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Rabies Remains a Persistent and Growing Public Health Challenge in Pakistan



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Rabies is one of the most neglected chronic endemic diseases and preventable infectious disorders in the Indo-Pak subcontinent. It is a viral zoonotic disease with dogs as the primary source of transmission, and is a significant problem in many developing countries. According to the World Health Organization, over 55,000 people die from rabies each year, with more than 31,000 of those deaths occurring in Asia, primarily affecting children [1]. Pakistan, ranks third in the world for rabies fatalities, with approximately 2,490 deaths annually. Rabies is primarily transmitted through dog bites. Initial symptoms include fever, pain, hallucinations, photophobia, and when virus enters brain, it damages CNS and spinal cord leading to paralysis, coma, and death. While preventive treatments such as post-exposure prophylaxis (PEP) can save lives, a lack of awareness, non-vaccinated dogs, limited access to healthcare, and poor clinical diagnosis is leading cause of morbidity and mortality, particularly in rural areas [2,3].

Previous studies in Pakistan have revealed high burden of rabies in both urban and rural populations with 19.13% rabies-related deaths. Most of these deaths were in villages. The findings also indicated a poor prophylaxis with 40% bite victims visiting hospitals, 24% seeking spiritual remedies, and 11% receiving no treatment at all. This highlights a critical gap in healthcare access with diagnosis of rabies, and the need for raising awareness about the importance of seeking medical treatment for dog bites in rural communities [4].

Another study in Pakistan pointed out that cases of dog bites particularly in Karachi are underreported with many of their victims failing to receive medical attention. Karachi was also declared as a hot spot in terms of rabies exposure, mainly because of the huge number of stray dogs listed there. This highlights the pressing need to implement specific disease-controlling interventions, including enhancing access to healthcare and awareness of the risks of rabies and the need to obtain medical treatment in a timely manner. [5].

Additionally, lack of coordinated efforts, such as mass immunization, effective dog control efforts, has worsened the spread of rabies in Pakistan. Despite the strategies that have been outlined by the World Health Organization (WHO) and other international organizations to eliminate human rabies spread by dogs by the year 2030, Pakistan is not doing much to do so. Studies show that regular and accessible rabies prophylaxis, including mass dog vaccination, substantial publicity, and the establishment of well-equipped diagnostic centers are needed to control and prevent the disease [6]. A coordinated strategy of government agencies, health organization and local communities can be an important aspect of controlling rabies to avoid future deaths. The government should focus on the human vaccination, and on controlling the dog population, including making rabies vaccines readily available and by injection in hospitals especially in rural locations, and by taking measures to control dog population.



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Original Article



Centrorhynchus saluni n.sp. (Acanthocephala: Centrorhynchidae) from the Greater Coucal *Centropus sinensis* (Cuculiformes: Cuculidae) in Saleh Pat, Sukkur, Sindh, Pakistan

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ABSTRACT

The Greater Coucal (*Centropus sinensis*) is a widespread, non-migratory resident bird of Pakistan that inhabits diverse habitats, including agricultural landscapes and forested areas.

Objectives: To describe a new species of *Centrorhynchus* from the Greater Coucal in Sindh, contributing to the knowledge of acanthocephalan diversity in Pakistan and the global taxonomy of the genus. **Methods:** Three Greater Coucals (*Centropus sinensis*) were collected through purposive sampling during routine avian surveys in Saleh Pat, Sukkur District, Pakistan. Gastrointestinal tracts were dissected and screened under a stereomicroscope. Worms were relaxed in hot water, fixed in AFA, stained with borax carmine, dehydrated through graded ethanol, cleared, and mounted in Canada balsam. Line drawings (Olympus BH2-DA) and photomicrographs (OMAX 10 MP) were prepared. Diagnostic features were consistent across specimens. The approach aligns with established acanthocephalan taxonomy protocols, where complete specimens validate new species, and the study focused on description rather than ecological statistics. **Results:** Seven acanthocephalans were recovered from the intestines of two Greater Coucals (*Centropus sinensis*), representing a new species, *Centrorhynchus saluni* n. sp. Male measured 6.27–6.70 mm, and female 7.41–7.92 mm. Diagnostic features include specific proboscis and receptacle dimensions, cylindrical lemnisci longer than the receptacle, tandem to slightly oblique testes, six elongated cement glands, and a posterior bursa in male. Female possess a uterine bell with eggs measuring 0.075–0.085 × 0.030–0.038 mm. **Conclusions:** *Centrorhynchus saluni* n. sp., discovered in the Greater Coucal from Sindh, Pakistan, enhances knowledge of *Centrorhynchidae* diversity and introduces new diagnostic traits.

INTRODUCTION

The Greater Coucal (*Centropus sinensis*) is a widespread, non-migratory resident bird of Pakistan that inhabits diverse habitats, including agricultural landscapes and forested areas. Its diet consists primarily of insects, caterpillars, and small vertebrates such as the saw-scaled viper (*Echis carinatus*), house lizard (*Hemidactylus frenatus*), and skinks (*Eutropis* spp.). Occasional feeding on snails, fruits, seeds, nestlings, and bird eggs has also been recorded [1]. Birds (Class Aves) represent one of the most abundant vertebrate groups and serve as important

indicators of ecological health. Globally, approximately 11,276 avian species are recognized [2], with Pakistan harboring nearly 778 bird species [3]. Helminth parasites form a diverse group of metazoans infecting both humans and animals, with significant ecological and veterinary importance [4]. Their survival and transmission depend on multiple biotic and abiotic factors, including host-parasite interactions, environmental conditions, and dispersal strategies [5, 6]. Within this group, acanthocephalans are obligate intestinal parasites characterized by a proboscis



armed with hooks, enabling firm attachment to host intestines. The genus *Centrorhynchus* Lühe, 1911 is globally distributed, occurring mainly in avian predators, including raptors and corvids, with a few records from other avian groups. Despite its wide distribution, the genus remains poorly documented in South Asia, including Pakistan. Only limited studies have reported acanthocephalans from avian hosts in the country, including those by Galaktionov and Atrashkevich [7], Amin et al. [8], Bilqees and Aly [9], Birmani et al. [10], Khan and Bilqees [11], Khan et al. [12], Khan et al. [13], Bushra et al. [14] and Naz et al. [15].

This study aims to describe a new species of *Centrorhynchus* from the Greater Coucal in Sindh, thereby contributing to the knowledge of acanthocephalan diversity in Pakistan and the global taxonomy of the genus.

METHODS

Three Greater Coucals (*Centropus sinensis*) were collected through purposive sampling during routine avian surveys in Saleh Pat, Sukkur District, Pakistan (Jan 2024 to July 2025). This targeted approach was used because this bird species is a known host for *Centrorhynchus* parasites in the region. Three hosts were captured during routine surveys. Gastrointestinal tracts were dissected and screened under a stereomicroscope. Worms were relaxed in hot water, fixed in AFA, stained with borax carmine, dehydrated through graded ethanol, cleared, and mounted in Canada balsam. Line drawings (Olympus BH2-DA) and photomicrographs (OMAX 10 MP) were prepared. Measurements are in millimeters (mm) and identifications followed standard *Centrorhynchus* literature. This study is a taxonomic description of a new acanthocephalan species, *Centrorhynchus saluni* n. sp., based on morphological and morphometric analyses. The research follows a descriptive parasitological approach, focusing on the identification and characterization of the parasite recovered from the Greater Coucal (*Centropus sinensis*) in Saleh Pat, Sukkur, Sindh, Pakistan. While only three hosts were examined, this meets taxonomic requirements because seven intact parasite specimens were recovered, enabling full morphological characterization diagnostic features were consistent across specimens the approach aligns with established acanthocephalan taxonomy protocols where complete specimens validate new species; and the study focused on description rather than ecological statistics Three live specimens of the Greater Coucal (*Centropus sinensis*) (Cuculiformes: Cuculidae) were collected during routine bird surveys using mist nets and hand capture with the assistance of local bird trappers. Captured birds were transported in ventilated cages to the field station and handled following ethical guidelines to minimize stress and ensure humane treatment. Birds were euthanized following accepted protocols, and complete

necropsies were performed under laboratory conditions. The gastrointestinal tracts were removed and examined under a stereomicroscope, yielding seven acanthocephalans belonging to the genus *Centrorhynchus* Lühe, 1911 from two of the three birds. Recovered specimens were transferred to 0.9% saline solution for cleaning and relaxation, then fixed under mild cover glass pressure in alcohol-formalin-acetic acid (AFA). Worms were stained with borax carmine, dehydrated through a graded ethanol series, cleared, and mounted permanently in Canada balsam. Illustrations were prepared using an Olympus BH2-DA drawing attachment, and photomicrographs were taken with an OMAX digital trinocular LED microscope (10 MP resolution). All measurements are given in millimeters (mm), and identifications were made using standard taxonomic literature for the genus. Family: *Centrorhynchidae* Lühe, 1909. Genus: *Centrorhynchus* Lühe, 1911 Species: *Centrorhynchus saluni* n. sp. (Figures 1–2). Type host: Greater Coucal *Centropus sinensis* (Cuculiformes: Cuculidae). Type locality: Saleh Pat, Sukkur, Sindh, Pakistan. Site of infection: Large intestine. Number of hosts examined: 3. Number of hosts infected: 2. Number of specimens recovered: 7.

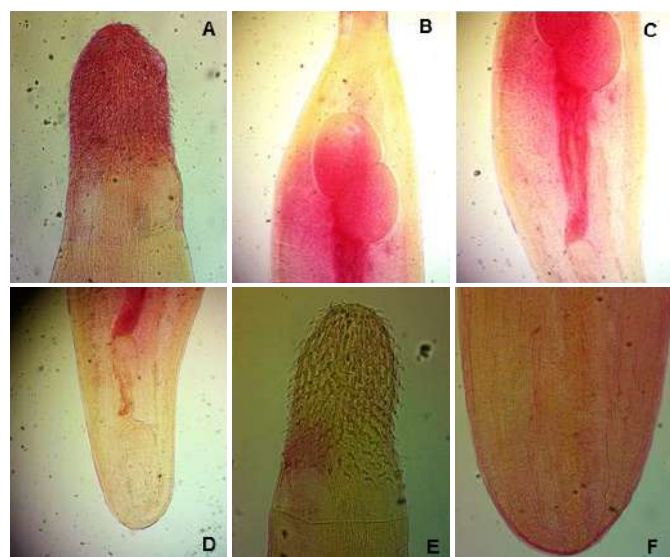
RESULTS

The body is fusiform and aspinose. Proboscis cylindrical, not divided into two sections, with 30–34 longitudinal rows of hooks, each row bearing 12–13 hooks. Proboscis receptacle claviform, extending up to the middle of the proboscis. Neck short. Lemnisci cylindrical, longer than the proboscis's receptacle. Testes are tandem to slightly oblique, situated in the anterior part of the trunk. Cement glands are elongated, six in number. Bursa is present at the posterior extremity. Seven acanthocephalans were recovered from the intestines of two Greater Coucals (*Centropus sinensis*) in Sindh, Pakistan, representing a new species, *Centrorhynchus saluni* n. sp. The species is characterized by a fusiform, aspinose body and a cylindrical proboscis bearing 30–34 longitudinal rows of 12–13 hooks. Male measured 6.27–6.70 mm, and female 7.41–7.92 mm. Diagnostic features include specific proboscis and receptacle dimensions, cylindrical lemnisci longer than the receptacle, tandem to slightly oblique testes, six elongated cement glands, and a posterior bursa in males. Females possess a uterine bell with eggs measuring 0.075–0.085 × 0.030–0.038 mm (Table 1).

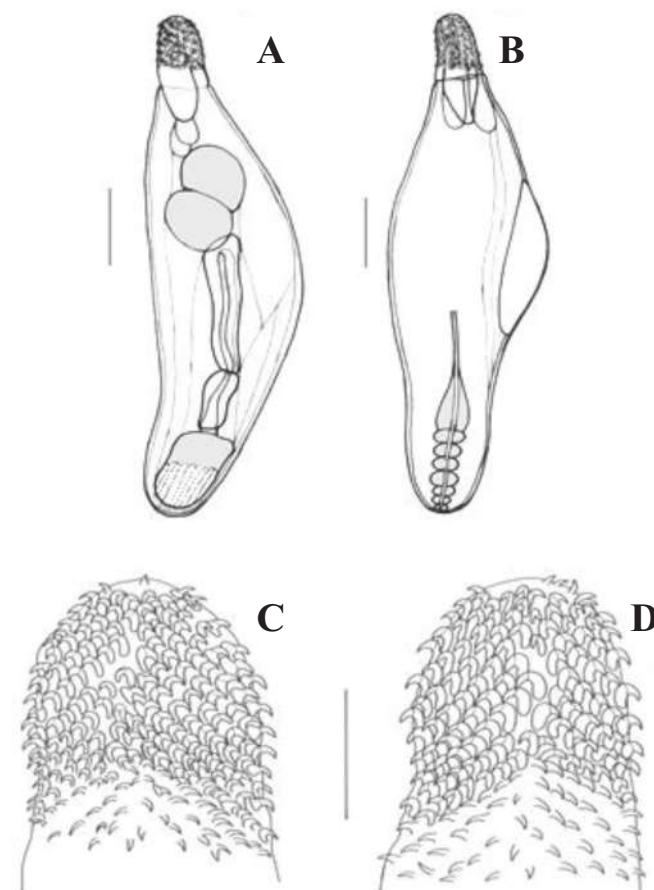
Table 1: Measurements (in millimeters) of Male and Female *Centrorhynchus saluni* n. sp

Character	Male	Female
Entire worm	6.27-6.70	7.41-7.92
Proboscis	0.15-0.66 × 0.59-0.75	0.60-0.77 × 0.70-0.77
Neck	Short	Short
Proboscis receptacle	0.75-0.91 × 0.25-0.36	0.72-0.83 × 0.85-0.94
Lemnisci	0.65-0.91	0.85-0.91
Anterior testis	0.78-0.91 × 0.55-0.66	-
Posterior testis	0.82-0.84 × 0.56-0.68	-
Bursa	1.00-1.05 × 0.50-0.59	-
Eggs	-	0.075-0.085 × 0.030-0.038

The proboscis of the male shows a distinct arrangement of hooks in longitudinal rows, which represents a key diagnostic feature of the species. The anterior trunk region displays the proboscis receptacle, paired lemnisci, and testes, highlighting the internal organization of male reproductive structures. The middle trunk reveals the series of tubular cement glands, important for copulatory processes, while the posterior trunk shows the bursa. In the female, the proboscis exhibits a similar hook pattern to the male, whereas the posterior trunk depicts the uterine bell, representing the reproductive system of the female. A. Proboscis of male showing arrangement of hooks and neck; B. Anterior portion of trunk of male showing proboscis receptacle, lemnisci and testes; C. Middle portion of trunk of male showing tubular cement glands; D. Posterior portion of trunk of male showing bursa; E. Proboscis of female showing arrangement of hooks and neck; F. Posterior portion of trunk of female showing uterine bell (Figure 1).

**Figure 1:** *Centrorhynchus saluni* n. sp. A. Proboscis of Male, B. Anterior Portion of Trunk of Male, C. Middle Portion of Trunk of Male, D. Posterior Portion of Trunk of Male, E. Proboscis of Female, F. Posterior Portion of Trunk of Female

The complete body form of both male and female worms is shown, clearly demonstrating sexual dimorphism in overall body structure. Enlarged views of the proboscis in both sexes highlight the arrangement and gradation of hooks, with males and females exhibiting comparable patterns but with minor variations. Scale bars indicate the relative sizes, with 1 mm for the whole worms and 0.3 mm for the proboscis enlargements. Scale bars: A and B. 1 mm; C and D. 0.3 mm (Figure 2).

**Figure 2:** *Centrorhynchus saluni* n. sp. A. Entire Male; B. Entire Female; C. Proboscis of Male Worm Enlarged; D. Proboscis of Female Worm Enlarged

In Pakistan, several species of *Centrorhynchus* have been reported from avian hosts (Table 2).

Table 2: Different Species of Genus *Centrorhynchus* Lühe, 1911 Reported from Pakistan

Species	<i>C. saluni</i> n. sp.	<i>C. cribbi</i>	<i>C. globirostris</i>	<i>C. faciutum</i>	<i>C. gibsoni</i>	<i>C. nickoli</i>
Reference	Present species	[14]	[8]	[9]	[16]	[12]
Body	Fusiform, spinose, 6.27–7.92	Elongated, 23.0–24.12 X 1.12–1.34	long, cylindrical with prominent anterior ovoid dilation, 12.50–23.75 X 0.47–0.95	Cylindrical, with conspicuous swelling near anterior end, 10.0–11.1 X 1.2–1.5	elongate with anterior end pointed and posterior rounded, 14.6–16.3 X 3.08–3.64	—
Proboscis	Cylindrical, not divided into two parts, 0.15–0.77 X 0.59–0.77	Small, globular not divided, 1.0–1.12 X 0.32–0.36	Globular, tilted ventrad, not divided, 603–700 X 364–468	Nearly cylindrical, 0.72–0.74 X 0.18–0.19	cylindrical 0.50–0.57	—
Longitudinal rows of hooks	30–34	14–16	24–25	12–13	14	16
Hooks in each row	12–13	12–14	10–11	6–18	20	20–24
Proboscis receptacle	Claviform, inserted inside proboscis up to middle of it, 0.72–0.91 X 0.25–0.94	Elongated inserted at middle of proboscis, 1.20–1.46 X 0.56–0.57	double-walled, about twice as long as proboscis, 1.16–1.50 X 0.17–0.32	0.91–0.92 X 0.21–0.22	relatively small, elongate	—
Neck	Short	—	Neck prominent	Absent	—	—
Lemnisci	Cylindrical, larger than proboscis receptacle	long, slightly sub-equal, right measuring 2.96–3.04 X 0.16, left 3.0–3.14 X 0.16–0.17	Lemnisci digitiform, equal, about three times as long as proboscis receptacle, 1.51–2.37	about 3–4 times longer than length of proboscis receptacle extending well beyond it but not reaching posterior end of body	Sub-equal	—
Testes	Tandem to slightly oblique, situated in the anterior half of the body	Situated in anterior half of body, anterior, 1.24–1.32 X 0.64–0.80 while posterior 0.96–1.20 X 0.80–0.84	Relatively large, elliptical, not contiguous, anterior testis 0.60–1.50 X 0.27–0.45, posterior testis 0.57–1.40 X 0.19–0.62	oval, tandem, sometimes overlapping, located in anterior swelling of trunk, 0.05–0.5 X 0.04–0.42	—	—
Cement glands	06 long, 0.652–0.916	04 tubular, 11.10–12.80 X 0.36–0.40	04 tubular, 6.25–13.12 X 0.10–0.32	03	—	—
Host	<i>Centropus sinensis</i>	<i>Centropus sinensis</i>	<i>Centropus sinensis</i>	<i>Butastur teesa</i>	<i>Corvus splendens</i>	<i>Coracias garrulus</i>
Locality	Saleh Pat, Sukkur, Sindh	Nausharo Feroze, Sindh	Oderolal, Sindh	Karachi, Sindh	Oderolal, Sindh	Mirpurkhas, Sindh

DISCUSSION

The genus *Centrorhynchus* Lühe is a well-defined group of parasitic worms from the class Acanthocephala that mature in the alimentary canals of birds [17]. In Pakistan, several species of *Centrorhynchus* have been reported from avian hosts. *C. cribbi* Siyal et al. from *Centropus sinensis* (Nausharo Feroze, Sindh) differs from the present species in having a larger elongated body, 14–16 longitudinal rows of hooks, elongated proboscis receptacle, long subequal lemnisci, and four cement glands [14]. *C. globirostris* Amin et al. also from *C. sinensis* (Oderolal, Sindh), has a globular proboscis tilted ventrad, 24–25 longitudinal rows of hooks with 10–11 per row, a proboscis receptacle twice as long as the proboscis, non-contiguous testes, and four cement glands [8]. *C. fuscum* (Westrumb, 1821), recorded by Bilqees and Khan from *Butastur teesa* (Karachi, Sindh), is characterized by a

cylindrical trunk, 12–13 longitudinal rows of hooks, larger lemnisci crossing mid-body, five cement glands, and a larger bursa [9]. *C. gibsoni* Khan, Ghazi and Bilqees, from *Corvus splendens* (Oderolal, Sindh) has a smaller proboscis divided by slight insertion, 14 rows with 20 hooks per row, smaller proboscis receptacle, and unequal lemnisci [16]. *C. nickoli* Khan, Bilqees and Ghazi, 2001 from *Coracias garrulus* (Mirpurkhas, Sindh) differs by its smaller cylindrical body, proboscis divisible into two portions, 16 rows with 20–24 hooks per row, larger double-walled proboscis receptacle, and larger lemnisci [12]. Outside Pakistan, numerous species differ markedly from the present taxon. For example, *C. aluconis* (Müller, 1780) Lühe, 1911 from various European raptors has 26–30 rows with 15–18 hooks per row and longer lemnisci; *C. amphibius* Das, 1950 from *Buteo buteo* (Bulgaria) has a cylindrical body, larger proboscis

with 32 rows of 20 hooks, and longer proboscis receptacle; *C. globocaudatus* (Zeder, 1800) Lühe, 1911 from falcons in Bulgaria has a long neck and larger testes with 30 rows of 18–19 hooks; *C. falconis* (Johnston and Best, 1943) Golvan, 1956 from *Spilornis cheela* (Sri Lanka) has 38–40 rows with 27–29 hooks and larger testes; and *C. buteonis* (Schränk, 1788) Kostyler, 1914 from Sri Lanka and India bears 35 rows with 23–24 hooks per row and a larger proboscis divisible into two parts. Indian records also reveal diagnostic differences: *C. clitorideus* (Meyer, 1931) [18] from *Athene brama* has a proboscis with 30–32 rows and 19–21 hooks per row, elongated testes, and a funnel-shaped bursa; *C. milvus* [19] from *Milvus migrans* govinda possesses 36 rows with 22–23 hooks; *C. atheni* [20] resembles the present species in body size but has a more elongated proboscis, smaller eggs, and digitiform genital appendages; *C. lucknowensis* [21] from *Haliastur indus* bears only three cement glands; and *C. sikkimensis* Bhattacharya, 2003 from eagles of India is the largest species, with 40–42 rows and 28–30 hooks per row, central ganglion, and two cement glands. These morphological comparisons demonstrate that *Centrorhynchus saluni* n. sp. differs from all previously described congeners in its combination of body size, cylindrical proboscis with 30–34 rows of 12–13 hooks, claviform proboscis receptacle, six cement glands, and the relative dimensions of the lemnisci and reproductive structures. The present study is based on only three specimens of the Greater Coucal (*Centropus sinensis*), two of which were infected, yielding seven worms. Although sufficient for species description, this small host sample size limits the ability to assess prevalence, host-parasite relationships, and intraspecific variation. Future studies with larger sample sizes and broader geographic coverage will be necessary to confirm the distribution, host range, and morphological variability of *C. saluni* n. sp.

CONCLUSIONS

The study concluded that a new acanthocephalan species, *Centrorhynchus saluni* n. sp., infects the Greater Coucal (*Centropus sinensis*) in Sindh, Pakistan. Distinctive features, including proboscis hook arrangement, reproductive structures, and sexual dimorphism, differentiate it from related species. This study adds to the understanding of avian acanthocephalan diversity in the region.

Authors Contribution

Conceptualization: BAS

Methodology: BAS

Formal analysis: BAS, FS

Writing review and editing: BAS, FS, NAB

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article



Acute Toxicity and Behavioral Anomalies in *Labeo Rohita* Due to Exposure of Expired Permethrin, Malathion and Dichlorovos Mixture

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ABSTRACT

Evaluation of waterborne toxicity is essential to estimate aquatic pollution. Generally, hazardous contaminants are a greater threat to freshwater organisms. **Objectives:** To measure the tolerance limit of *Labeo rohita* to expired permethrin, malathion, and dichlorovos mixture at different concentrations. **Methods:** Freshwater fingerlings of *L. rohita* were exposed to sub-lethal (0.3mg/l) and lethal (2.3mg/l) concentrations of permethrin, malathion, and dichlorovos mixture, with three replications under constant water temperature (16-30°C), total water hardness (225mgL⁻¹) and pH (6.8-7.7) for 96-hr. Probit analysis was used to calculate the Lethal concentration (LC50) and (LC100) of 96-hour exposure of the pesticide mixture for fish. Correlation analyses were conducted to identify relationships between the variables under study. Physicochemical parameters of water (Electrical conductivity (EC), Dissolved oxygen (DO), Oxidation-reduction potential (ORP), and Salinity) were recorded on a 12-hr basis. **Results:** For 96 hours, the mean values of LC50 and LC100 of *L. rohita* were calculated as 1.53 ± 0.028 and 3.05 ± 0.083 mgL⁻¹, respectively. *L. rohita* exhibited behavioural changes such as erratic swimming, loss of equilibrium, heavy breathing, and abnormal posture due to the toxic effects of the pesticide mixture. Correlation analysis indicates that increasing concentrations of permethrin, malathion, and dichlorovos pesticides significantly degrade water quality, reducing DO while increasing ORP, salinity, and EC. **Conclusions:** It was concluded that an expired pesticide mixture is more lethal to aquatic life than an unexpired pesticide mixture. So, it is important to take precautionary measures before discarding expired pesticides in aquatic ecosystems.

INTRODUCTION

Evaluation of waterborne toxicity is essential to estimate aquatic pollution. Generally, hazardous contaminants are a greater threat to freshwater organisms [1]. The investigation of freshwater poisoning addresses the detrimental impacts of exposure to harmful substances, especially pesticides and insecticides, in aquatic ecosystems. These highly toxic substances are mainly chemical pesticides, that destroy aquatic ecosystems. Insecticides are highly lethal and despite their acute toxicity, they are commonly used throughout the agricultural and other private sectors [2]. The ability of a poisonous material to cause harm to an organism with only one, brief, encounter is known as acute toxicity. The

primary purpose of the acute toxicity test for pesticides is to rapidly determine the doses that cause fish to suffer immediate and permanent harm [3]. The most popular acute toxicity assays, such as LC50 and LC100, show the danger to the test organisms concerning exposure time intervals and death rates [4]. Compared to many other pesticides, permethrin is seen to be a safer choice because it quickly metabolizes and is eliminated by living things, leaving it out of the environment. A recent study revealed that photo-degradation products of permethrin could prove a greater threat to aquatic creatures than the element itself [5]. Malathion pollution has propagated to many significant water sources. Therefore, people who



inadvertently ingest fish infected with malathion possess a greater likelihood of suffering from health problems caused by malathion toxicity, notably Parkinson's disease, neurocognitive challenges, weight gain, and related metabolic diseases [6]. Organophosphates, like dichlorvos, behave as strong nerve poisons that can be highly dangerous to fish and other organism aquatic ecosystem. It works by limiting synaptic transmission in cholinergic neurons by inhibiting the acetylcholinesterase (Ache) activity. The organism perishes untimely as a result of parasympathetic challenges spurred due to this disruption of nerve activity [7]. Fish provide vital means of protein for human beings, but they may become affected by chemical pesticides, that may interfere with their capacity to evolve, endure, and propagate. The primary culprit of aquatic pollution is agricultural cultivation. Exposure to pollutants for a prolonged amount of time might harm fish tissues, reduce metabolism, and diminish disease resistance [2]. *L. rohita* grows best in temperatures over 14°C. It blooms quickly; in a year, under normal growing scenarios, and normally reaches a total length of about 35–45 cm and a weight of 700–800 g. Its native habitat is shallow external sections of flooded rivers, where spawning occurs under an ideal temperature range of 22 to 31°C [8]. Pesticides are used to get rid of a variety of pests, such as insects, and rodents in order to improve the quantity and caliber of agricultural output [9]. These pesticides are extremely toxic and harmful to aquatic life as well as the quality of the water [10]. When crops are sprayed to eradicate pests, pesticides pose a serious hazard to non-target creatures, especially fish. These substances have the potential to kill fish by affecting their metabolic functions and causing health problems [11]. Fish exposed to pesticides on a long-term basis suffer from oxidation, mutagenesis, suppression of acetylcholinesterase function, carcinogenic effects, and changes in histopathology and development [12]. Moreover, pesticides make fish habitats less suitable and cause behavioral changes in fish, which increases the likelihood that the fish will be preyed upon [13]. The presence of expired pesticides in ecosystems introduces unique toxicological challenges that are not observed with unexpired formulations [14]. The quantity of expired pesticides in Pakistan's North West Frontier Province is estimated to exceed 5,000 tons, based on inventories and surveys [15]. The degradation of pesticides can result in their movement through soil and water or cause them to volatilize, reaching neighboring or distant locations [16]. This study aims to assess the toxicity of expired pesticide mixtures to *Labeo rohita* under controlled laboratory conditions.

METHODS

An experimental study design was conducted at the Department of Zoology, University of Sialkot, from September 2023 to February 2024. The juveniles of freshwater fish *Labeo rohita*, measuring about 6–7 cm in length, were acclimatized for 7 days in the laboratory under constant conditions. During acclimatization, water parameters were checked twice daily, and fish were fed to satiation on the feed (3.00Kcalg⁻¹ digestible energy and 37% digestible protein). Acute toxicity tests on *L. rohita* were conducted for 96 hours under controlled conditions to determine tolerance limits, recording LC50 and LC100 values at 4-hour intervals. All glassware and aquaria were washed before the experiment. Aquaria were filled with tap water, maintaining water hardness (225 mg/L), pH (6.8–7.7), and temperature (16–30°C). Ten *L. rohita* fish of similar weight were stocked per aquarium, with three replications for each pesticide concentration, while control fish were kept in pesticide-free water. Solutions of expired permethrin, malathion, and dichlorvos were mixed in a 1:1:1 ratio to prepare the pesticide mixture. To prepare a 75ml mixture, 25ml of permethrin, 25ml of malathion, and 25ml of dichlorvos solution were mixed in a measuring flask. The test concentration was started from a zero with an increment of 0.02mgL⁻¹. During the acute toxicity tests, concentrations of permethrin, malathion, and dichlorvos pesticide mixtures in an aquarium were gradually increased. 96 hr LC50 and the lethal concentration value of the permethrin, malathion, and dichlorvos for *L. rohita* were checked by Probit analysis. The statistical analysis method was used to check the physical chemistry of water parameters and fish mortality rate. Tuckey's Student Newman Keul test, correlation was also applied to check the relationship of pesticide mixture concentration and physicochemical parameters of water [17].

RESULTS

Increased fish mortality occurred from a gradual rise in the concentration of pesticides in the mixture. In all replicated aquaria, however, 50% of fish death was found at a dosage of 1.5 mgL⁻¹, whereas 100% of fish mortality occurred at a concentration of 2.3 mgL⁻¹. The percent mortality of *Labeo rohita* at various concentrations of a mixture of pesticides was recorded during a 96-hour exposure period for all three replications. 96-hour LC50 and the lethal concentration (LC100) values of the permethrin, malathion, and dichlorvos mixtures for the *Labeo rohita* during the first replication were calculated at 1.40 ± 0.029 and 2.985 ± 0.084 mgL⁻¹, respectively, with 95% confidence ranges of 1.344–1.458 and 2.833–3.167. Its Deviance Chi-square score, on the other hand, was calculated as 34.71, with the goodness of a fit test (p) 0.02. The 96 hr. LC50 and the lethal concentration values for the second replication were $1.61 \pm$

0.028mgL⁻¹ and 3.13 ± 0.085mgL⁻¹, respectively, with a computed Chi-square value of 48.50 and p-value of 0.02. 96hr LC50 and the lethal concentration (LC100) values for the 3rd replication was 1.588 ± 0.027mgL⁻¹, and 3.042 ±

0.080mgL⁻¹, respectively. It had a deviance Chi-square score of 47.67 and a goodness of fit test p-value of 0.04 (Table 1).

Table 1: Calculated 96hr LC50 and The Lethal Concentration (Mean ± SD) of Pesticide Mixture for the *Labeo Rohita*

Fish Species	Mean 96-hr Lc50 (mg ⁻¹)	95% Confidence Interval (mg ⁻¹)		Mean Lethal Concentration (mg ⁻¹)	95% Confidence Interval (mg ⁻¹)
<i>Labeo Rohita</i>	Replication I	1.40 ± 0.029	1.344-1.458	2.985 ± 0.084	2.833-3.167
	Replication II	1.61 ± 0.028	1.560-1.670	3.13 ± 0.085	2.979-3.318
	Replication III	1.588 ± 0.027	1.534-1.644	3.042 ± 0.080	2.897-3.217
*Mean ± SD		1.53 ± 0.028		3.05 ± 0.083	

In the present study, *Labeo rohita* indicated behavioural alterations such as erratic swimming and loss of equilibrium after being exposed to expired permethrin, malathion, and dichlorvos mixture. They were assembled in a single corner of the tank, then remained at the bottom, regularly rising to the surface, displaying heavy breathing, and heightened opercula movement, indicating distress and neurological impairment with their scales removed. These changes are likely due to the neurotoxic effects of the expired pesticide mixture, which disrupts normal nervous system function, leading to symptoms such as hyperactivity, lethargy, and abnormal posture. At the pesticide mixture concentrations of 2.3 and 0.1mgL⁻¹, the dissolved oxygen levels in test media (for *Labeo rohita*) were found lowest (5.14 ± 0.65mgL⁻¹) and maximum (7.69 ± 0.94mgL⁻¹) respectively. The dissolved oxygen (DO) content of the test media altered significantly as the pesticide mixture concentration increased. The DO content was decreased gradually as the concentration of the pesticide mixtures increased. At pesticide mixture concentrations of 0.1 and 2.3mgL⁻¹, maximum and minimum mean values of oxidation-reduction potential (ORP) in the test media were calculated as 67.11 ± 8.68 and 139.8 ± 16.9 mV, respectively. The lowest electrical conductivity value (917 ± 8.71µScm⁻¹) was found at a concentration of 0.1mgL⁻¹, while the highest value (968 ± 17.0µScm⁻¹) was recorded at a concentration of 2.3mgL⁻¹ pesticide mixture. The salinity of the test medium fluctuated after acute pesticide mixture exposure due to varying exposure doses. At pesticide mixture exposure levels of 2.3 and 0.1mgL⁻¹, the maximum and minimum salinity values (for *Labeo rohita*) test medium were calculated as 0.56 ± 0.045 and 0.44 ± 0.005 mgL⁻¹, respectively. The maximum salinity in a medium was found at 2.3mgL⁻¹ pesticide mixture concentration, while the lowest was found at 0.1mgL⁻¹ (Table 2).

Table 2: Physicochemical Parameters (Means ± SD) of Test Media Used for the *Labeo Rohita* During the Acute Toxicity Test with Various Concentrations.

Conc. (mg ⁻¹)	Temperature (C°)	pH	Hardness (mg ⁻¹)	EC (µScm ⁻¹)	DO (ppm)	ORP (mV)	Salinity (ppt)
0.1	16.8 ± 0.51	6.90 ± 0.25	222 ± 0.57	917 ± 8.71	7.69 ± 0.94	67.11 ± 8.68	0.44 ± 0.005
0.3	16.8 ± 0.51	6.90 ± 0.25	222 ± 0.57	917 ± 8.71	7.69 ± 0.94	67.11 ± 8.68	0.44 ± 0.005
0.5	16.8 ± 0.51	6.90 ± 0.25	222 ± 0.57	917 ± 8.71	7.69 ± 0.94	67.11 ± 8.68	0.44 ± 0.005
0.7	17.2 ± 1.15	7.41 ± 0.19	225 ± 0.01	958 ± 29.7	5.69 ± 0.27	99.30 ± 29.3	0.47 ± 0.001
0.9	17.2 ± 1.15	7.41 ± 0.19	225 ± 0.01	958 ± 29.7	5.69 ± 0.27	99.30 ± 29.3	0.47 ± 0.001
1.1	17.2 ± 1.15	7.41 ± 0.19	225 ± 0.01	958 ± 29.7	5.69 ± 0.27	99.30 ± 29.3	0.47 ± 0.001
1.3	18.3 ± 0.30	7.06 ± 0.25	223 ± 0.02	940 ± 22.9	7.01 ± 1.10	126.3 ± 8.59	0.55 ± 0.047
1.5	18.3 ± 0.30	7.06 ± 0.25	223 ± 0.02	940 ± 22.9	7.01 ± 1.10	126.3 ± 8.59	0.55 ± 0.047
1.7	18.3 ± 0.30	7.06 ± 0.25	223 ± 0.02	940 ± 22.9	7.01 ± 1.10	126.3 ± 8.59	0.55 ± 0.047
1.9	19.1 ± 0.11	6.96 ± 0.05	226 ± 0.03	968 ± 17.0	5.14 ± 0.65	139.8 ± 16.9	0.56 ± 0.045
2.1	19.1 ± 0.11	6.96 ± 0.05	226 ± 0.03	968 ± 17.0	5.14 ± 0.65	139.8 ± 16.9	0.56 ± 0.045
2.3	19.1 ± 0.11	6.96 ± 0.05	226 ± 0.03	968 ± 17.0	5.14 ± 0.65	139.8 ± 16.9	0.56 ± 0.045

DISCUSSION

Agricultural activities release a combination of pollutants into water sources including pesticides, fertilizers, manure from farm animals, and sediments. The widespread use of various pesticides and insecticides in farming activities leads to water contamination, causing environmental and health issues. Pesticide application has been reported to cause fatalities among aquatic animals [18]. In the current

investigation, the mortality rate in fish was increased with increasing concentration of pesticide mixture and time exposure. The median lethal concentration (LC50/96h) and the lethal concentration (LC100/96h) for various toxicants were employed to assess the sensitivity and the survival capability of a test organism. In the current study, acute toxicity experiments have been performed on *Labeo rohita*

at different concentrations, to determine LC50 and the lethal concentrations of permethrin, malathion, and dichlorvos expired mixture to fish. The median lethal concentration (LC50) suggests that expired formulations, such as dichlorvos, fenvalerate, lambda-cyhalothrin, pretilachlor, tebuconazole, hexaconazole, capta may exhibit higher toxicity than other formulations. pH changes in these expired pesticides can result in byproducts that cause acute toxicity to fish species like *L. rohita* [19]. Calculated 96-hr LC50 values of chlorpyrifos and dichlorvos of 0.753 mgL⁻¹ and 12.964 mgL⁻¹ for *Tor putitora*. The median lethal concentration (LC50) of dichlorvos and paraquat against juveniles of *Clarias gariepinus* were calculated as 730 µg l⁻¹ and 50 µg l⁻¹ respectively. In the present study, the LC50 and LC100 for *L. rohita* were 1.53 ± 0.028 and 3.05 ± 0.083 mgL⁻¹. As a comparative analysis, the current study verified that an expired pesticide mixture is highly lethal to aquatic life than an unexpired pesticide mixture [20].

CONCLUSIONS

It was concluded that increasing concentrations of the permethrin, malathion, and dichlorvos pesticides mixture significantly affect water quality parameters, characterized by a decrease in dissolved oxygen and increases in oxidation-reduction potential, salinity, and electrical conductivity, which collectively stress the aquatic environment and adversely impact the survival of *L. rohita*.

Authors Contribution

Conceptualization: ZA

Methodology: ZA

Formal analysis: NM

Writing review and editing: NM

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article



Antibiotic Resistance Profiles of Lactic Acid Bacteria Isolated from Dairy Products in Lahore, Pakistan

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ABSTRACT

Antibiotic resistance (AR) among lactic acid bacteria (LAB) in dairy products is an emerging concern due to their potential role as reservoirs of transferable resistance genes. While LAB are generally recognized as safe and widely used in fermentation, resistant strains may compromise food safety and public health. **Objectives:** To isolate and identify lactic acid bacteria (LAB) from popular dairy products (raw milk, yogurt, and cheese) in Lahore, Pakistan. **Methods:** This laboratory-based cross-sectional experimental study included a total of 60 dairy samples (raw milk, yogurt, and cheese) collected from local markets in Lahore, Pakistan. LAB were isolated and identified using standard microbiological and molecular methods, and their susceptibility to commonly used antibiotics was evaluated. **Results:** LAB were recovered from the majority of samples, predominantly *Lactobacillus* spp. Resistance was most frequently observed against tetracycline, erythromycin, and ampicillin, whereas vancomycin and chloramphenicol showed lower resistance levels. The multiple antibiotic resistance (MAR) index was used to estimate "resistance pressure," referring to the extent of environmental or selective exposure to antibiotics that may promote resistance development. Overall, a subset of isolates exhibited multidrug resistance, indicating potential public health implications. **Conclusions:** This study highlights that LAB isolated from dairy products in Lahore harbor resistance to clinically important antibiotics. Although LAB are beneficial in food production, their potential role as reservoirs of resistance genes calls for monitoring programs and responsible antibiotic practices in dairy farming.

INTRODUCTION

Despite lactic acid bacteria (LAB) being generally recognized as safe and widely used as starter cultures or probiotics, there remains a major research gap regarding their role as reservoirs of antibiotic resistance genes (ARGs) in Pakistan, as most studies have focused on pathogenic bacteria rather than beneficial LAB. Antibiotic resistance (AR) is a growing threat to human health, animal welfare, and food safety, accelerated by the misuse of antibiotics in agriculture, veterinary practice, and food production, which promotes multidrug-resistant (MDR) bacteria. Studies worldwide have reported ARG carriage among LAB: Floris et al found that 40.7% of 54 LAB strains from raw milk and dairy products carried at least one ARG,

commonly resisting vancomycin and tetracyclines, with some strains exhibiting multidrug resistance [1, 2]. The Multiple Antibiotic Resistance (MAR) index, often >0.2 in bacteria from high-antibiotic-pressure environments, serves as a useful risk indicator [3]. LAB from fermented foods in Nigeria and human sources showed resistance to multiple antibiotic classes and carried determinants like tetM and ermB [4]. Antibiotic-resistant bacteria have been reported primarily among pathogens: *E. coli* from raw milk in Khyber Pakhtunkhwa resisted amoxicillin and ceftriaxone [5], *Staphylococcus aureus* from raw milk in Lahore with subclinical mastitis resisted penicillin and amoxicillin [6], and *Enterococcus* species from poultry in

Karachi were resistant to tetracycline and erythromycin with virulence markers [7]. However, data specific to LAB and ARG carriage in dairy products in Pakistan remain scarce, despite the critical role of dairy in nutrition and the economy.

This study aims to isolate and identify lactic acid bacteria from raw milk, yogurt, and cheese in Lahore, Pakistan, assess their antibiotic resistance profiles, and evaluate their potential as reservoirs of antibiotic resistance genes.

METHODS

This laboratory-based cross-sectional experimental study was designed to isolate, identify, and characterize the antibiotic resistance profiles of lactic acid bacteria from dairy products. The sample size was determined based on similar microbiological surveys of dairy products [8], and the balanced distribution (20 per product type) was designed to enable a comparative analysis of LAB prevalence and resistance profiles across common dairy matrices. The study was conducted from October 2024 to December 2024 at the Government Graduate College, Township Lahore, Pakistan. Samples were aseptically collected in sterile containers, transported on ice, and processed within 4 h of collection to minimize contamination and microbial loss. Each sample type was analyzed in triplicate to ensure reproducibility and accuracy of results. Ten milliliters of each liquid sample and 10 g of each solid sample were serially diluted in sterile phosphate-buffered saline (PBS; pH 7.2). Aliquots (100 µL) of appropriate dilutions were spread onto de Man, Rogosa, and Sharpe (MRS) agar plates for the selective isolation of lactic acid bacteria. Plates were incubated anaerobically at 37 °C for 48 h using GasPak jars. Distinct colonies were picked, purified by repeated streaking, and preserved in 20% glycerol stocks at 80 °C until further analysis. All plating and biochemical assays were performed in triplicate under aseptic conditions for reproducibility. Pure isolates were subjected to Gram staining and catalase testing. Carbohydrate fermentation profiles were evaluated using standard biochemical assays to provide preliminary identification. Bacterial genomic DNA was extracted using a Qiagen kit (Germany) according to the manufacturer's protocol. Universal primers targeting the 16S rRNA gene were employed for PCR amplification: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). To confirm phenotypic identification, all 48 isolates that were recovered and biochemically characterized were subjected to molecular identification by 16S rRNA gene sequencing. The amplification cycle consisted of an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. A final

elongation step was performed at 72 °C for 7 minutes. PCR products were separated on a 1.5% agarose gel, after which representative amplicons were purified and sequenced. The obtained sequences were compared with reference databases using BLAST (NCBI) for species identification. Antimicrobial susceptibility testing of the isolates was carried out using the Kirby-Bauer disc diffusion technique on Mueller-Hinton agar, in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2023) recommendations. For lactobacilli, the medium was enriched with 5% defibrinated sheep blood to enhance growth. The antibiotic panel included ampicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), and vancomycin (30 µg). To reflect the regional antibiotic usage pattern in Pakistan's dairy and veterinary sectors, the selected antibiotics represent the most frequently used antimicrobial classes, namely β-lactams (ampicillin), tetracyclines (tetracycline), macrolides (erythromycin), amphenicols (chloramphenicol), and glycopeptides (vancomycin). Following incubation at 37 °C for 24 h under anaerobic conditions, inhibition zones were measured, and the results were categorized as resistant, intermediate, or susceptible based on CLSI breakpoints. Isolates resistant to three or more classes of antibiotics were classified as multidrug resistant (MDR). The Multiple Antibiotic Resistance (MAR) index was used to assess the exposure of bacterial isolates to antibiotic pressure. The MAR index for each isolate was calculated using the formula.

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics tested}}$$

A MAR index greater than 0.2 indicates exposure to environments with high or frequent antibiotic use. All statistical analyses were carried out in SPSS (version 26.0). Relationships among sample sources, bacterial isolates, and antimicrobial resistance profiles were examined using Chi-square and Fisher's exact tests. Results with a p-value of 0.05 or less were interpreted as statistically significant.

RESULTS

A total of 60 samples (20 raw milk, 20 yogurts, and 20 cheese) were analyzed. Overall, 48 samples (80.0%) yielded LAB growth, with the highest recovery rate from yogurt (90.0%), followed by cheese (80.0%) and raw milk (70.0%). A statistically significant difference was observed among sample types ($\chi^2=6.57$, $p=0.038$), indicating that LAB recovery varied with the type of dairy product. Among the isolates, *Lactobacillus* spp. (72.9%) predominated over *Enterococcus* spp. (27.1%) (Table 1).

Table 1: Frequency of Bacterial Isolates Recovered from Dairy Samples (N=60) (Simulated Data)

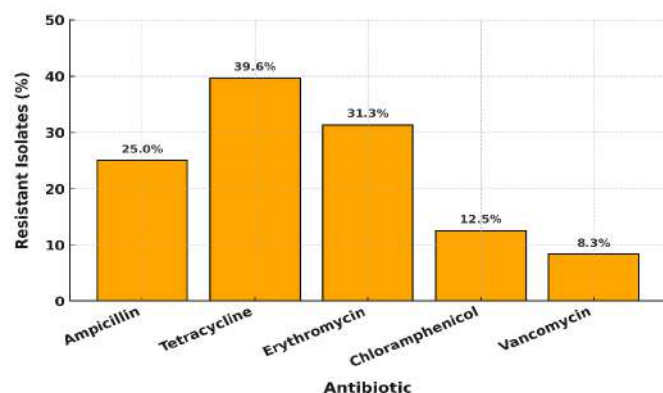
Sample Type	No. of Samples	Positive Isolates N (%)	<i>Lactobacillus</i> spp. n (%)	<i>Enterococcus</i> spp. n (%)
Raw milk	20	14 (70.0%)	10 (71.4%)	4 (28.6%)
Yogurt	20	18 (90.0%)	15 (83.3%)	3 (16.7%)
Cheese	20	16 (80.0%)	10 (62.5%)	6 (37.5%)
Total	60	48 (80.0%)	35 (72.9%)	13 (27.1%)

The overall resistance trend showed the highest resistance to tetracycline, followed by erythromycin and ampicillin, while chloramphenicol and vancomycin exhibited comparatively lower resistance. Statistical analysis revealed no significant association between sample type and specific antibiotic resistance patterns ($p=0.52$) (Table 2).

Table 2: Antibiotic Susceptibility Pattern of LAB Isolates (N=48) (Simulated Data)

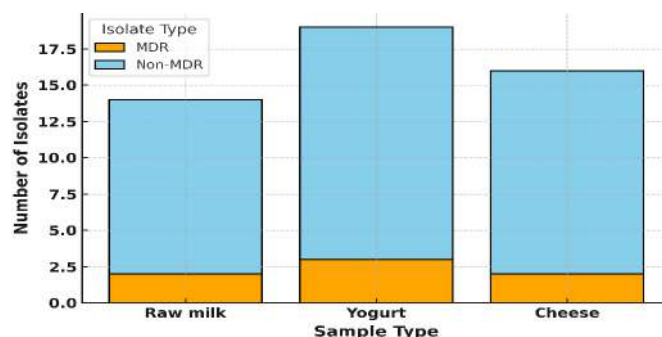
Antibiotic	Resistant N (%)	Intermediate N (%)	Susceptible N (%)
Ampicillin	12 (25.0%)	5 (10.4%)	31 (64.6%)
Tetracycline	19 (39.6%)	6 (12.5%)	23 (47.9%)
Erythromycin	15 (31.3%)	8 (16.7%)	25 (52.1%)
Chloramphenicol	6 (12.5%)	4 (8.3%)	38 (79.2%)
Vancomycin	4 (8.3%)	3 (6.3%)	41 (85.4%)

Percentage of lactic acid bacteria isolates resistant to different antibiotics (N= 48). The highest resistance was observed against tetracycline (39.6%), followed by erythromycin (31.3%) and ampicillin (25.0%). Lower resistance frequencies were recorded for chloramphenicol (12.5%) and vancomycin (8.3%). These findings indicate that tetracycline and erythromycin remain the most compromised antibiotics against LAB isolated from dairy products (Figure 1).

**Figure 1:** Percentage of Resistant Isolates Per Antibiotic, N=48 Isolates

The MAR index for all isolates ranged from 0.0 to 0.6, with a mean ($\pm$ SD) of 0.18 ± 0.14 . Notably, 31.3% (15/48) of the isolates had a MAR index greater than 0.2, indicating a high risk of origin from environments where antibiotics are frequently used. MDR frequency varied among species, being higher in *Enterococcus* spp. (23.1%) compared to

Lactobacillus spp. (11.4%). However, this difference did not reach statistical significance ($p=0.216$). These proportions indicate that MDR occurrence is species-dependent, though limited sample size may have constrained statistical power (Figure 2).

**Figure 2:** Distribution of MDR and Non-MDR Isolates by Sample Type

DISCUSSION

In this study, lactic acid bacteria (LAB) were isolated from raw milk, yogurt, and cheese samples with an overall isolation rate of 80%, demonstrating a high prevalence of culturable LAB in commonly consumed dairy products [8]. Our focus was specifically on *Lactobacillus* and *Enterococcus* spp. because these genera are both ecologically dominant in dairy environments and genetically distinct in terms of their metabolic and resistance traits. *Lactobacillus* species are key fermenters and probiotic candidates, while *Enterococcus* species, though occasionally beneficial, are also known opportunistic pathogens with high potential for horizontal transfer of resistance genes. Other LAB genera (such as *Leuconostoc*, *Pediococcus*, or *Streptococcus*) were excluded to avoid confounding results, since they differ markedly in ecological niches, intrinsic resistance mechanisms, and genomic organization. Grouping genetically and ecologically diverse bacteria could obscure genus-specific resistance patterns and complicate interpretation. While our primary focus was on these two taxa, similar investigations into *Enterococcus* from dairy products have revealed substantial antimicrobial resistance. For example, a study on raw goat and sheep milk and cheeses reported high frequencies of MDR *Enterococcus faecalis* and *E. faecium*, with many isolates resistant to tetracycline, streptomycin, erythromycin, and penicillin [9]. The prevalence of resistance in our isolates to tetracycline (39.6%), erythromycin (31.3%), and ampicillin (25.0%) aligns with previous finding, which observed similar resistance profiles among *Enterococcus* from bovine milk [10, 11]. In some dairy environments, bacteria from multiple genera (e.g., *Enterococcus*, *Staphylococcus*, *Streptococcus*) not only show resistance but also biofilm-forming capacity, which can enhance

persistence and complicate sanitation protocols. For instance, dairy farm isolates in southern Brazil demonstrated both resistance traits and significant biofilm production [12, 13]. Moreover, cheese-derived *Enterococcus* isolates from Urfa cheese in Turkey have been found to carry virulence genes (like *gelE*, *asa1*, *esp*) and exhibit a considerable rate of multidrug resistance. As reported by Karayigit, ~20.4% of *E. faecalis* and ~16.3% of *E. faecium* strains are MDR, and many isolates have a MAR index > 0.2. Our observed MDR rate of 14.6% is somewhat lower than that of cheese-derived *Enterococcus* studies, but the presence of MDR in LAB remains significant because these organisms can act as reservoirs of transferable resistance genes within the food chain. Mechanistically, resistance in these isolates may be driven by plasmid-encoded determinants and efflux systems, particularly those conferring resistance to tetracycline (*tet(M)*, *tet(L)*) and macrolides (*erm(B)*). Environmental exposure to residual antibiotics in animal feed or on dairy equipment surfaces may create selective pressure that favors resistant strains. The persistence of resistant LAB in dairy matrices suggests potential biofilm-mediated protection and stress-induced gene regulation that supports survival under sublethal antibiotic exposure. The MAR index phenomenon (isolates with high MAR values) suggests that environmental or processing pressures favor antibiotic resistance, as observed in the Urfa cheese study [14-17]. This indicates that contamination or repeated low-dose antibiotic exposure during production may select for resistant phenotypes rather than geographic or retail factors. Our statistical analysis revealed a significant association between sample type and bacterial recovery, with cheese and yogurt yielding higher LAB counts compared to raw milk, reflecting the favorable growth conditions provided by fermentation. However, the association between market location and resistance profile was non-significant ($p > 0.05$), suggesting that antimicrobial resistance is influenced more by intrinsic or production-level factors than by retail handling or local market differences. This trend aligns with findings from other dairy microbiota studies that emphasize processing and microbial ecology as stronger determinants of resistance dissemination. Although LAB are generally recognized as safe, the detection of antibiotic-resistant and MDR strains in consumer dairy products has local relevance in Pakistan, where dairy consumption is high and regulation of antibiotic use in livestock remains inconsistent [18-20]. Resistant LAB can transfer resistance genes to pathogenic bacteria in the human gut through conjugation or transformation, posing indirect risks to consumers and contributing to the regional antimicrobial resistance burden. The presence of virulence determinants in some isolates also raises food

safety concerns, particularly for immunocompromised individuals. This study was limited by the small sample size and reliance on phenotypic testing without molecular identification of resistance genes. Whole-genome sequencing and plasmid profiling were not performed, which could have clarified resistance mechanisms and gene transfer potential. Future studies should apply genomic tools to better characterize the resistome in LAB.

CONCLUSIONS

This study found antibiotic-resistant LAB in Lahore dairy, with tetracycline (39.6%), erythromycin (31.3%), and ampicillin (25.0%) resistance, and 14.6% multidrug resistance. Based on phenotypic testing, the results indicate potential resistance dissemination. Incorporating these data into risk assessments highlights the need for molecular surveillance, antibiotic control, and improved dairy hygiene.

Authors Contribution

Conceptualization: TB, AM, NM

Methodology: TB, AM, NM

Formal analysis: AM, NM

Writing review and editing: TB, AM

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article



A Field-Based Observational Study on Antiparasitic Control Measures and Outcomes in Captive Species at Lahore Safari Park

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ABSTRACT

There are a large number of wild animals that die, both in their natural home and in the confines of captivity, especially as a result of parasitism. **Objectives:** To evaluate the use of antiparasitic deworming on wild animals in the Safari Park, Lahore, which is a captive environment, as well as to analyze the mortality of various animals. **Methods:** This was a prospective observational analytic study that was done in Safari Park, Lahore. Animals were classified as carnivores, herbivores, and birds and were treated with Albendazole or Fenbendazole depending on their species, size, and diet. These administration routes were oral, injectable, and oral feed-based. Worming was done every quarter under the guidance of the Punjab Wildlife Department, according to international guidelines, whereby effective and safe parasites in captive wildlife are treated. **Results:** Albendazole (5 liters) was given to herbivores and one liter to the birds, whereas Fenbendazole was given to carnivores. There was no mortality (0.00), which ascertained the efficacy of both treatments. Statistical test revealed no significant difference in the mortality of carnivores, herbivores, and birds ($F(2,14) = 1.27$, $p = 0.31$), and no difference in the efficacy of Albendazole and Fenbendazole ($t = 0.89$, $p = 0.39$). There is a negative correlational relationship between dosage and parasitic stress ($r = 0.42$, $p < 0.05$), which is a sign of improved health outcomes. Overall, 19 carnivores, 423 herbivores, and 1,075 birds were successfully treated under veterinary supervision. **Conclusions:** Systematic antiparasitic management with Albendazole and Fenbendazole prevented mortality, ensuring effective, sustainable parasite control in Safari Park's captive wildlife.

INTRODUCTION

A zoological garden, often referred to as a zoo, safari park, wildlife sanctuary, or animal home, is a place where animals can be viewed in cages, as well as bred and researched [1, 2]. The first zoo was opened in 3400 BC. Zoos are like public parks, enabling visitors to learn and see the wild animals and their environment on planet Earth [3]. Zoos have crucial educational and conservation functions, and animals are taken care of by trained personnel who attend to their welfare [4]. The common and domestic animals in zoos can be carriers of different parasitic diseases [5, 6]. To manage these parasitic diseases, animals should be constantly fed with antiparasitic drugs [7, 8]. Intestinal parasites cause health complications in zoos and in other

wildlife species [9]. Fecal and ectoparasites may lead to high rates of mortality, especially of animals and birds being introduced into zoos [10]. The occurrence of this issue is also widespread in other zoological and wildlife centers across the globe, where endo- and ectoparasites are very common [11-13]. Parasite infections are major causes of health and productivity challenges to animals globally, and they are thus considered a major problem for agriculture. Animals can be exposed to internal parasites that are present in the body and external parasites, ticks, mites, lice, fleas, and flies, which exist on the body. The infestations lead to high costs of production of livestock [14]. The world has been utilizing different antiparasitic



drugs to control parasites in animals. They are, however, not sure to be used continuously because of reasons like the appearance of drug-resistant parasites, the cost of medications, limited availability, and the worry that food products contain drug remnants [15]. Helminthiasis has been controlled in herds or flocks using vaccines that can interfere with the life cycle of some helminth parasites [16]. Moreover, useful vaccines were invented and tested for their effectiveness against other parasite diseases. The parasitic diseases can be targeted by the implementation of vaccination strategies in two different modes: to safeguard the animals in a flock or herd that are the most vulnerable or to lower the rate of larval lumping within pastures, which consequently reduces the rate of infection of the vulnerable animals [17]. Animals are known to cause parasitic diseases that cause diverse pathological conditions. As an example, gastric parasite stages decline the functional mass of gastric glands, resulting in the formation of non-acidic gastric juice when parietal cells are replaced by cells lacking differentiation ability that rapidly divide [18]. This leads to poor feed consumption and loss of weight. Some parasites like *Haemonchus contortus* and *Ancylostoma* species feed on a lot of blood, leading to clinical anemia. Their blood-sucking behaviors, as well as blood leakage caused by ruptured mucosa and loss of epithelial cells of the gastrointestinal tract, are correlated with an augmented morbidity and mortality in animals. The case of blood loss through *Haemonchus* and *Fasciola*, among other species of intestinal helminths and protozoa, may result in multiple clinical episodes. Besides, parasites like *Fasciola*, *Schistosoma*, Lungworm, Ascarid, and filariids have been linked with organ damage caused by mechanical damage or inflammatory response, leading to severe disease pathways and diminished productive and reproductive performance. Moreover, systemic illnesses are known to be caused by parasites like *Trypanosoma*, *Toxoplasma*, and *Babesia* species [18]. In animals that are parasitized, the growth and death of animals may be stunted, and huge amounts of money may be lost, especially when the level of parasite infestation is high, with intestinal nematodes being the primary cause of production loss in ruminants [19-21]. Both external and internal parasites must be well managed during the lifetime of the animal since all age groups are susceptible to infections. Deworming procedures are based on legislative rules, veterinary advice, and personal risk factors, including hunting habits, previous exposure to parasites, and diet, and professional consultation is advisable [22]. When maintaining captive breeding and wildlife management, frequent surveillance, early detection, parasite management, and hygiene measures play a significant role in animal health, welfare, and conservation. As a descriptive study, it sought to document the current

practices, associated mortality outcomes, and overall health status of the animal collection and drugs used at Safari Zoo Lahore to control parasites and conserve captive fauna.

This study aims to evaluate the anti-parasitic protocols and drugs used at Safari Zoo Lahore to control parasites and conserve captive fauna.

METHODS

This prospective observational analytic study was carried out in the period between March 2024 and August 2024 in the Punjab Wildlife Parks Department (Safari Zoo Lahore), Raiwind Road, Lahore, Pakistan. The main objective was to describe the current management practice on anti-parasites and its performance; no hypothesis was set in advance. The sampling method was a full-fledged census-based method where the sample included all the animals that met the inclusion criteria within the study duration. Newly introduced, quarantined, or medically ill animals were excluded. The total number of animals used was 1,517: 19 carnivores, 423 herbivores, and 1,075 birds (27 species). The feeding habit was used to classify the animals: herbivores, carnivores, and birds. Pakistan Safari, African Safari, Desert Safari, Salt Range, Lion Safari, Avian Safari, and Pheasantry provided semi-natural homes to animals. De worming was done quarterly in January, April, July, and October under licensed veterinary care as per international zoo requirements. The standardized sheet was used to collect data prospectively, with species, number of animals, drug (Albendazole or Fenbendazole), administration route (oral, feed, or water), dose, and date. A variety of total animal counts was done by the park inventory and direct headcounts. Health and mortality monitoring after treatment was done daily for two weeks after every round of deworming. The treatment was done with Albendazole (Alba 10 Plus) and Fenbendazole (Panacur 10). The dosage of 1 ml was given per 10 kg body weight. Albendazole was applied through sprayed chickpeas to the herbivores and administered orally or in water (with Vitamin C) to the birds, and through meat to the carnivores. To measure natural mortality and parasitic stress, a small non-treated control group ($n = 35$; 10 herbivores, 5 carnivores, 20 birds) was monitored under the same conditions. Preexisting and post-treatment fecal samples were taken and analyzed using direct smear and flotation methods under the microscope. The prevalence of parasites improved from 21.4 to 2.8 pre- and post-treatment, respectively. The statistical analysis was done with the help of Microsoft Excel 365 (descriptive statistics: mean, standard deviation, percentages) and SPSS version 26.0. The One-way ANOVA compared the groups in terms of mortality, the independent t-tests were used to determine drug efficacy, and the Pearson correlation was used to test

the relationship between dosage and decrease in parasitic stress. The level of significance taken was $p < 0.05$.

RESULTS

A total of five one-liter bottles of Albendazole were used as an antiparasitic treatment for herbivores, and one bottle was used for birds. No mortality occurred following deworming. These practices helped to reduce the overall mortality of animals at Safari Zoo Lahore. The carnivore population included lions (*Panthera leo*), tigers (*Panthera tigris*), leopards (*Panthera pardus*), and hyenas (*Crocuta crocuta*). Lions and tigers were the most dominant carnivores, representing nearly 60% of the total carnivore population (Figure 1).

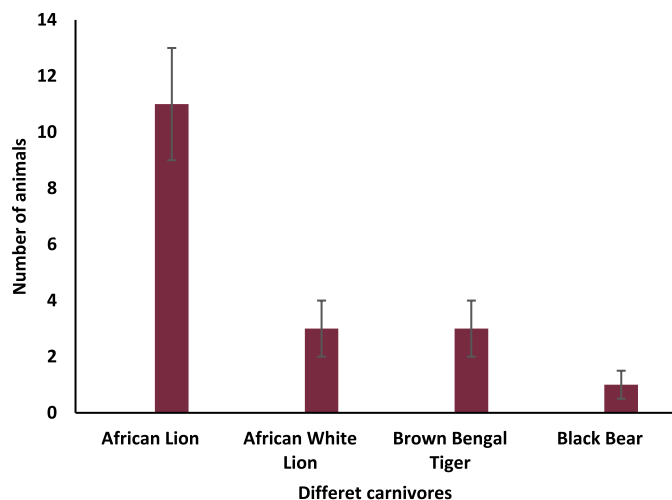


Figure 1: Data of Carnivore Species at Safari Zoo Lahore

The herbivore population comprised deer, nilgai, zebras, and antelopes. Spotted deer (*Axis axis*) and nilgai (*Boselaphus tragocamelus*) were the most abundant, accounting for more than half of all herbivores, followed by blackbuck and chinkara (Figure 2).

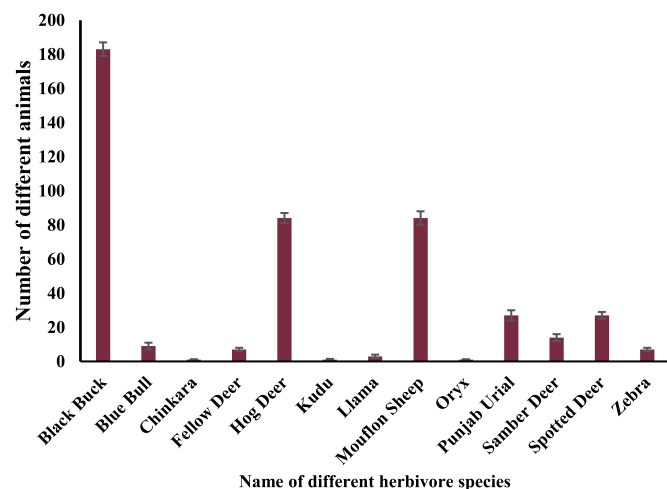


Figure 2: Data of Herbivore Species at Safari Zoo Lahore

A total of 27 bird species ($n = 1,075$) were recorded. Some birds were housed in the Pheasantry, while others were

moved to the Avian Safari. Bobwhite Quail and Chukar Partridge populations were noticeably higher. An Albendazole dose (5 mg/kg) was administered orally to partridges and quails for two consecutive days, with water intake monitored. Overall, the deworming program effectively reduced parasitic burden and improved animal health and survival at Safari Zoo Lahore (Figure 3).

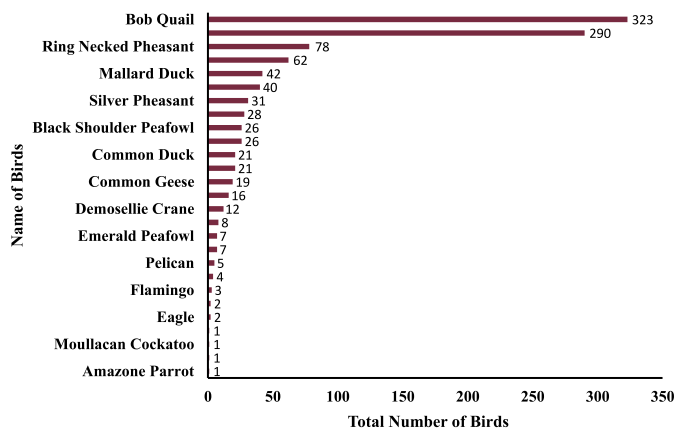


Figure 3: Number and Types of Birds at Safari Zoo Lahore

DISCUSSION

The major roles of zoos and aquariums are based on education, research, and conservation [23, 24]. These facilities are also important in ex-situ conservation, which is the maintenance of animal populations in areas outside their natural environment [25, 26]. In the current paper, Safari Zoo Lahore has shown that regular antiparasitic control, especially with the use of Albendazole, helped keep animals healthy as well as minimize their deaths. They used five one-liter bottles of Albendazole with herbivores and one with birds, with no deaths after deworming and a 12 percent net percentage decrease in mortality [27, 28]. This stresses the practical significance of prophylactic veterinary care in confinement settings. The research established that herbivores were more susceptible to parasitic diseases since they do graze, and they share the same enclosures with carnivores, thus the importance of worming on a regular schedule. Controlled doses of Albendazole also have a benefit on birds, especially Bobwhite Quail and Chukar Partridge, which had no complications after the treatment. The results confirm that systematic antiparasitic intervention is an efficient and secure management strategy for keeping various animal populations in captivity healthy. Even though the antiparasitism program produced positive results, some limitations were observed. The observational study did not have a laboratory-based identification of the parasites, quantitative fecal examination, or hematological confirmation of the reduction of infections. Moreover, seasonal changes and possible risks of re-infection were not eligible to be assessed during the course of the study.

Additional research that includes fecal egg count reduction tests (FECRT) and molecular identification of parasites would give a more detailed evaluation of the efficacy and resistance of drugs.

CONCLUSIONS

The findings had no post-therapeutic mortality and better survival after the use of Albendazole. Sustainable parasite control in captivity depends on maintaining hygiene, regular deworming, and tracking the success of the treatment. Further studies must be directed to the combination of laboratory diagnostics and comparative drug testing to streamline the treatment regimen and minimise the risk of parasitism even more.

Authors Contribution

Conceptualization: MMH

Methodology: MMH

Formal analysis: RY

Writing review and editing: RY

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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Original Article



Mixed Infection by *Anaplasma marginale* and *Anaplasma centrale* in Buffalo: A Short Follow-Up of a Case

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ABSTRACT

The buffaloes with mixed infections of *Anaplasma marginale* and *A. centrale* may cause severe clinical manifestation and difficulty in treatment, particularly in the field setting when diagnostic facilities are limited. **Objectives:** To report about a case of mixed *Anaplasma* infection in a buffalo heifer, explain clinical manifestations, laboratory diagnosis, response to treatment, and emphasize on the significance of evidence-based management. **Methods:** A buffalo heifer with clinical presentation of fever, emaciation, weight loss, shortness of breath, cough, anemia, fatigue, isolation with the herd, and anorexia were studied. Giemsa-stained thin blood smears were used to conduct laboratory diagnosis to confirm the presence of *A. marginale* and *A. centrale*. Stool was examined on metazoan parasites. The animal was given three normal doses of oxytetracycline with antipyretics and multivitamins separated by an interval of 24 hours. It was also observed that prior empirical treatments were done by local veterinary technicians. **Results:** Laboratory analysis proved the presence of both *A. marginale* and *A. centrale*. Stool analysis had a negative metazoan parasite. The buffalo never responded to the oxytetracycline treatment that was administered to him, and antipyretics made his symptoms only short-lived. Earlier treatments that were not validated in a laboratory were not effective. **Conclusions:** The case in question highlights the need to test anaplasmosis unambiguously in the laboratory in terms of appropriate diagnosis and therapeutic management. It also points out the issues which are related to lack of standardization of treatment practices and poor diagnostic facilities in the field setting.

INTRODUCTION

Anaplasmosis (Gall-sickness) is a domestic/wild ruminant rickettsial infection of red blood cells which is transmitted by vectors [1, 2]. It is the disease of some species of the genus *Anaplasma* such as *A. marginale*, *A. centrale*, *A. ovis*, *A. platys*, *A. phagocytophilum*, and *A. bovis* [3]. The most important of these are *A. marginale* and *A. centrale* that have been known to cause different levels of hemolytic anaemia, fever, emaciation and low productivity in bovines. An anaplasmosis, which is more severe, is caused by *A. marginale* and a milder infection is caused by *A. centrale* [4, 5]. It is primarily transmitted by ticks belonging to the genera *Boophilus*, *Dermacentor* and *Rhipicephalus*, but

can also be spread mechanically by biting flies or with contaminated equipment (e.g. needles, dehorning or tagging tools) [6, 7]. Dual infections of two species of *Anaplasma* are extremely infrequent, but can complicate case diagnosis and treatment care [8, 9]. The disease occurs in four phases, including incubation, developmental, convalescent, and carrier, the incubation period takes 3–9 weeks. Acute anaemia and clinical manifestation of the disease occur in the developmental phase, and finally it cures but with the carrier status lifelong [10, 11]. The diagnosis of bovine anaplasmosis in Pakistan rests on clinical manifestations rather than on

confirmatory laboratory tests thereby causing misdiagnosis and wrong treatment [12, 13]. The current case report of a rare mixed infection of *A. marginale* and *A. centrale* in a buffalo heifer, which was confirmed by the examination of the Giemsa-stained blood smears and highlights the importance of correct diagnosis and evidence-based treatment in the field.

This study aimed describe a case of mixed *Anaplasma* infection in a buffalo heifer, report on clinical presentation, laboratory diagnosis, response to treatment and emphasize the role of evidence-based management.

METHODS

It was a case study of clinical case report. A buffalo heifer 2.5–3 years old with apparent clinical features indicative of anaplasmosis was observed around the University of Peshawar, Pakistan, between 18 August 2017 and 28 August 2017. The ethical approval was taken from Institutional Review Board with IRB no: 10719. As per the description of the owner, the animal was bought on 07/08/2017 in the local livestock market as a business animal. Clinical symptoms have manifested themselves in the course of purchase, and the owner confused them with temporary effects of transport stress and extreme summer heat. Monitoring of the case involved a 10 days' follow-up on the case after initial identification and after which the animal was sold. The rectal temperature measures were taken daily, and mucous membranes of the vagina and eyes were observed on the presence of anemia. A stool sample on 18/08/2017 was tested on the microscope with helminth ova on a Lugol iodine stained smear preparation. In case of blood examination, the ear vein was pricked with a thin slide, and it was stained with freshly prepared Giemsa working solution. A second blood smear was prepared by using erythrocytes that were subjected to the Ficoll density gradient centrifugation technique that reduced contamination of leukocytes and debris. Thirdly, Oxytetracycline (24-hour intervals) was administered in combination with antipyretics and multivitamins in three standard doses to the animal. The surface of the whole body was thoroughly checked on the presence of ticks which are the main vectors of anaplasmosis but the presence of ticks was not found in the clinical examination. In the treatment process, high levels of uncertainty in veterinary practice were experienced. The owner initially visited a local dispenser 09/08/2017, which was two days after the purchase and was provided with unspecified injectable medications, details of which were not disclosed to the owner. This first dispenser was not successful, and another dispenser was consulted who prescribed one injection of an unspecified antibiotic and an antipyretic. Subsequently, the case was assumed by the study which rolled out a regular healing cycle of oxytetracycline and supportive antipyretics after the diagnosis was

established. The study was only able to administer three doses with intervals of 24 hours and the owner withdrew the treatment. This was due to cessation because he was advised by another veterinarian who used Imdidcarb, an antiprotozoal medication. This tendency to alternate between unqualified dispensers and veterinarians is a typical and alarming tendency among livestock owners, which, in most cases, is determined by desperation and ignorance, a drowning man is holding on to a straw. Illiterate and poor peasants and farmers are still very susceptible to exploitation by the quacks and the veterinary practitioners who have limited experience. It is also aggravated by the lack of well-equipped husbandry healthcare facilities and veterinary hospitals, which emphasizes the necessity of more powerful veterinary outreach and community education.

RESULTS

The animal presented persistent severe symptoms including fever, emaciation and weight loss, short breath, cough, anaemia, fatigue, isolation from the herd, frequent sitting in water, and anorexia. Anaemia (A), short breath (B), weight loss (C), and fluctuations in body temperature during 10-day follow-up (D). Over the 10-day follow-up period, the body temperature fluctuated between 102°F and 104°F. The animal was in the acute developmental stage of infection. It did not respond to the standard three-dose treatment regimen of oxytetracycline. However, administration of antipyretics provided temporary relief, easing shortness of breath and apparent discomfort, which might be attributed to a transient reduction in body temperature and improved oxygen solubility in blood (Figure 1).



Figure 1: Clinical Symptoms of Anaplasmosis in Buffalo

A Giemsa-stained thin blood smear examination revealed a mixed infection with *A. marginale* and *A. centrale*, while the stool smear was found negative for helminth ova. (A) Vials of medication are administered by the local dispenser. (B) *A. marginale* (white arrows) and *A. centrale* (blue arrows) observed in Giemsa-stained thin smear at 1000× magnification (oil immersion). (C) Vacuolar artefacts in stool smear (orange arrows). Scale bar = 10 µm. The Giemsa-stained smear prepared from density gradient-

isolated red blood cells (RBCs) gave a clearer and more distinct picture as compared to the direct smear obtained from venepuncture. The latter sample exhibited greater contamination with skin debris, even after thorough cleansing of the area with an alcohol swab before sampling. No external tick infestation was observed during examination (Figure 2).

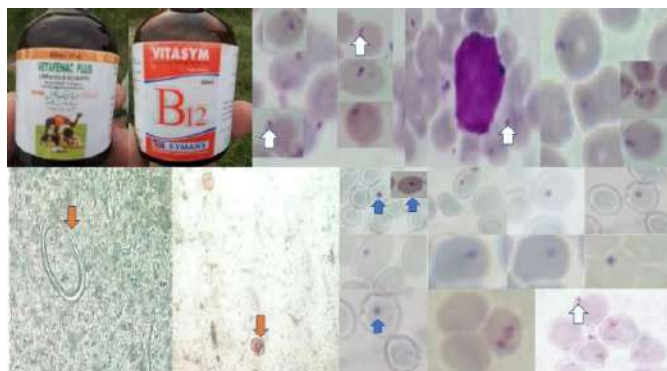


Figure 2: Microscopic and Treatment Observations

DISCUSSION

Microscopic observation in Romanowsky-or Giemsa-stained thin blood or tissue smears is one of the several diagnostic techniques used for *Anaplasma* species [14, 15]. It is the oldest and most conventional diagnostic method, and requires considerable technical expertise and experience on the part of laboratory technicians [16, 17]. It has been reported that with only 10^6 /mL RBC infectivity, the diagnostic sensitivity becomes markedly limited. Despite these limitations, it remains the most widely adopted tool for diagnosing anaplasmosis, especially effective in identifying the acute phase of infection [18, 19]. Viewing the results of our study, separation of the RBCs by the density gradient centrifugation technique is an effective method to eliminate leukocytes and creates a smear which is more homogenous and artefact-free. The detection of intraerythrocytic *Anaplasma* species is much more evident with only erythrocytes observable by the microscope. Elution of the impurities and precipitates of the supernatant also reduce the possibility of artefactual misinterpretation. These smears are consequently of special use in the identification of intraerythrocytic *Anaplasma* species. The age of animals infected had a high correlation with the severity of clinical symptoms. More severe in cattle aged 1-3 years and the best morbidity in cattle aged above 3 years with an estimated rate of mortality of 30-50%. This age-symptom correlation is of special importance to our study. The heifer had serious clinical manifestations, which were in line with past observations [20]. The current case has shown that acute anaplasmosis was not responsive to common treatment programs with oxytetracycline and imidocarb, just as it was observed in previous research [21]. Such non-response

can be explained by some potential causes, such as antimicrobial resistance, a highly developed infection, or a mixed infection of many *Anaplasma* species. The ignorance of livestock owners and the limited regulation are some of the factors that have enhanced the growth of unqualified and inexperienced veterinary practitioners, especially in the rural communities. Though veterinary ethics and animal welfare principles are included in professional training, they tend to be less than optimal when they are followed in the field. Other veterinarians can make their treatment choices based on presumptions of the economic limitations of their owner by choosing less expensive or less thorough treatment courses of action that can affect both the effectiveness of treatment and the welfare of the animal [22]. In the current case, it was noted that the animal had first been dispensed by unqualified veterinary dispensers who failed to explain to the owner what type of therapy, the dosage, and the use of the therapy was, and who was a poor and illiterate peasant.

CONCLUSIONS

The present study warns of impure veterinary healthcare arrangements in Khyber Pakhtunkhwa and Pakistan in general. Farmers' awareness regarding veterinary health medicine is extremely poor, due to which they become easy prey to quack and inexperienced veterinarians and veterinary technicians. Laboratory diagnosis of veterinary diseases is extremely poor due to a lack of diagnostic facilities (only the Veterinary Research Institute (VRI) in the whole Peshawar district has the facility).

Authors Contribution

Conceptualization: QJ

Methodology: QJ, MUH, FA, FS, JS

Formal analysis: FS, JS

Writing review and editing: I, MU

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Conflicts of Interest

All the authors declare no conflict of interest.

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