ISSN: 2008-8140
E-ISSN: 2322-3618

Urmia University

VETERINARY RESEARCH FORUM

Volume 14, Number 11
November 2023

An International Monthly Journal Published by Faculty of Veterinary Medicine, Urmia University, Urmia, I.R. Iran

In the name of God

Veterinary Research Forum (Vet Res Forum)

ISSN: 2008-8140, E-ISSN: 2322-3616

Founder
Faculty of Veterinary Medicine, Urmia University

Publisher
Urmia University

Abstracting and Indexing:

Web of Science (JCR; Impact factor = 1.00; Q3), Science Citation Index Expanded (Web of Science Core Collection), Current Contents Agriculture, Biology & Environmental Sciences | Essential Science Indicators | Zoological Record (Additional Web of Science Indexes), Scopus (SNIP = 0.581), SciMago (SJR = 0.28; Q2), Islamic World Science Citation Center (ISC; Impact factor = 0.116; Q2), PubMed, PubMed Central (PMC), Regional Information Center for Science and Technology (RICeST), Directory of Open Access Journals (DOAJ), ProQuest, CAB Abstracts (CABI), Index Veterinarius (CABI), Veterinary Bulletin (CABI), VetMed Resource (CABI), Magiran, Genamics Journal Seek, Scientific Information Database (SID), Academic Journals Database, Google Scholar, Index Scholar, Index Copernicus (IC), Medical Journals Links, Free Medical Journals (Amedeo), Journal Rate, Road, InfoBase Index and Publons.

Veterinary Research Forum is issued under the license of 124/2036 dated July 13, 2009 from the Ministry of Culture and Islamic Guidance. This journal has achieved the rating of Scientific-Research, by Commission for Evaluation of Iranian Scientific Journals, the Ministry of Science, Research and Technology, from Vol. 2, No. 1, March 2011 onward.

Editorial Office: Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
P.O. Box: 57153-1177.
Tel: +98 44 3277 0508, Fax: +98 44 3277 1926

Email: vrf@urmia.ac.ir , vrf.urmia@gmail.com

Web: http://vrf.iranjournals.ir/

Table of Contents

Board of Scientific Reviewers

1. Introduction and Editorial Policies

The **Veterinary Research Forum (Vet Res Forum)** publishes worldwide contributions on all aspects of veterinary science and medicine, including anatomy and histology, physiology and pharmacology, anatomic and clinical pathology, parasitology, microbiology, immunology and epidemiology, food hygiene, poultry science, fish and aquaculture, anesthesia and surgery, large and small animal internal medicine, large and small animal reproduction, biotechnology and diagnostic imaging of domestic, companion and farm animals. Authors who submit manuscripts to the journal should carefully read these "*Instructions for Authors*" when preparing their manuscripts, because compliance with these instructions will help reduce delays in manuscript processing. A manuscript is received with the understanding that it and all revisions have been approved by all authors and that neither the manuscript nor any of its parts has been published or is under concurrent consideration by any other publication. The corresponding author must provide a signed statement to this effect. All authors of *VRF* will be required to sign a written statement transferring copyright to the VRF prior to publication of any manuscript or letter. Requests to copy, reprint, or use portions of published material (including information in figures and tables) should be addressed to the editor-in-chief.

2. Editor-in-Chief

Farshid Sarrafzadeh-Rezaei. DVM, DVSc
Professor in Veterinary Surgery
Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. P.O. Box: 57153-1177
E-mail: f.sarrafzadeh@urmia.ac.ir

3. Submission of Manuscripts

Veterinary Research Forum is proud to announce launch of its official website at http://vrf.iranjournals.ir/.

Open access policy, online submission/tracking, web-based peer review system, and search feature are the fundamental features of the new website.

Manuscripts must be in English and Microsoft Word format (.doc/.docx). The manuscript (including references, and figure legends) must be double space typed, using 12-point Times New Roman font, 3.5 cm margins, and left justification. Manuscripts should be arranged as follows: title page, abstracts, body of manuscript, acknowledge-ments, conflict of interest, references, and figure legends. Please note that the names of the authors and their affiliations should not be included in the main document. The title page must include the title and the first name, middle initial, and last name of each author, along with professional affiliations and official email account of the authors at the time of the study should be indicated. If an author's affiliation has changed since the study was performed, the author's new affiliation should be identified. The name of the corresponding author should also be included on the title page. Software programs that automatically create endnotes, footnotes, and references should not be used. Each line and page of the manuscript must be numbered, beginning from the title page. Tables should be saved as separate files (.doc/.docx format).

Authors are required to provide the names, affiliations, and contact information (full mailing address, e-mail address) of two potential reviewers related to their manuscripts; though choice of reviewers is at the discretion of the editor-in-chief.

4. Authorship

Individuals should be listed as authors only if they 1) made a substantial contribution to conception and design of the study, the acquisition of data used in the study, or analysis and interpretation of that data; 2) were involved in drafting or revising the manuscript critically for important intellectual content; and 3) will have an opportunity to approve subsequent revisions of the manuscript, including the version to be published. Each individual listed as an author must have participated sufficiently to take public responsibility for the work. Acquisition of funding, collection of data, or general supervision of the research team does not, alone, justify authorship.

5. Acknowledgments

Acknowledgments can be used to identify important specific contributions from individuals who do not qualify for authorship. In particular, individuals who have contributed intellectually to the study or report but whose contributions do not justify authorship may be named and their function or contribution described. In general, this includes individuals who provided technical assistance (e.g., individuals who per-formed special tests or research) and individuals who provided assistance with statistical analyses. Acknowledgments should not include individuals whose only contribution to the study or report involved the routine performance of their normal job duties and who did not offer any unusual intellectual contribution or technical expertise. The acknowledgments should not be used simply as a method of expressing gratitude to individuals who had a minor role in the study. Acknowledgments of non-specific groups and un-identifiable groups are not allowed. Individuals named in acknowledge-ments must have given their permission to the authors to be listed, because readers may infer their endorsement of the data and conclusions.

6. Funding

Authors are expected to acknowledge all sources of funding or financial support and to disclose to the editor any financial interests (including ownership, employment, consultancy arrangements, and service as an officer or board member) they have with companies that manufacture products that are the subject of their research or with companies that manufacture competing products.

7. Humane Animal Care and Use

All research studies involving animals must have been performed with great respect to the animal welfare. Any unnecessary manipulation or stress should be strictly avoided. A manuscript containing information that suggests that animals were subjected to adverse, stressful, or harsh conditions or treatments will not be considered for publication unless the authors demonstrate convincingly that the knowledge gained was of sufficient value to justify these conditions or treatments. If animals were euthanized, the method of euthanasia must be indicated.

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming the informed consent was obtained from each subject or subject's guardian. All animal or human studies should be used after approval of the experimental protocol by a local ethics committee. The ethics committee approval number and date should be stated in the Materials and Methods section.

8. Style

For questions of style, refer to the latest edition of the *Veterinary Research Forum*. For anatomic terms, use anglicized versions of official terms listed in the *Nomina Anatomica Veterinaria*. Refer to the latest editions of the *American Drug Index* and *USP Dictionary of USAN and International Drug Names* for proper spelling of chemical and drug names and to the latest edition of *Dorland's Illustrated Medical Dictionary* for proper spelling and use of medical terms. Refer to *Bergey's Manual of Determinative Microbiology* for spelling and correct taxonomic classifications of microorganisms. Use of abbreviations should be kept to a minimum. Except for standard abbreviations and units of measure, all abbreviations used three or more times in the text, figures, and tables of a manuscript should be listed at the beginning of the manuscript, along with their definitions. These abbreviations should then be used without expansion. Abbreviations that appear only in the figures or tables should be defined in the table or figure legend. Abbreviations should not be used to begin a sentence. In addition, abbreviations in the abstract must be expanded at first mention, with the abbreviation given in parentheses after first mention of the expanded term. Products, equipment, and drugs should be identified by chemical or generic names or descriptions. A trade name may be included in a lettered footnote if that specific product, equipment, or drug was essential for the outcome. Body weights and temperatures must be reported in metric units. All dosages must include route of administration and interval (e.g., 10.00 mg kg^{-1}, IV, Alfasan, Netherlands). The manufacturer name with the city, state, and country must be provided for any brand name drugs. Numerical data should be expressed with decimal places (e.g., 10.00 mL).

9. Categories of Manuscripts

Authors may submit manuscripts for publication in the **Original Articles** (including original studies, retrospective studies, and descriptive studies), **Review Articles**, **Short Communications** and **Clinical Reports**.

A manuscript based on original research in which animals had a naturally developing or experimentally induced disease or condition will be considered for publication as an **Original Article**. A manuscript based on evaluation of case records accumulated during a specific period will be considered as a **Retrospective Study**. A manuscript in which a new technique, treatment, or diagnostic method is described will be considered as a **Descriptive Study**. A **Review Article** is usually written by scholars and experts in the field who have published at least five research papers on the subject in the native and/or international journals. In review articles, the results of published researches are closely evaluated, the strong and weak points are stated, and suggestions for further improvement may be made. A **Short Communication** is for a concise, but independent report representing a significant contribution to veterinary science and medicine and must report completed work, not preliminary findings: they are an alternative format for describing smaller pieces of work. A manuscript dealing with any number of clinical cases will be considered as a **Clinical Report**.

10. Manuscript Preparation

With the exception of review articles, all manuscripts submitted to the Original Research section must include an **Abstract** of 150 to 250 words. Authors should list three to five alphabetically ordered keywords which appropriately represent the contents of their manuscripts. Keywords are a tool to help indexers and search engines find relevant papers. If database search engines can find your journal manuscript, readers will be able to find it too. This will increase the number of people reading your paper, and likely lead to more citations. However, to be effective, keywords must be chosen carefully. They should be preferably not included in title of manuscript. According to the Commission for Evaluation of Iranian Scientific Journals policy, domestic authors are required to include a Farsi abstract (150 to 250 words) and keywords, in addition to the usual English abstract. For foreign authors unfamiliar with Farsi, their abstracts will be translated by the journal's language editors, free of charges.The text for an **Original Article** is organized under the following headings: Introduction, Materials and Methods, Results, and Discussion. The **Introduction** section should supply sufficient pertinent background information to allow readers to understand and interpret results. It must include the rationale for the study, the investigators' hypothesis, and a clear statement of the purpose of the study. The **Materials and Methods** section should describe the experimental design in sufficient detail to allow others to reproduce the study. A subsection detailing statistical

methods used to summarize data and test hypotheses and the level of significance used for hypothesis testing should be provided. The **Results** section should provide data that are clearly and simply stated without discussion or conclusion. Tables and figures should be cited parenthetically. The same data should not be presented in both table and graph form or repeated in the text. Data should not be repeated un-necessarily in text, tables and figures. The **Discussion** section should focus on findings in the manuscript and should be brief, containing only discussion that is necessary for interpretation of findings.

Authors are recommended to consider the following instructions when preparing their manuscripts:

- **Review Articles:** Body of article (Introduction to the end of Discussion) must not exceed 5000 words. The entire article should not exceed 30 A4 pages double-spaced, including figures, tables, and references (maximum of 60 references). Review articles can include a maximum of five figures and/or tables (i.e. any combination of tables and figures up to a total of five overall, not five figures and five tables).
- **Original Articles:** Body of article must not exceed 4000 words. The entire article should not exceed 25-30 A4 pages double-spaced, including figures, tables, and references (maximum of 50 references). Original articles should have no more than four figures and/or tables (total).
- **Short Communications:** Body of article must not exceed 2000 words with no more than 20 references. Figures, tables, or both, should not exceed two.
- **Case Reports:** Body of article must not exceed 1500 words. The maximum number of tables is two and the maximum number of figures is six. References should be limited to 15.

11. References

Authors bear primary responsibility for accuracy of all references. Excessive citations should be avoided and only essential resources should be listed. References should be numbered consecutively in the order in which they are first mentioned in the text (not in alphabetic order). References in non-English languages should be translated into English, and the original language has to be mentioned in square brackets [French], for example. Journal titles in the Reference section should be abbreviated according to the National Library of Medicine (NLM) and *Index Medicus*. The following is the style used for common types of references:

Journal article with one, two, or three authors:

Uzun A, Cengiz N, Kavaklı. Morphological and micro-scopical examination of the rat brachial plexus [Turkish]. Turk J Vet Anim Sci 2001; 25(3): 397-402.

Journal article with more than three authors:

Behfar M, Sarrafzadeh-Rezaei F, Hobbenaghi R, et al. Adipose-derived stromal vascular fraction improves tendon healing in rabbits. Chin J Traumatol 2011; 14(6): 329-335.

Book with a single author:

Gretchen LH. Animal tissue techniques. 4th ed. San Francisco, USA: WH Freeman 1979; 51-119.

Book with multiple authors:

Aronoff GR, Berns JS, Brier ME, et al. Drug prescribing in renal failure. 4th ed. Philadelphia, USA: American College of Physicians 1999; 39-45.

Chapter in a book with editors:

Nordin M, Lorenz T, Campello M. Biomechanics of tendons and ligaments. In: Nordin M, Frankel VH (Eds). Basic biomechanics of musculoskeletal system. 3rd ed. Maryland, USA: Lippincott Williams & Wilkins 2001; 102-125.

Conference proceedings:

Davidson EB. Treatment of mammary tumors in dogs and cats. In proceedings: North American veterinary conference. Orlando, USA 2003; 1036-1038.

Thesis:

Koyun M. The helminthes fauna of some fishes in Enne Dam Lake. Phd Thesis, Uludag University Science Institution. Bursa, Turkey: 2000.

Electronic material:

Animal and plant health inspection service website. Bovine spongiform encephalopathy (BSE). Available at: www.aphis.usda.gov/ipa/issues/bse/bse.html. Accessed Feb 18, 2020.

Manual:

CLSI. Autoverification of Medical Laboratory Results for Specific Disciplines. 1st ed. CLSI guideline AUTO15. Wayne, PA: Clinical and Laboratory Standards Institute; 2019.

12. Figures

All figures should be saved as separate electronic files (jpg); figures should not be embedded in the manuscript. Simple figures such as line drawings, bar graphs, and line graphs prepared in Excel should be saved as Excel files (.xls). Line drawings and graphs that were not prepared in Excel should be scanned on a flatbed scanner. Figures created with software programs that use proprietary graphic formats (e.g., SigmaPlot, Statistix) cannot be used; most such software programs have the capability to save figures in one of the aforementioned formats. Minimum resolution for line drawings and charts is 1,000 dpi. Figures that consist of images (e.g., photo-graphs, photomicro-graphs, and radiographs) should be scanned on a flatbed scanner at a resolution of at least 300 dpi. Files should be saved as jpg. If images were obtained with a digital camera, please submit the electronic file, rather than printing out the image and scanning it. Color figures should be submitted in CMYK, rather than RGB, format to prevent color shift during production. Limit figures to those that reduce or clarify the text. Text and symbols should be large enough that they will still be legible when the figure is reduced to one column width during publication. To ensure high-quality reproduction, symbols used in graphs should be limited to open and closed circles, triangles, and squares; axes should be labeled in Helvetica or Arial font. Keys to symbols may be placed in a small box inserted into the unused portion of graphs. For figures that consist of multiple parts, individual parts of the figure should be identified by capital letters embedded in the figure, rather

than by describing the location of the part in the legend (e.g., top right). Each figure can consist of 4 sub-panels. Figures should be numbered consecutively according to the order in which they have been first cited in the text. Original radiographs and transparencies will not be accepted for review or publication. Figure legends must be given at the end of the manuscript. Sufficient information should be included to allow the figure to be understood without reference to the text. When applicable, stains used for histologic sections should be indicated along with the original magnification in the legend. The Journal reserves the right to crop, rotate, reduce, or enlarge the photographs to an acceptable size. Preferably, micro-graphs should have a scale bar, rather than a magnification factor in figure legend. Labels, numbers, and symbols should be clear and of uniform size. The lettering for figures should be large enough to be legible after reduction to fit the width of the columns. Symbols, arrows, or letters used in photo-micrographs should contrast with the back-ground. Titles and detailed explanations belong in the legends for illustrations not on the illustrations themselves. Authors wishing to use any previously published figures must submit written permission from the copyright holder.

13. Tables

Submission of excessive tabular data is discouraged, and tables should be limited to those containing data important to understanding and interpreting results of the study. Authors will be asked to delete tables containing data that could be given more succinctly in the text. Do not use tables that focus on findings in individual animals. Table pieces (such as Table 1a and 1b) are not allowed. Authors wishing to use any previously published tables must submit written permission from the copyright holder. Each table should be numbered consecutively in the order of first citation in the text. Tables should be drawn in left-to-right direction. Supply a brief title of no more than 15 words for each, but place explanatory matter in the footnotes (not in the heading).

14. Review Process

All manuscripts submitted to the journal are subject to review. Journal reserves the right to reject a submission for any reason without explanation. Manuscripts are reviewed initially by a VRF scientific editor. Those with insufficient priority for publication are rejected promptly. Manuscripts considered for publication are sent to a minimum of three experts for external review. Identity of reviewers and authors are kept confidential. Authors are expected to respond to reviewer comments and make appropriate revisions within 30 days. Revised manuscripts may be re-reviewed. Manuscripts that pass review are accepted for publication provided that authors respond meaningfully to questions and concerns raised by VRF scientific editor. Authors should note that a previously rejected manuscript by VRF would not be re-evaluated.

15. Errata

The journal makes every effort to publish error-free manuscripts, providing authors with page proofs before publication. There are occasions, however, when it is necessary to publish a correction. Errata may be sent directly to vrf@urmia.ac.ir.

The author will be assessed page charge(s) for errata that result from author error. The journal will cover the costs of errata that are a result of the editing process.

16. Misconduct

The Journal only accepts research papers that are original works, no part of which has been published or is being considered for publication elsewhere except as brief abstracts. Duplicate publication, falsification, plagiarism, or fabrication will be considered actionable misconduct. Misconduct does not include honest error or honest differences in interpretations or judgments of data. If scientific misconduct is confirmed by institutional review, the Editor-in-Chief will report this to VRF's Publications and Communications Committee (PCC) Chair and the VRF Executive Director. Appropriate action will be decided in consultation with the VRF Office of Publications. Violations considered severe may warrant official withdrawal of a published article or rapid rejection of a manuscript at any stage before publication. If no scientific misconduct is found, the manuscript (if unpublished) will be scheduled for publication. For errors in a published article not deemed to be misconduct, an Erratum or Letter to the Editor will be published. If there is a disagreement about the results of the investigation, the Editor-in-Chief reserves the right to restrict the author(s) from publishing in the journal for a specified period of time.

17. Submission/Publication Fee

From March 15 th , 2023, manuscripts being accepted for publication in the journal are subjected to pay a mandatory charge for domestic and overseas authors as follows:

1) Domestic authors: 7,200,000 IRR for Review/Original Articles, and 3,600,000 IRR for Short Communications and Case Reports.

2) Overseas authors: 200.00 USD for all kinds of manuscripts, including Review/Original Articles, Short Communications, and Case Reports.

This charge covers expenses for peer review, journal publication, and online hosting and archiving. Color figures (maximum 4 figures) are published free of extra charge if the use of color is judged to be necessary for scientific reasons. Extra charge for more figures will be declared to the corresponding authors in the final acceptance letter. The charge form will be sent to the corresponding author on acceptance of an article for publication in the journal. Please be advised that the publication of the accepted manuscripts is dependent upon payment of the charge.

18. Complaint Policy

The authors who may have a complaint against any of the aspects of their interaction with Veterinary Research Forum should write an e-mail to the Editor-in-Chief. The Editor-in-Chief aims to acknowledge the complaint within 7 days after receiving it. Further, he should explain to the author the procedure which he will be undertaking to resolve the matter.

19. Disclaimer

The statements and opinions expressed in this journal are solely those of their respective authors and they do not necessarily reflect the views, nor are they endorsed by VRF. Veterinary Research Forum and its publisher do not guarantee the accuracy of, and are in no way responsible for, any content on the papers.

20. Plagiarism

Plagiarism ranges from the unreferenced use of others' published and unpublished ideas, including research grant applications to submission under "new" authorship of a complete paper, sometimes in a different language. It may occur at any stage of planning, research, writing, or publication: it applies to print and electronic versions. All the manuscript submitted to VRF are checked by iThenticate for possible plagiarism. The authors are expected to check their manuscript for plagiarism before submission.

21. Conflicts of Interest

Conflicts of interest comprise those which may not be fully apparent and which may influence the judgment of author, reviewers, and editors. They have been described as those which, when revealed later, would make a reasonable reader feel misled or deceived. They may be personal, commercial, political, academic or financial. "Financial" interests may include employment, research funding, stock or share ownership, payment for lectures or travel, consultancies and company support for staff. Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state "The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

22. Changes to authorship

Authors are expected to consider carefully the list and order of authors before submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only before the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the corresponding author: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors after the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

23. Copyright Policy

Last Updated: March 15th, 2023

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 583 - 588
doi: 10.30466/vrf.2023.1975795.3701
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Isolation and molecular characteristics of a recombinant feline calicivirus from Qingdao, China

Yongxiang Liu[1]*, Lide Qin[2], Xiaoliang Hu[3], Yanmei Jiang[1]

[1] Department of Veterinary Medicine, College of Life Sciences and Food Engineering, Hebei University of Engineering, Handan, China; [2] Department for Safety Supervision of Animal Products, China Animal Health and Epidemiology Center, Qingdao, China; [3] Department of Veterinary Medicine, Faculty of Agriculture, Forestry and Food Engineering, Yibin University, Yibin, China.

Article Info

Article history:

Received: 06 December 2022
Accepted: 20 May 2023
Available online: 15 November 2023

Keywords:

Feline calicivirus
Isolation
Recombination

Abstract

Feline calicivirus (FCV) is a highly contagious pathogen seriously affecting the upper respiratory tract and producing oral diseases in the feline. Despite widespread vaccination, the prevalence of FCV remains high. In this study, the FCV qingdao (qd)/2019/china was isolated from a domestic feline oropharyngeal swab collected from Qingdao, China. The virus was purified using the plaque assay and identified using the Polymerase chain reaction and indirect immunofluorescence assay methods, the capsid amino acid, *VP1* of qd/2019/china, showed sequence identity with the other isolates ranging between 83.90% (ym3/2001/jp) and 91.10% (CH-JL4). The sequence of the capsid amino acid revealed qd/2019/china to be closely related to CH-JL4 and clustered with CH-JL4 in the phylogenetic tree. The phylogenetic analysis indicated that the complete genomes (GenBank® accession No. MZ322896) of qd/2019/china and CH-JL4 were also classified into the same cluster. The recombination analysis with Simplot indicated that the qd/2019/china originated from the recombination of CH-JL4 and HRB-SS, and the region 3,821 - 5,301 nt originated from HRB-SS. Further, the region 3,821 - 5,301 nt were found to belong to the protease-polymerase (PP) of HRB-SS. Here, we isolated a new recombinant virus, FCV qd/2019/china. Therefore, these results would be beneficial for better understanding of the evolution and epidemiology of FCV.

Introduction

Feline calicivirus (FCV) is one of the most common contagious feline pathogens causing oral ulcers, ocular conjunctivitis and infectious upper respiratory tract disease.[1] The FCV can also infect other members of the Felidae like tigers, cheetahs[2] and lions.[3] It has also been isolated from dog feces.[4-6] Traditionally, FCV infections are usually associated with acute, mild and self-limiting diseases,[7] hence, do not inflict much harm. Therefore, FCV infections do not inflict significant harm if the secondary infection is well-controlled.

The virus belongs to the genus *Vesivirus* of the family *Caliciviridae*[8] comprising many viruses that cannot be readily cultured on cells such as the Norovirus, Sapovirus and Rabbit hemorrhagic disease virus which limit their pathogenic study. The FCV, on the contrary, can be readily cultured on the passaged feline cell lines such as CRFK and F81. There are no strict restrictions on the specific passaged numbers and demonstrating obvious cytopathic effect (CPE), hence, FCV has been established as an *in vitro* model for *Caliciviridae* research.[1]

The FCV is a single-stranded positive-sense RNA virus with a 7.80 kb long genome covered by a capsid protein, however, devoid of an envelope.[9] It has an icosahedral capsid diameter of about 27.00 - 40.00 nm. Due to lower fidelity, the FCV genome bears a high mutation rate and evolves rapidly. The immune system pressure constitutes the most important reason for the mutation power source contributing to its enhanced virulence and vaccination failure.[10]

The low fidelity of the protease and polymerase (PP) constitutes another reason for the mutation power source. The PP is the RNA-dependent RNA polymerase (RdRp) of FCV. The FCV has only one serotype and there is significant diversity in the strains isolated from different countries, regions and periods that needs further investigation.

***Correspondence:**

Yongxiang Liu. PhD
Department of Veterinary Medicine, College of Life Sciences and Food Engineering, Hebei University of Engineering, Handan, China
E-mail: liuyongxiang@hebeu.edu.cn

Random genetic drift, mutation and recombination are known to be the most common ways of viral evolution and variation. The high plasticity of the RNA genome in the RNA viruses has a high mutation rate during replication due to low fidelity, lack of proofreading and post-replicative repair activities of the viral RNA polymerase.[11] Recombination events like canine enteric coronaviruses,[12,13] porcine reproductive and respiratory syndrome virus,[14,15] and influenza virus[16] have been reported in several viruses, however, there have been very few studies on the recombination of FCV.

However, in recent years, the health of cats has been seriously threatened by the highly lethal strains due to the high degree of FCV variability.[17] Occasional outbreaks of virulent-systemic FCV (VS-FCV) infections have been reported not only in the USA but also in Europe during recent years and also in different regions of China like Heilongjiang and Shanghai.[18] The VS-FCV has been characterized by cutaneous edema, ulcerations of the head and feet, occasionally jaundice, and even death. Owing to the limited knowledge of FCV genetics and variation, the isolation and identification of new isolates and the analysis of the FCV genome sequence are of particular significance.

This study reported the isolation and sequence analysis of a recombinant FCV isolate qd/2019/china from the oropharyngeal swab of a dead domestic cat that exhibited serious mouth and tongue ulceration.

Materials and Methods

Sample information and treatment. In October 2019, a dead domestic cat oropharyngeal swab was submitted for laboratory investigation. The apparent clinical symptom comprised the eyes and nose covered with purulent discharge, severe tongue damage, the whole tongue layer falling off and ulceration of the hard palate. Necropsy of the cat showed hemorrhagic enteritis and lung edema. The oropharyngeal swab was collected with 0.80% sodium chloride (Beijing Solarbo Technology Co. Ltd., Beijing, China) and stored at – 80.00 ˚C until FCV was isolated.

Isolation and purification of the virus. The protocol for virus isolation follows the usual operation. Briefly, the CRFK cells (ATCC : CCL94) were grown in DMEM (Gibco, Carlsbad, USA) containing 10.00% fetal bovine serum (FBS) and 1.00% penicillin-streptomycin (Gibco). The cells were incubated at 37.00 ˚C in a 5.00% CO_2 humidified cabinet. The collected oropharyngeal swab was treated with a 0.22 μm filter and inoculated into the CRFK monolayer after which the CPE was observed at 1st passage (P1) virus generation. When the CPE was obvious, the cultures were freeze-thawed 2 cycles to release the virus then harvested and stored at – 80.00 ˚C. The virus stocks were purified using the plaque assay three cycles to reach out for purified virus. The CRFK cells were grown in DMEM containing 10.00% FBS and 1.00% penicillin-streptomycin on 6-well plate allowing the cells to reach the 80.00% - 90.00% confluency. The growth medium was removed and diluted virus was added to each well using multiple wells per dilution. The cells were coated with overlay medium, containing 1.00% agar No. 1 (Oxoid, Basingstoke, UK) and DMEM. The cell monolayers were observed every day for the presence of foci or plaques, the single foci or plaque were collected with a sterile pipette tip. At last, the virus titer of the purified FCV P2 virus generation was 10^7 median tissue culture infectious doses (TCID50) per 100 μL.

Identification of the virus. The viral RNA was extracted from the cell culture supernatant using a TIANamp virus DNA/RNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol, the cDNA was synthesized from the mRNA using the cDNA synthesis kit (TaKaRa, Tokyo, Japan), and the template for Polymerase chain reaction (PCR) identification was used. The PCR primer was synthesized according to the previous report,[19] *FCV-F*: 5′-GTAAAAGAAATTTGAGACAAT-3′, FCV-R:5′-TACTGAAGWTCGCGYCT-3′. The virus isolates were further identified by indirect immunofluorescence assay using a cat polyclonal antibody against FCV.[20]

Genome cloning and sequencing. The genome of the FCV strain qd/2019/china was cloned using a LA Taq PREMIX (LA Taq™ Version 2.0; Takara, San Jose, USA). The primer was obtained based on the conserved regions by multiple sequence alignment analysis of several China FCV isolates (Table 1). The 5′- and the 3′-end sequences were obtained using the 5′ and 3′ rapid amplification of the cDNA ends (5′ and 3′ RACE) kit (Invitrogen, Waltham, USA). The PCR products were cloned into the pMD18-T vector and the positive clones were used for sequencing and BLAST analyses to identify the related reference viruses of the qd/2019/china strain with the differential part of the genome and complete genome. The BLAST analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to identify the related reference viruses and used to study the qd/2019/china strain.

Table 1. Primers used in the genome cloning.

Primer	Sequence（5′ - 3′）	Position *
FCV-TY-20F	GTAAAAGAAATTTGAGACAA	1 - 20
FCV-TY-2440-R	ATGTTGATTGGCGGGTAGTTC	2440 - 2420
FCV-TY-2420-F	GAACTACCCGCCAATCAACAT	2420 - 2440
FCV-TY-5333-R	TTAGCGCAGGTTGAGCACAT	5333 - 5314
FCV-TY-5314-F	ATGTGCTCAACCTGCGCTAA	5314 - 5333
FCV-TY-7709-R	CCCTGGGGTTAGGCGCGA	7692 - 7709

* The position of primers was related to that in FCV strain CH-JL4 (Genbank® accession No. KT206207.1).

Sequence analysis. The capsid gene, *VP1* is the most variable gene in FCV genome. The phylogenetic analysis was usually undertaken based on the alignment of the *VP1* amino acid sequence. The sequence similarity was analyzed using DNAstar software (DNASTAR, Madison, USA). The

phylogenetic analysis of the full-length FCV genome and capsid gene were performed using the MEGA Software (version 6.0; Biodesign Institute, Tempe, USA). The bootstrap values were calculated according to 1,000 replicates of the alignment.

Recombination detection. From the blast analysis, we found that the complete genome of qd/2019/china had the highest similarity with GXNN01-19 (85.70%). While the different parts of the qd/2019/china genome were found to depict results in different search results, suggesting the possibility of recombination of different isolates. Then, the similarity comparisons and phylogenetic analyses of the full-length and part of FCV strain alignments were performed to detect the recombination signals in the FCV genomes. The sequence used was downloaded from NCBI and the Simplot 3.5.1 program with a 500 bp window and a 20 bp step and was used in this study to verify the recombination.

Results

Virus isolation and identification. The PCR assay was carried out to identify the FCV and depicted positive results. The viruses like FCoV, FPLV, and FHV-1 were not detected in the swab. Then the mouth swab was inoculated into the CRFK cells. The CPE could be easily observed 24 hr post-infection. After three rounds of plaque purification, the CPE was found to stably appear on the CRFK cells. The FCV was still positive with the PCR detection. Besides, the qd/2019/china isolate was further identified in the infected CRFK cells using a cat polyclonal antibody against FCV (previously identified by our lab). In this study, one FCV strain qd/2019/china was isolated from the mouth swab and was purified with the plaque assay method (Fig. 1).

Viral capsid amino acid sequence analysis. The genome of the qd/2019/china strain was successfully sequenced. Since the capsid protein was significantly important for the study of FCV evolution and immune escape, the sequence of the capsid amino acid was analyzed first. About 38 FCV isolates capsid protein sequences were retrieved from NCBI and DNAstar was used to analyze the sequence identity. The comparative pairwise analysis of the complete genome sequence of qd/2019/china *VP1* was conducted with 38 *VP1* sequences. The FCV isolates showed sequence identity ranging from 83.90% (ym3/2001/jp) to 91.10% (CH-JL4) with qd/2019/china that twas consistent with the previously reported genetically different FCV strains. The evolution of the qd/2019/china strain was further investigated by constructing a *VP1* amino acid sequence phylogenetic tree using MEGA. The evolutionary history was inferred using the maximum-likelihood method. The percentage of replicate trees with the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above the branches.[21] The analysis involved 38 amino acid sequences. The evolutionary tree demonstrated that the qd/2019/china was on the same cluster as CH-JL4. This was consistent with the *VP1* sequence analysis and the complete genome BLAST analysis. The qd/2019/china, CH-JL4, F65, UTCVM-H2, GX01-13, SH2014, UTCVM-H1 were in the same higher cluster (Fig. 2A). Since the FCV CH-JL4 challenge experiment on the kittens indicated typical clinical symptoms which meant that it had a stronger virulence. Since the qd/2019/china was also isolated from the dead cat, it might also be a virulent strain which needed further systematic virus challenge experiments for substantiation.

Complete genome sequence analysis. The complete genome sequence was submitted to GenBank® (accession No. MZ322896) to further understand the genetic origin of the qd/2019/china. The complete genome phylogenetic analysis was conducted using the MEGA6.0 (Fig. 2B). The FCV qd/2019/china strain was closely related to the CH-JL4 sequence and located in the same cluster as CH-JL4. Besides, the other Chinese isolates (SH, XH, WZ-1, HRB-SS, GX01-13), UTCVM-H2 and FCV/DD/2016/GE were in higher-level clusters with the qd/2019/china. The Chinese isolates were segregated into major genetic groups in the phylogenetic trees and designated as genogroups I and II. The strains qd/2019/china, CH-JL4, SH, XH, WZ-1, HRB-SS, GX01-13, UTCVM-H2 and FCV/DD/2016/GE were belonged to the genogroups I.[22]

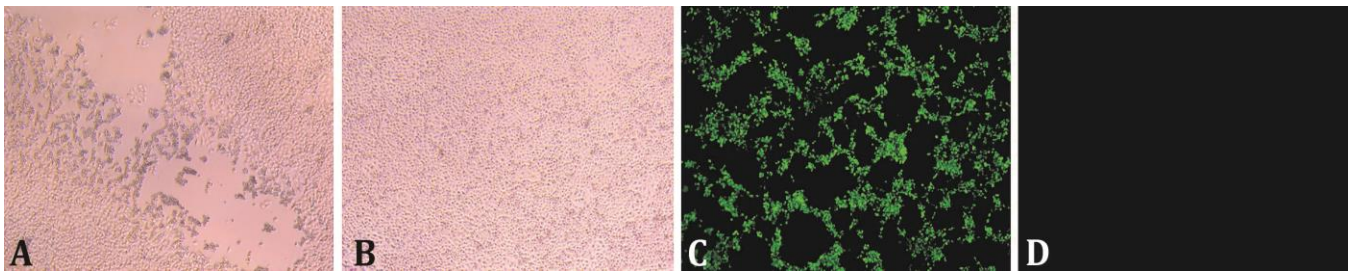

Fig. 1. Virus isolation and identification. **A)** The oropharyngeal swab was inoculated in the CRFK cells. Typical cytopathic effect (CPE), cell aggregation, rounding, falling off and enhanced shading was found (40×), **B)** The physiological saline inoculated CRFK showed no CPE (40×), **C)** Bright green fluorescence was found in the F81 cells infecting FCV (100×), and **D)** Green fluorescence was not found in the normal CRFK cells (100×).

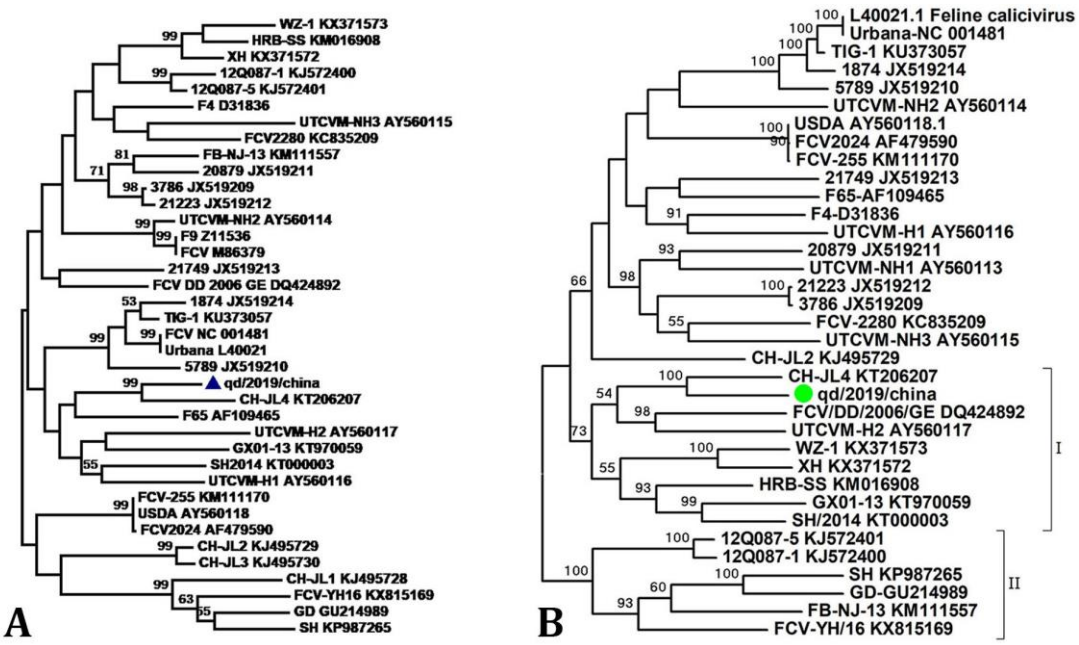

Fig. 2. Sequence analysis of FCV qd/2019/china. **A)** Phylogenetic tree of the feline calicivirus (FCV) *VP1* based on the amino acid sequence. The qd/2019/china was in the same cluster with CH-JL4. The phylogenetic tree was constructed using the maximum-likelihood method based on the JTT matrix-based model. The 1,000 bootstrap repetitions were performed for each analysis. The positions that contained gaps and/or missing data were eliminated. Bootstrap values > 50.00% are indicated, and **B)** The phylogenetic tree of the virus genome was constructed using the neighbor-joining method with 1,000 bootstrap replicates and the Kimura 2-parameter model in the MEGA 6.0 software package. The qd/2019/china is labeled with a green circle. GenBank® accession numbers are indicated on the branches. Bootstrap values > 50.00% are indicated.

Recombination analysis. The result of the BLAST showed that the different part of the FCV genome showed the highest similarity with the different FCV isolate. Given the potential significance of genetic recombination during the evolution of the picorna-viruses,[23] the recombination analysis was conducted with SimPlot. The results provided strong statistical support for an FCV qd/2019/china recombination event. The SimPlot graph revealed the breakpoints that separated the genome of qd/2019/china into three regions of which the two fragments arose from CH-JL4 (regions 1 - 3,821 nt and 5,301 - 7,707 nt) and one fragment originated from HRB-SS (region 3,821 - 5,301 nt). The 3,821 - 5,301 nt was the part of the PP gene based on the FCV CH-JL4 (Fig. 3A). To further validation of the detected recombination signals, a phylogenetic tree of recombination region (the corresponding loci in the genome of qd/2019/china (3821 - 5301 nt) were constructed containing the genogroups I member (CH-JL4, SH, XH, WZ-1, HRB-SS, GX01-13, UTCVM-H2 and FCV/DD/2016/GE) as shown in the tree. The strain qd/2019/china was in the same cluster as HRB-SS (Fig. 3B).

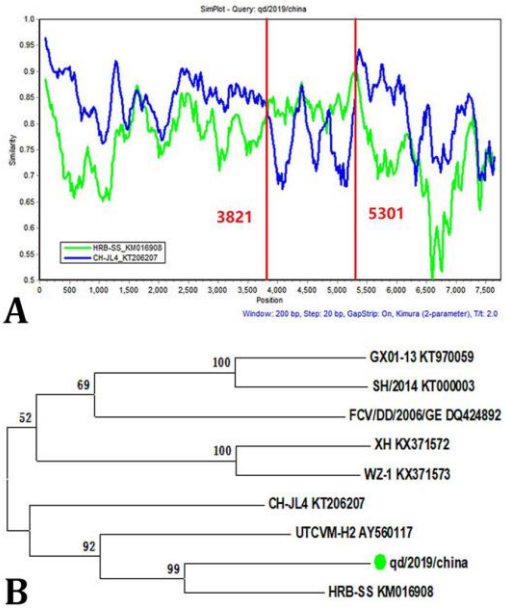

Fig. 3. A) Recombination analysis was based on the whole genome of qd/2019/china. Reference strains, HRB-SS (green), and CH-JL4 (blue) were used as putative parental strains. The X-axis indicates the location of the query sequence and Y-axis indicates the percentage of identity, and **B)** The phylogenetic analysis is based on the genome region 3821 - 5301 nt. The phylogenetic trees were constructed using the MEGA 6.0 software and the maximum likelihood algorithm, with 1,000 bootstrap replications and the Kimura 2-parameter substitution model.

Discussion

The FCV is one of the most common pathogens of domestic cats[1] which accounts for severe respiratory and oral diseases (stomatitis and gingivitis). Despite the cats

being increasingly vaccinated, many strains of the viruses are still isolated in China.[1,24-26] The FCV is a single positive strand RNA virus having high genomic variability for their RdRP low-fidelity. However, the detailed mechanisms of the emergence of the new genetically distinct FCV strains remain unknown.[23] In this study, an FCV qd/2019/china was isolated from a dead cat. This was the first FCV isolate from Qingdao, the eastern coastal areas of China. The sequence analysis of the capsid protein, *VP1*, indicated qd/2019/china to have the highest similarity with CH-JL4 (84.50%).

The northeast part of China has a well-developed pet breeding industry which transports large numbers of cats and dogs to the other parts of the country promoting the spread of the virus. The qd/2019/china might have originated from Jilin indicating that transporting cats across the regions might potentially promote the virus transmission and evolution. Almost at the same time as our first submission, the phenomenon of recombination between different FCV strains had been reported in September 2021. It can be seen that the reorganization of FCV is not a single phenomenon which needs more attention.

The recombination of the virus is a prevalent mechanism of virus evolution reporting the existence of a recombinant FCV circulating within a naturally infected population of cats. The quasispecies represents an effective adaptive strategy for the virus in an extremely heterogeneous viral population evolving with a better replicative capacity.[26] However, the recombination of the FCV virus across the different regions has never been reported. Here, the qd/2019/china might be a recombinant of HRB-SS (from Harbin) and CH-JL4 (from Jilin).

Although there is no licensed live-attenuated FCV vaccine in China, the non-licensed live-attenuated FCV vaccine cannot be prohibited, since it is believed by some clinical veterinarians to be better than the killed vaccine. Here, based on the evidence that the FCV virus can recombine between the different viruses, the virus in the live vaccine was found to be a potential recombinant strain suggesting that the live FCV vaccine registration should be more rigorous.

The FCV shows evolutionary rates higher than those of the other viruses and approximately 1.30×10^{-2} to 2.60×10^{-2} substitutions per nucleotide occur per year in the variable regions of the FCV capsid protein.[27] Thus, the high genetic plasticity of the virus has emerged new variants.[28] The PP protein is the RdRp encoded by FCV and is indispensable for the replication of the viral genome.[29] The PP and its precursor protein also possess the cysteine proteinase activity responsible for the proteolytic processing, cutting the ORF1 encoded large nonstructural proteins into five nonstructural proteins- p5.6, p32, p39, p30, VPg, PP. The PP proteins of FCV 2280 and F9 share 94.00% amino acid identity, however, the strain 2280 grows faster than the strain F9. The challenge experiments in cats showed the strain 2280 to be more virulent than the strain F9. The PP is one of the most important nonstructural protein of FCV. The N-terminal domain of PP can inhibit the host cell protein transcription,[30] therefore, recently PP was found to effectively reduce the mRNA expression by promoting the degradation of the host mRNAs.[31]

In this study, we found that the region (3,821 - 5,301 nt) of qd/2019/china was recombined from HRB-SS and the region (3,821 - 5,301 nt) belonged to the pp region. The PP is a crucial protein that ensures the fidelity of RNA replication, hence, the replacement or change of PP might greatly change the viral characteristics promoting the evolution of FCV and promoting the emergence of new FCV isolates. Besides, the recombination has been associated with the expansion of the range of the viral host,[32] as many FCV isolates were isolated from non-cat feline.[1]

In this study, an FCV strain qd/2019/china was isolated from Qingdao, China. The phylogenetic analysis of the FCV genome revealed that it had the highest similarity with CH-JL4. The recombination analysis revealed that it might be a recombinant virus of CH-JL4 and HRB-SS and that the recombination area belonged to the PP region. Our results thus, provided new evidence highlighting the contribution of viral recombination to FCV evolution.

Acknowledgments

This work was funded by the Natural Science Foundation of Hebei province （grant No. C2021402005), Science and Technology Research Project of Hebei Colleges and Universities (grant No. ZC2021214). The authors would like to thank all the reviewers who participated in the review and MJEditor (www.mjeditor.com) for their linguistic assistance during the preparation of this paper.

Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

References

1. Tian J, Liu D, Liu Y, et al. Molecular characterization of a feline calicivirus isolated from tiger and its pathogenesis in cats. Vet Microbiol 2016; 192: 110-117.
2. Evermann JF, Heeney JL, Roelke ME, et al. Biological and pathological consequences of feline infectious peritonitis virus infection in the cheetah. Arch Virol 1988; 102(3-4): 155-171.
3. Kadoi K, Kiryu M, Iwabuchi M, et al. A strain of calicivirus isolated from lions with vesicular lesions on tongue and snout. New Microbiol 1997; 20(2): 141-148.
4. Martella V, Pratelli A, Gentile M, et al. Analysis of the capsid protein gene of a feline-like calicivirus isolated

from a dog. Vet Microbiol 2002; 85(4): 315-322.
5. Di Martino B, Di Rocco C, Ceci C, et al. Characterization of a strain of feline calicivirus isolated from a dog faecal sample. Vet Microbiol 2009; 139(1-2): 52-57.
6. Martella V, Pinto P, Lorusso E, et al. Detection and full-length genome characterization of novel canine vesiviruses. Emerg Infect Dis 2015; 21(8): 1433-1436.
7. Battilani M, Vaccari F, Carelle MS, et al. Virulent feline calicivirus disease in a shelter in Italy: a case description. Res Vet Sci 2013; 95(1): 283-290.
8. Rohayem J, Bergmann M, Gebhardt J, et al. Antiviral strategies to control calicivirus infections. Antiviral Res 2010; 87(2): 162-178.
9. Fino VR, Kniel KE. UV light inactivation of hepatitis A virus, Aichi virus, and feline calicivirus on strawberries, green onions, and lettuce. J Food Prot 2008; 71(5): 908-913.
10. Prikhodko VG, Sandoval-Jaime C, Abente EJ, et al. Genetic characterization of feline calicivirus strains associated with varying disease manifestations during an outbreak season in Missouri (1995-1996). Virus Genes 2014; 48(1): 96-110.
11. Domingo E, Escarmís C, Sevilla N, et al. Basic concepts in RNA virus evolution. FASEB J 1996; 10(8): 859-864.
12. Chen S, Liu D, Tian J, et al. Molecular characterization of HLJ-073, a recombinant canine coronavirus strain from China with an ORF3abc deletion. Arch Virol 2019; 164(8): 2159-2164.
13. Decaro N, Mari V, Campolo M, et al. Recombinant canine coronaviruses related to transmissible gastroenteritis virus of Swine are circulating in dogs. J Virol 2009; 83(3): 1532-1537.
14. Wang HM, Liu YG, Tang YD, et al. A natural recombinant PRRSV between HP-PRRSV JXA1-like and NADC30-like strains. Transbound Emerg Dis 2018; 65(4): 1078-1086.
15. Liu D, Zhou R, Zhang J, et al. Recombination analyses between two strains of porcine reproductive and respiratory syndrome virus in vivo. Virus Res 2011; 155(2): 473-486.
16. He CQ, He M, He HB, et al. The matrix segment of the "Spanish flu" virus originated from intragenic recombination between avian and human influenza A viruses. Transbound Emerg Dis 2019; 66(5): 2188-2195.
17. Hu CJ, Chang WS, Fang ZS, et al. Nanoparticulate vacuolar ATPase blocker exhibits potent host-targeted antiviral activity against feline coronavirus. Sci Rep 2017; 7(1): 13043. doi: 10.1038/s41598-017-13316-0.
18. Guo H, Miao Q, Zhu J, et al. Isolation and molecular characterization of a virulent systemic feline calicivirus isolated in China. Infect Genet Evol 2018; 65: 425-429.
19. Meli ML, Berger A, Willi B, et al. Molecular detection of feline calicivirus in clinical samples: A study comparing its detection by RT-qPCR directly from swabs and after virus isolation. J Virol Methods 2018; 251: 54-60.
20. Wu H, Zhang X, Liu C, et al. Antiviral effect of lithium chloride on feline calicivirus in vitro. Arch Virol 2015; 160(12): 2935-2943.
21. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985; 39(4): 783-791.
22. Zhou L, Fu N, Ding L, et al., Molecular characterization and cross-reactivity of Feline Calicivirus circulating in Southwestern China. Viruses 2021; 13(9): 1812. doi: 10.3390/v13091812.
23. Lukashev AN. Recombination among picornaviruses. Rev Med Virol 2010; 20(5): 327-337.
24. Zhao Y, Chen X, Ying Y, et al. Isolation and phylogenetic analysis of three feline calicivirus strains from domestic cats in Jilin Province, China. Arch Virol 2017; 162(9): 2579-2589.
25. Liu C, Liu Y, Liu D, et al. Complete genome sequence of feline calicivirus strain HRB-SS from a cat in Heilongjiang Province, Northeastern China. Genome Announc 2014; 2(5): e00698-14. doi: 10.1128/genomeA.00698-14
26. Coyne KP, Reed FC, Porter CJ, et al. Recombination of feline calicivirus within an endemically infected cat colony. J Gen Virol 2006; 87(Pt 4): 921-926.
27. Coyne KP, Gaskell RM, Dawson S, et al. Evolutionary mechanisms of persistence and diversification of a calicivirus within endemically infected natural host populations. J Virol 2007; 81(4): 1961-1971.
28. Coyne KP, Christley RM, Pybus OG, et al. Large-scale spatial and temporal genetic diversity of feline calicivirus. J Virol 2012; 86(20): 11356-11367.
29. Wei L, Huhn JS, Mory A, et al. Proteinase-polymerase precursor as the active form of feline calicivirus RNA-dependent RNA polymerase. J Virol 2001; 75(3): 1211-1219.
30. Wu H, Zu S, Sun X, et al. N-terminal domain of feline calicivirus (FCV) proteinase-polymerase contributes to the inhibition of host cell transcription. Viruses 2016; 8(7): 199. doi: 10.3390/v8070199
31. Wu H, Huang J, Liu Y, et al. Feline calicivirus proteinase-polymerase protein degrades mRNAs to inhibit host gene expression. J Virol 2021; 95(13): e00336-21. doi: 10.1128/JVI.00336-21.
32. Simon-Loriere E, Holmes EC. Why do RNA viruses recombine? Nat Rev Microbiol 2011; 9(8): 617-626.

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 589 - 594
doi: 10.30466/vrf.2023.1978088.3703
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

The autumn activity patterns and time budgets of Forest musk deer (*Moschus berezovskii*) in captivity

Songwei Lin[1,2,3], Liquan Shen[1], Haoxiang Gao[1], Jiayi Wu[1], Qingxin Lv[1], Xin Zhou[1], Junsheng Li[2,3]*, Xiuxiang Meng[1,4,5]*

[1] School of Environment and Natural Resources, Renmin University of China, Beijing, China; [2] Command Center for Comprehensive Survey of Natural Resources, China Geological Survey Bureau, Beijing, China; [3] Institute of Ecology, Chinese Research Academy of Environmental Sciences, Beijing, China; [4] College of Science, Tibet University, Lhasa, China; [5] School of Animal Science, Xichang University, Xichang, China.

Article Info

Article history:

Received: 05 December 2022
Accepted: 16 March 2023
Available online: 15 November 2023

Keywords:

Activity pattern
Activity peak
Captive
Moschus berezovskii
Time budget

Abstract

Activity patterns and time budgets play a crucial role in the successful farming and management of animals. In this study, the behavior patterns of 53 forest musk deer (*Moschus berezovskii*) were analyzed from October 2nd to 16th, 2021, throughout the day and night. The results showed a distinct dawn–dusk activity rhythm in the captive forest musk deer with a peak activity observed at dawn (07:00 - 10:00) and dusk (16:00 - 19:00). Additionally, there were smaller activity peaks lasting less than an hour during the nighttime (00:00 - 04:00). Comparing behavior ratios between peak and off-peak periods, it was evident that all behaviors, except rumination (RU), showed significant differences. Furthermore, no significant differences were found in the behavior ratios of the forest musk deer between the daytime and night-time. During the daytime, the percentages of time spent performing locomotion (32.87 ± 3.38%), feeding (14.43 ± 1.81%), and RU (5.62 ± 1.46%) were slightly higher compared to the night-time. Based on these findings, it is important to match the management strategies for musk deer farming with the animals' activity patterns and behavioral rhythms. Doing so can enhance farming outputs and contribute to the welfare of captive forest musk deer.

Introduction

Musk deer (*Moschus* sp.) are critically endangered ungulates and they are in urgent need of conservation. Farming has been recognized as an effective *ex situ* conservation approach.[1] Musk deer farming has been carried out successfully in China since 1958, with forest musk deer (*Moschus berezovskii*) being the primary species reared in the captivity. Currently, captive rearing is considered one of the most important measures for the *ex situ* conservation of musk deer. Under artificial feeding conditions, the activity time allocation of captive musk deer differs from that of their wild counterparts and is influenced by various factors.[2] Some previous studies have reported the seasonal activity patterns of captive alpine musk deer (*Moschus chrysogaster*).[2]

Under captive conditions, alpine musk deer exhibit a distinct morning activity peak in the summer. However, this morning peak is not evident in autumn and winter. Instead, captive alpine musk deer show an activity peak around noon during these seasons.[2] Many other ungulate species live in one or more stable groups, and behaviors such as running or foraging are affected by the group dynamics. These behaviors often demonstrate certain degree of synchronization, which helps maintain group cohesion.[3-4] On the contrary, forest musk deer are solitary ungulates that predominantly inhabit high-altitude closed-cone coniferous and broad-leaved mixed forests.[5]

Activity time budgets are a fundamental biological characteristic of animals and reflect behavioral adaptations to the environment. The pattern of activity plays a crucial role in the life history strategies of both wild and domestic herbivores.[6,7] Studying the circadian

***Correspondences:**

Junsheng Li. PhD
Command Center for Comprehensive Survey of Natural Resources, China Geological Survey Bureau, Beijing, China & Institute of Ecology, Chinese Research Academy of Environmental Sciences, Beijing, China
E-mail: lijunshen@cgs.gov.cn
Xiuxiang Meng. PhD
School of Environment and Natural Resources, Renmin University of China, Beijing, China | College of Science, Tibet University, Lhasa, China | School of Animal Science, Xichang University, Xichang, China
E-mail: meng2014@ruc.edu.cn

rhythms of animals has always been a significant aspect of research in the field of behavioral ecology.[8,9] Research on animal circadian activity patterns and their influencing factors can broaden the understanding of animal behavior patterns,[10] and help clarify how animals adapt to their environment.[11-14] However, traditional methods, like manual scan sampling, have limitations in terms of time and data collection capabilities, as well as small sample sizes.

In this study, we used infrared cameras and scan sampling to explore the 24hr activity patterns and time budgets of a forest musk deer population after *ex-situ* conservation more effectively through data analysis. Autumn is just before the reproduction season for forest musk deer; therefore, it is crucial to know the activity patterns and time budgets of musk deer in autumn, which will benefit successful reproduction attempts, population increases, and *ex situ* conservation.

Materials and Methods

Research area. This study was carried out from October 2nd to 16th, 2021, at the Huailai Musk Deer Farm in Xinglinpu, Hebei Province, China. The farm is located in a mid-temperate semi-arid region known for its temperate continental monsoon climate, which exhibits significant temperature variations between day and night. The farm lies at the highest latitude of any musk deer farm in the world (E115°38′48″, N40°33′32″). In October, the local temperatures range from 0.00 to 30.00 ˚C, with average daily low and high temperatures of 5.00 and 17.00 ˚C, respectively. There were 3 days of precipitation during the study (October 3rd, 12th, and 16th). However, the amount of the rainfall was minimal with a little effect on the observations.

Animals. The study included a total of 53 captive forest musk deer (25 males and 28 females) in the Huailai musk deer farm. They were all born in captivity and descendant from the southern species of China. The study area consisted of six enclosures, each consisting of one 10.00 m^2 cell and 50.00 m^2 exercise area containing 6 - 10 deer (four enclosures each holding 10 forest musk deer; one enclosure holding six forest musk deer; and one enclosure holding seven forest musk deer). The enclosures featured a natural soil base that was covered with ground vegetation and trees. All deer were adults aged 3 years old, and were maintained by one deer-keeper and fed twice a day, at 08:00 and 18:00. The diet was supplemented with artificial feed containing approximately 40.00% corn, 25.00% wheat, and 25.00% beans, which was mixed onsite. Seasonal vegetables were also provided occasionally, and water was available *ad libitum*. Interaction with the human keeper was limited to 20 min per day, for feeding, cleaning, and other management duties. All deer were individually identified by a numbered plastic ear tag. This study was approved by the Academic Committee of the School of Environment and Natural Resources, Renmin University of China (No. 2021010 of proposal).

Behavioral sampling and data collection. Excessive ambient lighting at night can have an impact on animal behavior, such as causing over-expression and resulting in inaccurate data.[15,16] To minimize this interference, our study did not use any artificial lighting at night-time. Additionally, infrared cameras (Hikvision, Hangzhou, China) were installed on the diagonal of each barn to cover the entire barn area without blind spots. This allowed us to monitor the behavior 24 hr a day from October 2nd to October 16th with minimal environmental light interference for the captive forest muck deer, a particularly timid species. Scan sampling was used to record each individual's behavior at 30-min intervals. [4,17] Each scan was watched for up to 30 sec. The study ethogram was adapted from previous studies on the time allocation of alpine musk deer activities,[2,18] with the behaviors defined in Table 1. All behavioral sampling was performed by the same observer.

Statistical analysis. During the experiment, the maximum change in day duration was 34 min. To ensure the comparability of the incidence of behaviors between periods, the inter-variation of day duration was not considered when dividing periods. Hence, the daytime period remained fixed as 06:25 - 17:32 throughout the experiment, with the remaining time each day considered as night-time. Behavior samples were analyzed by individuals and, for each behavior, the behavior ratio was calculated (the duration of behavior was divided by the total sampling duration)[17] The average rate of the total population was then determined. The behaviors of locomotion (LO), rumination (RU) and feeding (FE) were merged to calculate the activity rate (the duration of these three behaviors was divided by the total sampling duration), and the period whose activity rate exceeded the average was defined as the activity peak. The Shapiro-Wilk test was used to assess the normality of data (behavior

Table 1. Ethogram of captive forest musk deer.

Behaviors	Abbreviations	Definition
Locomotion	LO	In locomotion, including walking, chasing, grooming, conflict behavior, and exploring the environment
Feeding	FE	Feeding or drinking water
Rumination	RU	Ruminating, including standing-ruminating and lying-ruminating
Lying still	LS	In a prone posture and resting although not ruminating, with the head on the shoulder or ground
Others	OT	Included other infrequent behaviors, such as excretion and tail-pasting

The observer was aware of the work protocol.

ratios). If the data were normally distributed, either an ANOVA (to explore the difference between different periods defined by activity rate) or a t-test (to explore the difference between daytime and night-time) was used to explore the difference. If the data were non-normally distributed, either the Kruskal-Wallis H test (to explore the difference between different periods defined by activity rate) or the Mann-Whitney U test (the difference between daytime and night-time) was used. Statistical analyses were completed using SPSS Software (version 25.0; IBM Corp., Armonk, USA) and all reported statistical probabilities were two-tailed at $p \leq 0.05$. Excel (version 2108; Microsoft Corp., Redmond, USA) was used to draw the diagrams.

Results

Distribution pattern of autumn activities. The behavior data of LO, FE, and lying still, and the logarithmically transformed RU data, were all normally distributed ($p > 0.05$). Lying still (LS) accounted for the highest percentage of the time budget (50.10 ± 2.72%, n = 48), followed by LO (31.39 ± 2.08%, n = 48) and FE (12.14 ± 1.17%, n = 48). Rumination accounted for the lowest percentage of time amongst the four main behaviors (4.82 ± 0.83%, n = 48). The distribution of the percentage of each behavior exhibited by captive forest musk deer over 24 hr is shown in Figure 1 with LO and LS found to be the main behaviors of the forest musk deer and FE and ruminating behaviors presented together.

Activity peak. As shown in Figure 2, throughout the study, the average daily activity rate was (43.53 ± 2.69%, n = 18). There were dawn activity peaks (07:00 ~ 10:00, 65.26 ± 2.35%, n = 18) and dusk activity peaks (16:00 ~ 19:00, 61.83 ± 7.27%, n = 18), and at midnight (0:00 ~ 04:00) there were multiple small, short-duration peaks of night activity. Among them, the activity peak duration at midnight (0:00 - 1:00) was relatively longer (1 hr), and its activity rate was (54.18 ± 6.75%, n = 18).

As shown in Table 2, the activity rates of the three peak periods of captive forest musk deer activities were significantly higher than those of the off-peak (rest) period (33.46 ± 2.19%, n = 30; F = 18.62, df = 3, $p < 0.001$). Comparing the activity rate of peak periods, there were no significant differences between them ($p > 0.05$).

Table 2. The activity rate (mean ± SE) of forest musk deer in active and inactive periods.

Time	Activity rate (%)	Activity period
00:00 - 01:00	54.18 ± 6.75[a]	Midnight peak
07:00 - 10:00	65.26 ± 2.35[a]	Dawn peak
16:00 - 19:00	61.83 ± 7.27[a]	Dusk peak
Rest of time	33.46 ± 2.19[b]	Off-peak
All day average	43.53 ± 2.69[a]	-

[a] indicates non-significant difference ($p > 0.05$), and [b] indicates highly significant difference ($p < 0.01$).

Behavior rate comparison. In the distribution of behavior ratios between peak and off-peak periods throughout the day, LO and FE exhibited significant differences in each time (Table 3). The further comparison found that the incidence of LO in the three peak periods was significantly higher than that in the off-peak period (F = 7.21, df = 3, $p < 0.001$). There was no significant difference in LO between the three peaks ($p > 0.05$), with the highest behavior ratio occurring within three hr of the peak at dawn (44.56 ± 3.55%). Similarly, FE was significantly higher during the peak periods than in the off-peak periods (F = 14.12, df = 3, $p < 0.001$), although there was no significant difference between peaks. However, RU with a lower behavior ratio exhibited no significant difference between different periods ($p > 0.05$) and peaked (5.62 ± 4.15%) in the time from 1.5 hr before to 1.5 hr after sunset. In addition, for inactive behavior LS, there were significant differences between periods (F = 20.85, df = 3, $p < 0.001$), and LS during off-peak periods (60.44 ± 2.20%) was much higher than that of peak periods.

Daytime and night-time differences in behaviors. The average behavior ratios are shown in Table 4. Deer LS values during the daytime (45.62 ± 4.63%) were slightly lower than that at night (54.22 ± 2.88%); however, the difference was not significant ($p > 0.05$). Other behaviors were slightly higher during the daytime but showed no significant difference.

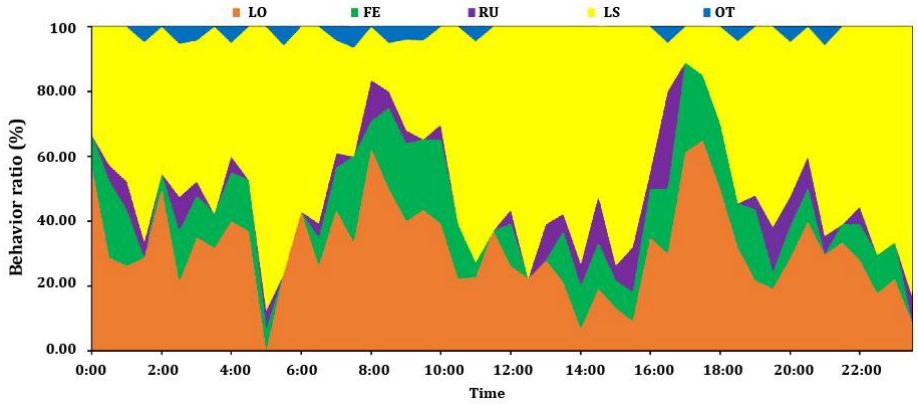

Fig. 1. The behavioral pattern of captive forest musk deer (LO: locomotion; FE: feeding; RU: ruminating; LS: lying still; OT: other behaviors).

Table 3. Incidence of various behaviors in active and inactive periods. Data are presented as mean ± SE.

Time	Locomotion (%)	Feeding (%)	Ruminating (%)	Lying still (%)
00:00-01:00	37.27 ± 9.96[a]	16.91 ± 4.13[a]	4.49 ± 2.51[a]	41.34 ± 4.25[a]
07:00-10:00	44.56 ± 3.55[a]	20.70 ± 2.70[a]	4.31 ± 1.58[a]	26.95 ± 2.99[a]
16:00-19:00	42.10 ± 6.30[a]	19.74 ± 1.75[a]	5.62 ± 4.15[a]	31.18 ± 6.75[a]
Rest of time	25.43 ± 2.01[b]	8.03 ± 1.05[b]	4.79 ± 0.85[a]	60.44 ± 2.20[b]

[a] indicates non-significant difference ($p > 0.05$) and [b] indicates highly significant difference ($p < 0.01$).

Table 4. Statistical analysis of day-night differences in behaviors. Data are presented as mean ± SE.

Time	Locomotion (%)	Feeding (%)	Ruminating (%)	Lying still (%)
Daytime	32.87 ± 3.38	14.43 ± 1.81	5.62 ± 1.46	45.62 ± 4.63
Night-time	30.04 ± 2.55	10.03 ± 1.41	4.09 ± 0.86	54.22 ± 2.88
All day average	31.39 ± 2.08	12.14 ± 1.17	4.82 ± 0.83	50.10 ± 2.72

There are non-significant differences among the data ($p > 0.05$).

Discussion

In this study, the 24h activity budgets showed that there was a dawn-dusk activity peak in captive forest musk deer in autumn. The activity rate at dawn (7:00 - 10:00) and dusk (16:00 - 19:00) were significantly higher than that in off-peak periods. Moreover, forest musk deer seem to have a clear schedule that distinguishes between activity and rest time. The activity rhythms of animals mainly include diurnal, nocturnal, and crepuscular activity, and they can also be affected by environmental factors, such as the photoperiod and temperature. This is closely related to the individual's internal physiological processes such as eating, digestion, ruminating, and hunger/satisfaction.[19] Animals use activity time allocation to achieve a balance between foraging and predation risks[20] and to avoid excessive water and energy consumption.[21]

The dawn and dusk peaks were close to the local sunrise (06:15 - 06:29) and sunset (17:59-17:37) times during the study. Similar bimodal activity patterns with obvious dawn–dusk peaks have been identified for most free-range ungulates in the northern hemisphere, such as goat antelope*(Rupicapra rupicapra*), white-tailed deer (*Odocoileus virginianus*),[22] red deer (*Cervus elaphus*),[3,8,23,24] Spanish ibex (*Capra pyrenaica*),[25] moose (*Alces alces*),[26,27] and Rocky Mountain elk (*Cervus elaphus*).[24,28] Two activity peaks found in this study were of relatively long duration, both reaching three hours, and the related activities were mainly LO and FE. The stable and long-term high frequency of activities indicated that the high frequency of activities during this period was due to group behaviors rather than a fixed individual habit. During the observation, we also found that the continuity of activity peaks may have been due to alternate eating in the musk group, which means taking turns in a certain order.[18] This order conforms to the habits of musk deer FE, ruminating, re-eating, and re-ruminating.[2] The FE time at the musk deer farm was close to the set sunrise and sunset times; thus, there was a peak in activity under the combined action of the two, and related issues still need to be further studied.

In addition, the study found that there were many short midnight peaks in the activity of forest musk deer, with narrow peaks and frequent alternation, of which the midnight (00:00 - 01:00) small peak was particularly significant. Animal activity can be divided into nocturnal, diurnal, and twilight activity, as well as some transitional forms in its activity. The midnight peak may be the deer's adaptation to the season or environment. Similar results have been shown in previous studies. Red deer have demonstrated a constant small peak of midnight activity, in addition to the normal peaks of morning and evening activity.[19] Meng *et al.* reported that captive alpine musk deer were active in the morning and evening, and also had a small peak of midnight activity.[2] Some scholars have attempted to prove that forest musk deer also have a midnight activity peak, but definitive evidence has not yet been found.[18] The current study confirms that forest musk deer also have this habit. In our study, we performed a full review of these periods and found that forest musk deer's activities were not just ordinary walking or exploring the environment during these midnight peaks, but mainly social behaviors such as chasing, grooming, and conflict behavior. Also, the midnight peak is also a manifestation of the self-protection mechanism of some herbivores in special ecological systems, whose levels of alertness are higher at night than during the day, meaning that activity disturbance activities are greater. The duration and positioning of animal activity peaks may be affected by a variety of factors;[29] therefore, further control studies should be conducted to determine the influences on captive forest musk deer caused by more potential factors.

Day-night differences in activities of captive forest musk deer were investigated in this study. Results showed that there were no significant differences in the activity patterns between daytime and night-time in autumn. Furthermore, no long-lasting behaviors were observed either at night-time or during the daytime. A study by Xue *et al.* on the daytime and night-time activities of forest musk deer in spring showed that the intensity of night activity of forest musk deer in spring was higher than that in the daytime, [18] which is similar to that of captive alpine musk deer in summer.[2] Activity is essential for animals to

adapt to ambient temperature,[30-32] and individuals demonstrate flexibility to temperature.[33] It has been reported that the shorter activity cycle in moose (*Alces alces*) is related to the increase in temperature at noon.[34-35] Additionally, this study found that the activity intensity of forest musk deer was greatly influenced by temperature change. At high temperatures, musk deer usually rest as a means of thermoregulation. Even though the temperature at night was lower than the daytime, the temperature at the musk deer farm was relatively mild in the early autumn and the forest musk deer showed midnight activity peaks, which may have led to the lack of significant differences in behavior ratios between daytime and night-time.

This study also found that, under captive conditions, musk deer had short rest times at night, between 0.5 and 1 hr, with a high frequency of changes in their resting positions. This relates to variations among individuals in the group. Forest musk deer in wild environments are highly solitary; however, when socially enclosed in musk deer farms, the interactions among individuals will mutually influence the whole group. Individual LO at night disturbs others and lead to group disturbance, which could explain why the LO behavior was not significantly reduced. In addition, due to the fixed FE time (08:00 and 18:00), there may not have been enough food after midnight (00:00), causing the forest musk deer to actively search for food in the enclosure and eat sporadic turf or food residues. This could also be a reason for the increased activity. After eating at night, individuals often expressed certain RU behaviors. During the FE process, musk deer often stood with vigilance for a short time (about 5 min) before continuing to feed, which may indicate that, after captive breeding and multigenerational reproduction, the forest musk deer born on the farm still expressed the same alertness behaviors developed in wild musk deer. This study was conducted outside of the mating season of captive forest musk deer, and the most active behaviors observed were related to foraging and FE. It is recommended that, in the management of musk deer farming practices, population size, stock density, and sufficient food provision should be taken into consideration in order to maintain stable and active populations.

Acknowledgments

This work was funded by the National Key R&D Program of China (2022YFF1301405) and the Nature Science Foundation of China (32170489, 32211530443, 31672300).

Conflict of interest

The authors declare no financial or conflict of interest that could inappropriately influence this study.

References

1. Yang Q, Meng X, Xia L, et al. Conservation status and causes of decline of musk deer (*Moschus* spp.) in China. Biol Conserv 2003; 109: 333-342.
2. Meng X, Yang Q, Feng Z, et al. Preliminary studies on active patterns during summer, autumn and winter seasons in captive alpine musk deer [Chinese]. Acta Theriol Sin 2002; 22(2): 87-97.
3. Georgii B, Schröder W. A radiotelemetric study of the activity of female red deer (*Cervus elaphus* L.) [German]. Z Jagdwiss 1978; 24(1): 9-23.
4. Altmann J. Observational study of behavior: sampling methods. Behaviour 1974; 49(3): 227-267.
5. Gao Y, Duszynski DW, Yuan F, et al. Coccidian parasites in the endangered Forest Musk Deer (*Moschus berezovskii*) in China, with the description of six new species of Eimeria (Apicomplexa: Eimeriidae). Parasite 2021; 28: 70. doi: 10.1051/parasite/2021067.
6. Owen-Smith N, Hopcraft G, Morrison T, et al. Movement ecology of large herbivores in African savannas: current knowledge and gaps. Mamm Rev 2020; 50(3): 252-266.
7. Renecker LA, Hudson RJ. Estimation of dry matter intake of free-ranging moose. J Wildl Manage 1985; 49(3): 785-792.
8. Gwinner E. Circannual rhythms in animals and their photoperiodic synchronization [German]. Naturwissenschaften 1981; 68(11): 542-551.
9. Nelson RJ, Bumgarner JR, Liu JA, et al. Time of day as a critical variable in biology. BMC Biol 2022; 20(1): 142. doi: 10.1186/s12915-022-01333-z.
10. Jacobs PJ, Bennett NC, Oosthuizen MK. Locomotor activity in field captured crepuscular four-striped field mice, *Rhabdomys dilectus* and nocturnal Namaqua rock mice, *Micaelamys namaquensis* during a simulated heat wave. J Therm Biol 2020; 87: 102479. doi: 10.1016/j.jtherbio.2019.102479.
11. Ridout MS, Linkie M. Estimating overlap of daily activity patterns from camera trap data. J Agric Biol Environ Stat 2009; 14: 322-337.
12. Guillera-Arroita G, Morgan BJ, Ridout MS, et al. Species occupancy modeling for detection data collected along a transect. J Agric Biol Environ Stat 2011; 16: 301-317.
13. Oliveira-Santos LGR, Antunes PC, Zucco CA, et al. Suitable animal movement indexes or just geometric correlations? A comment on Püttker et al. 2012. J Mammal 2013; 94(4): 948-953.
14. Rowcliffe JM, Kays R, Kranstauber B, et al. Quantifying levels of animal activity using camera trap data. Methods Ecol Evol 2014; 5(11): 1170-1179.
15. Neethirajan S. Transforming the adaptation physiology of farm animals through sensors. Animals (Basel) 2020; 10(9): 1512. doi: 10.3390/ani10091512.
16. Margulis SW. Relationships among parental

inbreeding, parental behaviour and offspring viability in oldfield mice. Anim Behav 1998; 55(2): 427-438.
17. Komers PE, Birgersson B, Ekvall K. Timing of estrus in fallow deer is adjusted to the age of available mates. Am Nat 1999; 153(4): 431-436.
18. Xue C, Meng XX, Xu HF, et al. Activity rhythm and behavioral time budgets of the captive forest musk deer (*Moschus berezovskii*) in spring [Chinese]. Acta Theriol Sin 2008; 28: 194-200.
19. Bowyer RT. Activity, movement, and distribution of Roosevelt elk during rut. J Mammal 1981; 62(3): 574-582.
20. Vieira EM, Baumgarten LC, Paise G, et al. Seasonal patterns and influence of temperature on the daily activity of the diurnal neotropical rodent *Necromys lasiurus*. Can J Zool 2010; 88(3): 259-265.
21. Kronfeld-Schor N, Dayan T. Activity patterns of rodents: the physiological ecology of biological rhythms. Biol Rhythm Res 2008; 39(3): 193-211.
22. Higdon SD, Diggins CA, Cherry MJ, et al. Activity patterns and temporal predator avoidance of white-tailed deer (*Odocoileus virginianus*) during the fawning season. J Ethol 2019; 37: 283-290.
23. Ikeda T, Takahashi H, Igota H, et al. Effects of culling intensity on diel and seasonal activity patterns of sika deer (*Cervus nippon*). Sci Rep 2019; 9(1): 17205. doi: 10.1038/s41598-019-53727-9.
24. Ensing EP, Ciuti S, de Wijs FA, et al. GPS based daily activity patterns in European red deer and North American elk (*Cervus elaphus*): indication for a weak circadian clock in ungulates. PLoS One 2014; 9(9): e106997. doi: 10.1371/journal.pone.0106997.
25. Granados JE, Ros-Candeira A, Pérez-Luque AJ, et al. Long-term monitoring of the Iberian ibex population in the Sierra Nevada of the southeast Iberian Peninsula. Sci Data 2020; 7(1): 203. doi: 10.1038/s41597-020-0544-1.
26. Bao H, Zhai P, Wen D, et al. Effects of inter-and intra-specific interactions on moose habitat selection limited by temperature. Remote Sens 2022; 14(24): 6401. doi: 10.3390/rs14246401.
27. Cederlund G, Bergström R, Sandegren F. Winter activity patterns of females in two moose populations. Can J Zool 1989; 67(6): 1516-1522.
28. Roberts CP, Cain III JW, Cox RD. Identifying ecologically relevant scales of habitat selection: diel habitat selection in elk. Ecosphere 2017; 8(11): e02013. doi:10.1002/ecs2.2013.
29. Vazquez C, Rowcliffe JM, Spoelstra K, et al. Comparing diel activity patterns of wildlife across latitudes and seasons: Time transformations using day length. Methods Ecol Evol 2019; 00: 1-10. doi:10.1111/2041-210X.13290.
30. Beever EA, Hall LE, Varner J, et al. Behavioral flexibility as a mechanism for coping with climate change. Front Ecol Environ 2017; doi:10.1002/fee.1502.
31. Huey RB, Tewksbury JJ. Can behavior douse the fire of climate warming? Proc Natl Acad Sci USA 2009; 106(10): 3647-3648.
32. Huey RB, Kearney MR, Krockenberger A, et al. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. Philos Trans R Soc Lond B Biol Sci 2012; 367(1596): 1665-1679.
33. Sassi PL, Taraborelli P, Albanese S, et al. Effect of temperature on activity patterns in a small Andean rodent: behavioral plasticity and intraspecific variation. Ethology 2015; 121(9): 840-849.
34. Van Ballenberghe V, Miquelle DG. Activity of moose during spring and summer in interior Alaska. J Wildl Manage 1990; 54(3): 391-396.
35. Demarchi MW, Bunnell FL. Forest cover selection and activity of cow moose in summer. Acta Theriol (Warsz) 1995; 40(1): 23-36.

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 595 - 599
doi: 10.30466/vrf.2023.1998950.3826
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Prevalence and genotypes of *Giardia duodenalis* in shelter dogs of southeastern Türkiye

Burçak Aslan Çelik[1]*, Özgür Yaşar Çelik[2], Akın Koçhan[3], Adnan Ayan[4], Özlem Orunç Kılınç[5], Gürkan Akyıldız[6], Kıvanç İrak[7], Özge Oktay Ayan[8], Kerem Ercan[2]

[1] Department of Parasitology, Faculty of Veterinary Medicine, Siirt University, Siirt, Türkiye; [2] Department of Internal Medicine, Faculty of Veterinary Medicine, Siirt University, Siirt, Türkiye; [3] Department of Internal Medicine, Faculty of Veterinary Medicine, Dicle University, Diyarbakır, Türkiye; [4] Department of Genetics, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, Van, Türkiye; [5] Özalp Vocational School, Van Yüzüncü Yıl University, Van, Türkiye; [6] Department of Basic Health Sciences, Faculty of Health Sciences, Marmara University, İstanbul, Türkiye; [7] Department of Biochemistry, Faculty of Veterinary Medicine, Siirt University, Siirt, Türkiye; [8] Department of Parasitology, Van Yüzüncü Yıl University, Faculty of Medicine, Van, Türkiye.

Article Info

Article history:

Received: 25 March 2023
Accepted: 17 June 2023
Available online: 15 November 2023

Keywords:

Giardia duodenalis
Nested polymerase chain reaction
Shelter dog
Türkiye

Abstract

Giardia duodenalis is a protozoan parasite found in humans and several mammals. This parasite spreads worldwide and is generally recognized as a zoonotic agent being reported to be one of the most common causes of diarrhea in humans and animals. In this study, it was aimed to determine the prevalence and genotypes of *G. duodenalis* in shelter dogs in Diyarbakır province being located in the southeastern Anatolia region of Türkiye. Native-Lugol method and nested polymerase chain reaction analyses of 100 fecal samples showed a prevalence of 3.00 and 4.00%, respectively. The prevalence was higher in females and in those younger than 1 year. Sequence analysis revealed the presence of zoonotic assemblage B, assemblage D and assemblage E. The detection of zoonotic assemblage B in this study suggests that dogs may be a reservoir for human giardiasis. Further molecular research is needed to determine the genotype diversity of *Giardia* as well as its possible role in the transmission of this parasite to humans.

Introduction

Giardia spp. include six species characterized by diverse host ranges. Of these, *Giardia duodenalis* (synonyms: *Giardia lamblia, Giardia intestinalis*) is the only human-infective *Giardia* species, widespread worldwide, associated with diarrhea in humans and domestic and wild mammals.[1-6]

Giardia duodenalis is reported to have at least eight different genotypes (A - H) according to genetic characteristics and host range.[1,7-10] Of these, assemblages A and B; although seen in many mammals, are mainly associated with human infections.[6,8-10] The remaining assemblages (C - H) have a limited host spectrum and are considered host specific.[6,7,9] However, assemblages C, D, E and F have been reported to be isolated with low prevalence in humans.[6] Assemblages C and D occur in dogs,[2,7-10] assemblage E in ruminants,[1,8,9] assemblage F in cats,[7,9,10] assemblage G in mice and rats[1,9] and assemblage H in marine mammals.[1,6,7]

Microscopic study,[7,11,12] indirect fluorescence antibody test (IFAT),[13] enzyme-linked immunosorbent assay (ELISA)[3,9,11,13] and polymerase chain reaction (PCR)[1,2,5,7,10,12-14] methods are used in the diagnosis of the disease. *Giardia* has two morphological forms including trophozoites and cysts being responsible for transmission.[15] Transmission of *G. duodenalis* occurs by fecal-oral ingestion of the contaminated food or water.[3,4,9,15] Reportedly, this parasite can cause growth and developmental retardation in children even in asymptomatic cases.[6] It is estimated that approximately 200 million people in Asia, Africa and Latin America have *Giardia* infection.[9]

In studies conducted around the world, the prevalence of *Giardia* was reported to be 31.33% in Brazil,[16] 11.20 - 15.50% in Korea,[1,15] 25.20 - 56.80% in Thailand,[10,13] 20.50% in Italy,[12] 1.90% in Poland,[4] 13.00 - 39.00% in Canada,[3,17] 4.50 - 11.00% in China,[2,14] 16.40 - 36.50% in Spain,[7,8] 75.55% in Iraq,[11] and 11.90 - 24.50% in Israel.[9,18] In Türkiye, the infection was first reported by Burgu[19] and the prevalence was recorded as 2.48 - 18.80%.[20-23]

***Correspondence:**

Burçak Aslan Çelik. PhD
Department of Parasitology, Faculty of Veterinary Medicine, Siirt University, Siirt, Türkiye
E-mail: burcakaslan@siirt.edu.tr

This study aimed to determine the prevalence and genotypes of *G. duodenalis* in shelter dogs in Diyarbakır province, Türkiye.

Materials and Methods

Study area and sample collection. Ethical clearance for the present study was obtained from the Dicle University Health Sciences Application and Research Centre, Diyarbakır, Türkiye (Approval Number: E-35582840-020). This study was carried out in Diyarbakır province (the main characteristics of its climate are high temperature and dryness) located in the southeastern Anatolia region of Türkiye (38° 02′ 33″ N, 40° 04′ 43″). The animals of the study consisted of 100 dogs of different breeds and sexes in Diyarbakır Municipality Animal Care and Rehabilitation Centre, Türkiye. The feces (non-diarrheic) were directly collected from the rectum of the dogs with disposable latex gloves and placed in individual sample containers. The sex and age (taken from the centre records) of the dogs were recorded and brought to the laboratory for examination.

Microscopic examination. The Nativ-Lugol technique was used to check all samples for the presence of *Giardia* cysts. A drop of saline solution was placed on one side of the clean slide and a drop of Lugol solution was placed on the other side. With the help of a plastic stick, rice grain sized pieces of faeces were taken from different parts of the faeces and homogenised on the slide. The coverslipped preparations were examined with the 40× objective of the microscope (Leica, Hamburg, Germany).[24]

DNA extraction. The DNA extraction was performed using GeneMATRIX Stool DNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer's protocol. The obtained DNAs were stored at – 20.00 ˚C until further analysis.

Nested PCR. In the nested PCR analysis, the β-giardin gene region of 753 bp was amplified using the primers described by Cacciò *et al*.,[25] (G7 F 5'- AAGCCCGACGACGA CCTCACCCGCAGTGC-3' forward and G759R 5'- GAGG CCGCCCTGGATCTTCGAGACGAC-3' reverse). Nested PCR was then performed using the primers described by Lalle *et al.*,[26] (BG1F 5'- GAACGAGATCGAGGTCCG-3' forward and BG2R 5'-CTCGACGAGTTCGTGTGTT-3' reverse). In this study, the PCR product obtained in our previous study, being confirmed by sequence analysis as *G. duodenalis* assemblage B, was used as a positive control.[24] The PCR products obtained were stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Seoul, South Korea) and images were obtained on 1.50% agarose gel.

DNA sequence analysis and phylogeny. Positive PCR samples were sequenced forward and reverse. The DNA sequences were individually checked, aligned and analyzed in BioEdit Sequence Alignment Editor (version 7.2.5; Tom Hall, Carlsbad, USA).[27] The edited formats of the DNA sequences were compared with the databases using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool to determine the assemblages.[28] In addition, data sets were created using the *β-giardin* gene sequences obtained from the NCBI GenBank database and the DNA sequences were obtained as a result of the study. The data sets were aligned in the BioEdit program and the model test was performed using the maximum likelihood statistical method in the IQ-TREE program (version 1.6.12; http://www.iqtree.org). The phylogenetic tree was created with 1,000 bootstraps according to the Bayesian information criterion optimal model and it was shown which assemblages the study samples were related to.[29,30]

Statistical analysis. The data obtained in the study were analyzed using the SPSS Software (version 16.0; SPSS, Inc., Chicago, USA) program. The relationship between grouped variables was calculated using the Chi-square test. The difference was considered statistically significant when $p < 0.05$.

Results

Microscopic examination of all samples revealed 3.00% (3/100) *Giardia* spp. cysts (Fig. 1). Nested PCR analysis revealed specific bands of 511 bp in 4 (4.00%) of the samples (Fig. 2). The highest prevalence was found in females (4.55%) and in those younger than one year (5.88%, $p > 0.05$; Table 1). Sequence analysis revealed that two samples overlapped with assemblage E (99.78% and 100%), one sample overlapped 99.78% with assemblage D and one sample overlapped 100% with assemblage B. The phylogenetic tree shows the placement of the specimens (Fig. 3).

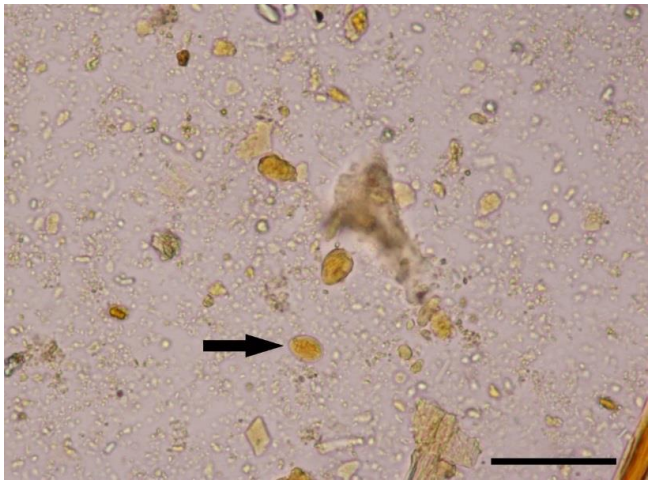

Fig. 1. Photomicrograph of the *Giardia duodenalis* cyst (arrow), (bar = 50 µm).

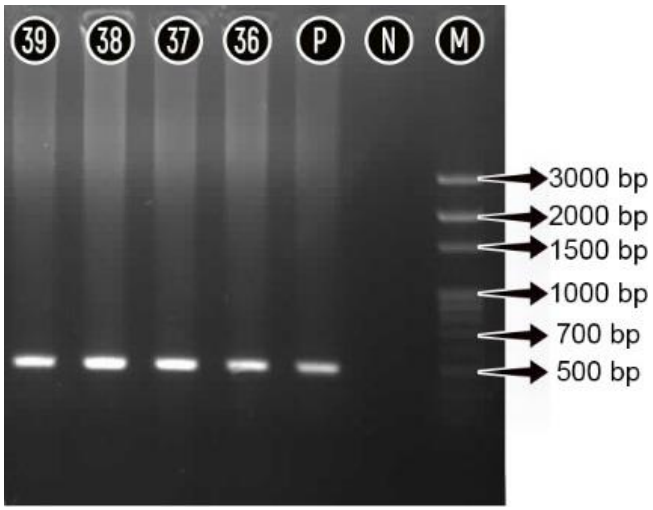

Fig. 2. Polymerase chain reaction products of *Giardia duodenalis*. Lane M: DNA marker (511 bp); Lane N: Negative control; Lane P: Positive control; Lanes 36 - 39: *G. duodenalis*.

Table 1. Prevalence of *Giardia duodenalis* infection in examined dogs (n) according to the sex and age.

Variables	Examined dogs	Infected dogs (%)	*p*-value
Sex			
Female	44	2 (4.55)	0.805
Male	56	2 (3.57)	
Age (year)			
≤ 1	34	2 (5.88)	0.491
> 1	66	2 (3.03)	
Total	100	4 (4.00)	

Discussion

Dogs are important companions in many homes around the world, contributing to the physical, social and emotional development of children and the well-being of their owners.[31] However, dogs are recognized as natural reservoirs of several zoonotic parasitic infections.[7] This increases the risk of human exposure to zoonotic parasites.[31] Therefore, it is important to determine the prevalence of infectious agents with zoonotic potential having the risk of transmission to humans and other animals.

It has been reported that IFAT has the highest sensitivity and specificity for *Giardia* detection and is considered as a gold standard test.[32] However, in the study conducted by Traub *et al*.,[13] it was reported that more prevalence was detected by PCR method, being similar to this study.

The prevalence determined in this study was higher than some previous reports,[4,23] similar to some of them[2,19] and lower than others.[1,3,9,10,12,16,17] The reasons for the difference between the studies may be due to the factors affecting the prevalence of the parasite, such as the age of the dogs, living conditions, animal density, nutritional and immune status and diagnostic methods.[12]

Giardia cysts may endure conditions with high humidity, low temperature, little sunshine exposure and low salinity for months.[10] The location where this study was carried out is located in the hot and dry region of Türkiye. The main characteristics of its climate are high temperature and dryness.[33] This explains the low prevalence rate obtained in this study.

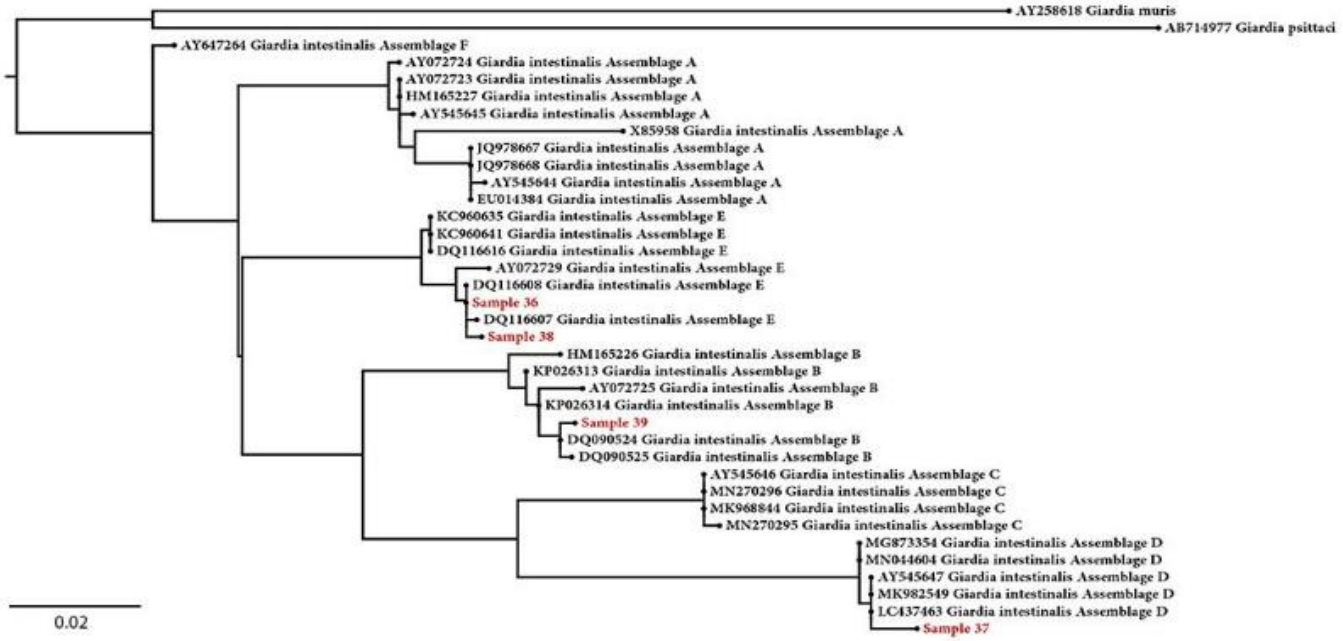

Fig. 3. Phylogenetic relationships of *Giardia duodenalis* isolates using maximum likelihood method analysis based on *β-giardin* gene region. Numbers at the nodes represent the bootstrap values (1,000 bootstrap). *Giardia psitacci* and *Giardia muris* were used as outgroup.

Assemblages C and D have been reported as host-specific genotypes in dogs.[4,5,22] Also, assemblage A,[2,7,17,34] assemblage B,[7,17] assemblage C,[1,4,9,10,14,17,34] assemblage D[1,4,9,10,17,34] and assemblage E[8,14] were reported in dogs. In this study, one of the 4 positive samples was zoonotic assemblage B, one was dog-specific assemblage D and two were assemblage E, being interestingly seen especially in ruminants. The reason for the occurrence of assemblage E may be due to the fact that these dogs lived in rural areas with dense farm animal populations.[35]

While some studies have reported higher prevalence in female dogs,[7,10,11,14,16] others reported in males.[1,2,15,18,22] In this study, a higher prevalence was found in female dogs. This result is similar to the former findings of the researchers.[7,10,11,14,16] The reason for the higher prevalence in females may be due to the decreased immunity of these animals during certain periods of their physiological cycle.[36]

In several studies, it was reported that a higher prevalence was detected in young animals compared to the adult ones.[10,11,14,16,18] In this study, similar to the previous findings, a higher prevalence was found in animals younger than one year (5.88%) in comparison with animals older than one year(3.03%). This may be due to the fact that the immune system is not developed in young animals and they cannot form an effective immune response to eliminate the infection.[11,37]

The detection of assemblages D and E as well as zoonotic assemblage B in this study suggests that dogs may be a source of giardiasis in humans. Therefore, it is important that those working with dogs, including veterinarians and shelter workers, be aware of this potential risk and take appropriate precautions to prevent infection. Further molecular epidemiological research is also needed to determine the genotype diversity of *Giardia* in dogs as well as its possible role in the transmission of this parasite to humans.

Acknowledgments

The authors are grateful for support of the Preclinical Science Department of Faculty of Veterinary Medicine, Siirt, Türkiye.

Conflicts of interest

The authors state no conflict of interest.

References

1. Kim HY, Lee H, Lee SH, et al. Multilocus genotyping and risk factor analysis of *Giardia duodenalis* in dogs in Korea. Acta tropica 2019; 199: 105113. doi: 10.1016/j.actatropica.2019.105113.
2. Li J, Zhang P, Wang P, et al. Genotype identification and prevalence of *Giardia duodenalis* in pet dogs of Guangzhou, Southern China. Vet Parasitol 2012; 188(3-4): 368-371.
3. Olson ME, Leonard NJ, Strout J. Prevalence and diagnosis of Giardia infection in dogs and cats using a fecal antigen test and fecal smear. Can Vet J 2010; 51(6): 640-642.
4. Solarczyk P, Majewska AC. A survey of the prevalence and genotypes of *Giardia duodenalis* infecting household and sheltered dogs. Parasitol Res 2010; 106(5): 1015-1019.
5. Uiterwijk M, Nijsse R, Kooyman FNJ, et al. Comparing four diagnostic tests for *Giardia duodenalis* in dogs using latent class analysis. Parasit Vectors 2018; 11(1): 439. doi: 10.1186/s13071-018-3014-2.
6. Zhang W, Zhang X, Wang R, et al. Genetic characterizations of *Giardia duodenalis* in sheep and goats in Heilongjiang Province, China and possibility of zoonotic transmission. PLoS Negl Trop Dis 2012; 6(9): e1826. doi: 10.1371/journal.pntd.0001826.
7. Adell-Aledón M, Köster PC, de Lucio A, et al. Occurrence and molecular epidemiology of *Giardia duodenalis* infection in dog populations in eastern Spain. BMC Vet Res 2018; 14(1): 26. doi: 10.1186/s12917-018-1353-z.
8. Dado D, Montoya A, Blanco MA, et al. Prevalence and genotypes of *Giardia duodenalis* from dogs in Spain: possible zoonotic transmission and public health importance. Parasitol Res 2012; 111(6): 2419-2422.
9. Salant H, Kuzi S, Navarro D, et al. Prevalence and molecular characterization of *Giardia duodenalis* in dogs in Israel. Comp Immunol Microbiol Infect Dis 2020; 73: 101548. doi: 10.1016/j.cimid.2020.101548.
10. Tangtrongsup S, Scorza AV, Reif JS, et al. Seasonal distributions and other risk factors for *Giardia duodenalis* and *Cryptosporidium* spp. infections in dogs and cats in Chiang Mai, Thailand. Prev Vet Med 2020; 174: 104820. doi: 10.1016/j.prevetmed.2019.104820.
11. Naser A, Wadood IA. Detection of Giardia infection in dogs of Basrah City. Bas Vet Res 2017; 16(2): 159-171.
12. Scaramozzino P, Di Cave D, Berrilli F, et al. A study of the prevalence and genotypes of *Giardia duodenalis* infecting kennelled dogs. Vet J 2009; 182(2): 231-234.
13. Traub RJ, Inpankaew T, Reid SA, et al. Transmission cycles of *Giardia duodenalis* in dogs and humans in Temple communities in Bangkok - - a critical evaluation of its prevalence using three diagnostic tests in the field in the absence of a gold standard. Acta Trop 2009; 111(2): 125-132.
14. Li W, Li Y, Song M, et al. Prevalence and genetic characteristics of *Cryptosporidium*, *Enterocytozoon bieneusi* and *Giardia duodenalis* in cats and dogs in Heilongjiang province, China. Vet Parasitol 2015; 208(3-4): 125-134.
15. Liu J, Lee SE, Song KH. Prevalence of canine giardiosis in South Korea. Res Vet Sci 2008; 84(3): 416-418.

16. Huber F, Bomfim TC, Gomes RS. Comparison between natural infection by *Cryptosporidium* sp., *Giardia* sp. in dogs in two living situations in the West Zone of the municipality of Rio de Janeiro. Vet Parasitol 2005; 130(1-2): 69-72.
17. Uehlinger FD, Greenwood SJ, McClure JT, et al. Zoonotic potential of *Giardia duodenalis* and *Cryptosporidium* spp. and prevalence of intestinal parasites in young dogs from different populations on Prince Edward Island, Canada. Vet Parasitol 2013; 196(3-4): 509-514.
18. Kuzi S, Argentaro SE, Baneth G. Prevalence of *Giardia duodenalis* infection, co-morbidities and associated risk factors in dogs admitted to a veterinary teaching hospital in Israel. Comp Immunol Microbiol Infect Dis 2020; 68: 101401.doi: 10.1016/j.cimid.2019.101401.
19. Burgu A. Distribution of *Giardia canis* in dogs in Ankara and its importance for public health [Turkish].Ankara Üniv Vet Fak Derg 1979; 26(03-04): 184-194.
20. Denizhan V, Karakuş A. Prevalence of gastrointestinal protozoon in stray dogs in the Van Province [Turkish]. Dicle Üniv Vet Fak Derg 2019; 12(1): 25-29.
21. Dumanlı N. A survey of the incidence of protozoer agents in dogs in Elazığ vicinity [Turkish]. Ankara Üniv Vet Fak Derg 1984; 31(3): 383-387.
22. Gultekin M, Ural K, Aysul N, et al. Prevalence and molecular characterization of *Giardia duodenalis* in dogs in Aydin, Turkey. Int J Environ Health Res 2017; 27(3): 161-168.
23. Uslu U, Ceylan C, Ceylan O, et al. Gastrointestinal protozoa and helminths detected in 2-6 months old puppies in Konya [Turkish]. Dicle Üniv Vet Fak Derg 2022; 15(2): 74-78.
24. Çelik BA, Çelik ÖY, Ayan A, et al. Molecular prevalence of *Giardia duodenalis* and subtype distribution (assemblage E and B) in calves in Siirt, Turkey. Egypt J Vet Sci 2023; 54(3): 457-463.
25. Cacciò SM, De Giacomo M, Pozio E. Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction–restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int J Parasitol 2002; 32(8): 1023-1030.
26. Lalle M, Pozio E, Capelli G, et al. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int J Parasitol 2005; 35(2): 207-213.
27. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. Nucleic Acids Symp Ser 1999; 41: 95-98.
28. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. J Mol Biol 1990; 215(3): 403-410.
29. Minh BQ, Nguyen MA, von Haeseler A. Ultrafast approximation for phylogenetic bootstrap. Mol Biol Evol 2013; 30(5): 1188-1195.
30. Trifinopoulos J, Nguyen LT, von Haeseler A, et al. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res 2016; 44(W1): W232-W235. doi: 10.1093/nar/gkw256.
31. Robertson ID, Irwin PJ, Lymbery AJ, et al. The role of companion animals in the emergence of parasitic zoonoses. Int J Parasitol 2000; 30(12-13): 1369-1377.
32. Tangtrongsup S, Scorza V. Update on the diagnosis and management of Giardia spp infections in dogs and cats. Top Companion Anim Med 2010; 25(3): 155-162.
33. Gedik GZ. Climatic design: an analysis of the old houses of Diyarbakir in the southeast region of Turkey. Archit Sci Rev 2004; 47(2): 145-154.
34. Leonhard S, Pfister K, Beelitz P, et al. The molecular characterisation of Giardia from dogs in southern Germany. Vet Parasitol 2007; 150(1-2): 33-38.
35. Harvey TV, Carvalho JPDS, Aquino MCC, et al. Giardiasis in children and dogs, and the first report of assemblage E in dogs from northeastern Brazil. Rev Bras Parasitol Vet 2023; 32(1): e012222. doi: 10.1590/S1984-29612023010.
36. Öner G, Ulutaş B. Prevalence of Cryptosporidium spp. in dogs in the Aegean Region. Anim Health Prod Hyg 2022; 11(1): 26-31.
37. Mundim MJ, Rosa LA, Hortêncio SM, et al. Prevalence of *Giardia duodenalis* and *Cryptosporidium* spp. in dogs from different living conditions in Uberlândia, Brazil. Vet Parasitol 2007; 144(3-4): 356-359.

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 601 - 606
doi: 10.30466/vrf.2023.1986019.3727
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Effect of saponin on spermatogenesis and testicular structure in streptozotocin-induced diabetic mice

Sadigheh Pashapour[1], Adel Saberivand[1]*, Amir Afshin Khaki[2], Maryam Saberivand[3]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran; [2] Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran; [3] Connective Tissue Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Article Info

Article history:

Received: 27 December 2022
Accepted: 06 February 2023
Available online: 15 November 2023

Keywords:

Diabetes
Oxidative stress
Saponin
Testis

Abstract

About a third of human infertility is related to male factors. Of these, idiopathic-related infertility is not curable. Diabetes mellitus is a metabolic disorder affecting male impotence and fertility by increased production of free radicals and oxidative stress. Saponin, a glycosidic compound found in many plants, improves sperm parameters. The present study investigated the effect of saponin on sperm oxidative stress and testicular structure in streptozotocin (STZ)-induced diabetic mice. The diabetes was induced by the administration of 150 mg kg^{-1} STZ via a single intra-peritoneal injection. All experimental mice were allocated to the following groups: Control group, diabetic control group, diabetic group administrated 100 mg kg^{-1} saponin daily and one healthy group administrated saponin daily for 56 days. At the end of the treatment period, serum levels of insulin, glucose and oxidative stress markers were measured. A histological evaluation of testicles was performed. Treatment of diabetic mice with saponin ameliorated testicular tissue damage as well as serum glucose and insulin concentrations. Furthermore, in the diabetic group, the serum concentration of malondialdehyde was increased; while, the activity of superoxide dismutase and glutathione peroxidase enzymes was reduced. The mean Johnsen's score and the diameter and thickness of seminiferous tubules were lower in the diabetic mice than control ones. However, these parameters were higher in the saponin-treated mice than controls. Overall, saponin administration rectified all examined parameters. The anti-oxidant role of saponin improves sperm parameters and diabetes-induced testicular oxidative damage.

Introduction

According to the report of the World Health Organization, infertility is a disorder occurring in 10.00 - 15.00% of couples, of which 30.00 - 40.00% are associated with the male factor.[1,2] Abnormal semen parameters due to the factors other than idiopathic reasons can be improved; while, treatment for poor idiopathic semen quality is not promising. [3]

Diabetes or diabetes mellitus is a chronic and endocrine disease, causing numerous concerns worldwide. Diabetes mellitus is a heterogeneous metabolic disorder caused by the lack of insulin production in the body or insulin resistance impairing male sexual ability and fertility.[4,5] Testicular dysfunction decreases the testicular weight along with sperm count and motility and changes the morphology of the seminiferous tubules. Testosterone levels are also reduced.[6] Diabetes increases the apoptosis rate (pro-apoptotic genes such as *Bax* up-regulation) in germ cells and also interrupts the spermatogenesis process.[5] In about 90.00% of diabetic patients, defects in sexual activity are seen as decreased libido and reduced fertility.[7]

Although the exact mechanism of diabetes mellitus is not well understood, the increase in the production of free radicals and increased oxidative stress are its major proposed damaging mechanisms.[7,5]

The presence of anti-oxidants such as vitamins or flavonoids in the diet can exert protective effects in diabetic patients.[8] Reactive oxygen species (ROS) over-production damages the mitochondrial membrane causing cytochrome C release, resulting in the apoptosis induction in testicular tissue cells.[7]

Saponins are glycosidic chemical compounds being abundant in many plants. Saponin is involved in protecting

***Correspondence:**

Adel Saberivand. DVM, PG Diploma, PhD
Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
E-mail: a.saberivand@tabrizu.ac.ir

the plant against germs and fungi. Although high doses of this substance are very toxic, several reports have indicated that saponin increases sperm motility and viability and hormone levels.[9,10]

This study aimed to investigate the effects of saponin on spermatogenesis, testicular tissue damage and blood biochemical and hormonal parameters in diabetic mice.

Materials and Methods

Sixty-four male mice weighing 25.00 to 30.00 g were obtained from the Animal House of Tabriz University of Medical Sciences, Tabriz, Iran, and kept for 2 weeks in standard conditions with 12 hr of light and adequate humidity. All procedures performed in studies involving animals were in accordance with the ethical standards of Tabriz University, Tabriz, Iran (Ethical code: 1398.027).

The animals were randomly divided into 4 groups of 16 and treated as follows:

Group 1: The control group (no injections); Group 2: The diabetic control group received a single intra-peritoneal injection of 150 mg kg^{-1} streptozotocin (STZ);[11] Group 3: The healthy control group received 100 mg kg^{-1} per day saponin via intra-peritoneal injection for 8 weeks;[2] Group 4: Treatment group receiving 150 mg kg^{-1} STZ (one injection) and 100 mg kg^{-1} per day saponin intra-peritoneally for 8 weeks.

At first, the glucose levels of all mice in both experimental and control groups were determined by a glucometer (Easy-Gluco 2657A; Complete Medical Supplies Inc., New York, USA). Then, to induce diabetes, 150 mg kg^{-1} per day of STZ was administered intra-peritoneally to groups 2 and 4. After 72 hr, blood glucose levels were measured again. After confirming that the mice were diabetic (blood glucose levels above 250 mg dL^{-1}), they received 100 mg kg^{-1} of saponin via intra-peritoneal injection once a day for 56 days.[12] At the end of the treatment period, all mice were anesthetized with a combination of 50.00 mg kg^{-1} ketamine (Panpharma, Luitré-Dompierre, France) and 10.00 mg kg^{-1} xylazine (Alfasan, Woerden, The Netherlands). Then, 2.00 to 3.00 mL of the blood samples were taken from the hearts of animals for biochemical assays.

In order to isolate sera, immediately after sampling, blood samples were centrifuged at 3,000 rpm for 10 min, and the harvested sera were stored at – 80.00 ˚C until used. The glucose concentration was measured by a commercial kit (Iran Pars Azmoon, Tehran, Iran). Serum concentrations of insulin were measured by the enzyme-linked immunosorbent assay (ELISA) using a standard commercial kit for mice (Mercodia Inc, Uppsala, Sweden) and reported as μg L^{-1}.

The lower abdominal area was incised under sterile conditions, and both testicles and epididymides were bilaterally removed and weighed. For histological examination, the right testicle was fixed in Bouin's fixative for 72 hr. Then, 5.00 μm sections were prepared,[13,5] and stained with the Hematoxylin and Eosin staining method. About 50 round seminiferous tubules were randomly examined by a light microscope (CX22; Olympus, Tokyo, Japan) with 400× magnification to determine the seminiferous tubule diameter, germinal epithelium height and spermatogenesis alterations.

Serum testosterone concentration was measured using a commercial ELISA kit (Demeditec Diagnostics, Kiel, Germany). Briefly, serum samples (25.00 μL) were incubated with 200 μL enzyme conjugate in pre-coated wells for 60 min at room temperature. Then, the wells were washed three times with 300 μL diluted irrigation solution and incubated with 200 μL substrate solution for 15 min at room temperature. The enzymatic reaction was ended by adding 100 μL stop solution, and the optical density of the solution in each well was recorded at 450 nm. The testosterone concentration was calculated using six standard concentrations and a four-parameter logistic curve fitting. The final testosterone concentration was obtained from each set of duplicates and expressed as ng mL^{-1}.

The superoxide dismutase (SOD) activity of serum samples was measured using a commercial kit (Ransod, Randox Laboratories Ltd., Crumlin, UK) according to the Arthur and Boyne.[14] In summary, this method is based on the generation of superoxide radicals by adding xanthine and xanthine oxidase to the sample and its reaction with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by the inhibition power of this reaction and expressed as U of SOD per 10.00 mg of protein. Protein was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, USA) according to the method described by Bradford.[15]

Glutathione peroxidase (GPx) activity was measured by a diagnostic kit (Randox) according to the Paglia and Valentine.[16] The oxidation of glutathione (GSH) is catalyzed by cumene hydroperoxide in this method. The oxidized GSH is immediately converted into the reduced form with concomitant oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP+ (oxidized form of NADPH) in the presence of glutathione reductase. Then, the decline in absorbance at 340 nm is calculated in a spectrophotometer (Thermo Fisher Scientific) and expressed as U L^{-1}.

To measure serum malondialdehyde (MDA) levels, first, 0.20 mL of serum was added to a microtube containing 3.00 mL of glacial acetic acid, following which 1.00% thiobarbituric acid (in 2.00% NaOH) was added to the microtube. The tube was then placed in the boiling water for 15 min. After cooling, the adsorption of the resulting solution was read in a spectrophotometer (Thermo Fisher Scientific) as pink at 532 nm.[17]

Statistical analysis. All statistical analyses were carried out using the SPSS software (version 19.0; IBM Corp., Armonk, USA). After ensuring the normal distribution of the variables, they were compared using a one-way analysis of variance. Tukey's *post hoc* test was applied to determine the differences between groups. The results were expressed as mean ± standard deviation. For all data, $p < 0.05$ was considered statistically significant.

Results

A significant increase was found in serum glucose levels in group 2 compared to the group 1 at the end of the study ($p < 0.05$). Additionally, a significant decrease was observed in serum glucose levels in group 3 in contrast to group 2 at the same time ($p < 0.05$; Table 1). This was true for one week before and one week after diabetes induction. The administration of the saponin to healthy mice (group 4) did not significantly alter glucose concentrations at any time of sampling.

Serum insulin evaluation indicated that group 2 (0.29 ± 0.02) had lower ($p < 0.05$) concentrations than group 1 (0.54 ± 0.02). Treatment of the diabetic mice with saponin (group 3) (0.37 ± 0.01) and healthy saponin-treated (group 4) (0.52 ± 0.02) improved ($p < 0.05$) the serum insulin level compared to the group 2 (0.52 ± 0.02 *versus* 0.37 ± 0.01; Fig. 1A).

The results of the histological evaluation showed that the mean Johnsen's score (MJS) was decreased ($p < 0.05$) in group 2 compared to the group 1 Table 1 and Fig. 2).

On the other hand, the MJS was higher ($p < 0.05$) in group 3 and group 4 than group 2. Histopathological examination showed that the diameter of seminiferous tubules was decreased ($p < 0.05$) in the group 2 compared to the group 1. Similarly, the thickness of seminiferous tubules was decreased ($p < 0.05$) in the group 2 compared to the group 1.

In addition, the diameter of seminiferous tubules was increased ($p < 0.05$) in the group 3 and group 4 compared to the group 2. In the same manner, the thickness of the seminiferous tubules was higher ($p < 0.05$) in the group 3 and group 4 than group 2 (Table 1).

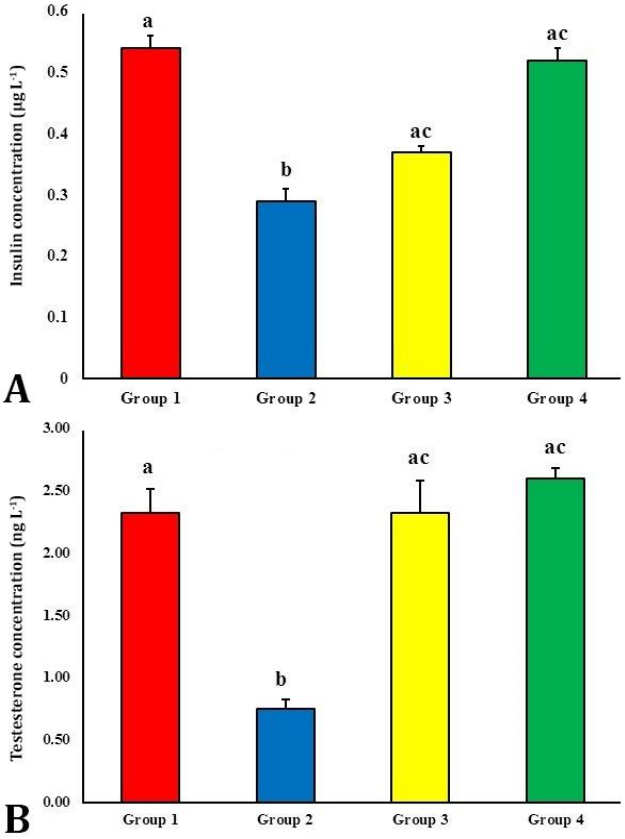

Fig. 1. The serum concentrations of **A)** insulin and **B)** testosterone in the group 1 (control), group 2 (diabetic control), group 3 (diabetic treated with 100 mg kg^{-1} saponin) and group 4 (healthy saponin treated mice). [abc] Indicate significant difference between control (Group 1) and other groups ($p < 0.05$).

Table 1. Concentrations of malondialdehyde, glutathione peroxidase, superoxide dismutase, and histological parameters of the testicles and glucose concentrations in week -1, week +1 and week +8 in testicular tissue, in the group 1 (control), group 2 (diabetic control), group 3 (diabetic treated with 100 mg kg^{-1} saponin) and group 4 (healthy saponin-treated mice).

Parameters	Group 1	Group 2	Group 3	Group 4
Malondialdehyde (U per 10 mg protein)	0.63 ± 0.06	2.30 ± 0.05*	1.54 ± 0.17*†	0.80 ± 0.05*†
Glutathione peroxidase (mmol L^{-1})	2.15 ± 0.01	0.84 ± 0.02*	1.72 ± 0.03*†	2.43 ± 0.06*†
Superoxide dismutase (U per 10 mg protein)	1.67 ± 0.03	0.79 ± 0.03*	1.38 ± 0.02*†	1.55 ± 0.25*†
Mean Johnsen's score	9.62 ± 0.36	4.35 ± 0.17*	7.35 ±0.54*†	9.55 ± 0.24*†
Seminiferous tubule diameter (µm)	262.42 ± 4.25†	140.11± 2.57*	190.50± 3.23*†	260.22 ± 1.70*†
Height of germianl epithelium (µm)	64.50 ± 1.23†	33.50 ± 2.03*	54.50 ± 2.15†	64.03 ± 1.05*†
Glucose week -1 (mg dL^{-1})	94.50 ± 5.34	100.20 ± 2.60*	95.70 ± 5.89*†	96.25 ±3.40†
Glucose week +1 (mg dL^{-1})	102.42 ± 7.25†	320.25 ± 4.07*	291.50 ± 4.03*†	93.80 ± 4.70†
Glucose week +8 (mg dL^{-1})	92.40 ± 9.34†	367.60 ± 38.73*	179.20 ± 3.20†	92.30 ± 8.05†

*† Indicate significant differences between the treatment versus control and diabetic groups, respectively ($p < 0.05$).

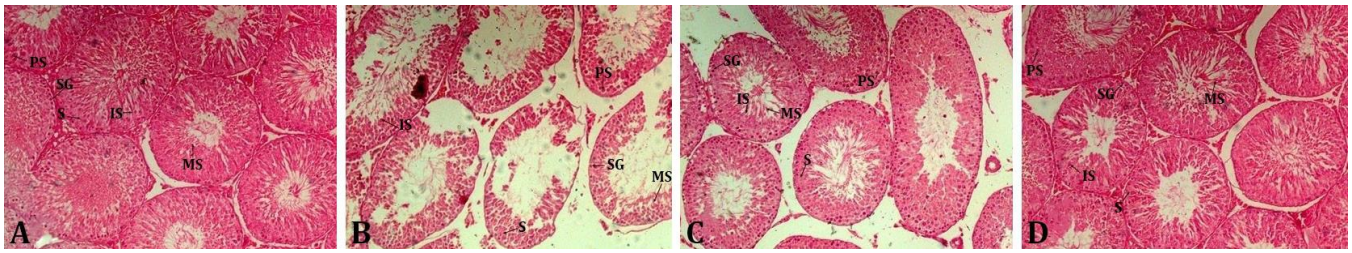

Fig. 2. The diameter and the thickness of the seminiferous tubules shown in the histological sections from **A)** group 1 (control, **B)** group 2 (diabetic control, **C)** group 3 (diabetic treated with 100 mg kg^{-1} saponin, and **D)** group 4 (healthy saponin treated mice. (Hematoxylin and Eosin, × 400). IS: Immature spermatid; MS: Mature spermatid; PS: Primary spermatocyte; S: Sertoli cell, and SG: Spermatogonium.

The serum testosterone concentrations were decreased ($p < 0.05$) in group 2 (0.75 ± 0.05) compared to the group 1 (2.31 ± 0.16). Moreover, the group 3 (2.32 ± 0.15) and group 4 (2.60 ± 0.18) showed higher ($p < 0.05$) serum testosterone concentrations compared to the group 2 (Fig. 1B).

As shown in Table 1, a substantial increase in the MDA levels was observed in the testes of group 2 compared to the group 1 ($p < 0.05$). The group 3 (and group 4 showed a dramatic decline in serum MDA levels compared to group 2 ($p < 0.05$). The SOD activity was decreased in group 2 compared to the group 1 ($p < 0.05$). The treatment of the diabetic group with saponin (group 3) elevated the activity of SOD enzyme in comparison with group 2 ($p < 0.05$). The SOD activity was also increased in group 4 compared to the group 2. The activity of the GPx enzyme was also decreased in group 2 compared to the group 1 ($p < 0.05$). Furthermore, group 3 and group 4 indicated higher GPx enzyme activity compared to the group 2 ($p < 0.05$).

Discussion

The present study examined the ameliorative effect of saponin on diabetes-induced injuries in male mice reproductive system. The findings of the present study showed that saponin declined the blood glucose and oxidative stress markers in the testes of diabetic mice. Diabetes produces testicular dysfunctions and reportedly, treatment with saponin improves these functional deficiencies via its anti-oxidant and anti-diabetic properties.[9,10] Accordingly, some studies have reported that treatment of STZ-induced diabetic mice with saponin reduces the blood glucose levels and increases the tissue sensitivity to insulin.[18,19] In another study, the saponin-contained fraction of the *Momordica charantia* plant stimulated insulin secretion in an *in vitro*, static incubation assay.[20] The hypoglycemic effect of saponin is related to its ability to increase the sensitivity of tissues to insulin.[19,21]

In diabetic patients, in addition to an enhanced amount of blood glucose, the balance between the generation and resolution of free radicals is also suspended. As a result, free radical levels increase and cause oxidative stress.[7,12] Oxidative stress results in cell injury via mechanisms such as lipid peroxidation and DNA and protein oxidative damages.[22] The results of the present study showed that diabetes remarkably incremented the MDA (a lipid peroxidation marker) levels in the testicular tissue of diabetic mice, indicating that lipid peroxidation had been elevated. This finding corresponds to the results of previous research on the effects of oxidative stress on the testis of diabetic mice.[5,7] Several studies in this context have reported an increase in lipid peroxidation and MDA level in the diabetic patients.[23] Other studies have reported that saponin scavenges the free radicals generated during lipid peroxidation.[24] Hence, the decline in testis MDA concentrations in the saponin-treated group may be related to the anti-oxidant effects of saponin. Akbarizare *et al*.,[25] have showed that saponin decreases the MDA level probably due to its anti-oxidant properties.

The activity of SOD dramatically declined in the diabetic mice in this study. These results confirm the findings of previous studies. The SOD is known as one of the most important enzymes of the anti-oxidant system. It mainly catalyzes the conversion of superoxide anion radicals to H_2O_2. Through this procedure, the toxicity of superoxide is decreased and no free radicals from super-oxide are produced.[22] The activity of SOD was remarkably enhanced in the serum of diabetic mice being treated with saponin in contrast to the diabetic control group in the present study. This is in line with the related literature. Hu *et al*.,[26] have showed that saponin increases the serum SOD levels and the protection against cisplatin-evoked intestinal injury via multiple ROS-mediated mechanisms.

In the present research, the GPx enzyme activity was intrinsically reduced in the diabetic mice compared to the control group. However, it was notably increased in the saponin-treated group compared to the diabetic control group. The GPx, an anti-oxidant enzyme, is another enzyme with detoxification effects against free radicals.[27] A decline in the activity of GPx in this study can be due to the increment in H_2O_2 generation because of glucose autoxidation and non-enzymatic protein glycation, causing oxygen free radicals production.[28] It is well-known that anti-oxidant therapy increases GPx activity.[29]

In the present study, the STZ-induced diabetes in mice resulted in alterations in the histological indices of testicular tissue. The treatment of the diabetic mice with saponin ameliorated most of the diabetes-induced deficits

as well as spermatogenesis. These alleviating effects in the treated animals were almost similar to those of the healthy control group (group 4).

The reduction or absence of insulin can also decrease testosterone concentrations causing testicular atrophy. Insulin itself, is known as an anti-apoptotic factor that can control testicular apoptosis and reproductive malfunction resulted from diabetes.[30]

In line with the findings of this study, previous studies have indicated that medicinal plants containing flavonoids can improve sperm quality and testosterone levels.[5,7,31] In a similar study, the increased rate of testicular germ cell death through apoptosis in STZ-induced diabetic rats was protected by *Dracaena arborea* aqueous extract containing saponins.[32] Feasible mechanisms complicated in the recuperation of testicular oxidative stress by saponin in diabetic mice can be described by its anti-oxidant property, decreased blood glucose and enhanced insulin secretion.[33]

The treatment of diabetic mice with saponin ameliorated diabetes-induced histological alterations in the seminiferous tubules. In this regard, the MJS and diameter and thickness of seminiferous tubules were decreased in the diabetic mice in the present study. These alterations are often important indicators of spermatogenic dysfunction alongside to the decreases in sperm production.[34] All these alterations could be due to the toxic effect of STZ on male reproductive system via decrease in testosterone concentrations and consequently interrupting testicular function.[35] This late event could resulted in the reduction and death of germ cells.[36] The oxidative-induced free radicals are proposed to explain the etiology and pathophysiology of the biological effects of diabetes mellitus. In this regard, the free radicals generated by STZ metabolism can damage DNA and chromosomes resulting in the cell death via apoptosis or necrosis.[35]

Moreover, the serum testosterone levels were decreased in diabetic mice, which may be related to the testicular tissue damage and Leydig cell injuries. However, treatment with saponin was able to ameliorate these damages. In this regard, Shoorei *et al*.,[7] have reported that diabetes induces testicular tissue damage and decreases testosterone levels in diabetic rats.

In conclusion, diabetes exerts a negative effect on the testis and sperm quality through oxidative stress. Saponin has a potent effect on the anti-oxidant system activation in reducing the oxidative stress induced by diabetes. However, further detailed researches are required to confirm these results.

Acknowledgments

This study was supported by the University of Tabriz, Tabriz, Iran. The authors wish to thank them for their financial support.

Conflict of interest

The authors declare no conflict of interest.

References

1. Modaresi M, Mesripour M, Asadi Morghmaleki M, et al. The effect of saffron extract on testis tissue [Persian]. Iran J Med Aroma Plants 2008; 24(2): 237-243.
2. Ameli M, Moghimian M, Saeb F, et al. The effect of clomiphene citrate and human chorionic gonadotropin on the expression of CatSper1, CatSper2, LHCGR, and SF1 genes, as well as the structural changes in testicular tissue of adult rats. Mol Reprod Dev 2019; 86(6): 738-748.
3. Durairajanayagam D. Lifestyle causes of male infertility. Arab J Urol 2018; 16(1): 10-20.
4. Abtahi-Evari SH, Shokoohi M, Abbasi A, et al. Protective effect of Galega officinalis extract on streptozotocin-induced kidney damage and biochemical factor in diabetic rats. Crescent J Med Biol Sci 2017; 4(3):108-114.
5. Shoorei H, Khaki A, Shokoohi M, et al. Evaluation of carvacrol on pituitary and sexual hormones and their receptors in the testicle of male diabetic rats. Hum Exp Toxicol 2020; 39(8): 1019-1030.
6. Shoorei H, Banimohammad M, Kebria MM, et al. Hesperidin improves the follicular development in 3D culture of isolated preantral ovarian follicles of mice. Exp Biol Med (Maywood) 2019; 244(5): 352-361.
7. Shoorei H, Khaki A, Khaki AA, et al. The ameliorative effect of carvacrol on oxidative stress and germ cell apoptosis in testicular tissue of adult diabetic rats. Biomed Pharmacother 2019; 111: 568-578.
8. Fang JY, Lin CH, Huang TH, et al. In vivo rodent models of type 2 diabetes and their usefulness for evaluating flavonoid bioactivity. Nutrients 2019; 11(3): 530. doi: 10.3390/nu11030530.
9. Gupta RS, Chaudhary R, Yadav RK, et al. Effect of saponins of *Albizia lebbeck* (L.) Benth bark on the reproductive system of male albino rats. J Ethnopharmacol 2005; 96(1-2):31-36.
10. Ji M, Minami N, Yamada M, et al. Effect of proto-panaxatriol saponin on spermatogenic stem cell survival in busulfan-treated male mice. Reprod Med Biol 2007; 6(2): 99-108.
11. Ventura-Sobrevilla J, Boone-Villa VD, Aguilar CN, et al. Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice. Proc West Pharmacol Soc 2011; 54: 5-9.
12. Shokri F, Shokoohi M, Niazkar HR, et al. investigation the spermatogenesis and testis structure in diabetic rats after treatment with Galega officinalis extract. Crescent J Med Biol Sci 2019; 6(1): 31-36.
13. Muratoğlu S, Akarca Dizakar OS, Keskin Aktan A, et al. The protective role of melatonin and curcumin in the

testis of young and aged rats. Andrologia 2019; 51(3): e13203. doi: 10.1111/and.13203.
14. Arthur JR, Boyne R. Superoxide dismutase and glutathione peroxidase activities in neutrophils from selenium deficient and copper deficient cattle. Life Sci 1985; 36(16): 1569-1575.
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
16. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70(1): 158-169.
17. Barghi B, Shokoohi M, Khaki AA, et al. Eugenol improves tissue damage and oxidative stress in adult female rats after ovarian torsion/detorsion. J Obstet Gynaecol 2021; 41(6): 933-938.
18. Pillion DJ, Amsden JA, Kensil CR, et al. Structure-function relationship among Quillaja saponins serving as excipients for nasal and ocular delivery of insulin. J Pharm Sci 1996; 85(5): 518-524.
19. Kwon DY, Kim YS, Ryu SY, et al. Platyconic acid, a saponin from Platycodi radix, improves glucose homeostasis by enhancing insulin sensitivity *in vitro* and *in vivo*. Eur J Nutr 2012; 51(5): 529-540.
20. Keller AC, Ma J, Kavalier A, et al. Saponins from the traditional medicinal plant Momordica charantia stimulate insulin secretion in vitro. Phytomedicine 2011; 19(1): 32-37.
21. Oishi Y, Sakamoto T, Udagawa H, et al. Inhibition of increases in blood glucose and serum neutral fat by Momordica charantia saponin fraction. Biosci Biotechnol Biochem 2007; 71(3): 735-740.
22. Ameli M, Hashemi MS, Moghimian M, et al. Protective effect of tadalafil and verapamil on testicular function and oxidative stress after torsion/detorsion in adult male rat. Andrologia 2018; 50(8): e13068. doi: 10.1111/and.13068.
23. Zhang P, Li T, Wu X, et al. Oxidative stress and diabetes: antioxidative strategies. Front Med 2020; 14(5):583-600.
24. Bian D, Liu M, Li Y, et al. Madecassoside, a triterpenoid saponin isolated from Centella asiatica herbs, protects endothelial cells against oxidative stress. J Biochem Mol Toxicol 2012; 26(10): 399-406.
25. Akbarizare M, Ofoghi H, Hadizadeh M. *In vitro* anticancer evaluation of saponins obtained from Spirulina platensis on MDA, HepG2, and MCF7 cell lines. Multidiscip Cancer Investig 2019; 3(4): 25-32.
26. Hu JN, Yang JY, Jiang S, et al. Panax quinquefolium saponins protect against cisplatin evoked intestinal injury via ROS-mediated multiple mechanisms. Phyto-medicine 2021; 82: 153446. doi: 10.1016/ j.phymed. 2020.153446.
27. Tremellen K. Oxidative stress and male infertility - - a clinical perspective. Hum Reprod Update 2008; 14(3): 243-258.
28. Huang JL, Jing X, Tian X, et al. Neuroprotective properties of Panax notoginseng saponins via preventing oxidative stress injury in SAMP8 mice. Evid Based Complement Alternat Med 2017; 2017: 8713561. doi: 10.1155/2017/8713561.
29. Francisco Javier O, Manuel R, Manuel RR. Regular physical activity increases glutathione peroxidase activity in adolescents with Down syndrome. Clin J Sport Med 2006;16(4): 355-356.
30. Schoeller EL, Schon S, Moley KH. The effects of type 1 diabetes on the hypothalamic, pituitary and testes axis. Cell Tissue Res 2012; 349(3): 839-847.
31. Shokoohi M, Khaki A, Shoorei H, et al. Hesperidin attenuated apoptotic-related genes in testicle of a male rat model of varicocoele. Andrology 2020; 8(1): 249-258.
32. Wankeu-Nya M, Florea A, Bâlici S, et al. *Dracaena arborea* alleviates ultra-structural spermatogenic alterations in streptozotocin-induced diabetic rats. BMC Complement Altern Med 2013; 13, 71. doi: 10.1186/1472-6882-13-71.
33. Lim JG, Park HM, Yoon KS. Analysis of saponin composition and comparison of the antioxidant activity of various parts of the quinoa plant (Chenopodium quinoa Willd.). Food Sci Nutr 2019; 8(1): 694-702.
34. Sainio-Pöllänen S, Henriksén K, Parvinen M, et al. Stage-specific degeneration of germ cells in the seminiferous tubules of non-obese diabetic mice. Int J Androl. 1997; 20(4): 243-253.
35. Amaral S, Oliveira PJ, Ramalho-Santos J: Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. Curr Diabetes Rev 2008; 4(1): 46-54.
36. Mallidis C, Agbaje I, McClure N, et al. The influence of diabetes mellitus on male reproductive function: a poorly investigated aspect of male infertility [German]. Urology A 2011, 50(1): 33-37.

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 607 - 614
doi: 10.30466/vrf.2023.561862.3618
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Trichoderma harzianum as fungicide and symbiont: is it safe for human and animals?

Amir Ali Shahbazfar[1]*, Marzieh Heidarieh[2], Samira Shahbazi[3], Hamed Askari[3]

[1] Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran; [2] Nuclear Science and Technology Research Institute, Tehran, Iran; [3] Department of Plant Protection, Nuclear Agriculture School, Nuclear Science and Technology Research Institute (NSTRI), Karaj, Iran.

Article Info

Article history:

Received: 10 September 2022
Accepted: 20 May 2023
Available online: 15 November 2023

Keywords:

Biological agents
Lab animals
Phytopathogenic fungi
Toxicity
Trichoderma harzianum

Abstract

Trichoderma species are considered as biological control agents against numerous phytopathogenic fungi. They are also helpful for plants as plant symbiont. This study aimed to identify harmful effects of *Trichoderma* in laboratory animals. In the first step, inhalation toxicity was studied. Six rats as control received a spray of bio-formulation without spores. Ten rats as treatment A received 1.00×10^6 colony-forming unit (CFU) of *Trichoderma* spores and ten rats as treatment B received 1.00×10^7 CFU per test of *Trichoderma* spores. The harmful effects of *Trichoderma* were obvious especially in the lungs, liver and kidney, and some blood parameters were abnormal. In the second step, we studied acute oral toxicity by gavage. Four rats as control received bio-formulation without spores. Six rats as treatment A received 1.00×10^6 CFU per test of *Trichoderma* spores. Six rats as treatment B received 1.00×10^7 CFU per test of *Trichoderma* spores. The harmful effects of *Trichoderma* were noticeable more in the liver and kidney tissues. For dermal toxicity study, two rabbits as control received bio-formulation without spores by rubbing on the surface of the skin. Treatment groups A and B received 1.00×10^6 and 1.00×10^7 CFU per test of *Trichoderma* spores, respectively (four rabbits for each group). The liver and kidney and some blood parameters were abnormal. *Trichoderma* has some harmful effects on tissues and organs and although it is a natural product, it should be used under cautions.

Introduction

Trichoderma species are considered as biological control agents against numerous phytopathogenic fungi, including *Fusarium oxysporum*, *Rhizoctonia solani* and *Macrophomina phaseolina.*[1-4] There are some reports of the usage of *Trichoderma* as an anti-parasite (*Toxocara canis*, *Leishmania amazonensis* and malaria) agent.[5-7] *Trichoderma* has been used for controlling human breast and cervical cancer.[8] Its species have been reported as pathogens of reptiles[9] and the list of infections that could be caused by *Trichoderma* spp. in humans is extensive.[10] *Trichoderma longibrachiatum* is the main human pathogen species within the genus and has been isolated with increasing frequency in recent years.[11] It produces toxic peptides called trilongins. Exposure to *T. longibrachiatum* cannot be treated with antimicrobial agents and dramatically weakens the immune system.[12] However, *Trichoderma* species also appear to belong to the growing list of emergent pathogens, with an increasing number of reports of invasive infections.[13,14] The problem is not limited to immunocompromised patients.[15] The antagonistic activity of *Trichoderma* isolates (NAS110) against plant pathogenic fungi: *R. solani*, *F. oxysporum*[16,17] *was* investigated in previous studies.[18-20]

To use *Trichoderma* as a fungicide and symbiotic, it is necessary to determine its potential side effects on the body of laboratory animals. This study aimed to determine the acute oral, dermal and pulmonary toxicity/pathogenicity of this agent according to the known scientific protocols.

Materials and Methods

***Trichoderma* preparation.** *Trichoderma harzianum* NAS110 was obtained from the microbial collection of Plant Pathology Laboratory (Nuclear Science and

***Correspondence:**

Amir Ali Shahbazfar. DVM, DVSc
Department of Pathobiology, Faculty of Veterinary medicine, University of Tabriz, Tabriz, Iran
E-mail: Shahbazfar@tabrizu.ac.ir

Technology Research Institute, Karaj, Iran). It was transferred to sterile potato dextrose broth under aseptic conditions and incubated at 28.00 ˚C for 72 hr. Then, the mycelial masses were transferred on potato dextrose agar medium and incubated in the same temperature conditions for 7 days. Spores were washed using saline solution from the surface of petri dishes and spore's suspension population was adjusted using a Neubauer slide (Paul Marienfeld, Lauda Konigshofen, Germany) at a concentration of 1.00×10^6 spore mL^{-1}. The spores were pelleted by centrifugation at 4,500 *g* for 10 min and washed twice in sterile saline solution. The washed spore suspension in distilled water was used as biomaterial of bio-formulation with components described as follow. Component of effective material (biomaterial) *T. harzianum* NAS110 included 2.00% *Trichoderma* spore, 98.00% of sucrose (Sigma-Aldrich, St. Louis, USA) as treatment A, and component of bio-formulation Trichofarm™ included 0.20% *Trichoderma* spore, 4.80% sucrose, 4.80% starch (Alphachemika Co., Mumbai, India), 0.20% Arabic gum (Sigma-Aldrich), and 90.00% kaolin (Sigma-Aldrich) as treatment B. All animals (rats and rabbits) were purchased from the animal house of Iran Pasteur Institute, Tehran, Iran. The study was carried out in accordance with the guidelines and regulations approved by the Animal Experimentation Committee of the Tabriz University (Approval Code: FVM.REC.1396.937; Approval Date: 02 October 2022), Tabriz, Iran.

Acute pulmonary pathogenicity (inhalation test). Twenty-six healthy young Wistar albino rats (13 of each sex) weighting 180 - 200 g (10 weeks old) were obtained and kept in metal and plastic cages. Food and water were *ad libitum*. The light and dark cycle was 12/12 hr and the temperature was set at 23.00 ˚C. After 1 week acclimatization period, they were divided as follows: Six animals (three of each sex) as control received a spray of bio-formulation without spores (Sucrose, Starch, Arabic gum, Kaolin with distilled water in the nose). Ten animals (five of each sex) as treatment A received 1.00×10^6 colony-forming unit (CFU) of *Trichoderma* spores. Ten animals as treatment B received 1.00×10^7 CFU of *Trichoderma* spores with intranasal route. The animals were then monitored for 21 days and were anesthetized 21 days after dosing by intraperitoneal injection of 70.00 mg kg^{-1} ketamine (Alfasan, Woerden, Netherlands) and 7.00 mg kg^{-1} xylazine (Alfasan) and after losing the pedal reflex, they were decapitated. Blood samples were collected of them. After necropsy, tissue specimens from the liver, kidney, spleen, heart and left lung were taken and fixed in buffered 10.00% formalin. After 1 week of fixation, the tissues were processed using a tissue processor and microscopic slides were made of them. The slides were stained by Hematoxylin and Eosin (H&E) and studied under a light microscope (ML2100; Krüss, Hamburg, Germany) and photomicrographs were created of them. The pathologic effects were classified as follow:

Liver) 0: No pathologic findings, 1: Slight hyperemia in sinusoids, 2: Hyperemia in sinusoids and central veins and/or hemorrhage, 3: Infiltrated inflammatory cells in tissue and/or cell degeneration, and 4: Scattered necrosis.

Heart) 0: No pathologic findings, 1: Hyperemia, 2: Hemorrhage and/or edema, 3: Infiltrated inflammatory cells in the tissue and/or cell degeneration, and 4: Scattered Necrosis.

Spleen) 0: No pathologic findings, 1: Hyperemia, 2: Hemorrhage, 3: Cell degeneration, and 4: Depletion of lymphoid tissue.

Kidney) 0: No pathologic findings, 1: Hyperemia, 2: Hemorrhage and /or protein secretion in renal tubules, 3: Cell swelling and/or degeneration of renal tubule epithelial cells and/or inflammation, and 4: Necrosis in the tubules.

Lung) 0: No pathologic findings, 1: hyperemia, 2: Hyperemia and slight hemorrhage and/or edema and emphysema, 3: epithelialization of type II pneumocytes, and 4: infiltrated inflammatory cells in tissue and/or scattered cell necrosis. The United States environmental protection agency preventing pesticides and toxic substances (7101) EPA712-c-96-318 February 1996. Microbial pesticide test guidelines OPPTS885-3150-Acute pulmonary toxicity/pathogenicity was followed.

Acute oral pathogenicity. Eighteen healthy young Wistar albino rats (nine of each sex) weighting 180 - 200 g (10 weeks old) were obtained and kept in metal and plastic cages. They were divided as follow: Four animals (two of each sex) as control received bio-formulation without spores, (sucrose, starch, Arabic gum and Kaolin) with distilled water with a stomach tube. Six animals (three of each sex) as treatment A received 0.20% (1.00×10^6 CFU) per test of washed spores of *Trichoderma* plus sucrose, starch, Arabic gum and kaolin with a stomach tube, six animals (three of each sex) as treatment B received 1.00×10^7 CFU per test of washed spores of *Trichoderma* plus sucrose with a stomach tube. Volume of suspension was 2.00 mL per 100 g body weight. The animals were fasted overnight before the test and after the substance had been administered, food was withheld for a further 3 - 4 hr. The animals were controlled for 21 days and were anesthetized using the previously mentioned protocol and after losing the pedal reflex were decapitated. Blood samples were collected from them. The rest of the procedure is like acute pulmonary toxicity. The United States environmental protection agency preventing pesticide and toxic substances (7101) EPA712-c-96-315 February1996. Microbial pesticide test guidelines OPPTS885-3050-Acute Oral Toxicity/pathogenicity was followed.

Acute dermal pathogenicity. Ten healthy young New Zealand white rabbits (five of each sex) weighting 2.50 - 3.00 kg were obtained and kept in metal cages. They received food and water *ad libitum*. The light and dark cycle was 12/12 hr and the temperature was set at

22.00 ˚C. After 1 week acclimatization period, they were divided as follow: Two animals (one of each sex) as control received bio-formulation without spores of *Trichoderma* by rubbing on the surface of the skin. Four animals as treatment A and four animals as treatment B. According to the protocol, acute dermal toxicity is the adverse effect occurring during or following 24 hr dermal exposure to a single dose of a test substance. The animals were monitored for 14 days and euthanized by an overdose of 60.00 mg kg^{-1} sodium thiopental (Hospira, Illinois, USA) 14 days after dermal exposure and blood samples were collected. The rest of the procedure was like the acute pulmonary toxicity. The United States environmental protection agency preventing pesticide and toxic substances (7101) EPA712-c-96-316 February 1996. Microbial pesticide test guidelines OPPTS885-3100-Acute Dermal Toxicity/pathogenicity was followed.

Blood analysis. The blood parameters and liver enzymes were measured in all dermal, oral and respiratory test groups. These parameters were white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count, carcinoembryonic antigen (CEA), glycosylated Hb, alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP).

Statistical analysis. Data of our grading system were processed with SPSS Software (version 22.0; IBM Corp., Armonk, USA). For processing the pathologic data, the Kruskal-Wallis and Mann-Whitney U tests were used. For data of blood parameters, one-way ANOVA with the Tukey test as post hoc were used. The confidence interval was less than 0.05 in all the tests.

Results

Acute pulmonary pathogenicity (inhalation test). After 21 days of trial and microscopic study, information was gathered and shown in Table 1. In the group with *T. harzianum* spores, some pathologic effects were observed in liver (hemorrhage, hyperemia, infiltration of inflammatory cells), kidney (hyperemia,) and lung (edema, infiltration of inflammatory cells, type II pneumocyte epithelialization) but not in spleen and heart. There were no statistical differences between two treatment groups (Fig. 1).

Acute oral pathogenicity. After 21 days of trial, microscopic study information was gathered and is shown in Table 1. In the group with *T. harzianum* spore's treatment, some pathologic effects were observed in the liver (hyperemia, infiltration of inflammatory cells) and kidney (Hyperemia, hyaline cast formation), but not in the spleen and heart. Although some slight pathologic changes were observed in the lung (hemorrhage, infiltration of inflammatory cells), changes in the lungs were not statistically different between the control and treatment groups. There were no statistical differences between the two treatment groups (Fig. 2). With the comparison of oral and respiratory route of administration, just lungs were more affected in respiratory route and pathologic effects (edema, infiltration of inflammatory cells, epithelialization in the lungs) were more severe with respiratory administration.

Acute dermal pathogenicity. After 14 days of trial, microscopic study information was gathered and is shown in Table 1. In the group with *Trichoderma* spore's treatment, some pathologic effects were observed in the liver (hyperemia, infiltration of inflammatory cells), kidney (hyperemia, cell swelling in renal tubules) and lung (hyperemia, emphysema, epithelialization of type II pneumocytes) but not in the heart. Although some slight pathologic changes were observed in the spleen, the changes were not statistically significant between control and treatment groups. There were no statistical differences between two treatment groups (Fig. 3).

Blood sample tests. Blood samples were taken at the time of euthanasia and different parameters (Liver enzymes: ALP, ALT, AST and Blood parameters: CBC, CEA and glycosylated Hb) were measured in them. Liver enzymes (AST, ALT, and ALP) showed enhancement in all the test groups (oral, dermal, and inhalation), but there were no statistical differences between the two *Trichoderma* groups. This meant the substance was harmful to the liver which was shown in pathology, as well. The WBC count, RBC count and Hb have been changed with the use of *Trichoderma*. The WBC count has been enhanced with the usage of *Trichoderma*. The RBC count and Hb have been increased after the use in all three forms (oral, dermal, and respiratory). Information was shown in Tables 2 and 3.

Table 1. Pathogenicity of *Trichoderma* spores in different organs after inhalation, oral and dermal exposure are shown as mean ± standard error of mean.

Organ	Route	Control	Treatment A	Treatment B
Liver	Inhalation	0.00	2.67 ± 0.42*	2.00 ± 0.44*
	Oral	0.00	2.45 ± 0.31*	2.13 ± 0.28*
	Dermal	0.00	2.25 ± 0.48*	2.55 ± 0.29*
Kidney	Inhalation	0.00	2.67 ± 0.21*	2.67 ± 0.21*
	Oral	0.00	1.80 ± 0.29*	1.74 ± 0.34*
	Dermal	0.00	1.75 ± 0.48*	1.75 ± 0.25*
Spleen	Inhalation	0.25 ± 0.25	0.33 ± 0.21	0.33 ± 0.21
	Oral	0.17 ± 0.17	0.27 ± 0.13	0.45 ± 0.16
	Dermal	0.33 ± 0.33	0.33 ± 0.33	0.75 ± 0.25
Heart	Inhalation	0.00	0.55 ± 0.22	0.83 ± 0.48
	Oral	0.00	0.61 ± 0.27	0.69 ± 0.27
	Dermal	0.00	0.00	0.00
Lung	Inhalation	0.00	0.83 ± 0.48*	2.10 ± 0.36*
	Oral	0.00	1.24 ± 0.39	0.66 ± 0.34
	Dermal	0.00	1.51 ± 0.29*	2.01 ± 0.73*

* indicates statistical difference with the group control at $p \leq 0.05$.

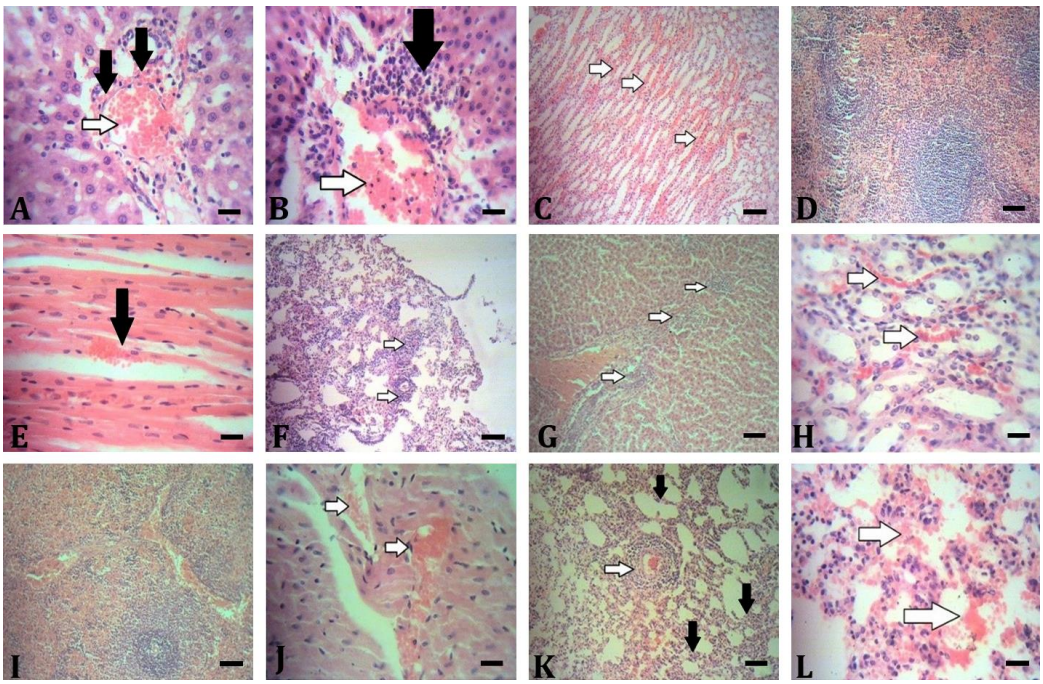

Fig. 1. Histopathogenicity of *Trichoderma* spore after inhalation exposure in different organs of treatments A (A – F) and B (G - L). **A)** Hemorrhage (black arrows) and hyperemia (white arrow) in the liver, **B)** Infiltration of inflammatory cells (black arrow) and hyperemia (white arrow) in the liver, **C)** Hyperemia in kidney (arrows), **D)** Normal spleen, **E)** Hyperemic heart muscle (arrows), and **F)** Infiltration of inflammatory cells in lung (arrows). **G)** Infiltration of inflammatory cells in liver (arrows), **H)** Hyperemia in kidney (arrows), **I)** Hyperemia in spleen (panoramic view), **J)** Hyperemia in heart muscle (arrows), **K)** Epithelialization of type II pneumocyte (black arrows) and Infiltration of inflammatory cells around a vessel (white arrow) in lung, and **L)** Edema in lung (arrows), (H&E staining, bars = 50.00 µm).

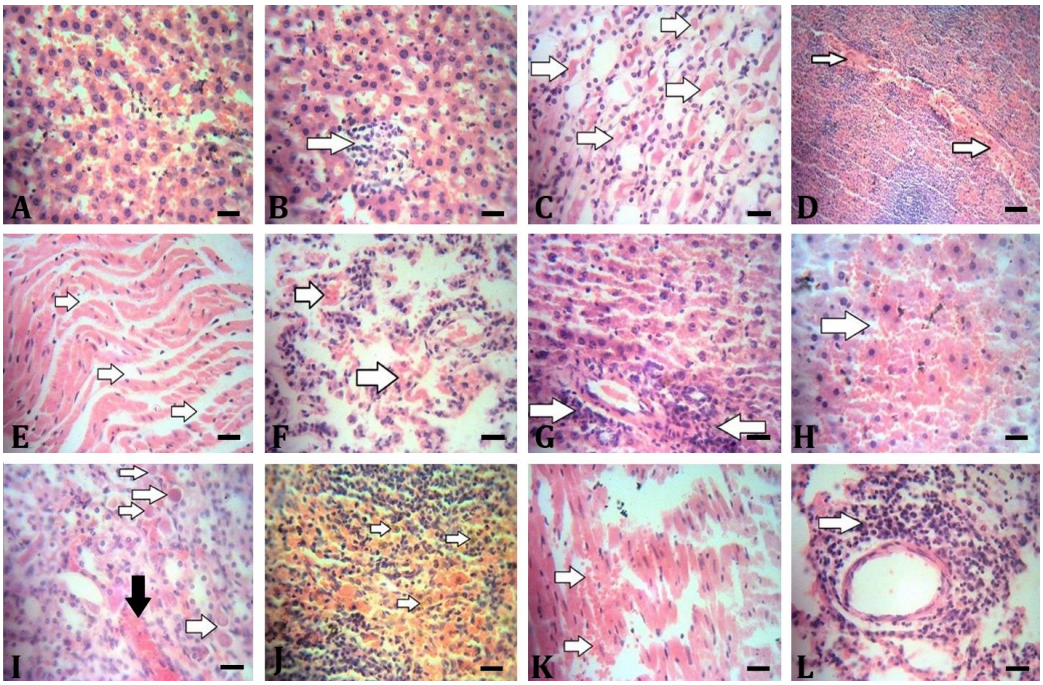

Fig. 2. Histopathogenicity of *Trichoderma* spore after oral exposure in different organs of treatments A (A – F), and B (G - L). **A)** Hyperemia in liver (panoramic view), **B)** Infiltration of inflammatory cells in liver (arrow), **C)** Protein secretion (hyaline cast and nephrotic syndrome) in renal tubules (arrows), **D)** Hyperemia in spleen (arrows), **E)** Edema in heart muscle (arrows), and **F)** Hemorrhage in lung (arrows). **G)** Infiltration of inflammatory cells in portal area of liver (arrows), **H)** Hemorrhage in liver (arrow), **I)** Protein secretion (hyaline cast and nephrotic syndrome) in renal tubules (horizontal white arrows) and hyperemia (transparent vertical arrow) in kidney, **J)** Hemosiderophages and hemosidrin pigments that is a sign of hemorrhage (arrows) in spleen, **K)** Hemorrhage in heart muscle (arrows), and **L)** Infiltration of inflammatory cells around a vessel in lung (arrow), (H&E staining, bar = 50.00 µm).

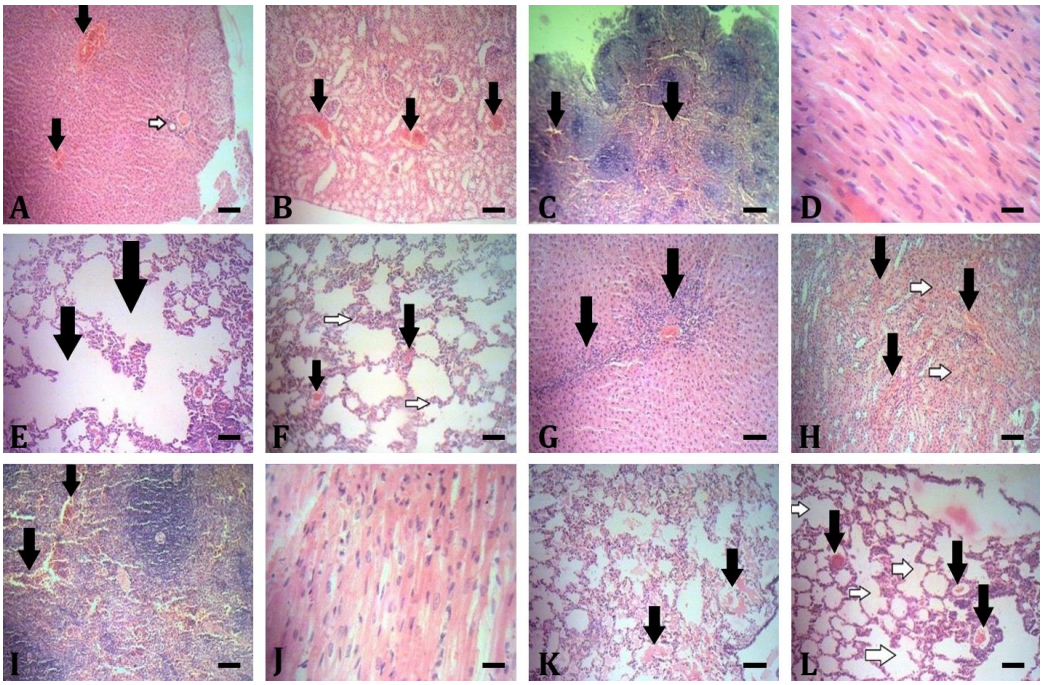

Fig. 3. Histopathogenicity of *Trichoderma* spore after dermal exposure in different organs of treatments A (A – F), and B (G - L). **A)** Hyperemia (black arrows) and slight infiltration of inflammatory cells in portal area of liver (white arrow), **B)** Hyperemia in kidney (arrows), **C)** Hyperemia in spleen (arrows), **D)** Normal heart muscle, **E)** Emphysema in lung (arrows), and **F)** Hyperemia (black arrows) and epithelialization of type II pneumocytes (white arrows) in lung. **G)** Infiltration of inflammatory cells in liver (arrows), **H)** Hyperemia (black arrows) and cell swelling in renal tubules (white arrows), **I)** Hyperemia (arrows) in spleen, **J)** Normal heart muscle, **K)** Pulmonary edema (arrows), and **L)** Hyperemia (black arrows) and emphysema (white arrows) in lung, (H&E staining, bar = 50.00 µm).

Table 2. Data of some blood parameters in experimental groups.

Groups	WBC ($\times10^3$ µL^{-1})	RBC ($\times10^6$ µL^{-1})	Hb (g dL^{-1})	HCT (%)	MCV (fL)	MCH (pg)	MCHC (%)	Platelet ($\times10^3$ µL^{-1})	Hb A1-C (%)
1-K	6.1 9 ± 0.97	7.62 ± 0.50	13.40 ± 0.11	36.50 ± 2.31	47.90 ± 1.70	16.00 ± 0.55	33.40 ± 2.60	432 ± 17.33	4.44 ± 0.17
2-K	4.72 ± 0.26	5.52 ± 0.17	15.50 ± 0.23	38.90 ± 2.30	45.70 ± 2.30	16.00 ± 0.46	35.00 ± 2.30	341 ± 23.00	4.03 ± 0.34
3-K	5.24 ± 0.24	7.14 ± 0.09	13.50 ± 0.26	36.80 ± 2.30	51.50 ± 1.70	17.20 ± 0.20	33.40 ± 2.00	234 ± 11.62	4.38 ± 0.10
4-T	3.59 ± 0.14[a]	9.72 ± 0.15[a]	15.40 ± 0.17[a]	41.40 ± 2.61	42.60 ± 1.30	15.80 ± 0.80	37.20 ± 1.70	440 ± 31.80	5.70 ± 0.24
5-T	2.80 ± 0.17[b]	9.58 ± 0.20	15.30 ± 0.09[b]	42.50 ± 2.45	44.40 ± 0.20	16.00 ± 0.88	36.00 ± 2.30	384 ± 25.40	5.75 ± 0.21
6-T	4.53 ± 0.17	8.67 ± 0.32[c]	14.00 ± 0.40[c]	42.10 ± 1.30	48.60 ± 2.70	16.10 ± 0.14	33.30 ± 2.10	207 ± 49.00	5.30 ± 0.09
7-F	3.30 ± 0.19[d]	9.07 ± 0.17[a]	15.40 ± 0.12[a]	43.20 ± 0.68	47.60 ± 2.20	17.00 ± 0.52	35.60 ± 3.20	389 ± 5.78	4.46 ± 0.11
8-F	3.07 ± 0.37[be]	9.68 ± 0.37[b]	14.90 ± 0.05b	43.10 ± 1.47	44.50 ± 2.80	15.40 ± 0.58	34.60 ± 2.88	332 ± 29.48	5.35 ± 0.23
9-F	4.38 ± 0.15[cf]	7.56 ± 0.50	14.90 ± 1.29[c]	43.90 ± 2.05	58.10 ± 3.03	19.70 ± 0.29	33.90 ± 2.21	441 ± 17.32	5.91 ± 0.21
Normal range	3.40 - 14.00	6.90 - 12.20	12.00 - 18.90	35.50 - 70.20	44.00 - 62.50	13.00 - 19.00	26.50 - 58.00	325 - 888	< 6.00

WBC: White blood cells, RBC: Red blood cells, Hb: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, and MCHC: Mean corpuscular hemoglobin concentration.
K: Formulation material without *Trichoderma* (1: Oral, 2: Dermal, 3: Respiratory).
T: *Trichoderma harzianum* NAS110 (bio-material)-1.00 × 10^7 colony-forming unit (4: Oral, 5: Dermal, 6: Respiratory).
F: Bio-formulation of *Trichoderma harzianum* NAS110 - 1.00 × 10^6 colony-forming unit (7: Oral, 8: Dermal, 9: Respiratory).
[a] indicates statistical significance with 1-k, [b] indicates statistical significance with 2-k, [c] indicates statistical significance with 3-k, [d] indicates statistical significance with 4-T, [e] indicates statistical significance with 5-T, and [f] indicates statistical significance with 6-T ($p < 0.05$).

Table 3. Data of liver enzymes and carcinoembryonic antigen test in experimental groups.

Groups	AST ($U\ L^{-1}$)	ALT ($U\ L^{-1}$)	ALP ($U\ L^{-1}$)	CEA ($ng\ mL^{-1}$)
1-K	148 ± 18.70	138 ± 15.20	330 ± 21.50	0.33 ± 0.07
2-K	440 ± 23.60	218 ± 10.68	142 ± 9.33	0.21 ± 0.02
3-K	320 ± 20.27	220 ± 16.70	165 ± 10.89	0.29 ± 0.02
4-T	153 ± 18.00	171 ± 13.10	296 ± 10.11	0.30 ± 0.03
5-T	943 ± 72.35	240 ± 10.31[b]	288 ± 0.17[b]	0.25 ± 0.02
6-T	332 ± 14.46[c]	401 ± 17.70	485 ± 6.00[c]	0.39 ± 0.04
7-F	361 ± 14.43[a]	233 ± 16.25[ad]	250 ± 11.54[a]	0.10 ± 0.02
8-F	597 ± 20.22[be]	108 ± 17.30[be]	290 ± 17.30[b]	0.33 ± 0.03
9-F	187 ± 11.78[f]	203 ± 18.40[f]	198 ± 17.90[f]	0.42 ± 0.12
Normal range	55.00 - 362	40.00 - 170	230 - 355	< 2.50

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, and CEA: Carcinoembryonic antigen.
K: Formulation material without *Trichoderma* (1: Oral, 2: Dermal, 3: Respiratory).
T: *Trichoderma harzianum* NAS110 (bio-material)-1.00×10^7 colony-forming unit (4: Oral, 5: Dermal, 6: Respiratory).
F: Bio-formulation of *Trichoderma harzianum* NAS110 - 1.00×10^6 colony-forming unit (7: Oral, 8: Dermal, 9: Respiratory).
[a] indicates statistical significance with 1-k, [b] indicates statistical significance with 2-k, [c] indicates statistical significance with 3-k, [d] indicates statistical significance with 4-T, [e] indicates statistical significance with 5-T, and [f] indicates statistical significance with 6-T ($p < 0.05$).

Discussion

With the usage of *Trichoderma harzianum*, different organs showed pathologic features but, in the liver, (hemorrhage, hyperemia, and infiltration of inflammatory cells), kidney (hyperemia, hyaline cast formation, cell swelling in renal tubules), and lung (edema, emphysema, infiltration of inflammatory cells, type II pneumocyte epithelialization) changes were statistically significant. Changes in the lungs were statistically significant just in the inhalation test group but pathologic findings existed in all the organs in all tests. Dose of 1.00×10^7 CFU showed more pathologic effects than dose of 1.00×10^6 CFU and the hazards were dose dependent. Liver enzymes (AST, ALT, and ALP) showed enhancement in all test groups (oral, dermal, and inhalation), however, there were no statistical differences in the two *Trichoderma* groups. This meant the substance was harmful to the liver which was shown in pathology, as well. The WBC count, RBC count and Hb have been changed with the use of *Trichoderma*. The WBC count has been enhanced with the usage of *Trichoderma*. The RBC count and Hb have been increased after the use in all three forms (oral, dermal, and respiratory).

Trichoderma species can cause localized infections such as pulmonary mycetoma, peritonitis, sinusitis, otitis, brain abscess and fatal disseminated disease.[11] The conidia of *Trichoderma* molds that are inhaled can interact with the airway epithelium causing symptoms

Trichoderma species can cause localized infections such as pulmonary mycetoma, peritonitis, sinusitis, otitis, brain abscess and fatal disseminated disease.[11] The conidia of *Trichoderma* molds that are inhaled can interact with the airway epithelium causing symptoms similar to those of Stachybotrys (black mold). The symptoms that follow the inhalation include, however, are not limited to sneezing, asthmatic attacks, prolonged coughing and infections of the lungs (e.g., pneumonia). *Trichoderma* species are a serious threat to immunocompromised patients. One species in this genus, *Trichoderma longibrachiatum*, is extremely harmful and toxic to human.[21]

In one case, thoracic tomodensitometry revealed bilateral pneumothorax, pneumopericardium and a dense lesion of the right apex.[14] Also, in one case *Trichoderma harzianum* was isolated from a pediatric patient with hematological malignancy (acute lymphoblastic leukemia). The child had pulmonary involvement and the mold was resistant to treatment.[22]

In a comprehensive search, 16 well-documented published cases of invasive *Trichoderma* infection have been found.[14] Skin lesions (necrotizing ulcerative) were observed in three patients.[14] *Trichoderma* species have also been isolated from food and contaminated food may explain the digestive involvement.[14,23] Direct microscopic examination of sputum, bronchoaspiration, and bronchoalveolar lavage fluid samples revealed the presence of fungal septate hyphae.[14] A recent case of otitis externa in ahealthy 12-year-old boy was resolved following treatment. In one research, *T. longibrachiatum* was administered to mice. The mortality of the infected mice was correlated with inoculum size.

The mortality rate of mice challenged with 1.00×10^4 and 1.00×10^5 CFU per animal was 25.00%; the rate was 62.50% in those challenged with 1.00×10^6 CFU per animal. All mice infected with 1.00×10^7 CFU per animal were died.[23] In another survey, inhaled spores could reach the alveoli and interacted with the airway epithelium. The fungal spores were found to trigger histamine release from the bronchiole associated lymphoid tissue Cells however, relatively high concentrations (0.10 - 2.00 $mg\ mL^{-1}$) were needed. A similar dose response was obtained in basophil histamine release. Mucosal mast cells from the airways were susceptible to the potentiating effect of *Trichoderma*.[24]

Some of these fungi that can act as human pathogens also produce mycotoxin. For example, trichodermin, a mycotoxin in the trichothecins group, is secreted by *Trichoderma brevicompactum*. Commonly, it contaminates the food source and consumption of these mycotoxin leads to vomiting and gastroenteritis.[25,26]

Trichoderma longibrachiatum is a human pathogen to immunosuppressed individuals, still other species can also cause infection in this type of individuals, such as

T. citrinoviride and *T. harzianum*. The list of illnesses that could be caused by *Trichoderma* spp. is extensive, including peritonitis and intra-abdominal abscess in patients undergoing continuous ambulatory peritoneal dialysis, liver infection, acute invasive sinusitis and disseminated infections of transplant recipients, brain abscess, skin infection, necrotizing stomatitis and pulmonary infections of patients with hematological malignancies, fungemia by contaminated saline, rhinosinusitis, pulmonary myeloma and fibrosis, hypersensitivity pneumonitis, endocarditis, otitis externa, cerebrospinal fluid infection and allergic reactions.[10,18] *Trichoderma* spp. bears a toxin called gliotoxin. Some of the effects of *Trichoderma* is referred to gliotoxin. Gliotoxin has high persistence in the water, soil and air, therefore, it is a risk to human health. This toxin is toxic for both animals and humans being lethal in relatively low concentrations.[27]

Gliotoxin can affect innumerable cell mechanisms leading to the death of the cells. The effects of gliotoxin on cells seem to be related not only to the concentration, but also to the cell type. In human and some animals, it is known that gliotoxin inhibits the function of the NADPH (reduced nicotinamide adenine dinucleotide phosphate) enzyme complex.[25] It can also induce apoptotic cell death of thymocytes, peripheral lymphocytes, macrophages and others.[15,28] According to references the prevalent species in the majority of *Trichoderma*-based products is *T. harzianum* (83.00%).[29]

This research showed that *T. harzianum* had pathologic effects on different organs and the blood with oral, dermal and respiratory usage and it must be used with care in agriculture.

Acknowledgments

This research was funded by The Nuclear Science and Technology Research Institute of Iran.

Conflict of interest

The authors declare no competing financial interest.

References

1. Sahampoor L, Zaker Tavallaie F, Fani SR, et al. *In vitro* efficiency of *Trichoderma harzianum* mutants in biocontrol of *Fusarium oxysporum f. sp. radicis-cucumerinum*. J Crop Prot 2020; 9(2): 285-300.
2. Harman GE. Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 2006; 96(2): 190-194.
3. Lava A, Babaeizad V, Tajick Ghanbary MA, et al. Effects of Trichoderma and endophytic fungus *Piriformospora indica* on cucumber physiology and Fusarium wilt disease of Cucumber. Annals of RSCB 2021; 25(4): 20742-20755.
4. Orojnia S, Habibi D, Shahbazi S, et al. Investigation the effect of different formulations of mutated trichoderma with gamma ray on soybean morphological indices [Persian]. Crop Physiol IZU Ahvaz 2021; 12(48): 97-115.
5. Abdel-Latif AMH, Haggag WM. Biocontrol potential of salinity tolerant mutants of *Trichoderma harzianum* against *Fusarium oxysporum*. Braz J microbiol 2006; 37(2): 181-191.
6. Lopes DS, Dos Santos UR, Dos Anjos DO, et al. Ethanolic extract of the fungus *Trichoderma asperelloides* induces ultrastructural effects and death on *Leishmania amazonensis*. Front Cell Infect Microbiol 2020; 10: 306. doi: 10.3389/fcimb.2020.00306.
7. de Souza Maia Filho F, da Silva Fonseca AO, Persici BM, et al. *Trichoderma virens* as a biocontrol of *Toxocara canis*: *in vivo* evaluation. Rev Iberoam Micol 2017; 34(1): 32-35.
8. Abd El-Rahman AA, El-Shafei SM, Ivanova EV, et al. Cytotoxicity of *Trichoderma* spp. cultural filtrate against human cervical and breast cancer cell lines. Asian Pac J Cancer Prev 2014; 15(17): 7229-7234.
9. Munoz FM, Demmler GJ, Travis WR, et al. *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. J Clin Microbiol 1997; 35(2): 499-503.
10. Mukherjee PK, Horwitz BA, Singh US, et al. Trichoderma: biology and applications. London, UK: CAB International 2013; 1-10.
11. Trabelsi S, Hariga D, Khaled S. First case of *Trichoderma longibrachiatum* infection in a renal transplant recipient in Tunisia and review of the literature. Tunis Med 2010; 88(1): 52-57.
12. Chouaki T, Lavarde V, Lachaud L, et al. Invasive infections due to *Trichoderma* species: report of 2 cases, findings of *in vitro* susceptibility testing, and review of the literature. Clin Infect Dis 2002; 35(11): 1360-1367.
13. Tang P, Mohan S, Sigler L, et al. Allergic fungal sinusitis associated with *Trichoderma longibrachiatum*. J Clin Microbiol 2003; 41(11): 5333-5336.
14. Akagi T, Kawamura C, Terasawa N, et al. suspected pulmonary infection with *Trichoderma longibrachiatum* after allogeneic stem cell transplantation. Intern Med 2017; 56(2): 215-219.
15. Scharf DH, Remme N, Heinekamp T, et al. Transannular disulfide formation in gliotoxin biosynthesis and its role in self-resistance of the human pathogen *Aspergillus fumigatus*. J Am Chem Soc 2010; 132(29): 10136-10141.
16. Ghasemi S, Safaie N, Shahbazi S, et al. The role of cell wall degrading enzymes in antagonistic traits of *Trichoderma virens* against *Rhizoctonia solani*. Iran J Biotechnol 2020; 18(4): e2333. doi: 10.30498/IJB.2020.2333.
17. Soufi E, Safaie N, Shahbazi S, et al. Gamma irradiation

induces genetic variation and boosting antagonism in *Trichoderma aureoviride*. Arch Phytopathol Plant Prot 2021; 54: 1649-1674.
18. Schuster A, Schmoll M. Biology and biotechnology of Trichoderma. Appl Microbiol Biotechnol 2010; 87(3): 787-799.
19. Shahbazi S, Zaker Tavallaie F, Daroodi Z. Morphological and molecular identification of *Fusarium* spp. associated with carnation *Dianthus caryophyllus* in Mahallat, Iran. J Crop Prot 2021; 10(3): 461-471.
20. Zin NA, Badaluddin NA. Biological functions of *Trichoderma* spp. for agriculture applications. Ann Agri Sci 2020; 65: 168-178.
21. Harman GE. Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T22. Plant Dis 2000; 84(4): 377-393.
22. Kantarcioğlu AS, Celkan T, Yücel A, et al. Fatal *Trichoderma harzianum* infection in a leukemic pediatric patient. Med Mycol 2009; 47(2):207-215.
23. Prelle A, Spadaro D, Garibaldi A, et al. A new method for detection of five alternaria toxins in food matrices based on LP-APCI-MS. Food Chem 2013; 140(1-2): 161-167.
24. Larsen FO, Clementsen P, Hansen M, et al. The indoor microfungus *Trichoderma viride* potentiates histamine release from human bronchoalveolar cells. APMIS1996; 104(9): 673-679.
25. Zeilinger S, Gruber S, Bansal R, et al. Secondary metabolites in *Trichoderma* – Chemistry meets genomics. Fungal Biol Rev 2016; 30(2): 74-90.
26. Woo SL, Ruocco M, Vinale F, et al. *Trichoderma*-based products and their widespread Use in agriculture. Open Mycol J 2014; 8: 71-126.
27. Vidal-García M, Redrado S, Domingo MP, Marquina P, et al. Production of the invasive Aspergillosis biomarker Bis (methylthio) gliotoxin within the genus *Aspergillus*: *In Vitro* and *in Vivo* metabolite quantification and genomic analysis. Front Microbiol 2018; 9: 1246. doi: 10.3389/fmicb.2018.01246
28. Vargas WA, Mukherjee PK, Laughlin D, et al. Role of gliotoxin in the symbiotic and pathogenic interactions of *Trichoderma virens*. Microbiology (Reading) 2014; 160(Pt 10): 2319-2330.
29. Topolovec-Pintarić S. Trichoderma: Invisible partner for visible impact on agriculture. IntechOpen 2019; doi:10.5772/intechopen.83363.

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 615 - 623
doi: 10.30466/vrf.2023.1978294.3704
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Whole-genome sequencing of foot-and-mouth disease virus serotype O/PanAsia-2/QOM-15 and comparison of its VP1-encoding region with two vaccine strains

Mehrnoosh Gadir[1], Seyed Mahmoud Azimi[2]*, Naser Harzandi[1], Behzad Hemati[1], Neda Eskandarzade[3]

[1] Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran; [2] Foot and Mouth Disease Reference Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran; [3] Department of Basic Sciences, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

Article Info

Article history:

Received: 15 December 2022
Accepted: 20 May 2023
Available online: 15 November 2023

Keywords:

FMDV serotype O
RNA-Seq
SNP discovery
VP1
Whole-genome sequencing

Abstract

Despite widespread vaccination against foot-and-mouth disease, many outbreaks still occur in endemic areas. We attempted to determine the genetic and antigenic properties of the O/PanAsia-2/QOM-15 foot-and-mouth disease virus new vaccine strain. Thus, whole-genome sequencing was used to identify vulnerable pinpoint sites across the genome. The VP1 sequence (1D gene) of the O/PanAsia-2/QOM-15 viral genome was then compared to the VP1 sequences of two previously used vaccine strains, O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146). The antigenic relationship of these three viruses was calculated by the two dimensional-virus neutralization test. At the nucleotide level, 47 single variants were identified, of which 19.00% were in the 5' untranslated region (UTR), 79.00% in the polyprotein region, and 2.00% in the 3' UTR region. Approximately half of the single nucleotide polymorphisms that have occurred in 1D gene resulted in amino acid (AA) substitutions in the VP1 structure. The single nucleotide polymorphisms also caused AA substitutions in other structural proteins, including VP2 and VP3, and some non-structural proteins (L^{pro}, 2C, and 3A). The O/PanAsia-2/QOM-15 shared higher sequence similarity with O/PanAsia-2 (91.00%) compared to O/PanAsia (87.30%). Evaluating r-value showed that the antigenic relationship of O/PanAsia-2/QOM-15 with O/PanAsia-2 (29.00%) was greater than that of the O/PanAsia (24.00%); however, all three viruses were immunologically distinct. After 10 years, the alteration of virus antigenicity and the lack of detectable adaptive pressure on VP1 sequence suggest that studying genetic dynamics beyond the VP1 region is necessary to evaluate FMDV pathogenicity and vaccine failure.

Introduction

Foot-and-mouth disease (FMD) is a contagious viral disease of livestock caused by the foot-and-mouth disease virus (FMDV) belonging to the genus *Aphthovirus*, a member of the family *Picornaviridae*.[1] Despite worldwide attempts to eliminate FMDV, FMD continues to be a major concern for animal health in many countries, having a negative impact on economics.[2,3] The FMDV genome consists of an 8.40-kb single-stranded RNA encoding a single open reading frame divided into three sections, known as P1, P2, and P3. Viral proteases cleave P1 to form four capsid proteins (VP1–VP4), while P2 and P3 are cleaved to form 10 non-structural proteins. The protein-coding sequences are located between two untranslated regions (UTRs): a large 5´UTR (1,300 nucleotide [nt]) and a short 3´UTR (90 nt) that have important roles in the initiation and regulation of RNA replication.[4] Among all capsid-encoding sequences, the 1D region encoding VP1 with 213 amino acid (AA) is of major importance.[5] Binding the G-H loop of VP1 to the integrin receptor on the cell surface is essential for the FMDV to infect the host, which is attributed to the conserved tripeptide motif (Arg, Gly, and Asp) located on the loop.[5] There are seven established FMDV serotypes: O, A, C, Asia1, and South African Territories "SAT 1, 2, and 3"; each diverges into genetically different strains, lineages, and specific geographical topotypes.[6] Infection or vaccination with one serotype does not provide cross-protection against other serotypes. Moreover, it does not provide complete immunity against

***Correspondence:**

Seyed Mahmoud Azimi. DVM, PhD
Foot and Mouth Disease Reference Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran
E-mail: m.azimi@rvsri.ac.ir

different subtypes or strains within a serotype.[2,7] Thus, precise antigenicity knowledge of circulating FMDV serotypes which could be predicted by amino acid (AA) changes resulting from single nucleotide polymorphism (SNP) in specific antigenic regions of the virus is critical for effective control strategies and vaccine development. Conventional approaches are either based on the VP1-encoding site or restricted to a limited number of sequences in the capsid-encoding region (P1).[7,8] Emerging whole genome sequencing (WGS) provides us with opportunities to study high-resolution genomics characterization not only of the P1 region, but also of SNP patterns in FMDV whole-genome sequences caused by evolutionary forces via mutations or recombination.[9]

The FMD is endemic in Iran, and outbreaks of two serotypes of FMDV have been frequently reported in this region.[10] In 2010, a large epidemic caused by the strain O/PanAsia-2 occurred in Iran, which was detected simultaneously in other Asian countries, such as China, Japan, South Korea, Thailand, Turkey, and the United Arab Emirates. From 2010 to 2016, O/PanAsia-2 (JN676146) antigenically covered the field isolated FMDV type O viruses. As a result, it was designated a vaccine strain and included in the vaccine. Despite widespread immunization against O/PanAsia-2, an epidemic of FMD type O was identified in 2016 in the Qom province and progressively spread to other areas. The outbreak was successfully stopped with the substitution of the new emerging strain O/PanAsia-2/QOM-15 into the vaccine.

In the present study, we attempted to sequence and characterize the whole genome of the vaccine strain FMDV O/PanAsia-2/QOM-15 and compare the VP1 sequence of this emerging virus to the previously used vaccine strains O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146).

Materials and Methods

Sample collection. The O/PanAsia-2/QOM-15 virus was retrieved from the FMDV master stock at the FMDV Reference Laboratory in 2021. This virus had been collected from the FMDV outbreak in 2016 in the Qom province, located in the central part of Iran.

RNA extraction. The working viral stock of O/PanAsia-2/QOM-15 was prepared by inoculating the IBRS-2 cell line. Based on the manufacturer's instructions, the supernatant of infected IBRS-2 cells was used for total RNA extraction using TRIzol® (Invitrogen, Friendswood, USA). The extracted viral RNA was quantified by a NanoDrop spectrophotometer (NanoDrop® ND-1000, Thermo Fisher Scientific Inc. Wilmington, USA) at wavelengths of 230, 260, and 280 nm. The extracted RNA was mixed with 20.00 µL sodium acetate 3.00 M (Merck KGaA, Darmstadt, Germany) plus 440 µL of pure ethanol and transferred for whole transcriptome sequencing (Macrogen Co., Seoul, South Korea).

Reverse transcription polymerase chain reaction (RT-PCR) verification. The O/PanAsia-2/QOM-15 viral RNA was reverse-transcribed into cDNA using supplied random hexamers (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and oligo (dT) by the Prime RT Premix 2x kit (Genet Bio, Daejeon, South Korea), according to the manufacturer's protocol. In the next step, the cDNA was subjected to polymerase chain reaction (PCR) for the 1D gene amplification. The primer sets used during reverse-transcription and PCR were *O–1C564F*: AATTACACATGGC AAGGCCGACGG and *NK72R*: GAAGGGCCCAGGGTTGGACTC, which were previously published by Knowles and Samuel.[11] The forward primer (*1C564F*) targeted the 1C gene at position 564 and the reverse primer (*NK72R*) targeted the 2A gene at region 34-48. The size of the expected amplicons after PCR was 800 bp. The PCR program included 5 min incubation at 95.00 ˚C followed by 35 cycles of 94.00 ˚C for 45 sec, 56.00 ˚C for 45 sec, and 72.00 ˚C extension time for 45 sec. The amplified DNA fragments were visualized by agarose gel electrophoresis 1.50% (Bio-Rad Laboratories Inc., Hercules, USA) and purified by the PCR product purification kit (AccuPrep® Gel Purification Kit; Bioneer, Daejeon, South Korea). Finally, PCR amplicons were subjected to sequencing (Macrogen). The final consensus of the 1D gene sequence was compared to any relevant sequence using the Nucleotide Biological Local Alignment Tool (BLAST) available in NCBI database.

Library construction and RNA-Seq analysis. The RNA purity and RNA integrity numbers were measured using the Tape Station System (Macrogen). Ribosomal RNA was depleted from the whole RNA and the remaining RNA was then used to construct cDNA libraries using the TruSeq Stranded Total RNA LT Sample Prep Kit with Ribo-Zero (Illumina Inc., San Diego, USA). Quality control of the cDNA libraries was performed using a DNA 1,000 chip on an Agilent Technologies 2,100 Bioanalyzer, and library concentrations were estimated using the Qubit standard quantification solution and calculator. Eligible libraries (with concentration greater than 10.00 nM) were sequenced using the Illumina NovaSeq instrument (Illumina), resulting in the generation of almost 30.00 million paired-end reads with an average length of 101 bp. CLC Genomics Workbench Software (version 20.0; Qiagen, Venlo, The Netherlands) was used to adapter and quality trim the reads using default parameters: (i) removal of short reads (15 nt cutoff), and (ii) max. ambiguities: 2. The CLC is a user-friendly bioinformatics tool that supports comprehensive analysis of next-generation sequencing (NGS) data, including de novo assembly of whole genomes.[12,13] The clean reads were mapped to the reference genome O/IRN/9/2016 (accession No. MT944981) and the mapped read collections were assembled using the *de novo* assembly option with a minimum contig length of 200 and a word size of 20 (Table 1).

Table 1. The O/PanAsia-2/QOM15 contig sequence (8185 nucleotide in length). The sequence was generated using CLC Genomics Workbench Software (version 20.0; Qiagen, Venlo, The Netherlands).

```
   1 cgctagggtc tcacccctag catgccaccg gcacctcctg cgttgcactc cacacttacg
  61 cacgtgcact cgcgggaatc gatggactgt tgttcaccca cctacagctg gactcacggc
 121 accgcgtggc cattttagct ggattgtgcg gacgaacacc gcttgcgcat ctcgcgtgac
 181 cggttagtac tcttaccacc ttccgcctac ttggtcgtta gcgctgtctt gggcactcct
 241 gttgggggtc gtccaacgct ccacgatctc ccccgtgtga cggactacgg tgatggggcc
 301 gcctcgtgcg agttggtcgt ctggtgtgct ttggctgtca ctcgaagccc acctttcacc
 361 cccccccccc cccccacgct taccgtcgtt cccgacgtta aagggatgaa accacaagct
 421 tgaaaccgtc ttgtctgacg ttaacgggct gtaaccacac gcttgtaccg cctttcccgg
 481 cgttaaaagg gatgtaacca caagctatac cttcgcccgg aagtaaaacg gcaactacac
 541 tcagttttgc ccgttttcat gagaaacggg acgtctgcgc acgaaacgcg ccgtcgctcg
 601 aggcagactt gcacaaacac gactcacgca agtttccaca accgacacac aacgtgcaac
 661 ttgaaacccc gcctggtctt tccaggtcta gaggggcgac actttgtact gtgattgact
 721 ccacgctcgg tccactggcg ggtactagta acaggactgt tgtttcgtag cggagcacga
 781 tggccgtggg acttcccccct tggtaacaag gacccacggg gccaaaagcc acgtccaaac
 841 ggacccatcg tgtgtgcaac cccagcacgg taactttact gtgaaaacta ctttaaggtg
 901 acactgatac tggtactcaa ccactggtga caggctaagg atgcccttca ggtaccccga
 961 ggtaacacgc gacactcggg atctgagaag gggactggga cttctttaaa agtgcccagt
1021 ttaaaaagct tctatgcctg aataggcgac cggaggccgg cgccttttcc ttaactaata
1081 ccagacttat gaatacaact gactgcttta tcgctctact gcacgctctt agagagatta
1141 aagcgctttt ccgtacacga acacaaggaa acatggaatt tacactctac aacggtgaga
1201 aaaagacctt ctactctagg cccaacaacc acgacaactg ttggttgaac accatccttc
1261 agctgtttag gtatgtcgat gaaccattct tcgactgggt ctatgattca cctgaaaacc
1321 ttactcttca agcaattagg caattagagg aactcaccgg ccttgaactg cacgaaggcg
1381 gaccacccgc tctcgtcatc tggaacatca aacatctgct ccacaccgga atcggcaccg
1441 cctcgcgacc tagtgaggtg tgtgtggttg acggtacgga catgtgtttg gctgatttcc
1501 atgctggtat cttcctgaaa ggacaagagc atgctgtgtt cgcctgcgtc acatccgatg
1561 ggtggtacgc gattgatgac gaggacttct acccctggac gccggacccg tccgacgttc
1621 tggtttttgt cccgtacgat caagagccac tcaacgggga atggcagaca aaggttcaga
1681 aacgactcaa aggcgccggg caatccagcc cggcgactgg gtcacagaac cagtcaggca
1741 acactggaag cattattaac aactactaca tgcaacagta ccagaactct atggacacac
1801 aacttggtga caacgctatt agcggaggct ccaacgaagg gtccacggac accacttcca
1861 cccacacaac caacacacag aacaacgact ggttttcaaa gctggccagt tccgctttta
1921 gcggcctttt cggcgctctt cttgccgaca aaaagaccga ggagaccacc cttctcgagg
1981 accgcatcct caccacccgt aacggacaca cgacctcgac aacccagtcg agtgttggag
2041 ttacctacgg gtacgcaaca gccgaggatt ttgtgagcgg accaaacaca tccggccttg
2101 agaccagggt tatgcaagca gagcggttct ttaaaaccca cttgttcgac tgggtcacca
2161 gtgacccgtt cggacgatgc cacctactgg aacttccgac tgaccacaaa ggtgtctacg
2221 gcagcctgac tgactcttat gcttacatga gaaacggttg ggatgtcgag gtcactgcag
2281 tgggaaatca gtttaacgga ggatgcctgt tggtggccat ggtgccagaa ctttgctcca
2341 tcaacaagag agagctgtac cagctcacgc tcttccccca ccagttcatc aacccccgga
2401 cgaacatgac ggcacacatt accgtgccct ttgttggcgt caatcgctac gaccagtaca
2461 aggtacacaa accttggacc ctcgtggtca tggtcgtggc cccgctgact gtcaacaatg
2521 aaggtgctcc acagatcaag gtttatgcca acatcgcccc taccagcgtg cacgtcgcgg
2581 gtgagttccc ttccaaggaa gggatcttcc ctgtggcatg tagcgttggt tacggcggtt
2641 ttgtgactac tgacccaaag acggctgacc ccgcctacgg gaaagttttc aaccccctc
2701 gcaacatgtt gccggggagg ttcaccaatt tccttgacgt ggctgaggcg tgtcctacgt
2761 ttctgcactt tgaaggcgac gtaccatacg tggtcacaaa gacggattcg gacaggatgc
2821 tctctcagtt tgacttgtct ttggcagcga agcacatgtc aaacaccttc ctggcgggtc
2881 ttgcccagta ctacacacag tacagtggca ccattaacct gcacttcatg ttcactggac
2941 ccactgacgc gaaagcgcgt tacatgattg catatgcccc acctggcatg gagccgccta
3001 agacacccga ggcagccgct cattgcattc atgcggagtg ggacactggg ttgaattcaa
3061 aatttacatt ctcaatccct tacctttcgg cagctgatta cgcgtacact gcgtctgaca
3121 ctgctgagac cacaaacgtg cagggatggg tttgcctgtt tcaaatcaca cacgggaagg
3181 ccgatggtga cgcacttgtc gttctggcta gcgccggtaa ggacttcgag ctgcggttac
3241 cagttgacgc tcgcacgcag accacctcca caggtgagtc agccgacccc gtgactgcca
3301 ctgttgaaaa ctacggtggc gagacacagg tccagagacg ccagcacacg gacgtttcgt
3361 tcgtgctgga cagatttgtg gaagtgacac cacgagatca aattaatgtg ctggacctga
3421 tgcaaacccc cgcccacact ttggtaggcg cgctcctccg caccgccacc tactatttcg
3481 cagatttaga ggtggcagtg aaacacgaag ggaaccttac ctgggtaccc aatggagcgc
3541 ctgagacggc gttgggcaac accaccaatc caacggccta ccgcaaggcg ccactcaccc
3601 gacttgcact gccctacacg gcaccacacc gtgtcttggc taccgtctac aacggggact
3661 gcaagtatgg cgagagccgc gcaaccaacg tgagaggtga cctacaagtg ttggcacaga
3721 aggcggccag aacgctgcct acctccttca actacggtgc cattaaagcc accagggtga
3781 ctgaactgct ttaccgcatg aagagggctg aaacatactg cccccggcct cttttggcca
3841 ttcagccgag tgaagcgaga cacaaacaaa agatagtggc acctgtgaaa caacttttga
3901 acttcgacct gctcaagttg gcaggggacg ttgagtccaa ccctgggccc ttcttcttct
3961 ccgacgtgag gtcgaacttc tccaaactgg tagacaccat caaccagatg caggaggaca
4021 tgtcaacaaa acacggaccc gactttaacc ggctggtgtc cgcgtttgag gaattggcca
4081 ctggagtgaa agctattagg actggtctcg acgaggccaa accctggtac aagctcatca
4141 aactcctgag ccgcctgtca tgcatggccg ctgtagcagc acggtcaaag gacccagtcc
4201 ttgtggccat tatgctagct gacaccggtc tcgagattct ggacagcacc tttgttgtga
4261 agaagatctc cgactcgctc tccagtctct ttcacgtgcc ggcccccgtc ttcagtttcg
4321 gagccccgat tctgttggcc gggttggtca aagtcgcctc gagtttcttc cggtctacac
4381 ccgaagacct tgagagagca gagaaacagc tcaaagcacg tgacatcaat gacattttcg
4441 ccattcttaa gaacggcgag tggctggtca agctgatcct tgctatccgc gactggatta
4501 aagcatggat tgcctcagaa gagaagtttg tcaccatgac agacttggtg cctggcatcc
4561 ttgaaaaaca gcgggacctc aacgacccaa gtaagtacaa ggaagccaag gagtggctcg
4621 acaacgcgcg ccaggcgtgc ttgaagagcg ggaacgtcca cattgctaac ctctgcaaag
4681 tggtcgcccc ggcacccagc aagtcgaggc ccgaacctgt agtcgtttgc ttccgtggca
4741 agtccggcca gggtaagagt ttcctagcga acgtgctcgc acaagcaatc tccacccact
4801 tcacgggcag aaccgattca gtttggtact gcccgcctga ccctgaccac ttcgacggtt
4861 acaaccagca gaccgttgtt gtgatggatg atctgggcca gaaccccgac ggcaaggact
4921 tcaagtactt cgcccagatg gtttcgacca cggggtttat cccgcccatg gcttcgctcg
4981 aggacaaagg taaacctttc aacagcaaag tcatcattgc caccaccaac ctgtactcgg
5041 gcttcacccc gagaaccatg gtgtgccccg atgcactgaa ccgaaggttt cactttgata
5101 ttgatgtgag tgccaaggac gggtacaaaa ttaacaataa attggacatt atcaaagctc
5161 ttgaagacac ccacaccaac ccagtggcaa tgttccagta tgattgtgcc cttctcaacg
5221 gcatggccgt tgagatgaag agaatgcaac aggacatgtt caagcctcaa ccgcccctcc
5281 agaacgtgta tcaactggtt caggaggtga ttgaacgggt tgagctccac gagaaagtgt
5341 cgagccatcc gatcttcaag cagatctcaa ttccttccca aaaatctgtg ctgtacttcc
5401 tcattgagaa aggtcagcat gaagctgcaa ttgaattctt tgaggggatg gtgcacgact
5461 ccatcaagga ggagctccga cctctcattc aacagacatc atttgtgaag cgcgctttca
5521 agcgcctgaa ggaaaatttt gagattgtcg ccctgtgttt gaccctcttg gcaaacatag
5581 taatcatgat ccgcgagact cgcaagagac aacagatggt ggatgatgca gtgaatgagt
5641 acattgagaa agctaacatc accacggacg acaagactct tgacgaggcg gaaaagaacc
5701 ctctggagac tagcggtgcc agcaccgttg gctttagaga gagaactctt ccaggacaaa
5761 aggcgagtga tgacgtgaac tccgagcccg ccaaacctgc aggagaacaa ccacaagctg
5821 aaggacccta cgccgggcca ctcgagcgtc agaaacctct gaaagtgcgc gccaagctgc
5881 cacagcagga ggggccctac gctggcccga tggagagaca gaaaccgctg aaggtaaaag
5941 caaaagcccc ggtcgttaag gaaggacctt acgagggacc ggtgaagaag cctgtcgctt
6001 tgaaagtgaa agcaaagaac ttgattgtca ctgagagtgg tgccccaccg accgacttgc
6061 aaaagatggt catgggcaac acaaagcctg ttgagctcat cctcgacggg aagacagtag
6121 ccatctgctg tgctactgga gtgtttggta ctgcctacct cgtgcctcgt catcttttcg
6181 cagagaagta tgacaagatc atgttggacg gcagagccat gacagacagt gactacagag
6241 tgtttgagtt tgagattaaa gtaaaaggac aggacatgct ctcagacgcc gcgctcatgg
6301 tgcttcaccg tgggaaccgc gtgcgtgaca tcacgaaaca cttccgtgat gttgcaaaga
6361 tgaagaaagg cacccccgtc gttggtgtga tcaacaacgc tgacgttggg agactgattt
6421 tctctggtga ggctcttacc tacaaagaca ttgtggtgtg catggatggt gacaccatgc
6481 ctggcctctt tgcctacaaa gccgccacca aggctggtta ctgtggaggg gccgttctcg
6541 caaaggacgg agccgagact ttcatcgtcg gcacccactc tgcaggaggc aatggagttg
6601 gatactgctc atgcgtttcc aggtccatgc ttcttaaaat gaaggcacac attgaccctg
6661 aaccacacca cgaggggttg attgttgaca ccagagatgt ggaagaacgc gtccacgtga
6721 tgcgcaaaac caagcttgcg cccaccgtag cgcacggtgt gttcaaccct gactttggcc
6781 ccgctgcctt gtccaacaag gacccgcggc tgaacgaagg tgttgtcctc gatgaagtca
6841 tcttctccaa acacaaaggg gacacaaaga tgacagagga agacaagaag ctgttccggc
6901 gctgtgctgc tgactacgcg tcacgcctgc actccgtgtt gggtacggca aatgccccat
6961 tgagcattta cgaggcaatc aaaggtgttg atggactcga cgccatggaa ccagacaccg
7021 cgcccggtct cccctgggcc ctccagggaa agcgccgtgg cgccctgatc gacttcgaga
7081 acggcacggt cggacccgaa gttgcagctg ccttagagct catggagaaa agagagtaca
7141 agtttgcttg ccagaccttc ctgaaggacg agattcgccc gatggagaaa gtgcgtgccg
7201 gcaagactcg cattgtcgat gttttgcccg tcgaacacat tctttacacc aggatgatga
7261 ttggcagatt ctgtgctcaa atgcactcaa acaacggacc gcaaattggc tcggcggtcg
7321 gttgtaaccc tgatgttgat tggcaaagat ttggcaccca ttttgctcag tacagaaacg
7381 tgtgggatgt ggactattcg gcctttgatg ccaaccactg cagtgacgca atgaacatca
7441 tgtttgagga ggtgtttcgc acggaattcg gtttccaccc gaacgcagag tggatcctga
7501 agactctcgt gaacacggaa cacgcctatg agaacaaacg catcactgtt gagggcggga
7561 tgccatctgg ctgttccgca acaagcatca tcaacacaat tctgaacaac atctatgtgc
7621 tctacgccct gcgtagacac tatgagggag ttgagctgga cacttacacc atgatctcct
7681 atggagacga tatcgtggtt gcaagtgatt acgatttgga cttcgaggct ctcaagcctc
7741 actttaaatc tcttggtcaa accatcactc cagctgacaa aagcgacaaa ggttttgttc
7801 ttggtcactc catcaccgat gtcactttcc ttaaaagaca cttccacatg gattatggaa
7861 ctgggtttta taaacctgtg atggcctcaa agacccttga ggctatcctc tcctttgcac
7921 gccgtgggac catacaggag aagttgatct ccgtggcagg actcgccgtc cactctggac
7981 ctgacgagta ccggcgtctc tttgagccct tccagggtct ctttgagatt ccaagctaca
8041 gatcacttta cctgcgttgg gtgaacgccg tgtgcggcga cgcataatcc ctcagatgtc
8101 acaattggca gaaagactct ggggcgagca gcgccgtagg agtgaaaagt ccgaaagggc
8161 ttttcccgct tcctaattca aaaaa
```

The consensus sequence of O/PanAsia-2/QOM-15 virus was generated and then registered in GenBank under the accession No. OK019689. Annotation of the genome was carried out using the virus reference genome (accession No. MT944981), and the final sequence of the O/PanAsia-2/QOM-15 was BLASTed against the NCBI database with an expected value threshold of 0.0001.

SNP discovery of the O/PanAsia-2/QOM-15. The whole-genome sequence of O/PanAsia-2/QOM-15 was mapped to the corresponding reference sequence O/IRN/9/2016 (accession No. MT944981), and SNPs were identified using the "Basic Variant Detection" tool implemented in the CLC Genomics Workbench Software. Table 2 presents the SNPs that caused AA substitutions and all variant frequencies detected in this study. The quality filter for SNP discovery was set to meet the following criteria: (i) neighborhood radius 5; (ii) min. central quality 20; (iii) min. neighborhood quality 15; and (iv) min. frequency of 5.00%.

1D gene sequencing. The evolution of the 1D gene sequence of serotype O, which was utilized as a vaccine strain for 10 years (2006 - 2016), was analyzed in this work. To address it, the 1D gene sequences of the two older vaccine strains, O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146), were compared with O/PanAsia-2/QOM-15 as a new vaccine strain candidate. The parent viral seeds of O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146) that were preserved in the archive of the virus seed bank were used for RNA extraction using a high-purification viral extraction kit (Roche). The amplification of the 1D gene was performed using the previously described protocol. The size of the expected amplicons after PCR was 800 bp.

Phylogenetic and variation analysis based on VP1-encoding region. The 1D gene sequences of O/PanAsia-2/QOM-15, O/PanAsia-2 (JN676146), and O/PanAsia (JQ321837) were aligned using the ClustalW approach implemented in Geneious Prime 2019 (Biomatters, Auckland, New Zealand). In the next step, aligned sequences (639 bp) were imported into the MEGA Software (version 7.0; Biodesign Institute, Tempe, USA) package to evaluate the 1D gene variability among the strains.[14] The evolutionary distance between the 1D gene sequences and the transition/transversion ratio bias (R) were determined using the maximum likelihood (ML) technique and the Kimura two-parameter model algorithm, respectively.[15]

Preparation of reference antiserum. A reference antiserum against the O/PanAsia-2/QOM-15 virus was acquired using a 10-month-old male calf. Based on the producer's recommendation, the calf was immunized with two doses of the commercial polyvalent vaccine (containing O/PanAsia-2/QOM-15, A05IR, and Asia1). Two doses of vaccine were injected 21 days apart, and 28 days after receiving the second dose, the calf blood anti body titer was measured by the seroneutralization test according to World Organisation for Animal Health (OIE) protocol.[2]

Antigenic characterization of the O/PanAsia-2/QOM-15 virus. To determine the antigenic similarity between O/PanAsia-2/QOM-15, O/PanAsia (JQ321837) and O/PanAsia-2 (JN676146), a two-dimensional virus neutralization test was carried out based on OIE instruction.[2] The antibody titers were calculated using the regression test, with the log10 of reciprocal antibody dilution required to produce 50.00% neutralization of 100 tissue culture viral infectious units (log10SN50%, 100TCID50 per mL).The antigenic relationship (r-value) was estimated using the following equation in Minitab Software (version 16.0; Minitab Inc., Pennsylvania, USA): (reciprocal neutralizing titer of reference antiserum against the O/PanAsia-2 or O/PanAsia) / (reciprocal neutralizing titer of the reference antiserum against the O/PanAsia-2/QOM-15). If the r-value between two viruses is calculated to be greater than or equal to 30.00%, the viruses have an antigenic relationship; otherwise, they are antigenically distinct.

Results

O/PanAsia-2/QOM-15 virus verification. The genomic RNA of O/PanAsia-2/QOM-15 virus was extracted from the supernatant of infected IBRS-2 cells. The concentration and purity (A_{260}/ A_{280} ratio) of viral RNA were determined to be 304 ng μL^{-1} and 2.10, respectively. A RT-PCR test was used to validate the O/PanAsia-2/QOM-15 strain by amplifying the 1D gene. By sequencing and blasting the virus's 1D gene in the GenBank, O/IRN/9/2016 (MT944981) was found to be a homologous virus, since the 1D gene sequences of these two viruses had 99.00% identity. This finding confirmed the virus for WGS and further analysis.

Whole-genome sequencing of the O/PanAsia-2/QOM-15. The whole-genome sequence of O/PanAsia-2/QOM-15 with an RNA integrity number of 8.30 was generated by RNA-Seq technology, and the clean reads were mapped to the reference genome O/IRN/9/2016 (accession No. MT944981). Among the total 30,371,798 reads, 1.06% belonged to the virus, and the unmapped reads (i.e., IBRS-2 cell) were discarded. The O/PanAsia-2/QOM-15 whole-genome sequence is available in GenBank under the accession No. OK019689.

SNP discovery of the O/PanAsia-2/QOM-15. The comparative analysis of the complete genome of O/PanAsia-2/QOM-15 and O/IRN/9/2016 showed 99.00% nucleotide identity with some SNPs (Table 2). We found 47 single variants in the O/PanAsia-2/QOM-15 genome sequence at the nucleotide level, including 9 (19.00%) in the 5'UTR region, 37 (79.00%) in the polyprotein region, and one (2.00%) in the 3'UTR area of the viral genome (Table 2).

Most variants were in the polyprotein region of the virus, with the largest share (44.00%) in the 1D gene region. Changes in the AA sequences of both structural and non-structural proteins were produced by 16 variations at the AA level (Table 2). There were no AA substitutions in the 5´ and 3´UTRs as well as in the non-structural regions including: P2, P3, 3B, 3C, and 3D (Table 2). In this article, the number of AA positions within the relevant viral proteins rather were used than the polyprotein. Three-dimensional homology-based modeling of O/PanAsia-2/QOM-15 VP1 with marked SNPs is shown in Figure 1. As shown, eight nucleotide substitutions result in five AA replacements in the VP1 structure including: I35M/V, K41E/N, D99G, H108R, and K171T, none of which are located exactly in three antigenic positions.

Table 2. Predicted SNPs in the whole genome of O/PanAsia-2/QOM-15 in comparison to O/IRN/9/2016 (MT944981) as a reference genome. The SNP discovery was performed using CLC Genomics Workbench Software.

No.	Reference position	Type	Reference	Allele	Frequency (%)	Overlapping annotations	AA change	Relevant viral protein
1	37	SNP	G	A	99.497	5' UTR: 5' UTR	-	-
2	67	SNP	C	T	98.863	5' UTR: 5' UTR	-	-
3	503	SNP	C	T	99.457	5' UTR: 5' UTR	-	-
4	609	SNP	T	C	99.749	5' UTR: 5' UTR	-	-
5	622	SNP	T	C	99.793	5' UTR: 5' UTR	-	-
6	698	SNP	C	T	30.514	5' UTR: 5' UTR	-	-
7	867	SNP	T	C	99.798	5' UTR: 5' UTR	-	-
8	881	SNP	C	T	99.508	5' UTR: 5' UTR	-	-
9	974	SNP	A	G	11.676	5' UTR: 5' UTR	-	-
10	1153	SNP	T	G	99.875	CDS: polyprotein	Ser19Ala	L^{pro}
11	1474	SNP	A	G	84.337	CDS: polyprotein	Met126Val	L^{pro}
12	1779	SNP	T	C	99.844	CDS: polyprotein	-	-
13	1800	SNP	C	T	99.744	CDS: polyprotein	-	-
14	1953	SNP	C	T	99.860	CDS: polyprotein	-	-
15	1980	SNP	T	C	99.251	CDS: polyprotein	-	-
16	2025	SNP	A	C	99.556	CDS: polyprotein	-	-
17	2122	SNP	G	A	99.749	CDS: polyprotein	Val56Met	VP2
18	2354	SNP	G	A	99.882	CDS: polyprotein	Ser133Asn	VP2
19	2376	SNP	T	C	99.838	CDS: polyprotein	-	-
20	2636	SNP	A	T	99.586	CDS: polyprotein	Asp9Val	VP3
21	2650	SNP	C	T	93.870	CDS: polyprotein	Leu14Phe	VP3
22	3147	SNP	T	C	99.771	CDS: polyprotein	-	-
23	3294	SNP	T	C	99.725	CDS: polyprotein	-	-
24	3373	SNP	A	G	99.778	CDS: polyprotein	Ile35Val	VP1
25	3375	SNP	A	G	65.956	CDS: polyprotein	Ile35Met	VP1
26	3391	SNP	A	G	64.643	CDS: polyprotein	Lys41Glu	VP1
27	3393	SNP	A	T	9.595	CDS: polyprotein	Lys41Asn	VP1
28	3566	SNP	A	G	94.370	CDS: polyprotein	Asp99Gly	VP1
29	3593	SNP	A	G	99.670	CDS: polyprotein	His108Arg	VP1
30	3600	SNP	A	G	99.413	CDS: polyprotein	-	-
31	3776	SNP	A	C	7.540	CDS: polyprotein	Lys171Thr	VP1
32	4080	SNP	A	G	99.823	CDS: polyprotein	-	-
33	4776	SNP	T	A	99.839	CDS: polyprotein	-	-
34	5143	SNP	A	G	46.200	CDS: polyprotein	Asn248Asp	2C
35	5162	SNP	C	T	99.428	CDS: polyprotein	Thr254Ile	2C
36	5211	SNP	C	T	99.785	CDS: polyprotein	-	-
37	5559	SNP	T	C	99.839	CDS: polyprotein	-	-
38	5592	SNP	G	A	99.795	CDS: polyprotein	-	-
39	5810	SNP	T	C	99.894	CDS: polyprotein	Val146Ala	3A
40	6480	SNP	A	T	99.613	CDS: polyprotein	-	-
41	6627	SNP	C	T	99.834	CDS: polyprotein	-	-
42	6900	SNP	A	G	99.779	CDS: polyprotein	-	-
43	7008	SNP	T	C	99.939	CDS: polyprotein	-	-
44	7335	SNP	C	T	99.885	CDS: polyprotein	-	-
45	7734	SNP	T	C	99.773	CDS: polyprotein	-	-
46	7740	SNP	C	T	99.635	CDS: polyprotein	-	-
47	8132	SNP	A	G	99.767	3' UTR: 3' UTR	-	-

The amino acid (AA) position has shown by number within relevant viral protein rather than the polyprotein.

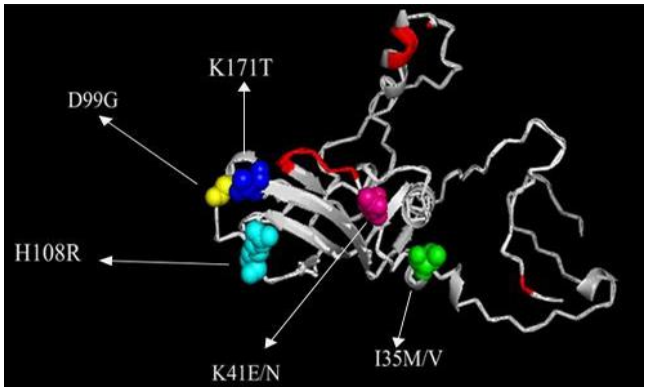

Fig. 1. Cartoon diagram of the O/PanAsia-2/QOM-15 VP1 based on homology modeling. Predicted SNPs resulting in AA substitutions are marked on the protein surface (VP1) in a sphere shape with different colors, while residues that participate in three antigenic sites are shown in red.

Genetic variability of the 1D gene and antigenic characterization of the O/PanAsia-2/QOM-15 virus. Evolutionary distance analyses were conducted using the MEGA7 package among three sequences of O/PanAsia-2/QOM-15, O/PanAsia-2, and O/PanAsia based on the VP1-encoding sequence with 639 positions. The transition/transversion ratio bias (R) was 3.83, and the maximum rate of different substitutions belonged to transversions A/G→C (30.41%). There was no significant evolutionary pressure on the VP1-encoding regions since dN/dS was interpreted as neutral. There were 59 nt variations in the O/PanAsia-2/QOM-15 1D gene compared to O/PanAsia-2 (JN676146), 81 nt in O/PanAsia-2/QOM-15 compared to O/PanAsia (JQ321837), and 65 nt in O/PanAsia-2 compared to O/PanAsia. The genetic distance between O/PanAsia-2/QOM-15 and O/PanAsia-2 and O/PanAsia was 0.10 and 0.14, respectively. O/PanAsia-2/QOM-15 shared more homology with O/PanAsia-2 than with O/PanAsia (91.00% vs. 87.30%). Similarly, O/PanAsia-2 had higher homology with O/PanAsia-2/QOM-15 (91.00%) than O/PanAsia (89.80%). The evolutionary distance between O/PanAsia-2 and O/PanAsia was also estimated 0.11. As shown in Table 3, the O/PanAsia-2/QOM-15 virus had a significant antigenic difference (r-value ≤ 30.00%) with both strains. The antigenic identity of O/PanAsia-2/QOM-15 virus with O/PanAsia-2 (29.00%) was higher than that of the O/PanAsia-2/QOM-15 virus against O/PanAsia (24.00%).

Discussion

When FMDV is circulating in the field, mutations and consequent changes in the virus's antigenic characteristics are critical adaptive strategies. Moreover, in the course of FMD vaccine preparation, repeated passages in cell culture subject the viral genome to "adaptive hot-spot" mutations. These mutations enable the virus to evolve and propagate more efficiently *in vitro*.[8,16] The emergence of new variations is significant, particularly when they occur in the sequences of structural proteins, since structural protein modifications can result in the introduction of pathogenic mutants that evade the host's immune system.[17] Major mutations comprise SNPs. However, the significant accumulation of these mutations, mainly in the antigenic sites, can impact considerably on virus antigenicity and vaccine efficiency. Evaluating SNPs in the O/PanAsia-2/QOM-15 sequence showed that mutations did not arise uniformly throughout the genome; approximately 80.00% of SNPs appeared in the polyprotein region, and half of them occurred in VP1 (Table 2). This finding is predictable since the highest variable residues (74.00%) in the structural protein VP1 play a significant role in developing escape mutants.[18,19] As shown in Figure 1, none of five the AA replacements in the VP1 structure (I35M/V, K41E/N, D99G, H108R, and K171T) are exactly in three antigenic positions. Previous studies have revealed five putative neutralizing antigenic sites in FMDV serotype O. Three of them (1, 3, and 5) were found on VP1, which included residues VP1-144, VP1-148, VP1-150, and VP1-208 in the C-terminal end of VP1, the critical residue VP1-149 in the G-H loop, and residues VP1-43, VP1-44, VP1-45, and VP1-48 in the B-C loop.[8,17,20] Many researchers have suggested that antigenic epitopes in FMDV genome are beyond those detected by monoclonal antibodies, and mutations in the residues near the antigenic regions can affect the binding of neutralizing antibodies.[20-23] For example, Sarangi *et al*. found that the VP1-41 near the B-C Loop antigenic region was substituted following immune selection *in vitro*.[21]

The Glu/Asn were previously replaced by Lys in the O/PanAsia-2/QOM-15 VP1-41 region, thus converting the VP1-41 positive AA to a negatively or neutrally charged residue as for a means of evading host antibody-mediated immunity (Table 2). As shown in Figure 1, another alteration detected in VP1 was at position 108 (His→Arg),

Table 3. The antigenic relationship (r-value) of three FMDV vaccine strains. The r-value was obtained from the two dimensional-virus neutralization test.

Taxonomy	O/PanAsia-2/QOM-15	O/PanAsia-2 (JN676146) Local name O2010	O/PanAsia (JQ321837)
Serotype	O	O	O
Topotype	Middle East-South Asia	Middle East-South Asia	Middle East-South Asia
Lineage	PanAsia-2	PanAsia-2	PanAsia
Sub lineage	QOM-15	FAR-09	-
O/PanAsia-2/QOM-15	100%	29.00%	24.00%
O/PanAsia-2 (JN676146)	-	-	26.00%

An r-value ≤ 30.00% indicates a significant antigenic difference between two relative viruses.

which increases the positive charge of the protein in the fivefold axis of the capsid for virus adaptation to cell culture if accompanied by the substitution at position 142.[8,18] However, our analysis did not detect any AA substitution at VP1-142, nor any change in AA net charge, since the positive charge AA arginine substituted the same charge histidine.

Table 2 also revealed, another detected AA alteration at VP2-133 (Ser→Asn) in antigenic site 2. At this site, there is a pocket made up of VP2 130–134 whose mutations affect the binding of G-H loop of VP1 to neutralizing antibodies.[24] Besides, substitution in VP2 may affect receptor tropism by altering the heparan sulfate binding site.[8,25] Since substitution at VP2-133 replaces the AA serine with the AA asparagine, both of which have the potential to form hydrogen bonds with the sugar moiety of heparan sulfate, this substitution does not appear to affect the overall binding process of the virus to its receptor.

Recently, a phylogenetic study based on VP2 sequence showed that in some FMDV serotypes (O, A, and Asia1), the VP2 sequence is less divergent among the three exposed capsid proteins (VP1-3).[26] There were four inter-serotypically conserved sites with high antigenicity values within the VP2 sequence: VP2 (1 - 14), VP2 (24 - 36), VP2 (48 - 55), and VP2 (114 - 124). Researchers have proposed these VP2 sequences as good candidates for developing serotype-independent diagnostic methods.[26] This finding is consistent with our study, as there are two AA substitutions within the VP2 sequence: VP2 - 133 (Ser→Asn) and VP2 - 56 (Val→Met), none of which were found in the four conserved fragments (Table 2).

Due to the great degree of conservation across capsid-encoding areas, mutations in VP4 are exceedingly uncommon.[27] Although two SNPs were identified in the VP4 sequence, none of these alterations resulted in an AA change (Table 2). This may be due to the internal location of this protein in the capsid, as mutation of this protein provides no beneficial advantages for the virus either in escaping the host immune system or selecting a cell surface receptor for viral entry.[28]

There were two AA substitutions in the L^{pro} region: L^{pro}-19 (Ser→Ala) and L^{pro}-126 (Met→Val), (Table 2). Both substitutions appear to be important, as a polar residue (serine) capable of forming a hydrogen bond has been substituted with a non-polar residue (alanine), and methionine has been substituted with a more hydrophobic residue (valine). There is also a single AA substitution in non-structural protein 3A-146 (Val→Ala). Certain evidence implies that non-structural protein sequences have variable sections; consequently, mutations in these regions may change host tropism or the severity of FMD in a particular host.[17] For example, it was shown that non-structural protein 3A, which is required for viral replication, is a genetic determinant of altered host tropism in a Taiwanese foot-and-mouth disease virus. Similarly, truncating 3A reduced the severity of FMD in cattle.[29] Pierce *et al*. studied polyprotein processing of FMDV and demonstrated that different precursors were produced due to a single mutation at the $3B_3$-3C junction.[30] Another study conducted by Yang *et al*. identified 70-nuceotide deletions in the S fragment and a single leucine insertion in L^{pro}-10 as novel determinants of restricted FMDV growth in bovine host cells.[31] They suggested that synergistic mutations in the S fragment and L^{pro} lead to host specificity of the FMDV serotype O strains.[31]

Three times changes in the vaccine strain over the ten-years period from 2006 to 2016 resulted in 59 nucleotide variations in the O/PanAsia-2/QOM-15 1D gene compared to O/PanAsia-2 (JN676146), 81 nucleotide variations in O/PanAsia-2/QOM-15 compared to O/PanAsia (JQ321837), and 65 nucleotide variations in O/PanAsia-2 compared to O/PanAsia. This result is unexpected, given that the O/PanAsia-2/QOM-15 had an extra 6 years to accrue point mutations. This result is corroborated by the r-value determined during the serological examination. As shown in Table 3, the antigenic identity of O/PanAsia-2/QOM-15 virus with O/PanAsia-2 was higher than that of the O/PanAsia-2/QOM-15 virus against O/PanAsia. Since the r-value of O/PanAsia-2/QOM-15 with O/PanAsia-2 and O/PanAsia was less than 30.00%, it was concluded that the O/PanAsia-2/QOM-15 virus had a significant antigenic difference with both strains (Table 3).

In conclusion, the fact that half of the SNPs are located outside the VP1-encoding region suggests that additional encoding sites contribute to altering the virus's antigenic characteristics. However, further study is required to ascertain the effect of variant sequences other than VP1 on FMDV pathogenicity.

Acknowledgments

This study was supported by grants from the Iran National Science Foundation (INSF; 97023106) and the Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran (2-18-18-033-000428).

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Zell R, Delwart E, Gorbalenya AE, et al. ICTV virus taxonomy profile: Picornaviridae. J Gen Virol 2017; 98(10): 2421-2422.
2. OIE. Foot-and-mouth disease (infection with foot and mouth disease virus). Paris, France: World Organization for Animal Health; 2022.
3. Compston P, Limon G, Häsler B. A systematic review of

the methods used to analyze the economic impact of endemic foot-and-mouth disease. Transbound Emerg Dis 2022; 69(5): e2249-e2260.
4. Belsham GJ. Towards improvements in foot-and-mouth disease vaccine performance. Acta Vet Scand 2020; 62(1): 20. doi: 10.1186/s13028-020-00519-1.
5. Dong H, Liu P, Bai M, et al. Structural and molecular basis for foot-and-mouth disease virus neutralization by two potent protective antibodies. Protein Cell 2022; 13(6): 446-453.
6. Brito BP, Rodriguez LL, Hammond JM, et al. Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. Transbound Emerg Dis 2017; 64(2): 316-332.
7. Upadhyaya S, Mahapatra M, Mioulet V, et al. Molecular basis of antigenic drift in serotype O foot-and-mouth disease viruses (2013–2018) from Southeast Asia. Viruses 2021; 13(9): 1886. doi: 10.3390/v13091886.
8. Dill V, Eschbaumer M. Cell culture propagation of foot-and-mouth disease virus: adaptive amino acid substitutions in structural proteins and their functional implications. Virus Genes 2020; 56(1): 1-15.
9. Brown E, Freimanis G, Shaw AE, et al. Characterising foot-and-mouth disease virus in clinical samples using nanopore sequencing. Front Vet Sci 2021; 8: 656256. doi: 10.3389/fvets.2021.656256.
10. Emami SJ, Bahonar AR, Mehrabadi MHF, et al. Evaluation of foot and mouth disease (FMD) vaccine using registered surveillance data. Trop Anim Health Prod 2022; 54(4): 215. doi: 10.1007/s11250-022-03204-9.
11. Knowles NJ, Samuel AR. RT-PCR and sequencing protocols for the molecular epidemiology of exotic virus diseases of animals. Institute of Animal Health, Pirbright Laboratory, Surry, UK. 1998 6; 37.
12. Sutton TDS, Clooney AG, Ryan FJ, et al. Choice of assembly software has a critical impact on virome characterisation. Microbiome 2019; 7(1): 12. doi: 10.1186/s40168-019-0626-5.
13. Ghorbani A, Samarfard S, Eskandarzade N, et al. Comparative phylogenetic analysis of SARS-CoV-2 spike protein - possibility effect on virus spillover. Brief Bioinform 2021; 22(5): bbab144. doi: 10.1093/bib/bbab144.
14. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016; 33(7): 1870-1874.
15. Lukas SS. Molecular characterization of foot-and-mouth disease Virus recently recovered in Zambezi region, Namibia. PhD Thesis. Sokoine University of Agriculture. Morogoro, Tanzania: 2020.
16. Mahravani H, Deljoo M. Assessment of foot and mouth virus subtype O2016 genetic alterations during successive passages in BHK monolayer. Iran J Virol 2019; 13(2): 1-10.
17. Li K, Wang C, Yang F, et al. Virus - host interactions in foot-and-mouth disease virus infection. Front Immunol 2021; 12: 571509. doi: 10.3389/fimmu.2021.571509.
18. Dill V, Hoffmann B, Zimmer A, et al. Adaption of FMDV Asia-1 to suspension culture: cell resistance is overcome by virus capsid alterations. Viruses 2017; 9(8): 231. doi: 10.3390/v9080231
19. Han SC, Guo HC, Sun SQ. Three-dimensional structure of foot-and-mouth disease virus and its biological functions. Arch Virol 2015; 160(1): 1-16.
20. Harmsen MM, Li H, Sun S, et al. Mapping of foot-and-mouth disease virus antigenic sites recognized by single-domain antibodies reveals different 146S particle specific sites and particle flexibility. Front Vet Sci 2023; 9: 1040802. doi: 10.3389/fvets.2022.1040802.
21. Sarangi LN, Mohapatra JK, Subramaniam S, et al. Antigenic site variation in foot-and-mouth disease virus serotype O grown under vaccinal serum antibodies in vitro. Virus Res 2013; 176(1-2): 273-279.
22. Lee HW, Yang CY, Lee MC, et al. The use of distinctive monoclonal antibodies in FMD VLP- and P1-based blocking ELISA for the seromonitoring of vaccinated swine. Int J Mol Sci 2022; 23(15): 8542. doi: 10.3390/ijms23158542.
23. Li K, Wang S, Cao Y, et al. Development of foot-and-mouth disease virus-neutralizing monoclonal antibodies derived from plasmablasts of infected cattle and their germline gene usage. Front Immunol 2019; 10: 2870. doi: 10.3389/fimmu.2019.02870.
24. Mahapatra M, Yuvaraj S, Madhanmohan M, et al. Antigenic and genetic comparison of foot-and-mouth disease virus serotype O Indian vaccine strain, O/IND/R2/75 against currently circulating viruses. Vaccine 2015; 33(5): 693-700.
25. Lee G, Hwang JH, Kim A, et al. Analysis of amino acid mutations of the foot-and-mouth disease virus serotype O using both heparan sulfate and JMJD6 receptors. Viruses 2020; 12(9): 1012. doi: 10.3390/v12091012.
26. Mishu ID, Akter S, Alam ASMRU, et al. In silico evolutionary divergence analysis suggests the potentiality of capsid protein VP2 in serotype-independent foot-and-mouth disease virus detection. Front Vet Sci 2020; 7: 592. doi: 10.3389/fvets.2020.00592.
27. Fish I, Stenfeldt C, Palinski RM, et al. Into the deep (Sequence) of the foot-and-mouth disease virus gene pool: Bottlenecks and adaptation during infection in Naïve and vaccinated cattle. Pathogens 2020; 9(3): 208. doi: 10.3390/pathogens9030208.
28. Asfor AS, Upadhyaya S, Knowles NJ, et al. Novel antibody binding determinants on the capsid surface of serotype O foot-and-mouth disease virus. J Gen Virol 2014; 95(Pt 5): 1104-1116.
29. Pacheco JM, Gladue DP, Holinka LG, et al. A partial

deletion in non-structural protein 3A can attenuate foot-and-mouth disease virus in cattle. Virology 2013; 446(1-2): 260-267.
30. Pierce DM, Hayward C, Rowlands D, et al. Insights into polyprotein processing and RNA-protein interactions in foot-and-mouth disease virus genome replication. J Virol 2023: 97(5): e0017123. doi: 10.1128/jvi.00171-23.
31. Yang F, Zhu Z, Cao W, et al. Genetic determinants of altered virulence of type O foot-and-mouth disease virus. J Virol 2020; 94(7): e01657-19. doi: 10.1128/JVI.01657-19.

ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (11) 625 - 630
doi: 10.30466/vrf.2023.560224.3650
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Effects of dietary resveratrol supplementation on digestive enzymes activities and serum biochemistry of rainbow trout (*Oncorhynchus mykiss*)

Alireza Afzali-Kordmahalleh[1], Saeid Meshkini[2]*

[1] Post-graduate of Veterinary Medicine, Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran;
[2] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Article Info

Article history:

Received: 24 October 2022
Accepted: 20 June 2023
Available online: 15 November 2023

Keywords:

Digestive enzymes
Rainbow trout
Resveratrol
Serum biochemistry

Abstract

The effects of resveratrol as an anti-oxidant in improving growth and health have been shown in several experiments. This study aimed to evaluate the effects of different dietary resveratrol inclusion levels on digestive enzymes activity and serum biochemistry of rainbow trout (*Oncorhynchus mykiss*). Accordingly, 225 juvenile rainbow trout with an average body weight of 10.00 ± 1.50 g were stocked in nine experimental units. The study was performed as a completely randomized design including three dietary levels of resveratrol as follows: 0.00, 400 and 800 mg kg^{-1} feed. During the experiment, fish were fed based on their respective body weight using standard feeding tables at three feeding times for 8 weeks. Nine fish were randomly selected from each treatment at the end of the 4th and 8th weeks of the experiment. Results revealed that supplementing 800 mg kg^{-1} feed resveratrol significantly increased lipase activity (31.40 ± 0.32 U mg^{-1} protein) compared to the control group (29.92 ± 0.52 U mg^{-1} protein) at the end of week eight. Also, at the same time, it increased serum high-density lipoprotein (123.04 ± 1.57 mg dL^{-1}) compared to the control group (97.055 ± 1.463 mg dL^{-1}). In addition, dietary supplementation of 800 mg kg^{-1} feed resveratrol effectively reduced serum alanine aminotransferase, alkaline phosphatase and aspartate aminotransferase activities along with glucose, cortisol and cholesterol. In conclusion, resveratrol can be used as a suitable food supplement to improve fish health by increasing digestive enzymes activities.

Introduction

Stress is one of the leading causes of growth retardation in fish farms especially in intensive cultures, making the fish use energy in other activities instead of conserving energy for growth and tissue synthesis.[1,2] Furthermore, stress may exhibit general effects in fish, such as changing serum total protein, albumin, glucose, cortisol, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), urea and triglyceride contents.[3,4] Reactive oxygen species (ROS) that cause these stresses are one of the body's most important free radicals mediating cellular mechanisms such as cellular transmission and transcriptional control.[5] Nevertheless, their excessive increase may lead to oxidative changes in cellular macromolecules, such as lipids, proteins and nucleic acids causing growth retardation or death in fish.[6] Moreover, stress decreases the ability of fish immune system and provides suitable conditions for pathogens to cause disease.[7] Studies have reported that increasing digestive enzymes activities may change the proportion of microbiota to beneficial ones by washing out the harmful microbiota in addition to efficient digestion and absorption of food.[8,9] Accordingly, fish breeders prefer using natural compounds to improve the function of fish immune system and confront stresses.[10,11]

Resveratrol is a lipid-soluble phytoalexin being present in fruits and vegetables effectively improving the immune system function. This natural anti-oxidant significantly reduces plasma triglycerides, free fatty acids, cholesterol and triglycerides entering the liver.[1,12] Resveratrol, being mostly as a glucuronide sulfate in plasma, effectively decreases the free radicals in body through enhancing the mitochondrial anti-oxidant enzymes level and changing the kinase activity as well as cellular signaling path improving the fish health.[13-17]

This study was conducted with the aim of investigating the effects of dietary supplementation of resveratrol on

***Correspondence:**

Saeid Meshkini. DVM, PhD
Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: s.meshkiniy@urmia.ac.ir

fish health and resistance to environmental stresses by evaluating digestive enzymes, liver enzymes and some of the above-mentioned biochemical parameters in rainbow trout.

Materials and Methods

Fish husbandry. A total of 225 juvenile rainbow trout with an average body weight of 10.00 ± 1.50 g were obtained from a local fish farm in Urmia, West Azerbaijan province, Iran. Fish were transferred to the Aquaculture Center of the Veterinary Medicine Faculty of Urmia University, Urmia, Iran. After a week of acclimation, fish were weighed to determine biomass and randomly divided into three experimental groups with three replications. Each treatment contained 75 fish in 300-L tanks (25 fish per tank) with a volume of 200 L of water per tank. During the experimental period (8 weeks), fish were hand-fed at 2.50% of their initial biomass three times a day. However, they were fasted for 24 hr before any handling or sampling in order to decrease fish metabolism and ammonia and maintain water quality. Water quality characteristics were as follows: pH: 7.30 - 7.50, dissolved oxygen: 8.50 ± 0.50 ppm and temperature: 14.00 ± 1.00 ˚C being daily monitored. All of the breeding water was replaced with filtered water every day. After an hr of feeding, uneaten feed was siphoned from the bottom of the tanks to maintain the water quality. All the experiments were performed based on the standard animal experimentation protocols of the Veterinary Ethic Committee of Urmia University, Urmia, Iran (Approval ID: IR-UU-AEC-3/34).

Diet preparation. Rainbow trout commercial fingerling food trout-1 (FFT1; including protein: 42.00%, crude fiber: 3.00%, fat: 14.00% and ash: 7.00%) was prepared as a basal diet from Faradaneh Company, Shahrekord, Iran. The experimental diets were consisted of a basal diet with inclusion of 400 and 800 mg kg^{-1} feed resveratrol according to the literature.[17,18] Resveratrol (purity > 99.00%) was purchased from Sigma-Aldrich (St. Louis, USA), weighed and added to the basal diet. Afterward, 4.00% gelatin was sprayed on the commercial food. The food was dried at room temperature and refrigerated at 4.00 ˚C until use. The control group was fed a basal diet containing 4.00% gelatin without any resveratrol supplementation. The experimental diets were weekly prepared.

Sampling. At the end of the weeks four and eight of the experiment, fish were anesthetized by dipping method using 100 ppm eugenol (Sigma-Aldrich) in aerated water 24 hr after the last meal.[19,20] Nine fish from each dietary treatment were sampled randomly (three from each experimental unit). Blood samples were taken from the caudal vein using syringes with a 22-gauge needle and reserved in non-heparinized micro-tubes for hematological analyses. Moreover, the intestine was removed, washed in physiological saline (0.90%) and stored in encoded tubes after removing its contents and visceral fat. All of these steps were performed on ice. Finally, these samples were kept at – 70.00 ˚C until digestive enzymes activities determination.

Digestive enzyme activities determination. Amylase activity was determined by the starch-hydrolyzing method, according to Bernfeld.[21] Starch is degraded to maltose by the amylase enzyme and measured using a dinitrosalicylic acid reagent (Sigma-Aldrich) through colorimetric and color intensity changes.[21] The hydrolysis of p-nitrophenyl myristate (Merck, Darmstadt, Germany) was used to determine the lipase enzyme activity using spectrophotometry (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).[22] Protease enzyme activity was measured using hydrolysis of 1.50% azocasein substrate in 50.00 mM Tris-HCl buffer (Merck) at pH of 7.50.[23,24]

Liver enzymes activities determination in serum. Blood samples were immediately centrifuged at room temperature (15 min at 3,000 rpm) and stored at – 20.00 ˚C. Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels was performed by colorimetric method; while, alkaline phosphatase (ALP) measurement was done by enzymatic method.[25]

Blood biochemical parameters measurement. Bradford method was used to measure serum soluble protein content. In this method, bovine serum albumin (Biowest, Nuaillé, France) was used as a standard. Also, to measure other biochemical parameters, blood samples were immediately centrifuged at room temperature (15 min at 3,000 rpm) and kept at – 20.00 ˚C until measurement.[26] Then, glucose, cholesterol, HDL, LDL, creatinine, urea, phosphorus, triglyceride and cortisol levels were measured using the spectrophotometer and Pars Azmoon kits (Tehran, Iran).[25]

Statistical analysis. Normality of data was examined using the Kolmogorov-Smirnov test and Levene's test was used to assess the homogeneity of variances. Data were analyzed using one-way analysis of variance and *post-hoc* Tukey HSD test by SPSS Software (version 20.0; IBM Corp., Armonk, USA). All tests were interpreted at a significance level of < 5.00%. The results were presented as mean ± standard deviation. Microsoft Office Excel (version 15.0; Microsoft Corp., Redmond, USA) was used to draw the charts.

Results

The 800 mg kg^{-1} resveratrol showed the highest levels of lipase, amylase and protease activities compared to the other treatments ($p < 0.05$; Fig. 1). At the end of the 8th week, statistical analysis showed a significant increase in lipase enzyme activity between the 800 mg kg^{-1} resveratrol treatment (31.40 ± 0.32 U mg^{-1} protein) and control groups (29.92 ± 0.52 U mg^{-1} protein; $p < 0.05$; Fig. 1). Also, 800 mg kg^{-1} resveratrol (37.53 ± 0.31 U mg^{-1} protein) showed a significant increase in protease enzyme activity

compared to the control group (36.28 ± 0.57 U mg^{-1} protein; $p < 0.05$; Fig. 1). However, no significant difference was observed in the amylase activity of fish between experimental groups ($p > 0.05$; Fig. 1).

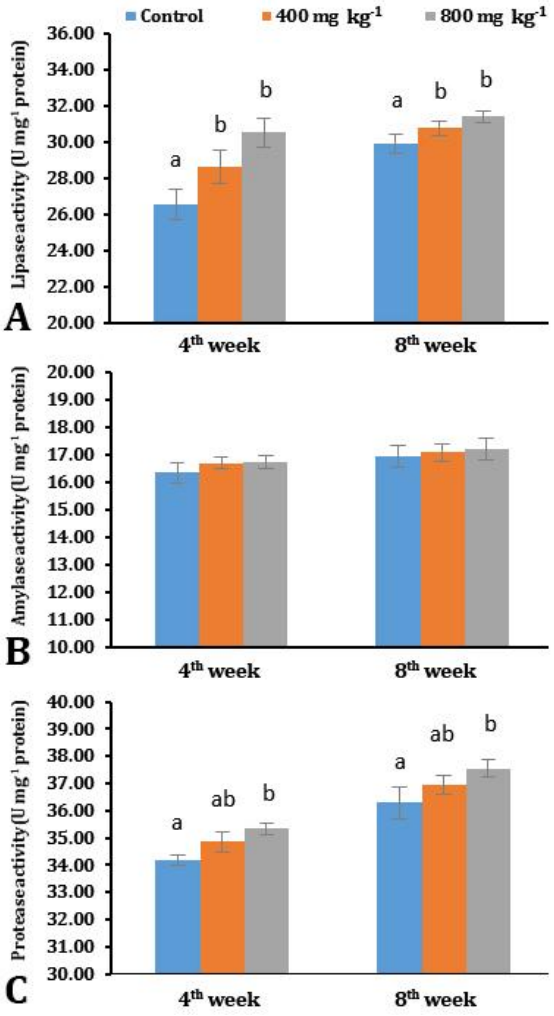

Fig. 1. Digestive enzymes activities at the end of weeks four and eight of the experiment. **A)** Lipase; **B)** Amylase; and **C)** Protease. [ab] Different letters indicate significantly differences at $p < 0.05$.

Dietary supplementation of 800 mg kg^{-1} resveratrol significantly reduced all measured liver enzymes activities compared to the control group in weeks 4 and 8 of the experiment ($p < 0.05$). The results indicated that ALT activity significantly decreased in fish fed with resveratrol ($p < 0.05$). However, no significant difference was observed between 400 and 800 mg kg^{-1} resveratrol treatments ($p > 0.05$; Fig. 2). Unlike 400 mg kg^{-1} treatment, the 800 mg kg^{-1} resveratrol showed a significant reduction in AST activity compared to the control group ($p < 0.05$; Fig. 2). Also, 800 mg kg^{-1} treatment significantly reduced ALP activity compared to the 400 mg kg^{-1} treatment and control groups at the end of the week eight ($p < 0.05$; Fig. 2). The blood biochemical para-meters showed no significant difference between the resveratrol treatment groups and control group regarding total serum protein and albumin contents ($p > 0.05$; Table 1). Furthermore, 800 mg kg^{-1} resveratrol significantly reduced glucose, cortisol, triglyceride and LDL in the blood compared to the control group ($p < 0.05$). However, no significant difference was observed between dietary supplementation of 400 and 800 mg kg^{-1} resveratrol regarding serum glucose and LDL contents ($p > 0.05$). Moreover, dietary 800 mg kg^{-1} resveratrol significantly reduced the serum urea content compared to the 400 mg kg^{-1} resveratrol and control groups at the end of week four ($p < 0.05$). The results also indicated that serum contents of HDL, phosphorus and creatinine were significantly increased in resveratrol treatment groups compared to the control group ($p < 0.05$). Additionally, the 800 mg kg^{-1} treatment showed a significant difference in serum HDL, phosphorus and creatinine compared to the 400 mg kg^{-1} treatment.

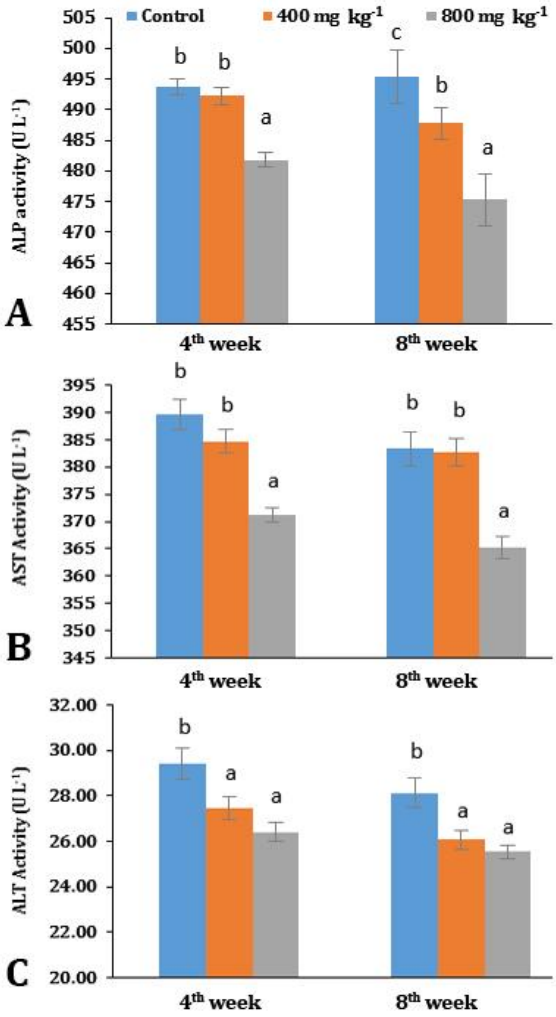

Fig. 2. Liver enzymes activities at the end of weeks 4 and 8 of the experiment. **A)** Alkaline phosphatase ALP; **B)** Aspartate amino-transferase (AST); and **C)** Alanine aminotransferase (ALT). [ab] Different letters indicate significantly differences at $p < 0.05$.

Table 1. Blood biochemical parameters of different experimental groups.

Parameters	Sampling time (Day)	Control	Resveratrol (400 mg kg^{-1})	Resveratrol (800 mg kg^{-1})
Total protein (g dL^{-1})	28	3.32 ± 0.18^{a}	3.42 ± 0.25^{a}	3.57 ± 0.26^{a}
	56	4.36 ± 0.30^{a}	4.62 ± 0.51^{a}	4.946 ± 0.42^{a}
Albumin (g dL^{-1})	28	1.66 ± 0.38^{a}	1.77 ± 0.35^{a}	1.98 ± 0.25^{a}
	56	1.88 ± 0.24^{a}	1.91 ± 0.27^{a}	2.16 ± 0.20^{a}
Glucose (mg dL^{-1})	28	68.60 ± 0.78^{b}	67.84 ± 0.51^{ab}	67.11 ± 0.42^{a}
	56	67.73 ± 1.09^{b}	66.32 ± 1.43^{ab}	64.74 ± 1.18^{a}
Cortisol (mg dL^{-1})	28	204.59 ± 1.73^{b}	204.38 ± 1.22^{b}	195.13 ± 1.40^{a}
	56	202.52 ± 1.49^{b}	200.40 ± 1.20^{b}	190.57 ± 1.81^{a}
Cholesterol (mg dL^{-1})	28	339.83 ± 4.60^{b}	338.40 ± 4.77^{b}	331.13 ± 3.11^{a}
	56	330.98 ± 9.39^{b}	321.34 ± 7.14^{b}	297.60 ± 9.54^{a}
High-density lipoprotein (mg dL^{-1})	28	94.83 ± 3.95^{a}	101.31 ± 1.12^{b}	104.18 ± 0.85^{c}
	56	97.05 ± 1.46^{a}	109.34 ± 2.54^{b}	123.04 ± 1.57^{c}
Low-density lipoprotein (mg dL^{-1})	28	155.26 ± 2.05^{b}	151.82 ± 1.90^{ab}	148.01 ± 1.64^{a}
	56	152.51 ± 2.35^{b}	150.40 ± 3.00^{ab}	146.38 ± 1.748^{a}
Creatinine (mg dL^{-1})	28	0.27 ± 0.02^{a}	0.36 ± 0.00^{b}	0.43 ± 0.01^{c}
	56	0.28 ± 0.00^{a}	0.30 ± 0.00^{b}	0.33 ± 0.00^{c}
Urea (mg dL^{-1})	28	5.19 ± 0.29^{b}	5.13 ± 0.27^{b}	4.10 ± 0.25^{a}
	56	5.89 ± 0.33^{b}	5.39 ± 0.12^{a}	5.07 ± 0.22^{a}
Phosphorus (mg dL^{-1})	28	15.66 ± 0.27^{a}	16.27 ± 0.12^{b}	16.55 ± 0.05^{c}
	56	17.78 ± 0.21^{a}	18.26 ± 0.00^{b}	18.38 ± 0.00^{c}
Triglyceride (mg dL^{-1})	28	328.01 ± 7.60^{a}	326.60 ± 4.81^{a}	321.63 ± 3.63^{a}
	56	352.79 ± 3.77^{c}	345.45 ± 2.59^{b}	330.83 ± 2.46^{a}

^{abc} Different superscript letters indicate significant differences at $p < 0.05$.

Discussion

Increased digestive enzymes activities lead to efficient digestion and absorption of food and increases in harmful microbiota washing out.[27] Various studies have been performed on herbal supplements or medicinal herbs containing anti-oxidants as gastrointestinal stimulants.[28,29] In the present study, 800 mg kg^{-1} resveratrol treatment significantly increased the activities of lipase and protease compared to the control group confirming the results of Liu *et al.*, showing that combined anti-oxidants (40.00 mg kg^{-1} vitamin C and 80.00 mg kg^{-1} vitamin E) could effectively improve digestive enzymes activities and growth of discus (*Symphysodon haraldi*).[30]

A study on the stem of *Hopea ponga* showed that one of the resveratrol oligomers, alpha-viniferin, had the most significant effect in preventing non-enzymatic reaction of sugars with protein.[31] It increased the activities of digestive enzymes such as glucosidase and amylase. Also, alpha-viniferin and trihydroxyphenanthrene glucoside, resveratrol oligomers, significantly increased glucose uptake into the cells. This raise occurred mainly due to the rearrangement of adenosine monophosphate-activated protein kinase, and eventually, by enhancing glucose transporter 4 activity, glucose transfer to the cell membrane will increase.[32,33]

Previous studies showed that some plant compounds stabilizing cell membranes could protect cells against destructive agents such as free radicals.[34,35] Thus, liver enzymes such as ALT, ALP and AST were significantly lower in fish fed with antioxidant-containing feed than fish fed by a basal diet.[34-36] Arinç *et al.*, investigated the mechanism of five flavonoids including resveratrol, inhibiting cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) and glutathione S-transferase in the fish liver. They discovered that resveratrol effectively inhibited the ethoxyresorufin-O-deethylase-dependent CYP1A1 enzyme.[37] As a result, this anti-oxidant, being also a potent cancer preventer, can effectively maintain liver health.[38-40] This study found that 800 mg kg^{-1} resveratrol treatment was the most effective one in significant lowering of ALT, ALP and AST activities compared to the control group.

Serum glucose and cortisol levels increase in stress conditions. Anti-oxidants such as resveratrol remove ROS and reduce oxidative stresses.[40] Although in the present study there were no differences between resveratrol treatments and the control group regarding the amounts of total protein and albumin, significant reduction of the amounts of cortisol and glucose was reported. Numerous studies have shown that grape products can increase adiponectin, a hormone being associated with increased blood HDL levels and reduced LDL levels.[41-43] In the current study, HDL levels in all three groups were significantly different, indicating that resveratrol in both doses of 400 and 800 mg kg^{-1} can effectively increase blood HDL. Resveratrol might help prevent damage to blood vessels, reduce LDL and prevent blood clots. There was no study regarding the effects of resveratrol on blood biochemical parameters of fish, such as urea, phosphorus, creatinine and triglyceride. However, several studies have shown that this anti-oxidant is a potent anti-glomerulonephritis food factor in humans, suppressing proteinuria, hypoalbuminemia and hyperlipidemia simultaneously. Moreover, resveratrol significantly lowered cholesterol and triglyceride levels in cholesterol-

fed rats compared to the control group.[12,44] As the results, except ALP, urea and triglyceride, the pattern of changes between experimental groups regarding digestive and liver enzymes as well as blood biochemical parameters was similar with each other in weeks four and eight.

In conclusion, the present study showed that resveratrol increased the activities of digestive enzymes including lipase and protease, which might result in improved digestion efficiency, better nutrients uptake and ultimately better fish performance. Resveratrol also reduced liver enzymes activities via assisting in hepatocytes detoxification, oxidative stress elimination and fish health promotion. In addition, resveratrol improved fish health by increasing HDL, lowering LDL and reducing fish stress through lowering blood glucose. It is recommended to include 800 mg kg^{-1} resveratrol in trout feed to improve fish health and performance.

Acknowledgments

The authors are thankful to the Research Deputy of Urmia University and the Veterinary Medicine Faculty of Urmia University, Urmia, Iran for the financial support.

Conflict of interest

The authors declare no conflict of interest regarding this study.

References

1. Terech-Majewska E, Siwicki AK, Hermańska S, et al. Influence of trans-resveratrol on macrophage and lymphocyte activity in rainbow trout (*Oncorhynchus mykiss*) – *in vitro* study. Cent Eur J Immunol 2018; 43(3): 241-247.
2. Naderi Farsani M, Meshkini S, Manaffar R. Growth performance, immune response, antioxidant capacity and disease resistance against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*) as influenced through singular or combined consumption of resveratrol and two-strain probiotics. Aquacu Nutr 2021; 27(6): 2587-2599.
3. Yousefi M, Hoseini SM, Vatnikov YA, et al. Rosemary leaf powder improved growth performance, immune and antioxidant parameters, and crowding stress responses in common carp (*Cyprinus carpio*) fingerlings. Aquaculture 2019; 505: 473-480.
4. Baharloei M, Heidari B, Zamani H, et al. Effects of heat shock protein inducer on Hsp70 gene expression and immune parameters during *Streptococcus iniae* infection in a Persian sturgeon fry. Vet Res Forum 2021; 12(4): 473-479.
5. Amoushahi M, Salehnia M. Reactive oxygen species level, mitochondrial transcription factor A gene expression and succinate dehydrogenase activity in metaphase II oocytes derived from *in vitro* cultured vitrified mouse ovaries. Vet Res Forum 2018; 9(2): 145-152.
6. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta 2016; 1863(12): 2977-2992.
7. Tort L. Stress and immune modulation in fish. Dev Comp Immunol 2011; 35(12): 1366-1375.
8. Dawood MA, Shukry M, Zayed MM, et al. Digestive enzymes, immunity and oxidative status of Nile tilapia (*Oreochromis niloticus*) reared in intensive conditions. Slov Vet Res 2019; 56(Suppl 22): 99-108.
9. Mirghaed AT, Yarahmadi P, Hosseinifar SH, et al. The effects singular or combined administration of fermentable fiber and probiotic on mucosal immune parameters, digestive enzyme activity, gut microbiota and growth performance of Caspian white fish (*Rutilus frisii kutum*) fingerlings. Fish Shellfish Immunol 2018; 77: 194-199.
10. Wunderlich AC, Zica É de OP, Ayres VF dos S, et al. Plant-derived compounds as an alternative treatment against parasites in fish farming: a review. Natural remedies in the fight against parasites. InTech; 2017. doi: 10.5772/67668.
11. Delmas D, Aires V, Limagne E, et al. Transport, stability, and biological activity of resveratrol. Ann N Y Acad Sci 2011; 1215: 48-59.
12. Zhu L, Luo X, Jin Z. Effect of resveratrol on serum and liver lipid profile and antioxidant activity in hyperlipid-emia rats. Asian-Aust J Anim Sci 2008; 21(6): 890-895.
13. Muzzio M, Huang Z, Hu SC, et al. Determination of resveratrol and its sulfate and glucuronide metabolites in plasma by LC–MS/MS and their pharmacokinetics in dogs. J Pharm Biomed Anal 2012; 59: 201-208.
14. Li X, Yao Y, Wang S, et al. Resveratrol relieves chlorothalonil-induced apoptosis and necroptosis through miR-15a/Bcl2-A20 axis in fish kidney cells. Fish Shellfish Immunol 2020; 107(Part B): 427-434.
15. Porcacchia AS, Moreira GA, Andersen ML, et al. The use of resveratrol in the treatment of obstructive sleep apnea and cancer: a commentary on common targets. J Clin Sleep Med 2022; 18(1): 333-334.
16. Tai YS, Ma YS, Chen CL, et al. Resveratrol analog 4-bromo-resveratrol inhibits gastric cancer stemness through the SIRT3-c-Jun N-terminal kinase signaling pathway. Curr Issues Mol Biol 2021; 44(1): 63-72.
17. Torno C, Staats S, Rimbach G, et al. Effects of resveratrol and genistein on nutrient digestibility and intestinal histopathology of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 2018; 491: 114-120.
18. King RE, Kent KD, Bomser JA. Resveratrol reduces oxidation and proliferation of human retinal pigment

epithelial cells via extracellular signal-regulated kinase inhibition. Chem Biol Interact 2005; 151(2): 143-149.
19. Holloway AC, Keene JL, Noakes DG, et al. Effects of clove oil and MS-222 on blood hormone profiles in rainbow trout *Oncorhynchus mykiss*, Walbaum. Aquacu Res 2004; 35(11): 1025-1030.
20. Guénette SA, Uhland FC, Hélie P, et al. Pharmaco-kinetics of eugenol in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 2007; 266(1-4): 262-265.
21. Bernfeld P. Amylases, α and β. Meth Enzymol 1955; 1: 149-158.
22. Iijima N, Tanaka S, Ota Y. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol Biochem 1998; 18: 59-69.
23. García-Carreño FL, Haard NF. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J Food Biochem 1993; 17(2): 97-113.
24. Chong AS.C, Hashim R, Chow-Yang L, et al. Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). Aquaculture 2002; 203(3-4): 321-333.
25. Borges A., Scotti LV, Siqueira DR, et al. Hematologic and serum biochemical values for jundiá (*Rhamdia quelen*). Fish Physiol Biochem 2004; 30: 21-25.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
27. Xia S, Zhao W, Li M, et al. Effects of dietary protein levels on the activity of the digestive enzyme of albino and normal *Apostichopus japonicus* (Selenka). Aquac Res 2018; 49(3): 1302-1309.
28. Zeilab Sendijani R, Abedian Kenari A, Smiley AH, et al. The effect of extract from dill *Anethum graveolens* on the growth performance, body composition, immune system, and antioxidant system of rainbow trout. N Am J Aquac 2020; 82(2): 119-131.
29. Bilen S, Altief TAS, Özdemir KY, et al. Effect of lemon balm (*Melissa officinalis*) extract on growth performance, digestive and antioxidant enzyme activities, and immune responses in rainbow trout (*Oncorhynchus mykiss*). Fish Physiol Biochem 2020; 46(1): 471-481.
30. Liu HP, Wen B, Chen ZZ, et al. Effects of dietary vitamin C and vitamin E on the growth, antioxidant defence and digestive enzyme activities of juvenile discus fish (*Symphysodon haraldi*). Aquac Nutr 2019; 25: 176-183.
31. Sasikumar P, Lekshmy K, Sini S, et al. Isolation and characterization of resveratrol oligomers from the stem bark of *Hopea ponga* (Dennst.) Mabb. and their antidiabetic effect by modulation of digestive enzymes, protein glycation and glucose uptake in L6 myocytes. J Ethnopharmacol 2019; 236: 196-204.
32. Rose PM, Saranya J, Eganathan P, et al. *In vitro* evaluation and comparison of antioxidant and anti-bacterial activities of leaf extracts of *Hopea ponga* (Dennst.) Mabberly. Int J Green Pharm 2013; 7: 177-181.
33. Lin MH, Hung CF, Sung HC, et al. The bioactivities of resveratrol and its naturally occurring derivatives on skin. J Food Drug Anal 2021; 29(1): 15-38.
34. Engwa GA. Free radicals and the role of plant phytochemicals as antioxidants against oxidative stress-related diseases. Phytochemicals: source of antioxidants and role in disease prevention. InTech; 2018. doi: 10.5772/intechopen.76719.
35. Ghafarifarsani H, Hoseinifar SH, Adorian TJ, et al. The effects of combined inclusion of *Malvae sylvestris*, *Origanum vulgare*, and *Allium hirtifolium boiss* for common carp (*Cyprinus carpio*) diet: Growth performance, antioxidant defense, and immunological parameters. Fish Shellfish Immunol 2021; 119: 670-677.
36. Adeli A, Shamloofar M, Akrami R. Dietary effect of Lemon Verbena (*Aloysia triphylla*) extract on growth performance, some haematological, biochemical, and non-specific immunity and stocking density challenge of rainbow trout juveniles (*Oncorhynchus mykiss*). J Appl Anim Res 2021; 49(1): 382-390.
37. Arinç E, Yilmaz D, Bozcaarmutlu A. Mechanism of inhibition of CYP1A1 and glutathione S-transferase activities in fish liver by quercetin, resveratrol, naringenin, hesperidin, and rutin. NutrCancer 2015; 67(1): 137-144.
38. Singh CK, George J, Ahmad N. Resveratrol-based combinatorial strategies for cancer management. Ann N Y Acad Sci 2013; 1290(1): 113-121.
39. Wu SL, Sun ZJ, Yu L, et al. Effect of resveratrol and in combination with 5-FU on murine liver cancer. World J Gastroenterol 2004; 10(20): 3048-3052.
40. Alfonso S, Gesto M, Sadoul B. Temperature increase and its effects on fish stress physiology in the context of global warming. J Fish Biol 2021; 98(6): 1496-1508.
41. Sierksma A, Patel H, Ouchi N, et al. Effect of moderate alcohol consumption on adiponectin, tumor necrosis factor-alpha, and insulin sensitivity. Diabetes Care 2004; 27(1): 184-189.
42. Pischon T, Girman CJ, Rifai N, et al. Association between dietary factors and plasma adiponectin concentrations in men. Am J Clin Nutr 2005; 81(4): 780-786.
43. Ghaedi E, Moradi S, Aslani Z, et al. Effects of grape products on blood lipids: a systematic review and dose-response meta-analysis of randomized controlled trials. Food Funct 2019; 10(10): 6399-6416.
44. Nihei T, Miura Y, Yagasaki K. Inhibitory effect of resveratrol on proteinuria, hypoalbuminemia and hyperlipidemia in nephritic rats. Life Sci 2001; 68(25): 2845-2852.

CLINICAL REPORT

Veterinary Research Forum. 2023; 14 (11) 631 - 633
doi: 10.30466/vrf.2023.2001992.3876
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Surgical management of an aspirated bone in a Shih Tzu terrier dog: a case report

Vahid Ganjiani*, Amin Bigham-Sadegh, Abdolhamid Meimandi-Parizi, Mohsen Nouroozi, Dariush Sarikhani, Parisa Afraz

Department of Clinical Sciences, Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran.

Article Info

Article history:

Received: 09 May 2023
Accepted: 17 June 2023
Available online: 15 November 2023

Keywords:

Dog
Foreign body
Tracheotomy

Abstract

Foreign bodies in the respiratory tract (trachea/bronchus) are very rare conditions; therefore, it is not on top of the list of differential diagnoses in patients with chronic or severe cough, but its occurrence should not be considered unexpected. A 2-year-old male Shih Tzu terrier mix dog (9.20 kg) with a history of dyspnea for 6 days was referred to the Veterinary Teaching Hospital of Shiraz University, Shiraz, Iran. In the clinical examination, respiratory distress and pain during palpation of the neck area were observed. Radiology indicated a triangular radiopaque object (bone) in the trachea of the animal. The size of the foreign body, the weakness of the grasping forceps of the device, and the edges of the bone being stuck in the trachea caused tracheoscopy failure after 30 min of trying. Surgical procedure (tracheotomy) was effective to remove the foreign body using Noyes alligator tissue forceps. Aspirated foreign body is not a common condition. In the case of early recognition and immediate treatment of the aspirated foreign body, the prognosis is usually good.

Introduction

Among previously reported foreign bodies, tracheal foreign bodies are very uncommon, but their occurrence is not impossible either. A foreign body in the airways (trachea/bronchus) causes acute symptoms, including severe cough and dyspnea.[1] Also, they are difficult to diagnose and treat.[1] Foreign bodies in the trachea and bronchi have been reported very rarely in animals, and probably most of the foreign bodies being aspirated come out with a lot of coughing, but in the patients that have been reported coughing was not effective. So far, cases have been reported in dogs, cats, horses, and chimpanzees.[2] There are different types of foreign objects, including bone fragments, cereal/wheat ears and plant materials, nails, pieces of rubber, teeth, stones, twigs, and bullets.[2] The most common clinical symptoms in cases with aspirated foreign bodies are severe cough, vomiting, and halitosis. Here, we have a very unusual case of foreign object in the trachea of a dog, being treated by tracheotomy.

Case Description

A 2-year-old male Shih Tzu terrier mix dog (9.20 kg) with a history of dyspnea for 6 days was referred to the Veterinary Teaching Hospital of Shiraz University, Shiraz, Iran. In the history taken from the owner of the animal, severe sudden coughs occurred about 6 days ago, but after that the coughs became intermittent and were getting worse in the mornings. In the clinical examination, respiratory distress and pain during palpation of the neck area were observed, but apart from these few signs, no other abnormalities such as cyanosis were observed (temperature: 38.30 ˚C; heart rate: 108 beats per min; respiratory rate: 22 breaths per min). In the radiology, a triangular radiopaque object (2.67 × 1.26 cm) was observed in the cervical trachea at the level of 6th cervical vertebra (near the thoracic inlet; Fig. 1A). These conditions were informed to the animal owner and written consent was obtained to continue the process of diagnosis and treatment. After the final diagnosis, a decision was made on emergency endoscopy.

Complete blood count assessment was performed (all parameters were within the normal range) and the patient was anesthetized for the endoscopy. Initially, 22.00 mg kg^{-1} cefazolin (Daana Pharmaceutical Co., Tabriz, Iran) was administered intramuscularly as a prophylactic antibiotic therapy. In the next step, sedation was induced using a mixture of intramuscular 0.05 mg kg^{-1} acepromazine (Alfasan, Woerden, The Netherlands) and 0.50 mg kg^{-1}

***Correspondence:**

Vahid Ganjiani. DVM, DVSc
Department of Clinical Sciences, Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran
E-mail: Vahid.ganjiani@hafez.shirazu.ac.ir

xylazine (Alfasan). After about 20 min when the patient calmed down, the cephalic vein was catheterized and fixed with adhesive tape. Then, anesthesia was induced with a combination of intramuscular 5.00 mg kg^{-1} ketamine (Bremer Pharma GmbH, Warburg, Germany) and 0.25 mg kg^{-1} midazolam (Exir Pharmaceutical Co., Borujerd, Iran). During anesthesia, the patient was receiving the Ringer solution at a rate of 10.00 mL kg^{-1} per hr (Shahid Ghazi Pharmaceutical Co., Tehran, Iran). Heart rate, respiratory rate, and oxygen saturation were constantly checked during anesthesia. During tracheoscopy, we tried to remove the foreign body with the grasping forceps, but the size of the foreign body, the weakness of the grasping forceps of the device, and the edges of the bone being stuck in the trachea caused tracheoscopy failure after 30 min of trying. In next step, the decision of the surgical team changed to an open approach. In the radiograph, the relative position of the foreign body was determined; so that, the tracheal incision was made in the same position. At this stage, anesthesia was changed from intravenous route to inhalation using 1.50% isoflurane (Minrad International Inc., Orchard Park, USA), but care was taken to insert the tracheal tube as far as possible from the foreign body, and its cuff was also filled. The patient was positioned in the dorsal recumbency, the midline in the neck area was shaved and scrubbed for surgery, and the incision site was draped. The cervical midline was incised, extending the incision from the middle to the sternum, the subcutaneous tissues were blindly dissected, and the sternocephalicus and sternohyoidus muscles were separated. After exposing the trachea, the Metzenbaum scissors were placed under the trachea to hold the trachea in the incision site (Fig. 1B). A transverse incision was made between two of the cartilaginous rings of the trachea on the ventral side; so that, the dorsal surface of the trachea remained intact. At this moment, isoflurane was offed, but the patient was receiving pure oxygen. Ketamine/midazolam mixture was prepared for re-induction, if the patient recovered during surgery. But, we noticed that the tracheal tube had pushed the foreign body forward by a few centimeters. The tracheal tube was slightly pulled back and the foreign body was removed with Noyes alligator tissue forceps with a slight pressure (Fig. 1C). In fact, after removing the foreign body, the reason for the failure of the tracheoscopy was found that the foreign body was large and stuck in the trachea. Indeed, the sharp edges of bone prevented its movement. A small amount of blood was also seen on the thin and sharp edges of the bone; so, the trachea was suctioned to remove any blood or pus. The incision site was sutured with a simple interrupted pattern using 2/0 Vicryl™ (Supa, Tehran, Iran). The muscles were placed together and sutured with a simple continuous pattern with the 2/0 Vicryl™ and the skin was sutured with intradermal pattern using the same material. The patient received fentanyl (5.00 µg kg^{-1}, IV; Caspian Tamin, Rasht, Iran) during surgery. For post-operative care, cephalexin syrup (20.00 mg kg^{-1}, q12hr, orally; Exir Pharmaceutical Co.) and meloxicam (0.10 mg kg^{-1}, q24hr, orally; Jalinous Pharmaceutical Co., Tehran, Iran) were administered. When the case was followed-up (two weeks after surgery), the respiratory distress was completely resolved and the suture site was healed without any complications.

Discussion

A foreign body in the trachea and bronchus, although rare, is life-threatening. The foreign body can enter the airways only when there are interferences with normal reflexes, such as taking a sudden inspiration during eating, running, or playing.[3] These reflexes are not yet complete in puppies and are not effective compared to adults; therefore, the prevalence of this complication has been more reported at young ages.[3] Although this disorder can occur in any breed, but it has been more reported in Labrador retriever breeds.[1] Even in some seasons of the year, the possibility of plant foreign bodies increases, because these are light and easily aspirated. In general, the ears of wheat and barley are known as foreign bodies penetrating deeply because their tentacles are one-sided and do not come out with coughing, but only move down and cause chemical and mechanical irritations.[1]

The size of the foreign body determines the place where it gets stuck, and this itself determines the intensity of the cough; the deeper foreign body, the less severe cough.[3] Depending on the size and type of foreign body, foreign body reaction may occur. If the edges of the foreign

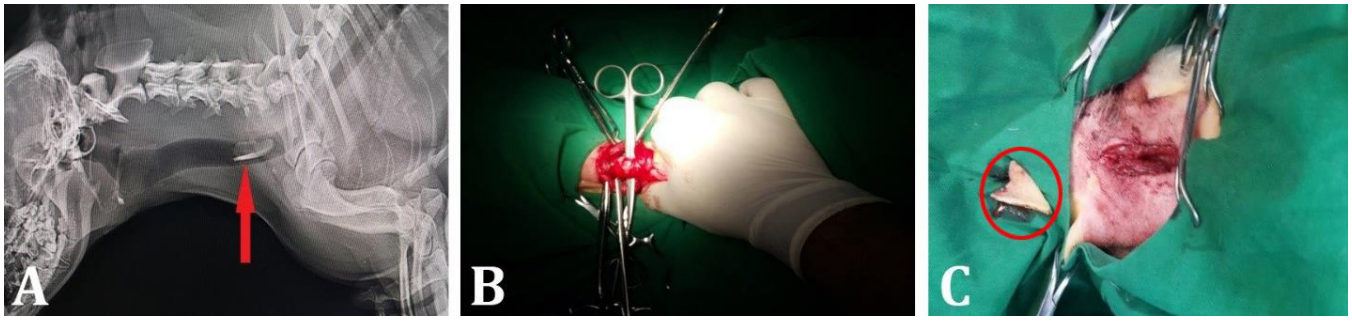

Fig. 1. Tracheotomy procedure performed to remove tracheal foreign body. **A)** The presence of the foreign body in the trachea; **B)** Trachea exposure through a midline incision; **C)** The extracted foreign body (red circle).

body are sharp, it can penetrate into the surrounding tissues and become chronic. If the foreign body enters the bronchus, it mainly enters the right bronchus; the main reason for that is unknown, but probably the right bronchus has a more direct path than the left one.[1] The common term used for respiratory foreign bodies is penetration syndrome, having symptoms such as intractable cough, sudden choking, breathlessness, and wheezing;[3] but the symptoms of this syndrome were not very evident in the current patient.

For a good outcome, rapid diagnosis and treatment should be done. The first step in the diagnosis and treatment of most cases is primary stabilization, including fluid therapy, oxygen therapy, sedation, and pain management. The diagnosis of this disorder is based on history, clinical symptoms and radiology. In the history, there are sudden and severe coughs occurring mainly after running in a dry and woody environment or at a park.[1] In the clinical examination, persistent or intermittent coughs being intensified after resting (also observed in current patient), hemoptysis, and halitosis are observed.[4] In chronic cases, leukocytosis, pyrexia, and lethargy may be seen. Cough is one of the main symptoms in the patient history and clinical examination. In fact, coughing is the first symptom of a respiratory foreign body in such a way that the absence of cough strongly rejects the entry of a foreign body into the airways. Radiology is effective in diagnosing of most foreign bodies, especially if the foreign body is radiopaque. Also, radiology is useful in examining the pulmonary status and the position of the foreign body. The combination of these three techniques greatly increases the power of diagnosis.

Several treatment methods have been reported so far to remove the respiratory foreign bodies; one of the simplest ways is to suspend the patient by its hindlimbs and shake it.[5] In these cases, two general treatment methods are defined consisting of minimally invasive techniques including tracheoscopy, bronchoscopy, and fluoroscopy and open procedure (surgery).[4] In some cases, when the foreign body is close to the larynx, it can be removed by reaching through the oral cavity (without surgery).[6] Depending on the location of the foreign body, surgery can be pre-sternal tracheotomy or thoracotomy. In thoracotomy, when the foreign body is palpable in the bronchus, the bronchotomy technique is used, and in cases where the foreign body has entered the lung tissue, the lung lobectomy is performed through the 4 - 6 inter-costal space. Rigid tracheoscopy/bronchoscopy is currently used as a standard technique in medical sciences, which is also considered as a standard technique in veterinary medicine. Initially, minimally invasive techniques should be chosen, because they have shorter recovery times and fewer complications. However, these techniques are not successful in all cases and the treatment should be surgery.[4] In a similar study, tracheotomy was performed to remove a foreign body (stone) being lodged in the cervical trachea at the level of 3rd - 4th cervical vertebrae.[7] Surgery has more complications, including technical difficulty, potential morbidity, hemorrhage, edema, and inflammation. Even after foreign body removal, a series of complications including pneumonia, pneumothorax, and laryngeal edema have also been reported.[4]

In conclusion, bronchial foreign bodies are rare, but they should be considered in the differential diagnoses of animals with chronic coughs. Bronchoscopy is an effective and non-invasive means of foreign bodies removal in animals that do not spontaneously cough them up. If the dog is too small, the foreign body too big, or if it cannot otherwise be removed via bronchoscopy, surgical intervention is required. The prognosis for survival and long-term return to function is good if the dog survives the immediate post-operative period.

Acknowledgments

The authors would like to thank the Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran, for the financial and technical supports of this study.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Brownlie SE, Davies J, Jones DGC. Bronchial foreign bodies in four dogs. J Small Anim Pract 1986; 27(4): 239-245.
2. Pacchiana PD, Burnside PK, Wilkens BE, et al. Primary bronchotomy for removal of an intrabronchial foreign body in a dog. J Am Anim Hosp Assoc 2001; 37(6): 582-585.
3. Musani MA, Khambaty Y, Jawed I, et al. An unusual foreign body in trachea. J Ayub Med Coll Abbottabad 2010; 22(1): 178-179.
4. Goodnight ME, Scansen BA, Kidder AC, et al. Use of a unique method for removal of a foreign body from the trachea of a cat. J Am Vet Med Assoc 2010; 237(6): 689-694.
5. McGlennon NJ, Platt D, Dunn JK, et al. Tracheal foreign body in a cat: a case report. J Small Anim Pract 1986; 27(7): 457-461.
6. Han MC, Sağlıyan A, Polat E. A case of tracheal foreign body encountered in a Kangal dog: clinical, radiological, endoscopic findings and treatment. FAVE Secc Cienc Vet 2021; 20(1): 47-49.
7. Jagan Mohan Reddy K, Gireesh Kumar V, Raghavender KBP. Surgical management of foreign body in trachea in Labrador dog. Int J Curr Microbiol App Sci 2017; 6(6): 2586-2588.

شاپا: ۸۱۴۰-۲۰۰۸
شاپای الکترونیک: ۳۶۱۸-۲۳۲۲

مجله بین المللی دانشکده دامپزشکی دانشگاه ارومیه

گفتمان پژوهش دامپزشکی

دوره چهاردهم، شماره یازدهم
آبان یک هزار و چهارصد و دو