gentamicin	10	21 (50.0)			8 (19.0)			13 (31.0)		
Florfenicol	30	20 (47.6)			6 (14.3)			16 (38.1)		
Streptomycin	10	10 (23.8)			14 (33.3)			18 (42.9)	49.36	
Tetracycline	30	9 (21.4)	80.30	<0.01#	14 (33.3)	11.08	0.27	19 (45.2)		<0.01#
Oxytetracycline	30	7 (16.7)			9 (21.4)			26 (61.9)		
Oxacillin	1	7 (16.7)			6 (14.3)			29 (69.0)		
Ampicillin	10	6 (14.3)			9 (21.4)			27 (64.3)		
Penicillin (in units)	10	2 (4.8)			10 (23.8)			30 (71.4)		
Vancomycin	30	0 (0.0)			10 (23.8)			32 (76.2)		
Total		107 (25.5)			92 (21.9)			221 (52.6)		

N = Concentration of antibiotics used; χ^2 =*Chi* Square test; # = Significant at $p < 0.05$

Table 2.
Percentage distribution of antibiotics susceptibility patterns of Gram-negative bacteria isolates from Gills of *Clarias gariepinus* from some fish farms in Kaduna State, Nigeria

Antibiotic	N (μg)	Sensitive (%)	χ^2	*p*	Intermediate (%)	χ^2	*p*	Resistance (%)	χ^2	*p*
Ciprofloxacin	5	29 (69.0)			5 (11.9)			8 (19.0)		
Gentamicin	10	28 (66.7)			3 (7.1)			11 (26.2)		
Florfenicol	30	26 (61.9)			4 (9.5)			12 (28.6)		
Streptomycin	10	11 (26.2)			13 (31.0)			18 (42.9)		
Tetracycline	30	8 (19.0)	121.10	< 0.01#	13 (31.0)	22.61	0.01#	21 (50.0)	75.12	< 0.01#
Oxytetracycline	30	6 (14.3)			12 (28.6)			24 (57.1)		
Ampicillin	10	3 (7.1)			11 (26.2)			28 (66.7)		
Vancomycin	30	5 (11.9)			7 (16.7)			30 (71.4)		
Oxacillin	1	6 (14.3)			5 (11.9)			31 (73.8)		
Penicillin (in units)	10	2 (4.8)			5 (11.9)			35 (83.3)		
Total		124 (29.5)			78 (18.6)			218 (51.9)		

N = Concentration of antibiotics used; χ^2 = *Chi* Square test; # = Significant at $P < 0.05$.

Discussion

Bacteria are an important component of the aquatic environment, and the interplay between these organisms and the changes in the habitat of the fish will lead to the exacerbation of disease in the fish farms, thereby causing great economic losses [17]. The identification of bacteria from *C. gariepinus* is very important as it provides information on the level of contamination in the fish, the culture environment, and the risk of transfer of the pathogens to humans to cause diseases like cholera, dysentery, and salmonellosis [18]. In this study, twelve different genera of bacteria known to cause disease in both fish and humans were isolated from *C. gariepinus* in the study. However, it differed from the findings of Uddin and Al-Harbi [19], who isolated 10 bacteria genera from polycultured common carp (*Cyprinus carpio*) and African catfish (*Clarias gariepinus*), Danba et al [20] isolated 5 genera from *C. gariepinus* from selected fish farms in Kano, Wamala et al. [21], isolated 15 in *Oreochromis niloticus* (Nile tilapia) and *Clarias gariepinus* (African catfish) in Uganda with most of the bacteria genera reported by Uddin and Al-Harbi [19]. Danba *et al* [20] and Wamala *et al* [21] reported findings similar to the present study. The differences in the genera and species of bacteria observed may be due to the different geographical locations, culture environments, species of fish, and different sampling and isolation methods.

Gram-negative bacteria were the most prevalent bacterial isolates from this study. This is similar to the findings of Tsfaye *et al.* [22] and Kousar *et al.* [23]. The isolation of E. *coli, Klebsiella pneumoniae*, *Proteus* mirabilis, *Staphylococcus aureus, Streptococcus* agalactiae, and *Vibrio* species from *C. gariepinus* is an indication of fecal contamination from livestock manure used for pond fertilization and the indiscriminate deposition of human and animal excreta into ponds and rivers that harbor fish or through the washing of land surfaces into water bodies during the rainy season [24]. Free-roaming animals, especially dogs, birds, and ruminants in the mixed farming system, contribute to the fecal contamination of surface water and ponds [25, 26]. *Staphylococcus aureus* isolates which were hemolytic on blood agar are known to be pathogenic to fish and their presence could be due to contamination of the fish by fish handlers during feeding, handling activities, and harvesting as observed also by Afolabi *et al.* [18]. The high presence of *Proteus mirabilis* in fish farms has been reported by Wanja, *et al.* [27] and was attributed to the use of poultry litter for fertilization of the ponds. The presence of these microorganisms poses a serious public health threat as some of the bacterial organisms isolated in this study, such as *Aeromonas hydrophila, Citrobacter freundi,* and *Pseudomonas aeruginosa* are known to be pathogenic to humans and are etiological agents of infectious diseases in fish, leading to mortalities in association with unfavorable environmental conditions in intensive fish farms [27, 28].

In this study, the antibiogram showed that most of the bacteria species isolated showed varying resistance

Table 3.
Multidrug resistance patterns and MAR Index of Gram-positive bacteria isolated from *Clarias gariepinus* from some fish farms in Kaduna State, Nigeria

Resistance patterns	No. of antibiotics involved	MAR	No of isolates	Bacteria species involved	Prevalence (%)	χ^2	*p*
OX, P	2	0.2	1	*Streptococcus agalactiae*	1 (2.4)		
OX, P, FFC	3	0.3	1	*Bacillus subtilis*			
TE, VA, P	3	0.3	1	*Staphylococcus aureus*			
OX, VA, P	3	0.3	1	*Bacillus subtilis*	4 (9.5)		
VA, CN, AMP	3	0.3	1	*Staphylococcus aureus*			
VA, P, FFC, OXE	4	0.4	1	*Corynebacteria aquaticum*			
VA, P, S, FFC	4	0.4	1	*Staphylococcus aureus*			
OX, P, AMP, OXE	4	0.4	1	*Corynebacteria aquaticum*			
OX, VA, AMP, OXE	4	0.4	2	*Staphylococcus* and *Bacillus subtilis*	6 (14.3)		
TE, VA, FFC, CIP	4	0.4	1	*Bacillus subtilis*			
OX, VA, P, CN, FFC	5	0.5	1	*Streptococcus agalactiae*			
OX, TE, VA, P, AMP,	5	0.5	1	*Staphylococcus aureus*			
OX, P, S, AMP, CIP	5	0.5	1	*Staphylococcus aureus*			
OX, CN, S, AMP, OXE	5	0.5	1	*Staphylococcus aureus*			
OX, VA, P, FFC, OXE	5	0.5	1	*Staphylococcus aureus*			
OX, VA, P, S, AMP	5	0.5	2	*Staphylococcus aureus* and *Bacillus subtilis*	13 (30.9)		
OX, VA, P, S, OXE	5	0.5	1	*Bacillus subtilis*			
OX, VA, S, AMP, OXE	5	0.5	1	*Streptococcus agalactiae*		22.56	<0.01#
OX, TE, S, AMP, CIP	5	0.5	1	*Bacillus subtilis*			
TE, VA, P, AMP, OXE	5	0.5	1	*Bacillus subtilis*			
VA, P, AMP, FFC, OXE	5	0.5	1	*Bacillus subtilis*			
OX, TE, CN, AMP, FFC, OXE	6	0.5	1	*Bacillus subtilis*			
OX, TE, P, S, AMP, OXE	6	0.6	1	*Bacillus subtilis*			
OX, VA, P, AMP, FFC, OXE	6	0.6	2	*Bacillus subtilis* and *streptococcus agalactiae*			
OX, TE, CN, AMP, FFC, OXE	6	0.5	1	*Bacillus subtilis*			
OX, TE, P, S, AMP, OXE	6	0.6	1	*Bacillus subtilis*			
OX, VA, P, AMP, FFC, OXE	6	0.6	2	*Bacillus subtilis* and *streptococcus agalactiae*	11 (26.1)		
OX, VA, P, CN, CIP, OXE	6	0.6	1	*Bacillus subtilis*			
TE, VA, P, CN, AMP, OXE	6	0.6	1	*Staphylococcus aureus*			
TE, VA, P, CN, S, FFC	6	0.6	1	*Staphylococcus aureus*			
TE, VA, P, S, AMP, OXE	6	0.6	1	*Staphylococcus aureus*			
OX, TE, VA, CN, S, FFC, OXE	7	0.7	1	*Staphylococcus aureus*			
OX, TE, VA, P, S, AMP, FFC	7	0.7	1	*Bacillus subtilis*	4 (9.5)		
OX, TE, VA, P, S, CIP, OXE	7	0.7	2	*Staphylococcus aureus* and *Corynebacteria aquaticum*			
OX, TE, VA, P, CN, AMP, CIP, OXE	8	0.8	1	*Streptococcus agalactiae*			
OX, TE, P, CN, AMP, FFC, CIP,OXE	8	0.8	1	*Staphylococcus aureus*	3 (7.1)		
OX,VA,P,CN,AMP,FFC,CIP,OXE	8	0.8	1	*Staphylococcus aureus*			

AMP: Ampicillin; CIP: Ciprofloxacin; FFC: Florfenicol; CN: Gentamicin; OXE: Oxytetracycline; OX: Oxacillin; P: Penicillin; S:Streptomycin ;TE : Tetracycline ; VA: Vancomycin. Multiple antibiotics resistance (MAR); χ^2 =*Chi* Square test ; # = Significant at $p < 0.05$.

Table 4.
Multidrug resistance patterns and MAR Index of Gram-negative bacteria isolated from *Clarias gariepinus* in some fish farms in Kaduna State, Nigeria

Resistance patterns	No. of antibiotics involved	MAR	No of isolates	Bacteria species involved	Prevalence (%)	χ^2	*p*
OX, AMP	2	0.2	1	*Pseudomonas aeruginosa*	1 (2.4)		
VA, AMP, CIP	3	0.3	1	*Pseudomonas aeruginosa*	1 (2.4)		
OX, P, S, OXE	4	0.4	1	*E. coli*			
OX, TE, P, FFC	4	0.4	1	*E. coli*			
OX, TE, VA, P	4	0.4	1	*Proteus mirabilis*	8 (19.0)		
OX, TE, P, S	4	0.4	1	*Proteus mirabilis*			
OX, VA, P, AMP	4	0.4	1	*Salmonella enterica*			
OX, VA, CIP, OXE	4	0.4	1	*Klebsiella pneumoniae*			
TE, VA, S, OXE	4	0.4	1	*E. coli*			
VA, P, AMP, OXE	4	0.4	1	*E. coli*			
OX, TE, VA, P, S	5	0.5	1	*Citrobacter freundi*			
OX, VA, P, S, FFC	5	0.5	1	*Proteus mirabilis*			
OX, TE, VA, P, AMP,	5	0.5	1	*Aeromonas hydrophila*			
OX, P, CN, FFC, OXE	5	0.5	1	*Salmonella enterica*			
OX, TE, P, CN, AMP,	5	0.5	1	*Proteus mirabilis*			
OX, VA, CN, AMP, OXE	5	0.5	1	*Citrobacter freundi*			
OX, VA, P, FFC, OXE	5	0.5	1	*Proteus mirabilis*			
OX, VA, P, AMP, OXE	5	0.5	1	*Shigella* species	18 (42.9)		
OX, VA, P, S, AMP	5	0.5	1	*E. coli*			
OX, VA, P, CIP, OXE	5	0.5	1	*Shigella* species		45.89	<0.01#
OX, VA, P, CN, AMP	5	0.5	1	*Citrobacter freundi*			
OX, P, AMP, CIP, OXE	5	0.5	1	*Pseudomonas aeruginosa*			
TE, VA, P, AMP, OXE	5	0.5	1	*Aeromonas hydrophila*			
TE, VA, P, S, OXE	5	0.5	1	*Pseudomonas aeruginosa*			
TE, VA, S, AMP, FFC	5	0.5	1	*Klebsiella pneumoniae*			
VA, P, CN, AMP, OXE	5	0.5	1	*Klebsiella pneumoniae*			
TE, P, CN, CIP, OXE	5	0.5	1	*Proteus mirabilis*			
TE, P, S, AMP, OXE	5	0.5	1	*E. coli*			
OX, VA, P, CN, AMP, FFC	6	0.6	1	*Pseudomonas aeruginosa*			
OX, TE, P, S, AMP, FFC	6	0.6	1	*E. coli*			
OX, TE, VA, P, AMP, OXE	6	0.6	1	*Pseudomonas aeruginosa*			
OX, VA, CN, S, AMP, OXE	6	0.6	1	*Proteus mirabilis*			
OX, VA, P, CN, S, OXE	6	0.6	1	*E. coli*		9 (21.4)	
OX, VA, P, S, AMP, OXE	6	0.6	1	*Proteus mirabilis*			
TE, VA, P, CN, S, OXE	6	0.6	1	*E. coli*			
TE, VA, P, S, AMP, FFC	6	0.6	2	*Proteus mirabilis, Shigella* species			
OX, TE, P, AMP, FFC, CIP, OXE	7	0.7	1	*E. coli*			
OX, TE, P, CN, FFC, CIP, OXE	7	0.7	1	*E. coli*			
OX, TE, P, S, AMP, CIP, OXE	7	0.7	1	*Salmonella enterica*		4 (9.5)	
OX, TE, VA, P, S, AMP, OXE	7	0.7	1	*Citrobacter freundi*			
OX, VA, P, S, AMP, FFC, CIP,OXE	8	0.8	1	*Proteus mirabilis*		1 (2.4)	

AMP: Ampicillin; CIP: Ciprofloxacin; FFC: Florfenicol; CN: Gentamicin; OXE: Oxytetracycline; OX: Oxacillin; P: Penicillin; S: Streptomycin ;TE : Tetracycline ; VA: Vancomycin. Multiple antibiotics resistance (MAR) ;χ^2 = *Chi* Square test; # = Significant at $p < 0.05$.

to penicillin, oxacillin, vancomycin, ampicillin, oxytetracycline, and tetracycline, but they were found to be sensitive to ciprofloxacin, gentamycin, and florfenicol, which was similar to the findings of [29, 30]. The susceptibility of the bacteria to ciprofloxacin, gentamycin, and florfenicol might be due to the less frequent utilization of these antibiotics in aquaculture. The resistance of the bacteria species could be due to the extensive and indiscriminate use of drugs like vancomycin, ampicillin, oxytetracycline, and tetracyclines which are easily accessible over-the-counter antibiotics and have been the hallmark of antimicrobial treatment administered either in feeds or in baths in fish farming [31]. More so, several of these drugs are non-biodegradable, leading to an increase in selective pressure and thus has resulted in an increase in the occurrence of drug resistance in fish-pathogenic bacteria [32]. The high prevalence of antibiotic resistance observed in Gram-positive bacteria in this study has also been reported by Ayadiran and Dahunsi [33], who reported the highest rate of multiple antibiotic resistance in Gram-positive bacteria. This could be due to the ubiquitous nature of the Gram-positive bacteria in the culture environment of the fish.

Multi-antibiotic resistance (MAR) indexing is well known as an efficient and less expensive method of tracking bacteria sources [34]. As a result, the MAR index is a useful method of ascertaining the risk of pollution that could threaten the life of an animal [35]. The multidrug resistance (MDR) of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics used. Varying antibiotic resistance patterns were observed for the different species of bacteria isolated in the study area. However, it was observed that bacterial species of the same genus displayed different antibiotic resistance patterns. Antibiotic resistance patterns may vary depending on the geographical location, management practice, and selective pressure [35], and these patterns change rapidly from time to time. The different patterns exhibited by different strains or species suggest how complex the understanding of the antibiotic's resistance is in the study area.

The MAR index analysis reveals that 97.3% of both Gram-positive and Gram-negative bacteria had a high MAR index value (> 0.2). This was similar to the findings of Kathleen *et al.* [35] and Adinortey *et al.* [31]. The high MAR index recorded indicates high contamination with antibiotics. The difference in the MAR index shows the impact of the use of antibiotics in the sampled fish farms. Diseases caused by bacteria with a high MAR index will be a great challenge to curb, leading to high mortalities and reduced profit on investment. Because bacteria possess multiple resistance mechanisms, this will aid in the reduction of antibiotic activity for both prevention and therapeutic purposes [36]. More so, the observed trend of multidrug-resistant strains poses a major public health concern globally, and there is a need to come up with effective policies and implementation plans to address these concerns.

In conclusion, the results from this study revealed the diversity of bacteria organisms within fish farms that are pathogenic to both fish and humans, which may pose a serious public health challenge to consumers when the fish are not properly cooked or handled. There is a high prevalence of antibiotic resistance, which may have environmental, public health, and global implications. Therefore, there is a need to implement optimal and more strict preventive management measures in fish farms that will prioritize adherence to practices as this will go a long way to helping produce healthy and wholesome fish as well as boost productivity. Controlled use of antibiotics in fish farming is very important, to avoid the occurrence and spread of antibiotic resistance and further complicate clinical management of the disease. Consequently, it has been strongly recommended that programs to monitor and regulate the usage of antimicrobial agents and the occurrence of antimicrobial resistance be advocated.

Materials & Methods

Study area and design

The study was carried out in Kaduna State, which is located at a geographic coordinate of latitude 10° 36' 33.54"N and Longitude 7° 25' 46.2144"E located in the northwestern part of Nigeria. It approximately occupies a total landmass of 48,473.2 square kilometers and has a population of more than 6 million people [37]. A cross-sectional study involving multistage random sampling of 15 active, grow-out fish farms from four local government areas (Sabo Gari, Kaduna North, Kaduna South, and Zaria Local Government Areas) of Kaduna State were sampled. Sampling was carried out based on the convenience and willingness of the fish farmers to participate in the study.

Fish sample collection

Samples of seventy-five live *Clarias gariepinus* (*C. gariepinus*), five fish per farm, were randomly selected from active productive grow-out farms within the study area. *C. gariepinus* fish with different total lengths of ≥ 12-35 cm and weights of 350 g – 1 kg were included in the study. The fish were caught using a fishnet from earthen ponds, plastic and concrete tanks between the hours of 06: 00 and 08:00 and put into a plastic bucket with a perforated cover containing water to ensure the survival of the fish samples.

They were later transported to the microbiology laboratory of the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, for further processing within 2 hours post-collection.

Each live fish was sacrificed (by brain spiking to minimize suffering) and placed on a clean stainless tray dorsally, and a swab (sterile cotton wool soaked in 70% alcohol) was used to clean the fish from the operculum to the abdominal area to reduce bacterial load. The operculum of the fish was lifted to expose the gills, and swabs of the gills were taken for bacterial isolation using sterile swab sticks.

Isolation and identification of bacterial isolates

The examination was conducted to isolate, identify, and confirm bacterial isolates from *Clarias gariepinus*. Conventional methods of bacterial isolation, such as growth and morphology on selective media, were employed. The sterile swab sticks were used to swab the gills of the sampled fish and were put into the nutrient broth and incubated at 37 °C for 24 hrs for the growth of microorganisms. After incubation, a loopful of the sample was picked with a sterilized loop and streaked on the Nutrient agar plate for the isolation and purification of bacteria colonies. MacConkey agar plate was used to grow Gram-negative organisms and to distinguish between lactose fermenter and non-lactose fermenter bacteria. Eosin methylene blue agar (Oxoid, UK) was used for the isolation of *E.* coli, *Citrobacter* species, and *Klebsiella* species. *Salmonella Shigella* agar (Oxoid, UK) for *Salmonella and Shigella* species [38, 39]. The agar plates were then incubated for 18-24 hours at 37 °C, and subculturing of the discrete colonies from the different agar plates onto fresh agar plates was carried out aseptically to obtain pure colonies of isolates. The hemolytic activity of the bacteria was determined on blood agar. The bacteria were then identified using morphological characteristics, Gram staining, and biochemical tests such as motility test, oxidase test, catalase test, triple sugar iron (TSI), indole test, urease test, citrate utilization test, methyl red test, oxidative fermentation test, Voges Proskauer test, nitrate reduction test, and gelatin liquefaction test [39]. All reagents for biochemical tests were prepared according to manufacturer instructions (Difco ®, Laboratories, USA and Oxoid ®, London, UK) and the results were interpreted using the manual for bacteria identification [38] and online ABIS (Advanced Bacteriological Identification Software) [40]. Antibiotic susceptibility test

The susceptibility to antimicrobial drugs was carried out on each of the identified bacterial isolates using the disc diffusion method on Mueller-Hinton agar plates (MHA) (Oxoid Basingstoke, UK) with inocula adjusted to an optical density of 0.5 McFarland standard unit [40]. Pure bacterial isolates were inoculated into the nutrient broth and incubated at 37 °C for 24 hrs. After that, the growth in the nutrient broth was inoculated and swabbed on Mueller-Hinton agar plates. Ten common antibiotics, including ampicillin (10 µg), ciprofloxacin (5 µg), florfenicol (30ug), gentamycin (10 µg), oxacillin (5 µg), oxytetracycline (30 µg), penicillin (10 units), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg), were dispensed on the swabbed plate using an automatic multi-disc dispenser (Bioanalyse) and incubated at 37 °C, for 18–36 h [42]. All the antibiotic discs used were supplied by Oxoid, UK. The results of the antibiotic susceptibility test were interpreted following standard measurement of zones of inhibition from the back of the agar plate to the nearest mm using a ruler and were interpreted as sensitive (S), intermediate (I), or resistant (R) according to the Clinical Laboratory Standard Institute [41].

Multiple Antibiotic Resistance (MAR) Index

The MAR index for each bacterial isolate was determined from the results of the disc diffusion method. It was calculated by dividing the total number of antibiotics to which the bacteria isolates were resistant by the total number of antibiotics used on the isolates. Multi-drug resistance was defined as resistance greater than or equal to four antimicrobials [43].

Data analysis

Data from the isolates were used for the determination of the prevalence rates of the bacterial isolates. The percentage resistance of the bacteria was also calculated for each of the antibiotics. The prevalence rate of bacteria isolates was ascertained as the number of times the bacteria organism was identified over the total number of times all the bacteria species were identified. The resistance rates for each antibiotic were calculated. The degree of resistance for each antibiotic from the different farms was compared using the *chi*-squared and student's *t*-test. Values of $p < 0.05$ with a 95% confidence interval were considered significant. A one-way ANOVA with *Tukey's* posthoc test was performed to compare the differences in antibiotic-resistant bacteria from the various sources.

Authors' Contributions

AAD., LS., and SO conceived and designed the experiments. AAD. and SAA carried out the experiments. SMD analyzed the data. LS and SO provided research space and equipment. SMD and OMD Contributed reagents/materials/analysis tools. AAD took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript

Acknowledgements

We are deeply grateful to the fish farmers and handlers for granting us access to their farms and the laboratory technologists for the work in the laboratory.

Competing Interests

The authors declare that there is no conflict of interest.

References

1. FAO. Regional review on status and trends in aquaculture development in Sub-Saharan Africa -2015. FAO Fisheries and Aquaculture circular, Rome, Italy. 2017; 1135/4

2. Assefa A, Abunna F. Maintenance of fish health in aquaculture: Review of epidemiological approaches for prevention and control of infectious disease of fish Vet Med Int. 2018; 2018:1-10.

3. Ugwem UG, Akinrotimi OA, Eseimokumo F. Haematological Responses of Wild Nile Tilapia Oreochromis niloticus after Acclimation to Captivity Jordan J Biol Sci. 2011; 4(4): 225 – 230.

4. Ozigbo E, Anyadike C, Adegbite O, Kolawole P. Review of Aquaculture Production and Management in Nigeria. Am J Exp Agric. 2014; 4(10):1137-1151.

5. Abanikannda OTF, Jimoh AA, Abagun AA, Badmus LA. Comparative study of growth parameters of African Catfishes as panacea for food security. Nigerian J Anim Sci. 2019; 21 (3): 179-192.

6. Miranda CD, Godoy FA, Lee MR. Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. Front Microbiol. 2018; 9:1284.

7. Wanja DW, Mbuthia PG, Waruiru RM, Bebora LC, Ngowi HA, Nyaga PN. Antibiotic and disinfectant susceptibility patterns of bacteria isolated from farmed fish in Kirinyaga County, Kenya. Int J Microbiol. 2020; 1–8.

8. Sarker J, Faruk MAR. Experimental infection of Aeromonas hydrophila in pangasius Progress. agric. 2016; 27(3):392-399.

9. Opiyo MA, Marijani E, Muendo P, Odede R, Leschen W, Charo-Karisa H. A review of aquaculture production and health management practices of farmed fish in Kenya. Int J Vet Sci Med. 2018; 6:141-148.

10. Chen Y, Zhu X, Yang Y, Han D, Jin J, Xie S. Effect of dietary chitosan on growth performance, hematology, immune response, intestine morphology, intestine microbiota and disease resistance in Gibel carp (Carassius auratus gibelio). Aquac Nutr. 2014; 20: 532–546.

11. Okocha RC, Olatoye IO, Adedeji OB. Food safety impacts of antimicrobial use and their residues in aquaculture. Public Health Rev. 2018; 39:21

12. Santos L, Ramos F. Analytical strategies for the detection and quantification of antibiotic residues in aquaculture fishes: A review. Trends Food Sci Technol. 2016; 52:16–30.

13. Ayukekbong JA, Ntemgwa M, Atabe AN. The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrob Resist Infect Control. 2017; 6:47.

14. Monteiro SH, Graziela CR, Moura A, Garcia F, Pilarski F. Antibiotic Residues and Resistant Bacteria in Aquaculture. Pharm Chem J. 2018; 5(4):127-147.

15. Smith P, Alday-Sanz V, Matysczak J, Moulin G, Lavilla-Pitogo CR, Prater D. Monitoring and surveillance of antimicrobial resistance in microorganisms associated with aquatic animals. Rev Sci Tech Off. Int Epiz. 2013; 32 (2): 583-593.

16 FAO. Monitoring and surveillance of antimicrobial resistance in bacteria from healthy food animals intended for consumption. Regional Antimicrobial Resistance Monitoring and Surveillance Guidelines – Volume 1. Bangkok. 2019.

17. Kousar R, Shafi N, Andleeb S, Ali N, Mazhar AT, Khalid S. Assessment and incidence of fish associated bacterial pathogens at hatcheries of Azad Kashmir, Pakistan. Braz J Biol. 2020; 80(3): 607-614.

18. Afolabi OJ, Oladele OO, Olususi FC. Assessment of bacterial loads of Clarias gariepinus (Burchell, 1822) obtained from cultured and natural habitats. JoBAZ. 2020; 81:32.

19 Uddim MN, Al-Harbi AH. Bacterial flora of polycultured common carp (Cyprinus carpio) and African catfish (Clarias gariepinus). Int Aquat Res. 2012; 4:10.

20 Danba EP, Bichi AH, Ishaku S, Ahmed MK, Buba U, Bingari MS. Occurrence of pathogenic bacteria associated with Clarias gariepinus in selected fish farms of Kumbotso Local Government Area of Kano State Nigeria. Pure and Applied Science. 2014; 7(2): 145-149.

21. Wamala SP, Mugimba KK, Mutoloki SØ, Evensen RM, Byarugaba DK, Sørum H. Occurrence and antibiotic susceptibility of fish bacteria isolated from Oreochromis niloticus (Nile tilapia) and Clarias gariepinus (African catfish) in Uganda. Fish Aquatic Sci. 2018; 21(6):2-10.

22. Tsfaye S, Kasye M, Chane M, Bogale B, Abebeagre Z. Preliminary Survey of Gram-negative bacterial pathogens from commonly caught fish species (Oreochromis niloticus, Cyprinus carpio and Clarias gariepinus) in Lake Hayiq, Ethiopia. Fish Aqua J. 2018; 9: 238.

23. Kousar R, Shafi N, Andleeb S, Ali N, Mazhar AT, Khalid S. Assessment and incidence of fish associated bacterial pathogens at hatcheries of Azad Kashmir, Pakistan. Braz J Biol. 2020; 80(3): 607-614.

24. Cabral JPS. Water microbiology, bacterial pathogens and water. Int J Env Res Pub He. 2010; 7: 3657-3703.

25. Green HC, Dick LK, Gilpin B, Samadpour M, Field KG. Genetic markers for rapid PCR-based identification of gull, Canada goose, duck and chicken feacal contamination in water. Appl Environ Microbiol. 2012;78 (2):503-510.

26. Mauffret A, Caprais M, Gourmelon M. Relevance of bacteroidales and F-specific RNA bacteriophages for efficient fecal contamination tracking at the level of a catchment in France. Appl Environ Microbiol. 2012;78 (15): 5143-5152.

27. Wanja DW, Mbuthia PG, Waruiru RM, Mwadime JM, Bebora LC, Nyaga PN and Ngowi HA. Bacterial pathogens isolated from farmed fish and source pond water in Kirinyaga County, Kenya. Int J Fish Aquat Stud. 2019; 7(2): 295-301.

28. Nahar S, Rahman MM, Ahmed GU, Faruk MR. Isolation, identification, and characterization of Aeromonas hydrophila from juvenile farmed Pangasius (Pangasianodon hypophthalmus). Int J Fish Aquat Stud. 2016; 4(4):52-60.

29. Gufe C, Hodobo TC, Mbonjani B, Majonga O, Marumure J, Musari S. Antimicrobial profiling of bacteria isolated from fish sold at informal market in Mufakose, Zimbabwe Int J Food Microbiol. 2019; 1-7.

30. Shahriar A, Akter T, Kobra AT, Emran TB, Mallick J,Dutta M. Isolation of pathogenic and non-pathogenic microbial stains from different types of sea fish samples and their quality assessment with antibiogram properties. Journal of Advances in

Microbiology. 2019; 19(1): 1-10.

31. Adinortey CA, Amewowor DHA, Otwe EP, Galyuon IKA, Asante DKA. Antibiotic susceptibility profile and occurrence of Escherichia coli isolated from clinical and environmental samples in Cape Coast, Ghana. Res J Microbiol, 2017; 12(3):170–176.

32. Watts JEM, Schreier HJ, Lanska L, Hale MS. The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Mar Drugs,. 2017; 15(6):158.

33. Ayandiran TA, Dahunsi SO. Microbial evaluation and occurrence of antidrug multi resistant organisms among indigenous Clarias spp in River Oluwa, Nigeria. J. King Saud Univ Sci. 2017; 29(1): 96-105.

34. Osundiya O, Oladele R, Oduyebo O. Multiple antibiotic resistance (MAR) indices of Pseudomonas and Klebsiella species isolates in Lagos University Teaching Hospital," African J Clin Exp Microbiol. 2013; 14(3): 164-168.

35. Kathleen MM, Samuel L, Reagan FC, Kasing EL, Lesley AM, Toh SC. Antibiotic Resistance of Diverse Bacteria from Aquaculture in Borneo Int J Microbiol.2016;2016:1-9.

36. Gazal LE, Brito, KCT, Kobayashi RK, Takayama N, Cavalli, LS, Otutumi et al. Antimicrobials and resistant bacteria in global fish farming and the possible risk for public health. Arquivos do Instituto Biológico. 2020; 87:1-11.

37. KSGC .2015. Kaduna State Geographical Center, www.kadunastate.gov.ng, retrieved 25-06 2019

38. Holt GJ, Krieg NR, Sneath PHA, Stanley JT, Williams ST. Bergey's manual of determinative bacteriology. 9th ed; Baltimore md; Williams and wikins. Pub.co, Marrylan. 1994; 786.

39. Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. Clinical Veterinary Microbiology, Oxford University Press, New York, NY, USA, 2nd edition 2013.

40. ABIS(Advanced Bacteriological Identification Software), 2018 (http://www.tgw1916.net/bacteria_logare_desktop.html). Accessed 12, March 2020.

41. CLSI, Performance Standards for Antimicrobial Susceptibility Testing. In: Information Supplement M100-S17. Clinical Laboratory and Standards Institute, Wayne, PA, USA. 2015; 76–79.

42. Bauer AW, Kirby WM, Sheriss JC, Turc M. Antibiotic susceptibility testing by standardized single method. Am J Clin Pathol. 1996; 45:493-496.

43. Oteo J, Lazaro E, de Abajo FJ, Baquero F, Campos J. Antimicrobial-resistant invasive Escherichia coli, Spain. Emerg. Infect Dis. 2005; 11(4):546-553.

How to cite this article
Adah AD, Lawal S, Oniye S, Adah AS, David SM, Obisesan OO. Antibiotic resistance patterns of bacteria isolated from *Clarias gariepinus* farms in Kaduna state, Nigeria. Iran J Vet Sci Technol. 2022; 14(1): 29-38.
DOI: https://doi.org/10.22067/ijvst.2022.72394.1080
URL: https://ijvst.um.ac.ir/article_42023.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Nov- 29
Accepted after revision: 2022- Apr- 10
Published online: 2022- Apr- 20

RESEARCH ARTICLE

DOI: 10.22067/ijvst.2022.73754.1099

Effects of subcutaneous progesterone injection as a short-time estrus synchronization protocol in ewes: a preliminary study

Behnam Valizadeh, Masoud Rajabioun, Mohammad Azizzadeh, Babak Khoramian Toosi

Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

ABSTRACT

The present study aimed to evaluate the effect of short-time progesterone injection subcutaneously (SC) on estrus synchronization of Baluchi ewes. All ewes received one dose of PGF2α (15 mg) and then were divided into two groups. In the P4 group, a combination of progesterone and propylene glycol was subcutaneously injected twice at the three-day interval, and a vaginal sponge was used in the control group for 6 days. On day 6, all ewes received 400 IU of eCG. 48h after eCG injection, two rams were introduced into the flock. Blood samples were taken daily from Day 0 to Day 13 to measure serum progesterone concentration. Ultrasonography was used to observe ovaries and monitor their changes at three-day intervals. In P4 and control groups, the estrus rate was 88.9% and 100% for P4 and control groups, respectively; the duration of estrus for the P4 and control groups was 8 and 8.5 days, respectively (p >0.05). No significant difference was observed in the size of the largest follicle and the number of follicles more than 2 mm in diameter between the treatment and control groups. Further studies with some changes and modifications are required for gaining acceptable fertility and prolificacy rates.

Keywords

Estrus, synchronization, ewe, progesterone, short-time protocol

Number of Figures: 3
Number of Tables: 3
Number of References:: 13
Number of Pages: 6

Abbreviations

CIDR: Controlled internal drug release
eCG: equine serum gonadotrophin

Corresponding author:
Babak Khoramian Toosi
Email: *khoramian@um.ac.ir*
Tel: *+98 (51)3880-5611*

Introduction

Several protocols are used to induce and synchronize estrus or/and ovulation in ewes. One of the most common methods used for estrus synchronization in flocks is using intra-vaginal progesterone combined with eCG treatment. In this method, intra-vaginal progesterone was used to simulate corpus luteum (CL) which its removal coincident with eCG injection can result in ovulation. Insertion of a CIDR in anestrus goats leads to an increase in serum progesterone concentration (more than 5 ng/ml) for 3 or 4 days, which is higher than that observed in the middle stage of the physiological luteal phase. After 6-day treatment, the concentration of serum progesterone has been reduced to less than 2 ng/ml and remained low until CIDR removal [1]. The profile of serum progesterone with the above-mentioned treatment protocols is different from what is observed during the normal estrous cycle; low at the beginning which increases and remains high until luteolysis (the end of the cycle). In ewe, sub-luteal level of progesterone leads to excessive growth of the largest follicle and its persistence, which causes an increase in the age of ovulatory follicles. In old methods, progesterone was used as long as corpus luteum existed (12 days), regardless of the stage of the cycle or follicular state of the ovary. Therefore, 12-day progesterone treatment could lead to ovulation of old follicles in those ruminants [2].

Long-term progesterone treatment effectively results in estrus synchronization but leads to different pregnancy rates. These protocols were designed before the 1990 decade, which is not in agreement with the current understanding of follicular dynamics [3].

Sareminejad et al (2014) reported that estrus rates in three study groups, using MAP sponge for 6 days, using MAP for 12 days, and a control group, were 93.33%, 91%, and 5.26%, respectively ($p > 0.05$) [4]. Ataman et al (2006) reported estrus rate during the breeding season; by using progesterone sponge for 7 or 12 days, the estrus rate was 100% and that of the non-breeding season was 93.3% and 86.6%, respectively ($p > 0.05$) [5]. Contreras-Solis et al (2008) indicated that subcutaneous progesterone injection with olive oil and propylene glycol could maintain plasma progesterone at a high level for 41 and 89 hours, respectively. In the propylene glycol group, plasma progesterone concentration was higher than 0.5 ng/ml for 52 hours [6]. Various investigations showed that treatment with a high dose of progesterone in reproductive programs for a short period could improve the control of follicular dynamics and pregnancy rate in small ruminants [7].

The present study aimed to investigate a low-cost and practical method for delivering progesterone as part of an estrus synchronization protocol in ewes.

Results

The The lowest serum progesterone concentration after eCG injection did not differ significantly between the two groups ($p > 0.05$) (88.9% vs 100% in P4 and control groups, respectively). Furthermore, the level of progesterone less than 0.5 ng/ml was considered as a criterion for ewes to be in estrus. The median, the first, and third quartiles of the duration of estrus were 8, 7, and 10.5 days in the treatment group, and 8.5, 7, and 11 days in the control group ($p > 0.05$). The median estrus duration was compared between the two groups and is shown in Figure 1. Time-interval between eCG injection and displaying estrus in the P4 and control groups were 5.3 and 4 days, respectively ($p > 0/05$).

By using ultrasonography at 25 days after removal of rams, 4 from 9 ewes in the P4 group and 8 from 10 ewes from the control group became pregnant (mean litter size was 1.25 and 1.37, respectively).

Results of the present study showed that two injections of progesterone along with propylene glycol (SC) at three-day intervals could maintain the progesterone level of more than 1 ng/ml in all ewes (Table 2). Also, the comparison of the mean progesterone concentration (ng/ml) between the two groups showed no significant difference within days 6 to 12 of the study ($p = 0.227$) (Figure 2).

Median, first quartile, and third quartile of the diameter of the largest follicle in the treatment group on day 6 were 6.27, 6.04, and 6.40, respectively. Median, first quartile, and third quartile of the diameter of the largest follicle in the control group were 6.06, 5.86, and 7.40, respectively. No significant difference was observed between these groups ($p > 0.05$) (Table 3).

The difference in follicle diameter and the number of follicles larger than 2 mm in diameter between the two groups was not statistically significant ($p > 0.05$) (Figure 3).

Discussion

According to new estrus synchronization methods, a 5- to 7-day P4 treatment seems to be sufficient for inducing estrus in ewes. The protocol used in the present study contained fewer injections and had less duration than the intra-vaginal method which made it more practical, cost-effective, simple, and quick. Dixon et al. (2006) reported that a high dosage of progesterone delivered by two CIDR-G devices for 12 days lead to considerable estrous response and a high fer-

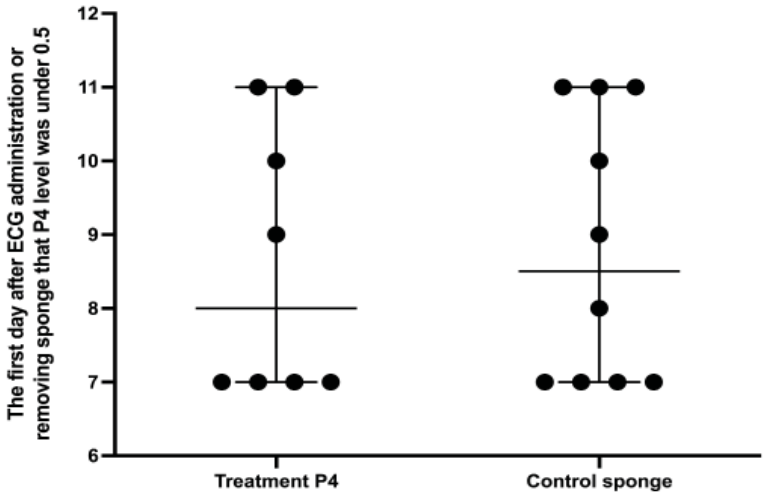


Figure 1.
Comparison of median and range of estrus time between two groups

Table 1.
Comparison of pregnancy rate, twinning and litter size between two groups

Groups	Non-pregnant sheep	single lamb	twins	Litter size
P4	5	3	1	1.25
Control	2	5	3	1.37

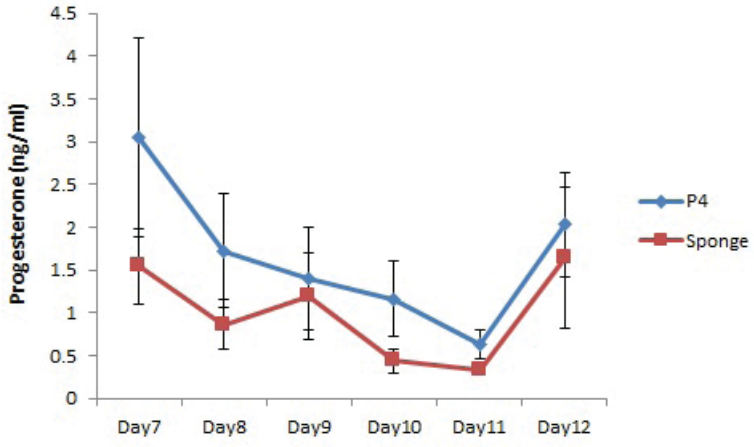


Figure 2.
Mean (±SE) of progesterone level during days 6 to 12 in control and P4 groups

Table 2.
The concentration of plasma progesterone from day 0 to 6 in P4 group (ng/ml)

Groups	Day 0*	Day 1	Day 2	Day 3**	Day 4	Day 5	Day 6
Mean	2.4	4.9	3.6	3.2	5.8	5.7	4.2
SD	2.2	2.7	1.9	1.8	1.7	1.6	2.4
Minimum	0.00	2.1	1.01	0.98	3.93	3.54	1.40
Maximum	7.026	10.44	7.44	7.49	9.74	9.06	7.92

* Day 0 was just before first progesterone injection
** Day 3 was just before second progesterone injection

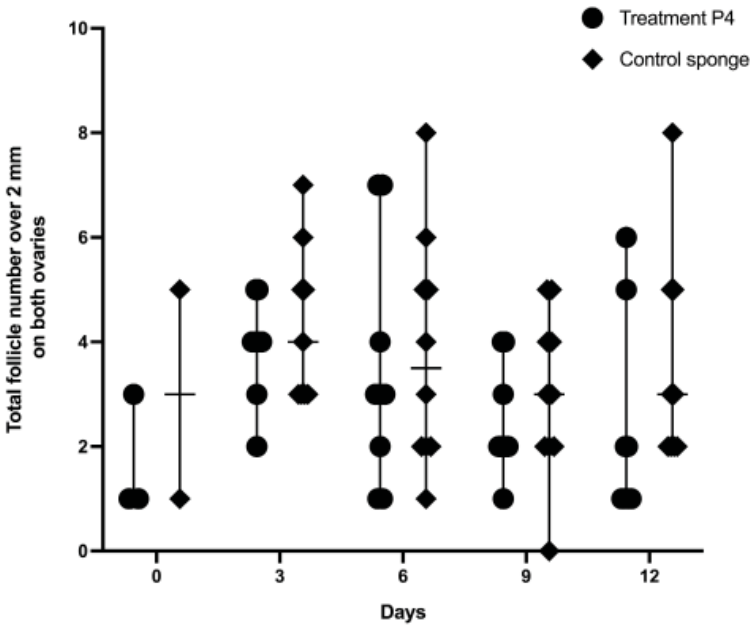


Figure 3.
The median and range of follicles over than 2 mm in two groups

Table 3.
Comparison of the largest diameter of the follicle (cm) from day 6 to 12 between two groups

Groups	Min	First quartile	Median	Third quartile	Max
P4	4.24	6.04	6.27	6.40	6.85
Control	4.62	5.86	6.06	7.40	8.81

tility rate. The mean plasma level of progesterone in ewes received two CIDRs simultaneously in their vagina for 12 days remained more than 1.4 ng/ml with a 97.7% estrus rate [8]. Pearce et al. (1985) reported that intra-vaginal sponges impregnated with 200 mg or 400, 500, or 600 mg progesterone resulted in the maintenance of plasma progesterone concentration of 1.5-4.9 ng/ml over a 12-day insertion period and create a condition similar to natural luteal phase (1.9-6.9 ng/ml) [9]. Results of the present study showed that in the P4 group the mean serum progesterone level during 6 days of synchronization protocol was 3.2-5.8 ng/ml with the minimum and maximum of 1.01 and 10.44 ng/ml. Considering mean progesterone concentration during the synchronization program, it showed that subcutaneous injection of progesterone in combination with propylene glycol could be an effective method for estrus synchronization in ewes. In the previous-mentioned study, 200 mg of progesterone was dissolved in corn oil and injected intramuscularly twice at 2.5 days intervals. Results showed that all ewes gave birth during the first 8 days of the parturition period, which indicated proper estrus synchronization [10]. In the present study, the estrus rate in the treatment and control groups was 88.9% and 100%, respectively; which no significant difference was observed between the two groups. Also, the median of the duration in which progesterone level remained less than 0.5 ng/ml in P4 and control groups were 2 and 2.5, respectively; moreover, the time that progesterone level reached its lowest level was day 3.5 and day 4.5 in P4 and control group, respectively. Robinson et al. removed sponge consisting of 10, 20, and 40 mg progesterone after 12 days and reported 29, 43-48, and 48-53 hours for the estrus occurrence peak, respectively which showed a relative delay in the peak of estrus occurrence, representing that by increasing progesterone, a rise in the plasma progesterone level was observed [11].

In this study, no difference in the size of the largest follicle and the number of follicles larger than 2 mm was observed during the observation of ovaries and follicles. This relative similarity in terms of the diameter of the largest follicles in the two groups was proportionate and was following the relative similarity in the estrus rate between the two groups. Johnson et al. (1996) indicated the larger follicle diameter in low progesterone concentration (1 ng/ml>) compared

to high progesterone concentration (1 ng/ml<) [12].

Results of the present study showed that plasma progesterone concentration in the P4 group was more than 1 ng/ml during the estrus synchronization protocol while we could not measure the plasma progesterone concentration during the synchronization protocol in the sponge group. An investigation showed that sheep with a low level of plasma progesterone (less than 1 ng/ml) had larger and older follicles compared to sheep with a high level of plasma progesterone, which resulted in a lower pregnancy rate [12]. Furthermore, the progesterone concentration during estrus in the control group was lower than in the P4 group which may lead to a higher estrus rate and pregnancy rate in the control group. In the artificial insemination technique, if the estrus occurs on certain days, the high fertilization rate will be predictable while in natural breeding programs shorter duration of estrus in the P4 group may lead to a lower pregnancy rate.

In conclusion, two subcutaneous injections of progesterone as a synchronization protocol may be used as a practical cost-effective method of estrus synchronization however, further studies with larger sample sizes and minor changes are required to achieve an acceptable fertility and prolificacy rates.

Materials & Methods

The present study was performed in the farm animal and poultry research center of the Ferdowsi University of Mashhad, Mashhad, Iran (latitude 35° 43' to 37° 8'N and the longitude 59° 4' to 60° 36'E). The selected ewes were not lactating and the study was performed at least three months post-lambing, also they had no observable general or reproductive disease. 5 ewes were excluded from the study due to illness or other problems and the investigation started with 19 sheep. The diet of ewes was balanced based on NRC. Ewes were divided into two groups randomly. The treatment group (P4): consisted of 9 ewes, which received 15 mg prostaglandin intramuscularly (vetalyse, Aburaihan, Iran) and after 3 hours 25 mg progesterone (Vetagestrone, Aburaihan, Iran) with 4mL propylene glycol injected subcutaneously. After 3 days, 400 IU eCG was injected intramuscularly and after 48 h, two rams were introduced into the flock and stayed for 5 days.

The control group: consisted of 10 ewes that received one dose of 15 mg prostaglandin (vetalyse, Aburaihan, Iran) intra-muscularly and after 3 hours an intra-vaginal progesterone sponge consisting of 60 mg MPA (Sponjavet, Hipra, Spain) was used for 6 days and 400 IU of eCG was injected intramuscularly after sponge removal (Gonaser, Hipra, Spain). After 48 h, two rams were introduced into the flock for 5 days.

MINDRAY DP-6600VET and rectal linear probe with frequencies of 7.5 and 10 MHz were used for ultrasonographic evaluation of ovaries. All ewes were 15-20 hours off-feed before the ultrasonographic examination. Ultrasonography of both ovaries was done from day 0 until day 13 and the follicular map was recorded. The follicles larger than 2 mm in diameter were mapped. Blood samples were collected daily for 13 consecutive days from the first day. The blood samples were centrifuged immediately at 3000 × g for 20 min, and collected blood serums were stored at −20 °C until progesterone measurement. The concentration of progesterone was determined by ELISA. (DRG, Germany). The P4 concentration of less than 0.5 ng/ml was considered as a criterion for ewes to be in estrus.

A Chi-square test was used to compare the estrus rate between the two groups. Mean progesterone levels were compared between two groups by using the ANOVA test. Mann-Whitney U test was used to compare the average diameter of the largest follicles, the number of follicles larger than 2 mm, and to compare the estrus duration and the number of embryos and lambs between two groups. Statistical analysis was performed using SPSS software. A p-value less than 0.05 was considered statistically significant.

Authors' Contributions

Research concept and design: BK, MR; Analyzed the data: MA; Performed the experiments: BV, BK, MR; wrote the paper: BV, BK, MR; All the authors read and approved the final manuscript.

Acknowledgements

The authors would like to gratefully acknowledge Dr. F. Fazel and Mr. A. Kargar for their assistance in this project. This work was financially supported by the Ferdowsi University of Mashhad (Grant number 3/33583, 14/11/1393). Funding was allocated to collection, analysis and interpretation of data.

Competing Interests

The authors declare that they have no conflict of interest.

References

1. Swelum AA, Saadeldin IM, Moumen AF, Ali MA, Alowaimer AN. Efficacy of controlled internal drug release (CIDR) treatment durations on the reproductive performance, hormone profiles, and economic profit of Awassi ewes. Small Ruminant Research. 2018; 166:47-52.

2. Knights M, Ramgattie R, Siew N, Singh-Knights D, Bourne G. Effectiveness of a short-term treatment with progesterone injections on synchrony of lambing and fertility in tropical hair sheep. Animal reproduction science. 2011;126(1-2):70-5.

3. Viñoles C, Forsberg M, Banchero G, Rubianes E. Effect of long-term and short-term progestagen treatment on follicular development and pregnancy rate in cyclic ewes. Theriogenology. 2001; 55(4):993-1004.

4. Sareminejad P, Tabatabaei S, Mamouei M, Mirzadeh K, Boujarpour M. The effects of short and long term medroxy progesterone acetate (MAP) sponge treatments on reproductive performance during the non-breeding season of arabian ewes. Iranian Journal of Applied Animal Science. 2014; 4(4):747-51.

5. Ataman MB, Akoz M, Akman O. Induction of synchronized oestrus in Akkaraman cross-bred ewes during breeding and anestrus seasons: the use of short-term and long-term progesterone treatments. Revue de médecine vétérinaire. 2006; 157(5):257-60.

6. Contreras-Solis I, Gomez-Brunet A, Encinas T, Gonzalez-Bulnes A, Santiago-Moreno J, Lopez-Sebastian A. Influence of vehicle on kinetics of exogenous progesterone administered either by subcutaneous and intramuscular routes to sheep. Research in veterinary science. 2008; 85(1):162-5.

7. Ungerfeld R, Rubianes E. Short term primings with different progestogen intravaginal devices (MAP, FGA and CIDR) for eCG-estrous induction in anestrus ewes. Small Ruminant Research. 2002; 46(1):63-6.

8. Dixon AB, Knights M, Pate JL, Lewis PE, Inskeep EK. Reproductive performance of ewes after 5-day treatment with intravaginal inserts containing progesterone in combination with injection of prostaglandin F2α. Reproduction in Domestic Animals. 2006; 41(2):142-8.

9. Pearce DT, Robinson TJ. Plasma progesterone concentrations, ovarian and endocrinological responses and sperm transport in ewes with synchronized oestrus. Reproduction. 1985; 75(1):49-62.

10. Knights M, Ramgattie R, Siew N, Singh-Knights D, Bourne G. Effectiveness of a short-term treatment with progesterone injections on synchrony of lambing and fertility in tropical hair sheep. Animal reproduction science. 2011; 126(1-2):70-5.

11. Robinson TJ, Smith JF. The time of ovulation after withdrawal of SC-9880-impregnated intravaginal sponges from cyclic Merino ewes. The time of ovulation after withdrawal of SC-9880-impregnated intravaginal sponges from cyclic Merino ewes. 1967.

12. Johnson SK, Dailey RA, Inskeep EK, Lewis PE. Effect of peripheral concentrations of progesterone on follicular growth and fertility in ewes. Domestic Animal Endocrinology. 1996; 13(1):69-79.

13. Evans AC. Ovarian follicle growth and consequences for fertility in sheep. Animal Reproduction Science. 2003; 78(3-4):289-306.

How to cite this article
Valizadeh B, Rajabioun M, Azizzadeh M, Khoramian Toosi B. Effects of subcutaneous progesterone injection on ewes as a short-time estrus synchronization protocol: A preliminary study. Iran J Vet Sci Technol. 2022; 14(1): 39-44.
DOI: https://doi.org/ 10.22067/ijvst.2022.73754.1099
URL:https://ijvst.um.ac.ir/article_42068.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Oct- 25
Accepted after revision: 2022- Mar- 11
Published online: 2022- Apr- 20

Case Report

DOI: 10.22067/ijvst.2022.73253.1094

Congenital First Pharyngeal Arch Anomaly in a Holstein Friesian Calf

Bahador Shojaei[a], Sina Delshad[a], Mehdi Farajli Abbasi[b], Maehdi Jaaferi[c], Mohammad Farajli Abbasi[d]

[a] Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Iran.
[b] Doctor of Veterinary Medicine, Mehr Pet Clinic, Tabriz, Iran.
[c] Department of Clinical Sciences, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Iran.
[d] Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran

ABSTRACT

The developmental error of the first pair of pharyngeal arch processes would result in a group of malformations related to the oral cavity. These malformations can be listed as agnathia, micrognathia, brachygnathia, syngnathia, cleft palate, campylognathia, parrot beak, and strophocephalia. Campylognathia is an anomaly in which the lower or upper jaw deviated from the midline. Clinical examination of a newborn female Holstein Friesian calf showed mandibular deviation, inferior and superior cheek teeth deformation, and some other facial malformations. No similar case was detected in the history of the herd. Precise consideration of malformed animals and the extent of involvement of different structures indicates the pattern of malformation, the time of teratogenic effect, and the motive embryonic structures. In the present case, the involvement of derivatives of both maxillary and mandibular processes shows the responsibility of the first pharyngeal arch for mentioned abnormalities. In humans, different types of abnormal development of the first pharyngeal arch have been reported and so, have been classified under the "first visceral arch syndrome". But according to the infrequency of reports, this classification has not been done in domestic animals.

Keywords

Congenital cleft jaw, pharyngeal arch, calf, anomaly, campylognathia

Number of Figures: 2
Number of Tables: 0
Number of References: 21
Number of Pages: 5

Abbreviations

TRP: Temperature, Pulse, Respiratory rate
BMP: Bone Morphogenetic Protein
FGF: Fibroblast Growth Factor
SHH: Sonic hedgehog
WNT: Wingless-related integration site

Corresponding author: Mohammad Farajli Abbasi
Email: m.fabbasi@kmu.ac.ir
Tel: +98914 1063701

Introduction

The mesenchyme of the first pair of pharyngeal arches gives rise to dorsal maxillary and ventral mandibular prominences. These facial structures converge and fuse in the midline enclosing an invagination of ectoderm, the stomodeum. The ventral mandibular prominences contribute to the formation of the lower jaw while the paired maxillary prominences procedure the upper jaw [1]. The developmental error of these structures, often due to the lack of migration of neural crest cells to the first pharyngeal arch, leads to a group of malformations related to the oral cavity [2]. The developmental error of these structures would result in a group of malformations related to the oral cavity. These malformations can be listed as agnathia, micrognathia, brachygnathia, Syngnathia, cleft palate, campylognathia [3,4], parrot beak, and strophocephalia [5]. Campylognathia is an anomaly in which the lower or upper jaw deviated from the midline [6]. It has been reported in geese [7], horses [8], sheep [4], and cows [9]. Lack of maternal protein and vitamins, infectious diseases, radiation, folic acid deficiency, teratogens, and endocrine disorders at the beginning of organogenesis lead to the formation of these malformations [10,11]. In humans, this anomaly has been reported as one of several malformations of the "First Arch Syndrome". But, in animals, no classification has been made on the pharyngeal arch abnormalities. In the present work, we aim to describe a Holstein Friesian calf with severe inferior campylognathia associated with the cleft palate and oligodontia from the Kerman province for the first time.

Case Presentation

A seven-day-old newborn female Holstein Friesian calf was referred to the veterinary hospital, Shahid Bahonar University of Kerman. On the clinical examination, mandibular deviation, respective upper and lower cheek teeth deformation, and malformation of the buccal and masseter regions on the left side of the face was seen (Figure 1). Regarding the history of the herd, it was the fifth delivery of the mother and the previous deliveries were normal, this was the first case in the herd, also no sign of poisoning was found and no medicine was used during pregnancy. TRP (Temperature, Pulse, Respiratory rate), and other vital signs have been normal. The calf had fed her mother milk. There was no symptom of respiration pneumonia, but milk was fed flow out from both nostrils during feeding. According to the severity of malformations, the animal was sacrificed and dissected. The necropsy was revealed a severe mandibular deviation to the left side of the face (Figure 2A). The ramus on the left side was short without any remnant of the condylar process (Figure 2B). The ipsilateral masseter muscle has a very poor configuration. The origin of the masseter on the facial bones was distorted and its insertion was seen as sparse fascicles on the abnormal mandibular ramus (Figure 2C). According to the shortening of the ramus, the caudal portion of the medial pterygoids was exposed. Both mandibular and maxillary oligodontia and Crooked teeth were seen in the right and left jaws. The rostral part of the hard palate was intact but a longitudinal cleft extended from the dental pad caudally to the pharyngeal region to involve both hard and soft palates (secondary cleft palate). The right external ear was intact. The left auricle has been formed but no external auditory meatus was detected. The left mandibular ramus was narrow and deformed. No other abnormalities were observed in the dissection of the brain and other organs. Also, the absence of ataxia, neurological symptoms such as turning around, head pressing, or any clinical neurological symptoms indicated the absence of this type of brain involvement and confirmed the anatomical evidence.

Figure 1.
A) Left view of the face of affected animal. B) Malformed buccal region results extension of the oral commissure nearby the external ear.

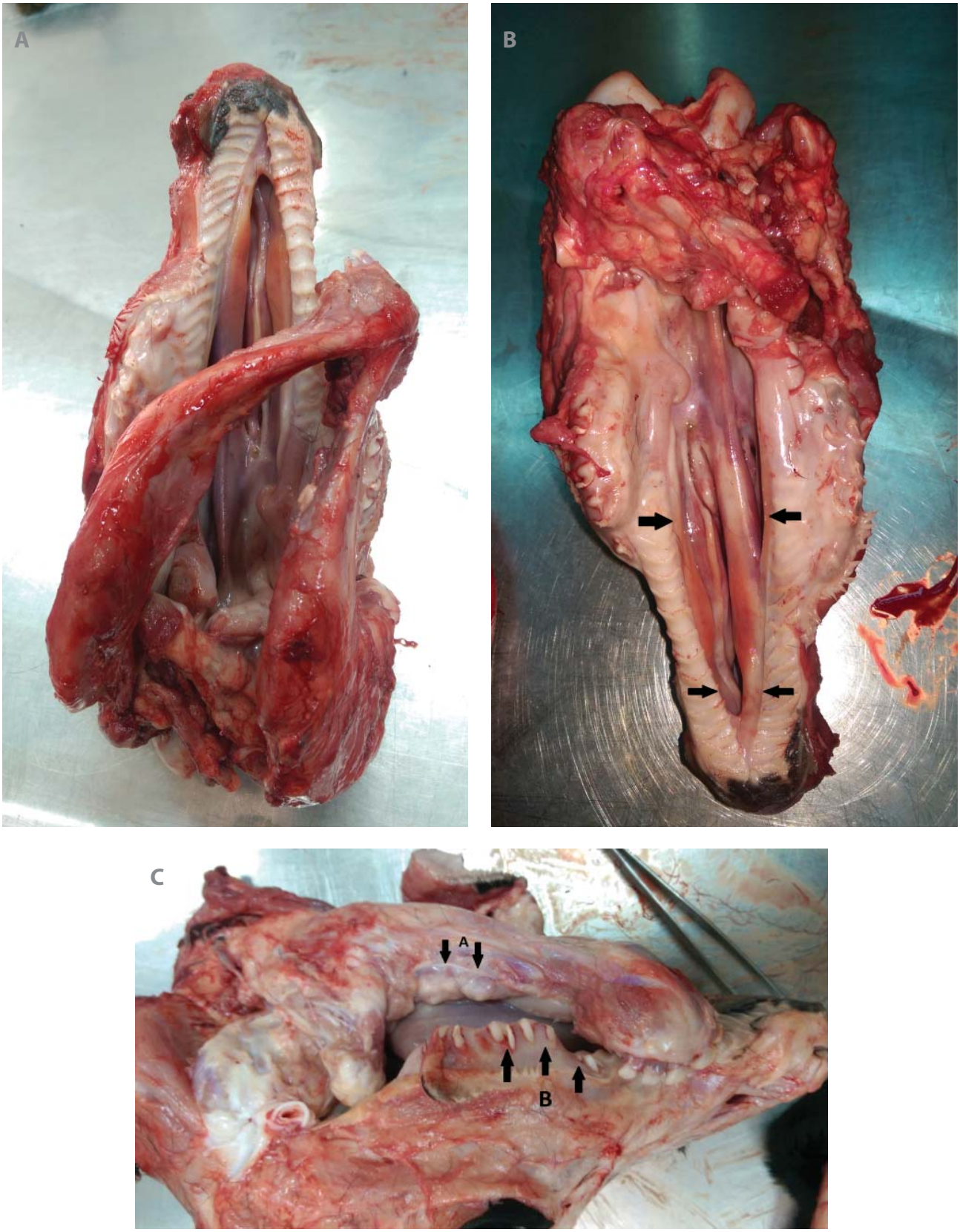


Figure 2.
A) Mandibular campylognathia. B) Secondary cleft palate (arrows). C) Exposition of the medial pterygoideus according to the malformation in the mandible. The oligodontia is also seen in the mandibular (arrowed A) and maxillary (arrowed B).

Results & Discussion

According to the interaction of several organ systems, the morphogenesis of the face seems very complicated [12]. It is known that the fetal organs such as the facial processes, the pharyngeal arches, and the developing brain, would deeply relate to the formation of the face in early embryonic life [12,13]. So, in the evaluation of the facial anomalies, attention should be paid to the derivatives of each embryonic precursor. Precise consideration of the malformed animals and the extent of involvement of different facial structures, revealed the pattern of malformation, the time of the effect of teratogen, and the motive embryonic structures [14]. In the present case, all of the detected disorders incriminate the first pharyngeal arch. Secondary cleft palate deviated mandible and its rudimentary ramus, malformed masticatory muscles, and oligodontia support the involvement of the maxillary and mandibular processes of this arch. Clefts of the face are developmental disorders due to failure of closure in facial processes such as the frontonasal, maxillary, and mandibular processes. They can be asymmetric unilateral or median symmetrical clefts. In the ruminants, the most reported type of these disorders is the secondary cleft palate, which is shaped due to failure of the growth of the maxillary processes of the first pharyngeal arches, but cleft palate accompanying with the campylognathia reported as a rare anomaly in the herbivores [8]. In the present case involvement of derivatives of both maxillary and mandibular processes shows the responsibility of the first pharyngeal arch for mentioned abnormalities. In humans, different types of abnormal development of the first pharyngeal arch have been reported and so, have been classified under the "first visceral arch syndrome" [15]. But according to the infrequency of reports, this classification has not been done in domestic animals.

Since the growth of teeth and the development of the craniofacial structures are affected by similar biological factors such as BMP, FGF, SHH, and WNT [16,17], therefore in many abnormal cases, malformation of these structures are accompanied by each other, as we saw in this case.

The critical period of fusion of facial fissure and palate took place at days 34 and 56 of gestation, respectively [18]. Although a genetic basis has been determined for the cleft palate (multifactorial or autosomal inheritance in the Charolais breed) and mandibular deformations (brachygnathia inferior) (polygenic inheritance in the Simmental breed) [8,19,20] as we did not find any similar case in the herd history and previous parturitions, also there was evidence of no poisoning and no change in diet, it may be stated that the present case has been exposed to environmental teratogens such as poisonous plants and viruses (e.g. Bluetongue virus) in the first two months of embryogenesis [21].

Ethics statement

This study was approved by the research ethics committee of the Shahid Bahonar University of Kerman, Iran (IR.UK.VETMED.REC). Animal care and all procedures were accomplished according to the instructions of care and use of laboratory animals.

Authors' Contributions

BS and SD performed review literature and manuscript writing. MFA and MFA performed the clinical examination and necropsy MJ examined the animal and performed review literature.

Competing Interests

The authors declare that there is no conflict of interest.

References

1. McGeady TA, Quinn PJ, Fitzpatrick ES, Ryan MT, Kilroy D, Lonergan P. Veterinary embryology. 2nd ed. John Wiley \& Sons; 2017. 274–288.

2. The Developing Human: Clinically Oriented Embryology, 9e - Keith L. Moore - Google Books [Internet]. 2019 [cited 2021 Jul 11]. p. 143–79. Available from: https://books.google.com/books?hl=en&lr=&id=OTaBDwAAQBAJ&oi=fnd&pg=PP1&ots=GoyHv9ri7z&sig=29Ch-4A6_MpoH2PtxDCKZtTH3QU#v=onepage&q&f=false

3. Flueck WT, Smith-Flueck JAM. Diseases of red deer introduced to Patagonia and implications for native ungulates. Anim Prod Sci. 2012; 52(8):766–73.

4. Shojaei B, Derakhshanfar A, Oloumi MM, Hashemnia S. Diprosopus, spina bifida and kyphoscoliosis in a lamb-a case report. Vet Arh. 2006; 76(5): 461–9.

5. Lejong M, Vanmuylder N, Louryan S. External and computed tomography analysis of a strophocephalic lamb. Morphologie. 2019; 103(341):122–5.

6. Shojaei B, Asadi A. Dicephalus, inferior brachygnathia, cleft palate and campylognathia in a calf. Iran J Vet Res. 2008; 4:117–22.

7. Lingnau A, Kaleta EF. Malformations of the upper beak (campylognathia superior) and adjacent bones within the splanchnocranium of domestic geese: an evaluation of cases that have been described during four centuries. Vet Hist. 2018; 19(2):163–76.

8. Flueck WT, Smith-Flueck JAM. Huemul heresies: beliefs in search of supporting data. 2. Biological and ecological considerations. Anim Prod Sci. 2012; 52(8): 694–706.

9. Leipold HW, Ojo SA, Huston K. Genetic Defects of the Skeletal System in Cattle. In: 1st World Congress on Genetics Applied to Livestock Production: Symposia. 1974; 35–40.

10. Hobbenaghi R, Dalir-Naghadeh B, Nazarizadeh A. Congenital multi-organ malformations in a Holstein calf. Vet Res Forum. 2015; 6(3): 257–260.

11. Oryan A, Shirian S, Samadian M.R. Congenital craniofacial and skeletal defects with arthrogryposis in two newborn male Holstein Friesian calves. Comp Clin Pathol. 2011; 20: 43–46.

12. Pruzansky S. Anomalies of face and brain. Birth Defects Orig Artic Ser. 1975; 11(7):183–204.

13. DeMyer W. Median facial malformations and their implications for brain malformations. Birth Defects Orig Artic Ser. 1975; 11(7): 155–81.

14. Moritomo Y, Tsuda T, MIYAMOTO H. Craniofacial skeletal abnormalities in anomalous calves with clefts of the face. J Vet Med Sci. 1999; 61(10): 1147–52.

15. McKenzie J. The first arch syndrome. Arch Dis Child. 1958; 33(171):477.

16. Seppala M, Fraser GJ, Birjandi AA, Xavier GM, Cobourne MT. Sonic hedgehog signaling and development of the dentition [Internet]. Vol. 5, Journal of Developmental Biology. Multidisciplinary Digital Publishing Institute; 2017 [cited 2021 Jul 11]; 6. Available from: https://www.mdpi.com/2221-3759/5/2/6/htm

17. Cobourne MT, Sharpe PT. Diseases of the tooth: The genetic and molecular basis of inherited anomalies affecting the dentition [Internet]. Vol. 2, Wiley Interdisciplinary Reviews: Developmental Biology. John Wiley & Sons, Ltd; 2013 [cited 2021 Jul 11]; 183–212. Available from: https://wires-onlinelibrary-wiley-com.ezp4.semantak.com/doi/full/10.1002/wdev.66

18. Dixon MJ. Treacher Collins syndrome. J Med Genet. 1995; 32(10):806.

19. Evans HE, WO S. Prenatal development of domestic and laboratory mammals: growth curves, external features and selected references. Anat Histol Embryol. 1973; 2:11–45.

20. Rousseaux CG. Congenital defects as a cause of perinatal mortality of beef calves. Vet Clin North Am Food Anim Pract. 1994; 10(1): 35–51.

21. Line BS. Congenital and Inherited Anomalies in Animals. 2020 [cited 2021 Jul 10];1–8. Available from: https://www.msdvetmanual.com/generalized-conditions/congenital-and-inherited-anomalies/congenital-and-inherited-anomalies-in-animals

How to cite this article

Shojaei B, Delshad S, Farajili Abbasi M, Jafari M, Farajili Abbasi M. Congenital First Pharyngeal Arch Anomaly in a Holstein Friesian Calf. Iran J Vet Sci Technol.2022; 14(1):45-49.
DOI: https://doi.org/ 10.22067/ijvst.2022.73253.1094
URL: https://ijvst.um.ac.ir/article_42069.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Jan- 05
Accepted after revision: 2022- Mar- 18
Published online: 202- Apr- 20

Case Report

DOI: 10.22067/ijvst.2022.74358.1104

Unilateral renal torsion in a lamb

Yaser Nozohour[a], Ghader Jalilzadeh Amin[a], Ali-Asghar Tehrani[b], Sajjad Rezaie[a], Alireza Moradian[a]

[a] Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
[b] Department of Pathology, Faculty of Veterinary Medicine, University of Urmia, Urmia, Iran.

ABSTRACT

In ruminant, the kidneys are a major excretory organ that eliminate metabolic byproducts and regulate water-electrolyte homeostasis and maintain acid-base balance in the body. Therefore, kidney abnormalities cause a life-threatening effect on ruminants' health. Renal torsion has not been reported in any animal species. In the present report, a case of a two-month-old male lamb that has unilateral renal torsion has been described. In necropsy, the twisted left kidney was severely congested and was enlarged in size. Histopathological studies revealed renal tubular and glomeruli necrosis. Vascular changes consisted of congestion and thrombi in the blood vessels. Abnormalities in the embryonic developmental period and increased mobility of the renal structure due to ligaments associated with a twisted kidney are suspected causes of the occurrence of renal torsion.

Keywords

Kidney, torsion, ruminant, urinary tract

Number of Figures: 2
Number of Tables: 0
Number of References: 12
Number of Pages: 4

Abbreviations

H&E: Hematoxylin and eosin:

Corresponding author: Ghader Jalilzadeh-Amin
Email: g. jalilzadeh@urmia.ac.ir
Tel: +98 (91)4417-3267

Introduction

The kidneys are located in pairs in the sublumbar and retroperitoneal regions. They regulate water-electrolyte homeostasis and maintain acid-base balance in the body [1, 2, 3]. Each kidney has a convex lateral border and a concave medial border [1, 2]. In the medial border of the kidney, there is a fossa called the hilus, in which the nerves, arteries, and ureters of the kidney are located. Around the kidneys, depending on the animal species, a mass of adipose tissue called perirenal fat is seen. These fat masses are involved in maintaining the kidneys in their position in the abdominal cavity [1, 2].

The rotation of the kidney around its vascular pedicle is a very rare condition. To our knowledge renal torsion has not been described in animals. In human beings, renal torsion is an unusual surgical complication and causes occlusion of vascular structure and parenchymal infarction [4,5,6]. Renal torsion was reported in humans after kidney-pancreas transplant surgery usually in infants which have a prune-belly syndrome or any condition that allows the organ to move freely within the pelvis [4, 5, 6]. Probable causes of renal torsion in humans include the excess vascular pedicle or ureteral length, and lack of adhesion, which allows for abnormal mobility of the kidney and a higher probability of twisting [4, 5]. Renal torsion causes severe abdominal pain and decreased urine output and may occlude the ureter, resulting in hydronephrosis or renal pelvic wall thickening [4, 5]. Therefore, any damage and dysfunction of the kidneys can lead to serious complications. Renal torsion in a lamb which has led to kidney damage has been reported for the first time.

Case Presentation

In the winter of 2021, a dead two-month-old lamb was submitted to the veterinary clinic of Urmia University for necropsy. According to the owner, the animal died suddenly and before that, it only showed a decrease in appetite and activity. Because of sudden death, it was not possible to obtain blood samples and laboratory tests. Post mortem examination of the lamb revealed the presence of unilateral renal torsion of the left kidney in the lamb (Figure 1). The kidney was dark brown and enlarged in size (renal length: 9.5 cm, width: 6.2 cm). Dimensions of the normal kidney were 6.3 × 3.8 cm. Appropriate tissue samples were taken from the kidney and were fixed in 10% neutral buffered formalin for 24 hr. The sections were stained by the hematoxylin and eosin (H&E) method. Histopathological studies revealed renal tubular necrosis characterized by pyknotic nuclei that sloughed into the lumen of the tubules (Figure 2). The glomeruli tuffs were atrophied. Severe vascular changes comprised congestion and thrombi in the blood vessels. The interstitial tissue was diffusely expanded by edema and hemorrhage (Figure 2).

Discussion

Renal torsion in lamb has not been reported in the literature. The compression on the vessels due to the torsion is such that arterial blood still gets into the organ because of their thick muscular wall but the pressure on the thinner-walled veins, restricts the blood outflow and venous blood accumulation, resulting in edema, congestion, hemorrhage and eventually necrosis [9, 10]. Ureteral obstruction is associated with urinary retention and destruction of the renal parenchyma, leading to enlarged kidneys [9, 10]. Unilateral ureteral obstruction can quickly cause the infiltration of inflammatory cells into the interstitial tissue of the kidney [9, 10]. Oxidative stress appears to play a key role in the onset and continuation of inflammation after obstruction, resulting in renal tubular damage and interstitial tissue fibrosis [11, 12]. The histopathological findings of this case show that renal torsion caused necrosis in renal tubules and glomeruli. According to the human medical literature, renal torsion can be an early or late complication and may be incomplete and intermittent [7, 8]. In the best of circumstances, renal torsion can be repaired; in the worst cases, the kidney is lost [7, 8].

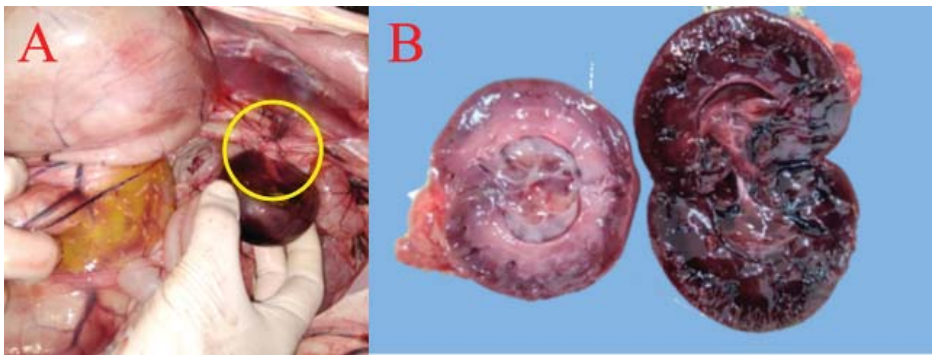


Figure 1.
The necropsy of the two-month-old lamb after sudden death. A) The axis of renal torsion around the vascular pedicle (circle). B) The sagittal sections of the normal and abnormal kidneys. Severe congestion and enlarged size are obvious.

Since this abnormal condition of kidney torsion has occurred at a very young age, it is likely to be due to a disorder in the fetal developmental stage. However, no increase in length was observed in the examination of ligaments related to the twisted kidney, therefore the possibility of torsion due to increased mobility of the renal structure seems unlikely, although it can be one of the causes.

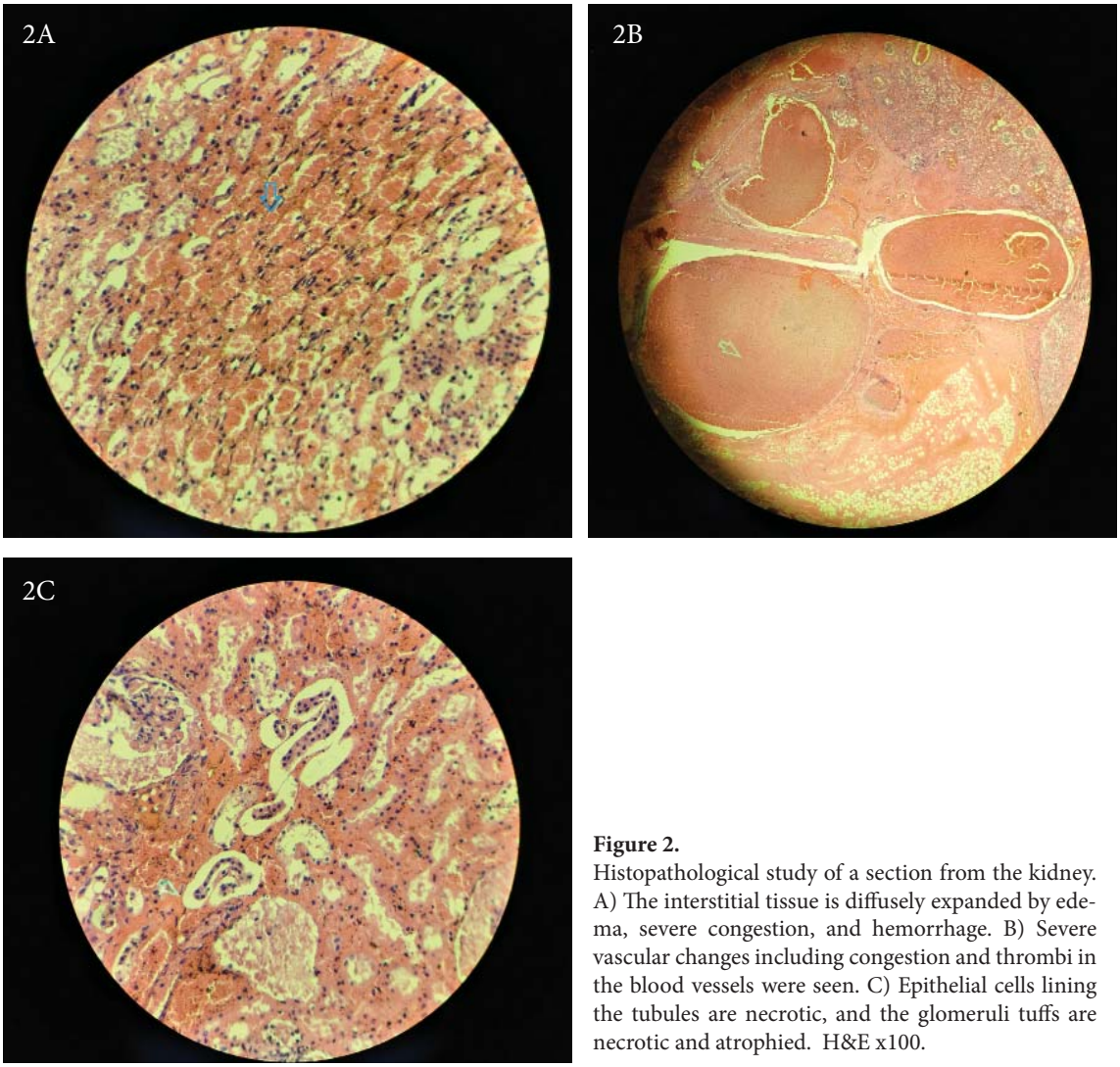


Figure 2.
Histopathological study of a section from the kidney. A) The interstitial tissue is diffusely expanded by edema, severe congestion, and hemorrhage. B) Severe vascular changes including congestion and thrombi in the blood vessels were seen. C) Epithelial cells lining the tubules are necrotic, and the glomeruli tuffs are necrotic and atrophied. H&E x100.

Authors' Contributions

Y.N., G.J., A.T., S.R., and A.M. contributed to sample preparation. A.B. took the lead in writing the manuscript. Y.N., G.J., A.T., S.R., and A.M. contributed to the interpretation of the results. G.J. took the lead in writing the manuscript.

Acknowledgements

We thank all the technical staff of the Veterinary Hospital of Urmia University.

Competing Interests

The authors declare no competing financial interest.

References

1. König HE, Bragulla H, Hans-Georg HG. Veterinary anatomy of domestic mammals: textbook and colour atlas. Schattauer Verlag; 2007.
2. Ebrahimi M A.Veterinary basic anatomy.1th ed. Sotodeh; 2011; 183-185.
3. Skotnicka E, Muszczyński Z, Dudzinska W, Suska M. A review of the renal system and diurnal variations of renal activity in livestock. Irish Veterinary Journal. 2007; 60(3):1-8.
4. Hsiao WC, Abt P, Amaral S, Levine M, LaRosa C. Late renal allograft torsion in a pediatric transplant recipient. Pediatric Transplantation. 2021:e14210.

5. Vincenzi P, Sivan S, Shah R, Goggins MO, Morsi M. Recurrent renal allograft torsion after simultaneous kidney and pancreas transplantation: is it still possible to salvage the graft? A case report. In Transplantation proceedings. 2021; (53): 2529-2535.

6. Sofue K, Vikraman DS, Jaffe TA, Chaubal GN, Bashir MR. Graft kidney torsion after simultaneous kidney-pancreas transplant: report of 2 cases and literature review. Journal of Computer Assisted Tomography. 2015; 39(4): 506-9.

7. Wong-You-Cheong JJ, Grumbach K, Krebs TL, Pace ME, Daly B, Chow CC, et al. Torsion of intraperitoneal renal transplants: imaging appearances. AJR. American Journal of Roentgenology. 1998; 171(5):1355-9.

8. Gutknecht M. Sonography in the diagnosis of renal transplant torsion. Journal of Diagnostic Medical Sonography. 2009; 25(5):231-8.

9. Klahr S. New insights into the consequences and mechanisms of renal impairment in obstructive nephropathy. American Journal of Kidney Diseases. 1991; 18(6):689-99.

10. Klahr S, Purkerson ML. The pathophysiology of obstructive nephropathy: the role of vasoactive compounds in the hemodynamic and structural abnormalities of the obstructed kidney. American Journal of Kidney Diseases. 1994; 23(2):219-23.

11. Moriyama T, Kawada N, Nagatoya K, Horio M, Imai E, Hori M. Oxidative stress in tubulointerstitial injury: therapeutic potential of antioxidants towards interstitial fibrosis. Nephrology Dialysis Transplantation. 2000; 15(suppl_6):47-9.

12. Zhou Z, Kang YJ. Cellular and subcellular localization of catalase in the heart of transgenic mice. Journal of Histochemistry & Cytochemistry. 2000; 48(5):585-94.

How to cite this article

Nozohour Y, Jalilzadeh-Amin G, Tehrani AA, Rezaie S, Moradian A. Unilateral renal torsion in a lamb. Iran J Vet Sci Technol. 2022; 14(1):50-53.
DOI: https://doi.org/ 10.22067/ijvst.2022.74358.1104
URL: https://ijvst.um.ac.ir/article_42022.html

Iranian Journal of Veterinary Science and Technology

Received: 2021- Sep- 21
Accepted after revision: 2021- Oct- 30
Published online: 2022- Mar- 01

Abstracts (in Persian)

ارزیابی فعالیت ضد باکتریایی cLF–کایمرا و پتانسیل هم افزایی آن با ونکومایسین در برابر استافیلوکوکوس اورئوس مقاوم به متی سیلین

سحر روشنک[۱]، فخری شهیدی[۱*]، عاطفه سرادقی توپکانلو[۱]، زانا پیرخضرانیان[۲]، محمد هادی سخاوتی[۲]

۱گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه فردوسی، مشهد، ایران.
۲گروه علوم دامی، دانشکده کشاورزی، دانشگاه فردوسی، مشهد، ایران.

چکیده

مصرف بی رویه آنتی بیوتیک ها باعث شیوع میکروارگانیسم های مقاوم به آنتی بیوتیک شده است. بنابراین نیاز مبرم به کشف عوامل ضدباکتریایی جدید یا ترکیبی از عوامل موجود، به عنوان یک راهکار درمانی ایمن برای مبارزه با عفونت های مختلف، وجود دارد. در مطالعه حاضر، اثر هم افزایی cLF-کایمرا که یک پپتید ضد میکروبی (AMPs) است و آنتی بیوتیک ونکومایسین با استفاده از روش چک برد علیه استافیلوکوکوس اورئوس مقاوم به متی سیلین (MRSA) مورد بررسی قرار گرفت. cLF-کایمرا دارای اثر ضد میکروبی بر MRSA و استافیلوکوکوس اورئوس حساس به متی سیلین (MSSA) بود (MIC به دست آمده به ترتیب ۵۱۲ و ۲۵۶ میکروگرم بر میلی لیتر بود). ترکیب cLF-کایمرا با ونکومایسین دارای اثر هم افزایی (CFI =۰/۳۷۵) بود. نتایج نشان داد که در غلظت FIC ، خروج مواد از سیتوپلاسمی از سلول های باکتریایی و تعداد سلول های زنده مانده، به ترتیب به طور قابل توجهی بیشتر و کمتر از زمانی است که از پپتیدها یا آنتی بیوتیک ها به تنهایی استفاده شد. تجزیه و تحلیل تصاویرمیکروسکوپی الکترونی در غلظت FIC ، آسیب شدید غشای سلول های باکتریایی را نشان داد. در نتیجه ، استفاده از cLF-کایمرا و ونکومایسین در این غلظت مصرف هر دو ماده را کاهش می دهد.

واژگان کلیدی

اثر هم افزایی، پپتیدهای ضد میکروبی، آنتی بیوتیک ها، باکتری های مقاوم

* نویسنده مسئول: فخری شهیدی
fshahidi@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Dec- 23
Accepted after revision: 2021- Mar- 05
Published online: 2022- Apr- 01

Abstracts (in Persian)

بررسی نقش گیرنده های اپیوئیدی مرکزی بر اخذ غذای ناشی از سروتونین در جوجه های گوشتی

بهروز رحمانی[1]، کیمیا مهدوی[1]، مرتضی زنده‌دل[1]، مینا خدادادی[1]*، مریم کشاورز[1]، علی باغبان‌زاده[1]، مجید شهابی[2]

۱ گروه علوم پایه، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.
۲گروه علوم بالینی، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.

چکیده

سروتونین و گیرنده های اپیوئیدی در تنظیم اخذ غذای پرندگان و پستانداران نقش دارند. مطالعه‌ی حاضر به منظور بررسی اثرات میانجی‌گری گیرنده های اپیوئیدی بر کاهش اخذ غذای ناشی از سروتونین در جوجه های گوشتی صورت گرفت. تعداد ۲۲۰ جوجه نر نوزاد نژاد گوشتی (راس ۳۰۸) در ۵ آزمون و هر آزمون در چهار گروه، شامل یک گروه کنترل و سه گروه تیمار دسته بندی شدند (۱۱ جوجه در هر گروه). میزان اخذ غذای تجمعی در جوجه‌های ۵ روزه در زمان های ۳۰، ۶۰ و ۱۲۰ دقیقه بعد از تزریق داخل بطن مغزی داروها اندازه‌گیری و تجزیه و تحلیل شد. بر اساس بررسی های حاصل از این مطالعه، تزریق داخل بطن مغزی سروتونین (۱۰ میکروگرم) به طور معنی داری میزان اخذ غذا را کاهش داد($p < ۰.۰۵$). همچنین تزریق داخل بطن مغزی آنتاگونیست گیرنده ی مو (mu) اپیوئیدی سبب کاهش معنی دار هیپوفاژی ناشی از سروتونین شد ($p < ۰.۰۵$). تزریق آگونیست گیرنده ی مو (mu) اپیوئیدی، هیپوفاژی القایی توسط سروتونین را به طور معناداری تقویت نمود ($p < ۰.۰۵$) درحالی که تزریق آنتاگونیست‌های گیرنده های کاپا (kappa) و دلتا (delta)، اثری بر هیپوفاژی ناشی از سروتونین نداشتند ($p \geq ۰.۰۵$). بر اساس نتایج به دست آمده، احتمالا کاهش اخذ غذای القایی توسط سروتونین به وسیله ی گیرنده ی مو اپیوئیدی در مغز جوجه ها میانجی‌گری می شود.

واژگان کلیدی

اخذ غذا، جوجه‌ی گوشتی، سروتونین، گیرنده‌های اپیوئیدی

* نویسنده مسئول: مینا خدادادی
mina.khodadadi@alumni.ut.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Jul- 12
Accepted after revision: 2022- Feb- 12
Published online: 2022- Mar- 01

Abstracts (in Persian)

ارزیابی مقاومت به فلوروکینولون ها و تعیین جهش ها در ژن های *gyrA* و *parC* در باکتری اشرشیاکلای جداشده از شیر خام گاو های شیری مبتلا به ورم پستان کلی فرمی در استان خراسان رضوی، ایران

مهدیه مهدوی[۱]، بهروز فتحی[۲*]، عبدا.. جمشیدی[۳]، بابک خرمیان[۴]

۱ فارغ التحصیل دامپزشکی، دانشکده دامپزشکی، دانشگاه فردوسی، مشهد، ایران.
۲گروه علوم پایه، دانشکده دامپزشکی، دانشگاه فردوسی، مشهد، ایران.
۳گروه بهداشت مواد غذایی و آبزیان، دانشکده دامپزشکی، دانشگاه فردوسی، مشهد، ایران.
۴گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه فردوسی، مشهد، ایران.

چکیده

مطالعه حاضر برای ارزیابی پروفایل مقاومت به فلوروکینولون ها و جهش در ناحیه مقاوم به کینولون ها در ژن های *gyrA* و *parC* اشریشیاکلای در ورم پستان کلی فرمی گاو صورت گرفت. با روش دیسک دیفیوژن، مقاومت به فلوروکینولون‌ها (نورفلوکساسین، سیپروفلوکساسین، انروفلوکساسین، لووفلوکساسین و افلوکساسین) در اشرشیاکلای جدا شده از (۱۰۰ نمونه شیر) گاوهای مبتلا به ورم پستان سنجیده شد. برای تعیین جهش‌های وابسته به مقاومت به فلوروکینولون‌ها در ژن‌های *gyrA* و *parC* اشرشیاکلای، دو جدایه که بیشترین مقاومت نسبی را به فلوروکینولون‌ها داشتند برای تکثیر و توالی‌یابی ناحیه مقاوم به کینولون‌ها در ژن‌های*gyrA* و *parC* مورد استفاده قرار گرفتند. روش دیسک دیفیوژن نشان داد جدایه‌های اشرشیاکلای بیشترین مقاومت نسبی را به افلوکساسین (۱۶.۷٪) و سپس به انروفلوکساسین و نورفلوکساسین (۱۵٪) داشتند درحالیکه مقاومت نسبی کمی به سیپروفلوکساسین و لووفلوکساسین (۳.۳۳٪) داشتند. جهش خاموش در ناحیه مقاوم به کینولون‌ها، در کدون‌های ۹۱ و ۱۰۰ و ۱۱۱، ۱۳۱ و ۱۳۲ ژن *gyrA* و در کدون ۹۱، ۱۵۷ و ۱۵۹ ژن *parC* در یک جدایه و جهش در نوکلئوتیدهای ۶۵، ۸۰ و ۸۳ ژن *gyrA* و نوکلئوتیدهای ۱۹۵، ۲۰۹ و ۲۱۲ ژن *parC* جدایه دیگر یافت شد. این نتایج نشان داد که میزان مقاومت نسبی باکتری اشرشیاکلای جدا شده از شیر خام گاوهای مبتلا به ورم پستان کلی فرمی نسبت به فلوروکینولون ها، بسیار پایین می باشد.

واژگان کلیدی

اشرشیاکلای ,*gene parC*, *gene gyrA* ، مقاومت به فلوروکینولون‌ها، ورم پستان

* نویسنده مسئول: بهروز فتحی
b-fathi@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Abstracts (in Persian)

Received: 2021- Nov-29
Accepted after revision: 2022- Apr-10
Published online: 2022-Apr-20

اثرات تزریق زیرجلدی پروژسترون در میش ها، به عنوان پروتکل همزمانسازی فحلی: یک مطالعه اولیه

بهنام ولی زاده، مسعود رجبیون، محمد عزیززاده، بابک خرمیان طوسی*

گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران.

چکیده

هدف از مطالعه حاضر ارزیابی اثرات کوتاه مدت تزریق زیرجلدی پروژسترون بر میزان همزمانسازی فحلی گوسفندان نژاد بلوچی می باشد. تمامی میش ها یک دوز پروستاگلندین (۱۵ میلی گرم) دریافت کردند و بصورت اتفاقی به دو گروه تقسیم شدند. در گروه اول، پروژسترون همراه با پروپیلن گلیکول بصورت زیرجلدی دوبار به فاصله ۳ روز دریافت کردند. در گروه کنترل یک اسفنج واژینال برای مدت ۶ روز قرار داده شد.

در روز ۶ تمامی گوسفندان ۴۰۰ واحد هورمون eCG دریافت کردند. ۴۸ ساعت پس از تزریق eCG دو قوچ به گله وارد شد. نمونه خون بصورت روزانه از روز صفر تا ۱۳ جهت اندازه گیری پروژسترون گرفته شد. اولتراسونوگرافی تخمدانها هر ۳ روز انجام گرفت. میزان فحلی بر اساس کاهش پروژسترون به پایین ترین میزان به ترتیب در گروه پروژسترون و کنترل ۸۸/۹ و ۱۰۰ درصد بود (p >۰.۰۵). براساس کاهش پروژسترون به زیر ۰/۵ نانوگرم در هر میلی لیتر میانگین زمان فحلی برای گروه پروژسترون و کنترل به ترتیب ۸ و ۸/۵ روز بود (p >۰.۰۵) . اختلاف معنی داری در سایز بزرگترین فولیکول و تعداد فولیکول های بزرگتر از ۲ میلی متر در تخمدانها بین دو گروه مشاهده نشد. مطالعات بیشتر و انجام تغییرات برای بدست آوردن میزان باروری و Prolificacy بالاتر مورد نیاز است.

واژگان کلیدی

همزمانی فحلی، میش، پروژسترون، پروتکل کوتاه مدت

* نویسنده مسئول: بابک خرمیان طوسی

khoramian@um.ac.ir

Iranian Journal of Veterinary Science and Technology

Received: 2021- Oct- 25
Accepted after revision: 2022- Mar- 11
Published online: 2022- Apr- 20

Abstracts (in Persian)

ناهنجاری مادرزادی زوج اول قوس حلقی در گوساله هلشتاین فریزین

بهادر شجاعی [۱]، سینا دلشاد [۱]، مهدی فرج لی عباسی [۲]، مهدی جافری [۳]، محمد فرج لی عباسی [۴*]

۱ گروه گروه علوم پایه، دانشکده دامپزشکی، دانشگاه شهید باهنر کرمان، کرمان، ایران.
۲ دکتر دامپزشک، کلینیک دامپزشکی مهر، تبریز، ایران.
۳ گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شهید باهنر کرمان ایران.
۴ مرکز تحقیقات علوم اعصاب، پژوهشکده نوروفارماکولوژی، دانشگاه علوم پزشکی کرمان، کرمان، ایران.

چکیده

خطای تکاملی جفت اول زائده های قوس حلقی منجر به ایجاد گروهی از ناهنجاری‌های مربوط به حفره ی دهان می‌شود. که از این ناهنجاری‌ها می توان به آگناتیا، میکروگناتیا، براکیگناتیا، سیگناتیا، کام شکاف دار، کمپیلوگناتیا، منقار طوطی و استروفوسفالی اشاره کرد. کمپیلوگناتیا یک ناهنجاری است که در آن فک پایین یا بالا از خط وسط منحرف شده است. معاینه بالینی یک گوساله ماده هلشتاین فریزین تازه متولد شده، انحراف فک پایین، تغییر شکل دندان های گونه تحتانی و فوقانی و برخی ناهنجاری های دیگر صورت را نشان داد. مورد مشابهی در تاریخچه گله مشاهده نشد. می توان با بررسی دقیق حیوانات بدشکل و میزان درگیری ساختارهای مختلف بدن آن ها به الگوی ناهنجاری، زمان اثر عوامل تراتوژنیک و ساختارهای جنینی محرک پی برد. در مورد حاضر درگیری مشتقات زائده های فک بالا و فک پایین تایید کننده این فرضیه است که قوس اول حلقی در ایجاد ناهنجاری های ذکر شده نقش اصلی داشته است. در انسان، انواع مختلفی از رشد غیرطبیعی قوس اول حلقی گزارش شده است و به همین دلیل تحت عنوان «سندرم قوس احشایی اول» طبقه بندی شده است. اما به علت گزارش های کم، این طبقه بندی در مورد حیوانات اهلی انجام نشده است.

واژگان کلیدی

شکاف مادرزادی فک، قوس حلق، گوساله، ناهنجاری، کمپیلوناتیا

* نویسنده مسئول: مهدی فرجلی عباسی
m.fabbasi@kmu.ac.ir

Iranian Journal of Veterinary Science and Technology

Abstracts (in Persian)

Received: 2022- Jan- 05
Accepted after revision: 2022- Mar- 18
Published online: 2022- Apr- 20

پیچ خوردگی یک طرفه کلیه در بره

یاسر نوظهور[1]، قادر جلیل زاده امین[1]*، علی اصغر طهرانی[2]، سجاد رضایی[1]، علیرضا مرادیان[1]

۱گروه بیماری های داخلی دام های بزرگ، دانشکده دامپزشکی دانشگاه ارومیه، ارومیه، ایران.
۲ گروه پاتولوژی، دانشکده دامپزشکی دانشگاه ارومیه، ارومیه، ایران.

چکیده

در نشخوارکنندگان، کلیه‌ها اندام اصلی دفع مواد زائد حاصل از متابولیسم مواد، تنظیم وتعادل الکترولیتها و تنظیم و تعادل اسیدیته بدن است. بنابراین اختلالات کلیه‌ها می تواند اثر تهدید کننده حیاتی بر روی سلامتی نشخوارکنندگان داشته باشد. پیچ خوردگی کلیه در هیچ گونه حیوانی گزارش نشده است. در این گزارش، یک راس بره نر دو ماهه که دچار پیچ خوردگی یک طرفه کلیه شده بود، توصیف گردیده است. در کالبدگشایی، کلیه پیچ خورده به شدت پرخون و اندازه آن بسیار بزرگ شده بود. مطالعات هیستوپاتولوژیکی نکروز توبولار و گلومرولی کلیه را نشان داد. تغییرات عروقی شامل، احتقان و ترومبوز در عروق خونی بودند. ناهنجاری در دوره رشد جنینی و افزایش تحرک ساختار کلیوی به دلیل رباط های مرتبط با کلیه پیچ خورده از علل مشکوک در وقوع پیچ خوردگی کلیه است.

واژگان کلیدی

کلیه، پیچ خوردگی، نشخوارکنندگان، دستگاه ادراری

* نویسنده مسئول: قادر جلیل زاده امین
g. jalilzadeh@urmia.ac.ir

Author Index

Author index

GUIDE FOR AUTHORS

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Guide for authors

SCOPE

Iranian journal of Veterinary Science and Technology (IJVST) publishes important research advances in veterinary medicine and subject areas relevant to veterinary medicine including anatomy, physiology, pharmacology, bacteriology, biochemistry, biotechnology, food hygiene, public health, immunology, molecular biology, parasitology, pathology, virology, large and small animal medicine, poultry diseases, diseases of equine species, and aquaculture. Articles can comprise research findings in basic sciences, as well as applied veterinary findings and experimental studies and their impact on diagnosis, treatment, and prevention of diseases. IJVST publishes four kinds of manuscripts: Research Article, Review Article, Short Communication, and Case Report.

Guide for Authors

GENERAL GUIDELINES

1. Submitted manuscripts should not be previously published elsewhere and should not be under consideration by any other journal.
2. The corresponding author should provide all co-authors with information regarding the manuscript, and obtain their approval before submitting any revisions.
3. The submitted manuscript should be accompanied by a written statement signed by the corresponding author on behalf of all the authors that its publication has been approved by all co-authors, stating that the whole manuscript or a part of it has not been published.
4. Ethics: Authors must state that the protocol for the research project has been approved by the Ethics Committee of the institution within which the work was undertaken. Authors are responsible for animal welfare and all statements made in their work.

OPEN ACCESS POLICY

Iranian Journal of Veterinary Science and Technology is a fully Open Access journal in which all the articles are available Open Access. There is no cost to the reader or author. All costs are covered by the Ferdowsi University of Mashhad Press.

COPYRIGHT

Copyright on any open access article in the Iranian Journal of Veterinary Science and Technology, published by Ferdowsi University of Mashhad Press is retained by the author(s).

- Authors grant Ferdowsi University of Mashhad Press a license to publish the article and identify itself as the original publisher.
- Authors also grant any third party the right to use the article freely as long as its integrity is maintained and its original authors, citation details, and publisher are identified.

The Creative Commons Attribution License 4.0 formalizes these and other terms and conditions of publishing articles. The Copyright assignment form can be downloaded from the IJVST website.

SUBMISSION

Authors should submit their manuscript in electronic format directly through the IJVST website (ijvst.um.ac.ir) along with a letter to the editor signed by the author to whom correspondence should be addressed. Please ensure that Email addresses are university/governmental addresses and full postal addresses are included on the title page of the manuscript. The following files and forms can be downloaded from the IJVST website:

Manuscript (template file can be downloaded from the IJVST website)
Title page (template file can be downloaded from the IJVST website)
Tables (template file can be downloaded from the IJVST website)
Endnote manuscript library file (Vancouver style can be downloaded from the IJVST website)

Copyright assignment form (can be downloaded from IJVST website)
Conflict of interest and author agreement form (can be downloaded from the IJVST website)

For further information, please contact the Editorial Office:
Iranian Journal of Veterinary Science and Technology
Email: ijvst@um.ac.ir;
Tel: +98 51 3880-3742

PREPARATION OF MANUSCRIPT

Manuscripts should be written in English, with Abstract in both English and Persian (where applicable), typewritten in MS Word program, double-spaced, in 12-point "Times New Roman" font on A4 paper size. Authors are requested to reserve margins of 2.5 cm all around the pages. Manuscript should also have line numbers. All pages of the manuscripts should also be enumerated.

Research Articles should contain Title page, Abstract, Keywords, List of Abbreviations, Introduction, Results, Discussion, Materials and methods, References, and Figure legends. Tables and figures should be appended as individual files.

Review Articles should contain Title page, Abstract, Keywords, List of Abbreviations, Introduction, appropriate sections dependeing to the subject, Conclusions and future directions. Tables and figures should be appended as individual files. The review article should provide an update on recent advances in a particular field. Authors wishing to submit review articles should contact the Editor with an outline of the proposed paper prior to submission.

Case Reports should include Title page, Abstract, Keywords, List of Abbreviations, Introduction, Case Presentation, Results and Discussion, and References. Case reports should not exceed 2000 words (excluding the references) and should include no more than two tables or figures. Tables and figures should be appended as individual files.

Short Communications should not exceed 2000 words (excluding the references) and include no more than two tables or figures. They should include Title page, Abstract, Keywords, List of Abbreviations, the text summarizing results with no other divisions, and References. Tables and figures should be appended as individual files.

Title Page

Full Title Page should include title (concise and informative), author(s) (including the complete name, department affiliation, and institution), running head (condensed title) (≤ 50 characters, including spaces), name and address of the authors to whom correspondence and reprint requests

should be addressed, Acknowledgements, Author contributions, and Conflict of interest.

Acknowledgements: Personal acknowledgement, sources of financial support, contributions and helps of other researchers and everything that does not justify authorship should be mentioned in this section, if required.

Author contributions: Authors are required to include a statement to specify the contributions of each author. The statement describes the tasks of individual authors referred to by their initials. Listed below is an example of author contributions statement:
Conceived and designed the experiments: HD, SS. Performed the experiments: SS. Analyzed the data: HD, SS, MMM, ARB. Research space and equipment: HD, MMM, ARB. Contributed reagents/materials/analysis tools: HD. wrote the paper: SS, HD.

Conflict of interest: All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state 'The authors declare that there is no conflict of interest'. This form can be downloaded from the IJVST website.

Abstract

Abstract (in English and Persian) no more than 250 words should contain the purpose of the study, findings and the conclusion made on the basis of the findings. Authors who are not native Persian speakers may submit their manuscript with an abstract in English only. Abbreviations and reference citations may not be used in the abstracts.

Keywords

For indexing purposes, each submitted manuscript should include three to seven keywords, following the abstract and preferably chosen from the Medical Subject Headings (MESH). Keywords should express the precise content of the manuscript.

Introduction

Introduction should be as concise as possible, and clearly explain the main objective and hypothesis of the investigation.

Results

Results indicate the results of an original research in a clear and logical sequence. Do not repeat data that are already covered in tables and illustrations. In manuscripts describing more than one animal, all animals should be assigned a case number.

Discussion

Discussion should include the answer to the question proposed in the introduction and empha-

size the new and important aspects of the study and the conclusions that follow from them. It could include the implication, application, or speculation of the findings and their limitations, relate the observations to other relevant studies, and links the conclusions with the goals of the study. Recommendations, when appropriate, may be included.

Materials and methods

Materials and methods should be described in sufficient details to allow other researchers to reproduce the results. Specify any statistical computer programs used .The methods of data collection and use of statistical analysis will be checked by the referees and if necessary, a statistician. Drugs and therapeutic agents, reagents, softwares and equipments should be given in the format: name (trade name, manufacturer name, city, country), e.g. Statview 5 (SAS Institute, Inc., Cary, NC, USA).

Animals: All animal experiments should comply with the ARRIVE (https://arriveguidelines.org/) guidelines and the authors should clearly indicate in the manuscript the ethical code of the study.

Gene names: The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

Mouse beta actin gene: *Actb*
Bovine beta actin gene: *ACTB*
Chicken beta actin gene: *actb*
Beta actin protein: ACTB

Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

The following information must be provided in the section:

Protocol for DNA/RNA extraction, including quantification and determination of purity;

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components;

qPCR: sequence of forward and reverse primers, probes, amplicon size, accession number of Genebank; thermocycler parameters (i.e. denaturation, annealing and extension steps, number of cycles, melting curves); validation of PCR products; non-template controls for reverse transcription and qPCR should be included in all reactions; and

Data analysis: details for the quantitative or relative analysis.

Use of antibodies: Authors must show that the antibodies are validated and their specificity sis con-

firmed.

References

Must be up-to-dated and limited to those that are necessary. Lists of references should be given in numerical order in the text, and in the reference list. Please use Vancouver style. To download the Vancouver Style follow the link in the IJVST website which could be used in the Endnote software.

Example piece of text and reference list :

An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan Goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.

5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005; 62(1):112-6.

6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015; 44(6):387-92.

Tables

Please submit tables as individual files and editable text and not as images. Place all table notes below the table body. Each table should have a title which is followed by explanation of results shown in the table. Use of vertical rules must be avoided. Tables should be self-explanatory, and clearly arranged. Tables should provide easier understanding and not duplicate information already included in the text or figures. Each table should be typewritten with double spacing on a separate file and numbered in order of citation in the text with Arabic numerals. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Explain any non-standard abbreviations in a footnote to the table.

Figures

Figures must be submitted in individual files (format: TIFF, Dimensions: Width: 789 – 2250 pixels

at 300 dpi Height maximum: 2625 pixels at 300 dpi, Resolution: 300 – 600 dpi, file size: less than 10 MB, Text within figures: Arial or Symbol font only in 8-12 point). The text and other labels should be placed in the figure as un-compressed layers. Each figure should have a title which is followed by explanation of results shown in the figure. Figures should be numbered in order of citation in the text with Arabic numerals.

For the use of bar diagrams the following publication should be consulted:

Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015; 13(4):e1002128.

The bar diagrams should be provided in color and in a well-designed and professional format. Please do not use different shades of gray. The axes of diagrams should have titles and units. Also, the source file of the image (Excel etc.) should be provided for typesetting.

Illustrations should be numbered as cited in the sequential order in the text, with a legend at the end of the manuscript. Color photographs are accepted at no extra charge. The editors and publisher reserve the right to reject illustrations or figures based upon poor quality of submitted materials.

If a published figure is used, the publisher's permission needs to be presented to the office, and the figure should be referenced in its legend.

Use of Italics

Gene symbols, Latin terms (i.e. *in vivo, in vitro, ex vivo, in utero, in situ*, and etc.) and species scientific names (using the binomial nomenclature), should be typed in italics, while the first letter of the genus name must be capitalized (i.e. *Homo sapiens*).

Publication Ethics

PUBLICATION ETHICS

Iranian Journal of Veterinary Science and Technology is aligned with COPE's (Committee on Publication Ethics) best practice guidelines for dealing with ethical issues in journal publishing and adopts the COPE guidelines. The journal members (editor, editorial board and the journal manager) have agreed to meet the purposes and objectives of the Journal.

Ethical guidelines for authors:

Authorship Criteria

IJVST requires authors to confirm that they and their co-authors meet all four criteria for authorship based on the guidelines of The International Committee of Medical Journal Editors (ICMJE) (verbatim as follows):

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The section "Author Contributions" in the manuscript should illustrate and clarify who contributed to the work and how. If a contributor does not meet all four above criteria should be acknowledged in the "Acknowledgements" section of the article.

Author agreements and conflict of interest

Written authorization from all authors for publication of the article is mandatory for IJVST to start the review process. This form entitled "Conflict of interest declaration and author agreement form" must be signed and completed by all authors. This statement and signatures certifies that all authors have seen and approved the manuscript being submitted. Also, the authors by signing this form warrant that the article is the Authors' original work, that the article has not received prior publication and is not under consideration for publication elsewhere, and that the corresponding author shall bear full responsibility for the submission.

Editors and members of editorial board as authors

Editor and members of editorial board are excluded from publication decisions when they are authors or have contributed to a manuscript.

Ethical guidelines for Peer reviewers

Iranian Journal of Veterinary Science and Technology (IJVST) follows and adheres to COPE Ethical Guidelines for Peer Reviewers. IJVST peer reviews all submitted manuscripts with contents in the scope of the journal. The process has been explained in the section "Peer Review Process".

Ethical guidelines for Editor

Iranian Journal of Veterinary Science and Technology regarding the responsibilities of the editors follows and adheres to COPE Ethical Guidelines for editors. The main guidelines are summarized in the guide to ethical editing from COPE.

Peer Review Process

PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

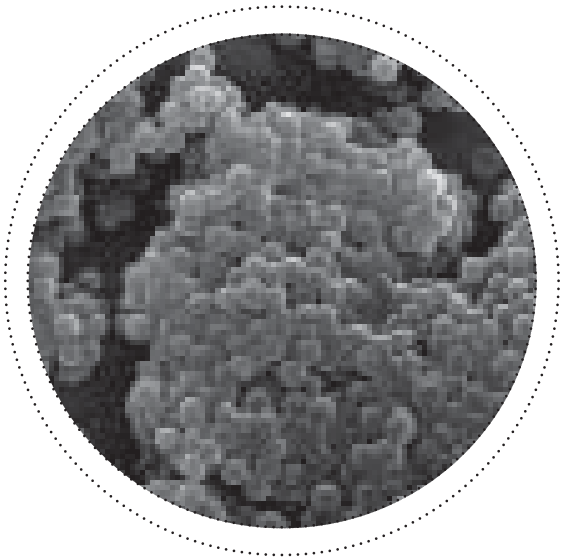
Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

Final Decision

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.



Iranian Journal of Veterinary Science and Technology

Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Azadi Square, Mashhad, IRAN
P.O. Box: 1793; Postal Code: 9177948974

Tel: 0098 51 3880 3742
Fax: 0098 51 3876 3852

Web: ijvst.um.ac.ir
Email: ijvst@um.ac.ir