FOLIA VETERINARIA

The scientific journal of the
UNIVERSITY OF VETERINARY MEDICINE AND
PHARMACY IN KOŠICE — Slovakia

ISSN 0015-5748
eISSN 2453-7837

2
LXVII • 2023

FOLIA VETERINARIA is a scientific journal issued by the University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia.
The journal is published quaterly in English (numbers 1–4) and distributed worldwide.

Contact: tel.: +421 915 984 669
e-mail: folia.veterinaria@uvlf.sk

Electronic Publisher: De Gruyter Poland, Bogumila Zuga 32A
01-811 Warsaw, Poland

ISSN 2453-7837 on-line
ISSN 0015-5748 print
EV 3485/09

Publisher's
Identification number: IČO 00397474

June 2023

FOLIA VETERINARIA

PUBLISHED BY
THE UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE
SLOVAKIA

Folia Veterinaria
Vol. 67, 2, 2023

VYDÁVA
UNIVERZITA VETERINÁRSKEHO LEKÁRSTVA A FARMÁCIE V KOŠICIACH
2023

FOLIA VETERINARIA, 67, 2, 2023

CONTENTS

sciendo

DOI: 10.2478/fv-2023-0011

FOLIA VETERINARIA, 67, 2: 1—10, 2023

DEFINITION, ASSESSMENT, HEALTH CONSEQUENCES AND MANAGEMENT OF EQUINE OBESITY: A REVIEW

Olumide O. Akinniyi[1], Anthony K. B. Sackey[1], Gabriel E. Ochube[2], Philip W. Mshelia[1], Kelvin O. Jolayemi[3]

[1]Department of Veterinary Medicine
[2]Department of Veterinary Surgery and Radiology
[3]Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Kaduna State Nigeria

olumide.akinniyi@gmail.com

ABSTRACT

Obesity is a state of abnormal or excessive fat accumulation. Obesity in horses increases the risk of developing a variety of health issues such as: insulin dysregulation (ID), reduced athleticism, colic, abnormal reproductive performance, laminitis, endotoxaemia, *diabetes mellitus*, hyperlipaemia, impaired thermoregulation, pituitary pars intermedia dysfunction, and osteochondrosis. Obesity is a significant health and welfare issue in horses that may go unreported or unnoticed due to a caregiver's (owner) neglect. Weight gain occurs when a horse's energy consumption exceeds his or her physical energy needs. Obesity is best assessed via necropsy or upon *in vivo* assessment with deuterium oxide administration, although this is not feasible in a clinical environment. In practice, obesity is assessed by: body condition scoring, cresty neck scoring, ultrasonographic assessment, morphometric measurements, or biochemical indicators in the blood. Dietary and exercise programs are the primary means of controlling equine obesity. Pharmacologic assistance (levothyroxine sodium and metformin hydrochloride), being a secondary approach, may be effective in some cases. Management involves a long-term plan that requires the horse's caregiver's effort and discipline, as well as the support and supervision of their veterinarian. This paper outlines the assessment, health consequences, and management of equine obesity.

Key words: equine metabolic syndrome; insulin dysregulation; laminitis; obesity

INTRODUCTION

Adipose tissue, along with being an energy store, is an active endocrine organ with roles in synthesizing and secreting hormones that impact metabolism [11]. Normal physiological responses lead to the deposition of adipose tissue. Health issues may arise when there is excess deposition of adipose tissue. Several pathological processes can develop as a result of excess adiposity, including: oxidative stress, inflammation, disturbances of cortisol metabolism, disturbances of lipid metabolism, vascular dysfunction, and stimulation of the hypothalamic-pituitary-adrenal axis. Obesity results from an imbalance between energy intake and energy expenditure [5].

Equine obesity is a major health and welfare issue in horses that may be under-reported or go unrecognized. Horse caregivers may sometimes misjudge the body condition of their horses, particularly if they are obese [39, 82]. Hence, veterinarians must be proactive in identifying obese horses and tutoring caregivers on the risks associated with obesity and how to effectively manage the condition. It is critical to pay attention to various aspects of equine obesity, due to the associated health risks. The epidemiology, health implications, assessment, and management of equine obesity are discussed in this article.

Obesity definition

Obesity is defined as adiposity to the point where health is directly or indirectly impacted. Obesity may be generalized or regional (focal); internal (accumulations of adipose tissue around and within muscles and organs), or external (palpable subcutaneous fat) [68].

Epidemiology of equine obesity

Weight gain in horses is dependent on the housing and feeding. Domesticated horses are largely physically inactive and fed diets that far exceed their energy requirements. Concerning general obesity, several studies were carried out in the United Kingdom with a prevalence ranging from 21% to 45% [33, 34, 70, 72, 82]. In Denmark, 24% of mature Icelandic horses are overweight or obese [39]; 24.5% in south-eastern Australia [62]; 8%–29% in Canadian horses [10, 44]; and a prevalence of 51% of horses in the United States [74]. Regional obesity (cresty neck score > 3/5) was present in 33% of domestic horses and ponies in the United Kingdom [32]. Cob-type, Draught-type, Welsh breeds, British natives [70], Morgan breeds, Spanish mustang breeds, Arabian breeds [27], Shetland ponies [62], Rocky Mountain Horses, Quarter Horses, Tennessee Walking Horses, Warmbloods, as well as mixed-breed horses, appear to be more susceptible to obesity in comparison to Thoroughbreds [74], indicating that many breeds of horses are affected [24]. Leisure horses and more dominant horses were more likely to be obese [32, 70].

Adverse effects of obesity on equine health

Obesity rates have risen dramatically as the role of horses has shifted away from productivity and toward companionship [1]. Obesity in horses has been linked to: impaired thermoregulation [13], exercise intolerance (reduced athleticism) [31, 42, 45], abnormal reproductive performance [36, 61], insulin dysregulation (ID) [37, 79], increased risk of laminitis [70], poor prognosis for recovery from laminitis [57], pituitary pars intermedia dysfunction (PPID) [40, 54, 65, 79], endotoxaemia [77], osteochondrosis [64], *diabetes mellitus* [40], hyperlipaemia [66], and colic. Colic results from intestinal obstruction caused by the development and growth of benign lipomas in the abdomen's mesenteric adipose tissue [30]. In some situations, mesenteric lipomas can grow a lengthy pedicle of attachment and become "pedunculated." Pedunculated lipomas tend to migrate about within the abdomen, obstructing and strangling intestines [41]. Affected horses have sudden and severe pain, shock, and death unless emergency surgery is performed.

EQUINE OBESITY ASSESSMENT

Benchmark methods for the evaluation of total body fat mass, such as deuterium oxide (D_2O) dilution and carcass dissection (necropsy), are, as of now, obviously not practicable in a clinical setting. Although the D_2O dilution technique is straightforward, it would have been valuable for clinical cases if the measurement of D_2O had become widely available [16, 68]. In practice, assessment of obesity can be subjective or objective. Rendle et al. [68] describe body condition score (BCS) and cresty neck score (CNS) as examples of subjective assessment, while morphometric measurements, diagnostic testing, and ultrasonography are examples of objective assessment. The measurement and estimation of body weight do not effectively quantify obesity but are highly beneficial in monitoring responses to diet.

Subjective assessment of obesity

1. Body condition score (BCS)

The body condition is a good indicator of the general health of a horse. The BCS helps one to know if a horse is too lean, too fat, or just within the normal body size. The scoring system by Henneke et al. [36] and modified by Kohnke [43] is a 9-point scale. It is widely used because it has been extensively validated. To carry out this body condition scoring system, it is required that six anatomically distinct body regions (neck, ribs, withers, shoulder loin, and tailhead) be assigned a score between

Fig. 1. Obese pony with body condition score of 9 (using Henneke body condition scoring)

1 and 9 separately, then the scores are averaged to obtain the overall body condition score. Under-condition (<4.5), moderate-condition (4.5–5.5), over-condition (6–6.5), and obese (≥7) horses (Fig. 1) are classified [17, 18, 62]. The 0–5-point body condition system was first described by Leighton–Hardman [47] and modified by Carroll and Huntington [8]. In this BCS system, 3 body areas (ribs and back, neck, and pelvis) are assessed for fat deposition on a scale of 0 (very poor) to 5 (very fat). To achieve the overall BCS, each of the three body parts is given a distinct score, with the pelvis score being altered by 0.5 points if it differs by 1 or more points from the back or neck scores. Several factors can affect condition scoring accuracy. Such factors include: coat length, conformation, evaluator bias, pregnancy, muscle development, and gut fill. The BCS systems were, in the ideal sense, created to assess subcutaneous fat tissue independent of muscle mass. In reality, it is not an easy task for even a highly experienced evaluator to differentiate muscle from adipose tissue, and in geriatric horses that have relatively low muscle mass, the fat mass could be underestimated.

Fig. 2. White arrows indicating an abnormal regional obesity of the neck (dropping neck) (cresty neck score of 5)

2. Cresty neck score

Cresty neck is an overabundance of fat along the top of the neckline. Carter et al. [9] developed a regional subjective scoring system specifically for the top of the neck region. Horses with cresty necks have been associated with a risk of metabolic health issues [9]. The cresty neck score is on a scale of 0 to 5, where 0 indicates no visual appearance of a crest, and 5 indicates an overabundance of fat along the top of the horse's neck (Fig. 2). Most horses within a range of 1 to 2 CNS have a small amount of fat coverage along the neck top line, while those within a range of 3 to 4 CNS have more fat coverage. A horse is regarded as having a cresty neck when the CNS falls between 3 and above [9]. Horses with a score of 5 (drooping neck) are rarely seen. Ideally, horses should have a CNS

of no more than 2. Horses with cresty neck may or may not have general obesity. One study found that obesity status (body condition score) and cresty neck condition were highly associated. In that study, 97.5 % of obese horses and 59.6 % of non-obese horses had cresty neck [52].

Objective assessment of obesity

1. Morphometric measurements

The estimation of equine body fat content using morphometric measurements (neck circumference, neck length, body mass index [BMI], belly girth, waist circumference, heart girth, and height to the withers) has been proven to be clinically applicable [15, 26] and has clear-cut advantages over subjective evaluation scores. They are helpful in the detection of small changes in body size, making it possible to obtain an accurate quantification when a horse's diet is modified [2, 15] and can be carried out by examiners [15]. The belly girth provides the most sensitive indicator of generalized fat loss in response to management modification [68].

2. Ultrasonography

Ultrasonography is known to be very sensitive for assessing ventro-abdominal retroperitoneal fat deposition [60, 71]. Ultrasound scanning is a useful tool in identifying horses that are lean but are diagnosed to have insulin dysregulation or showing signs of laminitis to identify TOFI horses (the acronym TOFI is applied by horse owners to horses with a lean EMS phenotype that appears Thin on the Outside but Fat on the Inside). The ultrasonography measures fat deposits on the ventral midline immediately caudal to the xiphisternum and around 10 cm on either side of the linea alba. This adipose reserve stores up rapidly during weight gain and, conversely, is one of the first to be lost during weight loss [68].

3. Diagnostic testing

Certain biomarkers, thought to be associated with obesity, in the blood are measured in laboratory tests [14]. There are no laboratory tests that can consistently predict when obesity may be harmful to one's health; nonetheless, diagnostic testing can be a valuable adjunct to clinical observations [68]. Even though serum leptin concentration corresponds well with adipose tissue mass, there is little correlation between laminitis risk and IR, limiting its utility over clinical assessment [4, 7]. Adiponectin production decreases with increasing adiposity, though this varies by breed and diet [3, 56], nevertheless, low total adiponectin concentration is linked to laminitis risk [81], suggesting that adiponectin could be a useful marker of adiposity. The measures of insulin dynamics do not indicate obesity per se, but they are a valuable tool for monitoring the risk of laminitis, which is linked to obesity and equine metabolic syndrome (EMS) [68].

EQUINE OBESITY MANAGEMENT

Controlled weight loss management is currently the mainstay of therapy for reversing obesity and lowering the risk of obesity-related diseases. Dietary restriction and increased physical activity are weight-loss strategies. Even when dietary provision is highly regulated, weight loss rates vary greatly between animals [2]. Medical treatment for obese horses is possible, but it should never be used as a substitute for dietary restrictions and exercise [14, 68]. Caregiver (owner) compliance and substantial weight loss often require the participation of a veterinarian. It's important to remember that weight-loss programs must be customized for the patient and take into account what is achievable for the caregiver. Furthermore, caregivers frequently believe that their horses require far more feed than they actually do [14]. To properly monitor weight loss, caregivers should not rely on the visual judgment of their horses' body weight; instead, they should employ a reliable means of bodyweight measurement such as weight on a scale, weight estimate using weight tape, or formula.

Dietary restrictions

When a horse's feed intake exceeds his or her physical energy needs, weight gain ensues. As a result, to lose weight, feed intake must be reduced, and/or physical energy demands must be increased. Obesity can be controlled by reducing the amount of digestible energy (DE) in the diet. A significant component of this strategy is limiting or removing pasture grass from the diet. When pasture access is maintained, quantifying the DE becomes nearly impossible. A horse may be allowed a limited daily duration of access to sparse pasture, or normal pasture with a grazing muzzle, to ensure his or her mental well-being [24, 49, 50]. Obese horses should be fed a forage diet supplemented with minerals and vitamins.

Hay with low non-structural carbohydrate (NSC) content should be chosen, which can be established by sending in a sample for analysis or acquiring forage with a stated nutrient analysis [27]. If the amount of NSC in hay exceeds 10%, it can be soaked in cold water for 60 minutes to reduce the water-soluble carbohydrate (WSC) content [12, 80]. However, recent studies found that results vary significantly between various hay samples [48, 51], indicating that this technique cannot be depended on to address the problem of high WSC concentrations in hay fed to obese ponies or horses. In overfed horses, removing all concentrates from the diet can occasionally be enough to cause weight loss. An obese horse should be given hay in an amount equal to 1.5% of the horse's ideal body weight. To ensure that the correct amounts of hay are fed, scales should be used. If an obese horse does not lose weight after 30 days of eating hay equivalent to 1.5% of its ideal body weight, the amount offered should be reduced to 1%. However, levels should not fall below 1%, and it should be emphasized that excessive calorie restriction can lead to hyperlipaemia, IR, and stereotyped behaviour that is inappropriate [27, 55]. Veterinarians' feeding advice should be brief and specific so that caregivers may incorporate the modifications into their daily routine [14]. If a specific recipe is offered that specifies exactly what quantity of exactly what feed is required, along with the product trade name, caregivers will be able to follow the guidance. Simply advising them to feed 1.5% body weight DM of a feed with less than 10% sugar is likely not to result in adequate compliance, as these values may be difficult to put into effect.

Physical exercise

An increase in physical exercise is recommended for obese horses to help them lose weight and enhance their insulin sensitivity [63, 73]. In addition to the intended fat reduction, weight loss will result in some unwanted muscle loss; frequent exercise may help to reduce muscle loss and may have an anti-inflammatory effect [58]. Improvements in insulin sensitivity linked to physical activity can exist even if weight loss does not occur or if there is no change in fat distribution [35]. Recommendations for ideal exercise levels are mostly subjective, yet any exercise program must be both explicit and practical to obtain compliance. Durham et al. [21] published a consensus statement with the following exercise recommendations for horses with insulin dysregulation or obesity:

- Horses with no clinical laminitis or lameness of any type should be exercised for more than 30 minutes at a low-to-moderate intensity (heart rates between 130 and 170 bpm; or canter to fast canter, ridden or unridden). This should be repeated at least 5 times every week.
- Horses with a history of clinical laminitis whose hoof lamellae are stable and fully recovered should be exercised for more than 30 minutes at a low intensity on a soft surface (heart rates between 110 and 150 bpm; fast trot to canter unridden). This should be repeated at least 3 times every week. It's crucial to keep an eye out for indicators of lameness.

Pharmacological aid

In obese and EMS horses, pharmaceutical treatment is usually not the first line of action. However, such treatment can be administered to a horse who has or is at risk of having laminitis and requires rapid weight loss or improved insulin sensitivity. Pharmaceutical treatment can also be used on a horse that has weight loss resistance despite following proper dietary modification guidelines [14]. Prescription of pharmaceuticals for the management of obesity and EMS when it is not essential is a serious concern, leading to caregivers neglecting dietary and management initiatives.

1. Levothyroxine sodium

Levothyroxine sodium helps in weight loss and has also been shown to increase insulin sensitivity [25, 28, 78]. Several studies found that administering levothyroxine to euthyroid horses resulted in significant weight loss and improved insulin sensitivity over 8 and 48 weeks with no cardiovascular side effects [25, 28, 78]. However, because these studies did not control the diets of the horses treated, more research is needed to ascertain the accurate and precise efficacy of levothyroxine. Morgan et al. [59] suggest that movement of horses administered levothyroxine should not be restricted because of the potential for welfare issues when given an activity-inducing medication. In the United States, levothyroxine is given indiscriminately and without sufficient consideration for the treatment of obesity and EMS, and it has even been stated that it is the most misused medicine in a horse practice [53]. Levothyroxine administration has been reported not as an alternative but as an adjunct to dietary management in horses that cannot be exercised [20], and has also been used in weight

loss-resistant cases and in severe cases of laminitis associated with IR that were not responding despite conventional management [68]. Recent research has shown that levothyroxine is safe at doses up to 10 times those used clinically [6]. Treatment should be given for 3 to 6 months at a dose of 0.1 mg.kg^{-1} PO SID. Treatment is continued until the goal body weight is reached, at which point the dose is reduced by half to 0.05 mg.kg^{-1} for 2 weeks, then by half again to 0.025 mg.kg^{-1} for another two weeks before treatment is stopped [23].

2. Metformin hydrochloride

Metformin is a biguanide drug that has been used in human medicine for more than 50 years as an oral antihyperglycemic agent [67]. Reduced hepatic glucose production, improved tissue sensitivity to insulin, and increased translocation of glucose type 4 (GLUT4) transporters to cell membranes in insulin-sensitive tissues are all known possible antidiabetic mechanisms of action [46]. Previous research found that when the medication was given at a dose of 15 mg.kg^{-1}, it improved basal measures of insulin sensitivity in a small number of insulin-resistant horses [19, 22]. However, other studies have found that the medication did not affect insulin sensitivity in both normal and insulin-resistant horses [75, 76]. Metformin has low oral bioavailability in horses, with just 7.1 % in unfed horses and 3.9 % in fed horses. In horses, the elimination half-life was less than 30 minutes, compared to 6 hours in humans [38]. Metformin may be beneficial for EMS through its actions in the gut or liver and its effects on the entero-insular axis. Metformin has been shown to reduce glucose absorption and reduce insulin spikes that occur after glucose ingestion in non-obese horses [69]. This could help reduce glucose absorption and limit post-prandial hyperinsulinemia, both of which are factors in the development of laminitis. However, more research is needed. Metformin is administered to horses in doses ranging from 15 to 30 mg.kg^{-1} q8–12h PO, with the drug ideally administered 30–60 minutes before feeding [21, 29].

CONCLUSIONS

Equine obesity is highly prevalent globally, especially in countries where horses are kept for companionship. It is a growing concern that has been overlooked by caregivers and must be identified and addressed for many health reasons. The practicable assessment of equine obesity relies on the subjective and objective recognition of excessive adipose deposits. Weight loss with a combination of dietary modification and exercise in horses without clinical laminitis is the mainstay of equine obesity management in horses. In selected cases, medical treatment with levothyroxine sodium and metformin may be considered.

REFERENCES

1. **Argo, C., 2015:** Equine obesity: beyond the equine metabolic syndrome. *Acta. Vet. Scand.*, 57, (Suppl.1), K2. DOI: 10.1186/1751-0147-57-S1-K2.
2. **Argo, C. M., Curtis, G. C., Grove-White, D., Dugdale, A. H., Barfoot, C. F., Harris, P. A., 2012:** Weight loss resistance: A further consideration for the nutritional management of obese Equidae. *Vet. J.*, 194, 2, 179–188. DOI: 10.1016/j.tvjl.2012.09.020.
3. **Bamford, N. J., Potter, S. J., Baskerville, C. L., Harris, P. A., Bailey, S. R., 2016:** Effect of increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds adapted to cereal-rich or fat-rich meals. *Vet. J.*, 214, 14–20. DOI: 10.1016/j.tvjl.2016.02.002.
4. **Bamford, N. J., Potter, S. J., Harris, P. A., Bailey, S. R., 2016:** Effect of increased adiposity on insulin sensitivity and adipokine concentrations in horses and ponies fed a high fat diet, with or without a once daily high glycaemic meal. *Equine Vet. J.*, 48, 3, 368–373. DOI: 10.1111/evj.12434.
5. **Becvarova, I., Pleasant, R. S., 2012:** Managing obesity in pasture-based horses. *Compend. Contin. Educ. Vet.*, 34, E1–E4.
6. **Bertin, F. R., Forsythe, L. E., Kritchevsky, J. E., 2019:** Effects of high doses of levothyroxine sodium on serum concentrations of triiodothyronine and thyroxine in horses. *Am. J. Vet. Res.*, 80, 6, 565–571. DOI: 10.2460/ajvr.80.6.565.
7. **Buff, P. R., Dodds, A. C., Morrison, C. D., Whitley, N. C., McFadin, E. L., Daniel, J. A., et al., 2002:** Leptin in horses: tissue localization and relationship between peripheral concentrations of leptin and body condition. *J. Anim. Sci.*, 80, 11, 2942–2948. DOI: 10.2527/2002.80112942x.
8. **Carroll, C. L., Huntington, P. J., 1988:** Body condition scoring and weight estimation of horses. *Equine Vet. J.*, 20, 1, 41–45. DOI: 10.1111/j.2042-3306.1988.tb01451.x.
9. **Carter, R. A., Geor, R. J., Staniar, W. B., Cubitt, T. A., Harris, P. A., 2009:** Apparent adiposity assessed by standardised scoring systems and morphometric measurements in

horses and ponies. *Vet. J.*, 179, 2, 204–210. DOI: 10.1016/j.tvjl.2008.02.029.

10. **Christie, J. L., Hewson, C. J., Riley, C. B., McNiven, M. A., Dohoo, I. R., Bate, L. A., 2004:** Demographics, management, and welfare of nonracing horses in Prince Edward Island. *Can. Vet. J.*, 45, 12, 1004.
11. **Coelho, M., Oliveira, T., Fernandes, R., 2013:** State of the art paper Biochemistry of adipose tissue: An endocrine organ. *Arch. Med. Sci.*, 9, 2, 191–200. DOI: 10.5114/aoms.2013.33181.
12. **Cottrell, E., Watts, K., Ralston, S., 2005:** Soluble sugar content and glucose/insulin responses can be reduced by soaking chopped hay in water. In *Proc. of the 19th Equine Science Society Symposia*, 293–298.
13. **Cymbaluk, N. F., Christison, G. I., 1990:** Environmental effects on thermoregulation and nutrition of horses. *Vet. Clin. North Am. Equine Pract.*, 6, 2, 355–372. DOI: 10.1016/S0749-0739(17)30546-1.
14. **Diaz, M. A., Jarvis, N., Menzies, N., Penrose, L., Rendle, D., Furtado, T., 2020:** Tackling obesity and related laminitis in equine patients. *Vet. Times*, 14.
15. **Dugdale, A. H., Curtis, G. C., Cripps, P., Harris, P. A., Argo, C. M., 2010:** Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares. *Equine Vet. J.*, 42, 7, 600–610. DOI: 10.1111/j.2042-3306.2010.00110.x.
16. **Dugdale, A. H., Curtis, G. C., Cripps, P. J., Harris, P. A., Argo, C. M., 2011:** Effects of season and body condition on appetite, body mass and body composition in ad libitum fed pony mares. *Vet. J.*, 190, 3, 329–337. DOI: 10.1016/j.tvjl.2010.11.009.
17. **Dugdale, A. H., Curtis, G. C., Harris, P. A., Argo, C. M., 2011:** Assessment of body fat in the pony: Part I. Relationships between the anatomical distribution of adipose tissue, body composition and body condition. *Equine Vet. J.*, 43, 5, 552–561. DOI: 10.1111/j.2042-3306.2010.00330.x.
18. **Dugdale, A. H., Grove-White, D., Curtis, G. C., Harris, P. A., Argo, C. M., 2012:** Body condition scoring as a predictor of body fat in horses and ponies. *Vet. J.*, 194, 2, 173–178. DOI: 10.1016/j.tvjl.2012.03.024.
19. **Durham, A. E., 2012:** Metformin in equine metabolic syndrome: an enigma or a dead duck? *Vet. J.*, 1, 191, 17–18. DOI: 10.1016/j.tvjl.2011.08.003.
20. **Durham, A. E., 2017:** Therapeutics for equine endocrine disorders. *Vet. Clin. North Am. Equine Pract.*, 33, 1, 127–139. DOI: 10.1016/j.cveq.2016.11.003.
21. **Durham, A. E., Frank, N., McGowan, C. M., Menzies-Gow, N. J., Roelfsema, E., Vervuert, I., et al., 2019:** ECEIM consensus statement on equine metabolic syndrome. *J. Vet. Intern. Med.*, 33, 2, 335–349. DOI: 10.1111/jvim.15423.
22. **Durham, A. E., Rendle, D. I., Newton, J. R., 2008:** The effect of metformin on measurements of insulin sensitivity and β cell response in 18 horses and ponies with insulin resistance. *Equine Vet. J.*, 40, 5, 493–500. DOI: 10.2746/042516408X273648.
23. **Frank, N., 2006:** Insulin resistance in horses. *Proc. AAEP*, 52. Accessed June 2022 at www.ivis.org.
24. **Frank, N., 2009:** Equine metabolic syndrome. *J. Equine Vet. Sci.*, 29, 5, 259–267. DOI: 10.1016/j.cveq.2010.12.004.
25. **Frank, N., Elliott, S. B., Boston, R. C., 2008:** Effects of long-term oral administration of levothyroxine sodium on glucose dynamics in healthy adult horses. *Am. J. Vet. Res.*, 69, 1, 76–81. DOI: 10.2460/ajvr.69.1.76.
26. **Frank, N., Elliott, S. B., Brandt, L. E., Keisler, D. H., 2006:** Physical characteristics, blood hormone concentrations, and plasma lipid concentrations in obese horses with insulin resistance. *J. Am. Vet. Med. Assoc.*, 228, 9, 1383–1390. DOI: 10.2460/javma.228.9.1383.
27. **Frank, N., Geor, R. J., Bailey, S. R., Durham, A. E., Johnson, P. J., 2010:** Equine metabolic syndrome. *J. Vet. Intern. Med.*, 24, 3, 467–475. DOI: 10.1111/j.1939-1676.2010.0503.x.
28. **Frank, N., Sommardahl, C. S., Eiler, H., Webb, L. L., Denhart, J. W., Boston, R. C., 2005:** Effects of oral administration of levothyroxine sodium on concentrations of plasma lipids, concentration and composition of very-low-density lipoproteins, and glucose dynamics in healthy adult mares. *Am. J. Vet. Res.*, 66, 6, 1032–1038. DOI: 10.2460/ajvr.2005.66.1032.
29. **Frank, N., Tadros, E. M., 2014:** Insulin dysregulation. *Equine Vet. J.*, 46, 1, 103—112. DOI: 10.1111/evj.12169.
30. **Garcia-Seco, E., Wilson, D. A., Kramer, J., Keegan, K. G., Branson, K. R., Johnson, P. J., et al., 2005:** Prevalence and risk factors associated with outcome of surgical removal of pedunculated lipomas in horses: 102 cases (1987–2002). *J. Am. Vet. Med. Assoc.*, 226, 9, 1529–1537. DOI: 10.2460/javma.2005.226.1529.
31. **Garlinghouse, S. E., Burrill, M. J., 1999:** Relationship of body condition score to completion rate during 160 km endurance races. *Equine Vet. J.*, 31, S30, 591–595. DOI: 10.1111/j.2042-3306.1999.tb05290.x.
32. **Giles, S. L., Nicol, C. J., Rands, S. A., Harris, P. A., 2015:**

Assessing the seasonal prevalence and risk factors for nuchal crest adiposity in domestic horses and ponies using the Cresty Neck Score. *BMC Vet. Res.*, 11, 1, 1–9. DOI: 10.1186/s12917-015-0327-7.

33. **Giles, S. L., Rands, S. A., Nicol, C. J., Harris, P. A., 2014:** Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. *PeerJ*, 2, 1, e299. DOI: 10.7717/peerj.299.
34. **Harker, I. J., Harris, P. A., Barfoot, C. F., 2011:** The body condition score of leisure horses competing at an unaffiliated championship in the UK. *J. Equine Vet. Sci.*, 5, 31, 253–254. DOI: 10.1016/J.JEVS.2011.03.058.
35. **Hawley, J. A., 2004:** Exercise as a therapeutic intervention for the prevention and treatment of insulin resistance. *Diabetes Metab. Res. Rev.*, 20, 5, 383–393. DOI: 10.1002/dmrr.505.
36. **Henneke, D. R., Potter, G. D., Kreider, J. L., Yeates, B. F., 1983:** Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet. J.*, 15, 4, 371–372. DOI: 10.1111/j.2042-3306.1983.tb01826.x.
37. **Hoffman, R. M., Boston, R. C., Stefanovski, D., Kronfeld, D. S., Harris, P. A., 2003:** Obesity and diet affect glucose dynamics and insulin sensitivity in thoroughbred geldings. *J. Anim. Sci.*, 81, 9, 2333–2342. DOI: 10.2527/2003.8192333x.
38. **Hustace, J. L., Firshman, A. M., Mata, J. E., 2009:** Pharmacokinetics and bioavailability of metformin in horses. *Am. J. Vet. Res.*, 70, 5, 665–668. DOI: 10.2460/ajvr.70.5.665.
39. **Jensen, R. B., Danielsen, S. H., Tauson, A. H., 2016:** Body condition score, morphometric measurements and estimation of body weight in mature Icelandic horses in Denmark. *Acta. Vet. Scand.*, 58, 1, 19–23. DOI: 10.1186/s13028-016-0240-5.
40. **Johnson, P. J., Messer, N. T., Ganjam, V. K., 2004:** Cushing's syndromes, insulin resistance and endocrinopathic laminitis. *Equine Vet. J.*, 36, 3, 194–198. DOI: 10.2746/0425164044877279.
41. **Johnson, P. J., Wiedmeyer, C. E., Messer, N. T., Ganjam, V. K., 2009:** Medical implications of obesity in horses–lessons for human obesity. *J. Diabetes Sci. Technol.*, 3, 1, 163–174. DOI: 10.1177/193229680900300119.
42. **Kearns, C. F., Mckeever, K. H., Kumagai, K., Abe, T., 2002:** Fat-free mass is related to one-mile race performance in elite standardbred horses. *Vet. J.*, 163, 3, 260–266. DOI: 10.1053/tvjl.2001.0656.
43. **Kohnke, J., 1992:** *Feeding and Nutrition: The Making of a Champion*. Birubi Pacific, 163–166.
44. **Kosolofski, H. R., Gow, S. P., Robinson, K. A., 2017:** Prevalence of obesity in the equine population of Saskatoon and surrounding area. *Can. Vet. J.*, 58, 9, 967–970.
45. **Lawrence, L., Jackson, S., Kline, K., Moser, L., Powell, D., Biel, M., 1992:** Observations on body weight and condition of horses in a 150-mile endurance ride. *J. Equine Vet. Sci.*, 12, 5, 320–324. DOI: 10.1016/S0737-0806(06)81345-6.
46. **Lee, J. O., Lee, S. K., Kim, J. H., Kim, N., You, G. Y., Moon, J. W., et al., 2012:** Metformin regulates glucose transporter 4 (GLUT4) translocation through AMP-activated protein kinase (AMPK)-mediated Cbl/CAP signaling in 3T3-L1 preadipocyte cells. *J. Biol. Chem.*, 287, 53, 44121–44129. DOI: 10.1074/jbc.M112.361386.
47. **Leighton-Hardman, A. C., 1980:** *Appendix 3. Weight estimation tables. Equine Nutrition*. Pelham Books, London, 106–107.
48. **Longland, A. C., Barfoot, C., Harris, P. A., 2009:** The loss of water-soluble carbohydrate and soluble protein from nine different hays soaked in water for up to 16 hours. *J. Equine Vet. Sci.*, 5, 29, 383–384. DOI: 10.1016/J.JEVS.2009.04.085.
49. **Longland, A. C., Barfoot, C., Harris, P. A., 2016:** Effects of grazing muzzles on intakes of dry matter and water-soluble carbohydrates by ponies grazing spring, summer, and autumn swards, as well as autumn swards of different heights. *J. Equine Vet. Sci.*, 40, 26–33. DOI: 10.1016/j.jevs.2015.09.009.
50. **Longland, A. C., Barfoot, C., Harris, P. A., 2016:** Efficacy of wearing grazing muzzles for 10 hours per day on controlling bodyweight in pastured ponies. *J. Equine Vet. Sci.*, 45, 22–27. DOI: 10.1016/j.jevs.2016.04.015.
51. **Mack, S. J., Dugdale, A. H., Argo, C. M., Morgan, R. A., McGowan, C. M., 2014:** Impact of water-soaking on the nutrient composition of UK hays. *Vet. Rec.*, 174, 18, 452. DOI: 10.1136/vr.102074.
52. **Martin-Gimenez, T., Aguirre-Pascasio, C. N., de Blas, I., 2004:** Beyond scoring systems: usefulness of morphometry considering demographic variables, to evaluate neck and overall obesity in Andalusian horses. *Animal*, 12, 3, 597–605. DOI: 10.1017/S1751731117001628.
53. **McFarlane, D., 2014:** Pharmacological treatment of equine endocrine diseases. *Equine Pharmacology*, John Wiley and Sons, Ames, 270–278.
54. **McGowan, C. M., Frost, R., Pfeiffer, D. U., Neiger, R., 2004:** Serum insulin concentrations in horses with equine Cushing's syndrome: response to a cortisol inhibitor and prognostic value. *Equine Vet. J.*, 36, 3, 295–298. DOI: 10.2746/0425164044877288.

55. **McGregor-Argo, C., 2009:** Appraising the portly pony: Body condition and adiposity. *Vet. J.*, 179, 2, 158–160. DOI: 10.1016/j.tvjl.2008.04.007.
56. **Menzies-Gow, N. J., Knowles, E. J., Rogers, I., Rendle, D. I., 2019:** Validity and application of immunoturbidimetric and enzyme-linked immunosorbent assays for the measurement of adiponectin concentration in ponies. *Equine Vet. J.*, 51, 1, 33–37. DOI: 10.1111/evj.12960.
57. **Menzies-Gow, N. J., Stevens, K., Barr, A., Camm, I., Pfeiffer, D., Marr, C. M., 2010:** Severity and outcome of equine pasture-associated laminitis managed in first opinion practice in the UK. *Vet. Rec.*, 167, 10, 364–369. DOI: 10.1136/vr.c3206.
58. **Menzies-Gow, N. J., Wray, H., Bailey, S. R., Harris, P. A., Elliott, J., 2014:** The effect of exercise on plasma concentrations of inflammatory markers in normal and previously laminitic ponies. *Equine Vet. J.*, 46, 3, 317–321. DOI: 10.1111/evj.12132.
59. **Morgan, R. A., Keen, J. A., McGowan, C. M., 2015:** Equine metabolic syndrome. *Vet. Rec.*, 177, 7, 173—179. DOI: 10.1136/vr.103226.
60. **Morrison, P. K., Harris, P. A., Maltin, C. A., Grove-White, D., Argo, C. M., 2017:** EQUIFAT: A novel scoring system for the semi-quantitative evaluation of regional adipose tissues in Equidae. *PLOS ONE,* 12, 3, e0173753. DOI: 10.1371/journal.pone.0173753.
61. **Nagy, P., Huszenicza, G., Juhasz, J., Kulcsar, M., Solti, L., Reiczigel, J., et al., 1998:** Factors influencing ovarian activity and sexual behaviour of postpartum mares under farm conditions. *Theriogenology*, 50, 7, 1109–1119. DOI: 10.1016/s0093-691x(98)00212-x.
62. **Potter, S. J., Bamford, N. J., Harris, P. A., Bailey, S. R., 2016:** Prevalence of obesity and owners' perceptions of body condition in pleasure horses and ponies in south-eastern Australia. *Aust. Vet. J.*, 94, 11, 427–432. DOI: 10.1111/avj.12506.
63. **Powell, D. M., Reedy, S. E., Sessions, D. R., Fitzgerald, B. P., 2002:** Effect of short-term exercise training on insulin sensitivity in obese and lean mares. *Equine Vet. J.*, 34, S34, 81–84. DOI: 10.1111/j.2042-3306.2002.tb05396.x.
64. **Ralston, S. L., 1996:** Hyperglycaemia/hyperinsulinaemia after feeding a meat of grain to young horses with osteochondritis dissecans (OCD) lesions. *Pferdeheilkunde*, 12, 320–322. DOI: 10.21836/PEM19960332.
65. **Reeves, H. J., McGowan, C. M., Lees, R., 2001:** Measurement of basal serum insulin concentration in the diagnosis of Cushing's disease in ponies. *Vet. Rec.*, 149, 15, 449–452. DOI: 10.1136/vr.149.15.449.
66. **Reid, S. W., Mohammed, H. O., 1996:** Survival analysis approach to risk factors associated with hyperlipaemia in donkeys. *J. Am. Vet. Med.*, 209, 8, 1449–1452.
67. **Rena, G., Pearson, E. R., Sakamoto, K., 2013:** Molecular mechanism of action of metformin: Old or new insights? *Diabetologia*, 56, 9, 1898–1906. DOI: 10.1007/s00125-013-2991-0.
68. **Rendle, D., McGregor Argo, C., Bowen, M., Carslake, H., German, A., Harris, P., et al., 2018:** Equine obesity: Current perspectives. *UK-Vet. Equine*, 2, Sup5, 1–9. DOI: 10.12968/ukve.2018.2.S2.3.
69. **Rendle, D. I., Rutledge, F., Hughes, K. J., Heller, J., Durham, A. E., 2013:** Effects of metformin hydrochloride on blood glucose and insulin responses to oral dextrose in horses. *Equine Vet. J.*, 45, 6, 751–754. DOI: 10.1111/evj.12068.
70. **Robin, C. A., Ireland, J. L., Wylie, C. E., Collins, S. N., Verheyen, K. L., Newton, J. R., 2015:** Prevalence of and risk factors for equine obesity in Great Britain based on owner-reported body condition scores. *Equine Vet. J.*, 47, 2, 196–201. DOI: 10.1111/evj.12275.
71. **Siegers, E. W., de Ruijter-Villani, M., Van Doorn, D. A., Stout, T. A., Roelfsema, E., 2018:** Ultrasonographic measurements of localized fat accumulation in Shetland pony mares fed a normal versus a high energy diet for 2 years. *Animal*, 12, 8, 1602–1610. DOI: 10.1017/S1751731111700325.
72. **Stephenson, H. M., Green, M. J., Freeman, S. L., 2011:** Prevalence of obesity in a population of horses in the UK. *Vet. Rec.*, 168, 5, 131. DOI: 10.1136/vr.c6281.
73. **Stewart-Hunt, L., Geor, R. J., McCutcheon, L. J., 2006:** Effects of short-term training on insulin sensitivity and skeletal muscle glucose metabolism in standardbred horses. *Equine Vet. J.*, 38, S36, 226–232. DOI: 10.1111/j.2042-3306.2006.tb05544.x.
74. **Thatcher, C. D., Pleasant, R. S., Geor, R. J., Elvinger, F., 2012:** Prevalence of overconditioning in mature horses in southwest Virginia during the summer. *J. Vet. Intern. Med.*, 26, 6, 1413–1418. DOI: 10.1111/j.1939-1676.2012.00995.x.
75. **Tinworth, K. D., Boston, R. C., Harris, P. A., Sillence, M. N., Raidal, S. L., Noble, G. K., 2012:** The effect of oral metformin on insulin sensitivity in insulin-resistant ponies. *Vet. J.*, 191, 1, 79–84. DOI: 10.1016/j.tvjl.2011.01.015.
76. **Tinworth, K. D., Edwards, S., Harris, P. A., Sillence, M. N., Hackett, L. P., Noble, G. K., 2010:** Pharmacokinetics

of metformin after enteral administration in insulin-resistant ponies. *Am. J. Vet. Res.*, 71, 10, 1201–1206. DOI: 10.2460/ajvr.71.10.1201.

77. **Tóth, F., Frank, N., Elliott, S. B., Geor, R. J., Boston, R. C., 2008:** Effects of an intravenous endotoxin challenge on glucose and insulin dynamics in horses. *Am. J. Vet. Res.*, 6, 1, 82–88. DOI: 10.2460/ajvr.69.1.82.
78. **Tóth, F., Frank, N., Geor, R. J., Boston, R. C., 2010:** Effects of pretreatment with dexamethasone or levothyroxine sodium on endotoxin-induced alterations in glucose and insulin dynamics in horses. *Am. J. Vet. Res.*, 71, 1, 60–68. DOI: 10.2460/ajvr.71.1.60.
79. **Vick, M. M., Adams, A. A., Murphy, B. A., Sessions, D. R., Horohov, D. W., Cook, R. F., et al., 2007:** Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *J. Anim. Sci.*, 85, 5, 1144–1155. DOI: 10.2527/jas.2006-673.
80. **Watts, K. A., Chatterton, N. J., 2004:** A review of factors affecting carbohydrate levels in forage. *J. Equine Vet. Sci.*, 24, 2, 84–87. DOI: 10.1016/J.JEVS.2004.01.005.
81. **Wray, H., Elliott, J., Bailey, S. R., Harris, P. A., Menzies-Gow, N. J., 2013:** Plasma concentrations of inflammatory markers in previously laminitic ponies. *Equine Vet. J.*, 45, 5, 546–551. DOI: 10.1111/evj.12031.
82. **Wyse, C. A., McNie, K. A., Tannahil, V. J., Murray, J. K., Love, S., 2008**: Prevalence of obesity in riding horses in Scotland. *Vet. Rec.*, 162, 18, 590–591. DOI: 10.1136/vr.162.18.590.

Received October 23, 2022
Accepted January 27, 2023

sciendo

DOI: 10.2478/fv-2023-0012

FOLIA VETERINARIA, 67, 2: 11—17, 2023

THE GENETIC ANALYSIS OF POTENTIAL NEW HEV-3 SUBTYPE IDENTIFIED ON PIG FARM IN SLOVAKIA

Božena Kočíková, Alica Pavlová, Katarína Dudášová, René Mandelík, Anna Jacková
Department of Epizootiology, Parasitology and Protection of One Health, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia

anna.jackova@uvlf.sk

ABSTRACT

Hepatitis E virus (HEV) represents the causative agent of acute viral hepatitis in the world. The virus belongs to the genus *Paslahepevirus*, species *Paslahepevirus balayani*, which contains 8 different genotypes and at least 14 reference HEV-3 subtypes. Recently we have identified three isolates in a Slovakian pig farm as a new unclassified HEV-3 subtype. The aim of this study was to sequence and analyze additional regions of HEV genome for PER5 isolate, a representative isolate of potential new HEV-3 subtype. The phylogenetic analyses of 749 bp and 828 bp DNA fragments amplified from ORF1 revealed that both sequences fall outside two major clades (3abchij and 3efg) of the HEV-3 genotype as well as the other HEV-3 subtypes. The nucleotide identity with other HEV-3 subtypes indicated low values within the range 79.2–84.1 % as well. The results provide further support for the circulation of a new HEV-3 subtype in the pig farm in Slovakia.

Key words: domestic pig; hepatitis E; HEV-3; sequencing; subtype

INTRODUCTION

Hepatitis E virus (HEV) probably represents the most common causative agent of acute viral hepatitis in the world [1, 10]. In general, the majority of HEV infections are asymptomatic, usually results in an acute self-limiting disease [23], otherwise in immunocompromised individuals HEV infection can become chronic [10]. The primary source of infection is through the faecal-oral route in developing countries. The secondary source, via consumption of raw or undercooked meats or meat products from reservoir animals represents an important risk factor for the emergence of hepatitis E in developed countries [23].

HEV is a single-stranded positive sense RNA with approximately 27–34 nm. The HEV genome (~7.2 kb) expresses three open reading frames (ORFs): ORF1 (~5,082 nucleotides), ORF2 (~1,983 nucleotides), and ORF3 (~300 nucleotides), which is partially overlapped with ORF2 [3, 11]. Virus belongs to the genus *Paslahepevirus*, species *Paslahepevirus balayani*, which contains 8 different genotypes [13, 18, 20, 25, 26, 29, 30]. HEV-3 genotype is found worldwide and represent the predominant genotype in Europe and America [17]. Moreover, HEV-3 genotype has a zoonotic potential and mainly domestic pigs, wild boars as well as deer are the source of infection for people. Humans may be potentially infected

via consumption of raw or undercooked animal products from HEV infected animals [4, 7, 14, 21, 22, 24].

The genetic analysis of HEV-3 revealed that this genotype is quite variable. Based on the phylogenetic analysis of hypervariable region in ORF1, the reference HEV subtypes were described [26]. HEV-3 genotype consists of subtypes divided to 2 main clades; one composed of six HEV-3 subtypes (abchij), the second, composed of three HEV-3 subtypes (efg), as well as ra subtype derived from rabbit genotype [7]. Except of mentioned subtypes, S m i t h et al. [24] provided update of the reference sequences and added three other HEV-3 subtypes namely HEV-3k, HEV-3l, and HEV-3m proposed as novel subtypes [5, 16, 24]. In addition, the novel HEV-3n subtype has been described recently [19].

In Europe, three HEV-3 subtypes (HEV-3c, HEV-3e, and HEV-3f) were the most commonly detected in human and animal samples [6, 17]. While HEV-3 isolates originating from domestic pigs in The Czech Republic, Hungary and Poland belonged mainly into HEV-3efg clade [2, 8, 12, 28], the epizootiological situation in Slovakia was different. In a previous study, we revealed that HEV isolates from Slovakian domestic pigs fall mainly into the HEV-3abchij clade [9]. We also identified the highly divergent HEV-3 isolates with relatively high nucleotide distances (p-distances 0.140–0.178) to other subtypes. This distance was higher than the limit for a new subtype ($P<0.123$) defined by S m i t h et al. [26]. The divergent isolates clustered outside of both major phylogenetic clades and thus remained not classified so far.

The aim of this study was a deeper genetic analysis of a representative PER5 isolate belonging to new unclassified HEV-3 subtype which was identified in Slovakian pig farm. The sequences of additional genomic regions of PER5 isolate were subject for further analysis by bioinformatics tools.

MATERIAL AND METHODS

Sample collection

PER5 isolate, a representative member of the new unclassified genetic subtype, used in this study was collected from randomly selected weaner pig (*Sus scrofa domestica*) originating from a farm in the Eastern Slovakia described by J a c k o v á et al. [9]. The veterinarian who inspected the farm did not recognize any clinical symptoms on a pig of which clinical sample was collected. Rectal swab from a pig was collected using swab applicator (Sarstedt AG & Co, Germany) with transport medium, thereafter delivered to the laboratory and processed.

HEV RNA extraction and reverse transcription

Rectal swabs were resuspended in 1 ml of 0.01 $mol.l^{-1}$ PBS (Merck Millipore Corp., USA) for 30 min., then vortexed at 2000 $rev.min^{-1}$ for 3 min and centrifuged at 14,000 × g for 5 min. RNA was isolated from 400 µl of solution prepared using the MagMAX™ Viral RNA Isolation Kit (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's protocol. The extracted RNA was stored at –80 °C. The cDNA was synthesized in a 20 µl reaction mix comprising 5 µl of extracted RNA, 0.5 mM dNTPs (Thermo Fisher Scientific, Inc., USA), 200 U RevertAid Premium reverse transcriptase with 1xRT buffer (Thermo Fisher Scientific, Inc., USA), 5 µM of gene specific reverse outer PCR primers [5, 15, 31] (Microsynth Austria, GmbH, Austria), 20 U RNase inhibitor (Invitrogen, Inc., USA) and filled with molecular biology grade water (Merck, GmbH, Germany).

Nested RT-PCR and sequencing

A total of twenty pairs of PCR primers available in the literature were used for the amplification of full-length HEV genome [5, 15, 31]. The PCR reaction mix (50 µl) was composed of 1× ThermoPol reaction buffer (New England Biolabs, Inc., USA), 0.2 mM dNTPs (Thermo Fisher Scientific, Inc., USA), 300 nM of outer/inner primers, 1 U Taq DNA polymerase (New England Biolabs, Inc., USA), 4 µl cDNA and molecular biology grade water (Merck, GmbH, Germany). The PCR was carried out under the following thermal profile: 1 cycle at 95 °C for 1 min, and 35 cycles with denaturation at 95 °C for 30 s, annealing (set at various temperature varying between 52–58 °C, which depended on the primers used) for 1 min, extension at 68 °C for 1 min and final extension at 68 °C for 5 min using Thermocycler C1000 (Bio-Rad Laboratories, Inc., USA). Electrophoresis was carried out in 2 % agarose gel, stained with GelRed™ (Biotium, Inc., USA) and visualized by Gel Doc EZ imager (Bio-Rad Laboratories, Inc., USA). The PCR products of expected size were purified and sequenced by the Sanger method using the PCR primers by a commercial company (Microsynth Austria, GmbH, Austria).

Phylogenetic analysis of HEV sequences

For bioinformatics analysis, the partial ORF1 sequences from PER5 sample were edited and aligned using the programs SeqMan, EditSeq and MegAlign (Lasergene, DNASTAR, Inc. USA). The sequences were first checked against the NCBI GenBank database using nucleotide BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide identity (nt. id.) was calculated using the program MegAlign. Than the phylogenetic trees were separately constructed using the cut off 749 bp and 828 bp nucleotide sequences (GenBank Acc. No. OQ378957 and OQ378956, respectively). The molecular evolution model tests were performed by MEGA11 [27]. The models with the lowest BIC scores (Bayesian Information Criterion) were used for phylogenetic analysis. The model tests indicated that The Maximum Likelihood analyses of 749 bp DNA fragment should be carried out using the Tamura-Nei model with Gamma distribution plus evolutionarily Invariable sites model (TN93+G+I). The Maximum Likelihood phylogenetic analysis of 828 bp fragment was set to the General time reversible model with Gamma distribution plus evolutionarily Invariable sites model (GTR+G+I). The bootstrap support values of branches were calculated from 1000 replicates. The HEV-1 Burma strain (M73218) was used as outgroup.

Ethical considerations

All procedures concerning the animals were performed in compliance with the national guidelines for animal care.

RESULTS

Despite various pair of primers from literature were applied to target parts of HEV genome, neither single PCR was successful to obtain DNA fragment from PER5 isolate. Fortunately, two nested PCRs using the primers referred as 3506-7 and 3154-77 by Meng et al. [15],

Fig. 1. Phylogenetic tree of HEV nucleotide sequences based on partial ORF1 (749 nt). Bootstrap values > 70 are indicated at their respective nodes. Slovak HEV PER5 sequence are indicated by a black circle. Twelve HEV-3 reference subtypes according to Smith et al. [19] are signed by corresponding letter of subtype. All sequences are denoted by name sequences, ISO code country of origin and NCBI GenBank Acc. No. in brackets. The scale bar indicates nucleotide substitutions *per site*

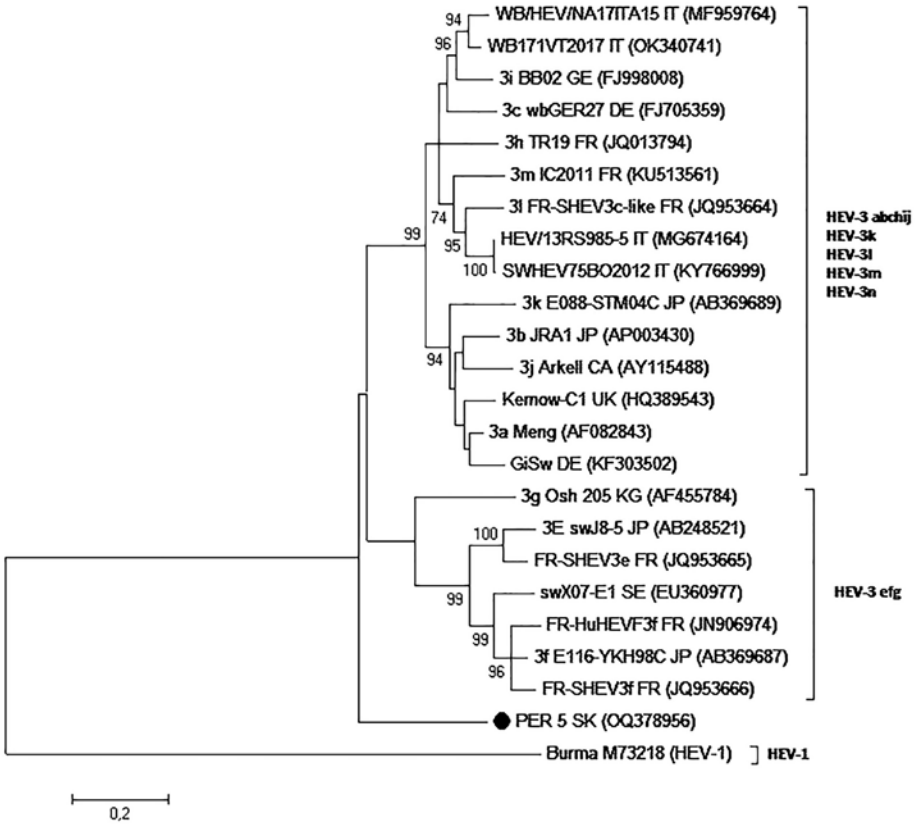

Fig. 2. Phylogenetic tree of HEV nucleotide sequences based on partial ORF1 (828 nt). Bootstrap values > 70 are indicated at their respective nodes. Slovak HEV PER5 sequence are indicated by a black circle. Twelve HEV-3 reference subtypes according to S m i t h et al. [19] are signed by corresponding letter of subtype. All sequences are denoted by name sequences, ISO code country of origin and NCBI GenBank Acc. No. in brackets. The scale bar indicates nucleotide substitutions *per site*

successfully amplified two regions of ORF1. They flanked the positions 3,209–4,324 (for the primer set 3506-7) and 4,189–5,181 (for the primer set 3154-77) in accordance with genome numbering for the representative Meng HEV-3a isolate (GenBank Acc. No. AF082843). The PCR products were sequenced and compared to sequences from the NCBI GenBank database. The blasting in GenBank indicated that DNA fragments were located in the RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) regions of HEV-3 genotype, respectively.

For next analysis, the 749 bp and 828 bp DNA fragments long were cut off from two regions amplified. The results of nucleotide identity showed that the Slovakian strain displayed low values: 79.2–84.1 % (749 bp fragment) and 80.4–83.9 % (828 bp) when compared to the established HEV-3 subtypes.

In the phylogenetic analysis, our two sequences were aligned with twenty-two HEV-3 subtype sequences, including reference subtypes proposed by S m i t h et al. [24] as well as other HEV-3 subtypes deposited in GenBank. The phylogenetic trees revealed similar results with both partial OFR1 sequences. The PER5 sequence analysed formed a part of the separate clade clustering outside of two major clades (3abchij and 3efg) of the HEV-3 genotype (Fig. 1, Fig. 2).

DISCUSSION

This study was focused on further genetic characterization of the representative PER5 isolate, which was identified in our study as a potentially new HEV-3 subtype [9]. We were able to amplify new regions in ORF1 which represent together with the sequences obtained previously [9] around one third (2,212 bp) of the HEV genome.

In our study, a total of twenty pairs of primers available in the literature were used for amplification of HEV genome [5, 15, 31]. The amplification of various genomic regions using single PCR primers was not successful most probably due to lower viral content in the tested sample.

However, two nested PCRs with primers proposed by M e n g et al. [15] provided sufficient amount of DNA for sequencing.

The results of nucleotide identity varied in both genomic regions of ORF1 (749 bp and 828 bp) in the range 79.2–84.1 % nt. id. indicating that PER5 isolate was not close related to the HEV-3 subtypes, which have been already classified. This fact is in agreement with low values of the nt. id. between subtypes already detected in other studies. For example, M i u r a et al. [16] obtained 81.0–87.0 % nt. id. of human HEV-3k with other HEV-3 subtypes in Japan. Similar, 81.4–89.9 % nt. id. of swine HEV-3l subtype as well as 80.9–88.6 % nt. id. of wild boar HEV-3n subtype with HEV-3 subtypes have been reported [5, 19].

Additional support for distinct feature of PER5 sequence provided the phylogenetic study. It is striking that in all four regions tested the same results of phylogenetic analysis were obtained [9, this study]. The sequences of PER5 isolate always formed a cluster separated of other identified subtypes, including new proposed HEV-3 (HEV-3k, HEV-3l, HEV-3m, and HEV-3n) subtypes. These data also support the idea that HEV-3 isolates circulating in a Slovakian pig farm belong to new subtype. The existence of unclassified HEV-3 subtypes was also described in other countries (Italy, Japan). They have been just recently proposed as new HEV-3 subtypes (HEV-3k, HEV-3l, HEV-3m, and HEV-3n) based on full-genome sequences [5, 16, 19, 24].

We realize that at present it is generally accepted that data based on nucleotide identity, p-distances between intra- and inter- subtypes or phylogenetic trees constructed with partial sequences do not completely fill in the criteria for the identification of new HEV-3 subtype [8]. So, for definitive conclusion that new HEV-3 subtype circulate in pig farms in Slovakia, the full-length genome sequence analysis is required [17, 26]. Next experiments in the laboratory will be focused to sequence the gaps to obtain complete genome sequence of HEV-3 PER5 isolate using PCR primers designed from our new sequence database created during this project.

CONCLUSIONS

In this study, we genetically characterized another regions of HEV genome in PER5 isolate from domestic pig. The phylogenetic analyses of two DNA fragments confirmed the same results; PER5 isolate clustered out of two major HEV-3 clades (3abchij and 3efg). To conclude, this study provides further evidence that the PER5 isolate from naturally infected domestic pig in Eastern Slovakia probably represents the HEV-3 subtype that has never been identified so far. The subsequent studies will be focused to obtain full genome sequence of unclassified Slovak PER5 isolate.

ACKNOWLEDGEMENTS

We would like to thank to professor Štefan Vilček from our university for his valuable discussion and editation of the text. The authors would like to thank Dagmar Matejová for the English language correction. This study was financially supported by the project "Open scientific community for modern interdisciplinary research in medicine (OPENMED)", ITMS2014+:313011V455 supported by the Operational Programme Integrated Infrastructure, funded by the ERDF.

REFERENCES

1. **Adlhoch, C., Wolf, A., Meisel, H., Kaiser, M., Ellerbrok, H., Pauli, G., 2009:** High HEV presence in four different wild boar populations in East and West Germany. *Vet. Microbiol.*, 139, 3–4, 270–278. DOI: 10.1016/j.vetmic.2009.06.032.
2. **Bigoraj, E., Paszkiewicz, W., Rzeżutka, A., 2021:** Porcine blood and liver as sporadic sources of hepatitis E virus (HEV) in the production chain of offal-derived foodstuffs in Poland. *Food Environ. Virol.*, 13, 3, 347–356. DOI: 10.1007/s12560-021-09475-z.
3. **Capai, L., Charrel, R., Falchi, A., 2018:** Hepatitis E in high-income countries: What do we know? And what are the knowledge gaps? *Viruses*, 10, 6, 285. DOI: 10.3390/v10060285.
4. **Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal., V., Gallian, P., et al., 2010:** Pig liver sausage as a source of hepatitis E virus transmission to humans. *J. Infect. Dis.*, 202, 6, 825–834. DOI: 10.1086/655898.
5. **De Sabato, L., Lemey, P., Vrancken, B., Bonfanti, L., Ceglie, L., Vaccari, G., et al., 2018:** Proposal for a new subtype of the zoonotic genotype 3 hepatitis E virus: HEV-3l. *Virus Research*, 248, 1–4. DOI: 10.1016/j.virusres.2018.02.010.

6. **De Sabato, L., Suffredini, E., Di Pasquale, S., La Rosa, G., De Santis, P., Giammarioli, M., et al., 2022:** Novel subtypes and unexpected heterogeneity of hepatitis E viral strains in wild boar captured in a small area in Central Italy. *Transbound. Emerg. Dis.*, 69, 5, e2541–e2550. DOI: 10.1111/tbed.14598.
7. **Doceul, V., Bagdassarian, E., Demange, A., Pavio, N., 2016:** Zoonotic hepatitis E Virus: Classification, animal reservoirs and transmission routes. *Viruses*, 8, 10, 270. DOI: 10.3390/v8100270.
8. **Forgách, P., Nowotny, N., Erdélyi, K., Boncz, A., Zentai, J., Szucs, G., et al., 2010:** Detection of hepatitis E virus in samples of animal origin collected in Hungary. *Vet. Microbiol.*, 143, 2–4, 106–116. DOI: 10.1016/j.vetmic.2009.11.004.
9. **Jacková, A., Dudášová, K., Šalamúnová, S., Mandelík, R., Novotný, J., Vilček, Š., 2021:** Identification and genetic diversity of hepatitis E virus in domestic swine from Slovakia. *BMC Vet. Res.*, 17, 1, 232. DOI: 10.1186/s12917-021-02936-4.
10. **Kamar, N., Dalton, H. R., Abravanel, F., Izopet, J., 2014:** Hepatitis E virus infection. *Clin. Microbiol. Rev*., 27, 1, 116–138. DOI: 10.1128/CMR.00057-13.
11. **Kenney, S. P., Meng, X. J., 2019:** Hepatitis E virus genome structure and replication strategy. *Cold Spring Harb. Perspect. Med.*, 9, 1, a031742. DOI: 10.1101/cshperspect.a031724.
12. **Kosínová, E., Bendova, J., Vašičková, P., Smitalová, R., Prodělalová, J., 2012:** The prevalence of hepatitis E virus in piglets on Czech pig production farms and phylogenetic analysis of recovered isolates. *Vet. Med.*, 57, 3, 115–120. DOI: 10.17221/5858-VETMED.
13. **Lee, G. Y., Poovorawan, K., Intharasongkroh, D., Sa-Nguanmoo, P., Vongpunsawad, S., Chirathaworn, C., et al., 2015:** Hepatitis E virus infection: Epidemiology and treatment implications. *World J. Virol.*, 4, 4, 343–355. DOI: 10.5501/ wjv.v4.i4.343.
14. **Lee, G. H., Tan, B. H., Teo, E. Ch. Y., Lim, S. G., Dan, Y. Y., Wee, A., et al., 2016:** Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk. *Gastroenterology*, 150, 2, 355–357.e3. DOI: 10.1053/j.gastro.2015.10.048.
15. **Meng, X. J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., et al., 1998:** Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J. Virol.*, 72, 12, 9714–9721. DOI: 10.1128/JVI.72.12.9714-9721.1998.
16. **Miura, M., Inoue, J., Tsuruoka, M., Nishizawa, T., Nagashima, S., Takahashi, M., et al., 2017:** Full-length genomic sequence analysis of new subtype 3k hepatitis E virus isolates with 99.97% nucleotide identity obtained from two consecutive acute hepatitis patients in a city in northeast Japan. *J. Med. Virol.*, 89, 6, 1116–1120. DOI: 10.1002/jmv.24743.
17. **Nicot, F., Dimeglio, C., Miguerez, M., Jeanne, N., Latour, J., Abravanel, F., et al., 2021:** Classification of the zoonotic hepatitis E virus genotype 3 into distinct subgenotypes. *Front. Microbiol.*, 11, 634430. DOI: 10.3389/fmicb.2020.634430.
18. **Nishizawa, T., Takahashi, M., Tsatsralt-Od, B., Nyamdavaa, K., Dulmaa, N., Osorjin, B., et al., 2021:** Identification and a full genome analysis of novel camel hepatitis E virus strains obtained from Bactrian camels in Mongolia. *Virus Res.*, 299, 198355. DOI: 10.1016/j.virusres.2021.198355.
19. **Pierini, I., Di Bartolo, I., Manuali, E., Pirani, S., Bazzucchi, M., Moscati, L., et al., 2021:** Hepatitis E virus (HEV) genotype 3 diversity: Identification of a novel HEV subtype in wild boar in Central Italy. *Transbound Emerg. Dis.*, 68, 4, 2121–2129. DOI: 10.1111/tbed.13860.
20. P**urdy, M. A., Drexler, J. F., Meng, X. J., Norder, H., Okamoto, H., Van der Poel, W. H. M., et al., 2022:** ICTV virus taxonomy profile: Hepeviridae 2022. *J. Gen. Virol.*, 103, 9. DOI: 10.1099/jgv.0.001778.
21. **Renou, C., Roque-Afonso, A. M., Pavio, N., 2014:** Foodborne transmission of hepatitis E virus from raw pork liver sausage, France. *Emerg. Infect. Dis.*, 20, 11, 1945–1947. DOI: 10.3201/eid2011.140791.
22. **Riveiro-Barciela, M., Míngues, B., Gironés, R., Rodriguez-Frías, F., Quer, J., Buti, M., 2015:** Phylogenetic demonstration of hepatitis E infection transmitted by pork meat ingestion. *J. Clin. Gastroenterol.*, 49, 2, 165–168. DOI: 10.1097/MCG.0000000000000113.
23. **Sooryanarain, H., Meng, X. J., 2019:** Hepatitis E virus: Reasons for emergence in humans. *Curr. Opin. Virol.*, 34, 10–17. DOI: 10.1016/j.coviro.2018.11.006.
24. **Smith, D. B., Izopet, J., Nicot, F., Simmonds, P., Jameel, S., Meng, X. J., et al., 2020:** Update: Proposed reference sequences for subtypes of hepatitis E virus (species Orthohepevirus A). *J. Gen. Virol.*, 101, 7, 692– 98. DOI: 10.1099/jgv.0.001435.
25. **Smith, D. B., Simmonds, P., Members of the International Committee on the Taxonomy of Viruses Study Group, Jameel, S., Emerson, S. U., Harrison, T. J., et al., 2014:** Consensus proposals for classification of the family Hepev-

iridae. *J. Gen. Virol.*, 95, 10, 2223–2232. DOI: 0.1099/vir.0.068429-0.

26. **Smith, D. B., Simmonds, P., Izopet, J., Oliveira-Filho, E. F., Ulrich, R. G., Johne, R., et al., 2016:** Proposed reference sequences for hepatitis E virus subtypes. *J. Gen. Virol.*, 97, 3, 537–542. DOI: 10.1099/jgv.0.000393.
27. **Tamura, K., Stecher, G., Kumar, S., 2021:** MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.*, 38, 7, 3022–3027. DOI: 10.1093/molbev/msab120.
28. **Vašíčková, P., Psikal, I., Widén, F., Smitalová, R., Bendova, J., Pavlík, I., et al., 2009:** Detection and genetic characterization of hepatitis E virus in Czech pig production herds. *Res. Vet. Sci.*, 87, 1, 143–148. DOI: 10.1016/j.rvsc.2008.10.013.
29. **Wang, Y., Toh, X., Ong, J., Teo, X. H., Bay, P., Fernandez, Ch. J., et al., 2020:** Serological prevalence and molecular characterization of hepatitis E virus in imported pigs in Singapore (2000–2019). *Transbound Emerg. Dis.*, 69, 2, 286–296. DOI: 10.1111/tbed.13977.
30. **Woo, P. C. Y., Lau, S. K. P., Teng, J. L. L., Cao, K. Y., Wernery, U., Schountz, T., et al., 2016:** New hepatitis E virus genotype in Bactrian camels, Xinjiang, China, 2013. *Emerg. Infect. Dis.*, 22, 12, 2219–2221. DOI: 10.3201/eid2212.160979.
31. **Xia, H., Liu, L., Linde, A. M., Belák, S., Norder, H., Widén, F., 2008:** Molecular characterization and phylogenetic analysis of the complete genome of a hepatitis E virus from European swine. *Virus Genes*, 37, 1, 39–48. DOI: 10.1007/s11262-008-0246-9.

Received February 3, 2023
Accepted March 22, 2023

sciendo

DOI: 10.2478/fv-2023-0013

FOLIA VETERINARIA, 67, 2: 18—23, 2023

INCIDENCE OF TRICHOTHECENES DEOXYNIVALENOL AND T-2 TOXIN IN POULTRY FEED MIXTURES

Michaela Harčárová, Pavel Naď
Department of Animal Nutrition and Husbandry, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia

michaela.harcarova@uvlf.sk

ABSTRACT

Trichothecenes are among the most important fusariotoxins. According to their chemical structure, they are divided into 4 groups (A–D). In terms of agriculture, trichothecenes of groups A and B are of greatest importance. In this study, the incidence of trichothecenes (deoxynivalenol and T-2 toxin) in mixed feed for broilers and turkeys were determined. Deoxynivalenol was detected in all analysed samples of feed mixture for broilers and turkeys (100 %) at an average concentration of 1.776 ppm; 0.675 ppm, respectively. T-2 toxin was present in 93.8 % of mixed feed for broilers at an average concentration of 36.625 ppb and in all of the tested samples (100 %) of turkey mixed feed (average level 25.899 ppb). The trichothecenes deoxynivalenol and T-2 toxin in feed samples for poultry did not exceed concentrations recommended by legislation.

Key words: ELISA; feed; mycotoxins; poultry; trichothecenes

INTRODUCTION

Trichothecenes represent a large group of secondary metabolites of microscopic filamentous fungi of the genus *Fusarium*. According to the characteristic functional chemical group (different substituents on C–3, 4, 7, 8 and 15), they are divided into four basic groups (A–D). The basis of the trichothecene molecule is a tricyclic sesquiterpene ring with a double bond between carbon C9, C10 and an epoxy ring in the position of carbon C12, C13, which is responsible for their toxicity. Trichothecenes are referred to as 12,13-epoxytrichothecenes [20]. The chemical structure of trichothecenes is variable and depends on the number and position of hydroxyl groups and also on the number and position of esterification groups. Trichothecenes of the genus *Fusarium* are relatively simple alcohols with short ester chains. The starting molecule in the biosynthesis of trichothecenes is trichodiene [13]. The occurrence of trichothecenes in grain is associated with cold and wet harvests and they are the most commonly found in wheat, barley and corn. In cereal grains are dominant trichothecenes of type A (T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol and verulol) and trichothecenes of type B (deoxynivalenol, acetylated forms of deoxynivalenol 3-ADON and 15-ADON, nivalenol and fusarenon-X) [9]. In terms of toxicity, trichothecenes are classified as gastrointestinal toxins, dermatotoxins, immunotoxins, hematotoxins and genotoxins [28]. Trichothecenes are resistant to inactivation during the processing of the grain. Therefore, the presence of trichothecenes in feed cannot be completely prevented and can be a cause of disruption of the productive

health of animals [8]. In poultry, trichothecene toxicosis is manifested acutely or chronically. The acute form of poisoning has a characteristic clinical picture, fast progress and is easily diagnosed. However, the chronic form manifests unspecific clinical symptoms and diagnosis is more difficult [26].

Deoxynivalenol – DON (Fig. 1) was first isolated in Japan in 1972 by Mooroko, as mentioned in the study by B r e i d e n b a c h et al. [2]. It is the most common worldwide distributed trichothecene [27]. Its producers are *F. graminearum, F. culmorum, F. sporotrichioides, F. poae* and *F. acuminatum* [25]. It occurs mainly in wheat, barley, corn, oats, rice, sorghum and millet. DON inhibits DNA and RNA synthesis at the level of ribosomes. It damages the expression of cytokines and increases the level of intracellular calcium, which leads to caspase activation and DNA and protein breaks with subsequent cell apoptosis [23]. In poultry, after feeding feed containing deoxynivalenol, a reduced weight of the egg itself, a deterioration in the quality of the eggshell, and residues in the egg was observed [21]. Progressive rickets was detected when broilers were fed trichothecene-containing feed for a long time [4].

T-2 toxin (Fig. 2) is produced by several species of *Fusarium* moulds, mainly *Fusarium sporotrichioides* and *Fusarium poae*. The occurrence of T-2 toxin has been recorded in cereals (corn, wheat, oats and barley), beans and soybeans [16].

T-2 toxin is readily metabolized to HT-2 toxin from which it differs structurally by the functional group at the C4 position. T-2/HT-2 toxins have been shown to produce numerous adverse effects on many animals, these two mycotoxins are frequently evaluated together [22]. T-2 toxin is a strong inhibitor of protein synthesis at the level of ribosomal RNA, disrupts DNA and RNA synthesis and induces immunosuppression [1, 12]. Trichothecene T-2 toxin is characterized by extreme skin and mucosal toxicity [15]. It is very toxic for poultry, in turkeys and chickens it causes necrosis on the beak, oral cavity, tongue and hard palate. Intoxication of poultry by T-2 toxin can lead to a reduction in weight gain, poor feathering, motor function impairment, and increased susceptibility to *E. coli* and *Salmonella* spp. [6, 14, 24].

The aim of this research was to determine concentrations of trichothecenes, deoxynivalenol and T-2 toxin in mixed feed for broilers and turkeys by immunoassay ELISA.

MATERIALS AND METHODS

Samples

A total of 20 samples of mixed feed for poultry were examined (16 samples were collected from mixed feed for broilers and 4 samples from mixed feed for turkeys)

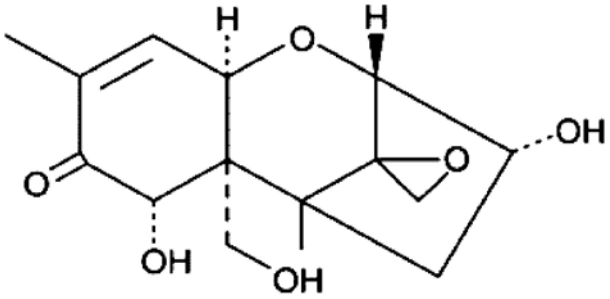

Fig. 1. Structural formula of deoxynivalenol

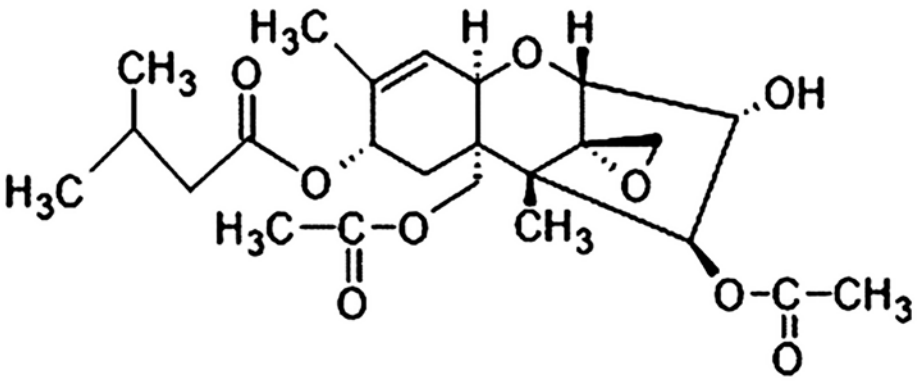

Fig. 2. Structural formula of T-2 toxin

(Tab. 1). The samples were obtained from commercial vendors in the form of pellets and were intended for different age categories of poultry. Mixed feed for broilers and turkeys was obtained from the producers from eastern Slovakia.

Determination of deoxynivalenol

The ELISA method was used to determine deoxynivalenol in samples of mixed feed for poultry. Analysis were performed using the Veratox 5/5 Quantitative DON Test (Neogen Corporation, Lansing, USA). Samples for the determination of deoxynivalenol were processed as follows: 50 g of each sample was ground and mixed with 250 ml of distilled water. The samples were mixed on a shaker for 3 minutes and then filtered through Whatman 1 filter paper (Cytiva, Kent, UK). The obtained filtrates from samples were diluted with distilled water (1 ml extract to 1 ml distilled water). Thus prepared samples were used in the ELISA analysis itself, which represents a direct competitive enzyme immunoassay. The principle of this method is the competition of unlabelled deoxynivalenol from samples and standards (standards with concentrations of deoxynivalenol 0, 0.25, 0.5, 1 and 2 ppm; $mg.kg^{-1}$) with enzyme labelled deoxynivalenol (conjugate) for antibody binding sites. After washing the samples, a substrate was added, which reacts with the conjugate to produce a blue colour. The more intense this colour reaction is, the less deoxynivalenol the sample contains. The resulting concentrations of deoxynivalenol (ppm) were determined spectrophotometrically at 650 nm using an ELISA reader (Dynex Technologies, Inc., Chantilly, USA).

Table 1. Number of analysed mixed feed for poultry

Mixed feed for broilers	Number of samples
BR1 (diet for fattening of broilers)	4
BR2 (diet for the growth of the broilers)	4
BR3 (final diet)	8
Mixed feed for turkeys	**Number of samples**
Morka Midi (diet for turkey from 9 to 12 weeks of age)	2
Morka Maxi (final diet)	2

Determination of T-2 toxin

The procedure for preparing samples for the determination of T-2 toxin was as follows: 25 ml of 70 % methanol was added to 5 g of the ground sample. The samples were shaken for 3 minutes on a shaker (Orbital Shaker Biosan) and filtered through a filter paper Whatman 1 (Cytiva, Kent, UK). After dilution with distilled water in a ratio of 1 : 1, they were prepared for quantitative determination using an ELISA kit. The procedure was performed according to manufacturer's protocol Veratox for T-2/HT-2 Quantitative Test (Neogen Corporation, Lansing, USA) The T-2 concentrations (ppb; $\mu g.kg^{-1}$) were determined at a wavelength of 650 nm by ELISA reader (Dynex Technologies, Inc., Chantilly, USA).

Statistical analysis

The statistical functions of the MS Excel software were used to evaluate the mean values and medians.

RESULTS

Table 2 shows the concentrations of trichothecenes deoxynivalenol and T-2 toxin in the mixed feed for poultry tested in this study. Deoxynivalenol in mixed feed for broilers was detected in all analysed samples (100 %) in a range of 0.435–1.829 ppm (average concentration 1.776 ppm). All tested samples of mixed feed for turkeys (100 %) were contaminated with deoxynivalenol in levels ranging from 0.283 to 1.067 ppm (average value 0.675 ppm). T-2 toxin

Table 2. Incidence [%] and concentrations of deoxynivalenol [$mg.kg^{-1}$] and T-2 toxin [$\mu g.kg^{-1}$] in poultry feed samples

Mixed feed	Parameter	DON	T-2 toxin
For broilers	n	16	16
	+/n	16/16 (100 %)	15/16 (93.8 %)
	Minimum	0.435	nd
	Maximum	1.829	42.285
	Average	1.776	36.625
	Median	1.179	17.419
For turkeys	n	4	4
	+/n	4/4 (100 %)	4/4 (100 %)
	Minimum	0.283	9.115
	Maximum	1.067	45.088
	Average	0.675	25.899
	Median	0.675	24.696

DON – deoxynivalenol; n – total number of samples; + – positive samples; nd – not detected

was present in 93.8 % of samples of mixed feed for broilers at an average concentration of 36.625 ppb and all the tested samples of mixed feed for turkey were contaminated (average value 25.899 ppb).

The recommended values of trichothecenes; deoxynivalenol and T-2 toxin were not exceeded in the tested samples.

DISCUSSION

Mycotoxin contamination of feed is a global agriculture problem [19]. Up to now, over 500 compounds have been identified as mycotoxins [17]. The most commonly studied mycotoxins with the greatest concern to human and animal health are aflatoxins, zearalenone, trichothecenes, patulin, ochratoxins, and fumonisins [11]. From the point of view of feed contamination, trichothecenes of groups A and B are of the greatest importance. Trichothecenes deoxynivalenol and T-2 toxin are predominantly found in cereals (wheat, maize), which are the basic part of feed mixtures for poultry. In the European Union, the maximum permitted content of deoxynivalenol in feed (EC Directive 2002/32/EC and EC Recommendations 2006/576/EC) is 5 $mg.kg^{-1}$ in supplementary and complete mixed feed and of T-2 toxin 250 $\mu g.kg^{-1}$ in mixed feed [5, 7, 29].

In our samples of poultry mixed feed, we recorded 100 % occurrence of deoxynivalenol in a range from 0.435 $mg.kg^{-1}$ to 1.829 $mg.kg^{-1}$. One hundred percent contamination of mixed feed for poultry with deoxynivalenol was also recorded by Cegielska-Radziejewska et al. [3]. However, DON was detected in the range of lower concentrations 0.003–0.099 $mg.kg^{-1}$ [3]. Greco et al. [10] reported that deoxynivalenol was found in 90 % of the analysed samples. According to Labuda et al., the mean DON content in positive samples (56 %) of mixed feed for poultry from Slovakia was 0.303 $mg.kg^{-1}$ [18]. In contrast, Magnoli et al. observed only 6 % deoxynivalenol contamination of poultry feeds in Argentina [19].

In our study, 93.8 % T-2 toxin contamination of mixed feed for broilers was confirmed. All tested samples of mixed feed for turkeys were contaminated by T-2 toxin. In the study of Labuda et al. [18], T-2 toxin was the most frequent mycotoxin detected. It was found in 90 % of the samples in relatively low concentrations ranging from 1 to 130 $\mu g.kg^{-1}$ (average value 13 $\mu g.kg^{-1}$). According to Greco et al. [10] T-2 toxin was detected in 38 samples out of 49 samples from Buenos Aires (78 %). However, in Poland, the presence of toxin T-2 was not detected in any of the total of 45 samples of feed mixture for poultry [3].

Deoxynivalenol and T-2 toxin in feed samples for poultry did not exceed maximum levels specified in the regulations and the concentrations detected were too small to cause any serious health problems to animals that consumed such feed.

CONCLUSIONS

The best protection against mycotoxins is the monitoring of their presence in feeds and foods. That means testing all along the pathway from initial harvest of grains to the finished product. Results indicate that the level of microbiological contamination in feeds for broiler chickens and turkeys produced in the Slovak Republic is within the requirements of the binding standards.

ACKNOWLEDGEMENT

This study was supported by the Scientific grant agency, VEGA project No. 1/0402/20 "Effect of additives in the nutrition of monogastric animals on production health, production parameters, products quality and environment."

REFERENCES

1. **Adhikari, M., Negi, B., Kaushik, N., Adhikari, A., Al-Khedhairy, A. A., Kaushik, N. K., Choi, E. H., 2017:** T-2 mycotoxin: Toxicological effects and decontamination strategies. *Monotargeted*, 8, 20, 33933–33952. DOI: 10.18632/oncotarget.15422.
2. **Breidenbach, M., Cramér, R., Lehrer, S., 2002:** Fungal allergy and pathogenicity. *Rev. Inst. Med. Trop.*, 44, 6. DOI: 10.1590/S0036-46652002000600012.
3. **Cegielska-Radziejewska, R., Stuper, K., Szablewski, T., 2013:** Microflora and mycotoxin contamination in poultry feed mixtures from western Poland. *Ann. Agric. Environ. Med.*, 20, 1, 30–35.
4. **Dänicke, S., 2002:** Prevention and control of mycotoxins in the poultry production chain: A European view. *World Poult. Sci. J.*, 58, 451–474. DOI: 10.1079/WPS20020033.

5. **Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed, 2022:** *Official Journal of the European Communities*, L 140/10, 2002. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32002L0032. Accessed January 13, 2023.
6. **Devreese, M., De Backer, P., Croubels, S., 2013:** Different methods to counteract mycotoxin production and its impact on animal health. *Vlaams Diergen. Tijds*., 82, 181–190.
7. **European Commission (EC) Commission Recommendation 576/2006/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding, 2006:** https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32006H0576. Accessed January 13, 2023.
8. **Foroud, N. A., Baines, D., Gagkaeva, T. Y., Thakor, N., Badea, A., Steiner, B., et al., 2019:** Trichothecenes in cereal grains – An update. *Toxins (Basel)*, 11, 11, 634. DOI: 10.3390/toxins11110634.
9. **Gab-Allah, M. A., Choi, K., Kim, B., 2023:** Type B trichothecenes in cereal grains and their products: Recent advances on occurrence, toxicology, analysis and post-harvest decontamination strategies. *Toxins*, 15, 2, 85. DOI: 10.3390/toxins15020085.
10. **Greco, M. V., Franchi, M. L., Rico Golba, S., L., Pardo, A. G., Graciela Noemí Pose, G. N., 2014:** Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. *Sci. World J.*, 1–9. DOI: 10.1155/2014/968215.
11. **Haque, M. A., Wang, Y., Shen, Z., Li, X., Saleemi, M. K., He, C., 2020:** Mycotoxin contamination and control strategy in human, domestic animal and poultry: A review. *Microb. Pathog.*,142, 104095. DOI: 10.1016/j.micpath.2020.104095.
12. **Hossam E., 2013:** Mycotoxins-induced oxidative stress and disease, mycotoxin and food safety in developing countries. In **Makun, H. A. (Ed.):** *InTech, Croatia*, 2013, 63–92.
13. **Chtioui, W., Balmas, V., Delogu, G., Migheli, Q., Oufensou, S., 2022:** Bioprospecting phenols as inhibitors of trichothecene-producing Fusarium: Sustainable approaches to the management of wheat pathogens. *Toxins*, 14, 2, 72. DOI: 10.3390/toxins14020072.
14. **Indresh, H. C., Umakantha, B., 2013:** Effects of ochratoxin and T-2 toxin combination on performance, biochemical and immune status of commercial broilers. *Vet. World*, 6, 11, 945–949. DOI: 10.14202/vetworld.2013.945-949.
15. **Janik, E., Niemcewicz, M., Podogrocki, M., Ceremuga, M., Stela, M., Bijak, M., 2021:** T-2 toxin – the most toxic trichothecene mycotoxin: Metabolism, toxicity, and decontamination strategies. *Molecules*, 26, 6868. DOI: 10.3390/molecules26226868.
16. **Ji, F., He, D., Olaniran, A. O., Mokoena, M. P., Xu, J., Shi, J., 2019:** Occurrence, toxicity, production and detection of Fusarium mycotoxin: A review. *Food Prod. Process. Nutr.*, 1, 6, 1–14. DOI: 10.1186/s43014-019-0007-2.
17. **Kolawole, O., Graham, A., Donaldson, C., Owens, B., Abia, W. A., Meneely, J., et al., 2020:** Low doses of mycotoxin mixtures below EU regulatory limits can negatively affect the performance of broiler chickens: A longitudinal study. *Toxins (Basel)*, 12, 7, 433. DOI: 10.3390/toxins12070433.
18. **Labuda, R., Parich, A., Berthiller, F., Tančinová, D., 2005:** Incidence of trichothecenes and zearalenone in poultry feed mixtures from Slovakia. *Int. J. Food Microbiol.*, 105, 19–25. DOI: 10.1016/j.ijfoodmicro.2005.06.005.
19. **Magnoli, C. E., Chiacchiera, S. M., R. Miazzo, R., 2002:** The mycoflora and toxicity of feedstuffs from a production plant in Cordoba, Argentina. *Mycotoxin Res.*, 18, 7–22. DOI: 10.1007/BF02946135.
20. **McCormick, S. P., Stanley, A. M., Stover, N. A., Alexander N. J., 2011:** Trichothecenes: From simple to complex mycotoxins. *Toxins*, 3, 7, 802–814. DOI: 10.3390/toxins3070802.
21. **Murugesan, G. R., Ledoux, D. R., Naehrer, K., Berthiller, F., Applegate, T. J., Grenier, B., et al., 2015:** Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies. *Poult. Sci.*, 6, 1298–315. DOI: 10.3382/ps/pev075.
22. **Nathanail, A. V., Varga, E., Meng-Reiterer, J., Bueschl, C., Michlmayr, H., Malachova, A., et al., 2015:** Metabolism of the *Fusarium* mycotoxins T-2 toxin and HT-2 toxin in wheat. *J. Agric. Food. Chem.*, 63, 35, 7862–72. DOI: 10.1021/acs.jafc.5b02697.
23. **Ostry, V., Dofkova, M., Blahova, J., Malir, F., Kavrik, R., Rehurkova, I., Ruprich, J., 2020:** Dietary exposure assessment of sum deoxynivalenol forms, sum T-2/HT-2 toxins and zearalenone from cereal-based foods and beer. *Food Chem. Toxicol.*, 139, 111280. DOI: 10.1016/j.fct.2020.111280.
24. **Pande, V. V., Kurkure, N. V., Bhandarkar, A. G., 2006:** Effect of T-2 toxin on growth, performance and haematobiochemical alterations in broilers. *Ind. J. Exp. Biol.*, 44, 1, 86–88.
25. **Pinto, A. C. S. M., De Pierri, C. R., Evangelista, A. G., Gomes, A. S. deL. P. B, Luciano, F. B., 2022:** Deoxynivalenol: Toxicology, degradation by bacteria, and phylogenetic analysis. *Toxins*, 14, 2, 90. DOI: 10.3390/toxins14020090.

26. **Resanović, R., Nešić, K., Nešić, V., Palić, T.D., Jaćević, V., 2009:** Mycotoxins in poultry production. *Zb. Matice Srp. Prir. Nauk.*, 116, 7–14. DOI: 10.2298/ZMSPN0916007R.
27. **Riley, R. T., Pestka, J., 2005:** Mycotoxins: Metabolism, mechanisms and biochemical markers. In **Diaz, D. E.:** *The Mycotoxin Blue Book*. University Press, Nottingham, 283 pp.
28. **Santin, E., 2005:** Mould growth and mycotoxin production. In **Diaz, D. E.:** *The Mycotoxin Blue Book*. University Press, Nottingham, 225–234.
29. **Stoev, S. D., 2015:** Foodborne mycotoxicoses, risk assessment and underestimated hazard of masked mycotoxins and joint mycotoxin effects or interaction. *Environ. Toxicol. Pharmacol.*, 39, 2, 794–809. DOI: 10.1016/j.etap.2015.01.022.

Received February 16, 2023
Accepted March 22, 2023

sciendo

DOI: 10.2478/fv-2023-0014

FOLIA VETERINARIA, 67, 2: 24—32, 2023

DENDRIMERS AS ANTIMICROBIAL AGENTS IN THE CENTRAL NERVOUS SYSTEM INFECTIONS. A REVIEW

Katarína Kucková[1], Mangesh Bhide[1, 2]

[1]Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice

[2]Institute of Neuroimmunology, Slovak Academy of Sciences v. v. i., Dúbravská cesta 9, 845 10 Bratislava Slovakia

mangesh.bhide@uvlf.sk

ABSTRACT

Bacterial meningitis is a serious infection of the central nervous system that affects people all over the world. *Streptococcus pneumoniae* and *Neisseria meningitidis* are the most common pathogens causing meningeal inflammation in Europe. Treatment with standard antibiotics is becoming ineffective, not only due to their inability to cross the blood-brain barrier, but also due to rising antibiotic resistance. As a result, novel therapeutics to combat the infection are required. A promising solution could be therapeutic nanomolecules, such as dendrimers, some of which have antimicrobial properties due to their chemical structure. Additionally, they may be decorated with a suitable therapeutic and central nervous system homing peptides to construct nano-drug delivery systems, which can effectively cross the blood-brain barrier. To synthesize safe dendrimeric nano-drug delivery system it is necessary to select the best dendrimer candidates with antimicrobial activity and to understand pharmacosafety, pharmacokinetics and dynamics. This review provides a brief overview of dendrimers and their antimicrobial properties as they have been studied in relation to the blood-brain barrier and existing antibiotics.

Key words: bacterial meningitis; blood-brain barrier; central nervous system; dendrimer; drug delivery

INTRODUCTION

Bacterial meningitis

Among the central nervous system (CNS) infections caused by various pathogens (bacteria, viruses, fungi), bacterial meningitis is the most common life-threating infection often ending in death [20, 39]. Approximately half of survivors have cognitive impairments that put them at risk of permanent disability [4, 61]. Meningitis has been recognized for over a century as an inflammation of the membranes that cover the brain and spinal cord – the meninges, particularly the pia mater and arachnoid mater [16, 23]. The pathogens responsible for approximately 80% of bacterial meningitis are Gram negative meningococcus *Neisseria meningitidis* and Gram positive pneumococcus *Streptococcus pneumoniae* [22]. Other bacteria that can colonize different mucosal surfaces, penetrate protective barriers, and cause infection include *Haemophilus influenzae* type b, *Escherichia coli* K1, *Mycobacterium tuberculosis, Listeria monocytogenes, Staphylococcus aureus, Salmonella, Klebsiella* spp. [11,58] or some species of spi-

rochete *Borrelia burgdorferi sensu lato* complex [34, 64]. *Streptococcus suis* is a bacterium that originally infects pigs and pork and cause a major problem in swine industry worldwide. Moreover, it is also a zoonotic pathogen responsible for several infections in humans, including meningitis [15, 69].

The ability to survive in blood and successfully evade host immunity through a variety of mechanisms (e. g. encapsulation, complement resistance) is critical for spreading from the primary site of infection into the brain via the blood-brain barrier (BBB). Three major meningeal pathogens (*S. pneumoniae, N. meningitidis, H. influenzae*) share molecular mimicry called "innate invasion", which acts against innate immune system [11, 41]. Exact mechanism how they are able cross the BBB is still not fully explored. Extracellular bacteria may induce signalling events after adhesion to endothelial cells, which may lead to transcytosis or disruption of tight junctions between the cells. *E. coli, Streptococcus* or *S. pneumoniae* produce toxins, which also damage cells. Then, bacteria can cross the BBB via paracellular way as well. Moreover, *N. meningitidis* or *S. pneumoniae* may reach the brain through olfactory nerve [40]. Other pathogens, for example, intracellular *M. tuberculosis* and *L. monocytogenes* use macrophages to pass through various membranes. This phenomenon is known as "Trojan horse" [35].

Blood-brain barrier and antibiotics

Blood-brain barrier is anatomically formed by brain capillary endothelial cells, pericytes, astrocytes end foot and nerve cells (microglia), shown in the Fig. 1 [18, 71]. Between brain endothelial cells, on their apical side, are tight junctions (claudins, occludins, junctional adhesion molecules) and adherens junction proteins, which are responsible for creating literally tight connections between the cells [49, 54]. Thanks to these connections, BBB is a highly selective membrane and crossing of ions, oxygen, glucose [8, 49] and other molecules (such as therapeutics) from the blood vessels into brain parenchyma is limited [17].

Despite the fact that, as previously stated, bacteria can cross this barrier in a variety of ways. Due to low number of immune cells and complement proteins in the brain, they can multiply rapidly in cerebrospinal fluid, particularly in the subarachnoid area. Immediately after bacteria cross the barrier, they may cause extensive damage. After disruption of BBB, its permeability is higher [11, 12, 41], thus neutrophils and macrophages are able to passage through. Subsequent activation of brain cells (microglia, astrocytes) and strong immune response of local cells and leukocytes from the blood, presence of pro-inflammatory cytokines, chemokines, proteolytic enzymes, oxidants and bacterial toxins as well, lead to damage of epithelial cells of brain barriers, neurones and inflammation of meninges surrounding the subarachnoid space [23, 61].

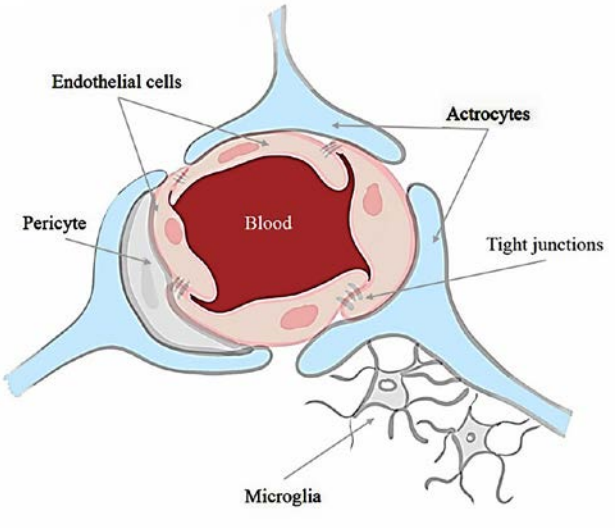

Fig. 1. Basic illustration of blood-brain barrier anatomy.
Original sketch, modified according to O m i d i et al. [49]

The use of antibiotics may decrease inflammation and restore balance of the BBB. However, using of β-lactam antibiotics may lead to cytokines and bacterial toxins release, which may dramatically increase inflammation response [12]. As a result, antibiotic treatment must be combined with other drugs that protect brain tissue by inhibiting several steps in the inflammatory cascade, such as dexamethasone [11]. The right choice of antimicrobial therapy depends on several factors that include mechanisms of antibiotic action, antimicrobial susceptibility, microbial growth rate, density and possible resistance to therapeutics. Patient age, status and type of infection are important as well. One of the most essential properties is the ability to cross the BBB into the subarachnoid space and act against bacteria. It is also affected by several physico-chemical factors, such as pH, structure of antibiotic, their protein binding ability or lipophilicity [12, 22]. Among the most used antibiotics to threaten meningococcal disease were sulfonamides, penicillin or chloramphenicol in the past [5]. D a v i s [9] published the list of

commonly used antibiotics for acute bacterial meningitis that included, for example, ciprofloxacin, cefepime, cefotaxime, gentamicin, meropenem, rifampin, vancomycin, also daptomycin and telavancin, but in high and long term dosages [9, 12]. Also, in the last few years, several new antibiotics have a potential to treat CNS infection, such as ceftriaxone, ceftobiprole, linezolid, moxifloxacin, trovafloxacin or tigecycline [46]. Additionally, thanks to synergistic effects of some of them, their appropriate combinations may be or are even desirable to be used [12].

Antimicrobial resistance is a natural phenomenon considering that several antibiotics are derived or naturally produced. Let us look at a few well-known examples: Actinomycetes produce secondary metabolites such as vancomycin, tetracycline, streptomycin and erythromycin [52], and, of course, mould *Penicilium notatum* produce penicillin [10]. History and mechanisms of antibiotic resistance are precisely described elsewhere [28, 31, 57, 70], discussion of those details would be beyond the scope of this mini-review. Antimicrobial resistance has also become a worldwide problem due to the overuse of antibiotics in therapy as well as feed supplements in husbandry or gain body weight [45]. *S. pneumoniae* has been resistant to penicillin for more than 60 years, and as a result, combination of two antibiotics (usually vancomycin and cephalosporin) is required instead of penicillin alone [22]. O p p e n h e i m published a brief overview in 1997 about increasing antibiotic resistance to drugs commonly used to treat meningococcal disease, which he saw as a future problem, particularly in the case of *N. meningitidis*. Therefore, more than 25 years ago, he suggested as a possible solution vaccination against meningococci and reducing of worldwide excessive using of antibiotics in the future [50]. Another report occurrence of multiresistant bacteria was emphasized in 1950s [36], and currently increasing multiresistant strains have been reported all over the world [60].

Nanomolecules in biomedicine

In biomedicine different types of nanomolecules can be used, for example, liposomes, micelles, metal nanoparticles, nanotubes, polymers or dendrimers [37]. Antimicrobial polymers in combination with other antimicrobial molecules, such as antibiotics, could be one of the most intriguing strategies for developing new nanotherapeutics [13]. More than 80 new nanoformulations are currently in preclinical testing and could be used as nano-drug delivery systems [27]. Dendrimers are among the nanoparticles that we are interested in.

Dendrimers

According to T o m a l i a et al. [68] their name consist of two Greek words: dendros (tree) and meros (part) [7]. Dendrimers are molecules with a regular geometrical structure [53] in size between 2 to 5 nm [17]. They have globular, radially symmetric, hyperbranched arrangement consisting of core, branching dendrones and various peripheral surface groups [38, 48], which defines their properties [37], as shown in the Fig. 2. In fact, polyamidoamine (PAMAM) dendrimer firstly synthesized by T o m a l i a et al. [68] is one of most studied, commercialized dendrimer from which many modification are constructed. The hydrophobic core of PAMAM is made of diamines (e. g. ethylene diamine), branches are made of ethylene diamine or methyl acrylate and on the surface are usually amide or carboxylic groups [67]. Unlike polymers synthesized by polymerization, dendrimers are synthetized differently, by two most commonly used methods. In divergent synthesis branches grows around the core layer by layer creating new "generation". On the contrary, in convergent synthesis they are made from periphery to the core [7, 48].

Polymer composition results in inner cavities containing one or more therapeutic agents [59]. Fig. 2 shows 3 possible basic interactions between dendrimer and drug: cleavable (ester, amid) bond (a), covalent bond (b) or as internal encapsulated part (c) part of structure. Therapeutic molecules (like antibiotics) could also be part of associated dendrimers. Covalent bond is very stable, thus the usage of dendrimers bounded with therapeutics by cleavable bond is more appropriate [7].

The physicochemical characteristic of nanomaterials (e. g. size, shape, surface properties, reactivity or biocompatibility) are crucial in stability, toxicity or biodistribution and drug delivery [37, 56]. Using dendrimers as a possible drug delivery system have a several advantages: safety, efficacy, regulated rate and location of drug release [2]. According to H u a n g and W u [26] the so-called "linkers" between drug and dendrimer may affect the activity and the drug release. Among these linkers are (1) ester bonds, which may be cleavable by ester enzymes in the cell, (2) groups unstable in acidic conditions of tumour or inflamed tissue (acetal/ketal, cis-aconityl, hydrazone groups) and (3) disulphide bond, which can be broken by glutathione

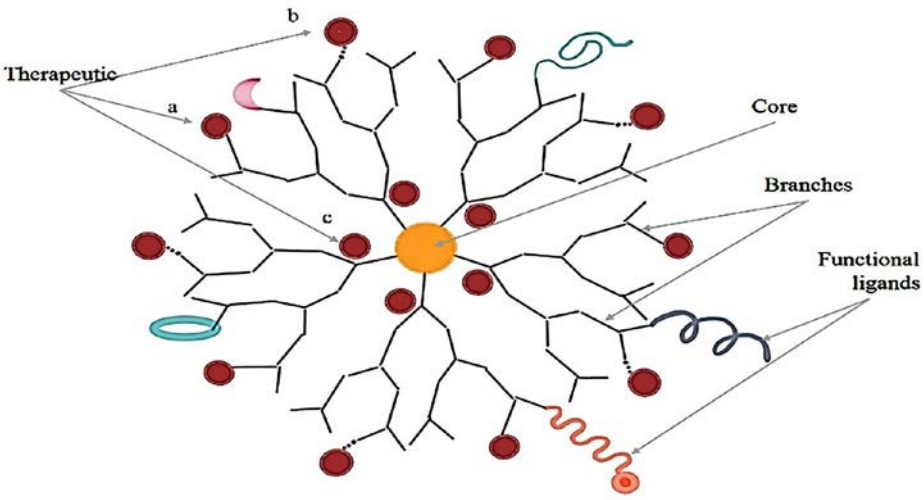

Fig. 2. Scheme of dendrimer structure and possible ways of therapeutics binding by a) cleavable (ester, amid) bond, b) covalent bond or as c) encapsulated therapeutic.
Original sketch, modified according to C a m i n a d e and T u r r i n [7].

in the cell [26]. Controlling dendrimer biodegradation into small fragments is also one of possible way to releasing drug at the specific target site. This intelligent delivery system may reduce the potential toxicity and side effects of drug-dendrimer molecule lower while increasing efficacy [26, 43].

In general, applying small molecules to the surface of nanoparticles opened up new avenues for potential applications because they determine their properties. These molecules can be antibodies, nanobodies, nucleic acids, aptamers, proteins or peptides, carbohydrates, signalling molecules etc. [63]. These functional ligands (Fig. 2) attached to branches influence the dendrimer`s reactivity to other receptor sites of cells, enzymes, peptides or pathogens, as well as the solubility of conjugated drug [59]. Nanobodies are small, single-domain and stable fragments of antibody with approximately 15 kDa weight [51]. Conjugation of specific nanobodies as one of functional group to the surface of dendrimer will allow active targeting of bacteria [21]. Similarly, using of aptamers, small oligonucleotides, is suitable option compared with antibody. They are able to penetrate through membranes, they have high specificity and stability in temperature and pH. Moreover, they have unlimited shelf life and may be diversified. Thus aptamers may be used as therapeutic agents or targeting component for drug delivery system [55]. Furthermore, several types of dendrimers are able to generate fluorescence under specific conditions (pH, oxidation). In order to improve this property and allow to study biodistribution and localization of nanoparticles in *in vitro* or *in vivo* systems by various imaging techniques, they can be associated with fluorescence dye [33, 42]. Dendrimers are used in a variety of medical applications, including tissue engineering, gene transfection, cancer treatment, drug delivery system, and as antiviral, antiparasitic, and antimicrobial agents [17, 48]. They have been used as therapeutics for about twenty years [53].

Despite extensive research into the functionality and safety of dendrimers, there is still the possibility of some negative effects. Among the negative properties of dendrimer is the possibility of toxicity not only to pathogens but also to human/animal cells. Another limitation is the inability to control drug`s incorporation and subsequent release [59]. Furthermore, structural changes – core, branches, surface – affect toxicity. Dendrimer of the third to fifth generation appears to be less harmful than the larger ones [67]. Often studied are polymers PEG (poly(ethylene)glycol) and PLGA (poly(lactic)-co-(glycolic) acid). They are biocompatible, biodegradable, low toxic and highly functionalized to using in drug delivery system to the brain [2]. PAMAM dendrimers conjugated with PEG showed lower toxicity while retaining antimicrobial activity [13].

They must be biodegradable, bio-pharmacologically safe, and highly selective in order to be effective as nano-drug delivery systems or therapeutics in clinical medicine. They should ensure low to no concentrations of captured therapeutics in non-targeted tissues while increasing concentration at the site of infection through precise drug

release, which could be accomplished using the methods described above [35]. They should also reduce treatment side effects and finally, avoid the development of antibiotic resistance [47, 63]. The advancement of nanobiotechnology should allow for the efficient use of targeted nano-drug delivery systems.

Antimicrobial effects of dendrimers

The use of dendrimers as antimicrobial agents has two effects. First, as previously stated, they can deliver a drug to the target site; second, they are therapeutics of themselves [56]. Dendrimer-drug conjugates are widely studied in many fields like cancer therapy, against inflammation, as antiviral or antimicrobial treatment [30, 32]. Choosing appropriate therapeutics to incorporate into dendrimer is a delicate issue. Aside from the chemical structure of the antibiotic and the dendrimer, the ability to fight effectively against G+ and G– bacteria is also important. For example, S v e n n i n g s e n et al. [66] synthesized PAMAM dendrimer-ciprofloxacin conjugate and observed synergic effect of this molecule against selected G+ or G– bacteria. It appears to be a beneficial solution, as drugs incorporation into dendrimers can increase solubility and bioavailability while decreasing dosage [59]. PAMAM or polypropylene imine (PPI) dendrimers with various terminal surface ligand modifications are the most studied dendrimers with positive effects against microbial or viral infection or inflammation [13, 14, 24]. Their biocidal effect is also determined by the size and generation of dendrimer branches. The main antibacterial mechanism is based on the cationic charge of dendrimer molecules, which can interact with negatively charged bacterial membranes. As a result, the membrane permeability increases, pores form, and bacteria lyse [17, 38]. G h o l a m i et al. synthesized a 7th generation PAMAM dendrimer and tested its antibacterial activity against a variety of G+ and G– bacteria (e. g. *E. coli, S. aureus, K. pneumonie, Acinetobacter baumannii*). They conclude, that this type of dendrimer is effective and tend to be a potential antimicrobial therapeutic [19]. C h e n et al. [29] also observed positive antibacterial effects of PPI dendrimers with quaternary ammonium groups. Not only cationic, but also modified anionic PAMAM dendrimers can be efficient against G+ bacteria [65].

Bacteria are able to recognize sugar on the surface of eukaryotic cells. Glycodendrimers decorated with various oligosaccharides on the surface remind eukaryotic cell, a result is bacteria binding to glycodendrimers instead their adhesion to the host cell [6]. Another type of effective dendrimeric nanomolecules are redox-active organometallic dendrimers. Antimicrobial properties of dendrimer conjugates with metal particles (e. g. silver, zinc or gold) enhance the stability and their bactericidal effect [2, 44]. They are able to produce free radicals and damage microbes. This mechanism with combination of positively charged molecule was successful against G+ bacteria (e. g. *S. aureus*) [1, 6]. Moreover, R i z v i et al. pointed out the possible usage of gold nanoparticles with combination of proper antibiotics, which could fight against pathogens causing bacterial meningitis (caused by in particular *S. pneumoniae* and *L. monocytogenes*) [58]. Interestingly, H o u et al. [25] and B a h a r et al. [3] observed positive antimicrobial effect of dendrimer against biofilm formed by *E. coli* or *Pseudomonas aeruginosa* respectively. The use of dendrimers may thus have a positive impact on the development of new antibiotic-resistant strains [6, 17], and it should be explored more.

Dendrimers ability to cross blood-brain

Drugs should be able to cross the BBB if pathogens can. Nonetheless, common therapeutics` passage and subsequent bioaccessibility are severely limited. This is one of the reasons why traditional antibiotics are almost ineffective in treatment CNS infections [59]. Thanks to nanostructure, dendrimers are able to cross various cell membranes and barriers, including BBB by paracellular or transcellular transport [17, 59]. The mechanism that was mentioned above also applies in this case. Molecules with positive charged surface may interact with negatively charged endothelial cell membrane and cross the barrier via adsorptive-mediated transcytosis (Fig. 3). Among other basic mechanisms of molecule transport, which are illustrated in the Fig. 3, belongs transcellular diffusion (1.), carrier-mediated transport (2.), passive or paracellular transport (3.) or receptor-mediated transport (4.) [54, 71, 72].

Receptor mediated transport, which is used for transmission of large molecules (e. g. proteins, hormones) is mostly utilized mechanism in bionanomedicine for the drug transport. Insulin, transferrin, low-density lipoprotein (LDL) receptors, and single domain llama antibodies are the most commonly studied [54]. The multifunctional receptors LRP1 and LRP2 are two of these LDL receptors. They can transport a variety of proteins, including lacto-

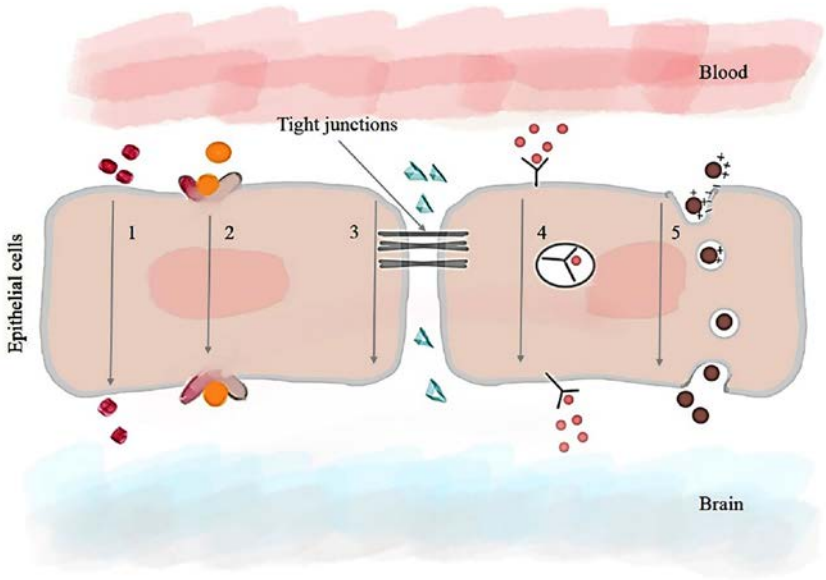

Fig. 3. Basic mechanisms of transport across blood-brain barrier. 1. Passive transcellular diffusion; 2. Carrier-mediated transport; 3. Paracellular transport; 4. Receptor-mediated transport; 5. Adsorptive-mediated transcytosis.
Original sketch, modified according to Velasco-Aguirre et al. [71]

ferrin, angiopep-2, and leptin peptide fragments [62]. Constructing nanoparticles, specifically dendrimers with these types of molecules on their surface as functional ligands, allows them to cross the BBB and have a direct effect on therapeutics at a target site. Furthermore, as previously stated, after pathogen entry into the CNS, tight junction proteins between endothelial cells are disrupted, increasing BBB permeability. As a result, dendrimers can more easily pass through the membrane via paracellular transport and attempt to fight the infection [72].

CONCLUSIONS

In conclusion, despite developed vaccines or antibiotics, bacterial meningitis poses a serious health risk. Pathogens adapt quickly, mutate and become resistant. To control meningitis globally, a new alternative antimicrobial strategy is required. We believe that using dendrimers as novel therapeutics is appropriate due to their advantageous structure as a nano-drug delivery agent to the central nervous system and health benefits. Given this information, a multidisciplinary approach is required to obtain stable, safe, and effective dendrimers for future treatment of bacterial meningitis in humans and animals.

ACKNOWLEDGEMENT

This study was supported by grants DSV-ITMS2014+ project code NFP313010V455, APVV-18-0259 and VEGA 1/0381/23.

REFERENCES

1. **Abd-El-Aziz, A. S., Agatemor, C., Etkin, N., Overy, D. P., Lanteigne, M., McQuillan, K., et al., 2015:** Antimicrobial organometallic dendrimers with tunable activity against multidrug-resistant bacteria. *Biomacromolecules*, 16, 11, 3694–3703. DOI: 10.1021/acs.biomac.5b01207.
2. **Ayub, A., Wettig, S., 2022:** An overview of nanotechnologies for drug delivery to the brain. *Pharmaceutics*, 14, 2. DOI: 10.3390/pharmaceutics14020224.
3. **Bahar, A. A., Liu, Z., Totsingan, F., Buitrago, C., Kallenbach, N., Ren, D., 2015:** Synthetic dendrimeric peptide active against biofilm and persister cells of Pseudomonas aeruginosa. *Appl. Microbiol. Biotechnol.*, 99, 19, 8125–8135. DOI: 10.1007/s00253-015-6645-7.
4. **Barichello, T., Generoso, J. S., Simoes, L. R. R., Goularte, J. S., Petronilho, F., Saigal, P., et al., 2016:** Role of microglial activation in the pathophysiology of bacterial meningitis. *Mol. Neurobiol.*, 53, 3, 1770–1781. DOI: 10.1007/s12035-015-9107-4.

5. **Bash, M. C., Matthias, K. A., 2017:** Antibiotic resistance in Neisseria. In **Mayers, D. L., Sobel, D. J., Ouellette, M., Kaye, K. S., Marchaim, D.:** *Antimicrobial Drug Resistance: Clinical and Epidemiological Aspects*, Volume 2. Springer Cham., 843–865.
6. **Borah, N., Gogoi, A., Saikia, J., 2022:** Dendrimeric entities as chemical alternatives toward antimicrobial therapy. In **Saha, T., Adhikari, M. D., Tiwary, B. K.:** *Alternatives to Antibiotics: Recent Trends and Future Prospects*. Springer, 379–400.
7. **Caminade, A. M.,Turrin, C. O., 2014:** Dendrimers for drug delivery. *J. Mater. Chem.*, 2, 26, 4055–4066. DOI: 10.1039/c4tb00171k.
8. **Coureuil, M., Lécuyer, H., Bourdoulous, S., Nassif, X., 2017:** A journey into the brain: insight into how bacterial pathogens cross blood-brain barriers. *Nat. Rev. Microbiol.*, 15, 3, 149–159. DOI: 10.1038/nrmicro.2016.178.
9. **Davis, L. E., 2018:** Acute bacterial meningitis. *Continuum* (Minneap. Minn.), 24, (5 Neuroinfectious Disease), 1264–1283. DOI: 10.1212/con.0000000000000660.
10. **Dodds, D. R., 2017:** Antibiotic resistance: A current epilogue. *Biochem. Pharmacol.*, 134, 139–146. DOI: 10.1016/j.bcp.2016.12.005.
11. **Doran, K. S., Fulde, M., Gratz, N., Kim, B. J., Nau, R., Prasadarao, N., et al., 2016**: Host-pathogen interactions in bacterial meningitis. *Acta Neuropathol.*, 131, 2, 185–209. DOI: 10.1007/s00401-015-1531-z.
12. **Ekizoğlu, M., 2017:** Infectious diseases of the brain. In **Gürsoy-Özdemir, Y., Bozdağ-Pehlivan, S., Sekerdag, E.:** *Nanotechnology Methods for Neurological Diseases and Brain Tumors*. Elsevier. 291–315.
13. **Falanga, A., Del Genio, V.,Galdiero, S., 2021:** Peptides and dendrimers: How to combat viral and bacterial infections. *Pharmaceutics*, 13, 1, 23. DOI: 10.3390/pharmaceutics13010101.
14. **Felczak, A., Wrońska, N., Janaszewska, A., Klajnert, B., Bryszewska, M., Appelhans, D., et al., 2012:** Antimicrobial activity of poly (propylene imine) dendrimers. *New J. Chem.*, 36, 11, 2215–2222.
15. **Feng, Y., Zhang, H., Wu, Z., Wang, S., Cao, M., Hu, D., et al., 2014:** *Streptococcus suis* infection: An emerging/reemerging challenge of bacterial infectious diseases? *Virulence*, 5, 4, 477–497. DOI: 10.4161/viru.28595.
16. **Flexner, S., 1907:** Experimental cerebro-spinal meningitis in monkeys. *J. Exp. Med.*, 9, 2, 142–167. DOI: 10.1084/jem.9.2.142.
17. **Garcia-Gallego, S., Franci, G., Falanga, A., Gomez, R., Folliero, V., Galdiero, S., et al., 2017:** Function oriented molecular design: Dendrimers as novel antimicrobials. *Molecules*, 22, 10. DOI: 10.3390/molecules22101581.
18. **Gauro, R., Nandave, M., Jain, V. K., Jain, K., 2021:** Advances in dendrimer-mediated targeted drug delivery to the brain. *J. Nanopart. Res.*, 23, 3, 76. DOI: 10.1007/s11051-021-05175-8.
19. **Gholami, M., Mohammadi, R., Arzanlou, M., Akbari Dourbash, F., Kouhsari, E., Majidi, G., et al., 2017:** *In vitro* antibacterial activity of poly (amidoamine)-G7 dendrimer. *BMC Infect. Dis.*, 17, 1, 395. DOI: 10.1186/s12879-017-2513-7.
20. **Giovan, R. A., Lavender, P. D., 2018:** Central nervous system infections. *Primary Care*, 45, 3, 505. DOI: 10.1016/j.pop.2018.05.007.
21. **He, Z. Y., Wei, X. W., Wei, Y. Q., 2017:** Recent advances of nanostructures in antimicrobial therapy. In **Grumezescu, A. M.:** *Antimicrobial Nanoarchitectonics: From Synthesis to Applications*. 167–194.
22. **Heckenberg, S. G., Brouwer, M. C., van de Beek, D., 2014:** Bacterial meningitis. *Handb. Clin. Neurol.*, 121, 1361–1375. DOI: 10.1016/b978-0-7020-4088-7.00093-6.
23. **Hoffman, O., Weber, R. J., 2009:** Pathophysiology and treatment of bacterial meningitis. *Ther. Adv. Neurol.*, 2, 6, 1–7. DOI: 10.1177/1756285609337975.
24. **Holmes, A. M., Heylings, J. R., Wan, K. W., Moss, G. P., 2019:** Antimicrobial efficacy and mechanism of action of poly(amidoamine) (PAMAM) dendrimers against opportunistic pathogens. *Int. J. Antimicrob. Agents*, 53, 4, 500–507. DOI: 10.1016/j.ijantimicag.2018.12.012.
25. **Hou, S., Zhou, C., Liu, Z., Young, A. W., Shi, Z., Ren, D., et al., 2009:** Antimicrobial dendrimer active against *Escherichia coli* biofilms. *Bioorg. Med. Chem. Lett.*, 19, 18, 5478–5481. DOI: 10.1016/j.bmcl.2009.07.077.
26. **Huang, D., Wu, D., 2018:** Biodegradable dendrimers for drug delivery. *Mater. Sci. Eng. C Mater. Biol. Appl.*, 90, 713–727. DOI: 10.1016/j.msec.2018.03.002.
27. **Huang, J., Li, Y. C., Orza, A., Lu, Q., Guo, P., Wang, L. Y., et al., 2016:** Magnetic nanoparticle facilitated drug delivery for cancer therapy with targeted and image-guided approaches. *Adv. Funct. Mater.*, 26, 22, 3818–3836. DOI: 10.1002/adfm.201504185.
28. **Chatterjee, A., Modarai, M., Naylor, N. R., Boyd, S. E., Atun, R., Barlow, J., et al., 2018:** Quantifying drivers of antibiotic resistance in humans: A systematic review. *Lan-*

cet Inf. Dis., 18, 12, E368–E378. DOI: 10.1016/s1473-3099(18)30296-2.

29. **Chen, C. Z., Beck-Tan, N. C., Dhurjati, P., van Dyk, T. K., LaRossa, R. A., Cooper, S. L., 2000:** Quaternary ammonium functionalized poly(propylene imine) dendrimers as effective antimicrobials: Structure-activity studies. *Biomacromolecules*, 1, 3, 473–480. DOI: 10.1021/bm0055495.
30. **Chis, A. A., Dobrea, C., Morgovan, C., Arseniu, A. M., Rus, L. L., Butuca, A., et al., 2020:** Applications and limitations of dendrimers in biomedicine. *Molecules*, 25, 17. DOI: 10.3390/molecules25173982.
31. **Christaki, E., Marcou, M., Tofarides, A., 2020:** Antimicrobial resistance in bacteria: Mechanisms, evolution, and persistence. *J. Mol. Evol.*, 88, 1, 26–40. DOI: 10.1007/s00239-019-09914-3.
32. **Kalhapure, R. S., Suleman, N., Mocktar, C., Seedat, N., Govender, T., 2015:** Nanoengineered drug delivery systems for enhancing antibiotic therapy. *J. Pharm. Sci.*, 104, 3, 872–905. DOI: 10.1002/jps.24298.
33. **Kimura, S., Harashima, H., 2020:** Current status and challenges associated with CNS-targeted gene delivery across the BBB. *Pharmaceutics*, 12, 12. DOI: 10.3390/pharmaceutics12121216.
34. **Kullberg, B. J., Vrijmoeth, H. D., van de Schoor, F., Hovius, J. W., 2020:** Lyme borreliosis: Diagnosis and management. *BMJ*, 369, m1041. DOI: 10.1136/bmj.m1041.
35. **Kumar, L., Verma, S., Vaidya, B., Mehra, N. K., 2017:** Nanocarrier-assisted antimicrobial therapy against intracellular pathogens. In **Ficai, A., Grumezescu, A. M.:** *Nanostructures for Antimicrobial Therapy*. Elsevier, 293–324.
36. **Kumar, M., Sarma, D. K., Shubham, S., Kumawat, M., Verma, V., Nina, P. B., et al., 2021:** Futuristic non-antibiotic the apies to combat antibiotic resistance: A review. *Front. Microbiol.*, 12. DOI: 10.3389/fmicb.2021.609459.
37. **Kumari, P., Luqman, S., Meena, A., 2020:** Nanomaterials: A promising tool for drug delivery. In **Daima, H. K., Navya, P. N., Ranjan, S., Dasgupta, N., Lichtfouse, E.:** *Nanoscience in Medicine*, 1, 39. 1–49.
38. **Ladd, E., Sheikhi, A., Li, N., van de Ven, T. G. M., Kakkar, A., 2017:** Design and synthesis of dendrimers with facile surface group functionalization, and an evaluation of their bactericidal efficacy. *Molecules*, 22, 6. DOI: 10.3390/molecules22060868.
39. **LaPenna, P. A., Roos, K. L., 2019:** Bacterial infections of the central nervous system. *Semin. Neurol.*, 39, 3, 334–342. DOI: 10.1055/s-0039-1693159.
40. **Le Guennec, L., Coureuil, M., Nassif, X., Bourdoulous, S., 2020:** Strategies used by bacterial pathogens to cross the blood-brain barrier. *Cell Microbiol.*, 22, 1, e13132. DOI: 10.1111/cmi.13132.
41. **Liechti, F. D., Grandgirard, D., Leib, S. L., 2015:** Bacterial meningitis: insights into pathogenesis and evaluation of new treatment options: a perspective from experimental studies. *Future Microbiol.*, 10, 7, 1195–1213. DOI: 10.2217/fmb.15.43.
42. **Longmire, M., Choyke, P. L., Kobayashi, H., 2008:** Dendrimer-based contrast agents for molecular imaging. *Curr. Top. Med. Chem.*, 8, 14, 1180–1186. DOI: 10.2174/156802608785849021.
43. **Mhlwatika, Z., Aderibigbe, B. A., 2018:** Application of dendrimers for the treatment of infectious diseases. *Molecules*, 23, 9. DOI: 10.3390/molecules23092205.
44. **Mintzer, M. A., Dane, E. L., O'Toole, G. A., Grinstaff, M. W., 2012**: Exploiting dendrimer multivalency to combat emerging and re-emerging infectious diseases. *Mol. Pharm.*, 9, 3, 342–354. DOI: 10.1021/mp2005033.
45. **Morrison, L., Zembower, T. R., 2020:** Antimicrobial resistance. *Gastrointest. Endosc. Clin. N. Am.*, 30, 4, 619–635. DOI: 10.1016/j.giec.2020.06.004.
46. **Nau, R., Djukic, M., Spreer, A., Ribes, S., Eiffert, H., 2015:** Bacterial meningitis: An update of new treatment options. *Expert Rev. Anti. Infect. Ther.*, 13, 11, 1401–1423. DOI: 10.1586/14787210.2015.1077700.
47. **Nazli, A., He, D. L., Liao, D., Khan, M. Z. I., Huang, C., He, Y., 2022:** Strategies and progresses for enhancing targeted antibiotic delivery. *Adv. Drug Deliv. Rev.*, 189, 114502. DOI: 10.1016/j.addr.2022.114502.
48. **Nikzamir, M., Hanifehpour, Y., Akbarzadeh, A., Panahi, Y., 2021:** Applications of dendrimers in nanomedicine and drug delivery: A review. *J. Inorg. Organomet. Polym. Mater.*, 31, 6, 2246–2261. DOI: 10.1007/s10904-021-01925-2.
49. **Omidi, Y., Kianinejad, N., Kwon, Y., Omidian, H., 2021:** Drug delivery and targeting to brain tumors: Considerations for crossing the blood-brain barrier. *Expert. Rev. Clin. Pharmacol.*, 14, 3, 357–381. DOI: 10.1080/17512433.2021.1887729.
50. **Oppenheim, B. A., 1997:** Antibiotic resistance in *Neisseria meningitidis*. *Clin. Infect. Dis.*, 24, S98–S101. DOI: 10.1093/clinids/24.Supplement_1.S98.
51. **Pardon, E., Laeremans, T., Triest, S., Rasmussen, S. G. F., Wohlkonig, A., Ruf, A., et al., 2014:** A general protocol for the generation of nanobodies for structural biology. *Nature Protocols*, 9, 3, 674–693. DOI: 10.1038/nprot.2014.039.

52. Perry, J., Waglechner, N., Wright, G., 2016: The prehistory of antibiotic resistance. *Cold Spring Harb. Perspect.*, 6, 6. DOI: 10.1101/cshperspect.a025197.

53. Pompilio, A., Geminiani, C., Mantini, P., Siriwardena, T. N., Di Bonaventura, I., Reymond, J. L., et al., 2018: Peptide dendrimers as "lead compounds" for the treatment of chronic lung infections by Pseudomonas aeruginosa in cystic fibrosis patients: *In vitro* and *in vivo* studies. *Infect. Drug Resist.*, 11, 1767–1782. DOI: 10.2147/idr.S168868.

54. Pulgar, V. M., 2018: Transcytosis to cross the blood brain barrier, new advancements and challenges. *Front. Neurosci.*, 12, 1019. DOI: 10.3389/fnins.2018.01019.

55. Rabiee, N., Ahmadi, S., Arab, Z., Bagherzadeh, M., Safarkhani, M., Nasseri, B., et al., 2020: Aptamer hybrid nanocomplexes as targeting components for antibiotic/gene delivery systems and diagnostics: A review. *Int. J. Nanomed.*, 15, 4237–4256. DOI: 10.2147/ijn.S248736.

56. Rajak, B. L., Kumar, R., Gogoi, M., Patra, S., 2020: Antimicrobial activity of nanomaterials. In **Daima, H. K., Navya, P. N., Ranjan, S., Dasgupta, N., Lichtfouse, E.:** *Nanoscience in Medicine*, 1, 39. 147–185.

57. Reygaert, W. C., 2018: An overview of the antimicrobial resistance mechanisms of bacteria. *Aims Microbiol.*, 4, 3, 482–501. DOI: 10.3934/microbiol.2018.3.482.

58. Rizvi, S. M. D., Hussain, T., Ahmed, A. B. F., Alshammari, T. M., Moin, A., Ahmed, M. Q., et al., 2018: Gold nanoparticles: A plausible tool to combat neurological bacterial infections in humans. *Biomed. Pharmacother.*, 107, 7–18. DOI: 10.1016/j.biopha.2018.07.130.

59. Santos, A., Veiga, F., Figueiras, A., 2020: Dendrimers as pharmaceutical excipients: Synthesis, properties, toxicity and biomedical applications. *Materials*, 13, 1. DOI: 10.3390/ma13010065.

60. Scorciapino, M. A., Serra, I., Manzo, G., Rinaldi, A. C., 2017: Antimicrobial dendrimeric peptides: Structure, activity and new therapeutic applications. *Int. J. Mol. Sci.*, 18, 3. DOI: 10.3390/ijms18030542.

61. Scheld, W. M., Koedel, U., Nathan, B., Pfister, H. W., 2002: Pathophysiology of bacterial meningitis: Mechanism(s) of neuronal injury. *J. Infect. Dis.*, 186, S225–S233. DOI: 10.1086/344939.

62. Somani, S., Dufes, C., 2014: Applications of dendrimers for brain delivery and cancer therapy. *Nanomedicine*, 9, 15, 2403–2414. DOI: 10.2217/nnm.14.130.

63. Spirescu, V. A., Chircov, C., Grumezescu, A. M., Andronescu, E., 2021: Polymeric nanoparticles for antimicrobial therapies: An up-to-date overview. *Polymers*, 13, 5. DOI: 10.3390/polym13050724.

64. Stanek, G., Wormser, G. P., Gray, J., Strle, F., 2012: Lyme borreliosis. *Lancet*, 379, 9814, 461–473. DOI: 10.1016/s0140-6736(11)60103-7.

65. Staneva, D., Angelova, S., Vasileva-Tonkova, E., Grozdanov, P., Nikolova, I., Grabchev, I., 2020: Synthesis, photophysical characterisation and antimicrobial activity of a new anionic PAMAM dendrimer. *J. Photochem. Photobiol.*, 403, 112878.

66. Svenningsen, S. W., Frederiksen, R. F., Counil, C., Ficker, M., Leisner, J. J., Christensen, J. B., 2020: Synthesis and antimicrobial properties of a ciprofloxacin and PAMAM-dendrimer conjugate. *Molecules*, 25, 6. DOI: 10.3390/molecules25061389.

67. Tarach, P., Janaszewska, A., 2021: Recent advances in preclinical research using PAMAM dendrimers for cancer gene therapy. *Int. J. Mol. Sci.*, 22, 6. DOI: 10.3390/ijms22062912.

68. Tomalia, D. A., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., et al., 1985: A new class of polymers – starbust-dendritic macromolecules. *Polymer J.*, 17, 1, 117–132. DOI: 10.1295/polymj.17.117.

69. van Samkar, A., Brouwer, M. C., Schultsz, C., van der Ende, A., van de Beek, D., 2015: *Streptococcus suis* meningitis: A systematic review and meta-analysis. *PLOS Negl. Trop. Dis.*, 9, 10, e0004191. DOI: 10.1371/journal.pntd.0004191.

70. Vega, N. M., Gore, J., 2014: Collective antibiotic resistance: mechanisms and implications. *Curr. Opin. Microbiol.*, 21, 28–34. DOI: 10.1016/j.mib.2014.09.003.

71. Velasco-Aguirre, C., Morales, F., Gallardo-Toledo, E., Guerrero, S., Giralt, E., Araya, E., et al., 2015: Peptides and proteins used to enhance gold nanoparticle delivery to the brain: preclinical approaches. *Int. J. Nanomed.*, 10, 4919–4936. DOI: 10.2147/ijn.S82310.

72. Zhu, Y. F., Liu, C. Y., Pang, Z. Q., 2019: Dendrimer-based drug delivery systems for brain targeting. *Biomolecules*, 9, 12. DOI: 10.3390/biom9120790.

Received February 27, 2023
Accepted March 30, 2023

sciendo

DOI: 10.2478/fv-2023-0015

FOLIA VETERINARIA, 67, 2: 33—41, 2023

IN VITRO ANTIFUNGAL ACTIVITY OF SELECTED ESSENTIAL OILS AGAINST *TRICHOPHYTON MENTAGROPHYTES*

Lucia Kottferová[1], Eva Čonková[2]
[1]Clinic of Birds, Exotic and Free Living Animals
[2]Department of Pharmacology and Toxicology
University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia

lucia.kottferova@uvlf.sk

ABSTRACT

The *in vitro* activity of *Cinnamomum cassia*, *Melaleuca alternifolia*, and *Cymbopogon citratus* essential oils and antimycotics clotrimazole, fluconazole, and ketokonazole against *T. mentagrophytes* was evaluated by the disc-diffusion method. The essential oils were tested at 5, 10, 20, 30, 50, and 100 % concentration. It was found that *Cymbopogon*, commonly called lemongrass, and cinnamon show stable results independent of the concentration used, whereas the efficacy of tea tree oil, significantly decreases with the decreasing concentration. When comparing the efficacy of antimycotics the largest zone of inhibition was obtained with clotrimazole, while fluconazole proved to be ineffective. We found that cinnamon and lemongrass are more effective than the antimycotics that we have used. Our study confirmed that some essential oils can be used for the treatment of dermatophytoses caused by *Trichophyton mentagrophytes* and some of them are more effective than the commercial drugs.

Key words: antimycotics; *Cinnamomi cassiae aetheroleum; Cymbopogon citratus aetheroleum*; dermatophytoses; *Melaleucae aetheroleum*

INTRODUCTION

Infectious diseases are regarded as a significant problem of health and are a major cause of morbidity and mortality worldwide. Dermatophytosis is quite common among infectious diseases [52].

Fungal infections of the skin are a common global problem. Pathogens responsible for skin mycoses are primarily anthropophilic and zoophilic dermatophytes from the genera *Trichophyton, Microsporum*, and *Epidermophyton*. There appears to be considerable inter- and intracontinental variability in the global incidence of these fungal infections [22, 29].

Clinical signs of trichophytosis are easily observable in humans and other animals. There are circular lesions observed on the skin that may also bind together and form large irregular structures and can have an average of a few centimetres in length [26]. The clinical forms of animal diseases are most often determined by the animal species, the animal´s age, health condition, and virulence process, and may also be determined by individual characteristics of the animal [17, 44]. The presence of inflammation or redness of the infected area and the loss of hair show typical symptoms of the infection [7].

The antifungal drugs that are routinely used the for the

treatment of deep mycoses include polyenes and azoles. An additional drug that sees infrequent use, primarily for life-threatening yeast infections, is flucytosine (5-fluorocytosine). For superficial mycoses, particularly infections caused by fungi, azoles, allylamines, and griseofulvin are most commonly used [23].

Many antifungal medications commonly used in the therapy of fungal diseases have poor efficacy indicating side effects, toxicity, and drug interaction and increasingly also appears the development of resistance to these medications [31, 40]. Treatment of dermatophytosis is quite expensive and time-consuming. Therefore, it is necessary to develop new drugs with improved efficacy and safety or evolve an alternative or combating infections [51]. Natural products have proven to be a source of new alternative active molecules. In many countries, particularly in the developing countries, the plants were primarily used to provide basic medical treatment [32].

The objective of this study was to compare the antifungal activity of essential oils (*Cinnamomi cassiae aetheroleum, Cymbopogon citratus aetheroleum, Melaleucae aetheroleum*) against *T. mentagrophytes* with activity of conventional antimycotics (clotrimazole, fluconazole, ketoconazole) used in clinical practice, using the disc-diffusion method.

MATERIALS AND METHODS

Essential oils

Antifungal activity was tested *in vitro* in three essential oils: cinnamon, tea tree, and lemongrass essential oils (Table 1) (HANUS – Herbal preparations, Nitra, the Slovak Republic) with a concentration of 100 %, 30 %, 20 %, 10 %, and 5 %. While diluting essential oils to the desired concentration, we proceeded exactly as in the preparation of emulsions. Gum Arabic was used as an emulsifier, which represented 30 % of the total concentration of the solution.

Antimycotics

The following commercial antifungal discs were used in the experiment (Biolab Slovakia s. r. o. Komárno, the Slovak Republic): clotrimazole 10 mcg; clotrimazole 50 mcg; fluconazole 25 mcg; ketoconazole 10 mcg.

Tested strain

For testing, we used the reference strain *Trichophyton mentagrophytes* CCM 8377/ATCC 9533 (the Czech Collection of Microorganisms, Brno, the Czech Republic).

Disc-diffusion method

The antifungal activity of essential oils and antimycotics were determined by the disc-diffusion method, according to a standard methodology M44-A2 clinical and laboratory standard institute CLSI for antimicrobial susceptibility testing. The pure culture of the *Trichophyton mentagrophytes* strain was prepared by passaging on Sabouraud dextrose agar with the addition of chloramphenicol. After a 10-day incubation period at room temperature (21–23 °C), a suspension of spores with a sterile saline solution was prepared. The inoculum density was adjusted using a densitometer (Denzi-Lameter, Pliva LaChema a. s., Brno, the Czech Republic) to the 0.5 McFarland-scale equivalent (i. e. $1 \times 10^6 – 5 \times 10^6$ CFU.ml^{-1}). Subsequently, the inoculum was applied with a sterile swab onto the surface of the nutrient medium twice in three directions, with 15 minutes between the two measurements. The discs

Table 1. The essential oils tested *in vitro* against *T. mentagrophytes*

Type of essential oil type	Latin name of the essential oil	Latin name of the mother plant	Latin name of the family
Cinnamon	*Cinnamomi cassiae aetheroleum*	*Cinnamomum cassia*	Lauraceae
Tea tree	*Melaleucae aetheroleum*	*Melaleuca alternifolia*	Myrtaceae
Lemongrass	*Cymbopogon citratus aetheroleum*	*Cymbopogon citratus*	Poaceae

associated with antimycotics, or essential oils at the appropriate concentration were transferred onto the prepared medium. The plates were incubated at a laboratory temperature for 10 days and then the results were counted. A zone of inhibition was created as the criterion for determining sensitivity, where the size reached at least twice the size of the disc. The experiment was repeated 3 times. When testing azole antimycotics, the sensitivity was evaluated based on the criteria in the manufacturer´s instructions.

Statistical analysis

The statistical analysis was performed by regression analysis. To test the differences among medium values of multiple files, the method ANOVA and Tukey's multiple comparison method were used. All statistical hypotheses were tested at a significance level of $\alpha \leq 0.05$.

RESULTS

The average values of the zones of inhibition against *T. mentagrophytes* are shown in Table 2. Lemongrass and cinnamon showed higher average values than the other tested substances. The median of the two essential oils reached even the highest possible value of 90 mm (i. e., the diameter of the Petri dish). We observed a decrease in the medium value of the lemongrass against cinnamon, which was caused by low values at 5 % concentration. Tea tree, fluconazole, and ketoconazole showed considerably lower values than the other tested substances. All of the substances, except the tea tree oil, resulted in very low values of the coefficient of variation, so their effects could be considered as stable.

Conventional antimycotics showed a stable effect since a low coefficient of variation was detected. Essential oils, cinnamon, and lemongrass reached the highest efficacy from the concentration of 10 %. The dose-dependent efficacy of essential oils concentration was verified by regression analysis separately for each essential oil (Tables 3, 4).

From Table 3 it is evident that the efficacy level of lemongrass did not depend on the concentration. The p-value of the linear coefficient was greater than 0.05 while that of the constant coefficient was very close to zero, thus the constant coefficient was significant.

From Table 4 it is evident that the efficacy value of the tea tree depends on the concentration. The p-value of the linear and constant coefficients was less than 0.05, and both coefficients were significant.

There was no statistical testing with the cinnamon, as the results of the measured values were equal to 90. Thus, a constant dependency was evident.

A one-way analysis by Tukey's method was employed for the multiple comparison efficacies of each concentration of essential oils for the concentrations ranging from 5 to 20 % (Table 5), 30 % (Table 6), and 100 %.

At a 100 % oil concentration it was impossible to find any differences among the substances since almost at every measurement the diameter of the inhibition zone reached 90 mm.

Table 2. The average values (mm) of the zones of inhibition against *T. mentagrophytes*

	Medium value	Median	Coefficient of variation	Susceptible/ resistant (S/R)
Lemongrass	78.53	90	0.3	S
Cinnamon	90	90	0	S
Tea tree	24.66	0	1.48	S
Clotrimazole 10	76.66	80	0.07	S
Clotrimazole 50	76.66	80	0.07	S
Fluconazole	0	0	0	R
Ketokonazole	28.66	29	0.08	S

Table 3. Linear regression for lemongrass

	Coefficients	Medium value error	t Stat	P-value
Constant coefficient	69.13	8.53	8.1	$3.302e^{-6}$*
Linear coefficient	0.30	0.20	1.48	0.163609

* – P ≤ 0.05

Table 4. Linear regression for tea tree

	Coefficients	Medium value error	t Stat	P-value
Constant coefficient	–7.93	3.82	–2.07	0.0096019*
Linear coefficient	0.99	0.09	10.78	$0.01581e^{-7}$*

* – P ≤ 0.05

Table 5. Analysis by Tukey´s method of 5–20 % concentration of essential oils

	Diameter difference [mm]	Lower estimate	Upper estimate	P-value
Lemongrass – tea tree	69.77	50.04	89.51	0*
Cinnamon – tea tree	88.88	69.15	108.62	0*
Cinnamon – lemongrass	19.11	–0.62	38.84	0.05

P ≤ 0.05

Table 6. Analysis by Tukey´s method for 30 % concentration of essential oils

	Diameter difference	Lower estimate	Upper estimate	P-value
Lemongrass – tea tree	60	31.07	88.92	0.0017169*
Cinnamon – tea tree	60	31.07	88.92	0.0017169*
Cinnamon – lemongrass	–1.421085E-14	–28.92	28.92	1.00

* – P ≤ 0.05

DISCUSSION

In vitro antimicrobial activity of essential oils of cinnamon was published in many studies [1, 5, 11, 12, 15, 18, 19, 21, 30, 35, 39, 42, 43, 45]. The antifungal effects of essential oil from *Cinnamomum longepaniculatum* leaves were studied with special reference to minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC), time-kill studies, and the mechanism of inhibition at an ultrastructural level against *Trichophyton mentagrophytes*, *Microsporum canis* and *Trichophyton gypseum* [47]. Our results also confirmed the antifungal effect of *Cinnamomum cassia*. In our experiment, the efficacy of *Cinnamomum cassia* against *Trichophyton mentagrophytes* did not depend on concentration of the essential oil. The average values of the zones of inhibition were determined at all tested concentrations at 90 mm as the maximum possible value.

In vitro and *in vivo* studies conducted in different countries demonstrated numerous beneficial medicinal effects

of *Cinnamomum zeylanicum* [41] and its anti-microbial activity [25]. T a o et al. confirmed that essential oil from *Cinnamomum longepaniculatum* showed a significant antifungal activity *in vivo*, weakening the virulence of three dermatophytes and killing fungi by destroying the cell membrane and organelles [47].

The aim of the study by C i s a r o v á et al. [9] was to assess the antifungal and anti-toxinogenic activity of 15 essential oils against three fungi of the genus *Aspergillus*. All essential oils exhibited activity against the tested strains of fungi.

G o z u b u y u k et al. [20] investigated the antifungal activity of *L. inermis* (henna) against six different dermatophyte species: *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, and *Trichophyton violaceum*. The antifungal activity of *L. inermis* was determined by agar diffusion method and henna was used in paste form. Henna paste showed high antifungal activity against all dermatophytes species.

The antifungal potential of tea tree was investigated *in vitro* against *Trichophyton rubrum* and *T. mentagrophytes* var. interdigitale, the most prevalent causes of tinea and onychomycosis infection in humans. There was observed a clear antifungal action on these organisms grown in culture. B e n g e r et al. [3] and C a s s e l l a et al. [8] detected the activity of *Melaleuca alternifolia* against the dermatophyte *Trichophyton rubrum*. The studies also confirmed that the oil was a potent antifungal agent and that the inverse relationship between essential oil concentration and fungal growth was not influenced by random variability. Our study showed that the efficacy of this essential oil against *T. Mentagrophyte*, was maximal at 100 % concentration (inhibition zone of 90 mm), but that the effectiveness was significantly decreased at a concentration of 30 %. The tea tree showed an inhibition zone diameter of 0 mm at concentrations ranging from 5 to 20 % at which it can be considered ineffective.

C o n c h a et al. [10] determined the activity of tea tree oil against 58 clinical isolates. This *in vitro* study indicated that tea tree oil may be useful in the treatment of yeast and fungal mucosal and skin infections.

Antimicrobial activities of methanol leaf extract of *Leucas* sp. were studied by B a b u et al. [2]. Antifungal susceptibilities of clinically isolated dermatophytes to methanol extracts of *Leucas aspera* and *Leucas zeylanica* leaves were investigated using the agar well diffusion method. The results obtained showed that all the extracts expressed remarkable antifungal activity. The maximum inhibition zone was recorded with *Penicillium* sp. while the minimum inhibition zone was recorded for *Candida tropicalis*. These results confirmed potential anti-dermatophytic activity of *Leucas* spp.

The antimycobacterial activity of aerial parts and essential oils of four *Salvia* species against *Mycobacterium tuberculosis* strains was analysed and the antifungal activity against dermatophytes *Microsporum gypseum* and *Trichophyton mentagrophytes* and also *Candida* species was compared with by broth microdilution method. The essential oils showed high antimycobacterial and antifungal activity [46].

The *in vitro* activity of twenty chemically defined essential oils against *Microsporum canis, Trichophyton mentagrophytes*, *T. erinacei, T. terrestre* and *Microsporum gypseum Microsporum canis*, *Trichophyton mentagrophytes, T. erinacei, T. terrestre* and *Microsporum gypseum* was assayed. More effective essential oils were *T. serpyllum, O. vulgare*, and *L. cubeba. F. vulgare* showed moderate efficacy against geophilic species such as *M. gypseum* and *T. terrestre* [34].

D e s a m et al. [13] studied the antifungal activity of mentha and peppermint essential oils. They showed a significant antifungal activity against *Alternaria alternaria, Fusarium tabacinum, Penicillum* spp. *Fusarium oxyporum* and *Aspergillus fumigates*.

The study conducted with south Indian medicinal plants involved their efficacy against three clinical fungal isolates *Trichophyton mentagrophytes, Epidermophyton floccosum,* and *Candida albicans*, using the agar well diffusion method. The extract of twenty plants used in this study acted as a good source of antibiotics against various fungal pathogens tested and exhibited a broad spectrum of antifungal activity [49].

Several authors investigated the antibacterial properties of *Cymbopogon citratus* [24, 36, 37]. K h a n and A h m a d [28] evaluated *in vitro* the antifungal activity of *Cymbopogon citratus* oil against azole-resistant strains of *Aspergillus* spp. and *Trichophyton* spp. They observed that *C. citratus* exhibited promising antifungal activity (zone of inhibition from 24.66 to 42.00 mm) and the killing potency against *Aspergillus fumigatus* and *Trichophyton rubrum*. Our results showed that the efficacy of *Cym-*

bopogon citratus did not depend on the concentration. It showed the highest possible efficacy against *T. mentagrophytes* at concentrations ranging from 10 to 100 %. The efficacy decreased (32.6 mm) at 5 % concentration.

B i a s i - G a r b i n et al [4] evaluated the *in vitro* antidermatophytic activity of 23 crude extracts from nine plant species found in Brazil. The extracts were tested by broth microdilution assay against the reference strains of *T. rubrum* and *T. mentagrophytes* and 33 clinical isolates of dermatophytes. All plants showed a fungicidal effect against both fungal species. Selected extracts of *Eugenia uniflora, Libidibia ferrea*, and *Persea americana* also exhibited a fungicidal effect against all clinical isolates of *T. rubrum* and *T. mentagrophytes* complex. This is the first report of the antifungal activity of *Schinus terebinthifolius, Piptadenia colubrina, Parapiptadeniarigida, Mimosa ophthalmocentra*, and *Persea americana* against both dermatophyte species.

O r c h a r d et al. reported high susceptibility of fungal pathogens, such as *Trichophyton mentagrophytes*, to combinations of essential oils. The potential combinations containing *Cinnamomum verum* or *Santalum austrocaledonicum* were studied in a clinical settings with the goal to decrease the need for systemic or prolonged antifungal treatments that may result in a treatment failure or resistance [38]. Essential oil mixtures may provide a better outcome than monotherapy in terms of side effects and toxicity, as well as reducing the emergence of resistant strains. Based on their antifungal and anti-inflammatory properties, these combinatorial strategies may be useful in supplementing conventional therapy by alleviating symptoms, promoting healing, and preventing spread of dermatophytosis [48].

Overall, we can summarize our findings that lemongrass and cinnamon have significantly better efficacy than tea tree, except for the concentration of 100 %, and their effect did not dependent on the tested concentration. The efficacy was the highest possible and the zone inhibition diameter was 90 mm. The statistical comparison shows that there are significant differences between lemongrass and cinnamon compared to tea tree. Z e n g, C h e n and L i a n g [53] studied the antifungal effects of fennel seed essential oil against *Trichophyton rubrum, Trichophyton tonsurans, Microsporum gypseum* and *Trichophyton mentagrophytes*. Fennel seed essential oil was investigated from various aspects, such as MIC and minimum fungicidal concentration, mycelia growth, spore germination, and biomass. The results indicated that this oil showed better antifungal activities than the commonly used antifungal agents fluconazole and amphotericin B.

Our experiments showed that the efficacy was less than 90 with each conventionally produced antifungal agent tested in our study and the essential oils such as cinnamon and lemongrass were more effective as antimycotics. D i o g o et al. [14] investigated the activity of different concentrations of terbinafine against *T. mentagrophytes* using the disc-diffusion method. The diameter of the inhibition zone was 40 mm and did not depend on the concentration of terbinafine. Comparing the effectiveness of conventional antimycotics, the largest inhibition zone was observed with clotrimazole (80 mm), but fluconazole proved ineffective against *T. mentagrophytes* and ketoconazole showed an inhibition zone of 29 mm in our experiment. The effectiveness of clotrimazole did not depend on concentration. K e y v a n et al. [27] conducted *in vitro* activity testing of six antifungal drugs against clinically important dermatophytes and reported that fluconazole exhibited the poorest activity while clotrimazole and terbinafine were the most effective antifungal drugs. E s t e b a n et al. [16] also tested by disc-diffusion method the antifungal susceptibility of six kinds of dermatophytes including *T. mentagrophytes* and confirmed the high efficiency of clotrimazole.

Despite promising results of recent studies [6, 33, 50], we agree with their authors that the potential toxicity of these products must be considered because EO have been shown to cause skin allergy and irritation. As a result, it is critical not only to investigate the effectiveness of essential oils, but also pay attention to their safety (harmlessness) as well as the potential associated risks.

CONCLUSIONS

Essential oils have long been known to be in a centre of attention for their antimicrobial effects because of their potential role as an alternative to antibiotics and antimycotics for therapeutic purposes. The *in vitro* experiment, based on descriptive statistics, indicated that lemongrass and cinnamon essential oils were effective against *T. mentagrophytes*, while the effectiveness of tea tree decreased significantly depending on concentration. When compar-

ing the efficacy of commercial antimycotics, the greatest commercial inhibition zone was observed with clotrimazole, and fluconazole has proved ineffective.

The disc-diffusion method used in this study is a simple and valuable method for the evaluation of the antifungal susceptibility of dermatophytes in *in vitro* experiments.

The antifungal effects of essential oils could be a very promising solution that can overcome the therapeutic shortcomings of antimycotic medication. More experiments are needed to examine the properties of these oils in order to devise effective and nonaggressive therapies for the treatment of dermatophytosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

The study was supported by the KEGA 009UVLF-4/2022.

REFERENCES

1. **Adam, K., Sivropoulou, A., Kokkini, S., Lanaras, T., Arsenakis, M., 1998:** Antifungal activities of *Origanum vulgare* subsp. *hirtum, Mentha spicata, Lavandula angustifolia,* and *Salvia fruticosa* essential oils against human pathogenic fungi. *J. Agric. Food Chem.*, 46, 1739–1745.
2. **Babu, A., Mohamed, M. S. N., Jaikumar, K., Anand, D., Saravanan, P., 2016:** *In vitro* antifungal activity of leaf extracts of *Leucas aspera* and *Leucas zeylanica*. *IJPSR*, 7, 752. DOI: 10.13040/IJPSR.0975-8232.7(2).752-56.
3. **Benger, S., Townsend, P., Ashford, R. L., Lambert, P., 2004:** An *in vitro* study to determine the minimum inhibitory concentration of *Melaleuca alternifolia* against the dermatophyte *Trichophyton rubrum*. *The Foot*, 14, 86–91.
4. **Biasi-Garbin, R. P., Demitto, F. D. O., Amaral, R. C. R. D., Ferreira, M. R. A., Soares, L. A. L., Svidzinski, T. I. E., Yamada-Ogatta, S. F., 2016:** Antifungal potential of plant species from Brazilian Caatinga against dermatophytes. *Revista do Instituto de Medicina Tropical de São Paulo*, 58. DOI: 10.1590/S1678-9946201658018.
5. **Biondi, D., Cianci, P., Geraci, C., Ruberto, G., Piattelli, M., 1993:** Antimicrobial activity and chemical composition of essential oils from Sicilian aromatic plants. *Flavour Fragr. J.*, 8, 331–337.
6. **Bogavac, M., Karaman, M., Janjušević, L., Sudji, J., Radovanović, B., Novaković, Z., et al., 2015:** Alternative treatment of vaginal infections – *in vitro* antimicrobial and toxic effects of *Coriandrum sativum* L. and *Thymus vulgaris* L. essential oils. *J. Appl. Microbiol.*, 119, 3, 697–710. DOI: 10.1111/jam.12883.
7. **Carrillo-Munoz, A. J., Giusiano, G., Ezkurra, P. A., Quindos, G., 2006:** Antifungal agents: Mode of action in yeast cells. *Rev. Esp. Quimioter*, 19, 130–139.
8. **Cassella, S., Cassella, J. P., Smith, I., 2002:** Synergistic antifungal activity of tea tree (*Melaleuca alternifolia*) and lavender (*Lavandula angustifolia*) essential oils against dermatophyte infection. *Int. J. Aromath.*, 12, 2–15.
9. **Císarová, M., Tančinová, D., Medo, J., Kačániová, M., 2016:** The *in vitro* effect of selected essential oils on the growth and mycotoxin production of *Aspergillus* species. *J. Environ. Health*, Part B, 51, 668–674. DOI: 10.1080/03601234.2016.1191887.
10. **Concha, J. M., Moore, L. S., Holloway, W. J., 1998:** Antifungal activity of *Melaleuca alternifolia* (tea-tree) oil against various pathogenic organisms. *J. Am. Podiatr. Med. Assoc.*, 88, 489–492.
11. **Cosentino, S., Tuberoso, C. I. G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., Palmas, F., 1999:** *In vitro* antimicrobial activity and chemical composition of *Sardinian thymus* essential oils. *Lett. Appl. Microbiol.*, 29, 130–135.
12. **Deans, S. G., Ritchie, G., 1987:** Antibacterial properties of plant essential oils. *Int. J. Food Microbiol.*, 5, 165–180.
13. **Desam, N. R., Al-Rajab, A. J., Sharma, M., Mylabathula, M. M., Gowkanapalli, R. R., Albratty, M., 2017:** Chemical constituents, *in vitro* antibacterial and antifungal activity of *Mentha Piperita* L. (peppermint) essential oils. *J. King Saud Univ. Sci*. DOI: 10.1016/j.jksus.2017.07.013.
14. **Diogo, H. C., Melhem, M., Sarpieri, A., Pires, M. C., 2010:** Avaliação do método de disco-difusão para determinação da eficácia da terbinafina *in vitro* em agentes de micoses superficiais e subcutâneas. *An. Bras. Dermatol.*, 85, 324–330. DOI: 10.1590/S0365-05962010000300005.
15. **Dorman, H. J. D., Deans, S. G., 2000:** Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J. Appl. Microbiol.*, 88, 308–316.
16. **Esteban, A., Abarca, M. L., Cabanes, F. J., 2005:** Compar-

ison of disk diffusion method and broth microdilution method for antifungal susceptibility testing of dermatophytes. *Med. Mycol.*, 43, 61–66. DOI: 10.1080/13693780410001711972.

17. **Fabikova, R., Kuttova, M., Huml, O., Cada, F., Stichova, S., 1995:** Evaluation of bacterial, mycological and parasitary findings from dermatological examination of dogs and cats in 1994 (In Czech). *Veterinarstvi*, 45, 447–451.
18. **Farag, R. S., Daw, Z. Y., Abo-Raya, S. H., 1989:** Influence of some spice essential oils on *Aspergillus parasiticus* growth and production of aflatoxins in a synthetic medium. *J. Food Sci.*, 54, 74–76.
19. **Ferhout, H., Bohatier, J., Guillot, J., Chalchat, J. C., 1999:** Antifungal activity of selected essential oils, cinnamaldehyde and carvacrol against *Malassezia furfur* and *Candida albicans. J. Essent. Oil Res.*, 11, 119–129.
20. **Gozubuyuk, G. S., Aktas, E., Yigit, N., 2014:** An ancient plant *Lawsonia inermis* (henna): Determination of *in vitro* antifungal activity against dermatophytes species. *J. Mycol. Med.*, 24, 313–318. DOI: 10.1016/j.mycmed.2014.07.002.
21. **Hammer, K. A., Carson, C. F., Riley, T. V., 1999:** Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.*, 86, 985–990.
22. **Havlickova, B., Czaika, V. A., Friedrich, M., 2008:** Epidemiological trends in skin mycoses worldwide. *Mycoses*, 51, 2–15. DOI: 10.1111/j.1439-0507.2008.01606.x.
23. **Hector, R. F., 2005:** An overview of antifungal drugs and their use for treatment of deep and superficial mycoses in animals. *Clin. Tech. Small Anim. Pract.*, 20, 240–249. DOI: 10.1053/j.ctsap.2005.07.005.
24. **Chandrashekar, K. S., Joshi, A. B., 2006:** Antifungal efficacy of essential oil of *Cymbopogon* species against some dermatophytes. *Ind. Perf.*, 50, 54.
25. **Jayaprakasha, G. K., Rao, L. J. M., 2011:** Chemistry, biogenesis, and biological activities of *Cinnamomum zeylanicum. Crit. Rev. Food Sci. Nutr.*, 51, 547–562. DOI: 10.1080/10408391003699550.
26. **Kaplan, W., Gump, R. H., 1958:** Ringworm in a dog caused by *Trichophyton rubrum. Vet. Med.*, 53, 139–142.
27. **Keyvan, P., Leila, B., Zahra, R., Manuchehr, S., Kamiar, Z., 2009:** *In vitro* activity of six antifungal drugs against clinically important dermatophytes. *Jundishapur J. Microbiol.*, 4, 158–163.
28. **Khan, M. S. A., Ahmad, I., 2013:** *In vitro* antifungal activity of oil of *Cymbopogon citratus* and citral alone and in combination with fluconazole against azole-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum. Phcog. Commn.*, 3, 29. DOI: 10.5530/pc.2013.3.6.
29. **Lakshmipathy, D. T., Kannabiran, K., 2010:** Review on dermatomycosis: Pathogenesis and treatment. *Nature*, 2, 726. DOI: 10.4236/ns.2010.27090.
30. **Manou, I., Bouillard, L., Devleeschouwer, M. J., Barel, A. O., 1998:** Evaluation of the preservative properties of *Thymus vulgaris* essential oil in topically applied formulations under a challenge test. *J. Appl. Microbiol.*, 84, 368–376.
31. **Martinez-Rossi, N. M., Peres, N. T., Rossi, A., 2008:** Antifungal resistance mechanisms in dermatophytes. *Mycopathologia*, 166, 369–383. DOI: 10.1007/s11046-008-9110-7.
32. **Meléndez, P. A., Capriles, V. A., 2006:** Antibacterial properties of tropical plants from Puerto Rico. *Phytomedicine*, 13, 272–276. DOI: 10.1016/j.phymed.2004.11.009.
33. **Mutlu-Ingok, A., Devecioglu, D., Dikmetas, D. N., Karbancioglu-Guler, F., Capanoglu, E., 2020:** Antibacterial, antifungal, antimycotoxigenic, and antioxidant activities of essential oils: An updated review (Open Access), *Molecules*, 25, 20, Art. No. 4711. DOI: 10.3390/molecules25204711.
34. **Nardoni, S., Giovanelli, S., Pistelli, L., Mugnaini, L., Profili, G., Pisseri, F., Mancianti, F., 2015:** *In vitro* activity of twenty commercially available, plant-derived essential oils against selected dermatophyte species. *Nat. Prod. Commun.*, 10. DOI: 10.1177/1934578X1501000840.
35. **Nelson, R. R., 1997:** *In vitro* activities of five plant essential oils against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium. J. Antimicrob. Chemother.*, 40, 305–306.
36. **Nwachukwu, I. N., Allison, L. N., Chinakwe, E. C., Nwadiaro, P., 2008:** Studies on the effects *Cymbopogon citratus, Ceiba pentandra* and *Loranthus bengwelensis* extracts on species of dermatophytes. *J. Am. Sci.*, 4, 58–67.
37. **Onawunmi, G. O., Yisak, W. A., Ogunlana, E. O., 1984:** Antibacterial constituents in the essential oil of *Cymbopogon citratus* (DC.). *Stapf. J. Ethnopharm.*, 12, 279–286.
38. **Orchard, A., van Vuuren, S. F., Viljoen, A. M., 2019:** Commercial essential oil combinations against topical fungal pathogens. *Nat. Prod. Comm.*, 14. DOI: 10.1177/1934578X1901400139.
39. **Paster, N., Juven, B. J., Shaaya, E., Menasherov, M., Nitzan, R., Weisslowicz, H., Ravid, U., 1990:** Inhibitory effect of oregano and thyme essential oils on moulds and foodborne bacteria. *Lett. Appl. Microbiol.*, 11, 33–37.
40. **Pfaller, M. A., Boyken, L., Hollis, R. J., Kroeger, J., Messer, S. A., Tendolkar, S., Diekema, D. J., 2008:** *In vitro* sus-

ceptibility of invasive isolates of *Candida* spp. to anidulafungin, caspofungin, and micafungin: Six years of global surveillance. *J. Clin. Microbiol.*, 46, 150–156. DOI: 10.1128/JCM.01901-07.

41. **Ranasinghe, P., Pigera, S., Premakumara, G. S., Galappaththy, P., Constantine, G. R., Katulanda, P., 2013:** Medicinal properties of 'true' cinnamon (*Cinnamomum zeylanicum*): A systematic review. *BMC Complement. Altern. Med.*, 13, 275. DOI: 10.1186/1472-6882-13-275.
42. **Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., Arsenakis, M., 1996:** Antimicrobial and cytotoxic activities of *Origanum* essential oils. *J. Agric. Food Chem.*, 44, 1202–1205.
43. **Smith-Palmer, A., Stewart, J., Fyfe, L., 1998:** Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett. Appl. Microbiol.*, 26, 118–122.
44. **Stewart, L. J., 1990:** Newly reported skin disease syndromes in the dog. *Veterinary Clinics of North America: Small Animal Practice*, 20, 1603–1613.
45. **Stiles, J. C., Sparks, W., Ronzio, R. A., 1995:** The inhibition of *Candida albicans* by oregano. *J. Appl. Nutr.*, 47, 96–102.
46. **Tan, N., Satana, D., Sen, B., Tan, E., Altan, H. B., Demirci, B., Uzun, M., 2016:** Antimycobacterial and antifungal activities of selected four salvia species. *Rec. Nat. Prod.*, 10, 593.
47. **Tao, C., Wei, Q., Yin, Z. Q., Zhou, L. J., Jia, R. Y., Xu, J., Deng, Y. X., 2013:** Antifungal activity of essential oil from *Cinnamomum longepaniculatum* leaves against three dermatophytes *in vitro*. *Afr. J. Pharm.*, 7, 1148–1152. DOI: 10.5897/AJPP11.013.
48. **Trifan, A., Luca, S. V., Bostănaru, A. C., Brebu, M., Jităreanu, A., Cristina, R. T., Mareş, M., 2021:** Apiaceae essential oils: boosters of terbinafine activity against dermatophytes and potent anti-inflammatory effectors. *Plants*, 10, 11, 2378. DOI: 10.3390/plants10112378.
49. **Uddandapu, P. K., Naidu, K. C., Rao, Y. V., 2016:** *In vitro* anti-fungal activity of few medicinal plants of Visakhapatnam region against clinically isolated dermatophytes. *Int. J. Bioassays*, 5, 4573–4578. DOI: 10.21746/ijbio.2016.05.008.
50. **Villar Rodríguez, J., Pérez-Pico, A. M., Mingorance-Álvarez, E., Mayordomo Acevedo, R., 2022:** Meta-analysis of the antifungal activities of three essential oils as alternative therapies in dermatophytosis infections. *J. Appl. Microbiol.*, 133, 2, 241–253. DOI: 10.1111/jam.15539.
51. **Wang, J., Li, J., Cao, J., Jiang, W., 2010:** Antifungal activities of neem (*Azadirachta indica*) seed kernel extracts on postharvest diseases in fruits. *Afr. J. Microbiol. Res.*, 4, 1100–1104.
52. **World Health Organization, 2004:** *The World Health Report Changing History. Statistical Annex. Deaths by Cause, Sex and Mortality Stratum in WHO Regions Estimates for 2002.* Geneva, Switzerland, WHO, 120–122.
53. **Zeng, H., Chen, X., Liang, J., 2015:** *In vitro* antifungal activity and mechanism of essential oil from fennel (*Foeniculum vulgare* L.) on dermatophyte species. *J. Med. Microbiol.*, 64, 93–103. DOI: 10.1099/jmm.0.077768-0.

Received April 5, 2023
Accepted May 15, 2023

sciendo

DOI: 10.2478/fv-2023-0016

FOLIA VETERINARIA, 67, 2: 42—50, 2023

WELFARE ASSESSMENT OF PACK DONKEYS IN AMARU, ZARIA ANCIENT CITY, KADUNA STATE, NIGERIA

Philip W. Mshelia[1], Henrietta M. Aji[1], Olumide O. Akinniyi[2], Richard E. Edeh[3]
[1]Department of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State
[2]Department of Veterinary Medicine, University of Ibadan, Oyo State
[3]Department of Veterinary Medicine, University of Jos, Plateau State
Nigeria

olumide.akinniyi@gmail.com

ABSTRACT

Donkey welfare is becoming a subject of much interest; for decades, people around the world have voiced rising worry about it. There are no studies on donkey welfare in Nigeria. This study aimed to assess the welfare state of donkeys in Amaru, Zaria Ancient City, Kaduna State, Nigeria. A cross-sectional study was done to purposefully select 38 pack donkeys for welfare assessment based on the first level of the Animal Welfare Indicators (AWIN) for donkeys, which covered behaviour, health, feeding, and environment and housing. Information regarding age and sex was collected. The analysed data were reported in terms of frequencies and percentages. The prevalence of donkeys with no social contact, avoidance behaviour, and walking downside was 2.6 %, 5.36 %, and 40.56 %, respectively. The most common health problems are integumentary alterations, which include an unhealthy hair coat (86.8 %), skin lesions (86.6 %), alopecia (60.5 %), and deep wounds (47.4 %). Only 5.3 % of the donkeys were lame. Around 42 % had a body condition score of 2 (moderate), while the remaining 57.9 % had a body condition score of 3 (ideal). The donkeys were poorly kept and housed, as evidenced by the high prevalence of unsatisfactory shelter dimensions (100 %), dirty bedding (89.5 %), and insufficient bedding (89.5 %). The current study found that welfare issues are serious challenges for donkeys in Amaru, Zaria Ancient City, Kaduna State, Nigeria. As a consequence of the current findings, it is recommended that donkey welfare, policies and legislative frameworks that support animal welfare and inspect animal facilities in Nigeria should to be reviewed and enforced.

Key words: pack donkey; skin lesions; welfare assessment

INTRODUCTION

Donkeys (*Equus asinus*), the first member of the Equidae family to be domesticated [27], are a cornerstone of human existence and have played a significant part in many economic activities throughout history. The global donkey population is estimated to be over 44 million, with half found in Asia, slightly more than a quarter in Africa, and the remainder primarily in Latin America [13].

Nigeria has a sizable donkey population (800,000) [18]. The cross-border movement of pastoral Fulani from Niger, Chad, Mali, Burkina Faso, and Cameroon has resulted in an increase in the donkey population in Nigeria [4]. There are

more donkeys in Nigeria's northern states. This is due to the savannah vegetation and the scarcity of disease vectors such as tsetse flies. Every year, around 16,000 donkeys are transported from the country's northern states to the southern states for meat production [4, 28].

Despite global mechanisation, working donkeys are still well deserving of the moniker "beasts of burden" in many developing countries, including Nigeria. They serve a crucial role in the transportation of people and commodities in arid and semi-arid areas where roads are inadequate or non-existent [15]. Approximately 95% of the world's donkey population is used for domestic labour in developing countries, as well as responsibilities related to transportation, agriculture, and various industries (e. g. construction) [12].

Animal welfare protection in Nigeria is faced with the following challenges: (1) the absence of laws to reflect true animal welfare; (2) outdated and low sanctions for those that violate the laws; and (3) lack of enforcement of these sanctions. These challenges have affected the welfare of animals, including donkeys. Despite donkeys' important contributions, they are assigned poor status in Nigeria and are hence the most neglected animals. Even some professionals, according to Dr. Philip Mshelia of Nigeria, do not treat donkeys ethically [11]. This resulted in a slew of welfare issues, including inaccessible water, feed, and shelter at work sites, as well as multiple skin lesions. Some methods of bobbling to restrain donkeys cause discomfort and inflict wounds [1]. Long-distance hauling without adequate padding and overloading causes donkeys to sustain external traumatic injuries. This abuse, mistreatment, and lack of veterinary care for donkeys have greatly contributed to their premature deaths, with the majority of them today having a working life expectancy of 4 to 6 years. However, in nations where animal welfare is practised, donkeys can live for up to 30 years [14].

According to research, working donkeys suffer from animal welfare issues such as gait abnormalities, joint swelling, broken skin, deep lesions, and dental difficulties [7, 16]. When their health deteriorates to the point where they are unable to work, they are frequently abandoned and left to die [28]. Donkey welfare is now a trending topic; for centuries, nations all over the world, including Nigeria, have expressed growing concern about it. The welfare status of donkeys in Nigeria has not been investigated.

The aim of the present study was to assess the pack donkey welfare state in Amaru, Zaria Ancient City, Kaduna State, Nigeria.

MATERIALS AND METHODS

Study area

The study was carried out in Amaru, Zaria Ancient City, Kaduna State, Nigeria. Zaria is a major city and a local government area in Kaduna State, Nigeria. It was once known as Zazzau and is one of the original seven Hausa city-states (Hausa Bakwai). Zaria is known to have a high number of local horses due to the presence of an emirate council located in the ancient city of Zaria, as well as donkeys for work. Zaria Local Government Ares (LGA) has a population of 736,000 people (according to the 2006 census). It is situated at 11°3' north latitude and 7°42' east longitude of the Greenwich meridian. It has a wet season lasting from April to September and a dry season from October to March.

Study design and sampling procedure

A cross sectional study was conducted. The places selected were based on donkey accessibility and population. A total of 38 pack donkeys were purposefully selected for this study.

Data collection

All of the pack donkeys sampled were stallions, ranging in age from 2 to 13 years. Donkeys were housed in groups in various families and worked up to six days a week, with working hours ranging from five to more than seven hours. For each pack donkey, a welfare assessment was made based on the first level of the Animal Welfare Indicators (AWIN) for donkeys [22]. The protocol included various questions, covering all aspects of feeding, health, behaviour, and environment and housing. Three (3) experienced veterinarians conducted the welfare assessment after the researchers developed a procedure to prevent inter-observer variance.

Welfare assessment based on behaviour

Donkey social interaction was assessed by observing the donkey's habitual environment and noting whether the donkey is housed with other donkeys or on its own. Access to other donkeys was noted while they were kept

alone. Donkeys that were segregated from other donkeys and had no physical contact with any other donkeys were considered to have no social contact; however, donkeys that were housed with other donkeys or that could have physical contact with other donkeys if housed alone were considered to have social contact.

Three tests were used to evaluate the human-animal relationship [22]:

a) The avoidance distance was measured by stepping three metres away from the donkey with the arm slightly lifted. When approaching the animal, if he attempted to move away, this was labelled avoidance behaviour.
b) Walking down side was the test in which the assessor was on the side of the donkey and softly placed his hand in the withers before calmly walking down the side of the animal. If the donkey tried to flee or kick, it was deemed a negative reaction.
c) Tail tuck was evaluated alongside the previous test; if the animal tucked its tail, it was considered present.

Welfare assessment based on health

The presence of skin alterations was noted and classified into four types: alopecia, skin lesions, deep wounds, and swelling. Donkeys were examined for swollen joints. Lameness was investigated: (1) by observing the animal's resting posture; (2) by watching the animal walk straight ahead. The assessment also included questioning the owners about whether the animals showed signs of lameness while working or afterward. Prolapses were assessed visually by examining the anus and vulva/penis. Nose, ocular, and vulva/penis discharges were graded as absent or present. The condition of the hair coat was checked across the body, taking extra care not to confuse a changing coat due to the changing season with a bad coat. Faecal soiling was assessed by observation of the hindlimbs. Hoof neglect was also examined by checking for signs such as severely overgrown hooves, toes backed up, and severe hoof cracks.

Welfare assessment based on feeding

The body condition score was evaluated by both observation and palpation, as described by The Donkey Sanctuary [33]. It was scored from 1 to 5. Body sites like the neck, ribs, rump, and hindquarters were carefully evaluated. Prominent bones were considered a sign of low BCS, being highly noticeable in animals with a score of 1, unlike an animal with a score of 5, in which no bone protuberances were felt and many fat deposits could be found. Donkeys with BCS of 1, 2, 3, 4, and 5 were regarded as poor, moderate, ideal, fat, and obese, respectively. The availability of drinking water was assessed based on the following: type of water points and cleanliness of water points. The types of water points were classified into no water point, trough, and automatic drinkers. The cleanliness of water points was classified into three categories: dirty, partially dirty, and clean.

Welfare assessment based on environment and housing

The environment and housing were assessed by taking into consideration bedding cleanliness, bedding quantity, shelter dimension, and signs of thermal stress. Bedding quantity was classified into no bedding, insufficient bedding (floor area not covered by the bedding is visible), and sufficient bedding. Bedding cleanliness was classified into dirty (wet and with the presence of overstayed faeces) and clean. Shelter was classified as satisfactory or not satisfactory, as described by M i n e r o et al. [22]. Regarding signs of thermal stress, donkeys were examined for a minute for the following signs: increased respiratory rate, flared nostrils, increased respiratory depth with head movement, sweating, apathy, and sunburn. Donkeys with more than three (3) of these signs were regarded as having signs of thermal stress [22].

Data analysis

All the data were entered in a Microsoft Excel spreadsheet (2019), and analysis was done using the Statistical Package for Social Sciences (SPSS®, version 26). The analysed data was reported in terms of percentages and frequencies.

Ethical approval

Ethical approval for the research was obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) with the approval number ABUCAUC/2022/043.

RESULTS

Age summary of pack donkeys assessed

A welfare assessment was carried out on 38 donkeys, with the youngest donkey being 2 years old (5.3%) and the oldest being 13 years old (2.6%). Most of the donkeys were between 5 and 9 years old (18.4%) (Table 1).

Welfare assessment based on behaviour

In terms of social interaction, 97.4% (37/38) had social contact, while only 2.6% (1/38) had no social contact (Fig. 1). Regarding avoidance distance, 94.7% (36/38) had no avoidance, while 5.3% (2/38) had avoidance behaviour. Out of the 38 donkeys, 15 (40.5%) were walking downside while 22 (59.5%) were not. The tail tuck was absent in all the donkeys in this study (Table 2).

Welfare assessment based on health

Out of the 38 pack donkeys assessed, none had swollen joints, discharge from the penis, or rectal prolapse. Ten (26.3%) of the 38 pack donkeys assessed had their hooves neglected by the owners, while the remaining 28 (73.7%) did not have their hooves neglected. Out of the 38 packed donkeys, 86.8% (33/38) had unhealthy hair coat conditions (the coat was extremely dirty, lacklustre, and had small hairless spots), while the remaining 13.2% (5/38) had healthy hair coat conditions. Regarding alopecia, 60.5% (23/38) had alopecia, while the remaining 39.5% (15/38) did not have alopecia. Regarding skin lesions, 86.8% (33/38) had skin lesions, while the remaining

Table 1. Age summary of pack donkeys assessed in Amaru, Zaria Ancient City, Kaduna State, Nigeria (n = 38)

Age [Years]	Frequency	Percentage [%]
2	2	5.30
3	1	2.60
4	3	7.90
5	7	18.40
5.50	1	2.60
6	2	5.30
7	3	7.90
7	1	2.60
7.50	1	2.60
8	2	5.30
9	7	18.40
10	4	10.50
11	2	5.30
12	1	2.60
13	1	2.60
Total	**38**	**100**

Table 2. Pack donkeys' social interaction and human-animal relationships in Amaru, Zaria Ancient City, Kaduna State, Nigeria (n = 38)

Welfare indicators	No	Yes
Social interaction		
Social contact	1 (2.6 %)	37 (97.4 %)
Human-animal relation		
Avoidance distance	36 (94.7 %)	2 (5.3 %)
Walking downside	22 (59.5 %)	15 (40.5 %)
Tail tuck	38 (100 %)	0

Table 3. Assessment of pack donkeys' health problems in Amaru, Zaria Ancient City, Kaduna State, Nigeria (N = 38)

Welfare indicators	No. of donkeys positive	Prevalence [%]
Hoof neglect	10	26.3
Unhealthy hair coat	33	86.8
Alopecia	23	60.5
Skin lesion	33	86.8
Deep wound	18	47.4
Swelling	8	21.1
Nasal discharge	2	5.3
Ocular discharge	7	18.4
Faecal soiling	3	7.9
Lameness	2	5.3
Swollen joint	0	0
Discharge from penis	0	0
Rectal prolapse	0	0

Fig. 1. Donkey with no social contact

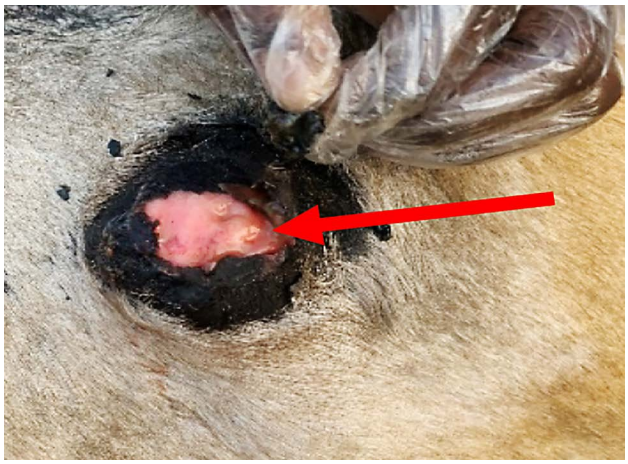

Fig. 2. Wound at the point of the hip (red arrow)

Fig. 3. Back wound (black arrow)

Fig. 4. Wound at the base of the tail (black arrow)

Fig. 5. Swelling on the neck region (black arrow)

Fig. 6. Unsatisfactory shelter dimension for 7 donkeys with insufficient bedding (red arrow)

13.2% (5/38) did not have skin lesions. Regarding deep wounds, 47.4% (18/38) had a deep wound (Figs. 2, 3, and 4), while the remaining 52.6% (20/38) did not have a deep wound. Regarding swellings, 21.1% (8/38) had swelling (Fig. 5), while the remaining 78.9% (30/38) did not have swelling. Out of the 38 packed donkeys, 2 (5.3%) had nasal discharge, while the remaining 36 (94.7%) did not. Out of the 38 packed donkeys, 7 (18.4%) had ocular discharge, and the remaining 31 (81.6%) did not have ocular discharge. Faecal soiling was absent in 92.1% (35/38) of the donkeys, while faecal soiling was present in the remaining 7.9% (3/38). Out of the 38 pack donkeys, 2 (5.3%) were lame, and the remaining 36 (94.7 %) were not lame (Table 3).

Welfare assessment based on feeding

Of the 38 pack donkeys assessed, 16 (42.1%) had a body condition score of 2 (moderate), while the remaining 22 (57.9%) had a body condition score of 3 (ideal). Out of the 38 pack donkeys assessed, 25 (65.8%) had no access to water points, and 13 (34.2%) had access to a water trough. Non had access to automatic drinkers. Regarding the cleanliness of water points, all had partially dirty water points.

Welfare assessment based on environment and housing

The shelter dimensions of all the donkeys (38; 100%) were not satisfactory (Fig. 6). Regarding bedding cleanliness, 89.5% (34/38) were dirty, while 10.5% (4/38) were clean. In terms of bedding quantity, out of the 38 packed donkeys assessed, 4 (10.5%) were housed with no bedding, while the remaining 34 (89.5%) were housed with insufficient bedding (Fig. 6). Signs of thermal stress were present in 94.7% (36/38) of the donkeys and absent in the remaining 5.3% (2/38).

DISCUSSION

The 38 pack donkeys evaluated were all stallions. Similar findings were reported in studies conducted in other developing countries, where the bulk of the donkeys used for work were stallions [15, 16, 31]. This clear-cut 100% of stallions might be connected to their purpose as working animals, as aggression and strength are more likely to be found in stallions. However, contradictory results were discovered in other studies conducted in Europe, where there was likewise a clear preponderance of females, followed by geldings [10, 26].

The donkeys in the present study ranged in age from 2 to 13 years. This shows that the donkeys are mature and capable of working and reproducing [16]. Several more studies have reported ages ranging from 3 to 13 years [15, 20, 32]. The most common ages of donkeys assessed in this study were 5 and 9 years old, which is similar to M o h a m e d et al. [23].

Because how an animal is handled has a direct impact on its welfare, assessing human-animal interactions is critical [17]. There was a low prevalence of avoidance behaviour in the present study. This is comparable to study by K u m a r et al. [16] work but different from that by C r u z [9]. Various authors described this behaviour as a result of the owner's improper handling [29]. However, it is also crucial to note that the tests were carried out by someone unfamiliar with the donkeys, which may result in a negative reaction. The low prevalence of donkeys with no social contact is similar to what C r u z [9] reported. Social contact is an important part of welfare evaluation, especially since donkeys are by nature social beings [5].

The most common health problems in the present study are integumentary alterations, which include an unhealthy hair coat, alopecia, deep wounds, and skin lesions. This is comparable to the work of F s a h a y e et al. [15] in Ethiopia. These integumentary alterations are frequently produced by a combination of multifactorial reasons. Environmental conditions, the type of harness material used (synthetic or natural), the fit of the harness, the owner's behaviour, the frequency of labour, and the load are among the risk factors that lead to the development of various types of wounds in pack donkeys [21].

The present study shows that the health problems with the lowest prevalence were ocular (18.4%) and nasal discharge (5.3%). The prevalence of ocular discharge is similar to studies in Chile (10%) and Ethiopia (17%) [15, 31]. Ocular or nasal discharges are not pathognomonic symptoms of any disease because they can occur in a variety of ailments, both local and generalised. This discharge can be present in ocular disease, respiratory issues, and dental problems [30, 34]; if it is observed, further investigation, including ophthalmic examination, respiratory auscultation, and dental examination, is required.

The current study discovered that 26.3 % of the donkeys assessed had some level of hoof neglect, which is similar to the results seen in the United Kingdom and Northeast Portugal [8, 9]. Hoof care is essential for optimal wellness since they bear all of the body's weight. Overgrown hooves were one of the most prevalent hoof alterations discovered in this study. This problem can result in lameness. The prevalence of lameness in our study was 5.3%, which was similar to the results obtained in Ethiopia [2]. Lameness percentages vary across several studies. In the same year, but in a different Ethiopian city, A m a n t e et al. [2] discovered that 12 % of working donkeys were lame. K u m a r et al. [16] discovered that 10 % of the donkeys were lame. Later, T e s f a y e et al. [32] reported a drop in lameness prevalence in 2016, and even better findings were discovered in 2018, with just 4.4 % of the donkeys being lame [15]. These findings are similar to those obtained in Europe; C o x et al. [8] discovered that 10 % of British donkeys were lame. Surprisingly, D a i et al. [10] reported the same findings as Mexico, with 2 % lameness detected [6]. Although the prevalence of lameness was not exceptionally high in any of the studies discussed, when lameness is diagnosed, it is critical to identify the source of the problem. Laminitis, tendonitis, and incorrect hoof trimming are some of the most common causes.

Healthy living and proper welfare require proper feeding. Without evaluating whether nutritional requirements are fulfilled or exceeded [3], the body condition score is an essential animal-based indicator in assessing the health state and welfare of working equids [25]. The current study found that donkeys' body condition ranged from moderate (BCS of 2) to ideal (BCS of 3). The prevalence of the ideal body condition (57.9 %) is comparable to M o l t u m o et al. [24] in Ethiopia, who reported a prevalence of 54.1 %. The absence of donkeys with BCS 4 (fat) contrasts with the report by C r u z [9] of a considerable number of donkeys with BCS 4 (fat). The difference may be attributed to a difference in management practises, working and environmental conditions, type of work, and working load.

The donkeys were poorly housed, as evidenced by the high prevalence of unsatisfactory shelter dimensions (100 %), dirty bedding (89.5 %), and insufficient bedding (89.5 %). Insufficient bedding results in poor faeces absorption, mechanical insults, and a lack of cushioning for the donkey's legs from trauma. These factors might have contributed to the integumentary alterations seen in this study.

The prevalence of donkeys showing indications of thermal stress was found to be low (5.3 %) in the present study. This is consistent with assertion by C r u z [9]. Thermal stress is a severe issue that is often underestimated in donkeys. According to studies, signs of heat stress generated by hot temperatures are more evident than those caused by cold temperatures [19]. Although heat stress is not always seen as significant as other abnormalities, it is crucial to note that aside from its implications for welfare, it can cause major health issues such as dehydration, organ dysfunction, and behavioural changes if left untreated. As a result, it is critical to train owners how to recognise thermal stress signs so that they may take action and prevent future suffering.

The present study discovered that welfare issues are significant problems for pack donkeys in Amaru, Zaria Ancient City, Kaduna State, Nigeria. As a result of the current findings, it is advised that the government and other non-governmental organisations (NGOs) promote comprehensive awareness creation on donkey welfare issues through training and extension services. To ensure donkey welfare, policies and legislative frameworks that support animal welfare and inspect animal facilities in Nigeria needs to be reviewed and enforced.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

REFERENCES

1. **Alujia, A. S., Lopez, F., 1991:** Donkeys in Mexico. In **Fielding, D., Pearson, R. A. (Eds.):** *Donkeys, Mules and Horses in Tropical Agricultural Development*. CTVM, Edinburgh, 1–7.
2. **Amante, M., Hunde, A., Endebu, B., Hirpa, E., Mamo, B., 2014:** Health and welfare assessment of working equine in and around Nekemte Town, East Wollega Zone, Ethiopia. *Am.-Eurasian J. Sci. Res.*, 9, 6, 163–174. DOI: 10.5829/idosi.aejsr.2014.9.6.86135.

3. **Aubert, A., 1999:** Sickness and behaviour in animals: A motivational perspective. *Neurosci. Biobehav. Rev.*, 23, 7, 1029–1036. DOI: 10.1016/S0149-7634(99)00034-2.
4. **Blench, R., 2004:** *The history and spread of donkeys in Africa*. Wageningen, ACP-EU Technical Centre for Agriculture and Rural Cooperation, 22–30.
5. **Burden, F., Thiemann, A., 2015:** Donkeys are different. *J. Equine Vet. Sci.*, 35, 5, 376–382. DOI: 10.1016/j.jevs.2015.03.005.
6. **Burden, F. A., Du Toit, N., Hernandez-Gil, M., Prado-Ortiz, O., Trawford, A. F., 2010:** Selected health and management issues facing working donkeys presented for veterinary treatment in rural Mexico: Some possible risk factors and potential intervention strategies. *Trop. Anim. Health Prod.*, 42, 597–605. DOI: 10.1007/s11250-009-9462-0.
7. **Burn, C. C., Dennison, T. L., Whay, H. R., 2010:** Environmental and demographic risk factors for poor welfare in working horses, donkeys and mules in developing countries. *Vet. J.*, 186, 385–392. DOI: 10.1016/j.tvjl.2009.09.016.
8. **Cox, R., Burden, F., Proudman, C. J., Trawford, A. F., Pinchbeck, G. L., 2010:** Demographics, management and health of donkeys in the UK. *Vet. Rec.*, 166, 18, 552–556. DOI: 10.1136/vr.b4800.
9. **Cruz, Z., Nóvoa, M., Leiva, B., Andrade, D., Quaresma, M., 2021:** Donkey welfare assessment in north-east Portugal. *Anim. Welfare*, 30, 1, 61–70. DOI: 10.7120/09627286.30.1.061.
10. **Dai, F., Dalla Costa, E., Murray, L. M. A., Canali, E., Minero, M., 2016:** Welfare conditions of donkeys in Europe: initial outcomes from on-farm assessment. *Animals*, 6, 1, 5. DOI: 10.3390/ani6010005.
11. **Donkey for Africa (DFA), 2018:** How ethically are donkeys treated? *Africa Network for Animal Welfare (ANAW)*, 10, 1–5.
12. **Farhat, S. F., McLean, A. K., Mahmoud, H. F., 2020:** Welfare assessment and identification of the associated risk factors compromising the welfare of working donkeys (*Equus asinus*) in Egyptian brick kilns. *Animals*, 10, 9, 1611. DOI: 10.3390/ani10091611.
13. **Fernando, P., Starkey, P., 2004:** Donkeys and development: Socio-economic aspects of donkey use in Africa. Donkeys, People and Development. *A Resource Book in the Animal Traction Network for Eastern and Southern Africa (ATNESA)*, 15 pp.
14. **Fred, O., Pascal, K., 2006:** Extension approaches to improve the welfare of working equines. *Kenya Network for Dissemination of Agricultural Technologies (KENDAT)*, 1–28.
15. **Fsahaye, S., Kumar, N., Kebede, E., Abebe, N., 2018:** Health and welfare assessment of working donkeys in and around Rama town, Tigray, Ethiopia. *Ethiop. Vet. J.*, 22, 1, 26–39. DOI: 10.4314/evj.v22i1.3.
16. **Kumar, N., Fisseha, K. K., Shishay, N., Hagos, Y., 2014:** Welfare assessment of working donkeys in Mekelle City, Ethiopia. *Glob. Vet.*, 12, 314–319.
17. **Luna, D., Tadich, T. A., 2019:** Why should human-animal interactions be included in research of working equids' welfare? *Animals*, 9, 2, 42. DOI: 10.3390/ani9020042.
18. **Mabayoje, A. L., Ademiluyi, Y. S., 2004:** A note on animal power and donkey utilization in Nigeria. *Animal Traction Network for Eastern and Southern Africa (ATNESA)*, 2 pp.
19. **Matthews, N. S., Taylor, T. S., Potter, G. D., 1998:** Physiologic responses during an exhaustive driving test in donkeys: effect of conditioning. *Appl. Anim. Behav. Sci.*, 59, 1–3, 31–38. DOI: 10.1016/S0168-1591(98)00118-X.
20. **McLean, A. K., Heleski, C. R., Yokoyama, M. T., Wang, W., Doumbia, A., Dembele, B., 2012:** Improving working donkey (*Equus asinus*) welfare and management in Mali, West Africa. *J. Vet. Behav.*, 7, 3, 123–134. DOI: 10.1016/j.jveb.2011.10.004.
21. **Mekuria, S., Mulachew, M., Abebe, R., 2013:** Management practices and welfare problems encountered on working equids in Hawassa town, Southern Ethiopia. *J. Vet. Med. Anim. Health*, 5, 9, 243–250. DOI: 10.5897/JVMAH10.017.
22. **Minero, M., Dalla Costa, E., Dai, F., Murray, L. A. M., 2015:** AWIN welfare assessment protocol for donkeys. IRIS, 69 pp. DOI: 10.13130/AWIN_donkeys_2015.
23. **Mohamed, Y. A., Mohamed, S. A., Mohamud, A. I., Mohamud, A. A., Jimale, K. A., Ibrahim, S. A., 2021:** Assessment of welfare and health conditions on working donkeys in Benadir region, Somalia. *Vet. Sci. Res.*, 7, 2, 121–128. DOI: 10.17582/journal.vsrr/2021.7.2.121.128.
24. **Moltumo, S., Mathewos, M., Fesseha, H., Yirgalem, M., 2020:** Assessment of welfare problems on working donkeys in Hosaena district, Hadiya zone, southern Ethiopia. *Vet. Med. Open J.*, 5, 1, 14–20. DOI: 10.17140/VMOJ-5-142.
25. **Pritchard, J. C., Barr, A. R. S., Whay, H. R., 2006:** Validity of a behavioural measure of heat stress and a skin tent test for dehydration in working horses and donkeys. *Equine Vet. J.*, 38, 5, 433–438. DOI: 10.2746/042516406778400646.
26. **Quaresma, M., Martins, A. M. F., Rodrigues, J. B., Colaço, J., Payan-Carreira, R., 2014:** Pedigree and herd characterization of a donkey breed vulnerable to extinction. *Animals*, 8, 3, 354–359. DOI: 10.1017/S1751731113002218.

27. **Rossel, S., Marshall, F., Peters, J., Pilgram, T., Adams, M. D., O'Connor, D., 2008:** Domestication of the donkey: Timing, processes, and indicators. *Proc. Natl. Acad. Sci.*, 105, 10, 3715–3720. DOI: 10.1073/pnas.07096921.
28. **Starkey, P., 1997:** Improving donkey utilisation and management. In *Report of the Workshop of the Animal Traction Network for Eastern and Southern Africa (ATNESA)*, Debre Zeit, Ethiopia. 1–59 pp.
29. **Swann, W. J., 2006:** Improving the welfare of working equine animals in developing countries. *Appl. Anim. Behav. Sci.*, 100 1–2, 148–151. DOI: 10.1016/j.applanim.2006.04.001.
30. **Swendsen, E. D., 1998:** *The Professional Handbook of the Donkey*. 4th edn., Whittet Books, 488 pp.
31. **Tadich, T., Escobar, A., Pearson, R. A., 2008:** Husbandry and welfare aspects of urban draught horses in the south of *Chile. Arch. Med. Vet.*, 40, 3, 267–273.
32. **Tesfaye, S., Deressa, B., Teshome, E., 2016:** Study on the health and welfare of working donkeys in Mirab Abaya district, southern Ethiopia. *A.J.A.D.*, 5, 2, 40–52. DOI: 10.5829/idosi.ajad.2016.40.52.
33. **The Donkey Sanctuary, 2014:** *Condition Scoring and Weight Estimation*. Available at https://www.thedonkeysanctuary.org.uk/sites/sanctuary/files/document/142-1423234830-donkey_health_and_welfare.pdf.
34. **The Donkey Sanctuary, 2019:** Diagnostic Methods. In **Evans, L., Lilly, G. (Eds.):** *The Clinical Companion of Donkey Dentistry*. ivis.org., 277 pp.

Received March 10, 2023
Accepted May 15, 2023

sciendo

DOI: 10.2478/fv-2023-0017

FOLIA VETERINARIA, 67, 2: 51—61, 2023

PHYTOBIOTICS AND THEIR ANTIBACTERIAL ACTIVITY AGAINST MAJOR FISH PATHOGENS. A REVIEW

Patrícia Hudecová, Jana Koščová, Vanda Hajdučková

Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia

patricia.hudecova@student.uvlf.sk

ABSTRACT

This review focuses on the characteristic of major fish bacteria, antimicrobial resistance and antibiotic substitution in aquaculture. Nowadays aquaculture represents the fastest growing industry leading to the use of intensive and semi-intensive methods resulting in outbreaks of bacterial diseases. Antibiotics are used to treat and prevent these bacterial diseases, which can cause an increase in resistance. Many antibiotics applied in aquaculture were originally used in human medicine and many countries do not have strict limits for their application. Annually, more than 700 000 people worldwide die from bacterial infections caused by bacteria resistant to antibiotics and even though European countries have strict rules for the use of antibiotics, the death rate resulting from resistance represents more than 33 000 people per year. One of the options how to resolve this problem is the replacement of antibiotics with bioactive compounds of plant origin that exhibit strong inhibitory activity against pathogenic bacteria.

Key words: antibiotic resistance; aquaculture; fish bacteria; phytobiotics

INTRODUCTION

Aquaculture is currently the most developing branch compared to other branches of the food industry. Based on the Food and Agriculture Organization [25], the global production of aquaculture products reached approximately 43 million tons in 2016 and about 61 million tons in 2020. This was due to the rapidly growing human population, which needs to be supplied with complete nutrients and aquaculture products are a good source of these [67]. They contain high-quality protein and omega-3 fatty acids, such as docosahexaenoic acid (DHA) that plays an important role in optimum brain development and neurodevelopment, and eicosapentaenoic acid (EPA) that has beneficial impact on cardiovascular health. Aquaculture products are rich in minerals like selenium, iron, zinc, calcium, potassium, phosphorus and also in vitamin A and D and some B vitamins [79]. Another advantage is related to the beneficial effect of aquaculture on global warming due to reduced hunting of wild fish and reduced marine pollution [67]. Development in fish farming technologies also contributed to the rapid growth of aquaculture [74]. On the other hand, the rapid increase has several disadvantages such as the use of intensive and semi-intensive farming methods, which involve higher concentration of

fish in a small water volume that is related to higher stress and a higher risk of bacterial diseases [67]. The bacteria most common in aquaculture are Gram-negative bacteria such as *Aeromonas salmonicida* [43], *Aeromonas hydrophila* [46], *Vibrio anguillarum* [27], *Vibrio alginolyticus* [38], *Edwardsiella tarda* [44], *Flavobacterium psychrophilum* [8] and *Yersinia ruckeri* [86]. Of the Gram-positive bacteria, the most common are *Staphylococcus* spp. and *Streptococcus* spp. [60]. All these bacteria can cause serious diseases, survive in aquatic environment without the host, increase mortality and cause great economic losses. These are the reasons why bacterial diseases have become the major obstacles to aquaculture. Antibiotics are used to prevent (prophylactic purpose) and treat (therapeutic purpose) these bacterial diseases but their increased use results in developing antibiotic resistance. Fish bodies absorb only 20–30% of the received antibiotics and the rest ends in the environment because fish are not able to metabolize them efficiently [70]. This is the reason why in aquaculture the fish, other aquatic animals and aquatic environment form reservoirs of resistant bacteria and the resistance is expanded by mutations in bacterial DNA or by horizontal gene transfer including conjugation with other bacteria, transduction with bacteriophage and transformation of free DNA [63, 68]. Since resistance to antibiotics is becoming more widespread every year, it is important to look for new methods to replace them. One of the perspective candidates are bioactive compounds of natural origin, which can be found in plants, animals, microorganisms and marine organisms. Bioactive compounds of plant origin include alkaloids, saponins, polyphenols, terpenes, essential oils and flavonoids [89] while polysaccharides, polyunsaturated fatty acids, coenzyme Q10, choline and peptides from milk protein belong to those typical for animals [88]. Many of the listed compounds are characterized by an anti-inflammatory, antioxidant, antitumor, and mainly by antimicrobial activities, however more studies are needed to improve their stability, obtain more knowledge about their mechanism of action, and about their impact on the environment, host microbiota and their stability in the feed [51].

THE MAIN BACTERIAL AGENTS IN AQUACULTURE

Aeromonas salmonicida

Aeromonas salmonicida is a Gram-negative bacterium also known as the oldest fish pathogen [43]. It is found in fresh and salt water where it causes disease in salmonids and non-salmonid fish, such as minnow, goldfish, carp, perch and catfish. The main clinical symptom is furunculosis, which is shown in Figure 1 and it also manifests as ulcerative dermatitis and granulomatous dermatitis [5]. The virulence factors of *A. salmonicida* are the A-layer, extracellular molecules as proteases, lipases, and type III

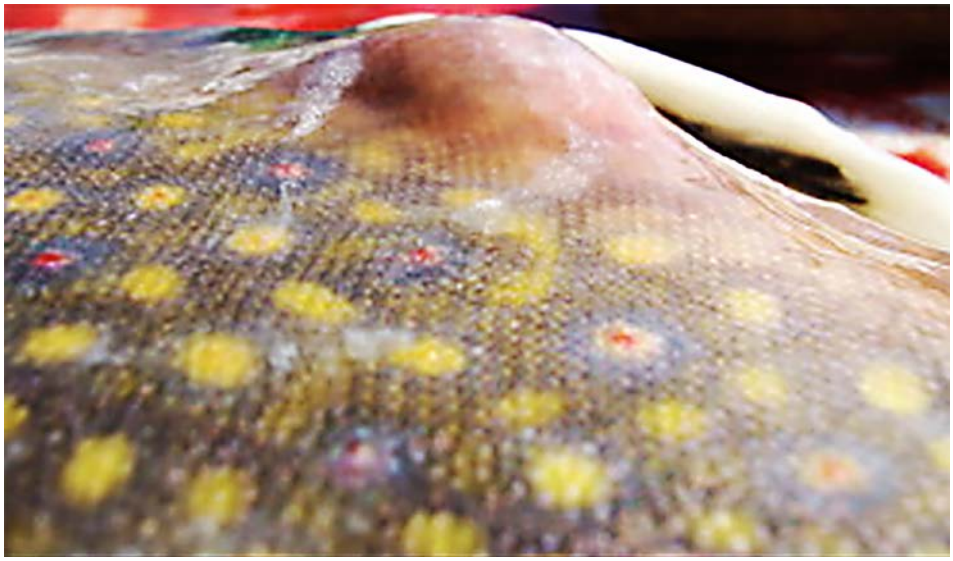

Fig. 1. Clinical manifestation of furunculosis [18]

secretion system [18]. The A-layer is also known as the S-layer and it occurs on the surface of the cell. It provides protection against the specific immune system and resistance to cytotoxicity of macrophage [54]. The extracellular molecules include serine protease, which causes muscle liquefaction leading to furunculosis or lipase, which participates in the formation of bleeding boils (Fig. 1) [18]. The type III secretion system enables the translocation of effector proteins, causing inhibition of phagocytosis and induction of apoptosis from the bacterial cytoplasm into the host cell [54].

Aeromonas hydrophila

Aeromonas hydrophila is characterized as a Gram-negative bacterium common in fresh water. It has been isolated from catfish, bass, carp and can be found also in marine fish species [17]. This bacterium is the causative agents of motile *Aeromonas septicaemia* known as tail and fin rot that is clinically manifested by haemorrhage, anaemia, ascetic fluid, abdominal distension and ulcerations [2]. The virulence factors include O-antigen that has several roles such as protection and adhesion against the host immune system, and cytotoxic enterotoxin that has the ability to destroy tissue culture cell lines and cause lysis of red blood cells. The virulence factors also include haemolysin, enzymes such as proteases, amylases, lipases and type III secretion system [14, 72, 80].

Vibrio anguillarum

Vibrio anguillarum, also known as *Listonella anguillarum*, is a Gram-negative bacterium causing big economy losses in marine, fresh and brackish water fish such as salmon, sea bass, cod and rainbow trout. Infection by *V. anguillarum* leads to haemorrhagic septicaemia called vibriosis that manifests with red spots, swollen and dark skin lesions, ulceration and haemorrhages of skin and fin [27]. Virulence factors of *V. anguillarum* include motility and chemotaxis, an iron uptake system, haemolysins and zinc metalloprotease that degrades the tissue in the host [30, 31].

Edwardsiella tarda

Edwardsiella tarda is characterized as a Gram-negative intracellular bacterium that can survive in an environment with 0–4% sodium chloride. It infects marine and fresh water fish worldwide and results in a systemic disease called edwardiellosis that manifests as exophtalmus, petechial haemorrhages on the skin and fin, rectal hernia, swelling of the abdominal surface and ascites [55]. The most important virulence factors of *E. tarda* are the type III and the type VI secretion systems. The type III secretion system allows to replicate intracellularly in phagocytes and evade phagocytosis and the type VI secretion system is only recently discovered secretion system, which contributes to bacterial virulence [37]. Other virulence factors include fimbrial adhesion-like protein, siderophore and exoenzymes, such as protease and catalase and outer membrane proteins (OMPs) that play a key role in the integrity and selective permeability of bacterial membranes [42, 83, 87].

Flavobacterium psychrophilum

Flavobacterium psychrophilum is a Gram-negative bacterium that is able to survive outside the fish in fresh water for a few months to several years [8]. This pathogen is characteristic for cold water fish species such as trout and salmonids and the infection leads into the cold water disease and rainbow trout fry syndrome (RTFS). The cold water disease in salmonids is manifested with erosion lesions on the caudal fin, necrosis and ulcerations of the skin on the lower jaw. Increasing pigmentation leads into the “black tail” and RTFS manifests with anaemia, haemorrhagic and protruding anus and skin erosions located mainly behind the dorsal fin or on the lateral side of the body [10, 41, 76]. Virulence factors consist of the type IX secretion system that is able to secrete proteins to the cell surface and beyond, cell surface adhesins, soluble and cell-associated peptidases, nucleases and more hydrolytic enzymes [7]. Another virulence factor is the gliding motility that allows movement of the cells over surfaces without the help of pili or flagellum and also include cytolytic toxins and proteases [23, 58].

Yersinia ruckeri

Yersinia ruckeri is a Gram-negative bacterium causing yersiniosis and enteric red-mouth disease mainly in salmonids and rainbow trout. The characteristic clinical symptoms of *Y. ruckeri* include haemorrhages on the surface of the body, redness at the base of the fins, along the lateral line and also in the head area [81]. The virulence factors include extra-cellular products, which have cytolytic and haemolytic activity, metalloprotease that causes degrada-

tion of fibronectin, actin and myosin [34]. Another virulence factors of *Y. ruckeri* are the type III secretion system, the type IV secretion system that is used to transport macromolecules across the bacterial cell membrane and the iron acquisition system which is involved in colonization and invasion of host tissues [81, 86].

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) is a characteristic trait of bacteria, viruses, parasites and fungi. The resistant agents have the ability to grow and spread when an antimicrobial medicine is present even though these medicines are normally effective against them [26]. There are several types of antimicrobial agents such as antibiotics, chemotherapeutics, food preservatives and disinfectants that are used against different microorganisms. Antimicrobial resistance is the main problem of healthcare organizations because the increasing use of antibiotics leads to the emergence of resistance [1]. Different origins of antibiotic resistance have been distinguished, such as natural, which is divided into intrinsic and induced resistance and then adaptive and acquired resistance. Intrinsic resistance is defined as a trait that is generally shared within a bacterial species and is always expressed. On other hand, induced resistance is normally occurring in the bacteria but is expressed only if there is some exposure to an antibiotic. Adaptive resistance is caused and induced by a specific environmental signal such as pH, stress or concentrations of ions. Acquired resistance develops in originally sensitive bacteria in two different ways. The first develops through mutation and the next by obtaining new genetic material from an exogenous source. It is also known as a horizontal gene transfer that includes transduction, transformation and conjugation [11, 16]. Aquatic environment is a well-suited reservoir of antibiotic resistance because water is a favourable environment for horizontal gene transfer and has a high infiltration capacity, which allows the contamination of clean water with unsanitary water containing antimicrobial compounds [6]. The ability of transfer of resistant pathogens from aquaculture to natural aquatic environment causes an increase in the resistance of fish, other aquatic animals and humans to antimicrobials that results in infections and failure of treatment [51]. In addition, many antibiotics used in aquaculture are also used in human medicine which promotes the risk of development of resistance in humans [60]. The antibiotics most frequently used in aquaculture worldwide, including the USA and European and Asian countries, are described in Table 1.

While European countries and USA have strict regulations for the use of antibiotics in aquaculture, in other countries like China; the limits for their use are poor. Studies showed a high rate of antimicrobial residues in various countries such as India, Indonesia, Bangladesh, Iran, Japan and Nigeria [12, 51]. Globally, approximately 10 259 tons of antimicrobials were used in aquaculture in 2017 and their consumption is expected to increase by 33% to around 13 600 tons in 2030 [71]. Because of the increasing antibiotic resistance in animal and human environments, it is necessary to look for new replacements to antibiotics. Annually, more than 700 000 human deaths worldwide, more than 35 000 deaths in the USA, 33 000 deaths in Europe and 58 000 deaths in India have been attributed to the increasing resistance of disease agents, and by 2050 around 10 million of deaths annually on account of antimicrobial resistance are expected [65, 85].

Table 1. The antibiotics most frequently used in aquaculture

Antibiotics			
Worldwide [57]	**European countries** [39, 67]	**Asian countries** [39]	**USA** [29]
Ampicillin	Erythromycin	Amoxicillin	Florfenicol
Erythromycin	Florfenicol	Chlortetracycline	Oxytetracycline
Florfenicol	Oxytetracycline	Florfenicol	Chloramphenicol
Streptomycin	–	Neomycin / Oxytetracycline	Erythromycin

ALTERNATIVES TO ANTIBIOTICS

New alternatives to antibiotics need to be discovered because of their negative impact on the environments, animals and humans. One of the promising alternatives are plants and their bioactive compounds known as phytobiotics, due to their antimicrobial, antioxidative, anti-inflammatory, antiparasitic and appetite stimulating effect [59]. Bioactive compounds represent secondary metabolites of the plant and due to their structural diversity and efficiency could be used as a drug candidate. The antibacterial activity of phytobiotics manifest with different mechanisms such as bacterial cell wall disruption, nucleic acid translation, transcription blockage, lysozyme and complement activity enhance but the efficiency depends on the target bacteria, phytobiotic and its dozen. Phytobiotics represent compounds such as flavonoids, tannins, alkaloids, terpenes, saponins and phenolic compounds. Their presence in the plant depends on the climate, the type of soil, the state of maturity of the plant and on the part of used plant [33, 48].

Terpenes

Terpenes represent the largest group of secondary metabolites, which show important functions in nature such as anti-inflammatory, antibacterial, antiviral and antiseptic function. Depending on the number of carbon atoms they can be divided into monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) and poly-terpenes (C>40). They occur in different parts of the plants, such as the flowers and fruits and they can be commonly found in tea, thyme, Spanish sage, cannabis and citrus fruits [15, 48].

Alkaloids

Alkaloids are widely spread compounds produced by plants, bacteria, fungi and animals. These compounds contain nitrogen atoms in their chemical structure. Only 20 % of plant species contains alkaloids and are known for their variety of functions. They have analgesic, anticancer, antiseptic, antibacterial, antifungal and antiviral functions [13, 66]. Plants belonging to the families Amaryllidaceae, Berberidaceae, Liliaceae, Leguminaceae, Papaveraceae, Ranunculaceae, and Solanaceae contain a large amount of alkaloids [9].

Phenolic compounds

Phenolic compounds have typical structure formed in an aromatic ring with one or more hydroxyl groups. They commonly occur in bark, seeds, leaves and flowers [32]. They can be divided into five subgroups: i.e., coumarins, flavonoids, phenolic acids, lignins and tannins. The major subgroup of phenolic compounds represents flavonoids which acts as antioxidants and also have anti-inflammatory, anti-mutagenic and anti-carcinogenic effects [53]. Coumarins are known for their influence on the central nervous system, antioxidant, antibacterial effect and for their role in cancer prevention [35]. Phenolic acids have a wide spectrum of actions: such as antioxidant, anti-inflammatory, anticancer, antimicrobial or immunoregulatory [62]. Another subgroup represents lignins, which protect polysaccharides in cell wall from microbial degradation. [82]. The last one, tannins, have anticancer, antioxidant, antimutagenic function [19].

Saponins

Saponins are important secondary metabolites that are characterized with antibacterial, anti-tumor, antioxidative, anti-inflammatory, antidiabetic and neuro-protective function. Depending upon the chemical structures they are divided into two classes: i.e., triterpenoid and steroidal saponins. Their high concentrations can be found in plants like quinoa, spinach, oat, beans, peas and citrus [49].

The main bioactive compounds and properties of some plants usable in aquaculture are described in Table 2.

Hayatgheib et al. [28] investigated the effect of phytobiotics against *Aeromonas salmonicida* subsp. *salmonicida* isolated from rainbow trout and discovered the effectiveness of the following herbs: *Cinnamomum zeylanicum; Origanum vulgare; Thymus vulgaris*. The main bioactive compounds, which showed suitable inhibitory effect belongs to the phenolic compounds (eugenol, carvacrol, thymol) and to the aldehydes (cinnamaldehyde). Also study involving *Thymus vulgaris* confirmed a strong inhibitory effect against *Vibrio anguillarum*. Major components with antibacterial effect represent phenolic compounds (thymol, carvacrol) and monoterpenes (p-Cymene) [47]. The studies conducted by Musa et al. and Nya et al. showed benefits of *Allium sativum* toward inhibition of *Staphylococcus aureus, Aeromonas hydrophila* and *Edwardsiella tarda*. Allicin represent the compound with antibacterial activity that *Allium sativum* contain [45,

Table 2. List of bioactive compounds of plant origin used in aquaculture

Plant scientific name	Common Name	Biological active compounds	Properties	References
Allium sativum	Garlic	Saponins, phenolic compounds, polysaccharides and organic sulphides	Antibacterial, anti-inflammatory, antifungal	[33, 36, 73]
Cinnamomum verum	True cinnamon tree	Phenolic and terpenoids compounds, tannins	Antibacterial, anticancer, anti-inflammatory	[4, 64, 84]
Cymbopogon citratus	Lemongrass	Flavonoids, phenolic compounds, terpenoids	Antibacterial, antifungal	[20, 52]
Eupatorium odoratum	Siam weed	Flavonoids, phenolic compounds	Antibacterial, antiviral	[33, 69]
Ocimum sanctum	Tulsi	Flavonoids, tannins, saponins	Antibacterial, antifungal	[40, 56]
Origanum onites	Greek oregano	Terpenoids, flavonoids, phenolic compounds	Antibacterial, antiviral, anti-inflammatory	[20, 75, 78]
Origanum vulgare	Oregano	Phenol compounds, flavonoids	Antibacterial, antifungal	[20, 61]
Thymus vulgaris	Thyme	Phenolic compounds	Antibacterial, anti-inflammatory, antiviral, immune stimulation	[3, 22]
Zingiber officinalis	Ginger	Flavonoids, phenolic compounds saponins, tannins	Antibacterial, antiviral, antifungal, anti-inflammatory	[24, 77]

50]. De Rezende et al. [21] focused on Nile tilapia's dietary supplementation with a blend of phytobiotics contained in *Lippia sidoides, Thymus vulgaris* and *Thymus zygis* with the phenol compounds (thymol) as their major component. Studies have shown showed improvement in tilapia's resistance against *A. hydrophila*, better biochemical responses and benefits for the immune system.

CONCLUSIONS

This review describes the necessity of dealing with antibiotic resistance as it represents a major obstacle in maintaining animal and human health. Due to the rapid development of the aquaculture industry, there is an increased incidence of bacterial diseases requiring application of antibiotics for preventive and treatment purposes. However, their application is not regulated appropriately in some countries, especially in Asia, and results in increase resistance of disease agents to antimicrobials. A suitable substitute for antibiotics could be phytobiotics especially due to their antibacterial effect but also other benefits such as: antiviral, anti-inflammatory and antifungal properties. In order to successfully replace antibiotics with phytobiotics, studies need to be performed to obtain more knowledge about their mechanisms of action, impact on the environment and the host microbiota and their stability in feed.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENT

This study was supported by the project VEGA No. 1/0731/21.

REFERENCES

1. **Abushaheen, M. A., Muzaheed, Fatani, A. J., Alosaimi, M. Mansy, W., George, M., et al., 2020:** Antimicrobial resistance, mechanisms and its clinical significance. *Dis. Mon.*, 66, 6, 100971. DOI: 10.1016/j.disamonth.2020.100971.
2. **Akmal, M., Rahimi-Midani, A., Hafeez-Ur-Rehman, M., Hussain, A., Choi, Tae-Jin, 2020:** Isolation, characterization, and application of a bacteriophage infecting the fish pathogen *Aeromonas hydrophila. Pathogens*, 9, 3, 215. DOI: 10. 3390/pathogens9030215.
3. **Alagawany, M., Farag, M. R., Abdelnour, S. A., Elnesr, S. S., 2021:** A review on the beneficial effect of thymol on health and production of fish. *Rev. Aquacult.*, 13, 1, 632–641. DOI: 10.1111/raq.12490.
4. **Alizadeh Behbahani, B., Falah, F., Lavi Arab, F., Vasiee, M., Tabatabaee Yazdi, F., 2020:** Chemical composition and antioxidant, antimicrobial, and antiproliferative activities of *Cinnamomum zeylanicum* bark essential oil. *Evid.-based Complement. Altern. Med.*, 2020. DOI: 10.1155/2020/5190 603.
5. **Austin, B., Austin, D. A., 2016:** Aeromonadaceae representative (*Aeromonas salmonicida*). In *Bacterial Fish Pathogens*. 6th edn., Springer, Cham, 215–321.
6. **Vincent, A. T., Gauthier, J., Derome, N., Charette, S. J., 2019:** The rise and fall of antibiotics in aquaculture. In *Microbial Communities in Aquaculture Ecosystems*. Springer, Cham, 3–8.
7. **Barbier, P., Rochat, T., Mohammed, H. H., Wiens, G. D., Bernardet, J. F., Halpern, D., et al., 2020:** The type IX secretion system is required for virulence of the fish pathogen *Flavobacterium psychrophilum*. *Appl. Environ. Microbiol.*, 86, 16, e00799–20. DOI: 10.1128/AEM.00799-20.
8. **Barnes, M. E., Brown, M. L., 2011:** A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. *Open Fish Sci. J.*, 4, 1–9. DOI: 10.2174/1874401x01104010040.
9. **Bhambhani, S., Kondhare, K. R., Giri, A. P., 2021:** Diversity in chemical structures and biological properties of plant alkaloids. *Molecules*, 26, 11, 3374. DOI: 10.3390/molecu les 26113374.
10. **Boyacioglu, M., Akar, F., 2012:** Isolation of *Flavobacterium psychrophilum* causing rainbow trout fry syndrome and determination of an effective antibacterial treatment in rainbow trout (*Oncorhynchus mykiss*) fry. *Kafkas Univ. Vet. Fak.*, 18, 197–203. DOI: 10.9775/kvfd.2011.5254.
11. **Reygaert, W. C., 2018:** An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol.*, 4, 3, 482–501. DOI: 10.3934/microbiol.2018.3.482.
12. **Caputo, A., Bondad-Reantaso, M. G., Karunasagar, I., Hao, B., Gaunt, P., Verner-Jeffreys, D., et al., 2022:** Antimicrobial resistance in aquaculture: A global analysis of literature and national action plans. *Rev. Aquac.*, 1–11. DOI: 10. 1111/raq.12741.
13. **Casciaro, B., Mangiardi, L., Cappiello, F., Romeo, I., Loffredo, M. R., Iazzetti, A., et al., 2020:** Naturally-occurring alkaloids of plant origin as potential antimicrobials against antibiotic-resistant infections. *Molecules*, 25, 16, 3619. DOI: 10.3390/molecules25163619.
14. **Chopra, A. K., Xu, X. J., Ribardo, D., Gonzalez, M., Kuhl, K., Peterson, J. W., et al., 2000:** The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. *Infect. Immun.*, 68, 5, 2808–2818. DOI: 10. 1128/iai.68.5.2808-2818.2000.
15. **Cox-Georgian, D., Ramadoss, N., Dona, C., Basu, C., 2019:** Therapeutic and medicinal uses of terpenes. In *Medicinal Plants: From Farm to Pharmacy*. Springer, 333–359. DOI: 10.1007/978-3-030-31269-5_15.
16. **Christaki, E., Marcou, M., Tofarides, A., 2020:** Antimicrobial resistance in bacteria: mechanisms, evolution, and persistence. *J. Mol. Evol.*, 88, 1, 26–40. DOI: 10.1007/s00239-019-09914-3.
17. **Citterio, B., Biavasco, F., 2015:** *Aeromonas hydrophila* virulence. *Virulence*, 6, 5, 417–418. DOI: 10.1080/21505594. 2015.1058479.
18. **Dallaire-Dufresne, S., Tanaka, K. H., Trudel, M. V., Lafaille, A., Charette, S. J., 2014:** Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet. Microbiol.*, 169, 1–2, 1–7. DOI: 10.1016/j.vetmic. 2013. 06.025.
19. **Das, A. K., Islam, M. N., Faruk, M. O., Ashaduzzaman, M., Dungani, R., 2020:** Review on tannins: Extraction processes, applications and possibilities. *S. Afr. J. Bot.*, 135, 58–70. DOI: 10.1016/j.sajb.2020.08.008.
20. **Dawood, M. A., El Basuini, M. F., Zaineldin, A. I., Yilmaz, S., Hasan, M. T., Ahmadifar, E., 2021:** Antiparasitic and antibacterial functionality of essential oils: An alternative approach for sustainable aquaculture. *Pathogens*, 10, 2, 185. DOI: 10.3390/pathogens10020185.
21. **de Rezende, R. A. E., Soares, M. P., Sampaio, F. G., Cardoso, I. L., Ishikawa, M. M., Dallago, B. S. L., et al.,**

2021: Phytobiotics blend as a dietary supplement for Nile tilapia health improvement. *Fish Shellfish Immunol.*, 114, 293–300.

22. **Dorojan, O. G. V., Cristea, V., Creţu, M., Dediu, L., Docan, A. I., Coadă, M. T., 2015:** The effect of thyme (*Thymus vulgaris*) and vitamin E on the *Acipenser stellatus* juvenile welfare, reared in a recirculating aquaculture. *AACL Bioflux*, 8, 2, 150–158.
23. **Duchaud, E., Boussaha, M., Loux, V., Bernardet, J. F., Michel, C., Kerouault, B., et al., 2007:** Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. *Nature Biotechnol.*, 25, 7, 763–769. DOI: 10.1038/nbt1313.
24. **Fazelan, Z., Vatnikov, Y. A., Kulikov, E. V., Plushikov, V. G., Yousefi, M., 2020:** Effects of dietary ginger (*Zingiber officinale*) administration on growth performance and stress, immunological, and antioxidant responses of common carp (*Cyprinus carpio*) reared under high stocking density. *Aquaculture*, 518, 734833. DOI: 10.1016/j.aquaculture.2019.734833.
25. **Food and Agriculture Organization of the United Nations:** *Global Fish Processed Products Production Statistics Quantity (1976–2020)*. Updated July 17, 2022. Accessed July 17, 2022. https://www.fao.org/fishery/statistics-query/en/trade_pp/trade_pp_quantity.
26. **Founou, R. C., Founou, L. L., Essack, S. Y., 2017:** Clinical and economic impact of antibiotic resistance in developing countries: a systematic review and meta-analysis. *PLOS ONE*, 12, 12, e0189621. DOI: 10.1371/journal.pone.0189621.
27. **Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., Rediers, H., 2011:** *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *J. Fish Dis.*, 34, 9, 643–661. DOI: 10.1111/j.1365-2761.2011.01279.x.
28. **Hayatgheib, N., Fournel, C., Calvez, S., Pouliquen, H., Moreau, E., 2020:** *In vitro* antimicrobial effect of various commercial essential oils and their chemical constituents on *Aeromonas salmonicida* subsp. *salmonicida*. *J. Appl. Microbiol.*, 129, 1, 137–145. DOI: doi:10.1111/jam.14622.
29. **Hays, L.:** *The Current State of Antibiotics in Aquaculture*. Updated May 4, 2020. Accessed May 4, 2020. https://www.qb-labs.com/blog/2020/5/4/the-current-state-of-antibiotics-in-aquaculture.
30. **Hickey, M. E., Lee, J. L., 2018:** A comprehensive review of *Vibrio (Listonella) anguillarum*: Ecology, pathology and prevention. *Rev. Aquac.*, 10, 3, 585–610. DOI: 10.1111/raq.12188.
31. **Karami, A. M., Ødegård, J., Marana, M. H., Zuo, S., Jaafar, R., Mathiessen, H., et al., 2020:** A major QTL for resistance to *Vibrio anguillarum* in rainbow trout. *Front. Genet.*, 11, 607558. DOI: 10.3389/fgene.2020.607558.
32. **Kumar, S., Abedin, M. M., Singh, A. K., Das, S., 2020:** Role of phenolic compounds in plant-defensive mechanisms. In *Plant Phenolics in Sustainable Agriculture*, Springer, 517–532. DOI: 10.1007/978-981-15-4890-1_22.
33. **Jana, P., Karmakar, S., Roy, U., Paul, M., Bera, A. K. S., 2018:** Phytobiotics in aquaculture health management: A review. *J. Entomol. Zool. Stud.*, 6, 4, 1422–1429.
34. **Kumar, G., Menanteau-Ledouble, S., Saleh, M., El-Matbouli, M., 2015:** *Yersinia ruckeri*, the causative agent of enteric redmouth disease in fish. *Vet. Res*., 46, 1, 1–10. DOI: 10.1186/s13567-015-0238-4.
35. **Küpeli Akkol, E., Genç, Y., Karpuz, B., Sobarzo-Sánchez, E., Capasso, R., 2020:** Coumarins and coumarin-related compounds in pharmacotherapy of cancer. *Cancers*, 12, 7, 1959. DOI: 10.3390/cancers12071959.
36. **Lee, J. Y., Gao, Y., 2012:** Review of the application of garlic, *Allium sativum*, in aquaculture. *J. World Aquac. Soc.*, 43, 4, 447–458. DOI: 10.1111/j.1749-7345.2012.00581.x.
37. **Leung, K. Y., Siame, B. A., Tenkink, B. J., Noort, R. J., Mok, Y. K., 2012:** *Edwardsiella tarda* – virulence mechanisms of an emerging gastroenteritis pathogen. *Microbes Infect.*, 14, 1, 26–34. DOI: 10.1016/j.micinf.2011.08.005.
38. **Liu, H., Wang, Y., Cao, J., Jiang, H., Yao, J., Gong, G., et al., 2020:** Antimicrobial activity and virulence attenuation of citral against the fish pathogen *Vibrio alginolyticus*. *Aquaculture*, 515, 734578. DOI: 10.1016/j.aquaculture.2019.734578.
39. **Lulijwa, R., Rupia, E. J., Alfaro, A. C., 2020:** Antibiotic use in aquaculture, policies and regulation, health and environmental risks: A review of the top 15 major producers. *Rev. Aquac.*, 12, 2, 640–663. DOI: 10.1111/raq.12344.
40. **Siva, M., Shanmugam, K. R., Shanmugam, B., Venkata, S. G., Ravi, S., Sathyavelu, R. K., et al., 2016:** *Ocimum sanctum:* A review on the pharmacological properties. *Int. J. Basic Clin. Pharmacol.*, 5, 558–565. DOI: 10.18203/2319-2003.ijbcp20161491.
41. **Madetoja, J., Nystedt, S., Wiklund, T., 2003:** Survival and virulence of *Flavobacterium psychrophilum* in water microcosms. *FEMS Microbiol. Ecol.*, 43, 2, 217–223. DOI: 10.1111/j.1574-6941.2003.tb01061.x.
42. **Maiti, B., Shetty, M., Shekar, M., Karunasagar, I., Karunasagar, I., 2011:** Recombinant outer membrane protein A

(OmpA) of *Edwardsiella tarda*, a potential vaccine candidate for fish, common carp. *Microbiol. Res.*, 167, 1, 1–7. DOI: 10.1016/j.micres.2011.02.002.

43. **Menanteau-Ledouble, S., Kumar, G., Saleh, M., El-Matbouli, M., 2016:** *Aeromonas salmonicida*: updates on an old acquaintance. *Dis. Aquat. Org.*, 120, 1, 49–68. DOI: 10.3354/dao03006.
44. **Miniero Davies, Y., Xavier de Oliveira, M. G., Paulo Vieira Cunha, M., Soares Franco, L., Pulecio Santos, S. L., Zanolli Moreno, L., et al., 2018:** *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. *Vet. Q.*, 38, 1, 99–105. DOI: 10.1080/01652176.2018.1540070.
45. **Musa, N., Wei, L. S., Seng, C. T., Wee, W., Leong, L. K., 2008:** Potential of edible plants as remedies of systemic bacterial disease infection in cultured fish. *Glob. J. Pharmacol.*, 2, 2, 31–36.
46. **Mzula, A., Wambura, P. N., Mdegela, R. H., Shirima, G. M., 2019:** Current state of modern biotechnological-based *Aeromonas hydrophila* vaccines for aquaculture: A systematic review. *BioMed Res. Int.*, 2019. DOI: 10.1155/2019/3768948.
47. **Navarrete, P., Toledo, I., Mardones, P., Opazo, R., Espejo, R., Romero, J., 2010:** Effect of *Thymus vulgaris* essential oil on intestinal bacterial microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum) and bacterial isolates. *Aquac. Res.*, 41, 10, e667–e678. DOI: 10.1111/j.1365-2109.2010.02590.x.
48. **Ndomou, S. C. H., Mube, H. K., 2023:** The use of plants as phytobiotics: A new challenge. In *Phytochemicals in Agriculture and Food*, IntechOpen. DOI: 10.5772/intechopen.110731.
49. **Nguyen, L. T., Farcas, A. C., Socaci, S. A., Tofana, M., Diaconeasa, Z. M., Pop, O. L., Salanta, L. C., 2020:** An overview of saponins – a bioactive group. *Bull. UASVM Food Sci. Technol.*, 77, 1, 25–36. DOI: 10.15835/buasvmcn-fst: 2019.0036.
50. **Nya, E. J., Dawood, Z., Austin, B., 2010:** The garlic component, allicin, prevents disease caused by *Aeromonas hydrophila* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.*, 33, 4, 293–300. DOI: 10.1111/j.1365-2761.2009.01121.x.
51. **Okocha, R. C., Olatoye, I. O., Adedeji, O. B., 2018:** Food safety impacts of antimicrobial use and their residues in aquaculture. *Public Health Rev.*, 39, 1, 1–22. DOI: 10.1186/s40985-018-0099-2.
52. **Oladeji, O. S., Adelowo, F. E., Ayodele, D. T., Odelade, K. A., 2019:** Phytochemistry and pharmacological activities of *Cymbopogon citratus*: A review. *Sci. Afr.*, 6, e00137. DOI: 10.1016/j.sciaf.2019.e00137.
53. **Panche, A. N., Diwan, A. D., Chandra, S. R., 2016:** Flavonoids: An overview. *J. Nutr. Sci.*, 5, e47. DOI: 10.1017/jns.2016.41.
54. **Paquet, V. E., Vincent, A. T., Moineau, S., Charette, S. J., 2019:** Beyond the A-layer: adsorption of lipopolysaccharides and characterization of bacteriophage-insensitive mutants of *Aeromonas salmonicida* subsp. *salmonicida*. *Mol. Microbiol.*, 112, 2, 667–677. DOI: 10.1111/mmi.14308.
55. **Park, S. B., Aoki, T., Jung, T. S., 2012:** Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Vet. Res.*, 43, 1, 1–11. DOI: 10.1186/1297-9716-43-67.
56. **Pattanayak, P., Behera, P., Das, D., Panda, S. K., 2010:** *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: An overview. *Pharmacogn. Rev.*, 4, 7, 95. DOI: 10.4103/0973-7847.65323.
57. **Pepi, M., Focardi, S., 2021:** Antibiotic-resistant bacteria in aquaculture and climate change: A challenge for health in the Mediterranean area. *Int. J. Environ. Res. Public Health*, 18, 11, 5723. DOI: 10.3390/ijerph18115723.
58. **Pérez-Pascual, D., Rochat, T., Kerouault, B., Gómez, E., Neulat-Ripoll, F., Henry, C., et al., 2017:** More than gliding: involvement of GldD and GldG in the virulence of *Flavobacterium psychrophilum*. *Front. Microbiol.*, 8, 2168. DOI: 10.3389/fmicb.2017.02168.
59. **Pérez-Sánchez, T., Mora-Sánchez, B., Balcázar, J. L., 2018:** Biological approaches for disease control in aquaculture: Advantages, limitations and challenges. *Trends Microbiol.*, 26, 11, 896-903. DOI: 10.1016/j.tim.2018.05.002.
60. **Preena, P. G., Swaminathan, T. R., Kumar, V. J. R., Singh, I. S. B., 2020:** Antimicrobial resistance in aquaculture: A crisis for concern. *Biologia*, 75, 9, 1497–1517. DOI: 10.2478/s11756-020-00456-4.
61. **Rashidian, G., Boldaji, J. T., Rainis, S., Prokić, M. D., Faggio, C., 2021:** Oregano (*Origanum vulgare*) extract enhances zebrafish (*Danio rerio*) growth performance, serum and mucus innate immune responses and resistance against *Aeromonas hydrophila* challenge. *Animals*, 11, 2, 299. DOI: 10.3390/ani11020299.
62. **Rashmi, H. B., Negi, P. S., 2020:** Phenolic acids from vegetables: A review on processing stability and health benefits. *Food Res. Int.*, 136, 109298. DOI: 10.1016/j.foodres.2020.109298.
63. **Rasul, M. G., Majumdar, B. C., 2017:** Abuse of antibiotics in aquaculture and it's effects on human, aquatic animal and

environment. *Saudi J. Life Sci.*, 2, 3, 81–88. DOI: 10.21276/haya.

64. **Rattanachaikunsopon, P., Phumkhachorn, P., 2010:** Potential of cinnamon (*Cinnamomum verum*) oil to control *Streptococcus iniae* infection in tilapia (*Oreochromis niloticus*). *Fish. Sci.*, 76, 2, 287–293. DOI: 10.1007/s12562-010-0218-6.
65. **Reverter, M., Sarter, S., Caruso, D., Avarre, J. C., Combe, M., Pepey, E., et al., 2020:** Aquaculture at the crossroads of global warming and antimicrobial resistance. *Nat. Commun.*, 11, 1, 1–8. DOI: 10.1038/s41467-020-15735-6.
66. **Roy, A., 2017:** A review on the alkaloids an important therapeutic compound from plants. *IJPB*, 3, 2, 1–9.
67. **Santos, L., Ramos, F., 2018:** Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. *Int. J. Antimicrob. Agents*, 52, 2, 135–143. DOI: 10.1016/j.ijantimicag.2018.03.010.
68. **Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., et al., 2008:** Aquaculture practices and potential human health risks: Current knowledge and future priorities. *Environ. Int.*, 34, 8, 1215–1226. DOI: 10.1016/j.envint.2008.04.009.
69. **Saravanan, K., Sivaramakrishnan, T., Praveenraj, J., Kiruba-Sankar, R., Suma, D., Devi, V., et al., 2019:** Phytochemical, antioxidant and antibacterial activity of *Chromolaena odorata* from Andaman Islands, India. *Andaman Sci. Assoc.*, 24, 2, 123–130.
70. **Sargenti, M., Bartolacci, S., Luciani, A., Di Biagio, K., Baldini, M., Galarini, R., et al., 2020:** Investigation of the correlation between the use of antibiotics in aquaculture systems and their detection in aquatic environments: A case study of the Nera river aquafarms in Italy. *Sustainability*, 12, 12, 5176. DOI: 10.3390/su12125176.
71. **Schar, D., Klein, E. Y., Laxminarayan, R., Gilbert, M., Van Boeckel, T. P., 2020:** Global trends in antimicrobial use in aquaculture. *Sci. Rep.*, 10, 1, 1–9. DOI: 10.1038/s41598-020-78849-3.
72. **Odeyemi, O. A., Asmat, A., Usup, G., 2012:** Antibiotics resistance and putative virulence factors of *Aeromonas hydrophila* isolated from estuary. *J. Microbiol. Biotechnol. Food Sci.*, 1, 6, 1339–1357.
73. **Shang, A., Cao, S. Y., Xu, X. Y., Gan, R. Y., Tang, G. Y., Corke, H., et al., 2019:** Bioactive compounds and biological functions of garlic (*Allium sativum* L.). *Foods*, 8, 7, 246. DOI: 10.3390/foods8070246.
74. **Sheng, L., Wang, L., 2021:** The microbial safety of fish and fish products: Recent advances in understanding its significance, contamination sources, and control strategies. *Compr. Rev. Food Sci. Food Saf.*, 20, 1, 738–786. DOI: 10.1111/1541-4337.12671.
75. **Spyridopoulou, K., Fitsiou, E., Bouloukosta, E., Tiptiri-Kourpeti, A., Vamvakias, M., Oreopoulou, A., et al., 2019:** Extraction, chemical composition, and anticancer potential of *Origanum onites* L. essential oil. *Molecules*, 24, 14, 2612. DOI: 10.3390/molecules24142612.
76. **Starliper, C. E., 2011:** Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *J. Adv. Res.*, 2, 2, 97–108. DOI: 10.1016/j.jare.2010.04.001.
77. **Talpur, A. D., Ikhwanuddin, M., Bolong, A. M. A., 2013:** Nutritional effects of ginger (*Zingiber officinale* Roscoe) on immune response of Asian sea bass, *Lates calcarifer* (Bloch) and disease resistance against *Vibrio harveyi*. *Aquaculture*, 400, 46–52. DOI: 10.1016/j.aquaculture.2013.02.043.
78. **Tepe, B., Cakir, A., Sihoglu Tepe, A., 2016:** Medicinal uses, phytochemistry, and pharmacology of *Origanum onites* (L.): A review. *Chem. Biodivers*., 13, 5, 504–520. DOI: 10.1002/cbdv.201500069.
79. **Thilsted, S. H., James, D., Toppe, J., Subasinghe, R., Karunasagar, I., 2014:** Maximizing the contribution of fish to human nutrition. In *ICN2 Second International Confrence on Nutrition: Better Nutrition Better Lives*, Rome, Italy, November 19–21, 1–16.
80. **Thurlow, C. M., Hossain, M. J., Sun, D., Barger, P., Foshee, L., Beck, B. H., et al., 2019:** The gfc operon is involved in the formation of the O antigen capsule in *Aeromonas hydrophila* and contributes to virulence in channel catfish. *Aquaculture*, 512, 734334. DOI: 10.1016/j.aquaculture.2019.734334.
81. **Tobback, E., Decostere, A., Hermans, K., Haesebrouck, F., Chiers, K., 2007:** *Yersinia ruckeri* infections in salmonid fish. *J. Fish Dis.*, 30, 5, 257–268. DOI: 10.1111/j.1365-2761.2007.00816.x.
82. **Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W., 2010:** Lignin biosynthesis and structure. *Plant Physiol.*, 153, 3, 895–905. DOI: 10.1104/pp.110.155119.
83. **Wang, Y., Zhang, X. H., Austin, B., 2010:** Comparative analysis of the phenotypic characteristics of high-and low-virulent strains of *Edwardsiella tarda*. *J. Fish Dis.*, 33, 12, 985–994. DOI: 10.1111/j.1365-2761.2010.01204.x.
84. **Weerasekera, A. C., Samarasinghe, K., de Zoysa, H. K. S., Bamunuarachchige, T. C., Waisundara, V. Y., 2021:** *Cinnamomum zeylanicum*: Morphology, antioxidant prop-

erties and bioactive compounds. In *Antioxidants-Benefits, Sources, Mechanisms of Action*. IntechOpen, 407–420.

85. **World Health Organization, 2019:** *New Report Calls for Urgent Action to Avert Antimicrobial Resistance Crisis*. Updated April 29, 2019. Accessed April 29, 2019. https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis.
86. **Wrobel, A., Leo, J. C., Linke, D., 2019:** Overcoming fish defences: The virulence factors of *Yersinia ruckeri*. *Genes*, 10, 9, 700. DOI: 10.3390/genes10090700.
87. **Xu, T., Zhang, X. H., 2014:** *Edwardsiella tarda*: An intriguing problem in aquaculture. *Aquaculture*, 431, 129–135. DOI: 10.1016/j.aquaculture.2013.12.001.
88. **Zhang, X., Chen, F., Wang, M., 2015:** Bioactive substances of animal origin. In **Cheung, P. C. K., Mehta, M. B.:** *Handbook of Food Chemistry*. Springer, Berlin, 1009–1033.
89. **Zhao, Y., Wu, Y., Wang, M., 2015:** Bioactive substances of plant origin. In **Cheung, P. C. K., Mehta, M. B.:** *Handbook of Food Chemistry*. Springer, Berlin, 967–1008.

Received March 24, 2023
Accepted May 22, 2023

sciendo

DOI: 10.2478/fv-2023-0018

FOLIA VETERINARIA, 67, 2: 62—77, 2023

HERD-LEVEL RISK FACTORS FOR LAMENESS, LEG INJURIES, THIN BODY CONDITION AND MASTITIS ON ALGERIAN DAIRY FARMS

Kechroud A. Abdelouahed[1], Merdaci Latifa[1], Miroud Kamel[1], Aoun Leila[1], Gherissi D. Eddine[2]
[1]Laboratory of Epidemio-Surveillance, Production and Reproduction, Health, Cellular Experimentation and Therapy of Domestic and Wild Animals, Department of Veterinary Sciences, Chadli Bendjedid University, El-Tarf
[2]Institute of Agronomic and Veterinary Sciences, Mohamed Cherif Messaadia University, Souk-Ahras
Algeria

ahmedalgerie41@hotmail.fr

ABSTRACT

This present study aims to investigate the relationship of herd characteristics and management practices with the prevalence of clinical and severe lameness, hock and knee injuries, thin cows, and mastitis at the herd level on Algerian dairy farms. Altogether 1210 dairy cows from 107 farms were examined and the clinical aspects related to studied affections were recorded. Multivariable regression models were built to analyse the relationship between the risk factors and the occurrence of health indicators. Overlay, the health outcomes were multifactorial, with pasture access ($P<0.001$), scraping frequency ($P<0.01$), and floor regularity ($P=0.05$) as the main factors causing lameness. Factors associated with both hock and knee injuries (score≥2) were lower cow number ($P<0.01$), more days spent on pasture ($P<0.001$), and poorer straw amount for bedding ($P<0.001$). Some farming practice, in particular, providing proper amount of concentrate feeds ($P<0.001$), higher scraping frequency ($P<0.01$), thicker bedding ($P<0.001$), and having younger dairy cows ($P=0.058$), were associated with a lower within-herd prevalence of thin cows. While, more days on pasture increased the percentage of under-conditioned cows ($P<0.01$). The inadequate hygienic conditions of the floor ($P<0.001$), improper milking procedures ($P<0.05$), and shorter dry period ($P<0.05$) increased the clinical mastitis occurrence. Our results highlighted the specific management practices responsible for increasing health risks and provided useful information for the farmers and veterinarians to make preventive and controlling strategies for lameness, leg injuries, low body condition and mastitis on dairy farms.

Key words: Algeria; dairy cow; hock lesions; lameness; management practice; mastitis

INTRODUCTION

Animal health is one of the daily concerns of breeders. It has a direct impact on animal welfare, the working conditions of the farmers, and the technical and economic performance of farms. Mastitis and lameness are, respectively, in the first and third positions of the most important diseases, reproductive disorders come in second position [17]. They account for nearly 37% of the dairy unit's health expenditure [17]. They are considered as the most important livestock related diseases with essential impact on the dairy farm's performance: a drop in productivity, early culling, discarding milk during processing, and increasing open days and low fertility, increasing veterinary costs, negative repercussions on working time and work-

ing conditions, a shortfall in the price of milk; therefore, they represent half of the economic impact generated by herd diseases [13, 21, 22]. Moreover, these diseases appear as priority targets to reduce the use of antibiotics in dairy herds. The analysis of the incidence of the predisposing factors associated with these diseases; such as temporal, genetic, breeding practices and behavioural factors represents an important point of descriptive epidemiology of these diseases and they have been the subject of several recent studies [10, 34]. In addition, the dairy cattle body condition has significant implications on the health and performance of dairy cows. In contrast, inappropriate body condition score (BCS) has been associated with lameness [24], hypocalcaemia [38], low milk production, and fertility [39, 40], whereas, negative energy balance is mainly due to nutrition deficiency. Guaranteeing farm performance and animal welfare depends on better prevention of these diseases. There are many risk factors, but the living environment of dairy cows is a central element.

In the literature, vast information is available about the main health disorders affecting dairy cows. However, it remains difficult for a farmer to control overall health in his herd because controlling one disease can exacerbate the situation with another. As a result, a global assessment of the health status is the best strategy for improving animal welfare, taking into account the major disorders.

This present study was conducted to investigate the relationship of herd characteristics and management practices with the prevalence of clinical and severe lameness, hock and knee injuries, thin cows, and mastitis on dairy farms in eastern Algeria.

MATERIALS AND METHODS

Farm selection and description

This study was conducted on 107 Algerian dairy farms from Souk Ahras region, one of the best ranked dairy-producing region in Algeria. The farms were visited once by the same assessor from October 2020 through March 2021.

Two predominate types of climates in the study area: sub-humid in the north with an average annual precipitation of 700 mm and semi-arid in the south with a precipitation of 250 mm per year. Montbeliard cattle dominate the studied farms, with a mean herd size of 22 ± 13 (min = 7, max = 91). The cows were mainly kept in a tied housing system with a concrete floor, and 90.1 % of farms used straw for bedding as the amount was 0.3–3.8 kg per cow every day after removing the contaminated one. Dry cows were housed under the same conditions. Cows were milked two times a day. The average milk production was 15.6 litres per cow per day, and most farms used the resting pen for milking purposes. Cows had 220 days of pasture access on average per year. Besides, they received green or hay fodder and were supplemented with a varied amount of commercial concentrate and a vitamin-mineral mixture, with a feeding frequency of 2–3 times a day and a feeding interval of 7.6 h.

Dairy herd-related risk factors

Direct observation and interview with the farm manager were carried out to collect information regarding herd characteristics, feeding practices, prevention against disease, milking procedures, and condition of the floor and livestock areas (Tables 1 and 2).

Herd characteristics

Milking herd size was obtained by interview with the farm manager. Information regarding lactation period length, dry period length and herd average parity were collected from the data available in the records and through interview with the farmer.

Feeding practices

Concentrate feed, pasture access, feeding method, and feeding frequency were collected through interview with the farm manager. Feeding method was characterised as balanced ration when the cows received calculated feed ration based on stage and number of lactation, estimated ration when dry cows received a reduced amount of concentrate without calculation, or standard ration when all animals fed the same ration regardless of their physiological stages and their production.

Condition of the floor and livestock areas

Litter provision and cleaning frequency were collected through interview with the farm manager. Lying down and rising, floor regularity, floor slipperiness, light intensity, air quality, and sharp objects and obstacles were noted after direct observation. Cattle on each farm were observed when lying down and rising of their own volition; their behaviour was judged as unrestricted, mildly restricted or

Table 1. Descriptive statistics of quantitative risk factors involved in the studied dairy farms (n = 107)

Variable	Mean ± SD	Median (Q1–Q2)
Herd characteristics		
Herd size (HS; heads)	13 ± 9	11 (8–15)
Lactation period length (LPL; days)	319.2 ± 35	314.8 (292.4–334.6)
Dry period length (DPL; days)	59.6 ± 18.9	60.8 (45.6–76)
Herd average parity (HAP; lactations' number)	3.2 ± 1.2	3.1 (2.4–3.8)
Feeding practices		
Concentrate feed (CF; $kg.cow^{-1}.day^{-1}$)	6.8 ± 2.3	7 (5–9)
Pasture access (PA; days)	226.6 ± 59.3	220 (180–289)
Condition of the floor and livestock areas		
Litter provision (LP; $kg.cow^{-1}.day^{-1}$)	1.6 ± 0.9	1.6 (1–2.2)

Table 2. Descriptive statistics of qualitative breeding practice related risk factors involved in the studied dairy farms (n = 07)

Variable	Category
Feeding practices	
Feeding method (FM)	Estimated ration; Standard ration; Balanced ration
Feeding frequency (FF; $time.day^{-1}$)	2; 3
Prevention against disease	
Main calving location (MCL)	Isolated pen; Pasture; In barn with herd
Checking uterus involution(CUI)	No; Occasional; Systematic
Frequency of hoof trims (FHT)	Never; If necessary
Hoof trimming personnel (HTP)	None; Dairy personnel; Professional
Vaccination (V)	No; Yes
Deworming (D)	No; Yes
Milking procedures	
Teat preparation before milking (TPBM)	Wet towel; Washing; Pre-dipping
Teat drying (TD)	No; Collective towel; Individual towel
Subclinical mastitis screening (SMD)	No; Yes
Condition of the floor and livestock areas	
Cleaning frequency (CFr; $time.day^{-1}$)	1; 2; > 2
Lying down and rising (LDR)	Restricted; Mildly restricted; Unrestricted
Floor regularity (FR)	Uneven; Plane
Floor slipperiness (FS)	Not slippery; Slippery
Light intensity (LI)	Weak; Sufficient; Good
Air quality and air flow (AQAF)	Bad; Medium; Good air quality
Sharp objects and obstacles (SOO)	No; Yes

restricted depending on the ease with which animal can lie down and rise from a lying position. Light intensity was evaluated as weak, sufficient or good based on the luminosity in the facility. Moreover, air quality ranges from good to bad depends on the accumulation of gaseous effluents for example, ammonia and hydrogen sulphide.

Prevention against disease

Information regarding the main calving location, checking uterus involution, frequency of hoof trims, hoof trimming personnel, vaccination against diseases (FMD, rabies, anthrax) and deworming (anthelmintics) were recorded following interview with the farmer.

Milking procedure

The method of teat preparation before milking (humid towel, washing, pre dipping), whether teat drying with collective towel, individual towel or not practiced, and if there was any subclinical mastitis screening were all recorded through interview with the farmer.

Evaluation of cows

A total of 1210 cows were examined on 107 farms, of which 76% were lactating and 24% dry cows. On farms with more than 20 cows, at least 15 cows were selected at random, in farms with 13–20 cows, at least 10 cows were examined, and in farms with 12 or fewer cows all were selected.

The cows were evaluated for lameness, hock and knee injuries, body condition score (BCS) and clinical mastitis. The locomotion score proposed by Thomsen et al. [47] was noted on a 5-point scale with clinical lameness scores$\geq$3, and severe lameness scores$\geq$4. Hock and knee injuries were scored using a 4-point scale based on the tarsal and carpal joints condition [18]. Then, a cut-off score ($\geq$2) was used to estimate the prevalence of cows with injuries within and across herds. BCS was measured to all observed cows according to the system developed by Vasseur et al. [49]. The scale ranges from 1 to 5 for very thin and very fat cows, respectively. The percentage of cows with BCS$\leq$2 was recorded as a cut-off score representing a thin cow. The clinical mastitis included three levels: severe, moderate and mild [36]. The three levels were evaluated according to the general health condition (fever, anorexia, or lethargy), signs of udder inflammation (swelling, heat, or hardness) and abnormal appearance of milk (watery, clots, or pus), respectively. All these cases were considered as clinical mastitis and were included in the calculation of the prevalence of this health issue per herd.

Statistical analysis

All statistical analyses were performed by IBM SPSS version 26.0. Descriptive statistics were presented as means, standard deviation (SD) and medians with interquartile range (IQR; 1st–3rd quartiles) for quantitative parameters and frequency and percentages for qualitative parameters. The farm was considered a sampling unit, with the outcomes of interest being the percentage of clinically lame cows ($3\leq$score<4), severely lame cows ($\geq$4), percentage of cows with hock injuries ($\geq$2), percentage of cows with knee injuries ($\geq$2), percentage of cows that were thin (BCS$\leq$2), and percentage of clinical mastitis. Variables with less than 5% per category were not considered for analysis. Multivariable analyses included six linear regression models that were constructed to evaluate influence of housing and management factors on health parameters. First, each predictor was screened by univariable nonparametric analysis. Because several numerical health outcomes were not distributed normally according to the Shapiro-Wilk test, the Spearman test was used in the case of quantitative independent variables and the Kruskal-Wallis and Mann-Whitney tests in the case of categorical variables. Factors with $p<0.2$ in the screening were included in subsequent modelling.

Independent variables were also tested for multicollinearity. If two predictors were highly correlated ($r>0.65$), the one with the strongest correlation with the dependent variable in the univariable analysis was selected. Then, a multivariable linear regression model was constructed for each outcome using Stepwise backward procedures. In the final models, only factors with a P-value<0.1 were chosen. The normality distributions of the residuals were examined by the Kolmogorov-Smirnov test ($P=0.2$).

RESULTS

Fig. 1 shows the mean proportion of affected animals by thin body condition score, lameness, hock and knees injuries and clinical mastitis within the 107 studied dairy herds. The results of the present survey allow us to rank the diseases from the most to the least incident as follows:

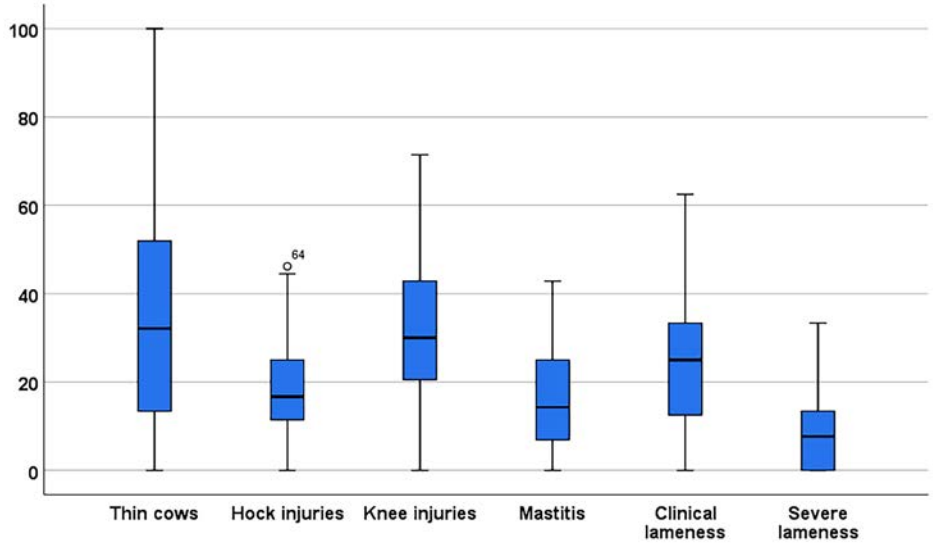

Fig. 1. Box-plots of the health outcomes in 107 dairy cattle herds from the eastern Algeria

Table 3. Results of univariable analysis for preselection of quantitative predictors in the multivariable regression models

Factors	P-value					
	Thin cows	Hock injuries	Knee injuries	Clinical mastitis	Clinical lameness	Severe lameness
Herd characteristics						
HS	0.493	< 0.001*	< 0.001*	0.107*	0.003*	0.059*
LPL	0.002*	–	–	0.010*	–	–
DPL	< 0.001*	–	–	< 0.001*	–	–
HAP	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Feeding practices						
CF	< 0.001*	< 0.001*	0.001*	0.001*	0.008*	0.002*
PA	0.006*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Condition of the floor and livestock areas						
LP	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

HS – Herd size (heads); LPL – Lactation period length (days); DPL – dry period length (days); HAP – herd average parity (lactations' number); CF – Concentrate feed ($kg.cow^{-1}.day^{-1}$); PA – Pasture access (days); LP – Litter provision ($kg.cow^{-1}.day^{-1}$)
* – Factors included in subsequent multivariable analysis (P < 0.2).

body condition (leanness), knee injuries, clinical lameness, hock injuries, clinical mastitis, and severe lameness. The median of thin cows (BCS ≤ 2) was 32.1 % (Q1 = 13.4, Q2 = 51.9). The overall median proportion of lame cows was 25 % (Q1 = 12.5, Q2 = 33.3), of which 7.7 % (Q1 = 0, Q2 = 13.4) had severe lameness. The median prevalence of hock injuries was 16.7 % (Q1 = 11.4, Q2 = 25), and of knee injuries was 30 % (Q1 = 20.5, Q2 = 42.9). The median prevalence of clinical mastitis was 14.3 % (Q1 = 6.9, Q2 = 25).

Tables 3–6 present univariable analysis for preselection of quantitative and qualitative risk factors in the multiple regression analysis.

Table 4. Results of univariable analysis for preselection of qualitative predictors regarding feeding practices and milking procedures in the multivariable regression models

Risk factor	P-value					
	Thin cows	Hock injuries	Knee injuries	Clinical mastitis	Clinical lameness	Severe lameness
Feeding method						
Estimated ration Standard ration Balanced ration	0.003*	–	–	–	–	–
Feeding frequency						
2 3	< 0.001*	0.704	0.365	0.317	0.375	0.581
Teat preparation before milking						
Wet towel Washing Pre-dipping	–	–	–	< 0.001*	–	–
Teat drying						
No Collective towel Individual towel	–	–	–	0.001*	–	–
Subclinical mastitis screening						
No Yes	–	–	–	< 0.001*	–	–

* – Factors included in subsequent multivariable analysis (P < 0.2).

Table 5. Results of univariable analysis for preselection of qualitative predictors regarding prevention against disease in the multivariable regression models

Risk factor	P-value					
	Thin cows	Hock injuries	Knee injuries	Clinical mastitis	Clinical lameness	Severe lameness
Main calving location						
In a calving pen At pasture In barn with herd	< 0.001*	0.020*	0.012*	0.002*	0.014*	0.009*
Checking uterus involution						
No Occasional Systematic	< 0.001*	–	–	–	–	–
Frequency of hoof trims						
Never If necessary	–	–	–	–	0.763	0.671
Hoof trimming personnel						
None Dairy personnel Professional	–	–	–	–	0.306	0.198*
Vaccination						
No Yes	0.044*	–	–	–	–	–
Deworming						
No Yes	0.937	–	–	–	–	–

* – Factors included in subsequent multivariable analysis (P < 0.2).

Table 6. Results of univariable analysis for preselection of qualitative predictors regarding condition of the floor and livestock areas in the multivariable regression models

Risk factor	P-value					
	Thin cows	Hock injuries	Knee injuries	Clinical mastitis	Clinical lameness	Severe lameness
Cleaning frequency						
1 2 >2	< 0.001*	0.009*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Lying down and rising						
Restricted mildly restricted unrestricted	0.038*	0.006*	0.036*	0.007*	0.044*	0.039*
Floor regularity						
Uneven Plane	–	0.015*	0.063*	< 0.001*	0.043*	0.064*
Floor slipperiness						
Not slippery Slippery	–	< 0.001*	< 0.001*	–	0.018*	0.002*
Light intensity						
Weak Sufficient Good	–	0.001*	0.01*	–	0.016*	0.015*
Air quality and air flow–						
Bad Medium Good air quality	< 0.001*	< 0.001*	0.011*	< 0.001*	< 0.001*	< 0.001*
Sharp objects and obstacles						
No Yes	–	< 0.001*	0.004*	< 0.001*	< 0.001*	< 0.001*

* – Factors included in subsequent multivariable analysis (P < 0.2).

Lameness models

Among the 16 variables tested during the univariable step, 15 were retained in the multivariable model. Finally, 8 variables were associated with lameness (Table 7). Other variables that were not meet the criteria but considered for inclusion in the model included frequency of hoof trims and hoof trimming personnel. Furthermore, herd-level factors associated with clinical lameness were lesser days of access to pasture (P<0.001), lower frequency of floor cleaning (P<0.01), lower daily liter quantity (P=0.017), and concentrate feeds (P=0.062). Hoof trimming performed by dairy personal was associated with more occurrence of lameness compared with that performed by professionals (P=0.07). In addition, dairy farms with poor air quality (P<0.001),

Table 7. Results of multivariable linear regression model regarding percentage of clinical lameness (R2 = 0.657, R2 adjusted = 0.625, F = 20.635; n = 107)

Model	Estimate	SE	t	P-value
(Constant)	55.489	8.420	6.590	< 0.001
PA	–0.128	0.019	–6.679	< 0.001*
LP	–3.006	1.234	–2.435	0.017*
CF	–1.037	0.549	–1.889	0.062*
HTP (reference: Professional)				
Dairy personnel	3.846	2.097	1.834	0.070*
CFr (reference: >2)				
1	8.979	2.943	3.051	0.003*
AQAF (reference: Good)				
Bad	12.055	3.010	4.004	< 0.001*
Medium	5.015	2.379	2.109	0.038*
SOO (reference: None)				
Yes	5.351	2.666	2.008	0.047*
FR (reference: Plane)				
Uneven	5.204	2.627	1.981	0.050*

PA – Pasture access (days); LP – Litter provision (kg.cow^{-1}.day^{-1}); CF – Concentrate feed (kg.cow^{-1}.day^{-1});
HTP – Hoof trimming personnelCFr – Cleaning frequency; AQAF – Air quality and air flow; SOO – Sharp objects and obstacles; FR – Floor regularity
*– Herd-level factors significant at P < 0.1 that were retained in the final model

Table 8. Results of final multivariabl e linear regression model regarding percentage of severe lameness (R2 = 0.655, R2 adjusted = 0.619, F = 18.245; n = 107)

Model	Estimate	SE	t	P-value
(Constant)	9.882	6.156	1.605	0.112
PA	–0.041	0.012	–3.323	< 0.001*
HAP	1.587	0.599	2.648	0.009*
CF	–0.580	0.333	–1.739	0.085*
LP	–2.541	0.740	–3.435	< 0.001*
MCL (reference: Isolated pen)				
In barn with herd	2.579	1.180	2.186	0.031*
FR (reference: Plane)				
Uneven	3.302	1.561	2.116	0.037*
CFr (reference: >2)				
1	3.197	1.804	1.773	0.079*
AQAF (reference: Good)				
Bad	2.878	1.499	1.920	0.058*
HTP (reference: Professional)				
Dairy personnel	2.635	1.263	2.086	0.040*
SOO (reference: None)				
Yes	6.710	1.601	4.190	< 0.001*

PA – Pasture access (days); HAP – Herd average parity (lactations' number); CF – Concentrate feed (kg.cow^{-1}.day^{-1}); LP – Litter provision (kg.cow^{-1}.day^{-1});
MCL – Main calving location; FR – Floor regularity; CFr – Cleaning frequency; AQAF – Air quality and air flow; HTP – Hoof trimming personnel; SOO – Sharp objects and obstacles
*– Herd-level factors significant at P < 0.1 that were retained in the final model.

uneven floor (P=0.05), and sharp objects and obstacles (P=0.047) were more likely to have a higher prevalence of severe lameness. From the 16 variables that met criteria in the univariable screening for severe lameness, 10 were retained in the final model (Table 8). Other variable that did not meet the criteria but was considered for inclusion in the model included frequency of hoof trims. Dairies with high average parity (P<0.01), low access pasture (P<0.01), amount of concentrate feeds (P=0.085) and litter provision (P<0.001), uneven floor (P=0.037), improper scraping frequency (P=0.079), main calving location in barn with herd (P=0.031), poor air quality (P=0.058), hoof trimming by dairy personnel (P=0.040), and sharp objects and obstacles (P<0.001) were more likely to have a higher prevalence of severe lameness.

Hock and knee injury models

Fourteen variables were screened by univariable analysis for the prevalence of hock injuries, with 6 being retained in the final multivariable model (Table 9). Hock injury was positively affected by parity (P<0.001) and weak (P=0.078) or medium light intensity in the cowshed (P<0.032), however was negatively affected by herd size (P<0.01), litter provision (P<0.001), pasture access (P<0.001), and concentrates amount (P=0.017). Among the 14 variables introduced in the multivariable model for knee injuries, 7 were introduced in the final model (Table 10). Knee injuries were positively associated with parity (P=0.06), restricted lying down/rising movements (P=0.091), bad air quality (P=0.076), and scraping frequency (P=0.041) and were negatively associated with herd size (P<0.001), litter provision (P<0.001), and pasture access (P<0.001).

Thin cow models

A total of 16 explanatory variables were retained in the multivariable model of the prevalence of thin cows, of which seven were found to be associated with the outcome (Table 11). The prevalence of thin cows was greater in farms with older cows (P=0.058), more pasture access (P<0.01), longer lactation period (P<0.01), lower concentrates amount (P<0.001), litter provision (P<0.001), and scraping frequency (P<0.01). Moreover, cows that received a balanced ration had lesser occurrence of being thin than others received estimated (P<0.01) or standard ration (P<0.01).

Mastitis models

Six factors showed the significance of the risk factor for mastitis likelihood where the percentage of mastitis increased with fewer days of access to pasture (P<0.001), lesser dry period (P=0.019), and scraping frequency (P<0.001). Other factors that showed the significance of the risk factor for mastitis occurrence were washing teat (P<0.01) or using humid towel (P<0.001) before milking compared with pre dipping and teat drying with a collective towel (P<0.016) in comparison with individual towel (Table 12).

Table 9. Results of Final multivariable linear regression model regarding percentage of hock injuries (R2 = 0.671, R2 adjusted = 0.648, F = 28.816; n = 107)

Model	Estimate	SE	t	P-value
(Constant)	42.821	5.594	7.655	< 0.001
HS	–0.208	0.073	–2.867	0.005*
HAP	2.338	0.648	3.610	< 0.001*
PA	–0.085	0.012	–7.201	< 0.001*
CF	–0.876	0.360	–2.435	0.017*
LP	–3.015	0.800	–3.770	< 0.001*
LI (reference: Good)				
Weak	3.819	2.142	1.783	0.078*
Sufficient	3.177	1.457	2.181	0.032*

HS – Herd size (heads); HAP – Herd average parity (lactations' number); PA – Pasture access (days); CF – Concentrate feed (kg.cow^{-1}.day^{-1}); LP – Litter provision (kg.cow^{-1}.day^{-1}); LI – Light intensity
*– Herd-level factors significant at P < 0.1 that were retained in the final model

Table 10. Results of final multivariable linear regression model regarding percentage of knees injuries (R2 = 0.621, R2 adjusted = 0.594, F = 23.162; n = 10

Model	Estimate	SE	t	P-value
(Constant)	64.465	6.437	10.014	< 0.001
HS	–0.462	0.119	–3.878	< 0.001*
PA	–0.101	0.017	–5.832	< 0.001*
HAP	1.934	1.018	1.900	0.060*
CF	-7.557	1.177	-6.420	< 0.001*
LDR (reference: unrestricted)				
Restricted	4.459	2.615	1.705	0.091*
CFr (reference: >2)				
1	6.408	3.087	2.076	0.041*
AQAF (reference: Good)				
Bad	3.811	2.123	1.795	0.076*

HS – Herd size (heads); PA – Pasture access (days); HAP – Herd average parity (lactations' number);
CF – Concentrate feed (kg.cow^{-1}.day^{-1}); LDR – Lying down and rising; CFr – Cleaning frequency; AQAF – Air quality and air flow.
*Herd-level factors significant at P < 0.1 that were retained in the final model

Table 11. Results of final multivariable linear regression model regarding percentage of thin cows (R2 = 0.706, R2 adjusted = 0.682, F = 29.184; n = 107)

Model	Estimate	SE	t	P-value
(Constant)	44.330	19.190	2.310	0.023
LPL	0.118	0.044	2.669	0.009*
PA	0.076	0.028	2.699	0.008*
HAP	2.954	1.538	1.920	0.058*
CF	–6.574	0.853	–7.706	< 0.001*
LP	–7.444	1.814	–4.103	< 0.001*
FM (reference: Balanced ration)				
Estimated ration	–18.895	6.825	–2.769	0.007*
Standard ration	–23.757	7.754	–3.064	0.003*
CFr (reference: >2)				
1	12.506	4.458	2.805	0.006*

LPL – Lactation period length (days); PA – Pasture access (days); HAP – Herd average parity (lactations' number);
CF – Concentrate feed (kg.cow^{-1}.day^{-1}); LP – Litter provision (kg.cow^{-1}.day^{-1}); FM – Feeding method; CFr – Cleaning frequency
* – Herd-level factors significant at P < 0.1 that were retained in the final model

Table 12. Results of final multivariable linear regression model regarding percentage of mastitis (R2 = 0.58, R2 adjusted = 0.546, F = 16.936; n = 107)

Model	Estimate	SE	t	P-value
(Constant)	26.533	4.759	5.575	< 0.001
DPL	-0.105	0.044	-2.378	0.019*
PA	-0.090	0.014	-6.390	< 0.001*
TPBM (reference: Pre-dipping)				
Wet towel	10.051	2.464	4.080	< 0.001*
Washing	6.898	2.238	3.082	0.003*
TD (reference: Individual towel)				
Collective towel	4.921	2.000	2.461	0.016*
CFr (reference: >2)				
1	12.480	2.806	4.448	< 0.001*
2	7.328	1.942	3.773	< 0.001*
AQAF (reference: Good)				
Bad	4.059	1.872	2.168	0.033*

DPL – Dry period length (days); PA – Pasture access (days); TPBM – Teat preparation before milking; TD – Teat drying; CFr – Cleaning frequency; AQAF – Air quality and air flow
* – Herd-level factors significant at P < 0.1 that were retained in the final model

DISCUSSION

The current study was performed to explore potential associations between animal-based health indicators (lameness, BCS, legs injuries, and mastitis) and farm characteristics regarding herd, housing, and management practices.

Lameness

Lameness is widely reported as one of the important welfare impairments facing the dairy cow, with several housing characteristics and management practices influencing the prevalence of lameness. Pasture was negatively associated with the prevalence of clinical and severe lameness on Algerian dairy farms. The benefits of pasture for hoof health and mobility have been shown in several studies, as cows in pasture-based systems had less dirty hind limbs and demonstrated lower lameness compared to those in zero-grazing systems [1, 25]. Furthermore, the daily amount of litter was found to be negatively related to the prevalence of lameness. It must be thermally insulated from the ground, cushioned, and keep the bedding area dry to avoid excessive bacterial development [45, 51]. Factors such as poor bedding and abrasive floors reduced lying time [48], increased the risk of hoof horn lesions [20], and lameness in dairy cows [15]. Furthermore, a higher herd average parity was associated with severe lameness. Previous reports supported this result [12, 42] with a particular result from D i p p e l et al. [12], suggesting that heifers showed lesser lameness than older cows. As expected, less scraping frequency was a potential risk factor for lameness, which was confirmed by another study [28]. Floor dirtiness exposes legs to contamination by manure and promotes softening of hoofs and bacterial transmission [34, 37]. However, having a hygienic condition reduces the occurrence of digital infection and lameness [9, 37]. In contrast, the current study found that farms where hooves are trimmed by dairy personnel have more lame cows than farms where hooves are trimmed by professional. Proper hoof trimming prevents the appearance of overgrown hoof, improves even postures, and straightens the limbs [16, 43]. In addition, it is very important to check the floor condition in order to highlight any protruding and irregular parts that could injure the animal's hooves [16, 34].

Providing a lower amount of concentrates was associated with a higher prevalence of severe lameness. Moreover, nutrition deficiency increased the possibility of poor body condition in the studied cows. According to many authors, dropped BCS was a factor causing lameness [19, 29]. Leaner cows have lesser fat thickness of digital cushion, which is associated with reduced protective function as a shock absorber and therefore increased the hoof lesion and lameness [6, 35].

One of the strongest associations occurred with the calving location. A lower proportion of severely lame cows were on farms where cows calved in a separate pen compared with farms where cows calved in the main pen with the herd. According to OIE [51], calving areas should be thoroughly cleaned and covered with fresh bedding before each calving. Alternatively, we can assume that farmers who have better care for their cows use a calving pen when they are about to calve, and the association with the proportion of lame cows could come from these management practices. Among the risk indicators, good air quality decreased the percentage of lame cows. Ventilation serves the purpose of renewing the ambient air in order to evacuate the gases and humidity present in the building and to partially regulate the interior temperature, however, with poor ventilation, litters are always humid and often difficult to keep clean, which promotes the proliferation of pathogens on the bedding [33].

Hock and knee injuries

Herein, the prevalence of knee injuries was higher than hock injuries, which may be explained by the lying behavior of cows, where both knees are in contact with the floor at the same time, whereas cows have to put their hocks alternately on the floor, thus, decreasing the pressure on their tarsal joints. Common risk factors for both hock and knee lesions were herd size, access to pasture, amount of bedding, and herd average parity. Risk factors that differed for skin lesions on the hock and the knee were the amount of concentrates, light intensity, lying down/rising behaviours, scraping frequency, and air quality. Farms that allowed cow grazing had a lesser prevalence of hock lesions than farms with a zero grazing system [1]. During longer periods in housing, cows are more likely to come into contact with barn objects, therefore, they are in greater danger of injuries. According to Heyerhoff et al. [23] and Kester et al. [26], lack of bedding and abrasive surfaces are strongly related to hock and knee lesions, which indicate that thick bedding prevents cattle from serious integument damage. Furthermore, several authors showed that the prevalence of hock lesions increased with age and parity [27, 41], whereas others found no association [3].

In the literature, poor nutrition can lead to a deficit of minerals and other essential elements for tissue recovery, causing swelling and eventual necrosis [4]. On the other hand, thin cows have less fat and thin protective tissues, so they are at greater risk of wounds in the joint areas, especially while lying down on hard floors [29]. Cattle in housing which do not have adequate access to natural light must be provided with additional lighting that follows the natural periodicity and adequate for their well-being. Access to surrounding areas should be well lighted [51], because the walkways and alleys often contain foreign materials and sharp objects that may induce traumatic lesions [7]. Moreover, all cattle in a pen must have enough space to simultaneously lie down and rest [51]. Restricted space allowances in the resting areas and around the feed bunk possibly promote agonistic behaviours; therefore, these conditions promote slips and falls as a result of rapidly moving to avoid dominant cows. Also, the frequency of scraping has a strong impact on the cleanliness of alleys, stalls, and cows [31]. Meanwhile, a dirty environment weakens the protective effect of the skin by promoting its maceration and the development of bacteria.

Thin cows

We observed an increase in thin cow prevalence as the days in pasture increased. Mee and Boyle [32] observed that an insufficient grass allowance at pasture may be a risk for negative energy balance and weight loss. Throughout pasture season, cows may need additional nutrition indoors to balance their diet [14]. At the herd level, feeding deficiency increased the likelihood of poor body condition in the studied farms. DesRoches [11] observed that low values of BCS (<2.5) indicate that dietary supplies don't fulfil energy needs.

A lower amount of bedding was significantly linked to a higher proportion of cows with poor body condition. Tucker et al. [48] demonstrated that a lack of straw bedding and discomfort during rest reduced lying times, which may have an effect on BCS [5, 50]. Dairy farms that used balanced rations had fewer thin cows than those on which the farmer used estimated or standard rations

because balanced rations supply the proper amount and proportions of nutrients during different stages of lactation and gestation. OIE [51] reported that cattle must have access to a balanced feed ration, quantitatively and qualitatively adapted and in accordance with their physiological needs.

Mastitis

In the present study, mastitis in Algerian animals is believed to be associated with some of the herd characteristics and management practices, including the length of the dry period, number of days in pasture, teat preparation before milking, teat drying, scraping frequency, and ventilation. C o l l i e r et al. [8] reported that shortening or omitting the dry period possibly influence the mammary health and promote the occurrence of mastitis. Furthermore, intramammary infections are considerably increased during late gestation in continuously milked cows [2]. Interestingly, the mastitis percentage was lower in "younger herds," i.e. herds with a lower average parity. Aged or multiparous animals are more vulnerable to a variety of diseases, including mastitis [46]. Pasture access for cows was negatively associated with the prevalence of clinical mastitis. Lesser grazing has been linked with poor hygiene conditions in cows [25, 37], as well, S c h r e i n e r and R u e g g [44] recorded a significant association between udder dirtiness and the probability of intramammary infection. Our findings on the effect of scraping frequency on udder health back up previous findings that increased scraping frequency reduced mastitis pathogen contamination [30]. In the farms where teat preparation included pre-dipping, the prevalence of mastitis was lower when compared to the other farms using humid towels. Besides, teat drying with individual towels would reduce the percentage of mastitis compared to collective towels. Adequate sanitation of milking practices and proper attention to mammary gland hygiene are important measures that should be respected to prevent udder infection.

CONCLUSIONS

This study's findings highlight common factors for health outcomes in particular; parity, litter amount, and cleaning frequency were commonly associated with lameness, hock and knee injuries, thin cows, and mastitis in Algerian dairy farms. Furthermore, grazing reduced lameness, tarsal and carpal joint lesions, whereas increased pasture access had a negative impact on cow body condition. The hygienic conditions, in particular during milking, the floor condition and the livestock areas showed a significant impact on the occurrence of clinical mastitis. Also, the results of the study showed a lower percentage of cows with hock and knee joint injuries on the farms that used thicker straw bedding. Finally, these findings can be used to obtain improvements in dairy cattle health through improving husbandry and management practices and eliminating the potential sources of lameness, integument damage, and intramammary infections.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

REFERENCES

1. **Adams, A. E., Lombard, J. E., Fossler, C. P., Román-Muñiz, I. N., Kopral, C., 2017:** Associations between housing and management practices and the prevalence of lameness, hock lesions, and thin cows on US dairy operations. *J. Dairy Sci.*, 100, 2119–2136. DOI: 10.3168/jds.2016-11517.
2. **Annen, E. L., Stiening, C. M., Crooker, B. A., Fitzgerald, A. C., Collier, R. J., 2008:** Effect of continuous milking and prostaglandin E2 on milk production and mammary epithelial cell turnover, ultrastructure, and gene expression. *J. Dairy Sci.*, 86, 1132–1144.
3. **Barrientos Araneda, A. K., 2012:** *Hock Injuries in Freestall Housed Dairy Cows* (Doctoral dissertation, University of British Columbia), Accessed 19th October, 2021, on https://open.library.ubc.ca/media/download/pdf/24/1.0073480/1.
4. **Bass, M. J., Phillips, L. G., 2007:** Pressure sores. *Curr. Probl. Surg.*, 44, 101–143. DOI: 10.1067/j.cpsurg.2006.12.007.
5. **Bewley, J. M., Boyce, R. E., Hockin, J., Munksgaard, L. Eicher., S. D., Einstein, M. E., et al., 2010:** Influence of milk yield, stage of lactation, and body condition on dairy cattle lying behaviour measured using an automated activity monitoring sensor. *J. Dairy Res.*, 77, 1–6. DOI: 10.1017/s0022029909990227.
6. **Bicalho, R. C., Machado, V. S., Caixeta, L. S., 2009:** Lame-

ness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. *J. Dairy Sci.*, 92, 3175–3184. DOI: 10.3168/jds.2008-1827.

7. **Chesterson, R. N., 2015:** The lame game – Chile vs New Zealand – can we both be winners? In *18th International Symposium and 10th Conference on Lameness in Ruminants*. Valdivia, Chile, 26–29.
8. **Collier, R. J., Annen-Dawson, E. L., Pezeshki, A., 2012:** Effects of continuous lactation and short dry periods on mammary function and animal health. *Animal*, 6, 403–414. DOI: 10.1017/ S1751731111002461.
9. **Cook, N. B., Nordlund, K. V., 2009:** The influence of the environment on dairy cow behavior, claw health and herd lameness dynamics. *Vet. J.*, 179, 360–369. DOI: 10.1016/j. tvjl.2007.09.016.
10. **Daros, R. R., Weary, D. M., von Keyserlingk, M. A., 2022:** Invited review: Risk factors for transition period disease in intensive grazing and housed dairy cattle. *J. Dairy Sci.*, 105, 4734–4748. DOI: 10.3168/jds.2021-20649.
11. **Des Roches, A. D. B., Veissier, I., Boivin, X., Gilot-Fromont, E., Mounier, L., 2016:** A prospective exploration of farm, farmer, and animal characteristics in human-animal relationships: An epidemiological survey. *J. Dairy Sci.*, 99, 5573–5585. DOI: 10.3168/jds.2015-10633.
12. **Dippel, S., Dolezal, M., Brenninkmeyer, C., Brinkmann, J., March, S., Knierim, U., et al., 2009:** Risk factors for lameness in cubicle housed Austrian Simmental dairy cows. *Prev. Vet. Med.*, 90, 102–112. DOI: 10.1016/j.prevetmed. 2009.03.014.
13. **Dolecheck, K., Bewley, J., 2018:** Animal board invited review: Dairy cow lameness expenditures, losses and total cost. *Animal*, 12, 1462–1474. DOI: 10.1017/s1751731118000575.
14. **European Food Safety Authority (EFSA), 2009:** Scientific opinion of the panel on animal health and welfare on a request from European Commission on welfare of dairy cows. Effects of farming systems on dairy cow welfare and disease. *EFSA J.*, 1143, 1–38. DOI: 10.2903/j.efsa.2009.1143r.
15. **García-Munoz, A., Singh, N., Leonardi, C., Silva-del-Río, N., 2017:** Effect of hoof trimmer intervention in moderately lame cows on lameness progression and milk yield. *J. Dairy Sci.*, 100, 9205–9214. DOI: 10.3168/jds.2016-12449.
16. **Gervais, F., Bizeray-Filoche, D., Briand, P., Duvauchelle, A., Frennet, J. L., Leruste, H., et al., 2017:** *Impact des sols de circulation sur la santé des pieds des vaches: comment l'évaluer, le diagnostiquer et l'améliorer?* Institut de l'Élevage, Collection Synthèse, 48 pp. Accessed 16th April, 2022 on https://idele.fr/detail-article/impact-des-sols-de-circulation-sur-la-sante-des-pieds-de-vaches.
17. **Gervais, F., Capdeville, J., David, V., Duvauchelle-Wache, A., Gautier, J. M., Le Clainche, D., et al., 2018:** *Des vaches laitières en bonne santé: moins d'antibiotiques avec de bonnes pratiques d'élevage et des bâtiments adaptés.* Institut de l'Élevage, Collection Synthèse, 88 pp. Accessed 15th April, 2022 on https://idele.fr/detail-article/des-vaches-laitieres-en-bonne-sante.
18. **Gibbons, J., Vasseur, E., Rushen, J., de Passillé, A. M., 2012:** A training programme to ensure high repeatability of injury scoring of dairy cows. *Anim. Welf.*, 21, 379–388.
19. **Green, L. E., Huxley, J. N., Banks, C., Green, M. J., 2014:** Temporal associations between low body condition, lameness and milk yield in a UK dairy herd. *Prev. Vet. Med.*, 113, 63–71. DOI: 10.1016/j.prevetmed.2013.10.009.
20. **Griffiths, B. E., Grove White, D., Oikonomou, G., 2018:** A cross-sectional study into the prevalence of dairy cattle lameness and associated herd-level risk factors in England and Wales. *Front. Vet. Sci.*, 65. DOI: 10.3389/fvets.2018.00065.
21. **Guimaraes, J. L. B, Brito, M., Lange, C. C., Silva, M. R., Ribeiro, J. B., Mendonca, L. C., et al., 2017:** Estimate of the economic impact of mastitis: A case study in a Holstein dairy herd under tropical conditions. *Prev. Vet. Med.*, 142, 46–50. DOI: 10.1016/j. prevetmed.2017.04.011.
22. **Gussmann, M., Steeneveld, W., Kirkeby, C., Hogeveen, H., Farre, M., Halasa, T., 2019:** Economic and epidemiological impact of different intervention strategies for subclinical and clinical mastitis. *Prev. Vet. Med.*, 166, 78–85. DOI: 10.3168/jds.2018-14939.
23. **Heyerhoff, J. Z., LeBlanc, S. J., DeVries, T. J., Nash, C. G. R., Gibbons, J., Orsel, K., et al., 2014:** Prevalence of and factors associated with hock, knee, and neck injuries on dairy cows in freestall housing in Canada. *J. Dairy Sci.*, 97, 173–184. DOI: 10.3168/jds.2012-6367.
24. **Hoedemaker, M., Prange, D., Gundelach, Y., 2009:** Body condition change ante-and postpartum, health and reproductive performance in German Holstein cows. *Reprod. Domest. Anim.*, 44, 167–173. DOI: 10.1111/j.1439-0531. 2007. 00992.x.
25. **Hund, A., Logrono, J. C., Ollhort, R. D., Kofler, J., 2019:** Aspects of lameness in pasture based dairy farms. *Vet. J.*, 244, 83–90. DOI: 10.1016/j.tvjl.2018.12.011.
26. **Kester, E., Holzhauer, M., Frankena, K., 2014:** A descriptive review of the prevalence and risk factors of hock lesions

in dairy cows. *Vet. J.*, 202, 222–228. DOI: 10.1016/j.tvjl.2014.07.004.

27. **Kielland, C., Ruud, L. E., Zanella, A. J., Østerås, O., 2009:** Prevalence and risk factors for skin lesions on legs of dairy cattle housed in freestalls in Norway. *J. Dairy Sci.*, 92, 5487–5496. DOI: 10.3168/jds.2009-2293.
28. **King, M. T. M., Pajor, E. A., LeBlanc, S. J., DeVries, T. J., 2016:** Associations of herd-level housing, management, and lameness prevalence with productivity and cow behaviour in herds with automated milking systems. *J. Dairy Sci.*, 99, 9069–9079. DOI: 10.3168/jds.2016-11329.
29. **Lim, P. Y., Huxley, J. N., Green, M. J., Othman, A. R., Potterton, S. L., Brignell, C. J., et al., 2015:** Area of hock hair loss in dairy cows: Risk factors and correlation with a categorical scale. *Vet. J.*, 203, 205–210. DOI: 10.1016/j.tvjl.2014.11.005.
30. **Lowe, J. L., Stone, A. E., Akers, K. A., Clark, J. D., Bewley, J. M., 2015:** Effect of alley-floor scraping frequency on *Escherichia coli, Klebsiella* species, environmental *Streptococcus* species, and coliform counts. *Prof. Anim. Sci.*, 31, 284–289.
31. **Magnusson, M., Herlin, A. H., Ventorp, M., 2008:** Short communication: Effect of alley floor cleanliness on free-stall and udder hygiene. *J. Dairy Sci.*, 91, 3927–3930. DOI: 10.3168/jds.2007-0652.
32. **Mee, J. F., Boyle, L. A., 2020:** Assessing whether dairy cow welfare is "better" in pasture-based than in confinement-based management systems. *N. Z. Vet. J.*, 68, 168–177. DOI: 10.10 80/00480169.2020.1721034.
33. **Mille, S., Anderbourg, J., Mounaix, B., Roy, C., Assié, S., Benayas, E., et al., 2016:** *Des veaux allaitants en bonne santé, conduite d'élevage adaptée et bâtiments bien conçus*. Accessed 4th October, 2021 on https://www.agrireseau.net/documents/96332/des-veaux-allaitants-en-bonne-sante-conduite-d_elevage-adaptee-et-batiments-bien-con%C3%A7us.
34. **Moreira, T. F., Nicolino, R. R., Meneses, R. M., Fonseca, G. V., Rodrigues, L. M., Facury Filho, E. J., et al., 2019:** Risk factors associated with lameness and hoof lesions in pasture-based dairy cattle systems in southeast Brazil. *J. Dairy Sci.*, 102, 10369–10378. DOI: 10.3168/jds.2018-16215.
35. **Newsome, R. F., Green, M. J., Bell, N. J., Bollard, N. J., Mason, C. S., Whay, H. R., et al., 2017:** A prospective cohort study of digital cushion and corium thickness. Part 2: Does thinning of the digital cushion and corium lead to lameness and claw horn disruption lesions? *J. Dairy. Sci.*, 100, 4759–4771. DOI: 10.3168/jds.2016-12012.
36. **Pinzón-Sánchez, C., Ruegg, P. L., 2011:** Risk factors associated with short-term post-treatment outcomes of clinical mastitis. *J. Dairy Sci.*, 94, 3397–3410.
37. **Relun, A., Lehebel, A., Bruggink, M., Bareille, N., Guatteo, R., 2013:** Estimation of the relative impact of treatment and herd management practices on prevention of digital dermatitis in French dairy herds. *Prev. Vet. Med.*, 110, 558–562. DOI: 10.1016/j.prevetmed.2012.12.015.
38. **Roche, J. R., Berry, D. P., 2006:** Periparturient climatic, animal, and management factors influencing the incidence of milk fever in grazing systems. *J. Dairy Sci.,* 89, 2775–2783. DOI: 10.3168/jds.s0022-0302(06)72354-2.
39. **Roche, J. R., Lee, J. M., Macdonald, K. A., Berry, D. P., 2007a:** Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. *J. Dairy Sci.*, 90, 3802–3815. DOI: 10.3168/jds.2006-740.
40. **Roche, J. R., Macdonald, K. A., Burke, C. R., Lee, J. M., Berry, D. P., 2007b:** Associations among body condition score, body weight, and reproductive performance in seasonal-calving dairy cattle. *J. Dairy Sci.*, 90, 376–391. DOI: 10.3168/jds.s0022-0302(07)72639-5.
41. **Rutherford, K. M., Langford, F. M., Jack, M. C., Sherwood, L., Lawrence, A. B., Haskell, M. J., 2008:** Hock injury prevalence and associated risk factors on organic and nonorganic dairy farms in the United Kingdom. *J. Dairy Sci.*, 91, 2265–2274. DOI: 10.3168/jds.2007-0847.
42. **Rutherford, K. M., Langford, F. M., Jack, M. C., Sherwood, L., Lawrence, A. B., Haskell, M. J., 2009:** Lameness prevalence and risk factors in organic and non-organic dairy herds in the United Kingdom. *Vet. J.*, 180, 95–105. DOI: 10.1016/j.tvjl.2008.03.015.
43. **Sadiq, M. B., Ramanoon, S. Z., Shaik Mossadeq, W. M., Mansor, R., Syed-Hussain, S. S., 2020:** Claw trimming as a lameness management practice and the association with welfare and production in dairy cows. *Animals*, 10, 1515. DOI: 10.3390/ani10091515.
44. **Schreiner, D. A., Ruegg, P. L., 2003:** Relationship between udder and leg hygiene scores and subclinical mastitis. *J. Dairy Sci.*, 86, 3460–3465. DOI: 10.3168/jds.s0022-0302(03)73950-2.
45. **Séité, Y., Guiocheau, S., Gautier, B., Foisnon, O., Coutant, S., Prudhomme, J. F., et al., 2012:** *Concevoir et installer des logettes. Institut de l'élevage* (Ed), 12 pp. Accessed 10th October, 2021 on https://www.gie-elevages-bretagne.fr/admin/upload/concevoir_et_installer_des_logettes_avril_2012_BAT.pdf.

46. **Suresh, M., Muhammed Safiullah, A. H, Kathiravan, G., Narmatha, N., 2017:** Incidence of clinical mastitis among small holder dairy farms in India. *Atatürk University J. Vet. Sci.*, 12, 1–13.

47. **Thomsen, P. T., Munksgaard, L., Tøgersen, F. A., 2008:** Evaluation of a lameness scoring system for dairy cows. *J. Dairy Sci.*, 91, 119–126.

48. **Tucker, C. B., Weary, D. M., Von Keyserlingk, M. A. G., Beauchemin, K. A., 2009:** Cow comfort in tie-stalls: Increased depth of shavings or straw bedding increases lying time. *J. Dairy Sci.*, 92, 2684–2690. DOI: 10.3168/jds.2008-1926.

49. **Vasseur, E., Gibbons, J., Rushen, J., de Passillé, A.M., 2013:** Development and implementation of a training program to ensure high repeatability of body condition score of dairy cow in animal welfare assessments. *J. Dairy Sci.*, 96, 4725–4737

50. **Westin, R., Vaughan, A., de Passille, A. M., DeVries, T. J., Pajor, E. A., Pellerin, D., et al., 2016:** Cow- and farm-level risk factors for lameness on dairy farms with automated milking systems. *J. Dairy Sci.*, 99, 3732–3743. DOI: 10.3168/jds.2015-10414.

51. **World Organisation for Animal Health (OIE), 2022:** *Terrestrial Animal Health Code*. Accessed 10th December, 2022, on https://www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/.

Received April, 6, 2023
Accepted May 22, 2023

sciendo

DOI: 10.2478/fv-2023-0019

FOLIA VETERINARIA, 67, 2: 78—88, 2023

KNOWLEDGE, ATTITUDE AND PRACTICES AMONG PASTORALISTS AND ANIMAL HEALTH WORKERS ON TICK-BORNE SPOTTED FEVER GROUP RICKETTSIOSIS IN PLATEAU STATE, NIGERIA

Henry E. Nnabuife[1], Bernard M. Matur[2], Ndudim I. Ogo[1], Obed N. Goselle[2], Joshua Kamani[1], Georgina S. Mwansat[2], Rebecca Weka[1], Yinka Adedeji[1], Henry Osemeka[1], Jolly Amoche[1], Oko K. Egwu[1]

[1]National Veterinary Research Institute (NVRI), Vom, Plateau State
[2]Department of Zoology, Faculty of Natural Sciences, University of Jos, Jos, Plateau State
Nigeria

ekennabusimple@yahoo.com

ABSTRACT

Tick-borne Spotted Fever Group Rickettsioses (SFGR) is a serious disease reported in low income communities of many developed countries. The dearth of information on the existence of SFGR in most developing countries particularly in Sub Saharan Africa (SSA) including Nigeria, has been attributed to many factors chiefly amongst which are; poor knowledge of the disease, limitation of diagnostic capacity, leading to misdiagnosis and under reporting of some infectious zoonotic diseases such as SFGR. The aim of this study was to evaluate the pastoralists (livestock farmers) and animal health workers' knowledge, attitudes and perceptions/practices towards ticks and tick-borne SFGR affecting livestock in 3 Local Government Areas (LGA): i.e., in Plateau State, Nigeria and including Jos the Capital City of the State. A total of 141 semi-structured questionnaires were administered to respondents including 90 pastoralists and 51 health workers from different veterinary clinics. Interestingly, the pastoralists expressed no knowledge about SFGR, although 61.36 % reported a history of tick infestation. Among the animal health workers, about 76.47 % expressed some knowledge about the disease, but lacked knowledge of the diagnosis of the disease. The findings indicate that there was the need for an increase awareness on SFGR and its predisposing factors among relevant stakeholders in the Plateau State, Nigeria.

Key words: animal health workers; Nigeria; pastoralists; plateau; SFGR; tick-borne

INTRODUCTION

Tick-borne rickettsioses are zoonotic infections caused by obligate intracellular bacteria of the genus *Rickettsia*. The infections are endemic in many African countries and represent a public health challenge [38]. Members of the genus *Rickettsia* can be classified into four phylogenetic groups for which Spotted Fever Group Rickettsioses (SFGR) is one of them [24].

Ixodid ticks are the main vectors and reservoirs of the SFGR and they transmit the infections to domestic animals, wild life and humans [32, 35]. Rickettsioses are considered zoonotic diseases because they are transmissible from animal to humans and are considered vector-borne zoonoses

because they are transmitted by the bite of various arthropods including ticks, lice, fleas and mites [8]. Also, rickettsioses have been viewed as emerging and re-emerging diseases with an almost worldwide distribution [23, 36]. Ungulates play a very important role as the primary hosts for the ticks while humans are accidental and dead-end hosts [6]. *Rickettsia* is a large genus encompassing at least 30 recognized species of which 19 are considered to be human pathogens [2, 24]. In some African countries, such as South Africa, four tick-borne SFG *Rickettsia* species have been implicated as the cause of human diseases, including *R. africae* which causes African Tick Borne Fever (ATBF), *R. conorii* which causes the Mediterranean Spotted Fever (MSF), *R. aeschlimannii* which causes innominate rickettsioses, and *R. sibirica* subsp. *Mongolitimonae*, which causes lymphangitis-associated rickettsiosis (LAR) [22, 28, 31]. In Nigeria, though, some of these zoonotic SFG Rickettsiae species (*R. africae, R. aeschlimannii, R. conorii* subsp. israelensis and *R. massilliae*) had been reported [12, 13, 18, 19, 20, 26, 27, 33] in ixodid ticks collected from animal hosts and vegetation, there hasn't been any record of *Rickettsia* species in humans. Generally, the information about the circulation and distribution of rickettsiae-infected tick species in Nigeria is scarce and fragmented.

Most people are exposed to ticks and tick-borne rickettsioses during occupational and recreational activities in rural areas, and the epidemiology of each tick-borne rickettsial disease is reflected by the geographic distribution and seasonal activity of the tick vectors [7, 15]. In Nigeria, the extensive or semi-intensive practice of the production system by livestock farmers, in which the animals are allowed to roam freely and fend for themselves occasionally, depending on the season, exposes the animals to tick infestation and tick-borne pathogens such as SFG rickettsioses.

SFG rickettsioses are considered second only to malaria and they also play an important role in public health as the most commonly diagnosed diseases in travellers returning from sub-Saharan Africa with systemic febrile illness [9, 11]. Common symptoms of SFG rickettsioses in humans may include fever, headache, intense myalgia, skin rash, occasional eschar formation at the site of the tick bite, with the capability of deadly consequence in acute cases if they are not treated [3, 29, 30, 39]. The early treatment of rickettsial infections can prevent complications from exacerbating, reduce the risk of dying, and shorten the recovery time. The use of antibiotics such as doxycycline (preferred) or chloramphenicol in the early treatment of rickettsial infections have been reported to be effective [10].

In spite of the abundance and wide distribution of the tick vectors, there is insufficient or scarcity of epidemiological information, limited diagnostic capacity, and poor knowledge of the SFG rickettsioses in rural communities of sub-Saharan Africa [29]. This could be attributed to the fact that most tropical rickettsioses are often misdiagnosed as malaria, typhoid or acute febrile diseases.

Domestic animals such as dogs, cats, goats and sheep can serve as important hosts of SFGR infections to humans due to their close relationship and associated activities with humans [28].

Despite the vast epidemiological and predisposing reported factors of SFGR, there is no available literature on the pastoralists/livestock farmers' and health workers' knowledge, attitudes and practices on ticks and tick-borne SFG rickettsioses in Nigeria. This study, therefore, sought to examine socio-demographic characteristics, knowledge, attitudes and practices of pastoralists (livestock farmers) and animal health workers towards SFGR and its vector ticks in Plateau State, North Central Nigeria.

MATERIALS AND METHODS

Ethical clearance

Approval for this study was granted by the Animal Use and Care Committee (AUCC), National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria, No. AEC/02/62/18.

Consent was sought from pastoralists/livestock farmers before interviews using a language of their choice and alongside, animals were sampled for ticks after obtaining verbal consent from them. In the same vein, verbal consent was also sought from the veterinary personnel in the veterinary clinics before the interviews. They were all assured of the confidentiality of the information provided during the interviews.

Study area

The study was carried out in three Local Government Areas (LGAs) – Jos-South, Kanke, and Shendam, of Plateau State, Nigeria. Randomized sampling through balloting was used to determine the study areas.

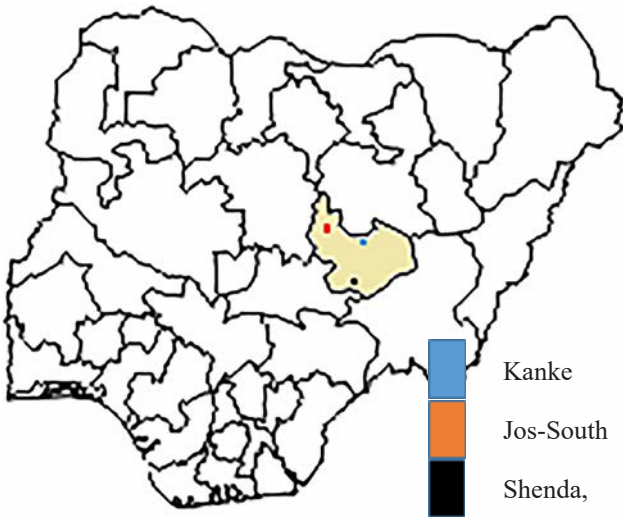

Fig.1. Map of Nigeria showing the study areas in Plateau State

Plateau State (9°00′–10°30′ N and Latitude 8°30′–09°30′ E) with an estimated population of about 5.5 million inhabitants, occupies an area of approximately 26,899 km^2 in the central part of Nigeria [1, 17]. Although they are situated in the tropical zone, a higher altitude of about 1238 m above sea level, means that Plateau State has a near temperate climate with temperature of between 13 and 22 °C. The mean annual rainfall varies between 131.75 cm (52 in.) in the southern part to 146 cm (57 in.) on the Plateau. The study was carried out in three LGAs representing the three Senatorial Districts of Plateau State, Nigeria, and including Jos the capital city of the state (Fig. 1). The different major languages spoken in these LGAs include the following: Jos-South (Berom), Kanke (Ngas, Myet, Boghom, Taroh and Badawa) and Shendam (Geomai).

Study design

The questionnaire was administered to two groups of people, the livestock farmers, and the health workers (veterinary personnel). The interviews were conducted on the livestock farmers in three LGAs, Jos-South, Kanke and Shendam representing the three Senatorial districts of Plateau State, including Jos capital city where big and established veterinary clinics are located in the state.

A total of one hundred and forty-one (141) semi-structured questionnaires were administered to respondents including 90 livestock farmers, 30 each from the three LGAs, and fifty-one (51) veterinary personnel from different veterinary clinics which include; National Veterinary Research Institute, Vom Clinic, ECWA Veterinary clinic, Bukuru, University of Jos Veterinary Teaching Hospital, Jos and MAAS Private Veterinary clinic, Jos.

Data collection

The administration of the semi-structured questionnaire was done with the help of research assistants who understand the local dialects of the livestock farmers for easy translation for those not conversant with the English language. The veterinary clinics' data collection commenced immediately after that of the livestock farmers' and all these were carried out between September and December, 2018.

The questionnaire focused on specific issues for each group of respondents. For the livestock farmers (pastoralists) these included; socio-demographic characteristics, knowledge of tick infestations and likely diseases transmitted/caused in livestock and man, diseases of importance shared with the livestock, zoonotic diseases including tick-borne especially SFG rickettsioses and their clinical manifestations (symptoms) and preventive measures taken against zoonotic infections. For the veterinary personnel, they included: socio-demographic characteristics, the types of animals received in the clinics, zoonotic tick-borne diseases found in the animals received, knowledge of SFG rickettsioses, clinical signs and animals diagnosed of SFG rickettsioses, drugs of choice, and laboratory diagnostic methods for SFGR.

Data Analysis

The data generated from the respondents were analysed using Epi-Info 2000 Software version 3.3.2, Excel spreadsheet (Microsoft Inc.) and SPSS version 25.0 (PASW Inc.). Percentages were calculated as the ratio between the pastoralists'/animal health workers' response to the interviews and the total number of pastoralists/animal health workers interviewed. The percentage of each response was calculated separately and presented in Tables. The associations of perception/practice with demographic variables were tested using Chi-square statistic. Values of $P \leq 0.05$ were considered significant.

RESULTS

Pastoralists

Socio-demographic information

Of the 90 pastoralists interviewed, 30 were from each LGA which include Jos South, Kanke, and Shendam.

Most of the respondents across the three LGAs were males with 81(90.0%) while females were 9 (10.0%). Also, the data show that most of the household heads were men with 77.7% (70/90). Overall, across the three study areas, the largest group with 30/90 (33.33%) of the respondents were aged 51–60, followed by ages 41–50 having 22/90 (24.44%), while the least were those of ages less than 20 (1.11%). The highest level of education attended by the respondents was secondary school with 39/90 (43.33%), followed by primary school 33/90 (36.67%) and the least were tertiary school level 9/90 (10.00%). About 10.00% had no formal education (Table 1).

Major livestock kept by the respondents in the study areas were sheep, goats and cattle. Of which, out of 90 households 76/90 (84.44%) kept goats, 54/90 (60%) kept sheep and 22/90 (24.44%) kept cattle. In respect of

Table 1. Pastoralists: Socio-demographic information

	Frequency	Percent [%]
Sex of respondents		
Female	9	10.00
Male	81	90.00
Total	**90**	**100.00**
Position of respondents in household		
Head	70	77.78
Spouse	8	8.89
Son	11	12.22
Daughter	1	1.11
Total	**90**	**100.00**
Age of respondents		
< 20	1	1.11
21–30	9	10.00
31–40	19	21.11
41–50	22	24.44
51–60	30	33.33
> 60	9	10.00
Total	**90**	**100.00**
Educational qualification		
None	9	10.00
Primary	33	36.67
Secondary	39	43.33
Tertiary	9	10.00
Total	**90**	**100.00**

	Frequency	Percent [%]
Types of livestock kept		
Goats		
Yes	76	84.44
No	14	15.56
Total	90	100.00
Sheep		
Yes	54	60.00
No	36	40.00
Total	90	100.00
Cattle		
Yes	22	24.44
No	68	75.56
Total	**90**	**100.00**
Types of livestock kept in respect of LGA		
LGA/Goats		
Jos South	170	24.25
Kanke	275	39.23
Shendam	256	36.52
Total	**701**	**100.00**
Sheep		
Jos South	115	24.31
Kanke	132	27.91
Shendam	226	47.78
Total	**473**	**100.00**
Cattle		
Jos South	67	33.67
Kanke	57	28.64
Shendam	75	37.69
Total	**199**	**100.00**

LGA – Local Government Area

LGAs, goats were kept more by households in Kanke with 275 (39.32%), followed by Shendam 256 (36.23%) while Jos South was the least with 170 (24.25%). However, in Shendam LGA, sheep and cattle were found to be kept more by households than in Jos-South and Kanke, with sheep recording 226 (47.78%) and cattle 75 (37.69%), respectively (Table 1). Apart from sheep and goats, other livestock kept included rabbits, chickens, dogs, pigs, cats etc. by much proportion of the households except dogs that were kept by almost all the households especially in Kanke LGA, where dog meat is also used as their special delicacy.

Of the 90 respondents, 54 (61.36%) accepted that their animals were affected by ticks, while 34 (38.64%) didn't accept. Out of the 54 (61.36%) who accepted 12 (22.22%) were from Jos south, while 21 (38.89%) were from Kanke and Shendam each.

The respondents who were aware about tick-borne zoonotic diseases were few 27 (33.75%). They however listed diseases such as babesiosis, anaplasmosis, and even trypanosomosis that is not a tick-borne disease. In respect of LGA, 5 (18.52%) were from Jos South, 10 (37.04%), while Kanke and Shendam recorded the highest with 12 (44.44%).

Less than 50% of the respondents 32 (39.51%) across the three study areas admitted that they had been bitten by ticks. For example, seven (21.88%) were from Jos South, 8 (25.00%) from Shendam, and the highest 17 (53.13%) were from Kanke who admitted having been bitten by ticks. In addition, some revealed having been discomforted or felt itchy upon the bite by ticks.

Interestingly, out of the 90 respondents, across all the three LGAs, none had knowledge of the disease SFG rickettsiosis in livestock or humans (Table 2).

Some practices by pastoralists were identified as potential risk factors that can predispose them to SFG rickettsioses. These included: (i) sharing living accommodations with livestock by most households which can potentially facilitate transmission of diseases through tick bites; (ii) Self-treatment of livestock by most pastoralists due to limited or absence of veterinary facilities in the study areas (iii); keeping of dogs which have been reported around the world as the potential carriers of SFGR by almost all the households, etc.

The respondents were asked of the possibility of transmitting livestock diseases to humans through sharing of the same household with livestock. Out of the 33 that re-

Table 2. Respondents' knowledge, attitudes and practices/perceptions on ticks, tick-borne diseases (TBDs) and spotted fever group rickettsioses (SFGR)

	Frequency	Percent [%]
Are your animals infested by ticks?		
Yes	54	61.36
No	34	38.64
Total	88	100.00
LGA		
Jos South	12	22.22
Kanke	21	38.89
Shendam	21	38.89
Total	**54**	**100.00**
Are you aware of disease that ticks can transmit to livestock?		
Yes	27	33.75
No	53	66.25
Total	80	100.00
LGA		
Jos South	5	18.52
Kanke	10	37.04
Shendam	12	44.44
Total	**27**	**100.00**
Are there times when you are bitten by ticks?		
Yes	32	39.51
No	49	60.49
Total	81	100.00
LGA		
Jos South	7	21.88
Kanke	17	53.13
Shendam	8	25.00
Total	**32**	**100.00**
Have you heard of the disease called Rickettsiosis?		
No	90	100.00
Total	**90**	**100.00**

LGA – Local Government Area

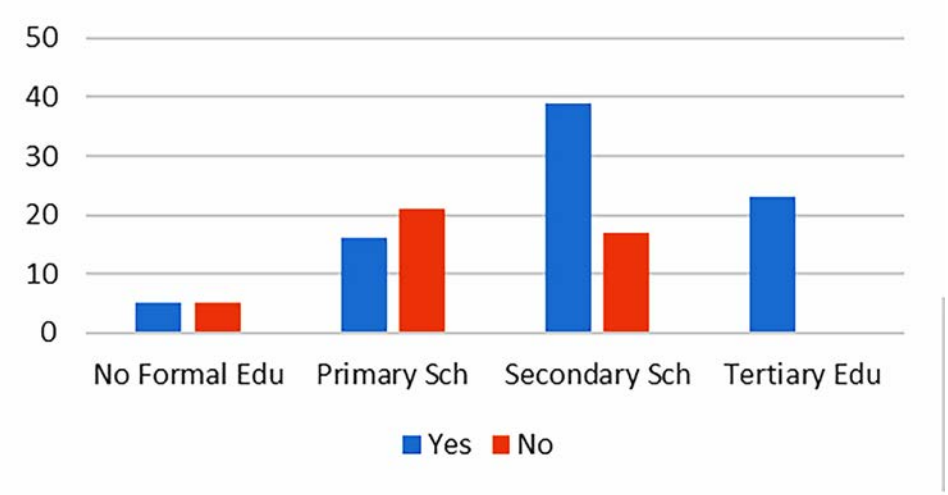

Fig. 2. Educational level and animal tick control practices

sponded, 18/33 (54.55%) agreed that sharing the same house with livestock can transmit livestock diseases to humans while 15/33 (45.45%) disagreed. The practice or perception of the respondents in seeking medical attention after observing some seemingly symptoms and signs of rickettsioses was not encouraging. Out of 26 that responded only 3 (11.54%) sought medical attention while the remaining 23 didn't seek medical attention. These 3 were from Jos South (2) and Kanke (1). The respondents were further asked if they ever considered taking measures to prevent tick bites on themselves (pastoralists). Out of 46 that responded 21 (45.65%) accepted taking measures to prevent tick bites while 25 (54.35%) had no consideration for that. On the other hand, some respondents also considered taking measures to prevent tick bites against their animals. Out of the 44 that responded, 29 (65.91%) expressed taking measures to prevent tick bites against their animals while the remaining 15 (34.09%) did not.

Though, there was no statistically significant association between educational qualifications and responses ($P > 0.05$), those with higher educational qualification were more likely to consider taking steps to reduce tick infestation in their farms (Fig. 2)

Animal health workers (professionals)

Socio-demographic information

The semi structured questionnaire was administered to animal health workers in different clinics at different locations in the Plateau state. The clinics visited included the following: NVRI, Vom, Veterinary clinics, ECWA Veterinary clinics, Bukuru, Veterinary teaching hospital (VTH), Polo, Jos and MAAS Veterinary clinics, Jos.

Of the 51 health workers interviewed, 34 (66.67%) were males while 17 (33.33%) were females. Also, out of the 51 respondents, VTH had the highest number of respondents with 31 (60.78%), followed by NVRI, Vom Veterinary clinics with 11 (21.57%), ECWA Veterinary clinics 7 (13.73%) and the least was MAAS Veterinary clinics with 2 (3.92%).

Out of the 51 respondents interviewed, the majority of the workers responded were within the age range of 31–40 with 26/51 (50.98%), followed by the age range of 41–50 with 11/51 (21.57%) while the age range less than 20 had no respondent. Out of 51 respondents, 17 (33.33%) were veterinary doctors, followed by technologists 16 (31.37%), technicians 11 (21.57%), animal health officers 4 (7.84%), scientists 2 (3.92%) and others with 1 (1.96%).

Forty-nine out of 50 (98.60%) that responded said they encounter tick infestations on animals they receive, while only 1/50 (1.4%) respondent said no. From their responses 44/50 (88.0%) listed dog as the most commonly infested animal with ticks followed by sheep and goats with 2/50 (4%) each, and birds and geese the least with 1/50 (2.0%) each too. Out of the 51 respondents, 50/51 (98.04%) were aware of diseases that ticks transmit to animals (as they mentioned the diseases such as babesiosis, anaplasmosis, ehrlichiosis, borreliosis, etc.) while only 1/51(1.96%) was not aware. About 86.00% (43/50) of the respondents said that some of the listed diseases cause itching or discomfort to animals, while only 14% (7/50) answered no. At least more than half 76.47% (39/51) of the respondents had knowledge of rickettsiosis while 23.53% (12/50) had no information about it. Unfortunately, knowledge of that disease is not enough as almost all didn't know the diag-

nostic methods of it except one out of 38 (2.63%) that listed Enzyme Linked Immunosorbent Assays (ELISA). Furthermore, none of the respondents had come across the disease on the animals and let alone finding it necessary to confirm this disease in animals.

Comparing the responses between the pastoralists (livestock farmers) and animal health workers (professionals), however indicates that the professionals were much more aware of rickettsioses than the farmers (Fig. 3). There is statistically significant relationship between the two groups ($P \leq 0.05$) (Table 5).

Table 3. Socio-demographic data of animal health workers

	Frequency	Percent [%]
Sex of respondents		
Female	17	33.33
Male	34	66.67
Total	**51**	**100.00**
Clinic/Hospital		
ECWA Vet	7	13.73
MAAS Vet	2	3.92
VOM Vet	11	21.57
VTH Polo	31	60.78
Total	**51**	**100.00**
Age of respondents		
≤ 20	0	0.00
21–30	10	19.61
31–40	26	50.98
41–50	11	21.57
51–60	2	3.92
≥ 61	2	3.92
Total	**51**	**100.00**
Position of respondents in the clinic		
Veterinary doctor	17	33.33
Animal health officer	4	7.84
Scientist	2	3.92
Technician	11	21.57
Technologist	16	31.37
Others	1	1.96
Total	**51**	**100.00**

Table 4. Knowledge, attitudes and practices on ticks, tick-borne diseases (TBDs) and spotted fever group rickettsioses (SFGR)

	Frequency	Percent [%]
Do you encounter tick infestations on animals received?		
Yes	49	98.00
No	1	2.00
Total	**50**	**100.00**
If yes, which animal is most commonly infested?		
Birds	1	2.00
Dogs	44	88.00
Geese	1	2.00
Goats	2	4.00
Sheep	2	4.00
Total	**50**	**100.00**
Are you aware of any disease(s) that ticks can transmit to animals?		
Yes	50	98.04
No	1	1.96
Total	**51**	**100.00**
Does any of the diseases listed cause itching/discomfort to animals?		
Yes	43	86.00
No	7	14.00
Total	**50**	**100.00**
Have you heard of the disease called rickettsiosis?		
Yes	39	76.47
No	12	23.53
Total	**51**	**100.00**

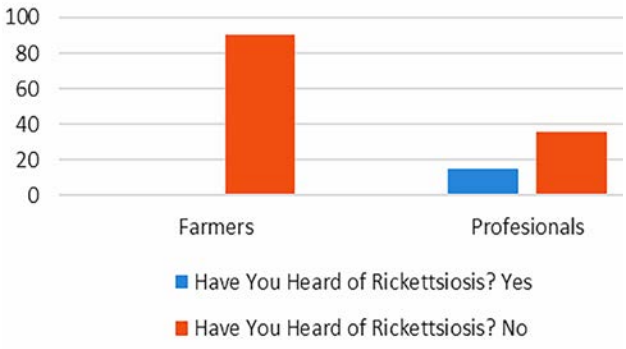

Fig. 3. Knowledge of rickettsiosis between pastoralists and animal health workers

Table 5. Association of knowledge of tick-borne rickettsioses between pastoralists and professionals

Response	Professionals (%)	Pastoralists [%]	Chi	P-value
Yes	39 (76.47)	0 (0.00)	91.4	2.2e-16
No	12 (23.53)	90 (100.00)	–	–
Total	**51 (100.00)**	**90 (100.00)**	**–**	**–**

DISCUSSION

This study demonstrated that among the pastoralists in the three study areas, males remain the livestock owners more than females. These findings are in line with previous reports that traditional livestock are usually owned by males as they can manage and face challenges such as feeding, handling and treatment of the animals better [14, 21, 25, 40]. Similar reports of the dominance of the livestock industry by males compared to females have been documented in South Africa [40], Zimbabwe [37], Nigeria [16] and Tanzania [5].

The majority of the livestock farmers in this study were above 50 years of age who seem to depend on farming as their source of income [4]. These findings can be compared with those of [40] and [34] who reported that elderly people are found more in the rural areas depending on farming for their source of income [21] while the younger people move to the urban areas for better job opportunities which are limited in the rural areas.

From this study, it is evident that there is no knowledge about SFGR amongst the livestock farmers/pastoralists, unlike the animal health workers who expressed knowledge about the disease, but, didn't have knowledge about the diagnostic methods of this disease apart from one veterinary doctor that listed Enzyme Linked Immuno-sorbent Assay (ELISA). These findings are comparable with those of N d h l o v u et al. [25] who reported low knowledge about SFGR amongst the pastoralists and health workers. These results of lack of knowledge about the SFG rickettsioses could be attributed to the low level of literacy amongst the pastoralists as the majority had only secondary 39/90 (43%) and primary 33/90 (36%) education level. This lack of knowledge about the diseases especially amongst the pastoralists raise concerns about the potential risks posed by the SFG rickettsioses to these livestock keepers. This lack of knowledge is in agreement with the findings of [25] and [37] who observed that more than 90% of the farmers participating in farming in Zimbabwe had secondary education level and [4, 21, 40] whose reports revealed farmers with only a primary education level that led to improper tick control. Owing to these facts, no doubt there is every possibility that this disease will be circulating unnoticed in the study areas between the animals and humans, since the facilities for investigation of the presence of the disease is not available in the veterinary hospitals and clinics visited. The disease could be amongst the "fevers of unknown origin" recorded in most medical facilities. The interview, according to some veterinary doctors created awareness or awaken their thoughts towards the disease and its diagnosis which had never been thought of in the past, as they promised to notify the relevant authorities.

Sharing living accommodations with animals among other things were identified as some practices that can predispose the pastoralists to zoonoses which include SFGR. Low level of knowledge towards many zoonotic diseases appears to be common in many African countries including Nigeria, raising concern about their under-diagnosis and under-reporting as also reported by [25]. Some, also were spotted even sharing their plate of food especially with dogs, and dogs have been incriminated in many research reports as one of the major hosts of the SFGR. Sharing living accommodations with livestock can promote transmission of SFGR as it can expose the livestock owners to tick-bites through close contact. This is also in agreement with the work of [25] and [40].

CONCLUSIONS AND RECOMMENDATIONS

The lack of knowledge on SFG rickettsiosis amongst the pastoralists raises concern about the potential risks of these zoonoses amongst local populations. This portends a

grave danger to both the pastoralist and his animals, who may contract and harbour the disease unknowingly, but focus on other maladies like malaria, typhoid etc. that have similar symptoms as rickettsiosis thus, causing more harm than good. In the same vein, the low knowledge on SFG rickettsioses and significantly no understanding of diagnosis of the said disease amongst the most respondents in the veterinary hospitals/clinics indeed raises alarm and calls for worry in Nigeria.

We therefore, recommend regular awareness about ticks and tick-borne diseases especially rickettsiosis to pastoralists/livestock farmers in the study areas. Also, inclusion of rickettsiosis as one of the differential diagnosis for fevers of unknown origin in the veterinary clinics or hospitals in Nigeria.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

We thank the management of the National Veterinary Research Institute (N.V.R.I.), Vom for their supports so far. We are also indebted to the head of Parasitology Division, N.V.R.I., Vom, Dr. (Mrs.) Rebecca Yakubu Arin, and all the staff of the Division for their assistance in one way or the other in the course of the study. Mr. Patrick Adeleye of Planning Department, N.V.R.I., Vom is also appreciated for Figure 1.

REFERENCES

1. **Adekunle, J. O., 2004:** *Politics and Society in Nigeria's Middle belt: Borgu and the Emergence of a Political Identity*. Afr. Wld. Pres., Inc., Trenton, N. J., U.S.A.
2. **Anstead, C. A., Chilton, N. B., 2013:** Detection of a novel *Rickettsia* (Alphaproteobacteria: Rickettsiales) in rotund ticks (*Ixodes kingi*) from Saskatchewan, Canada. *TTBDs.*, 4, 3, 202–206.
3. **Chen, L. F., Sexton, D. J., 2008:** What's new in Rocky Mountain spotted fever? *Infect. Dis. Clin. North Am.*, 22, 415–432.
4. **Chenyambuga, S. W., et al., 2010:** Knowledge and perceptions of traditional livestock keepers on tick-borne diseases and sero-prevalence of *Theileria parva* around Lake Victoria Basin. *Livestock Res. Rur. Dev.*, 22, 7. Available at http://www.lrrd.org/ lrrd22/7. Accessed November 13, 2019.
5. **Covarrubias, K., Nsiima, L., Zezza, A., 2012:** *Livestock and Livelihoods in Rural Tanzania: A Descriptive Analysis of the 2009 National Panel Survey*. http://www.africalivestockdata.org/sites/africalivestockdata.org/files/PAP_LIV_TZ_LSMS-ISA_July_!2. pdf.
6. **Dantas-Torres, F., Chomel, B. B., Otranto, D., 2012:** Ticks and tick-borne diseases: A one health perspective. *Trends Parasitol.*, 28, 437–46. DOI: 10.1016/j. pt.2012.07.003.
7. **Demma, L. J., Traeger, M. S., Nicholson, W. L., Paddock, C. D., Blau, D. M., Eremeeva, M. E., et al., 2005:** Rocky Mountain spotted fever from an unexpected tick vector in Arizona. *New Eng. J. Med.*, 353, 587–594.
8. **Fournier, P. E., Karkouri E. L., Leroy, K., Robert Q., Giumelli C., Renesto B., et al., 2009:** Analysis of the *Rickettsia africae* genome reveals that virulence acquisition in *Rickettsia* species may be explained by genome reduction. *BMC Genomics*, 10, 166. DOI: 10.1186/1471-2164-10-166.
9. **Freedman, D. O., Weld, L. H., Kozarsky, P. E., Fisk, T., Robins, R., Sonnenburg, F. V., et al., 2006:** Spectrum of disease and relation to place of exposure among ill returned travellers. *New Eng. J. Med.*, 354, 2, 119–130.
10. **Holman, R. C., Paddock, C. D., Curns, A. T., Krebs, J. W., McQuiston, J. H., Childs, J. E., 2001:** Analysis of risk factors for fatal Rocky Mountain spotted fever: evidence for superiority of tetracycline for therapy. *J. Infect. Dis.*, 184, 1437–44. DOI: 10.1086/324372.
11. **Jensenius, M., Davis, X., von Sonnenburg, F., 2009:** Multicentre geo sentinel analysis of rickettsial diseases in international travellers, 1996–2008. *Emerg. Infect. Dis.*, 15, 1791–1798.
12. **Kamani, J., Baneth, G., Gutiérrez, R., Nachum-Biala, Y., Mumcuoglu, K. Y., Harrus, S., 2018:** *Coxiella burnetii* and *Rickettsia conorii*: Two zoonotic pathogens in peridomestic rodents and their ectoparasites in Nigeria. *TTBDs.*, 9, 86–92
13. **Kamani, J., Baneth, G., Apanaskevich, D. A, Mumcuoglu, K. Y., Harrus, S., 2015:** Molecular detection of *Rickettsia aeschlimannii* in *Hyalomma* spp. ticks from camels (*Camelus dromedarius*) in Nigeria, West Africa. *Med. Vet. Entomol.*, 29, 205–209. DOI: 10.1111/mve.12094.
14. **Katiyatiya, C. L. F., Muchenje, V., Mushunje, A., 2014:** Farmers' perceptions and knowledge of cattle adaptation to

heat stress and tick resistance in the eastern cape, South Africa. Asian-Australas. *J. Anim. Sci.*, 27, 1663–1670. DOI: 10.5713/ajas.2014.14174.

15. **Katswara, T., Mukaratirwa, S., 2021:** Knowledge, attitudes and practices on African tick bite fever of rural livestock communities living in a livestock-wildlife interface area in the Eastern Cape Province of South Africa. *BMC Infect. Dis.*, 21, 497. DOI: 10.1186/s12879-021-06174-9.
16. **Kristjanson, P., Waters, A., Johnson, N., Tipilda, A., Njuki, J., Baltenweck, I., et al., 2014:** Livestock and women's livelihoods. *Gender in Agriculture*. Springer Netherlands, Dordrecht, 209– 233. DOI: 10.1007/978-94-017-8616-4_9.
17. **Lee, V. H., 1972:** Ecological aspects of the Jos Plateau, Nigeria. *Bull. WHO*, 46, 641–644.
18. **Lorusso, V., Wijnveld, M., Majekodunmi, A. O., Dongkum, C., Fajinmi, A., Dogo, A. G., et al., 2016:** Tick-borne pathogens of zoonotic and veterinary importance in Nigerian cattle. *Parasit. Vect.*, 9, 217. DOI: 10.1186/s13071-016-1504-7.
19. **Lorusso, V., Picozzi, K., de Bronsvoort, B. M., Majekodunmi, A., Dongkum, C., Balak, G., et al., 2013a:** Ixodid ticks of traditionally managed cattle in Central Nigeria: Where *Rhipicephalus (Boophilus) microplus* does not dare (yet?). *Parasit. Vect.*, 6, 171.
20. **Lorusso, V., Gruszka, K. A., Majekodunmi, A., Igweh, A., Welburn, S. C., Picozzi, K., 2013b:** *Rickettsia africae* in *Amblyomma variegatum* ticks, Uganda and Nigeria. *Emerg. Infect. Dis.*, 19, 1705–1707.
21. **Mdungela, N. M., Bahta, Y. T., Jordaan, A. J., 2017:** Indicators for economic vulnerability to drought in South Africa. *Dev. Trop. Anim. Hlth. Prod. Pract.*, 27, 1050–1063. DOI: 10.1080/09614524. 2017.1361384.
22. **Mediannikov, O., Diatta, G., Fenollar, F., Sokhna, C., Trape, J. F., Raoult, D., 2010:** Tickborne rickettsioses, neglected emerging diseases in rural Senegal. *PLOS Negl. Trop. Dis.,* 4, 9. DOI: 10.1371/journal.pntd.0000821.
23. **Merhej, V., Angelakis, E., Socolovschi, C., Raoult, D., 2014:** Genotyping, evolution and epidemiological findings of *Rickettsia* species. *Infect. Gen. Evol.*, 25, 122–137. DOI: 10.1016/j.meegid.2014.03.014.
24. **Merhej, V., Raoult, D., 2011:** Rickettsial evolution in the light of comparative genomics. *Biol. Rev. Camb. Philos. Soc.*, 86, 2, 379–405. DOI: 10.1111/j.1469-185X.2010.00151.x.
25. **Ndhlovu, D. N., Masika, P. J., 2013:** Ethno-veterinary control of bovine dermatophilosis and ticks in Zhombe, Njelele and Shamrock resettlement in Zimbabwe. *Trop. Anim. Hlth. Prod.*, 45, 525–532. DOI: 10.1007/s11250-012-0253-7.
26. **Nnabuife, H. E., Matur, B. M., Ogo, N. I., Goselle, O. N., Shittu, I., Mkpuma, N., et al., 2023:** *Rickettsia africae* and *Rickettsia massiliae* in ixodid ticks infesting small ruminants in agro-pastoral settlements in Plateau State, Nigeria. *Exp. Appl. Acarol.*, 89, 1, 117–130. DOI: 10.1007/s10493-022-00769-w.
27. **Ogo, N. I., Fernández de Mera, G. I., Galindo, R. C., Okubanjo, O. O., Inuwa, H. M., Agbede, R. I. S., et al., 2012:** Molecular identification of tick-borne pathogens in Nigerian ticks. *Vet. Parasitol.*, 187, 572–577.
28. **Parola, P., Paddock, C. D., Socolovschi, C., Labruna, M. B., Mediannikov, O., Kernif, T., et al., 2013:** Update on tick-borne rickettsioses around the world: A geographic approach. *Clin. Microbiol. Rev.*, 26, 657–702. DOI: 10.1128/CMR.00032-13.
29. **Parola P., Paddock, C. D., Raoult, D., 2005:** Tick-borne rickettsioses around the world: Emerging diseases challenging old concepts. *Clin. Microbiol. Rev.*, 18, 719–756.
30. **Parola, P., Raoult, D., 2001:** Ticks and tick-borne bacterial diseases in humans: An emerging infectious threat. *Clin. Infect. Dis.*, 32, 897–928.
31. **Pretorius, A., Birtles, R. J., 2004:** *Rickettsia mongolotimonae* infection in South Africa. *Emerg. Infect. Dis.*, 10, 125–126.
32. **Raoult, D., Roux, V., 1997:** Rickettsioses as paradigms of new or emerging infectious diseases. Clin. Microbiol. Rev., 10, 694–719.
33. **Reye, A. L., Arinola, O. G., Hübschen, J. M., Muller, C. P., 2012:** Pathogen prevalence in ticks collected from the vegetation and livestock in Nigeria. *Appl. Environ. Microbiol.*, 78, 2562–2568.
34. **Scholtz, M. M., Bester, J., Mamabolo, J. M., Ramsay, K. A., 2008:** Results of the national cattle survey undertaken in South Africa, with emphasis on beef. *Appl. Anim. Husb. Rural Dev.*, 1, 1–9.
35. **Shaw, S. E., Day, M. J., Birtles, R. J., Breitschwerdt, E. B., 2001:** Tick-borne infectious diseases of dogs. *Trends Parasitol.*, 17, 2: 74–80.
36. **Sun, J., Lin, J., Gong, Z., Chang Y., Ye, X., Gu, S., et. al., 2015:** Detection of spotted fever group Rickettsiae in ticks from Zhejiang Province, China. *Exp. Appl. Acarol.*, 65, 403–411. DOI: 10.1007/s10493-015-9880-9.
37. **Sungirai, M., et al., 2016:** Communal farmers' perceptions of tick-borne diseases affecting cattle and investigation of

tick control methods practiced in Zimbabwe. *TTBDs*, 7, 1–9. DOI: 10.1016/j.ttbdis.2015.07.015.

38. Vanegas, A. R., Keller, C. A., Krueger, A., Tanyi, M. K., 2018: Molecular detection of spotted fever group Rickettsiae in ticks from Cameroon. *TTBDs*, 9, 4. DOI: 10.1016/j.ttbdis.2018.03.022.

39. Walker, D. H., 2007: Rickettsiae and rickettsial infections: The current state of knowledge. *Clin. Infect. Dis.*, 45, (Suppl. 1), S3944.

40. Yawa, M., Nyangiwe, N., Jaja, I. F., Kadzere, C. T., Marufu, M. C., 2020: Communal cattle farmer's knowledge, attitudes and practices on ticks (Acari: Ixodidae), tick control and acaricide resistance. *Trop. Anim. Hlth. Prod.*, 52, 6, 3005–3013. DOI: 10.1007/s11250-020-02319-1.

Received March 23, 2023
Accepted May 29, 2023

sciendo

DOI: 10.2478/fv-2023-0020

FOLIA VETERINARIA, 67, 2: 89—101, 2023

BRAIN GROSS ANATOMY AND CEREBELLAR HISTOLOGY OF THE CATTLE EGRET (*BUBULCUS IBIS*)

Idris A. Azeez[1], Jamiu O. Omirinde[1], Jerry J. Chomo[1], James O. Olopade[2]
[1]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Jos
[2]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Ibadan
Nigeria

azeezi@unijos.edu.ng

ABSTRACT

The structural parts of brain are similar in all vertebrates, but they differ in their complexity and organization. The avian brain for instance, is organised differently compared to mammals, with variations existing in the relative size and location of their structures. The cattle egret (*Bubulcus ibis*) is a cosmopolitan avian species native to Africa. Despite their economic importance as excellent sentinels in assessing environmental pollutants, there is a dearth of information on their neuroanatomy. Hence, we here investigated their gross morphological features and morphometric parameters, and the cerebellar histology. Twelve transcardially perfused juvenile cattle egrets were studied, with the body and brain weights and brain linear measurements considered. The brain weight constituted 0.46 % of the total body mass, with a strong positive correlation recorded between the body and brain weights (r = 0.9204). Morphologically, the brain was lissencephalic, with the corpus callosum absent and the olfactory bulbs rudimentary with no discernible division into the olfactory bulb, olfactory tract and olfactory lobe. We observed prominent sagittal eminence and vallecula telencephali, as well as an obvious fovea limbica on the dorsal and lateral surfaces of the cerebral hemispheres, respectively. The diencephalic structures were completely covered by telencephalon, with the pineal gland occupying the polygonal space between cerebrum, optic lobe and cerebellum. The mesencephalic tectum appeared as a large oval bilaterally bulging structure with prominent optic tracts and constituted the bulk of the midbrain. There was a dorsal extension of the fourth ventricle into the cerebellum known as the ventriculus cerebelli and the cerebellar histology presented a persistent external granular layering suggestive of a potential for adult neurogenesis. Our data has added relevant literature on the cattle egret brain and could prove useful in comparative, developmental and evolutionary avian neuroanatomy.

Key words: avian; brain stem; cattle egret; cerebellum; cerebral hemispheres; neuro-morphometry

INTRODUCTION

The nervous system of vertebrates is divided according to functional or structural features. Functional classifications are the somatic and autonomic nervous system, and these are concerned with conscious and involuntary processes, respectively [9]. Meanwhile, structurally, the nervous system is divided into: the peripheral nervous system (PNS) comprising the cranial, spinal and autonomic nerves, and the enteric nervous system; and the central nervous system (CNS), constituted by the brain and spinal

cord [11]. The brain controls and regulates many important functions and is regarded as one of the most important organs of the body [50].

Comparatively, the structural parts of the brain are similar in all vertebrates, but they differ in their complexity and organization [45]. The avian brain, for instance, is organised differently compared to mammals, with variations existing in their structural sizes and/or locations [33]. Functionally, the avian nervous system obtains information through sensory receptors about their environment, analyse, respond and store the processed information and, also it coordinates the motor impulses to skeletal muscles as well as to the viscera [7, 27].

The cattle egret (*Bubulcus ibis*), an avian species of the heron, is found all over the world, with a least concern conservation status [48]. They belong to the order Pelecaniformes, family Ardeidae. They are native to Africa with a wide distribution in tropical and subtropical African regions including Nigeria, and are mostly found in dry grassy habitats, in association with wildlife and livestock [32]. The remarkable migration of the cattle egret from its origins in Africa to its present geographical range including Asia, the Americas and Australasia, during the 20th century has been well documented [29]. They are associated with both small and large flocks, feeding in loose aggregates. The birds feed on insects, earthworms, spiders, frogs and most especially ticks present on cattle and other large herbivores [16, 20, 32].

Generally, cattle egrets are colonial in nature and whitish. Many populations of cattle egrets are highly migratory and dispersive and this has helped the species' range expansion. The massive and rapid expansion of the cattle egret's range is due to its relationship with humans and their domesticated animals [48]. Both sexes of this bird are similar, with the male being slightly bigger and also possessing marginally longer breeding plumes than the female. The juvenile age group lacks coloured plumes and has a black beak [32], while the adult possesses greyish-yellow feet and sharply pointed short yellow bill [44]. The Western cattle egret is a medium sized bird, possessing short-legs and thick necks, in comparison to other egrets [20].

There is a dearth of literature on the documentation of the nervous system of the cattle egret. Literature search till date revealed published articles in this regard to include studies on the cattle egrets' cerebellum line drawing [23], Wulst volume [53], and more recently, the histology of the hippocampal complex [17] and craniofacial morphometrics [1]. Despite considerable advances toward the understanding of comparative avian neuroanatomy, there is hardly any literature yet on the comprehensive descriptions of the gross morphology of the cattle egret's brain and their neuromorphometric correlates, as well as their cerebellar histology. Hence, we here investigated the gross morphological features and morphometric parameters of the juvenile cattle egret's brain, as this is crucial to the exploration of the physical attributes of this species brain for easy identification and elucidation of some of its morpho-functional features, as well as consolidate the literatures on comparative avian neuroanatomy.

MATERIALS AND METHODS

Ethical considerations

Care was taken to ensure the birds experienced neither undue pain nor discomfort. The ethical clearance for the use of this bird was sought and granted by the Animal Care and Use Committee of the University of Jos, Nigeria (reference code: UJ/FPS/F17-00379). Also, the experimental protocols used were in conformity with the National Institute of Health Guide for the Care and Use of Animals (NIH Publications No. 80–23) and the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Authors ensured that all ethical issues concerning plagiarism, approval to publish, errors in fabrication, double publication, and submission were adhered to.

Animals

The juvenile age group (n = 12, mixed sexes) of the captive and clinically healthy cattle egrets were sourced from Jos metropolis (GPS coordinates: 9°59'21.5"N; 8°52'49.7"E), a guinea savannah region of Nigeria [30]. They were aged according to the detailed description by [32] and [44]. The birds were procured alive at their habitat and immediately transported to the Gross Anatomy Laboratory of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Jos, Nigeria.

Perfusion and brain excision

The birds were sedated by the administration of ketamine (40 mg.kg^{-1}) and xylazine (10 mg.kg^{-1}) through the intravenous route, as described by [28]. The animals were

perfused transcardially in compliance with the protocol of [3] using 0.9% saline solution (for initial blood rinsing), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution at pH 7.4. The brains were carefully removed from the skull, and post-fixed for 48 hrs in 4% paraformaldehyde in 0.1M PB.

Gross morphological description and morphometrics

The gross anatomical observations were made on the brain in both *in situ* and *ex situ* positions. Gross images were captured using a Sony® digital camera (DSC-H300 20.1 megapixels with 35× Optical Zoom). The live bird weight was obtained using the mechanical laboratory weighing scale sensitive to 0.1 gm (Camry Emperor table scale J1712420834, China), while brain weight was obtained using the analytical weighing balance (Ohaus GmbH, Nänikon, Switzerland), sensitive to 0.01 gram, following its careful dissection from the cranium (*ex situ*). Linear gross morphometric parameters (Table 1) were measured with the aid of vernier caliper. The nomenclature here adopted in this study for the anatomical descriptions was as described in [38].

Schematic brain illustrations

The sketch of the cattle egret brain (for Figures 1A-C and 2Di, Ei and Ii) was made using Adobe Illustrator software (Adobe Inc., 2022. Adobe Illustrator version 27.0; available at https://adobe.com/products/illustrator). The pen tool was used to trace out the sketches from the bitmap image, to create a vector diagram. The images were imported into the artboard created, the pen tool was then used to trace out the edges of the image and also the various parts were traced out. Each of the different parts were then given different colours using the selection and colour swatch tools.

Definitions of measured gross parameters

The gross anatomical structures measured are concisely stated and defined as follows:

- Weight of the animal (WOA) (g): live bird weight to the nearest gram
- Weight of the brain (BOW) (g): weight of the whole brain without the meninges

Table 1. Mean ± SD values of measured parameters of the brain of the juvenile cattle egret

Measured parameters	Mean ± SD	No. of animals (n)
Body weight (WOB) [g]	538.33 ± 78	12
Brain weight (BRW) [g]	2.45 ± 0.34	12
Length of brain (LOB) [mm]	25.80 ± 1.98	12
Depth of brain (DOB) [mm]	16.86 ± 1.20	12
Length of cerebrum (LOC) [mm])	16.78 ± 1.15	12
Height of cerebrum (HOC) [mm]	10.83 ± 0.91	12
Width of cerebrum (WOC) [mm]	21.46 ± 1.42	12
Height of cerebellum (HCB) [mm]	8.97 ± 1.29	12
Length of cerebellum (LCB) [mm]	12.11 ± 0.68	12
Optic lobe length (OLL) [mm]	9.52 ± 0.49	12
Optic lobe width (OLW) [mm]	5.61 ± 0.32	12
Olfactory bulb length (OBL) [mm]	3.37 ± 0.56	12
Olfactory bulb width (OBW) [mm]	2.26 ± 0.19	12

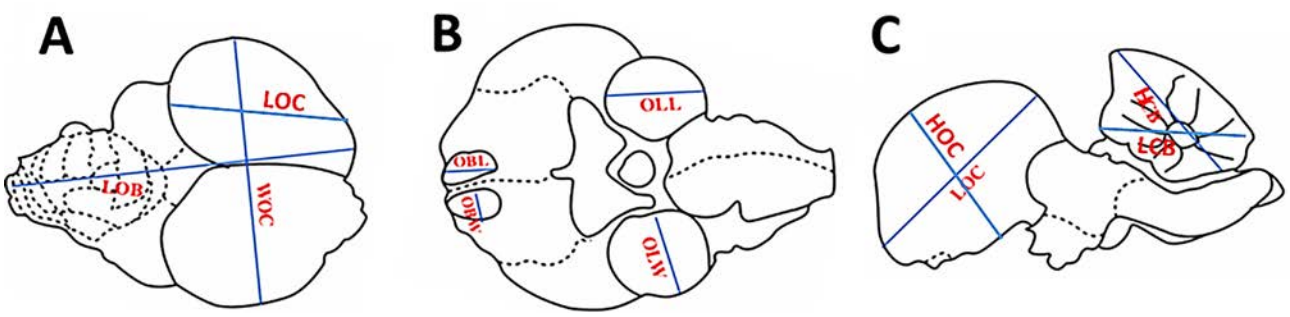

Fig. 1. Schematic illustrations of the measurements of major subdivisions of the brain of the juvenile Cattle egret (*Bubulcus ibis*)
Some of the measured gross parameters are depicted:
A – dorsal view: LOB: length of brain; WOC: width of cerebrum; LOC: length of cerebrum; **B** – ventral view: OBL: length of left olfactory bulb; OBW: width of left olfactory bulb; OLL: length of optic lobe; OLW: width of optic lobe; **C** – mid-sagittal view: LOC: length of cerebrum; LCB: length of cerebellum; HOC: height of cerebrum; HCB: height of cerebellum

- Length of brain (LOB) (mm): the maximum length from the tip of the olfactory bulb to the caudal most portion of the cerebellar cortex
- Depth of brain (DOB) (mm): the distance from the highest point of the brain (i.e., the topmost part of the cerebellum) to the lowest point of the brain (i.e., the most ventral part of the cerebrum/optic lobe)
- Length of cerebrum (LOC) (mm): the maximum length from the tip of the frontal lobe to the caudal most portion of the occipital lobe of the cerebral cortex
- Height of cerebrum (HOC) (mm): maximum dorso-ventral length of the cerebral cortex
- Height of cerebellum (HCB) (mm): maximum dorso-ventral length of the cerebellar cortex
- Length of cerebellum (LCB) (mm): maximum rostro-caudal length of the lobes of the cerebellum
- Width of cerebrum (WOC) (mm): maximum length across the most lateral portion of the parietal lobes of the cerebral cortex
- Optic lobe length (OLL) (mm): maximum length of the lobes of the optic tectum
- Optic lobe width (OLW) (mm): maximum width of the lobes of the optic tectum
- Olfactory bulb length (OBL) (mm): length of the (right or left) olfactory bulb from the tip (most rostral part) of the bulb to its caudal-most part
- Olfactory bulb width (OBW) (mm): maximum length across the latero-medial axis of the (right or left) olfactory bulb.

Histological processing of cerebellum

Immediately after the birds were sacrificed, the cerebelli were removed and fixed in 4% phosphate-buffered paraformaldehyde (Loba Chemie PVT Ltd, India). The tissue samples were processed using the routine histological technique as described by [2]. Briefly, the samples were dehydrated in graded alcohol concentrations, cleared in xylene and infiltrated and paraffin wax embedded. Surgifield SM-202A rotatory microtome was used for the sectioning at 5 µm. The processed sections were stained using Haematoxylin (CDH lab reagent, New Delhi, India) and Eosin (Kem Light lab reagent, Mumbai, India). Photomicrographs were taken at ×40, 100 and ×400 magnifications through an Olympus microscope equipped with industrial digital camera using the imaging software, MII ImageView version 3.7.9229 (YSC Technologies, Fremont, CA, USA). Histological examination was carried out on the processed tissue samples and photomicrographs of the slides were taken.

Data analysis

The numeric raw data generated from the brain gross morphometry were analysed and expressed as mean ± SD, and the Pearson correlation coefficient (R two-tailed test; $P < 0.05$) was used to compare the means and the strength between the variables and their relationships. The GraphPad Prism software (GraphPad Prism version 7 for Windows, GraphPad Software, San Diego, California USA), and the JMP SAS/STATR software version 10.0 (SAS Institute Inc., Carry, NC, USA) were used for all statistical analyses.

RESULTS

Figures 1–2 reveal the various anatomical regions of the brain of *Bubulcus ibis* studied with detailed labelling of measured parameters. The data obtained for the mean ± SD values of the measured parameters of the brain of the juvenile cattle egret and their correlation analyses were shown in Tables 1 and 2, respectively. The low and high-power photomicrographs of the H&E-stained slides of cerebellum were shown in Figure 3. The descriptive terms here adopted for the gross brain features are in accordance with [26, 38], while that of the histological features were as described by [47] and [23].

Brain gross morphology

Figures 2A-I illustrates the different anatomical regions of the brains of the cattle egrets' studied. The brain was entirely located in the cranium (Figure 2A). The brain was entirely covered and tightly packed by meninges which was a tough fibrous sheath with a whitish colouration (Figure 2B). The *Bubulcus ibis* brain basically consists of the similar components with that of mammals and regionally, it is organized into: the forebrain (prosencephalon), mid-brain (mesencephalon) and the hindbrain (rhombencephalon).

For easy description, the brain of the juvenile cattle egret in this study was divided rostro-caudally and regionally into olfactory bulb, the cerebrum, the optic lobe (lobus opticus), the cerebellum and the pons and medulla oblongata (Figures 2Di, Ei and Ii), with the various anatomical

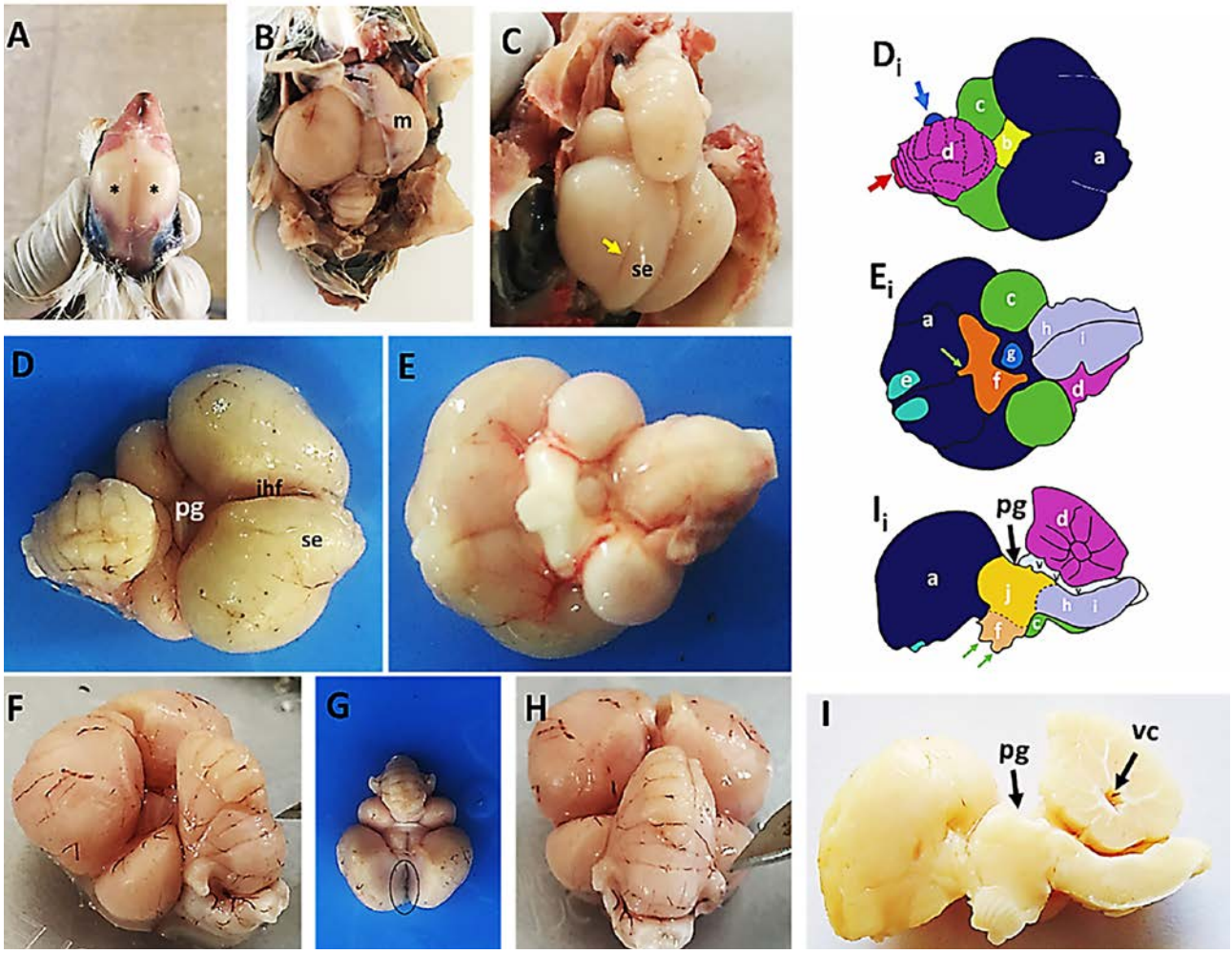

Fig. 2. Brain of the juvenile cattle egret (*Bubulcus ibis*) *in situ* and *ex-situ*

A – shows the cranial covering of the unexposed brain. Black asterisks indicate bony cranium; **B** – following the complete removal of the cranium, the portion partly covered with meninges and the exposed portion of the brain are shown. Black arrow shows the reflected part of the meninges while m is unreflected meningeal covering; **C** – show the dorsal view of the brain with the ventral part still situated in the skull (*in situ* position), with prominent appearance of "three superstructures" viz cerebrum, optic lobe and cerebellum. sagittal eminence (se), yellow arrow points to vallecula telencephalic; **D** and **G** – dorsal view, *ex situ*. Black circle placed at the interhemispheric fissure (ihc) in G indicates absence of the corpus callosum. Di illustrates prominent structures such as the cerebral cortex (a), pineal gland (b), optic tectum (c), cerebellum (d), while the blue arrow points to the auricula cerebelli and the broken white lines on the rostral part of cerebrum indicates the vallecula telencephali. Red arrow refers to the initial segment of the spinal cord. **E** – ventral view of the brain, Ei illustrates the olfactory bulb (e), optic chiasm (f), hypothalamic area (g), pons (h) and the medulla oblongata (i), while the green arrow points to the projection of the optic nerve; **F** – lateral view; **H** – caudal view; **I** and Ii mid-sagittal views – prominently show the diencephalon. Ventriculus cerebelli (vc); pineal gland (pg) (j), and the 4th ventricle (v)

Note: Di, Ei and Ii are reconstructed images

regions characterized by their own peculiarities. The anatomical characteristics of the different parts of the cattle egrets' brain are listed below:

Olfactory bulbs

Olfactory bulbs (*Bulbus olfactorius*) were very reduced and tapered to the rostro-ventral pole of the cerebral cortex. The bulbs were coplanar and conical-like in shape (Figure 2E). The olfactory bulbs were barely visible when the brain was viewed from a dorsal position (Figure 2D) but prominent in the ventral view (Figure 2E). Grossly, there was no discernible division of the olfactory brain into the olfactory bulb, the olfactory tract and the olfactory lobe. The olfactory bulb is paired and projected from the two hemispheres of the cerebrum, the two small bulges connected together by the indistinct olfactory tract. The olfactory bulb of the cattle egret assumed the same colouration as the entire brain structures, and rhinal fissure (which was a small depression) separated the olfactory bulbs from cerebral hemispheres.

Table 2. Pearson correlation coefficient (R two-tailed test) of means and strength (variables vs relationships) of measured parameters of the brain of the juvenile cattle egret

	BOW	BRW	LOB	DOB	LOC	HOC	WOC	HCB	LCB	OLL	OLW	OBL	OBW
BOW	–	0.92	0.77	0.33	0.27	0.45	0.55	0.44	0.81	0.45	0.59	0.33	-0.01
BRW	0.92	–	0.85	0.54	0.49	0.68	0.76	0.64	0.75	0.60	0.74	0.60	0.13
LOB	0.77	0.85	–	0.55	0.62	0.49	0.67	0.69	0.79	0.37	0.65	0.66	0.21
DOB	0.33	0.54	0.55	–	0.57	0.43	0.61	0.43	0.27	0.54	0.39	0.45	0.66
LOC	0.27	0.49	0.62	0.57	–	0.40	0.47	0.44	0.38	0.32	0.34	0.51	0.57
HOC	0.45	0.68	0.49	0.43	0.40	–	0.68	0.58	0.41	0.76	0.71	0.67	0.30
WOC	0.55	0.76	0.67	0.61	0.47	0.68	–	0.84	0.39	0.59	0.81	0.70	0.23
HCB	0.44	0.64	0.69	0.43	0.44	0.58	0.84	–	0.34	0.21	0.83	0.90	0.25
LCB	0.81	0.75	0.79	0.27	0.38	0.41	0.39	0.34	–	0.47	0.44	0.26	0.04
OLL	0.45	0.60	0.37	0.54	0.32	0.76	0.59	0.21	0.47	–	0.46	0.21	0.26
OLW	0.59	0.74	0.65	0.39	0.34	0.71	0.81	0.83	0.44	0.46	–	0.81	0.16
LOH	0.01	0.16	0.21	0.45	-0.04	0.24	0.23	0.44	0.15	0.05	0.29	0.44	0.25
OBL	0.33	0.60	0.66	0.45	0.51	0.67	0.70	0.90	0.26	0.21	0.81	–	0.24
OBW	-0.01	0.13	0.21	0.66	0.57	0.30	0.23	0.25	0.04	0.26	0.16	0.24	–
WHO	0.36	0.48	0.55	0.35	0.43	0.28	0.43	0.74	0.27	-0.11	0.53	0.76	0.30

Cerebral hemispheres

The cattle egret brain is lissencephalic, with the cerebral cortex (*pallium*) smooth-surfaced and lacking a discernable gyri and sulci. From a dorsal view, the shape of the cerebrum assumes that of an obtuse triangle, there was a hemicycle-like expansion of the left and right lateral parts, and a wide posterior part in contact with the most rostral portion of the optic lobe (Figures 2D, F, H). The external surface appeared smooth and the corpus callosum was conspicuously absent (Figure 2G). A prominent interhemispheric fissure separates the two cerebral hemispheres (Figure 2D). Each hemisphere presented a shallow groove with a caudo-lateral orientation, this groove is known as the *vallecula telencephalic* which has an appearance of a cambered super-sulcus, and was richly engrossed by large blood vessels that caudally tappers out (Figure 2Di). Between the interhemispheric fissure and the vallecula is an ellipse-shaped bilateral protuberance known as the sagittal eminence (*eminentia sagittalis*), and this appeared slightly elevated above the cerebral cortical portion lateral to it (Figures 2C-D, Di).

There was a prominent indention (due to their relatively large eyes of this bird) on the lateral aspects of the cerebral hemispheres known as the *fovea limbica*. The medial surface of the cerebrum was smooth and straight (Figure 2I). A transverse sulcus (*Fissura subhemispherica*) was present between the most caudal part of the cerebral hemispheres and the rostral part of the cerebellum it borders (Figures 2D-H).

Diencephalon

The diencephalic structures were located on the ventral aspect of the cerebral hemispheres and were completely covered by telencephalon. They were almost invisible in the dorsal and ventral views (Figures 2D-E) of the brain but prominent in the mid-sagittal/median section of the brain (Figure 2I). The diencephalic structures represent the rostral limit of the brain stem and they were completely covered by the cerebral hemispheres (*hemispherium cerebri*). The pineal gland is a small structure occupying the polygonal space at the junction of the transverse sulcus, cerebral hemispheres, optic lobe and the cerebellum (Figures 2D, G). The diencephalon was bilaterally linked with the mesencephalic tectum, with the pineal gland appearing to form its dorsal limit (Figures 2D, G, I).

Mesencephalic tectum

The mesencephalon of the cattle egret brain is chiefly constituted by the mesencephalic tectum (optic lobe), a monologue of the mammalian anterior colliculi. The mesencephalic tectum formed the bulk of the roof of the midbrain. The optic lobes were large, paired oval eminences protruding laterally and ventrally from the surface of the midbrain, and lying lateral to the more ventral tegmentum (Figures 2D-H). The midbrain bordered the rostral portions of the rhombencephalon (cerebellum and pons), with a transverse furrow delineating the boundary between the mesencephalon and the rostral rhombencephalon. The optic lobes receive the fibers (optic tract) of the optic chiasma at its convergence in the ventro-median line (Figure 2E). No obvious demarcation exists between the midbrain, pons and the medulla oblongata.

Cerebellum

The metencephalon comprises the pons and cerebellum, while the myelencephalon is constituted by the medulla oblongata. The cerebellum of the cattle egret encloses the fourth ventricle (*ventriculus quartus*) and forms its lateral walls and roof (Figure 2I).

The cerebellar cortex lies over the pons, medulla oblongata, and the median segment of the mesencephalon (Figures 2F-H). It is connected bilaterally to the brainstem by rostral and caudal cerebellar peduncles (one of these is evident in Figure 2I). The superficial layer of the cerebellum (cerebellar cortex) is grey matter and it covers the internal white matter (cerebellar medulla). There was a centrally-placed apparent vermis and laterally positioned auricles. Multiple strips of superficial and deep transverse sulcus distributed on the cerebellar vermis divide the cerebellar vermis into lobules. The cerebellar auricles are the paired bilateral posterio-lateral extensions of the cerebellum and were located at the ventrolateral surface of the cerebellar vermis (Figures 2D, F, H). Dorsally, there was an extension of the fourth ventricle into the cerebellum and this is known as the *ventriculus cerebelli* (vc) (Figure 2I).

Pons and medulla oblongata

The pons and medulla oblongata were located on the ventral surface of the cerebellum. They are the two posterior parts of the brain stem that extend from the most caudal part of the midbrain to the occipital foramen. A ventral middle fissure (a longitudinal supersulcus), the *fissura mediana ventralis* runs its course medially through the pons and the medulla oblongata (Figures 2E, Ei). The dorsal surface of the pons, jointly with the medulla oblongata, form the ventral wall for the fourth ventricle, with their ventral surface forming the lowest spot of the brain stem (Figures 2E, I). there appears to be no discernible borders delineating the boundaries of the midbrain, pons and medulla oblongata.

Brain weight and gross morphometry

Figure 1 illustrates the landmarks for the gross morphometric parameters taken and the results of the morphometric measurements were presented in Table 1 and the correlations analyses of the parameters were presented in Table 2.

The average recorded body weight was 538.33 ± 78 g, while the brain weight was 2.45 ± 0.34 g, implying that the brain constituted about 0.46 % of the total body mass (Table 1). The cerebral cortex length makes the most of the brain length with a contribution of about 52 %, followed by the cerebellum (about 38 %) and olfactory bulb (about 10 %) to the total brain length value, as shown in Table 1. The optic lobe of the cattle egret was about as twice longer than its wide, with the olfactory bulb length and width values sharing similarity in values.

The body weight was strongly positively correlated with the brain weight ($r = 0.92$; $P < 0.001$) and the length of the brain ($r = 0.77$; $P = 0.001$). As shown in Table 2, the brain weight was strongly positively correlated with the length of the brain ($r = 0.85$; $P < 0.001$), width of cerebrum ($r = 0.76$; $P = 0.004$) and the length of cerebellum ($r = 0.75$; $P = 0.005$). Also, the olfactory bulb length was strongly positively correlated with the height of cerebellum ($r = 0.90$; $P < 0.001$), and width of the optic lobe ($r = 0.81$; $P = 0.002$).

Cerebellar histology

The histological appearance of the cerebellum (Figures 3 A-B) was similar in all the examined animals. The cerebellar cortex had the three typical cerebellar layers (Figures 3 C-F) and there was the innermost cerebellar medulla (Figures 3 C-D). The molecular cell layer was the outermost layer and it is composed of small neurons and neuroglia. The Purkinje cell layer was sandwiched between the outer molecular and inner granular layers, and it presented a single row of predominantly flask/pear-shaped

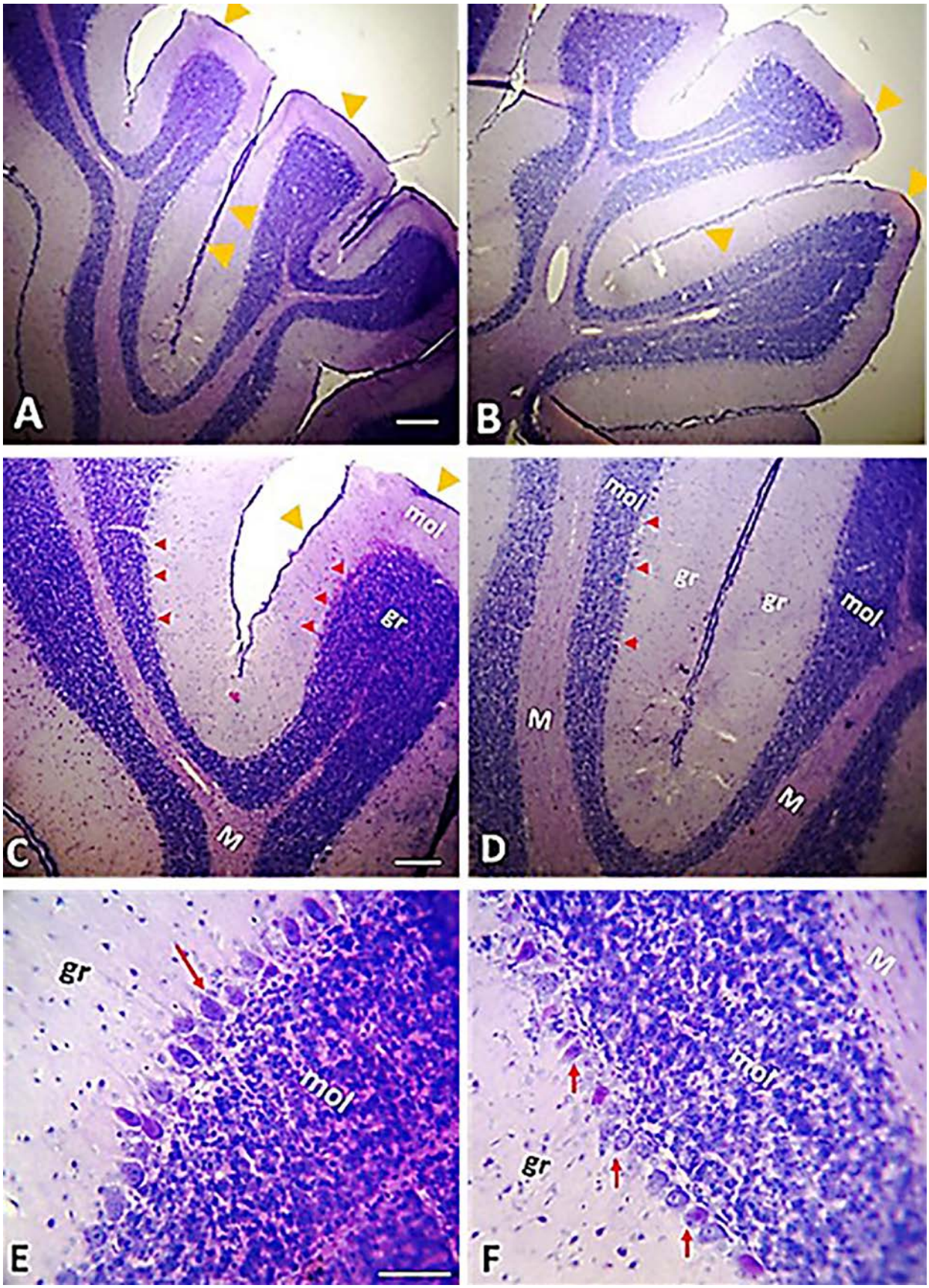

Fig. 3. Juvenile cattle egret cerebellum

A–D – low power photomicrographs. E–F – high power photomicrographs. External granular layer (yellow arrow heads), molecular layer (mol), inner granular layer (gr), Purkinje cell layer (red arrow heads), M – medulla, Purkinje cells (red arrows) Scale bars: A–B, 150 µm; C–D, 100 µm; E–F, 50 µm

cells and some round/oval-shaped cells, showing distinct nuclear materials and nucleoli, and bearing prominent dendrites with threadlike appearances (Figures 3 E-F). Of note, there were traces of persistent external granular layer, as evident in Figures 3 A-C. The inner granular layer presented numerous round granule cells, and the cerebellar white matter possessed sparse granule neurons enriched with cellular processes.

DISCUSSION

We here described the gross anatomical features and gross morphometry of the juvenile cattle egret (*Bubulcus ibis*) brain as well as the cerebellar histology. The present study showed that the brain of the cattle egret consists of prosencephalon, mesencephalon and the rhombencephalon and this conforms to the structural pattern of brain morphology in avians [26].

Grossly, the olfactory bulbs of the cattle egret were rudimentary and poorly developed (relatively to the other structures) with no discernible division of the olfactory brain into the olfactory bulb, the olfactory tract and the olfactory lobe, with the two small bulges connected together by the indistinct olfactory tract and were barely visible from a dorsal position but prominent in the ventral view. This shares similarity with the observation of rudimentary olfactory bulbs in Sparrowhawk (*Accipiter nisus*), Coturnix quail and domestic birds [4, 12, 31, 37], however, some diurnal birds such as the wild African parrot, canaries and vultures [36, 51], as well as nocturnal birds' species such as the Kiwi and Barn owl reportedly have well developed olfactory bulbs [31]. In most avian species, previous evidences suggested that structural parts associated with sense of smell were clearly atrophied and poorly developed relatively to some other species besides avians. Comparatively, the observation by U s e n d e et al., [50] in the reptilian African side necked turtle (*Pelusios castaneus*), revealed that the olfactory brain was elaborate, and presented an initial olfactory bulb, a distinct middle olfactory tract and a caudal olfactory lobe. This may not be unusual, as well developed and conspicuously evident olfactory brains in lateral, ventral and dorsal views are typical of reptiles, rodents and Siluriformes brains [35, 49, 54]. However, in similarity, completely inconspicuous and dorsally invisible olfactory bulbs have been reported in man and elephant [46].

Also, we reported herein that the cerebral hemispheres of the cattle egret appeared smooth, lacking gyri and sulci, a phenomenon known as lissencephaly. Generally, the avian brains are regarded as lissencephalic. Smooth-surfaced brains have been reported in birds such as the Coturnix, African ostrich, Sparrowhawk and domestic birds [4, 10, 12, 37, 43] in accordance with our findings. Of note, similar lissencephalic brains have also been reported in some rodents such as the African giant and grasscutter rats, reptiles, and sea turtles [8, 19, 39, 54].

We observed the prominent bulge of the sagittal eminence and obvious vallecula telencephali on the dorsal surface of the cerebral hemispheres, which conforms to the observations in the wild African Parrot [51] and Macaw [34], Common starling [10], African ostrich [43], Emu (a diurnal, flightless) and Barn owl (a nocturnal flying bird), but those features were reportedly less pronounced in the various breeds of chicken [5, 13, 14, 42], pigeon (a diurnal flying bird) and non-prominent in the Kiwi, a nocturnal flightless bird [31]. The vallecula in avian supports visual acuity, and its prominence could enhance the visualization of prey, especially in wild birds [51].

The cattle egret possessed a well-developed optic lobe which bulged laterally with prominent optic tracts appearing as large bands of the white matter on the ventro-medial parts of the optic lobes. The optic tracts crossed just cranial to the hypothalamic portion of the diencephalon bearing a connection with hypophysis to form the optic chiasma. This clearly conforms to the observations of grossly developed and highly visible mesencephalic tectum in the Sparrowhawk [4], Coturnix quail [12], African ostrich [25, 43], domestic birds [37] and the pigeon [18, 31] and helmeted Guinea fowl [52], but differs from the reportedly less developed optic lobes of the Barn owl, Kiwi, Humming birds and wild African parrot [31, 51]. Birds are strongly visual animals as a result of the extensive development of nuclei and pathways concerned with processing of visual signalling in the CNS [26]. This likely explains the large mesencephalic tectum (optic lobe) of the cattle egret which appeared as paired oval eminences forming the bulk of the midbrain.

It's been reported that morphometric analyses of organs may elucidate minute structural changes that may not be evident in ordinary qualitative analyses, and quantitative assessments have been used to unveil the morphologic and functional capabilities of brain regions in different animals [40]. Since the beginning of the 19th century,

documentations of the morphometric studies on various organs of avian species started and reports on it has been on the increase. So far in the last two decades, several comparative studies have been reported in which different variables such as the ecological and social attributes have been deployed to expound the variabilities in vertebrate species' brain sizes [47]. Also, environmental factors like an animal's habitat, lifestyle, and their morphological and physiological parameters, have been correlated to their evolutionary history [6, 43).

In this study, the average recorded body weight of the cattle egret was 538.33 ± 78 g, this is in line with the weight range (270–512 g) of the cattle egret as documented by Telfair and Raymond [48]. Our body weight data strongly positively correlated with the brain weight ($r = 0.92$; $P < 0.001$). The average length of brain here recorded for the cattle egret was 25.80 ± 1.98 mm, this value was similar to that of Sparrowhawk (24.3 mm) but higher than that of Coturnix quail brain (16 mm) [4, 12]. Significant increase in the length and width of the cerebral hemispheres with advancing age has been reported in the chicken [13, 42]. Interestingly, Peng et al. [43] and Karkoura et al. [25] reported higher values in the average mean length and width of optic lobes of adult African ostrich than we recorded in our study on the juvenile cattle egret. Wanmi et al. [52] reported that the mean weight of the midbrain of the helmeted Guinea fowl increased with advancing in age, hence the variability in morphometry of optic lobe and brain length might be due to age and/or species differences. Overall, our regression analysis data ($r^2 = 0.98$) indicates that the weight of the animal can be excellently predicted from parameters such as the brain length, cerebral width, cerebellar height, optic lobe width and the olfactory bulb length (data not shown).

The developmental form in various avian species has been reported to correlate with the differences in their relative brain volume [21]. The average brain weight of the cattle egret used in this study was 2.45 ± 0.34 g, this was slightly lower than average weight (3.00 ± 0.2 g) of the Sparrowhawk brain [4] and much lesser than that of the domestic fowl (3.34 ± 0.08 g) [15]. The juvenile cattle egret brain weight constituted about 0.46 % of the total body mass. This is in contrast to reports in some avian species, where the relative brain to body mass index was reported to be 0.015 % in the African ostrich, 0.26 % for the oriental white stork, 0.26 % in the Pekin duck, and the grey goose brain accounted for 0.25 % of the total body weight [43]. The above report suggests that the encephalic ratio of the cattle egret is the highest compared to the other listed birds. This could be due to the age categories of birds under consideration as we used juvenile age group in our study, whereas the adult age bracket was considered for the African ostrich study with the age groups for the other birds undisclosed by their authors. Also, relatively larger brains could be associated with the length or prolongation of the nestling stage of birds [21].

The gross anatomical organisation and histological layering of the cerebellum is similar in most vertebrates, though some differences have been reported according to species' general morphology and behavioural attributes [22, 43, 47]. Iwaniuk et al. [23] reported on the cerebellum of cattle egret using a line drawing. The authors showed that the cattle egret cerebellar folia organization followed the "typical" avian cerebellar morphology, with a presentation of eleven major folia. The result of our study on the juvenile age group was in conformity with the above report, and were have also shown the cellular organization of the cerebellar layer of this bird by the use of the H&E stain. Interestingly, the cerebellar histology of the juvenile cattle egret we here studied presented a persistent external granular layering suggestive of a potential for adult neurogenesis. The cerebellum is widely used in studies on motor system, this is because it is responsible for the maintenance of equilibrium and motor disorders are mostly associated with cerebellar dysfunctions. The cellular components of the cerebellum function as a control system located between motor and cerebellar dysfunctions pathways [41]. Our gross description of the cattle egret cerebellum aligned with the reports on that of the African ostrich, Sparrowhawk and domestic birds [25, 37, 43], but in contrast with the small, rounded/oval and unfolded cerebellum of the wild African parrot [51]. Of note, most large-sized birds (especially flightless or short distance flyers) reportedly possess relatively small-size of cerebellum [24].

The most emphasized and studied cells of the cerebellum in behavioural and cognitive studies are the Purkinje cells, especially with respect to their functional capability. A comparative study in the pigeon, turkey, ducks and starling revealed that increasing number of Purkinje cell resulted in an increase in behavioural complexity and cognitive ability [47]. The presence of complicated pleats in

the superficial grey matter of the cerebellum has been attributed to its' larger surface area. In the African ostrich, the cerebellum protrudes visibly upward and its length was reported to be about 1.5 times larger than its height [43]. These findings conform to our observation in the cattle egret, with cerebellum elevated above the cerebral hemispheres and the cerebellar length 1.4 times larger than the cerebellar height. Our histological profiling of the cattle egret cerebellum has added further data and relevant literature to comparative avian cerebellar histology.

CONCLUSIONS

The morphological, morphometric and histological studies on the cattle egret brain are very important for comparative and developmental studies and evolutionary neuroscience, as the research on cattle egret brain morphology during the different developmental phases is lacking in the literature. Hence, we believe that the macro-anatomical structures and histology of cerebellum of cattle egret here described has provided supplementary data to the basic information on the Avian CNS. However, further studies are required especially on the aging-associated morphological and morphometric indices, as studies across age groups are of great value to set standards of comparison for research. Also, the detailed description of the internal structure of the cerebrum as well as the embryonic development/foliation levels of the cerebellum in this species will be described in further studies. Our future research directions also entail the neurocellular and neurochemical profiling of the distinct cells in various regions of the cattle egret brain.

DECLARATION OF COMPETING INTEREST

All authors declare no conflicts of interest.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Ogunleye Olusola of the Department of Veterinary Anatomy, University of Jos for his technical assistance in the preparation of the histological slides.

REFERENCES

1. **Azeez, I. A., Ekeolu, O. K., Usende I. L., Adejumobi, O. A., Adetogun, A., 2022:** Anatomical investigation on the craniofacial and ocular morphometrics of the male western cattle egret (*Bubulcus ibis*). *Anat. J. Africa*, 11, 1, 2033–2042. DOI: 10.4314/aja.v11i1.2.
2. **Azeez, I. A., Hena, S. A., Ogwujo, P. O., Omirinde, J. O., Akinsola, O. M., Plang, N. J., Gosomji I. J., 2022:** Comparative skin anatomy of the Nigerian local dogs and pigs. *Savannah Vet. J.*, 5, 1, 20–27. DOI: 10.36759/svj.2021.158.
3. **Azeez, I. A., Olopade, F., Laperchia, C., Andrioli, A., Scambi, I., Onwuka, S. K., et al., 2016:** Regional myelin and axon damage and neuroinflammation in the adult mouse brain after long-term postnatal vanadium exposure. *J. Neuropathol. Exp. Neurol.*, 75, 9, 843–854. DOI: 10.1093/jnen/nlw058.
4. **Balkaya, H., Toprak, B., 2018:** External anatomical structures of sparrowhawk (*Accipiter nisus*) encephalon. *Ind. J. Anim. Res*., 52, 9, 1281–1284. DOI: 10.18805/ijar.11157.
5. **Batah, A. L, Ghaje, M. S., Aziz, S. N., 2012:** Anatomical and histological study for the brain of the locally breed chicken. *J. Thi-Qar Sci.*, 3, 3, 47–53.
6. **Bartyzel, B., Kobryn, H., Szara, T., Podbielska, I., Myslek, P., 2003:** Heart size in wood pigeon *Columba palumbus* (Linnaeus, 1758). *Veterinarija Ir Zootechnika T.*, 21, 43, 9–12.
7. **Burish, M. J., Kueh, H. Y., Wang, S. S. H., 2004:** Brain architecture and social complexity in modern and ancient birds. *Brain Behav. Evol.*, 63, 2, 107–24. DOI: 10.1159/000075674.
8. **Byanet, O., Onyeanusi, B. I., Ibrahim, N. D. G., 2009:** Sexual dimorphism with respect to the macro-morphometric investigations of the forebrain and cerebellum of the grasscutter (*Thryonomys swinderianus*). *Int. J. Morphol.*, 27, 2, 361–365.
9. **Catala, M., Kubis, N., 2013:** Gross anatomy and development of the peripheral nervous system. *Handb. Clin. Neurol.*, 115, 29–41. DOI: 10.1016/B978-0-444-52902-2.00003-5.
10. **Dhage, S. A., Shehan, N. A., Ali, S. A., Aziz, F. H., 2013:** Anatomical and histological study of cerebral in *Sturnus vulgaris. Bas. J. Vet. Res.*, 12, 2, 21–227. DOI: 10.33762/bvetr.2013.83674.
11. **Esteves, M., Almeida, A., Leite-Almeida, H., 2020:** Insights on nervous system biology and anatomy. *Handbook of Innovations in Central Nervous System Regenerative Medi-*

cine. Elsevier, 28 pp. DOI: 10.1016/B978-0-12-818084-6.00001-5.

12. **Fitzgerald, T. C., 1969:** *The Coturnix Quail, Anatomy and Histology*. Iowa State University Press, Ames, Iowa, 171–205.
13. **Gautam, A. K., Ray, S., Das, P., Mandal, A. K., Tudu, N. K., Singh, D., Islam, M., 2020:** Macroscopic study on the cerebrum of post hatch broiler chicken with reference to age. *J. Entomol. Zool. Stud*., 8, 3, 1926–1929.
14. **Gupta, S. K., Behera, K., Pradhan, C. R., Mandal, A. K., Shethy, K., Behera, D., et al., 2016:** Biometry of the different brain parts in Vencobb and Vanaraja birds. *Ind. J. Vet. Anat.*, 28, 1, 33–35.
15. **Gupta, S. K., Haswitha, S., Kaur, A., 2019:** Gross morphometrical study on the brain of fowl (*Gallus domesticus*). *Ind. J. Vet. Sci. Biotechnol.*, 14, 4, 33–35.
16. **Halidu, S. K., Wahab, M. K. A., Ibrahim, A. O., Chekezie, J., 2020:** Food and feeding ecology of Cattle Egret (*Bubulcus ibis*) in Federal College of Wildlife Management, New-Bussa, Niger state, Nigeria. *Int. J. Sci. High Technol.*, 22, 2, 152–156.
17. **Hamza, I. M., Mobarak, Y. M., Hafez, H. S., 2021:** Hippocampus anatomical structure and distribution of agrin proteoglycan and ryanodine receptor expression boost bird food caching behaviour. *Europ. Zool. J.*, 88, 1, 303–315.
18. **Husband, S. A., Shimizu, T., 1999:** Efferent projections of the ectostriatum in the pigeon (*Columba livia*). *J. Comp. Neurol.*, 406, 329–345.
19. **Ibe, C. S., Onyeansusi, B. I., Hambolu, J. O., 2014:** Functional morphology of the brain of the African giant pouched rat (*Cricetomys gambianus* Waterhouse, 1840). *Onderstepoort J. Vet. Res.*, 81, 1, 1–7. DOI: 10.4102/ojvr.v81i1.644.
20. **Ivory, A., 2000:** *"Bubulcus ibis"* (Online), Animal Diversity Web., https://animaldiversity.org/accounts/Bubulcus_ibis/.
21. **Iwaniuk, A. N., Nelson, J. E., 2003:** Developmental differences are correlated with relative brain size in birds: A comparative analysis. *Can. J. Zool.*, 81, 1913–1928. DOI: 10.1139/Z03-190.
22. **Iwaniuk A. N., Hard P. L., Wylie, D. R. W., 2006:** The comparative morphology of the cerebellum in caprimulgiform birds: Evolutionary and functional implications. *Brain Behav. Evol.*, 67, 53–8. DOI: 10.1159/000089120.
23. **Iwaniuk, A. N, Hurd, P. L., Wylie, D. R. W., 2007:** Comparative morphology of the avian cerebellum: II. Size of folia. *Brain Behav. Evol.*, 69, 3, 196–219. DOI: 10.1159/000096987.
24. **Kappers, C. U., 1936:** The cerebellum. *The Comparative Anatomy of the Nervous System of Vertebrates Including Man*. Vol. II. Heffner Publishing Company, New York, 696–860.
25. **Karkoura, A. A., Alsafy, M. A. M., Elgendy, S. A. A., Eldefrawy, F. A., 2015:** Morphological investigation of the brain of the African Ostrich (*Struthio camelus*). *Int. J. Morphol.*, 33, 4, 1468–1475.
26. **König, H. E., Korbel, R., Liebich, H. G., 2016:** *Avian Anatomy: Textbook and Colour Atlas*. 2nd edn., 5M Publishing Ltd, Sheffield, UK, 360 pp.
27. **Kubke, M. F., Wild, J. M., 2009:** Evolution of avian brains. In **Binder, M. D., Hirokawa, N., Windhorst. U. (Eds.), Butler, A. (Section Ed.):** *Encyclopedia of Neuroscience*. Springer-Verlag GmbH, Berlin, Heidelberg, 1312–1318.
28. **Lierz, M., Korbel, R., 2012:** Anaesthesia and analgesia in birds. *J. Exot. Pet. Med*., 21, 1, 44–58. DOI: 10.1053/j.jepm.2011.11.008.
29. **Maddock, M., Geering, D., 1994:** Range expansion and migration of the cattle egret. *Ostrich*, 65, 2, 191–203. DOI: 10.1080/00306525.1994.9639682.
30. **Majekodunmi, A. O., Fajinmi, A., Dongkum, C., Shaw, A. P. M., Welburn, S. C., 2014:** Pastoral livelihoods of the Fulani on the Jos Plateau of Nigeria. *Pastoralism*, 4, 1, 1–16.
31. **Martin, G. R., Wilson, K. J., Wild, J. M., Parsons, S., Kubke, M. F., Corfield, J., 2007:** Kiwi forego vision in the guidance of their nocturnal activities. *PLOS ONE*, 2, 2, e198. DOI: 10.1371/journal.pone.0000198.
32. **Mckilligan, N., 2005:** *Herons, Egrets and Bitterns: Their Biology and Conservation Status in Australia*. Csiro Publishing, Collingwood, Australia, 144 pp.
33. **Medina, L., Reiner, A., 2000:** Do birds possess homologues of mammalian primary visual, somatosensory and motor cortices? *Trends Neurosci.*, 23, 1, 1–12.
34. **Melo, L. F., Azarias, F. R., Walther, M. E. F., Aquino, J. B., Rodrigues, R. F., Melo, A. F., et al., 2022**: Comparative topographical description of the central nervous system of the macaw (*Ara ararauna*) and the owl (*Tyto furcata*). *Int. J. Morphol.*, 40, 3, 860–866.
35. **Musa, S. A., 2015:** *Morphological and Stereological Studies of the Olfactory Bulb and Cerebrum of the African Giant Pouched Rat* (*Cricetomys gambianus* Waterhouse 1840). PhD. Thesis, Department of Human Anatomy, College of Health Science, Ahmadu Bello University, Zaria, Nigeria, 141 pp.
36. **Nevitt, G., 1999:** Foraging by seabirds on an olfactory landscape. *Am. Sci.*, 87, 46–54.

37. **Nickel, R., Schummer, A., Seifirle, E., 1977:** *Anatomy of the Domestic Birds*. Verlag Paul Parey, Berlin, Hamburg, 114–142.
38. ***Nomina Anatomica Veterinaria*, 2017:** *Nomina Anatomica Veterinária*. 6th edn., prepared by the International Committee on Veterinary Gross Anatomical Nomenclature (I.C.V.G.A.N.) Published by the Editorial Committee Hanover (Germany), Ghent (Belgium), Columbia, MO (U.S.A.), Rio de Janeiro (Brazil), 178 pp.
39. **Nzalak, J. O., Byanet, O., Salami, S. O., Umosen, A. D., Maidawa, S. M., Ali, M. N., Imam, J., 2008:** Comparative morphometric studies of the cerebellum and forebrain of the African giant rat (AGR) (*Cricetomys gambianus*, Waterhouse) and that of grasscutter (*Thryonomys swinderianus*). *J. Anim. Vet. Adv.*, 7, 9, 1090–1092. DOI: javaa.2008.1090.1092.
40. **Olude, M. A., Mustapha, O. A., Olopade, J. O., 2016:** Morphological characterization of the African Giant Rat (*Cricetomys gambianus*, Waterhouse) brain across age groups: Gross features of cortices. *Niger. J. Physiol. Sci.*, 31, 133–138.
41. **Pal, B., Chowdhury, S., Ghosh, R. K., 2003:** Comparative anatomical study of the cerebellum of man and fowl. *J. Anat. Soc. Ind.*, 52, 32–37.
42. **Panigrahy, K. K., Behera, K., Mandal, A.K., Sethy, K., Panda, S., Mishra, S. P. et al., 2017**: Gross morphology and encephalization quotient of brain in male and female Vanaraja chicken at different age. *Explor. Anim. Med. Res.*, 7, 1, 28–32.
43. **Peng, K., Feng, Y., Zhang, G., Liu, H., Song, H., 2010:** Anatomical study of the brain of the African ostrich. *Turk. J. Vet. Anim. Sci.*, 34, 3, 235–241.
44. **Seedikkoya, K., Azeez, P. A., Abdul Shukkur, E. A., 2007:** Cattle egret as a biocontrol agent. *Zoos' Print J.*, 22, 10, 2864–2866. DOI: 10.11609/jott.zpj.1731.2864-6.
45. **Shimizu, T., Patton, T. B., Husband, S. A., 2010:** Avian visual behaviour and the organization of the telencephalon. *Brain Behav. Evol.*, 75, 3, 204–217.
46. **Shoshani, J., Kupsky, W. J., Marchant, G. H., 2006:** Elephant brain: Part I: Gross morphology, functions, comparative anatomy, and evolution. *Brain Res. Bull.*, 70, 2, 124–157. DOI: 10.1016/j.brainresbull.2006.03.016.
47. **Sur, E., Öznurlu, Y., Özaydin, T., Çolakoğlu, F., Ünsall, S., Yener, Y., 2011:** Comparative histometrical study of the cerebellum and the determination of some Agnor parameters in different avian species. *Bull. Vet. Inst. Pulawy*, 55, 261–265.
48. **Telfair, R. C., Raymond, C., 2006:** Cattle Egret (*Bubulcus ibis*). In **Poole, A. (Ed.):** *The Birds of North America*. Cornell Lab of Ornithology, Ithaca, Available at http://bna.birds.cornell.edu.hal.weber.edu:2200/bna/species/113doi:10.2173/bna.113.
49. **Usende, I. L., Oyelowo, F. O., Adamu, J. I., Azeez, I. A., Shokeye, I., Tags, S. Z., Ekeolu, O. K., 2020:** Investigation of some aspects of the neuro-morphometry of the African catfish (*Clarias gariepinus*). *J. Vet. Biomed. Sci.*, 2, 2, 19–29. DOI: 10.36108/jvbs/9102.20.0230.
50. **Usende, I. L., Attah, O. R., Oyelowo, F. O., Shokoye, I., Rassaq, A. A., Madubuike, S. A. 2022:** Morphology and morphometry of the brain of African side necked turtle (*Pelusios castaneus*): A preliminary investigation. *Sahel J. Vet. Sci.*, 19, 3, 20–27.
51. **Wanmi, N., Samuel, C. I., Abdullahi, S. S., 2018:** Anatomical investigation into the brain of wild African parrot (*Poicephalus sengalusversteri*): Gross and quantitative study. *Int. J. Brain Cogn. Sci.*, 7, 2, 31–35. DOI: 10.5923/j.ijbcs.20180702.01.
52. **Wanmi, N., Usendi, I. L., Gosomji, I. J., Pindar, D. B., 2019:** Histology of the midbrain of the grey breasted helmeted guinea fowl (*Numida meleagris galeata*) at post-hatch. *J. Vet. Med. Anim. Health*, 11, 4, 88–93. DOI: 10.5897/JVMAH2017.0600.
53. **Wylie, D. R., Gutiérrez-Ibáñez, C., Iwaniuk, A. N., 2015:** Integrating brain, behaviour, and phylogeny to understand the evolution of sensory systems in birds. *Front. Neurosci.*, 9, 281.
54. **Wyneken, J., 2007:** Reptilian neurology: Anatomy and function. *Vet. Clin. Exot. Anim.*, 10, 837–853. DOI: 10.1016/j.cvex.2007.05.004.

Received April 14, 2023
Accepted June 2, 2023