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Impact of Provision Food on the Foraging Ecology of Rhesus Macaque (*Macaca mulatta*) Inhabiting Margalla Hills National Park, Islamabad

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ABSTRACT

This study explores the behavioral and dietary impacts of food provisioning on Rhesus macaques in Margalla Hills National Park, Islamabad, focusing on variations across high-provisioning (HP) and low-provisioning (LP) areas, visitor densities, and age-sex groups. Over 720 hours of observation, including 2,329 scans, revealed that HP rhesus macaques spent significantly more time foraging (67.53%) compared to LP rhesus macaques (46.21%), with lower levels of resting (11.36% vs. 22.49%) and grooming (9.85% vs. 13.49%). HP rhesus macaques also exhibited reduced locomotion (6.17% vs. 29.62%) and a higher reliance on human-provided food. Behavior analysis across visitor densities indicated that moderate visitor levels (51-80) promoted active behaviors like foraging and locomotion, whereas very high visitor levels (> 80) led to increased resting and grooming. Foraging peaked at 51-80 visitors and declined at extreme visitor counts, while grooming and resting behaviors showed opposite trends. Post hoc analyses revealed distinct behavioral patterns based on visitor density, with significant variations in grooming, locomotion, and food consumption. Age and sex differences indicated that adult females with infants foraged less frequently than adult males, and juveniles exhibited the highest foraging activity. Dietary analysis confirmed significant differences, with HP macaques primarily consuming human food, while LP macaques relied more on natural sources. Locomotion patterns differed markedly between provisioning areas, with HP macaques showing concentrated movement and increased social interactions. These findings underscore the influence of provisioning and visitor density on macaque behavior, highlighting the need to carefully manage the human-wildlife interactions.

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Authors' Contribution
SB conceived and designed the study, conducted the fieldwork, performed data analysis, and wrote the first draft of the manuscript. SA contributed to field visits and data collection. KZ assisted in data collection and manuscript preparation. MK prepared all maps used in the study, including distribution maps. ARK supervised the research.

Key words
Rhesus macaques, Food provisioning, Visitor density, Behavioral patterns, Dietary differences

INTRODUCTION

Food provisioning to the free-ranging primate population is a ubiquitous phenomenon that occurs in a wide range of sociocultural situations. Provisioning refers to the supplemental feeding provided by humans. Food provisioning may have cultural and spiritual reasons that facilitate interaction between people and primates in different habitat settings such as temples, towns, or tourist sites (Medhi *et al*., 2007). Provisioning has both positive and negative outcomes. On the positive side, it can significantly boost tourism, providing economic benefits to local communities and fostering a greater appreciation and willingness to protect wildlife (Knight, 2009). On the other side, provisioning can have detrimental effects on primates. It often leads to changes in their natural behaviors, health issues, and altered social dynamics (Maréchal *et al*., 2016). Moreover, it can result in higher population densities, leading to increased competition and stress among individuals (Becker and Hall, 2014).

Globally, human provisioning has been shown to alter primate behavior and ecology in multiple ways. The primates showed a diversity of specialized foraging adaptations and preferences. Spatial variation in primate population abundance is influenced by the interplay of food quality and quantity, with fallback food availability modulating population size under differing nutritional needs and environmental conditions (Hanya and Chapman, 2013). Fruit availability significantly influenced their feeding behavior, challenging previous assumptions about their dietary habits and highlighting the importance of fruit in their ecology and behavior (Matsuda *et al*., 2009). A study conducted in Ghana, West Africa, found that Lowe's Rhesus macaque (*Cercopithecus lowei*) spent less time foraging on natural foods, increased rest, and showed higher aggression compared to groups relying solely on wild foods (Badiella-Giménez *et al*., 2021). Provisioning

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has influenced population dynamics and disease risk by altering contact rates and immunity (Grossberg *et al*, 2003). A study in Ouzoud, Morocco, demonstrated that Barbary rhesus macaques displayed habitat diversity, with a notable presence in various environments where they show habituation to humans and partial reliance on provisioned food (El-Alami *et al*., 2013).

The rhesus macaque is the non-human primate with the most diverse geographical and ecological range. Rhesus macaques are distributed throughout South Asian countries like Pakistan, Afghanistan, Bhutan, Bangladesh, Burma, and Nepal, as well as China, Vietnam, and Thailand. The rhesus macaque is widely distributed in the Margalla Hills National Park, Islamabad (Aslam *et al*., 2024).

In Asia, Studies found that Provisioning has a profound impact on the ecology of Rhesus macaques. Rhesus macaques in India at pilgrimage and tourist sites spent more time on anthropogenic food, thereby decreasing natural foraging and increasing aggressive behavior (Sengupta and Radhakrishna, 2018). A study in Bangladesh showed that activity patterns of different Rhesus macaque groups are influenced by habitat, season, and food availability. These urban-rural disparities are primarily driven by distinct food resource availability, showcasing behavioral flexibility crucial for managing commensal populations (Jaman and Huffman, 2013). Unregulated roadside provisioning of long-tailed monkeys in West Sumatra, Indonesia, has led to a rise in road traffic accidents (RTAs), causing fatalities and injuries to both animals and humans (Ilham *et al*., 2023; Zhoa *et al*., 2023).

Recent years have seen a rise in literature about Rhesus macaques in Pakistan. Aslam *et al*. (2024) documented the diet and food preferences of rhesus macaques in the highly biodiverse Margalla Hills National Park. Moreover, Ehtisham *et al*. (2021) examined the impact of tourism on the distribution of the rhesus macaques in the Galiyat area of Khyber Pakhtunkhwa. More recently, Hussain *et al*. (2025) studied the complex status of the rhesus macaques and the multifaceted challenges to their conservation in Machiara National Park, Azad Jammu and Kashmir. However, there is a notable lack of understanding of how explicitly tourist provisioning affects the feeding behavior and food choice of rhesus macaques in MHNP. This study addressed this significant gap

This study was conducted to assess the effect of tourist provisioning on the foraging ecology of the rhesus macaques in MHNP. More specifically, it assessed how the presence of visitors affects activity patterns and foraging strategies of rhesus macaques. In addition, the study investigated the effect of provisioning on the ranging behavior of rhesus macaques using distribution maps of group locations in high and low-provisioned areas to indicate differences in mobility. To address these objectives, behavioral and spatial data were collected.

MATERIALS AND METHODS

Study area

The current study was conducted in the Margalla Hills National Park (MHNP), located between (33.6° to 36.55° N and 72.8333° to 73.4333° E) situated in the Federal Capital city Islamabad, Pakistan (Fig. 1). It covers an area of 17,386 hectares, with elevation ranging from 456 to 1580 meters (m), and the landscape is rugged mountains. The climate of the study area is humid subtropical (Koppen climate classification). The region experiences two wet seasons because it lies in the monsoon zone; summer rains last from July to September, while winter rains last from January to March each year. The vegetation of the Margalla Hills range is classified as tropical dry mixed deciduous forest, dry subtropical semi-evergreen scrub, and subtropical pine forest (Khalid *et al*, 2019). The reported mammalian fauna of the park mainly includes the common leopard, Asiatic jackal, red fox, Asian palm civet, small Indian civet, yellow-throated marten, Indian pangolin, and rhesus macaque etc. (Anwar and Chapman, 2000).

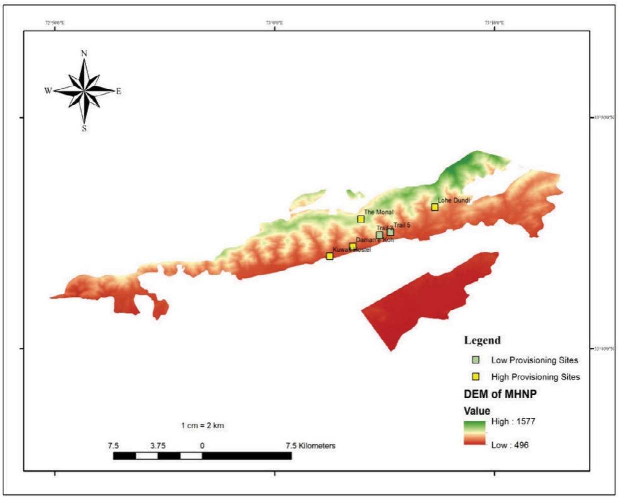

Fig. 1. Map of the study area (Margalla Hills National Park, Islamabad) showing different provisioning sites. Yellow colour boxes indicate four high provisioning sites, Damne koh, Monaal, Loh-e-dandi, and Faisal masjid (Kuwait hostel E 11). Green boxes show low provisioning sites (Trail 3 and Trail 5).

We divided our study area in the Margalla Hills National Park (MHNP) into high and low provisioning areas. The high provisioning area (HPA) included four sites with substantial human-provided food: Damne Koh, Loh-e-Dundi, the Monal restaurant area, and the Faisal Masjid. While the low provisioning area (LPG) consisted of trails three and five, where rhesus macaques primarily rely on natural food sources due to lower tourist presence and less supplemental feeding.

Data collection

The current study was conducted from September 2022 till July 2023. The field data were collected from December 2022 to February 2023, and then from May to July 2023. At the onset of the study, we conducted are naissance survey of the study area in the month of November 2022 to assess the population size and distribution of rhesus macaques, as well as the patterns of visitor activity across various locations within the Margalla Hills National Park (MHNP). The composition of focal animal groups and identification of key individuals at each age-sex classification Porter (2005) site is given, guided by (Table I).

The field visits were structured as 10 days each month, with five days at high-provisioning sites and five days at low-provisioning sites. An ethogram of rhesus macaques' behaviors was developed following Porter (2005) (Table II). We recorded the behavior of all visible individuals of the focal group, categorizing their activities into predefined behavioral categories: Grooming (GR), Locomotion (LC), Resting (RS), Provisioning by People (PBP), Snatching (SN), Scavenging (SC), and Natural Food (NF).

Observations were conducted from 06:00 to 18:00, encompassing the entire daylight period. We employed a two-tiered observational approach comprising scan sampling and focal animal sampling techniques to study the behavior and dietary habits of rhesus macaques (*Macaca mulatta*) in their natural habitat. Scan sampling, conducted at regular 15-min intervals following the methodology outlined by Altmann (1974), served as the primary method for recording specific behavioral activities. When an individual rhesus macaque was observed engaging in feeding behavior during a scan, we immediately initiated focal animal sampling on that individual. The focal observation lasted for a 15-min session, during which we closely monitored and recorded the specific food items being consumed and the duration of feeding.

Table I. Composition of rhesus macaque groups observed at high-provisioning areas (HPA) and low-provisioning areas (LPA) in Margalla Hills National Park. The table includes group ID, sampling site, total number of individuals, age-class distribution (infants, juveniles, sub-adults, adults), and notable/key individual characteristics.

Provisioning level	Sampling site	Total	Infants	Juveniles	Sub-adults	Adults	Notable Individual's characteristics
HPA	Daman-e-Koh	32	5	9	11	7	Adult female: Baby clung to the belly, short tail
	Loh-Dandi	17	3	3	6	5	Adult male: Head bigger than usual; Adult female: short tail, black scar, and injured leg
	Monal	15	4	3	5	3	Juvenile: Tail wrapped in rope; Adult female: spot near right eye
	Faisal Masjid	18	4	7	3	4	Sub-adult: Tail hook-shaped
LPA	Trail 3	22	5	2	6	9	Sub-adult: Coiled tail
	Trail 5	22	5	2	6	9	Adult female: Black saggy nipples

Table II. An ethogram of rhesus macaque (*Macaca mulatta*) behaviors used in the present study.

Ethogram behavior	Mode of foraging	Description
Foraging	Natural food	It included only feeding on natural resources in the locality
	Provisioned by people	Included all the food directly given by people
	Snatching	Included attacking people for food items
	Scavenging	Included scavenging through the dustbins and the ground for food residues
Resting		the animal remained stationary, adopting various postures such as standing, sitting, or lying down.
Locomotion		Included all animal movements, such as strolling, sprinting, swinging, jumping, etc.
Grooming		It included all individuals cleaning themselves or other troop members on the road.

Visitor numbers were recorded to assess the impact of human presence on the foraging behavior of Rhesus macaques. Visitor counts were conducted daily, with observations lasting two hours each in both the morning and afternoon. This approach ensured that visitor numbers were recorded during peak times when food provisioning typically occurred. The count of visitors was limited to those present within a 20-meter radius of the center of the focal rhesus macaque group. Based on the observed numbers, we have designed four categories: <20 visitors, 21-50 visitors, 51-80 visitors, and >80 visitors. The geographical coordinates for each sampled location were recorded using a handheld GPS device (Garmin eTrax Vista).

The collection of GPS coordinates was integral to our study design as it served the purpose of mapping the spatial distribution of rhesus macaques behaviors. By utilizing GPS locations, we gained insights into how the rhesus macaques behaviors varied across different areas within their habitat, allowing us to assess the impact of human-provided food on their movement patterns. The behaviors of rhesus macaques were digitally recorded using a mobile phone.

Data analysis

The collected data about the behaviors of rhesus macaques were analyzed using SPSS software (Version 23) by applying a t-test to check the activity pattern of the rhesus macaque population. A one-way ANOVA was applied to compare rhesus macaques' behaviors across areas, age-sex categories, and visitor numbers, followed by a post hoc test to identify specific group differences. A Chi-square test followed by post hoc (Holm) correction was applied to analyze the relationship in food items consumed by rhesus macaques in the Margalla Hills National Park. Finally, we mapped the daily locations of the rhesus macaques with the help of GPS coordinates by using Arc GIS software to show the difference in movement patterns at both high and low provisioning sites.

RESULTS

A total of 2329 scans were recorded in the current study, with 720 h, including 1335 observations for the high provisioning group (HPG), and 994 for the low provisioning group (LPG). The overall activity budgets of rhesus macaques differed between HPG and LPG groups and were further influenced by visitor levels (Table III). The HPG spent, on average, 67.53% of their time in foraging activities, which include all types of feeding, significantly higher than the LPG, which allocated 46.21% of their time to foraging ($p < 0.001$) (N=60, d.f= 58). The HPG showed significantly lower resting behavior (11.36%) compared to the LPG (22.49%) ($p < 0.001$) (N=60, d.f= 58). Grooming behavior was significantly lower in the HPG (9.85%) compared to the LPG (13.49%) ($p < 0.001$) (N=60, d.f= 58). The HPG exhibited a significantly lower moving behavior (6.17%) compared to the LPG (29.62%) ($p < 0.001$) (N=60, d.f= 58). Scavenging, feeding on natural food, feeding on human food, and snatching behaviors were also significantly different between the HPG and LPG ($p <0.001$) (N=60, d.f= 58) (Fig. 2).

Foraging (FG) varied significantly across visitor levels (F = 33.92, $p < 0.001$), being highest at 51–80 and 20–50 visitors, intermediate at >80 visitors, and lowest at <20 visitors. Among the foraging sub-categories, Natural food (NF) decreased as visitor numbers increased (F = 31.99, $p < 0.001$), whereas Provisioning by people

Table III. Activity patterns (%) of rhesus macaques in high-provisioning group (HPG) and low-provisioning group (LPG), under varying visitor levels (mean ± SD).

Behavior	HPG (%)	LPG (%)	<20 visitors	20–50 visitors	51–80 visitors	>80 visitors	F	p
Foraging (FG)	67.53 ± 11.85	46.20 ± 8.48	45.20 ± 7.37^{b}	69.97 ± 5.86^{a}	61.51 ± 1.39^{a}	35.64 ± 1.29^{c}	33.92	<0.001
Natural food (NF)	10.95 ± 3.73	33.62 ± 7.48	34.34 ± 6.96^{d}	12.35 ± 5.24^{b}	20.53 ± 1.26^{a}	7.30 ± 4.49^{c}	31.99	<0.001
Provisioning by people (PBP)	38.41 ± 10.89	4.60 ± 6.12	3.43 ± 2.33^{c}	38.53 ± 1.04^{a}	26.52 ± 2.10^{ab}	19.59 ± 7.94^{b}	34.21	<0.001
Snatching (SN)	12.65 ± 5.66	3.68 ± 3.31	3.16 ± 2.92^{c}	13.79 ± 4.39^{a}	9.17 ± 6.77^{b}	3.32 ± 0.57^{c}	22.05	<0.001
Scavenging (SC)	5.36 ± 3.70	3.70 ± 2.21	3.87 ± 2.42^{a}	5.15 ± 2.17^{a}	4.45 ± 2.87^{a}	5.42 ± 3.05^{a}	1.12	0.351
Grooming (GR)	9.85 ± 3.08	13.40 ± 3.20	13.58 ± 3.03^{ab}	9.38 ± 3.30^{c}	11.16 ± 3.25^{bc}	15.05 ± 0.12^{a}	7.85	<0.001
Locomotion (LC)	6.17 ± 3.37	12.41 ± 5.22	13.30 ± 4.99^{a}	6.49 ± 3.78^{bc}	8.04 ± 4.08^{b}	3.28 ± 0.43^{c}	11.97	<0.001
Resting (RS)	11.36 ± 10.68	22.49 ± 11.48	23.07 ± 1.10^{b}	8.08 ± 2.95^{c}	14.93 ± 1.15^{bc}	39.04 ± 1.54^{a}	14.81	<0.001

FG, Foraging (overall); Sub-categories: NF, Natural food; PBP, Provisioning by people; SN, Snatching; SC, Scavenging. Values within each row sharing the same superscript letter are not significantly different at $p < 0.05$ (Duncan post-hoc test).

Table IV. Activity patterns (%) of rhesus macaques across age–sex classes (mean ± SD).

Behavior	Adult male	Adult female	Adult female w/ baby	Sub-adult male	Sub-adult female	Juvenile	F	p
Foraging (FG)	58.10 ± 13.83^{b}	58.87 ± 12.36^{b}	40.90 ± 8.97^{a}	70.37 ± 1.91^{c}	61.73 ± 13.84^{c}	72.62 ± 3.68^{c}	8.31	<0.001
Natural food (NF)	24.06 ± 14.39^{b}	22.00 ± 12.03^{b}	27.39 ± 12.40^{b}	12.47 ± 2.86^{a}	23.00 ± 13.21^{b}	12.25 ± 4.56^{a}	1.67	0.157
Provisioning (PBP)	21.48 ± 21.10^{b}	24.65 ± 20.21^{b}	6.68 ± 7.94^{a}	33.15 ± 8.86^{c}	26.23 ± 23.03^{b}	37.53 ± 10.48^{c}	3.15	0.015
Snatching (SN)	7.73 ± 5.61^{b}	7.22 ± 4.41^{b}	2.63 ± 1.93^{a}	18.79 ± 6.78^{d}	8.67 ± 4.03^{b}	14.79 ± 1.59^{c}	11.42	<0.001
Scavenging (SC)	4.45 ± 2.24^{b}	4.23 ± 1.92^{b}	3.52 ± 2.33^{a}	6.83 ± 3.57^{c}	3.83 ± 2.06^{a}	7.08 ± 1.37^{c}	2.55	0.039
Grooming (GR)	12.66 ± 3.81^{b}	10.94 ± 1.92^{b}	12.79 ± 3.27^{b}	10.58 ± 1.41^{b}	10.77 ± 5.46^{b}	6.60 ± 2.13^{a}	2.23	0.064
Locomotion (LC)	10.15 ± 4.68^{a}	10.04 ± 6.98^{a}	10.03 ± 6.59^{a}	6.52 ± 4.44^{a}	8.84 ± 4.17^{a}	5.79 ± 1.74^{a}	0.76	0.586
Resting (RS)	14.01 ± 10.69^{b}	13.92 ± 5.87^{b}	31.12 ± 12.95^{c}	8.32 ± 2.40^{a}	14.53 ± 7.05^{b}	6.88 ± 1.64^{a}	8.12	<0.001

FG, Foraging (overall); Sub categories: NF, Natural food; PBP, Provisioning by people; SN, Snatching; SC, Scavenging. Values within each row sharing the same superscript letter are not significantly different at $p < 0.05$ (Duncan post-hoc test).

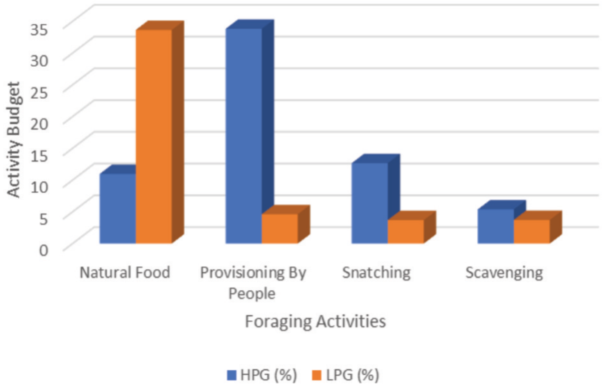

Fig. 2. Comparison between foraging activities of Rhesus Monkey between two groups: HPG, high provisioned group; LPG, low provisioned group.

(PBP) and snatching (SN) increased significantly (F = 34.21, $p < 0.001$; F = 22.05, $p < 0.001$). Scavenging (SC) did not differ with visitor level (F = 1.12, p = 0.351). Other behaviors were also affected: Grooming (GR) declined with increasing visitor numbers (F = 7.85, $p < 0.001$), Locomotion (LC) increased (F = 11.97, $p < 0.001$), and Resting (RS) was lowest at intermediate visitor levels and highest in the absence of visitors (F = 14.81, $p < 0.001$).

Age–sex classes also showed significant variation in activity patterns (Table IV). Foraging (FG) differed among groups (F = 8.31, $p < 0.001$), with juveniles and sub-adult males devoting the most time to foraging, while adult females with infants foraged the least. Within the sub-categories, Provisioning (PBP) and Snatching (SN) were significantly higher in juveniles and sub-adult males, and lowest in adult females with infants (F = 3.15, p = 0.015; F = 11.42, $p < 0.001$). Scavenging (SC) also varied significantly (F = 2.55, p = 0.039), being more common in juveniles and sub-adult males. Natural food (NF) did not differ significantly across groups (F= 1.67, p= 0.157).

Table V. Food item consumption of rhesus macaques at high-provisioning (HP) and low-provisioning (LP) sites. Percentages indicate the proportion of feeding scans in which each food type was consumed. Chi-square tests (A^2) were performed using the total feeding scans (N = 2,329) to examine differences in diet composition between HP and LP sites. Post-hoc comparisons were conducted using Holm correction to identify which food categories differed significantly.

Food item	HP (%)	LP (%)	Chi-square (χ^2)	p value	Post-hoc (Holm)
Fruit	9.19	32.46	143.0	<0.001	***
Flower	12.28	28.77	99.0	<0.001	***
Leaves	6.95	24.27	70.7	<0.001	***
Human food	64.94	8.82	688.6	<0.001	***
Water	6.61	7.77	1.2	0.27	ns

Significant differences are indicated as *** ($p < 0.001$); ns, non-significant.

Overall, dietary composition differed significantly between high and low provisioned rhesus macaque groups (A^2 = 794.79, df = 4, $p < 0.001$) (Table V and Fig. 2). Post-hoc pairwise chi-square tests with Holm correction revealed that LPG of rhesus macaques consumed a significantly higher proportion of natural food items: fruits (32.46%), flowers (28.77%), and leaves (24.27%), compared to HPG rhesus macaques (9.19%, 12.28%, and 6.95%), respectively ($p < 0.001$). In contrast, HPG relied predominantly on human-provisioned foods (64.94%), whereas LPG consumed only 8.82% of these items ($p < 0.001$). No significant difference was observed in the consumption of water between the two groups.

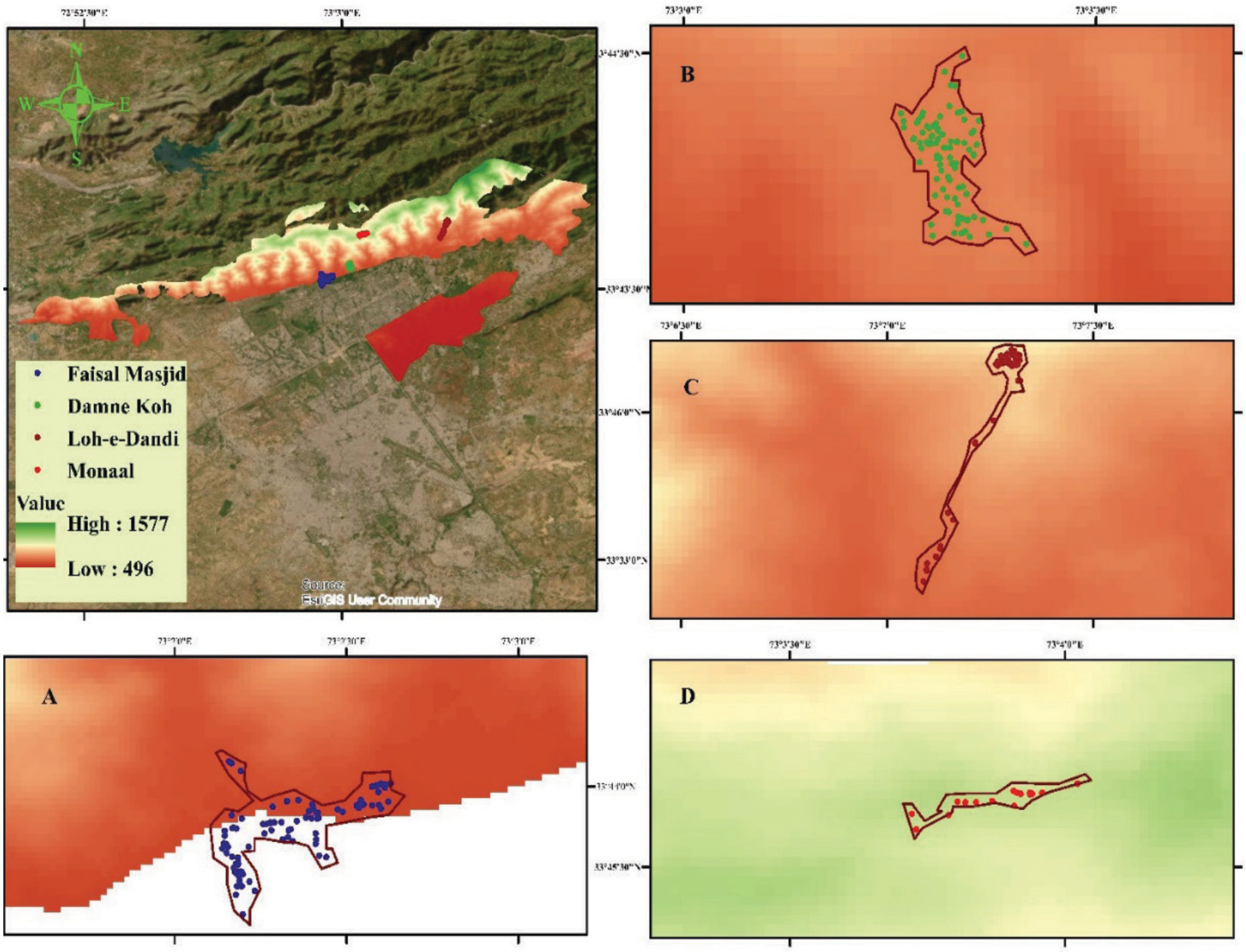

Fig. 3. Map of high provisioned sites; (A) Faisal masjid (Purple), (B) Damn-e-Koh (Green), (C) Loh-e-Dandi (Brown), (D) Monal (Red), and representing reduction in locomotion and movement pattern of rhesus macaques at Margalla Hills National Park, Islamabad.

*The delineated boundaries illustrate the locomotion patterns of macaques at the highly provisioned site.

Results of the current study showed that the rhesus macaques of the high-provisioned region limited their locomotion and became habituated to human food provided (Fig. 3). They spent most of their time around visitors at high-provisioned sites due to the availability of food without or with less expenditure of energy needed for foraging. In the high-provisioned areas, including Faisal Masjid, Damn-e-Koh, Loh-e-Dandi, and Monal, the rhesus macaques exhibit concentrated movement patterns, as indicated by the dense clustering of observation points. Conversely, in the low-provisioned areas represented by Trail 3 and Trail 5, the rhesus macaques demonstrate increased locomotion despite the scarcity of resources, with movement patterns appearing more dispersed as they search for food across a broader area (Fig. 4).

DISCUSSION

Overall analysis of the collected data indicated that the presence of visitors significantly influenced various behavioral types of the rhesus macaques in all sampling sites of the Margalla Hills National Park, Islamabad. The specific patterns of behavior varied depending on the number of visitors present, with different behaviors showing significant differences among the rhesus macaques groups. However, no significant differences were observed for scavenging behavior. Badiella-Giménez *et al.* (2021) reported that primates' routine foraging and social behaviors are highly impacted by the season and environment of their habitat.

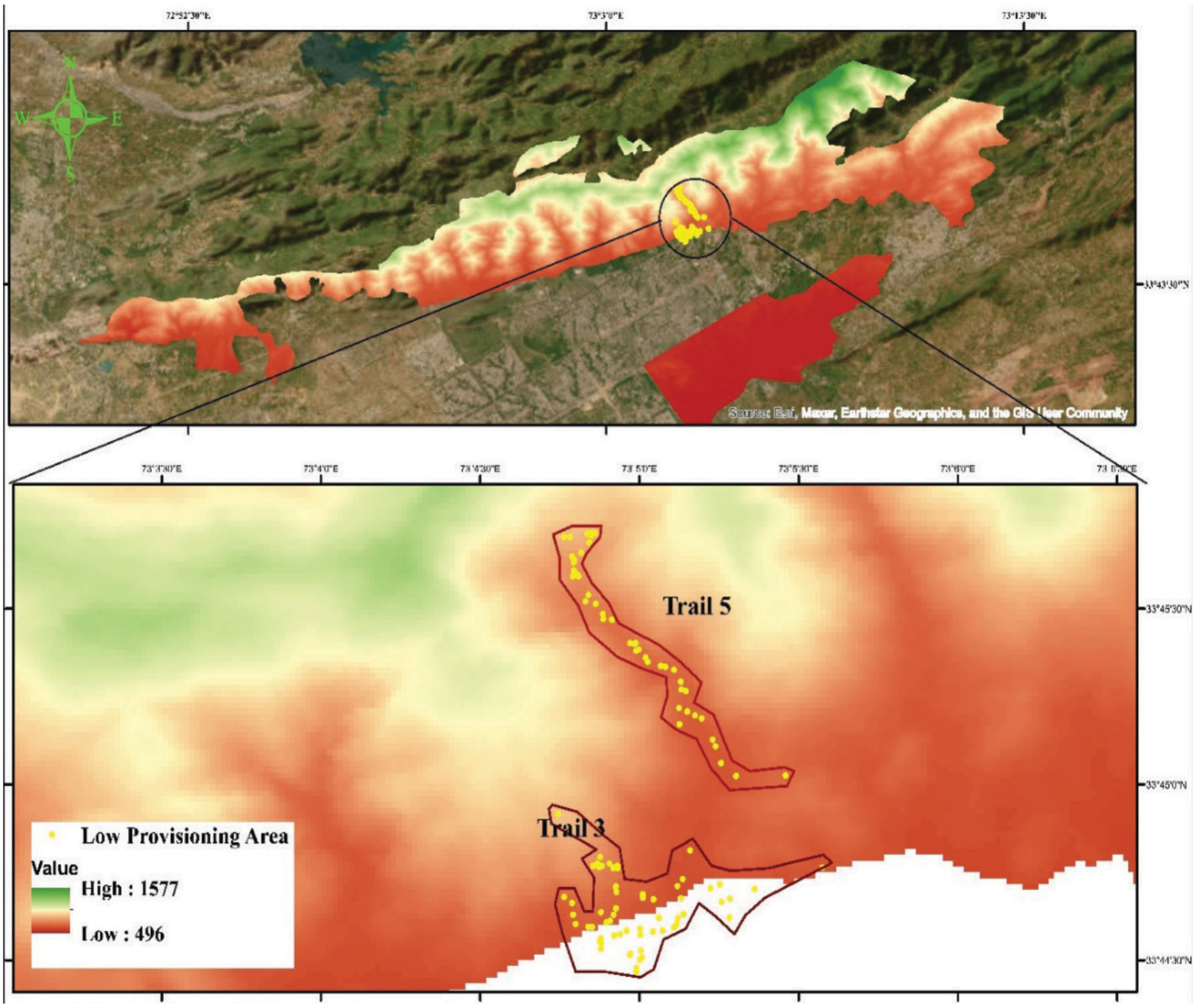

Fig. 4. Map of low-provisioned sites; Trail 3 and Trail 5 (yellow), representing an increase in locomotion and movement pattern of rhesus macaques at Margalla Hills National Park, Islamabad.
*The delineated boundaries illustrate the locomotion patterns of macaques at the low-provisioned site.

In the current study, we have investigated the impact of food provisioning to macaques by humans in the Margalla Hills National Park, Islamabad. Our results indicate that the foraging behavior of rhesus macaques has changed; the rhesus macaques spend more time at the sites around the visitors where they get food from them. Rhesus macaques in HPG have been found to spend 67% of their activity time in foraging activity around the visitors, as against the LPG rhesus macaques that spend 46% of their time in feeding behavior, which is significantly different. Our study observed that the LP group spent substantially less time grooming and more time actively foraging, a pattern consistent with findings reported by another researcher (Brennan *et al.*, 1985). The findings of the current study highlight that LPG and HPG rely more heavily on fruits and provisioned food, respectively, and this fact was previously reported Boutourlini's blue macaques in forested areas, that ate more fruit than those in fragmented forests (Tesfaye *et al*., 2013).

We found that the high provisioning group (HPG) spent significantly more time on provisioned food and spent less time on natural feeding, compared to the low provisioning group (LPG). This increased focus on human food in the HPG resulted in significantly less time spent on other activities, such as resting, grooming, and moving. Previous studies on activity budgets have demonstrated that Barbary macaques with high levels of provisioning spent less time in natural foraging (Unwin and Smith, 2010; Alami *et al*, 2012). Additionally, their frequent interactions with human visitors during feeding likely contributed to reduced time for stress-relieving activities like grooming. Instead, the competition over provisioned food often led to conflicts, as members of the HPG frequently engaged in fights during feeding sessions. This shift in behavior highlights the complex impact of human provisioning on both the foraging patterns and social dynamics of rhesus macaque groups.

The findings suggest that rhesus macaques adjust their behavior based on visitor density, with moderate visitor levels (51-80 visitors) promoting active behaviors like foraging and locomotion, while very high visitor levels (> 80 visitors) lead to increased resting and grooming, likely due to stress or competition for resources. When examining the impact of visitors' presence on the HP group, we discovered that, in comparison to when visitors weren't present, their presence significantly increased the feeding time and decreased the resting time, particularly during visits where the rhesus macaques are long-term hand-fed by visitors, as reported by Asquith (1989).

It was observed in the MHNP that in the case of overcrowding of people at the tourist sites, rhesus macaques felt threatened, so they reduced their locomotion and got hidden in plantation patches or moved in the forest as Ruel and Ayres (1999) documented that increased or decreased locomotion and grooming may reflect changes in stress levels or attempts to adjust to the new environmental conditions created by human presence. Snatching may be an adaptive reaction to new food resources brought by visitors, but provisioning by visitors might result in opportunistic eating behaviors and rivalry among individuals, as reported by several researchers (Fuentes, 2002; Robbins and Hohmann, 2006).

Our study reveals that the presence of humans leads to an increase in resting behavior among these rhesus macaques. This observation aligns with findings from various studies across different species, suggesting that animals may choose to rest more when faced with unexpected or potentially threatening human interactions. This behavior likely serves to reduce stress or conserve energy during such encounters, as noted by Hashmi and Sullivan (2020).

Adult females with infants in our study were observed to avoid areas with high human presence, which is in line with Maréchal *et al.* (2016) Maréchal *et al.* (2016) reported that provisioning can induce stress responses in rhesus macaques, leading to changes in behavior such as increased reliance on natural food sources and avoidance of human activity. Our findings that juveniles and sub-adults have a notable preference for human-provided sustenance align with observations by De Waal (1984) The research highlighted how provisioning by humans can influence rhesus macaques foraging behavior, particularly by increasing the reliance on human-provided resources among younger individuals. The observed differences in foraging behavior among the age-sex categories of Rhesus macaques in our study align with and extend findings from previous research. Our results indicate that adult females with infants exhibit reduced foraging activity compared to adult males, consistent with findings by Jaman and Huffman (2013). They observed that female rhesus macaques with infants exhibit lower foraging rates, as they prioritize infant care over foraging, leading to increased dependence on available natural food sources.

The findings of our study show that the movement of rhesus macaques gets restricted due to provisioning by humans, and the same was reported by Sengupta *et al*. (2015). Rhesus macaque groups in HPA exhibited reduced movement through the forest during provisioning, indicating that provisioning may contribute to shorter travel distances, and same fact was reported by other studies, which shows that the abundance and ease of access to food from visitors influenced the ranging patterns of *C. lowei* in BFMS (Badiella-Giménez *et al*., 2021; EI-Alami *et al*., 2012). Visitors use food as an attractant to approach and feed the rhesus macaques with provisioned food, which in turn results in a decrease in the rhesus macaques' home range size and daily travel distances (Asquith 1989).

CONCLUSION

These results highlight the significant influence of resource distribution on the locomotion and habitat use of rhesus macaques, underscoring the importance of future research that incorporates detailed travel and dispersal data to better understand the impact of human interventions on the ecological role of rhesus macaques in seed dispersal. Therefore, educational programs should be implemented to raise awareness of the negative ecological impacts, such as increased conflict, disease transmission, and animal road-kills. These programs should also encourage proper waste disposal and discourage wildlife feeding. Enhanced patrolling and enforcement by local authorities, along with penalties for feeding, can further curb this behavior. Additionally, a nationwide literacy campaign is recommended to educate the public on the detrimental effects of feeding rhesus macaques, as a lack of understanding of ecosystem dynamics contributes to ongoing environmental issues.

DECLARATIONS

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Ethical statement

We strictly followed the PMAS-Arid Agriculture University's Ethical Committee's protocol and guidelines (reviewed in the 26th meeting held on 06th January 2020) during the conduct of this research study. No animal, in any form, was harmed during this study.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI or AI-assisted technologies were used in the design of the study, data collection, data analysis, or interpretation of results.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Review Article

Coral Reef Communities of Astola Island: The First Marine Protected Area of Pakistan

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Key words
Arabian sea, Balochistan, Astola island, Coral reefs, Biodiversity, MPAs

ABSTRACT

This study emphasizes on the environmental, ecological, geographical, economical and cultural importance, while assessing the current situation, vulnerability and management of Astola island, as the first ever notified marine protected area of Pakistan. Declaring MPAs is important for the conservation of coral reefs and other associated biodiversity, which provides ecological, socioeconomic, and cultural outcomes while strengthening resilience against climate change. Since 2017, Government of Balochistan has declared three MPAs to date i.e, Astola, Churna and Miani Hor. Coral reefs in the Arabian sea meet unique environmental stressors, yet they entertain significant diversity with remarkable endemism and resilience. Astola island is home 29 hard, 01 black, and 08 soft coral, supporting diverse marine life. The fourth global mass coral bleaching event (2023-25), which has affected 83.9% of coral reef communities worldwide, raised concerns and elevates the importance for active conservation of coastal belts. Key challenges for proetction include insufficient resources, baseline data, limited stakeholder integration, coordinated monitoring systems, poor enforcement and lack of approved management plan. Overall, the coral reefs of Astola island represent ecologically and environmentally valuable yet vulnerable ecosystems. Functional and effective conservation needs adaptive management, scientific innovation, community engagement and international collaboration to ensure persistence of coral reefs under expanding anthropogenic and accelerating climate change.

INTRODUCTION

Marine protected areas (MPAs), which usually covers coral reefs, coastal zones, estuaries, open ocean water, mangroves, seagrass beds, are legally and designated marine areas which are managed to protect and preserve biodiversity, provide ecosystem services, and maneuver values of culture for present and coming generations (Horta e Costa *et al*., 2016). The main and principal goals of marine protected areas are preservation of coral reefs, protecting oceanic biodiversity, conservation of endangered species, reducing overexploitation or degradation of ecosystems, biomass and species recovery, increasing climate change resilience, implementation of sustainable strategies for fisheries, improve livelihood of local residents (Indriyani *et al*, 2025 Collins *et al*, 2021). Other key platforms offered by MPAs are include scientific evaluation, monitoring, educational approaches, and engagement of communities (Jiang *et al*., 2024). In Pakistan, total three marine protected areas have been declared so far which are

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Abbreviations
SST, Sea Surface Temperature; MPAs, Marine Protected Areas; SPSC, Small Polyp Stony Corals; LPSC, Large Polyp Stony Corals; SPSC, Small Poly Soft Corals; NPBC, Non Photosynthetic Black Corals; CBD, Convention on Biological Diversity; IUCN, International Union for Conservation of Nature; WWF, World Wide Fund for nature; CBOs, Community Based Organizations; NGOs, Non Governmental Organizations; NOAA, National Oceanic and Atmospheric Administration; CRW, Coral Reef Watch; DHW, Degree Heating Week; MCP, Marine Conservation Pakistan; CRCP, Coral Reef Conservation Program; NCORE, National Center for Coral Reef Research; GCRMN, *Global Coral Reef Monitoring Network;* ICRS, *International Coral Reef Society;*GFCR, *Global Fund for Coral Reefs;* CTI-CFF, Coral Triangle Initiative on Coral reefs, Fisheries and Food security; ICRAN, International Coral Reef Action Network; ICRI, International Coral Reef Initiative; BACI, Before After Control Impact.

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Table I. The longitude and latitude of Astola island.

Buffer zone		Area	Core zone		Area	Total area
Longitude (E)	**Latitude (N)**		**Longitude (E)**	**Latitude (N)**		
63°44' 53.0343"	25°12' 44.9818"	225.83 km^2	63°46' 22.2757"	25°11'23.7367"	175.64 km^2	401.47 km^2
63°56' 46.9657"	25°12' 44.9818"		63°55' 17.7240"	25°11'23.7367"		
63°56' 46.9657"	25°01' 55.0142"		63°55' 17.7240"	25°03' 16.2611"		
63°44' 53.0343"	25°01' 55.0142"		63°46' 22.2757"	25°03' 16.2611"		

The main and central focus of declaring MPAs is coral reefs protection, which are the most.

found in jurisdiction of Balochistan, these include Astola island, Chruna Island and Miani Hor.

Astola Island (Fig. 1) having an area of 401.47 km^2 (Table I), was declared as first marine protected area of Pakistan, by Governemt of Balochistan under section 34, 40, 46, 47, and 48of the Balochistan Wildlife (Protection, Preservation Conservation and Management) Act-2014, on 15th June, 2017. The fundamental principal aim of this declaration was to protect coral communities and other marine aquatic biological diversity along the coastal region of Balochistan (Astola island, First Marine Protected Area in Pakistan, 2018).

Fig. 1. Astola island geographic location.

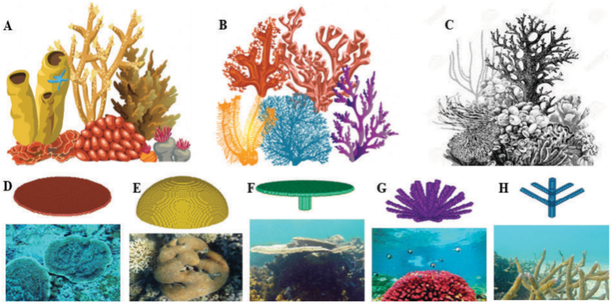

Fig. 2. Types and physical appearance of coral reefs. A, Scleractinian (Hard or Stony corals); B, Alcyonacea (Soft or Ahermatypic corals); C, Antipatharia (Black corals); D, Encrusting; E, Hemispherical; F, Tabular; G, Corymbose; H, Branching.

The main and central focus of declaring MPAs is coral reefs protection, which are the most environmentally, economically, culturally, and ecologically important, valuable but susceptible ecosystems. These MPAs are essential tools in mitigation of coral reefs degradation and secure the ecological, cultural, and economic benefits (Pakistan, 2016).

Coral reefs are formed by marine invertebrates called as coral polyps. The Corals are classified into hard or stony (subclass; hexacorallia/scleractinia), soft (subclass; octocorallia) and black (Fig. 2) within class Anthozoa and phylum Cnidaria (Peel, 2017 Rouzé *et al*, 2021). The hard corals are found in shallow water followed by soft coral and the black corals are inhabitant of deep-sea water (Fig. 3). These Cnidarians secrete the calcium carbonate skeletons that gather and accumulate over long period from 10,000 to 30 million years to form complex reef shapes and structures. Reefs are basically classified into Barrier, Fringing, Atolls and sometimes into Patch reef (Fig. 4). The barrier reef grows close to shorelines, fringing reef grows from shoreline forming a lagoon, atoll reefs grow and encloses the lagoon and patch reef growns within a large reef system on the seafloor in a lagoon (Davin *et al*., 2021).

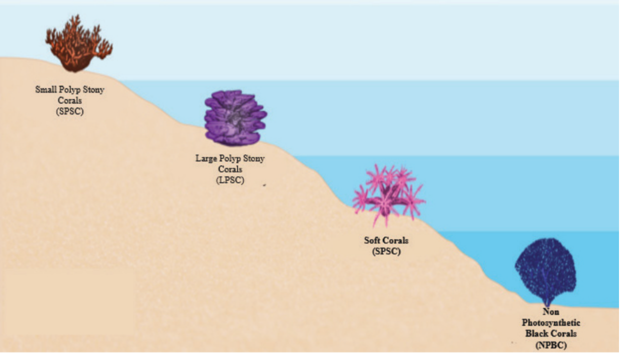

Fig. 3. Habitat of coral reefs in marine ecosystem.

The coral reefs are sometimes referred to as the rainforests of the sea and these are biologically most diverse ecosystems on earth. They cover only 0.1% of the ocean floor but according to an estimation it supports more than 25% of all marine species (Hoegh-Guldberg *et al*., 2017) including fish, sea turtles, mullusks, echinoderms, crustaceans, sea mammals, cnidarians, arthropods, and different types of land and sea birds (Eddy ;*et al*., 2021 Hughes *et al*., 2017).

Beside protection of biodiversity (Fig. 5), these structures also provide shelter to food security, coastal protection, add value to economies, arrange recreational opportunities, possess medicinal potential (Doney *et al*., 2020).

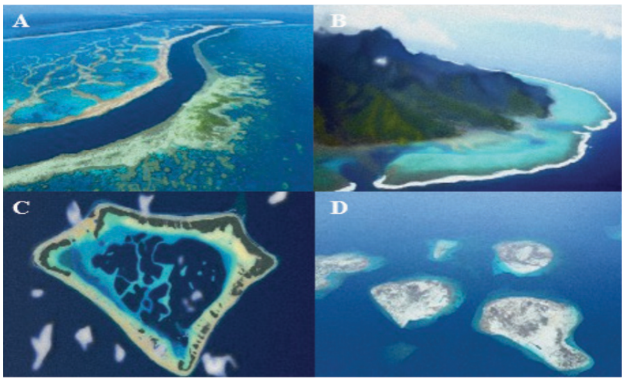

Fig. 4. A, Barrier reef; B, Fringing reef; C, Atoll reef; and D, Patch reef.

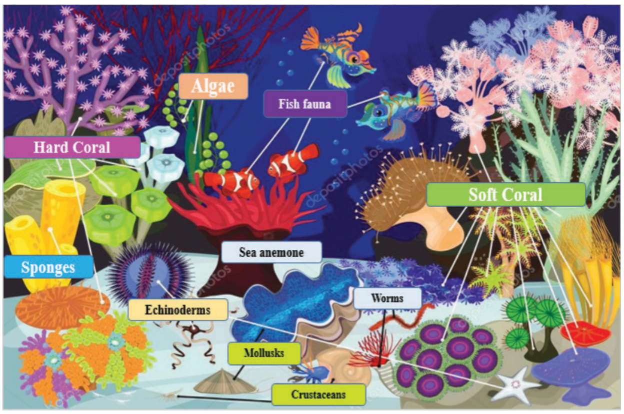

Fig. 5. Coral reef supports diverse biological diversity in marine ecosystem.

Despite of providing enormous supporting, cultural, provisioning, and regulating services, these reefs are facing environmental stressors and anthropogenic pressures such as climate change, ocean acidification, rise of ocean temperature, pollution, overfishing, nutrient runoff, habitat dismantling, coastal development etc. which make the reefs more prone and susceptible to devastation and the situation becomes more worsen when the stressors fuction synergistically. More than 50% cover of corals have been lost globally from last few decades due to rise in average temperature from ~1 to 1.5 °C (Souter *et al*., 2021).

GEOGRAPHICAL, ECOLOGICAL SIGNIFICANCE AND ASSOCIATED BIODIVERSITY IN ASTOLA

Astola is also locally known as Haftalar meaning seven hills (Fig. 6), a largest offshore island covering an area of 175.64km^2 (core zone) and 225.83km^2 (buffer zone) which expands to an area of 401.47km^2 (Table I), with 246 feet highest point above the sea level. The island is located about 39km off the coast near town of Pasni (Fig. 1) and the largest island in Pakistan (Tribune, 2017). North face of the island is cliff like with sharp vertical drops while the south face is more gently and naturally characterised by caves, vegetations. Island is facing freshwater scarcity and morely relying on rainfall or soil moisture (Shafqat, 2025 Spreebird, 2012).

Fig. 6. Different geographical aspects of Astola marine protected area (IUCN, 2017b, 2024; WWF, 2020).

The ecosystem of Astola island is diverse encircled with 20 to 25 species of stony several soft coral reefs (Tribune, 2017). Studies underwater show an abundance of marine life including roughly estimated 156 species of fish alongside invertebrates like lobsters, fan worms, sea urchins, other bio include algae (Zooxanthellae), fungi (endoliths, endosymbionts, and pathogens) etc. with no prominent sign of coral bleaching (Shafqat, 2025). The humback whale (*Megaptera novaeangliae),* Indo-Pacific humpback dolphin (*Sousa chinensis*), common bottlenose dolphin (*Tursiops truncatus*), Indo-Pacific bottlenose dolphin (*Tursiops aduncus*), spinner dolphin (*Stenella longirostris*), Risso's dolphin (*Grampus griseus*), blacktip shark (*Carcharhinus limbatus*), bull shark (*Carcharhinus leucas*), spot-tail shark (*Carcharhinus sorrah*) (Moazzam and Osmany, 2021). Whale shark (*Rhincodon typus*), common guitarfish (*Rhinobatos rhinobatos*), mobulids such as *Mobula eregoodoo*, *Mobula mobular*, *Mobula birostri, Mobula kuhlii*, *Mobula thurstoni, Mobula tarapacana* (Siddiqui *et al.*, 2025), parrotfish (*Scarine labrids*), hot-lip fish (*Plectorhinchus gibbosus*) and many more marine species have been usually sighted in the water of island. Reptiles such as endangered green turtle (*Chelonia mydas*), hawksbill turtle (*Eretmochelys imbricata*), spinny tail lizard (*Uromastyx* spp.), Bengal monitor lizard (*Varanus bengalensis*), collar lizard (*Crotaphytus collaris*), long tailed desert lizard (*Mesalina watsonana*), saw-scaled viper (*Echis carinatus astolae*), carpet viper (*Echis pyramidum*), cliff racer (*Platyceps rhodorachis*), skinks (Family Scincidae), nine types of different sea snakes are also reported in the island. Additionally, 19 terrestril and 87 aquatic bird species are found in and around the island. Some population of birds such as sooty gulls have reduced due to predation by feral cats and rats (Agency, 2017).Astola island provides habitat to about 82 species of terrestrial plants and remains limited due to arid conditions (IUCN, 2017a; Shafqat, 2025).

The Astola island remains uninhabited due to limited sources but it serves as base during the season for fishermen from September to May and during the period they are fishing lobsters, oyesters, crabs, and other marine products (Spreebird, 2012). The island is also characterized by lighthouse installed in 1982. Beside that it also carries cultural, religious, and historical significance where a small masjid named after Pir Khawaja Khizr (Fig. 7), Satadip hindu temple named after goddness Kali are features found in the island (IUCN, 2017a; Spreebird, 2012).

Fig. 7. Pir Khawaja Khizr masjid (IUCN, 2017b).

CORAL REEFS OF NORTHERN INDIAN OCEAN (ARABIAN SEA)

The coral reefs of the Arabian sea and northern Indian ccean fall within the broader biogeographic region of Indo-Pacific but constitute a unique sub region characterized by high level of salinity, variable irregular monsoon systems, and high temperature of sea surface (Obura *et al.*, 2020; Riegl and Purkis, 2015). This region, biogeographically includes habitats of coastal corals of indian ocean countries such as Pakistan, Iran, India, Oman, Maldives, Suadi Arabia etc. The gulf of Aden and Oman is acting major transitional section between the coral reefs of Red sea and Indian ocean (Cowburn *et al.*, 2019). The strong seasonal monsoons influence the surface of sea temperature, salinity, nutrient fluxes and upwelling and dominate the regional oceanography, these dealings creat challenging and dynamic environment for the growth of corals (Purkis *et al.*, 2025). The surface of the Arabian sea exceeds up to 31°C in on regular basis in summer and this rise in temperature reaches the coral bleaching thresholds (Arora *et al.*, 2019), while Omani and Pakistani coasts are injected cold nutrient rich water which enhancing productivity which is caused by upwelling but this can also kepp stress on asemblages of corals due to reduction in aragonite saturation (Spreter *et al.*, 2022). Though there are several challenges for the survival of coral species but the region provide suitable habitat and supports a notable diversity of 200 to 250 species out of total 6000 species of corals in the nothern Indian ocean. It is important to mention that the local diversity of coral species varies significantly due to availability of nutrients, turbidity, thermal variation and other historical stress events (Chanda *et al.*, 2022; Obura *et al.*, 2020). The recent scientific studies and surveys in the Arabian sea have appeared reduction in the cover of corals in some areas such as Lakshadweep islands, gulf of kutch, and gulf of mannar in India, Makran coast including Astola island in Pakistan, coast of Maldives, Sri Lanka, and gulf of UAE, Oman, and Iran (Vinoth *et al.*, 2012). The population of corals is effected by rising of Sea surface temperatures (SSTs) and anthropogenic pressures in these areas but yet isolated refugia persist in less impacted zones especially in open areas such as such as coasts of Balochistan (Ahmad *et al.*, 2024). When the coral communities of Arabian sea are compared to the coral triangle, though the diversity is lower, but exhibit high levels of resilience and regional endemism which highlights their importance in future preservation and climate adaptation strategies (Eakin *et al.*, 2019).

The toral stretch of coastal belt in Pakistan is between 900 to 1050 km covering both Sindh and Balochistan. This stretch has harbors scattered but yet ecologically important coral communities which is endemic to this region (Ahmad *et al.*, 2024). At the beginning the presence of corals and the reefs in these waters was in doubt but after attention of wildlife departments and field surveys conducted by academia in early 2010s total changed the perspective about the corals. 2 landmark study was conducted in 2013 by Amjad Ali and his collegues from university of Karachi and documented 29 hard, 1 black and 8 soft corals, dived into 18 different sites along the coasts of Balochistana and Sindh (Ahmad *et al*., 2024; Ali *et al*., 2014). In 2017, more broader survey was managed and the study was expanded which reported more than 50 coral species and other associated fauna including sponges, molluscs, crustaceans, anemones, echinoderms and 54 fish species. The investigating team also exposed data about 48 fossil coral species which is dating from pleistocene to holocene at uplift sites of Jiwani and Gwadar. Before notifying any formal MPA, the coral communitities faced threats from anthropogenic and environmental stressors.

In 2020 it has been studied that the health of reef has been facing declining due to different pressures such as pollution (29%), overfishing (24%), tourism (26%), climate change (16%), tourism (26%), and sedimentation (18%) (Ahmad *et al*., 2024). For protection, preservation and conservation strategies in marine protected areas, a sustainable and regulated fishing, pollution control, community engagement, limited ecotourism, integrated management and other nature based sustainable activities were pending. In coastal belt of Balochistan to protect these creattures, Asotla island, Churna island and Miani Hor are declared as marine protected areas by Government of Balochistan, Wildlife department (Ahmad *et al*., 2024; Khan, 2025a).

To protect the historical and delicate ecosystems of Astola island was declared as first MPA on 15th June, 2017 for aligning with international agreements and commitments under the Convention on Biological Diversity (CBD) and Aichi Biodiversity Targets (Service, 2001). The declaration mainly focus on protection, conservation, and management of fauna and flora in the region along with regulating sustainable use of resources in the island.

CORAL COMMUNITIES IN ASTOLA ISLAND

The coral communities around Astola island are important and remarkable for diversity and density mostly concentrated on the sheltered northern side where the stony coral reefs can constitute about 80% cover. The most documented and abundant coral species are *Favites complanata*, *Favites pentagona*, *Coscinaraea monile*, *Porites nodifera*, *Pocillopora damicornis*, *Porites harrisoni*, *Leptastrea pruinosa*, *Psammocora obtusangulata, Plesiastrea versipora*, *Psammocora superficialis*, *Acanthastrea hillae*, *Acanthastrea maxima*, *Montipora mollis*, and *Antipathes dichotoma* (Fig. 8) etc. that often occur as fringing and large monospecific patches (Ali *et al*., 2014, 2021).

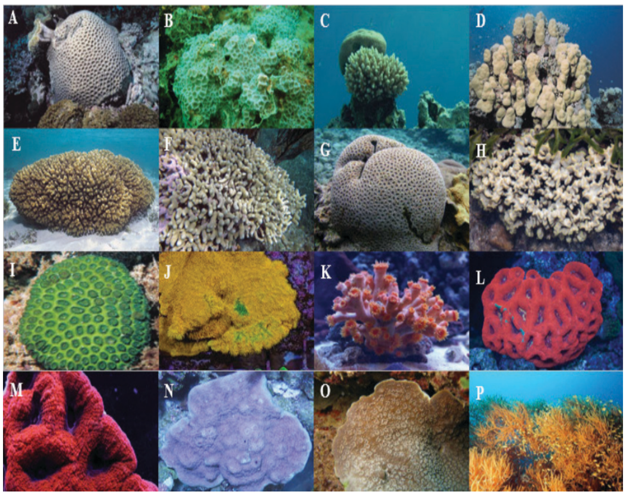

Fig. 8. Coral species: A, *Favites complanata*; B, *Favites pentagona*; C, *Coscinaraea monile*; D, *Porites nodifera*; E, *Pocillopora damicornis*; F, *Porites harrisoni*; G, *Leptastrea pruinose*; H, *Psammocora obtusangulata*; I, *Plesiastrea versipora*; J, *Psammocora superficialis*; K, *Goniopora columna*; L, *Homophyllia bowerbanki*; M, *Acanthastrea hillae*; N, *Acanthastrea maxima*; O, *Montipora mollis*; P, *Antipathes dichotoma.*

Scientific studies and surveys have recorded about different species of stony or hard and soft corals (Astola Island, First Marine Protected Area in Pakistan, 2018; WWF, 2020). In broader and specific terms, in regional assessments of the Astola island, (e.g., Ali *et al.* 2014) a scientific report shows 29 hard or stony corals of fourteen genera from eight different families, one black coral, and eight soft coral species of seven genera from three families (Ahmad *et al*., 2024).

According to recent studies the habitat of coral reefs around the island is in good condition and health, no concrete evidence of coral bleaching is detected (Khan, 2025b; WWF, 2020). The proto-reef structures are generally evident, though no true authentic reef formations have been recorded yet so far (Ahmad *et al.*, 2024). However, due to ongoing stressors and pressures from anthropogenic activities such as coral removal for ornament purposes, damages from fishing nets, lobster pots impact, careless skin and scuba diving etc. increaing negative impacts on corals and lead to degradation of communities. The most susceptable species of corals such as *Pocillopora damicornis* and *Porites nodifera* are facing threat and they are reducing their prevalence in the region around island (Ali *et al*., 2021), but the physiochemical conditions i.e. nutrient level, temperature, remain suitable for coral and associated fish communities for proper function of ecosystem in area (Ali *et al*, 2021; IUCN, 2017a WWF, 2020). Total assemblage type observed in expedition of the sheltered areas include up to 80% dense stony coral cover, proto reef structures formed from mono specific stands and patches, and soft coral dominating on more exposed sites (Ahmad *et al*., 2024). The habitat and health indicators observed during the study were favoring the communities of coral reefs and no bleachings were noted. The presence of proto coral reeds were implying active flourishing however due to human activities such as coral harvesting, fishing gear, diving, and sedimentation contributed to structural damage and disease prevalence which reduced the abundance of certain species and the mortality was documented locally. Overall, the habitat remained favorable for the health of communities though proper conservation and management measures are needed to reduce anthropogenic pressures (Ali *et al*., 2021).

THREATS TO CORAL REEFS AROUND ASTOLA ISLAND

Scientific study has predicted that 70% to 90% coral reefs in next 20 years due to main cause of warming of oceans, acidity and pollution (Nace, 2020). Due to human activities such as illegal coral mining, devastating fishing methods, careless scuba, pollution, and skin diving have been reported as major and outreach threats which is mean of physical injury and habitat degradation. Fishing gear and mechanical disturbance from lobster pots also add contribution to this cause (Ali *et al*., 2021; Khan, 2025). Beside that marine pollution such as oils leakge from engines, descarded nets, plastic debris, human wastes and other trashes scattered due to anthropogenic activities are expending the situation and making it poor for the communities of corals (IUCN, 2017b).

Other than anthropogenic risk, natural calamities in form of storms, temperature fluctuation, sedimentation also exaggerate dismantling of coral systems. The unusual natural storms with high intensity can break the structure of corals and sediment runoff the colonies from distant land. These smother corals and block their access to light (Donner and Potere, 2007).

Coral bleachings are noted in some regions of coastal belt in Balochistan which are triggered by rise in temperature and increasing vulnerability and relative health of ecosystem associated with coral reefs (Ahmad *et al*., 2024; Khan, 2025).

Global warming drived due to climate change exacerbates to coral communities of Astola island through rise in SST and acidification of ocean. Rise temperatures can trigger coral bleaching which make corals to expel symbiotic algae such as Zooxanthellae and this leads to loss of coloration and increase mortality rate of coral colonies (Donner and Potere, 2007; Kraft, 2014 Kurihara, 2020). On the other hand increase in atmospheric CO levels, the ocean pH is deceased which reduce availability of carbonates that are required for calcification of corals Donner and Potere, 2007). These stressors combine to reduce the resilience and threaten the long term survival of coral communities (Table II).

WWF-Pakistan funded scuba survey for study of coral communities in 2020 shows that the ecosystem around the Astola island is comparatively stable and functioning properly. During the study no signs of bleaching noted and documented abundant marine life such as different fish species, turtles, invertebrates, and rich coral habitats (WWF, 2020). The field experts caution that these ecosystems can deteriorate promtly without effective protection, conservation and management (ICSF, 2020 Khan, 2025).

Table II. Pressure hazards on coral communities in coastal belt of Balochistan.

Hazard	Particular
Anthropogenic pressure	Coral mining, destructive fishing, pollution, diving, scuba
Natural stressors	Sedimentation, storms, temperature fluctuation
Climate change	Thermal stress, ocean acidification
Current status	Coral reefs are relatively healthy, but vulnerable without management

MANAGEMENT IMPLICATION AND CONSERVATION

The conservation of coral reefs in Sindh and especially in Balochistan has moved forward but yet faces some intimation gaps. Astola Island has been declared as MPA in 2017 but still management plan even after lapse of eight years still pending for approval which is highlighting the slow operationalization (Azam, 2025). In 24th September, 2024, Churna island was declared as second MPA after approval from the Balochistan cabinet. In 2025, Miani Hor received approval from the Balochistan government to be designated as the third MPA of Pakistan, but its official notification is still pending. The expanding protection beyond small areas with low on water enforcement, buoy zoning, and visitor compliance systems (APP, 2025; Dawn, 2024; Tribune, 2017, 2024; WWF, 2025). The government wildlife departments and organizations working on nature seeks fishing communities for contributions to advocacy and surveillance for MPAs to get long term befinits and conservation of protected areas, yet combined management frameworks and mechanisms for benefit sharing remain poor (WWF, 2025). Standardized underwater surveys, fish assemblage studies, baseline information for coral cover, disease, water quality and monitoring capacity are incomplete (Fig. 9).

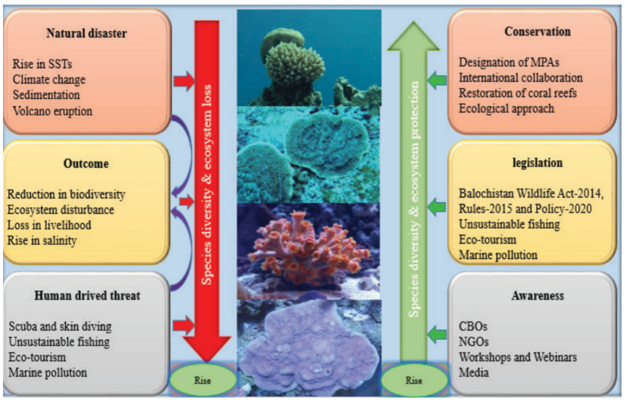

Fig. 9. The habitat of coral reefs is degraded with rise of natural and anthropogenic activities while it can be conserved and improved with the nature based strategies.

Peer reviewed site-specific studies are required further to extend adaptive conservation and management (Ahmad *et al*., 2024). These weaknesses can deteriorate the habitat for coral reefs. The National Oceanic and Atmospheric Administration (NOAA) reported the fourth mass global bleaching event in April 2024, and by mid-2025 documented bleaching-level heat stress across most coastal regions. The rapid rise in SSTs has prompted the implementation of bleaching response protocols and post-disturbance monitoring in MPAs in Pakistan (NOAA, 2025).

RECOMMENDATIONS AND FUTURE RESEARCH

The coastal belt of Pakistan still lacks contemporary consistent baseline data for health of corals, benthic cover, water quality, marine pollution, invasive species, local marine ecosystems. Developing unified scientific protocols such as phtogrammetry, annual temperature loggers (SSTs), study of eDNA for biodiversity, diseave and bleaching scoring is need of hour for data documentation at national (MCP, WWF, IUCN) and international platforms (CRCP, NCORE, NOAA, GCRMN, ICRS, GFCR, CTI-CFF, ICRAN, CRW, ICRI). These measures should be taken on most urgent basis because the recent fourth global mass bleaching event between January 2023 and May 2025 has caused bleaching-level heat stress in about 83.9% areas of the coral reefs in the 83 countries which is confirmed on April 15, 2024, which is 15.7% higher than third global mass bleaching event betwen 2014 to 2017. The global mass bleaching event has putting pressure on already vulnerable coral reefs in coastal belt of Pakistan and keeping corals at higher risk. The routine integration of satellite heat stress indices such as HotSpot and DHW with validation in water at MPAs should anchor as early warning system (Ahmad *et al*., 2024; NOAA, 2025).

Scientifically active rehabilitation and restoration should be trialed in controlled environment for coral gardening with heat tolerant genotypes, selective endemic species for assisting gene flow to raise temperature tolerant colonies, and assisted recruitment in designated sites. Contemporary researches and studies show selective breeding and heritable heat tolerance have enhanced the thermal performance of offspring and hand on trait variation shows heat tolerant phenotypes can be found in most reefs. In such cases the discovery is feasible by implying the local broodstock. Side by side resilience diagnostics which is omics-based health assays, disease screening symbiont typing etc. can discover colonies and sites likely to survive under environemntal stressors. The pilots should include designs based on robust BACI and 2 to 3 years survival tracking to quantify cost effectiveness before assessing (Chille *et al*., 2025; Denis *et al*., 2024; Quigley and van Oppen, 2022).

The Government and Wildlife Department of Balochistan must align MPA network with global bleaching response frameworks on enhance the capacity and funding. Regionally the documentation should be taken from the field in accordance to prescribed procedures of ICRI and Green fin global coral bleaching response toolkits. The data should be incorporated to alearting system of NOAA and CRW for refining local risk forecasts. Fourth global bleaching event has mobilized and catalyzed international organizations i.e. ICRI, COP, GFCR, etc. and participation in such international fora can help to unlock technical, finanicial, technological, environmental, logistical, legal, political protection, monitoring and restoration. Coordination and cooperation across Balochistan and Sindh wildlife departments, academia and NGOs should formalize coral monitoring consortium at intragovernmental, national, cross border regional and intergovernmental level which monitor the health of wild communities in MPAs at terrestrial and aquatic zones. The consortium also should publish annual research articles, reports, short communication, white paper etc. for close observation of coral reef ecosystems (ICSF, 2025; IUCN, 2024; Khan, 2025c; NOAA, 2024).

CONCLUSION

The diverse coral reefs of coastal belt of Pakistan especially in MPAs such as Astola island represent economically, ecologically, culturally, and environmentally unique valuable ecosystems that support habitat of endemic species, food security, coastal protection, biodiversity, fisheries. Yet these areas are highly susceptible and vulnerable to anthropogenic pressures and climate change impacts more specifically to rise in SSTs, acidification, loss of calcification, etc. that has already triggered fourth global mass bleaching event (2023-25). Despite the declaration of Astola island as MPA (including Churna and Miani Hor), yet lack of resources, inadequate management frameworks, lack of baseline authentic data, protection measures, weak community integration, minimize effective conservation efforts. However, in some coastal belts, the endemism, resilience and comparatively stable conditions highlight opportunities to the government and nature-based organizations for adaptive protection and management of sustainable fisheries, community-based stewardship, preservation of ecosystems, conservation of coral communities. Adoption of green fin glocal coral bleaching response toolkits and CRW protocols will help for monitoring, restoration and bleaching response and enhance the survival of coral reefs. Strengthening the national and international cooperation, aligning with standard conservation processes, and investing in scientific research driven restoration protocols are required to save the important coral reef ecosystems for future.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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A Collection of Hipparionines from the Middle Siwaliks (Late Miocene) of Pakistan

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Key words
Dhok pathan formation, *Proboscidipparion, Plesiohipparion, Hippotherium*, Mohal Pati, Siwaliks

ABSTRACT

The present study is focused on the collection of three toed horses known as hipparionines from the Late Miocene of the Siwalik Group of Pakistan. The specimens have been collected from the Dhok Pathan stratotype in the district Talagang and Mohal Pati village of district Jhelum, Punjab, Pakistan. The morphological traits of the specimens indicate that one specimen belongs to the genus *Proboscidipparion* and others were categorized as Hipparionini indet. but shows the features of some other genera such as *Plesiohipparion*, *Hippotherium* etc.

INTRODUCTION

The Siwalik Group or Siwaliks that range from 18 Ma to 0.6 Ma has produced an abundant mammalian fauna that has attracted researchers from around the world for nearly 200 years (Flynn *et al*., 2024). The ungulates are most frequent finds in this mammalian fauna and have been described extensively (Colbert, 1935; Khan *et al*., 2010, 2012). Two orders of the ungulates are of special interest because of their abundance, Artiodactyla (even toed ungulates) and Perissodactyla (odd toed ungulates) where perissodactyls are represented by three families; Chalicotheriidae, Rhinocerotidae and Equidae (Colbert, 1935; Khan *et al*., 2012; Hussain, 1971). The members of the family Equidae first appeared in the Late Miocene and have been considered important not only in taxonomic but stratigraphical context (Hussain, 1971; Wolf *et al*., 2013; Bernor *et al*., 2025). In other words, their presence in certain deposits in the Siwaliks is considered age specific and these are useful for relative dating. Traditionally, these were represented by two genera *Hipparion* and *Equus*, and by five species *Hipparion theobaldi*, *Hipparion antelopinum*, *Hipparion nagriensis*, *Hipparion* sp. and *Equus sivalensis* (Hussain, 1971; Bernor and Hussain, 1985; Hussain *et al*., 1992; Bernor *et al*. 2019). However, recent assessment of the Siwalik hipparionines from the late Miocene deposits by Wolf *et al*. (2013), resulted in the recognition of four genera *Cormohipparion*, *Sivalhippus*, *Hipparion*, and *Cremohipparion* and seven species *Cormohipparion* sp., *S. nagriensis*, *S. perimensis*, *S. theobaldi*, *S. anwari*, *Hipparion* sp. small and *Cremohipparion antelopinum*. Even more recently, the work of Jukar *et al*. (2018 2019) has recognized two more genera *Eurygnathohippus* and *Plesiohipparion* from the Indian subcontinent Siwalik, which resulted in further increase of equid diversity. Despite the extensive study of the equids, their taxonomy in the Siwaliks remains problematic and needs specific attention.

We are describing some hipprionine remains collected

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from the Dhok Pathan stratotype in the district Talagang and Mohal Pati village of district Jhelum, Punjab, Pakistan that will add further anatomical knowledge in this group to resolve the taxonomic issues of this group.

Mohal Pati is a small village located near the famous Bhandar Bone Bed of Pilgrim (1913) in district Jhelum, Punjab, Pakistan. It is located 1.5 km northeast of Bhandar, and 2.5 northeast of palaeontologically famous Hasnot village (Fig. 1). According to Pilgrim (1913), the sediments at the Bhandar Bone Bed are considered as the upper Dhok Pathan Formation, and Johnson *et al.* (1982) has palaeomagnetically determined that the age of these sediments is 6 to 7 Ma, representing Chron 4 of magnetic polarity time scale of LaBrecque *et al.* (1977) that corresponds to C3r of geomagnetic polarity time scale of Ogg (2020). This age corresponds well with the upper Dhok Pathan Formation as well as the upper late Miocene. Lithologically, the sediments are characterized by pale yellow silt/claystone and dark to light colored sandstone with minute quantity of conglomerates, consistent with the Dhok Pathan Formation of the Middle Siwalik. However, some of the specimens came from nearby area, Andar Kas, whose deposits are dated to Nagri Formation about 9.2 to 10 Ma (Johnson *et al.*, 1982).

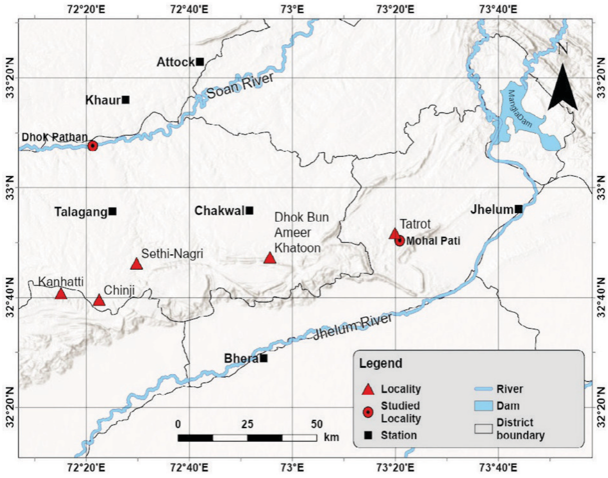

Fig. 1. Map showing the location of areas in Punjab, Pakistan from where new specimens have been collected.

Dhok Pathan village is located in Talagang district of Punjab, Pakistan at the bank of Soan River (Fig. 1). Pilgrim (1913) assigned the outcrops of near the Dhok Pathan Rest House as type locality of the Dhok Pathan Formation, and area from the Naragghi to Markhal village as the stratotype of the Dhok Pathan Formation. Lithologically, the sediments are characterized by pale yellow silt/claystone and dark to light colored sandstone alternating with thin beds conglomerates. The specimens described in this study came from two localities, Kundrali and Dhendar as reported by Aftab *et al*. (2022). Kundrali locality corresponds well to Barnum Brown locality 38 (B38) and Dhendar roughly corresponds to B40-B41 as shown in fig. 18 of Colbert (1935). The age of these localities is between 7.9 to 7.6 Ma as determined by Barry *et al*. (1980) corresponding to C4n.2n to C4n.1n of geomagnetic polarity time scale of Ogg (2020).

MATERIALS AND METHODS

The material includes 13 specimens comprising isolated upper and lower check teeth. The collection area of each specimen is given in parentheses. The surface collection was the primary method for the collection of specimens as recommended by Behrensmeyer and Barry (2005). After collecting or excavating the samples, these were wrapped in cotton for transportation to the laboratory. In the lab., the specimens were prepared by washing and removing sediments (silt/clay/mud or sand) using very fine needle. The partially broken specimens, found broken or broken while preparing, were glued using GMSA glue. After preparation, the specimens were catalogued using numerator and denominator and PUPC as institutional abbreviation. For example, in PUPC 23/129, PUPC stands for Punjab University Palaeontological Collection, 23 as year of collection, and 129 as number of the specimen collected in this year. We followed Bernor *et al*. (2010, 2017) and Wolf *et al* (2013) for taxonomy, and the terminology for the description of dental material is given in Figure 2 Measurement protocols follow Eisenman *et al*. (1988), and measurements were taken using digital Vernier caliper. Occlusal length and width were recorded in millimeters. The photos were taken using DSLR camera, Canon 6D, and photos were arranged in plates using Adobe Photoshop. The specimens are housed in Dr. Abu Bakr Fossil Display and Research Centre, Institute of Zoology, Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan.

SYSTEMATIC PALAEONTOLOGY

Order Perissodactyla Owen, 1848
Suborder Hippomorpha Wood, 1937
Family Equidae Gray, 1821
Tribe Hipparioninini Quinn, 1955
Genus *Proboscidipparion* Sefve, 1927
cf. *Proboscidipparion* sp.
(Fig. 3.1)

Referred material: PUPC 23/97, right p4 (Mohal Pati).

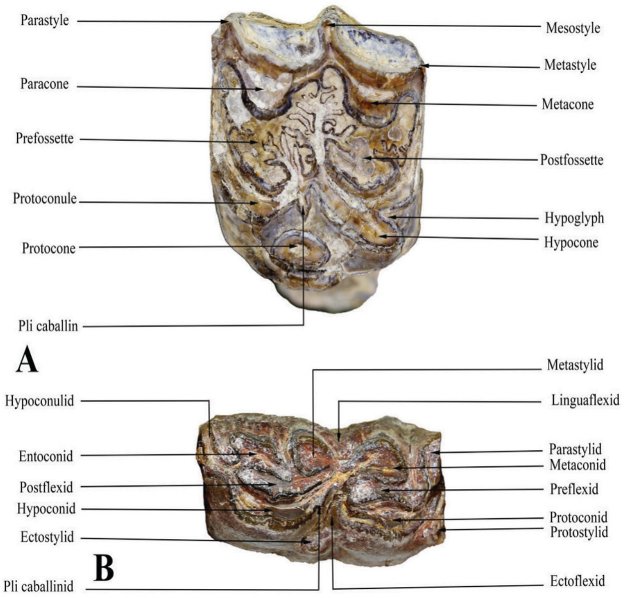

Fig. 2. Dental terminology being used in this study for the description of dental material. Topology of A, upper tooth, B, lower tooth.

Description

PUPC 23/97 is highly worn and partially broken p4 as judged by its dimensions (Fig. 3.1). Parastylid, protostylid, protoconid, and hypoconulid are partially broken. The metaconid is high lingually with slightly round anterior border, while metastylid is completely pointed and shows completely triangular morphology and both forming a houfenoid double knot. Both, the linguaflexid and ectoflexid are deep and U-shaped. The pre- and postflexid are compressed labiolingually, narrow and plicated. Its preserved length is 24 mm and width is 13.7 mm.

Comparative remarks

The described molar, PUPC 23/97, shows houfenoid double knot (metaconid and metastylid triangular in form and positioned more or less symmetrically), a character of *Eurygnathohippus*, *Plesiohipparion*, and *Proboscidipparion* (Qiu *et al*., 1987; Bernor and Lipscomb, 1991; Bernor and Sun, 2015). The specimen lacks the ectostylid that differentiates it from *Eurygnathohippus* and lacks the pli caballanid that isolates it from *Plesiohipparion* (Bernor and Sun, 2015), but associate the specimen to *Proboscidipparion*. Further, the molar has round anterior and lingual border of the metaconid that confirms it affinity to *Proboscidipparion* (as stated by)Bernor and Sun, 2015 and such a morphology is found in *Proboscidipparion pater* in late wear stage lower molar V 8172 described and figured by Bernor and Sun (2015 Fig. 3F3). PUPC 23/97 differs from *Sivalhippus* species in straighter posterior border of the metaconid and more houfenoid morphology. Based on these observations the specimen is described as cf.*Proboscidipparion* sp. until more specimens are recovered from the area.

Hipparionini indet

(Fig. 3.2-3.13)

Referred material

PUPC 23/113, right DP3 (Mohal Pati); PUPC 23/140, right M2 (Dhok Pathan); PUPC 23/114, right M2 (Mohal Pati); PUPC 23/136, right M2? (Dhok Pathan); PUPC 23/112, right M3 (Mohal Pati); PUPC 23/137, ?M? (Dhok Pathan); PUPC 23/111, partial upper molar (Mohal Pati); PUPC 23/115, partially broken left dp4 (Mohal Pati); PUPC 23/101, anterior portion of p2 (Mohal Pati); PUPC 23/99, left m1? (Mohal Pati); PUPC 23/100, right m1? (Mohal Pati); PUPC 23/134, left m3 (Dhok Pathan).

Description

PUPC 23/113 is a very small and partially broken deciduous upper premolar (Fig. 3.2). The meta- and hypocone are half, resulting in loss of metastyle and hypoglyph. It is completely unworn. The protocone is small and flat lingually, giving almost triangular appearance. Both the para- and mesostyle are equally developed and are thick. The fossettes are wide and deep. Its preserved length is 19.8 mm and width is 21.2 mm.

PUPC 23/140 is partially broken, slightly worn M2 (Fig. 3.3). The metacone is well preserved, rest of the cusps are partially broken. Only the lingual half of the protocone is preserved. The parastyle is partially preserved, the mesostyle is large and thick while the metastyle is weakly developed. The plications are weak due to early wear. Its preserved length is 22.4 mm, and preserved width is 19.4 mm.

PUPC 23/114 is a partially broken and highly worn M2 (Fig. 3.4). The paracone is completely broken and protocone is partially preserved. The protocone is elongate, compressed lingually and slightly round lingually. The posterior border of prefossette is slightly plicated while the anterior border of postfossette has only spur. Hypoglyph is deeply incised. Only metastyle is preserved. Its preserved length is 26.1 mm and width is 23.4 mm.

PUPC 23/136 is broken anteriorly, resulting in loss of most of the protoconule and paracone (Fig. 3.5). The enamel is also lost from most of the tooth. The metacone is elongated and narrows posteriorly. The fossettes are only sparsely plicated. The hypoglyph is very deep, isolating the hypocone. Its preserved length is 20.7 mm, and preserved width is 21 mm.

Fig. 3. cf. *Proboscidipparion* sp. 1, PUPC 23/97, right p4. Hipparionini indet. 2, PUPC 23/113, right DP3; 3, PUPC 23/140, right M2; 4, PUPC 23/114, right M2; 5, PUPC 23/136, right M2?; 6, PUPC 23/112, right M3; 7, PUPC 23/137, ?M?; 8, PUPC 23/111, partial upper molar; 9, PUPC 23/115, partially broken left dp4; 10, PUPC 23/101, anterior portion of p2; 11, PUPC 23/99, left m1?; 12, PUPC 23/100, right m1; 13, PUPC 23/134, left m3.
Views: a. occlusal, b. lingual, c. labial. Scale bar equal to 10 mm.

PUPC 23/112 is a partially worn M3 (Fig. 3.6). The protocone is isolated, compressed lingually, and tends to be oval in shape. The fossettes are wide and deep. The parastyle is thick and strong while the mesostyle is moderately thick, and the metastyle is weakly developed. The hypoglyph is deeply incised, isolating the hypocone. Its preserved length is 30.7 mm and width is 21.5 mm.

PUPC 23/137 is partially preserved upper molar (Fig. 3.7). It preserves the para- and metacone with some portion of the pre- and postfossette. The opposing border of the pre- and postfossette are complexly plicated. Its preserved length is 21.6 mm, and preserved width is 12.7 mm.

PUPC 23/111 is a partially preserved upper molar (Fig. 3.8). The posterior border of the prefossette and anterior border of postfossette are sparsely plicated. The para- and mesostyle are thick. The preserved length is 23.5 mm and width is 14.5 mm.

PUPC 23/115 represents a small sized deciduous lower premolar in which anterior lobe is broken resulting in loss of protoconid and associated structures, and metaconid (Fig. 3.9). The hypoconid is extremely large and partly wavy. The metastylid is preserved which is triangular in shape and it is pointed lingually. The entoconid is large and flat. The hypoconulid is large, lingually pointed. Its preserved length is 18.8 mm and width is 12 mm.

PUPC 23/101 represents paraconid, protoconid and metaconid of p2 (Fig. 3.10). It is highly worn. The paraconid is triangular and metaconid is oval. Its preserved length is 15.7 mm and preserved width is 11.7 mm.

PUPC 23/99 is partially broken and moderately worn m1 (Fig. 3.11). The apex of metastylid is completely lost while the metaconid is partially preserved. A thick protostylid is present. The linguaflexid is deep and V shaped, as well as ectoflexid. The hypoconilid is partially preserved. Its preserved length is 22.2 mm, and preserved width is 12.4 mm.

PUPC 23/100 is broken anteriorly resulting in loss of most of the protoconid, metaconid and hypoconulid, and complete loss of proto- and parastylid (Fig. 3.12). Pli caballanid is prominent. Both the linguaflexid and ectoflexid are shallow. It is moderately worn. Its preserved length is 22.3 mm, and preserved width is 11.8 mm.

PUPC 23/134 is slightly broken and in early wear (Fig. 3.13). The protostylid is broken and reaches half of the height of the tooth. The parastylid is slightly broken and weak. The proto- and hypoconid are almost equal in length and have round borders. Both the metaconid and metastylid are oval in shape, linguaflexid is deep and V shaped. The hypoconid is small and rectangular in shape. The hypoconulid is large and made prominently third lobe and has a large stylid. Its length is 25.2 mm and width is 10.5 mm.

Comparative remarks

The material described here as Hipparionini indet. is heterogeneous in nature and it is composed of different species. Due to the breakage of the material, most of the material cannot be identified to a generic of specific level, and some of the material corresponds to a small species (Fig. 3.6, 7, 13), *Hipparion/ Cormohipparion* sp. small described by Wolf *et al*. (2013) and Bernor *et al.* (2025) but the nature of this species is uncertain as discussed by the same authors that more material is needed for the precise allocation of the material reported to this species. We are tentatively placing all such cheek teeth in the referred material (PUPC 23/112, PUPC 23/115 and PUPC 23/134) that may correspond to *Hipparion/ Cormohipparion* sp. small. to Hipparionini indet. until more material is recovered to fully resolve the nature of the *Hipparion/Cormohipparion* sp. small. Among rest of the materials, PUPC 23/140 (Fig. 3.3) shows the confluence of the pre- and postfossette, a character of *Hippotherium* (Bernor *et al*., 2017) but the plications are less complex, and protocone and hypoglyph are not preserved to show their morphology. PUPC 23/136 shows weak fossette plications, trifid pli caballin, deeply incised hypoglyph, and elongate protocone that makes a pli posteriorly (Fig. 3.5), characters that can associate with *Cormohipparion* but the specimen is broken and slightly deformed, also it is in early wear. PUPC 23/113 seems DP3 but maybe just erupted M1/M2. It is unworn and does not show occlusal morphology in detail, however, besides the isolated protocone, a pli caballin is also visible (Fig. 3.2). Its morphology in this state is close to *Baryhipparion tchikoicum* described and figured by Deng et al. (2016, Fig. 3). Similarly, PUPC 23/137 (Fig. 3.7) is slightly worn M3 that can be compared with *Baryhipparion tchikoicum*. PUPC 23/115 is an incomplete dp4 (Fig. 3.9) whose affinity can be made with *Hipparion/Cormohipparion* sp. small or with *Baryhipparion*. Nevertheless, more material is needed to fully assess the presence of *Baryhipparion* in Siwaliks. PUPC 23/100 is partially broken (Fig. 3.12) that hinders its specific attribution; however, the preserved tooth morphology is similar to the specimens described above as *Eurygnathohippus* sp.

DISCUSSION

The Siwalik equids are extremely important in terms of biogeographic distribution, evolution and diversification of this group in Asia (Wolf *et al*., 2013; Sankhyan *et al*., 2023). The classification of the Siwalik equids remained a matter of problem and debate since their first discovery and opinins have been changed from time to time and from one researcher to other (e.g., Falconer and Cautley, 1849; Lydekker, 1876; Colbert, 1935; Hussain, 1971; Bernor and Hussain, 1985; Wolf *et al*., 2013; Sankhyan *et al*, 2023; Bernor *et al*., 2025). However, Wolf , *et al.* (2013) after the detailed analysis of all the Siwalik material from the Late Miocene have proposed the presence of four genera *Cormohipparion*, *Sivalhippus*, *Hipparion*, and *Cremohipparion* and seven species *Cormohipparion* sp., *S. nagriensis*, *S. perimensis*, *S. theobaldi*, *S. anwari*, *Hipparion* sp. small and *Cremohipparion antelopinum*. Even more recently, the work of Jukar *et al.* (2018 , 2019) has recognized two more genera, *Eurygnathohippus* and *Plesiohipparion* from the Indian subcontinent Siwalik, which resulted in further increase of equid diversity. Some years later, Sankhyan *et al*. (2023) identified the presence of the genus *Proboscidipparion* from the Haritalyangar, adding another genus of hipparionine equids in the Siwaliks. *Proboscidipparion* sp. is reported based on a lower molar in this study as well and from the Late Miocene. This genus is strictly Pliocene in its age range and predominantly known from China, as well as from the Turkey and England. This genus is recently reported from the Haritalyangar area (Sankhyan *et al*. 2023), but most of the specimens are from very latest Miocene deposits. Among the other specimens that could not be identified to either generic or specific level, Hipparionini indet., contain three specimens (PUPC 23/113, PUPC 23/137 and PUPC 23/115) that are potential candidates of yet another hipparionine genus, *Baryhipparion*, in the Siwaliks. However, more material is needed to confirm its occurrence in the Siwaliks. One of the specimens, PUPC 23/100 shows all the morphological features of *Cremohipparion* material described by Lydekker (1876) but such morphology is also found in the genus *Eurygnathohippus* described by Bernor *et al*. (2013, 2020). It is quite probable that the genus *Eurygnathohippus* predates all other records in the Siwaliks and is point of distribution to the other regions. However, more material is needed and ongoing taxonomic reassessment of the Siwalik hipparionines will answer to such questions related to taxonomy and biogeography of this group.

CONCLUSIONS

The present study focused on thirteen recently collected materials of the hipparionine remains resulted in the identification of the genus *Proboscidipparion* in the Siwaliks of Pakistan based on a lower molar after its previous identification in the Siwaliks of India by Sankhyan *et al*. (2023). The other materials is described as Hipparionini indet. awaiting new material. Some of the specimens in Hipparionini indet, are potential candidates of some other genera that have not been previously reported from the Siwaliks and new materials and/or reassessment of already collected material will reveal their exact taxonomic position.

DECLARATIONS

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Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Evaluation of Azo Dye Degradation Potential of *Aspergillus niger* and *Penicillium simplicissimum*

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Azo dye, Fungal strains, GC-MS analysis, Wastewater, Bioremediation

ABSTRACT

This study examines the potential of fungal strains for degrading azo dyes, providing an eco-friendly and efficient alternative to traditional methods. For the dye decolorization assay, two fungal strains were used to evaluate their degradation capability on Congo Red, yielding decolorization rates of 90% by *Aspergillus niger* and 91% by*Penicillium simplicissimum*, respectively, over 60 days. According to the decolorized dye's Gas Chromatography-Mass Spectrometry (GC-MS) examination, several dye-degraded products, including octadecanoic acid, benzoic acid, 3,4,5-trihydroxy, 9H-fluoren-9-one, and many others, were generated by the fungal strains. The azo dye-degradative compounds have potential applications as they possess antioxidant and antimicrobial properties. These fungal strains have promising potential to degrade toxic azo dyes into less hazardous compounds, as evidenced by dye-degraded products, and can be used to clean the environment from such toxic compounds/dyes.

INTRODUCTION

A number of environmental issues that negatively affect the soil, water, and biotic elements of the biosphere have been brought on by the persistent efforts to develop various industries, including the chemical, pharmaceutical, agricultural, and textile sectors (Dutta *et al.*, 2024). Azo dyes, the vibrant hues that are widely used in the textile, paper, and food industries, pose significant environmental and health risks due to their toxic and recalcitrant nature. Many contaminants persist in the environment and cause air, water and soil pollution and they are physical contaminants (plastic, heavy metals), chemical contaminants (dioxins, azo dyes, PAHs) or biological contaminants (bacteria, viruses etc.) (Ali *et al.*, 2023) and there are many methods to remove contaminants such as UV treatment, Filtration, Chemical precipitation and Distillation etc.

Dyes are widely used in many industries, and they are classified as water-soluble dyes (Acidic, Basic, Direct, and Reactive dyes) and water-insoluble dyes (Disperse, Vat, and Sulfur dyes). These kinds are resistant and they impose negative impacts on human health as well as on plant growth, leading to body impairments and growth-related disorders.

Azo dyes have become essential in today's world because they have entered every aspect of life, as natural dyes are expensive and tiring to apply. They are a commercially important family of azo compounds, i.e., compounds containing the C-N=N-C linkage. So, the release of azo dyes into the environment has become a significant concern due to their toxic and carcinogenic properties and is responsible for ADHD in kids, inefficient immune response generation in humans, type 1 hypersensitivity reactions among individuals, etc.

For the azo dyes decolorization, several methods are employed, including physical methods (adsorption, coagulation, flocculation), which use physical forces to break down dye molecules, chemical methods (ozonation and advanced oxidation processes), and biological methods using microorganisms to degrade complex organic compounds into simpler compounds. Biological agents like fungi and bacteria are the most reliable way to break down dyes. Because living things like these either adsorb or absorb colors into their cells, they lack any toxic substances (Ilyas and Rehman, 2013). In the current

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investigation, two fungal strains i.e., *Aspergillus niger* and *Penicillium simplicissimum* are used to evaluate their potential to degrade azo dye.

MATERIALS AND METHODS

Fungus strains and culturing media

Two fungus strains i.e., *Penicillium simplicissimum* and *Aspergillus niger* were obtained from Pakistan's first Fungal Culture Bank (FCBP), University of the Punjab.

A natural potato dextrose agar was made for the cultivation of strains. A kilogram of peeled potatoes was boiled in 1000 mL of tap water; distilled water was added as needed. After that, 20g of agar was added and then autoclaved. After that, 10mL of glucose solution was added. After mixing thoroughly, 2 mL of the chloramphenicol stock (9 mL of 70% ethanol and 0.25g of chloramphenicol powder were combined, and the volume was raised to 10mL using autoclaved distilled water) was added. Mueller-Hinton broth was prepared by dissolving 2 g beef extract, 17.5 g tryptone, and 1.5 g starch in one liter of distilled water for further growth of fungal strains. The media were subject to incubation for three days (*A. niger*) and seven days (*P. simplicissimum*) at 30 ± 2 °C.

Fungal decolorization of dyes

After preparing and autoclaving the Mueller-Hinton medium (200 mL), it was then inoculated with both strains of the fungi, namely *Penicillium simplicissimum* and *Aspergillus niger,* and incubated until the flasks were fully cultivated. After incubation, 0.01% of Congo Red dye was added to both the flasks. After nearly two months, the preparations were removed and centrifuged for five minutes at 14500 rpm. At 550 nm, the optical densities of the test and control samples for Congo Red were measured.

The formula was used to determine the percentage of dye decolorization (Vithalani and Bhatt, 2023).

$$\text{Decolorization percentage (\%)} = \frac{\text{The difference between the initial and final absorbance}}{\text{Initial absorbance}} \times 100$$

Wastewater degradation by fungi

Textile wastewater was collected and inoculated with the *A. niger* strain. This preparation was kept for one month, taken out, centrifuged at 14500 rpm, and then the optical density of control and test samples was measured at 550nm, and their percentage decolorization was calculated.

GC-MS analysis

The flasks having decolorized dye and red wastewater decolorized by the fungal strains were taken. The fungal mat was removed, and 1 mL from each preparation was taken into an Eppendorf tube separately. These were centrifuged at 14500 rmp for 5 min and finally underwent. For the examination of compounds produced by fungi, a Gas Chromatograph Mass Spectrometer from Agilent USA was used in conjunction with gas chromatography and mass spectrometry. With the test volume of 1 :L, the inlet temperature was set at 280°C. Helium, with dimensions of DB 5MS 30m, 0.25mm, or 0.25 :m, was included in the column and flowed at a rate of 1 mL/min. After being kept at 50°C for 1 min, the oven's temperature was progressively raised by 15°C each min or 320°C for 5 min. It ran for 24 min in total. The procedure lasted for roughly 4.8 min, with the transfer line temperature maintained at 280°C and the MS mode set to Scan (35–500).

RESULTS

Fungal dye decolorization

When the media containing the dye and fungi were examined after incubation, it was evident that the fungus strains enzymatic activity had caused a color shift from dark to light hues. The treated samples optical density values were lower than the control. *A. niger* degraded Congo Red by 90% within 60 days, whereas *P. simplicissimum* showed degradation upto 91% within 60 days of incubation (Fig. 1).

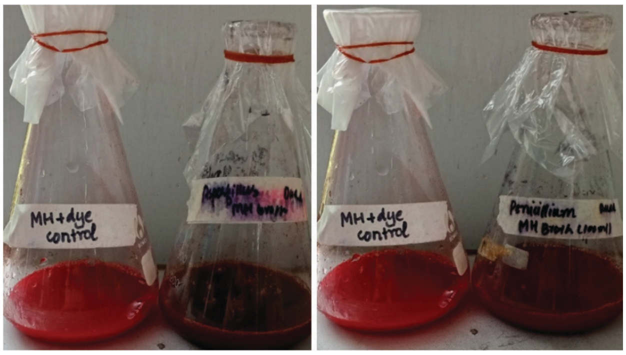

Fig. 1. Color change/degradation of azo dye in the presence of *A. niger* (left) and *P. simplicissimum* (right) as compared to the control.

GC-MS analysis of degradation product of Congo Red

Several compounds were produced by the action of *A. niger* when it degraded Congo Red, including Tetradecane, Eicosane, Nonadecane, 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4 dione, Octadecanoic acid, Benzoic acid, 3,4,5-trihydroxy, 9H-fluoren-9-one, 2,7-diamino, Ferrocene, (carboxyethynyl)-, trans-2,3,5-Trimethoxy-.beta.-methyl-.beta.-nitrostyrene, 5-Bromo 1-methylindole-2-carboxylic acid, and 1-benzylindole, etc. (Fig. 2A).

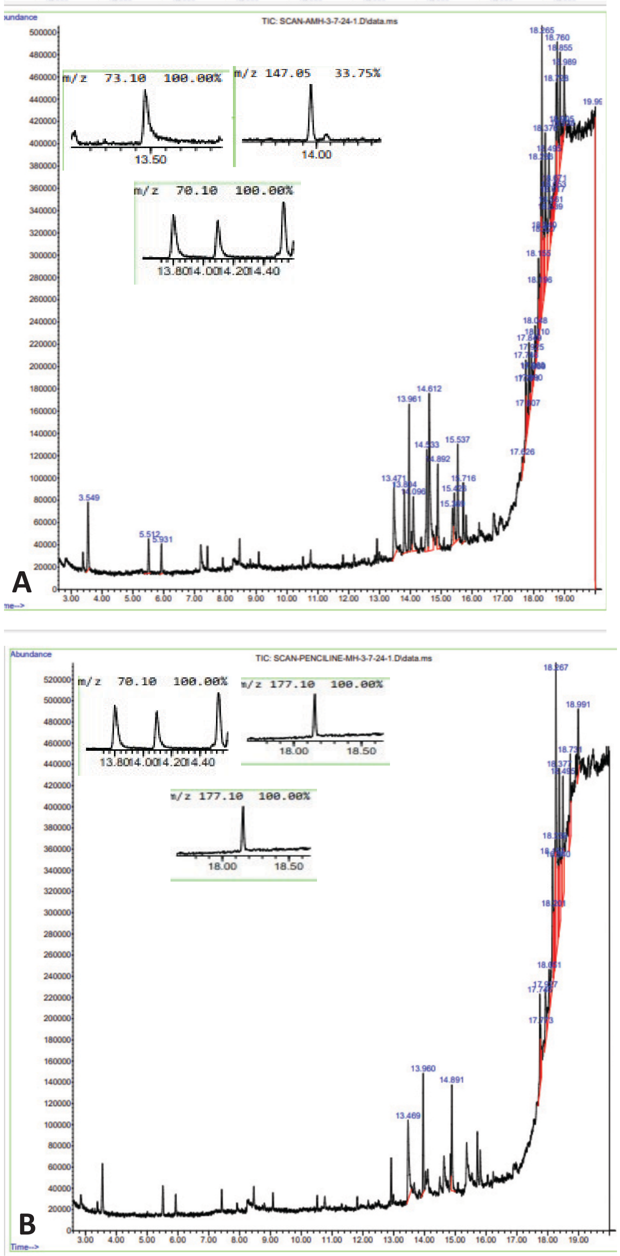

Fig. 2. Compounds produced by the action of (a) *A. niger* and (b) *P. simplicissimum* on Congo Red-containing medium.

The compounds produced by *P. simplicissimum* by its action on Congo Red included n Hexadecanoic acid, Benzene, 1-methoxy-4 (2-phenylethenyl)-, 1H-Pyrrolo[2,3-b]pyridine, 3-amino-2-(4-pyridyl)-, 9H-fluoren-9-one, 2,7-diamino, (2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans, 5-Phenylvaleric acid, 2-methyloct-5-yn-4-yl ester, Pimelic acid, di(2 chlorophenyl) ester, etc (Fig. 2B). A summary of all the compounds produced by both strains after comparing with control is given in . Table I

Wastewater degradation

Post-incubation, wastewater containing *A. niger* strain was observed, and there was a noticeable shift in hue from dark red to bright (Fig. 3). Fungal mat was removed; the sample was centrifuged at 14500 rpm and then the optical density of the control and test sample was taken and there was decrease in the absorbance value of the test sample. The percentage of decolorization was found to be 70%.

Fig. 3. Flask 1, containing only wastewater, acts as a control, whereas flask 2 contains decolorized wastewater treated with the fungal strain.

GC-MS analysis of degradation products of Congo Red by A. niger

Various compounds were found to be produced in the presence of the fungal strain, which can be evaluated by observing the chromatogram (Fig. 4). The extensive compounds produced by *A. niger* it degraded wastewater included 2,4,7-Trinitrofluorenone, 8-Methyl-6-nonenamide, Benzothiophene-3(carboxylic acid), 4,5,6,7-tetrahydro-2-amino-6-ethyl-, Imidazole, 4,5-di(2-furyl)-2-(3-indolyl), Phenylacetamide, ethyl ester, N-ethyl-N-(3-methylphenyl)-, 12-Chloro-14-azatetracyclo[7.6.1.0(2,7).0(13,16)]hexadeca-1(15),2(7),3,5,9(16),10,12-heptaen-8-one, Methyl 3-amino-2-methylbenzoate, N, N-diacetyl, Benzonitrile, m-phenethyl, Benzothiophene-3-carboxylic acid, 4,5,6,7-tetrahydro-2-amino-6-ethyl-, ethyl ester, 2-Pentyl-6-phenyl-1H-pyrazolo[1,2-a]cinnoline-1,3(2H)-dione, 2,6-Lutidine 3,5-dichloro-4-dodecylthio, 4-(4-Acetamidophenyl)-2-aminothiazole, 2-[2-[2-(4-Chloro-phenoxy)-ethylsulfanyl]-benzoimidazol-1-yl]-acetamide, 3-Hydroxy-4’-methoxy-6-methylflavone, trifluoroacetate.

Table I. GC-MS analysis of compounds generated by *A. niger* during azo dye degradation.

Degradation products produced by *A. niger*	**Activities reported**	**Reference**
1. Tetradecane	Antibacterial, antifungal	Abd El-Rahim *et al*. (2021)
2. Eicosane	Anti-inflammatory, analgesic, and antipyretic effects	Costa *et al*. (2025)
3. Nonadecane	Antimicrobial, antioxidant	-
4. 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	Not reported	-
5. Azetidine, 1,1'-methylenebis	Not reported	-
6. Hexahydropyrrolizin-3-one	Not reported	-
7. L-Proline, N-(hexanoyl)-, pentyl ester	Not reported	
8. 3-Cyclohexyl-L-alanine amide, N,N,N'N'-tetramethyl	Not reported	
9. Octadecanoic acid	Antibacterial	Rodríguez *et al*. (1999)
10. Benzoic acid, 3,4,5-trihydroxy	Antioxidant, antimicrobial	Hadibarata *et al*. (2013)
11. Carbonic acid, monoamide, N-(2-pentyl)-N-propyl, allyl ester	Not reported	-
12. 6-Chloro-2-(4-nitro-phenoxy)-4-phenyl-quinazoline	Anticancer, antimicrobial	Syafiuddin and Fulazzaky (2021)
13. Pent-4-enoylamide,2-methyl-N-(2-butyl)-N-nonyl	Not reported	-
14. 1-Benzylindole,4,5-trihydroxy, carboxylic acid	Indole derivatives are often bioactive; this specific compound is not found	-
15. 9H-Fluoren-9-one	Antimicrobial	Ameen *et al*. (2021)
16. Ferrocene	Anticancer, antiproliferative, antimalarial (via derivatives)	Tang *et al*. (2019)
Degradation products produced by *P. simplicissimum*		
1. 5-Azacytosine, N,N,N'-trimethyl	Not reported	-
2. 5-Cyclohexyl-3H-1,3,4-oxadiazole-2-thione	Not reported	-
3. 1H-(2,1,3)-Benzothiadiazine, 3,4-dihydro-, 2,2-dioxide	Not reported	-
4. Undec-10-ynoylamide, N-(2-pentyl)-N-decyl	Not reported	-
5. Valeramide, N-(2-butyl)-N-nonyl	Not reported	-
6. Acetamide, 2-phenyl-N-(2-butyl)-N-nonyl	Not reported	-
7. 18-Nor-estra-1,3,5(10),9(11)-tetraen-12-one		-
Indole, 3-(4-nitrophenylamino)-	Indole derivatives possess antiviral, anti-inflammatory, anticancer, anti- HIV, antioxidant, antimicrobial.	Singh and Dwivedi (2020)
8. Quinoline, 6-[difluoro[6-(1-methyl-1H-pyrazol-4-yl)-1,2,4-triazolo[4,3-b]pyridazin-3-yl]methyl]-	Not reported	-
9. 1,4-Anthracenedione, 6-nitro	Not reported	-
10. 1H-Pyrrolo(2,3-b)pyridine, 3-amino-2-(4-pyridyl)-	Not reported	-
11. Benzene, 1-methoxy-4-(2-phenylethenyl)-	Not reported	-
12. 9H-Fluoren-9-one, 2,7-diamino	Not reported	-
13. 5-Phenylvaleric acid, 2-methyloct-5-yn-4-yl ester	Not reported	-
14. Pimelic acid, di(2-chlorophenyl) ester	Not reported	-

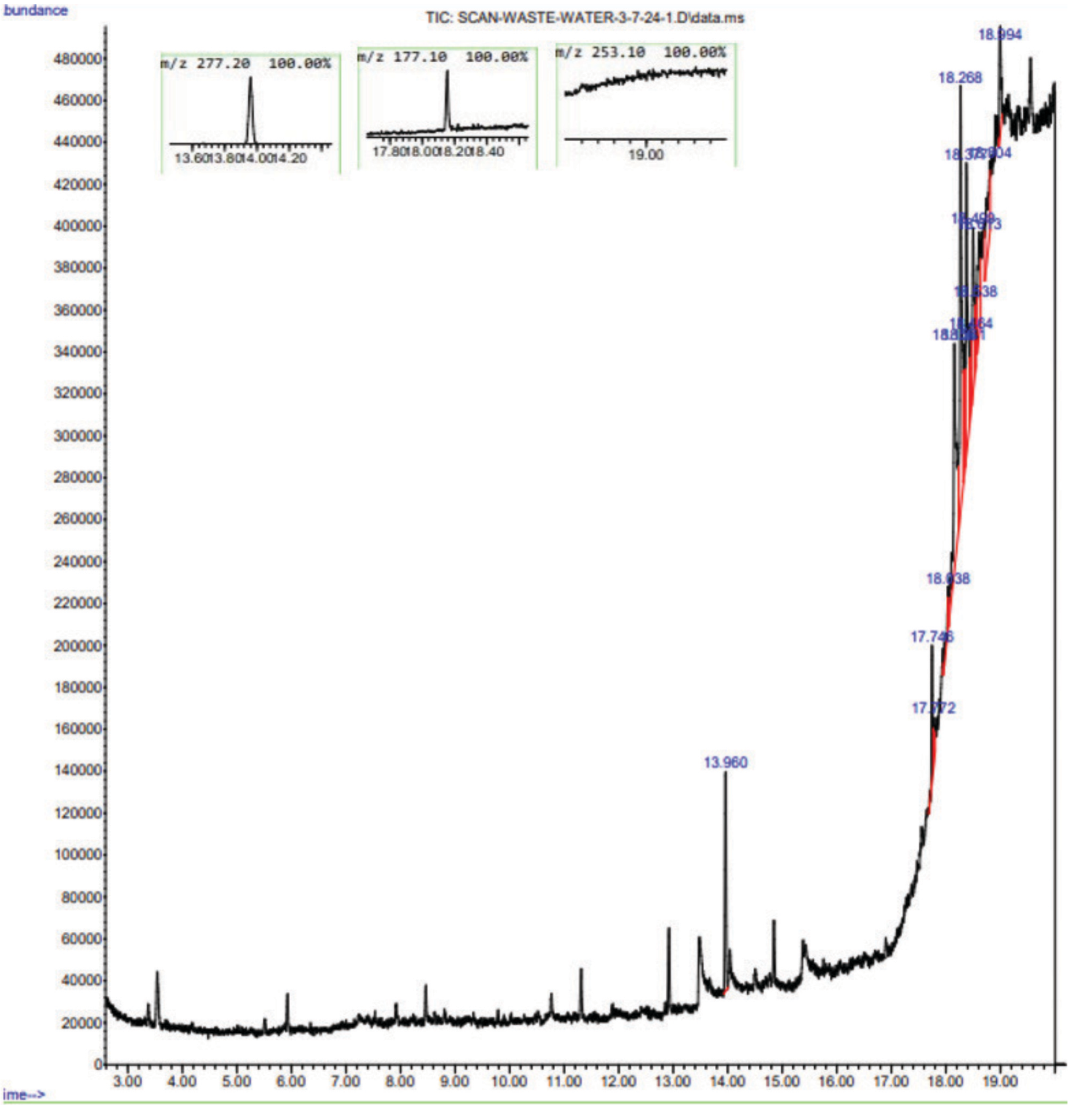

Fig. 4. Compounds produced in the presence of *A. niger* in wastewater containing Congo Red.

DISCUSSION

The wastewater from the textile industry contains many toxic and carcinogenic contaminants. Among them, the major contaminant is azo dye. The biodegradation of such harmful and carcinogenic contaminants is mediated by the utilization of microorganisms such as fungi, bacteria, and yeast, but the degradation of azo dyes using fungal strains has been a topic of increasing interest in recent years because fungi are capable of breaking down complex organic pollutants into less toxic compounds such as phenolic intermediates. These intermediates can further be degraded, which results in the complete mineralization of azo dyes. The optimum conditions required for the degradation of dyes by fungi are pH 5-6, temperature 25-30^{o}C, and the dye concentration 100-200 mg/L. However, fungal growth and dye degradation potential can further be enhanced by the use of co-substrates, such as glucose and yeast extract.

Many fungal strains have been reported in the degradation of dyes due to their degradation capabilities (Abd El-Rahim *et al*, 2021) because of the production of extracellular enzymes. It was reported that white rot fungi have been used for the breakdown of many dyes (Costa *et al*, 2025). According to one study, white rot fungi have a degradation potential of about 52% because of the excretion of specific extracellular enzymes like Mn-peroxidase, laccase, etc. (Rodríguez *et al*, 1999). *Pleurotus eryngii* F032 showed 93.56 % decolorization of Reactive Black 5 dye (Hadibarata *et al*., 2013). Fungus strains *Trichoderma koningiopsis, Pestalotiopsis* sp., and *Trichoderma citrinoviride,* were responsible for degrading Remazol Brilliant Blue R by 52.5%, 74.8%, and 33.1%, respectively (Syafiuddin and Fulazzaky, 2021). According to the study conducted in 2023, *A. niger* gave 90%, 70%, and 40% decolorization on Navy-Blue, Synozol yellow, and red, whereas *Trichoderma viride* gave 87%, 73%, and 36%, respectively, for 60 days (Ali *et al*., 2023).

In this study, *Aspergillus niger* and *Penicillium simplicissimum* were evaluated for their degradation potential. The maximum decolorization calculated by *A. niger* for Congo Red dye was 90% and by the *P. simplicissium* was 91% for the same dye. Acid Blue and Disperse Red 1 dyes were also broken down by numerous *Aspergillus* strains (Ameen *et al*., 2021).

In another study, the consortium of fungal and microalgae strains decolorized 98.09% of Disperse Red 3B dye (Tang *et al*, 2019). Another study reported the successful degradation of azo dyes using a consortium of *Fusarium* sp. and *Trichoderma* sp. (Singh and Dwivedi, 2020). The use of consortia offers several benefits which including increased degradation efficiency, reduced toxicity, and improved adaptability to different environmental conditions (Kumar *et al.*, 2021). The GC-MS analysis of Congo Red dye-polluted water treated with *A. niger* generated several compounds, including benzene dicarboxylic acid and benzene propanoic acid as degraded products (Singh and Dwivedi, 2022).

According to the study conducted in 2023, *A. niger* and *T. viride*-treated wastewater containing Navy-blue, Synozol yellow, and Synozol red dyes produces many compounds such as caprolactam, oleic acid, and isopropyl benzene, etc., which are observed by GC-MS analysis (Ali *et al*., 2023).

When *Aspergillus* sp. and *Chlorella sorokiniana* were combined, GC-MS analysis of a dye called Disperse Red produced a variety of compounds, including o-xylene, di-isobutyl phthalate, and acetone (Tang *et al*, 2019). When Trametes gibbosa treated polluted Alizarin Red wastewater, the compounds produced were analyzed and found to include 1-butylene and acrylaldehyde (Zhang *et al*., 2021).

According to a recent investigation, the degraded products by *T. viride* and *A. niger*were found to be Arsenous acid, caprolactam, tris (trimethylsilyl) ester, Diglycolamine, and many others. According to the current investigation, the *A. niger* produced Tetradecane, Eicosane, Nonadecane, 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a] pyrazine-1,4-dione, Octadecanoic acid, Benzoic acid, 3,4,5-trihydroxy, 9H-fluoren-9-one, and many others on degrading Congo Red dye. Whereas, *P. simplicissimum* on degrading Congo Red dye resulted in the formation of Hexadecanoic acid, 1H-Pyrrolo[2,3-b] pyridine, 3-amino-2-(4-pyridyl)-, 1-methoxy-4-(2-phenylethenyl)-, 9H-fluoren-9-one, 2,7-diamino, (2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans, 5-Phenylvaleric acid, 2-methyloct-5-yn-4-yl ester, Pimelic acid, di(2-chlorophenyl) ester.

Benzene, propanoic acid, and benzene dicarboxylic acid were the main byproducts of the degradation of Congo Red dye-polluted water treated with *A. niger* when it was examined through GC-MS (Singh and Dwivedi, 2022).

Moreover, in the current study, wastewater was treated with *A. niger,* which resulted in 70% decolorization, and GC-MS analysis of wastewater treated with *A. niger* produced 2,4,7-Trinitrofluorenone, 8-Methyl-6-nonenamide, Benzothiophene-3-carboxylic acid, 4,5,6,7-tetrahydro-2-amino-6-ethyl-, ethyl ester, Imidazole, 4,5-di(2-furyl)-2-(3-indolyl), Phenylacetamide and many others. Despite the promising results, there are some limitations associated with the fungal degradation of azo dyes. These include a slow rate of degradation, the formation of toxic intermediates, and the need for optimal conditions.

Overall, these findings suggest that fungal strains and consortia provide a promising solution for the degradation of azo dyes. Further research is needed to fully understand the mechanisms of fungal degradation and to optimize the process for industrial applications, thus providing a feasible solution to a toxicity-free environment.

CONCLUSION

This research work focused on the breakdown/degradation of azo dyes by fungal strains. Both fungal strains i.e., *A. niger*and *P. simplicissimum*, degraded azo dye upto 90% and 91%, respectively, and proved to be excellent bioremediators of contaminants, including azo dyes. During this degradation process, various compounds, including octadecanoic acid, benzoic acid, 3,4,5-trihydroxy, 9H-fluoren-9-one, and many others, were generated as byproducts. These dye-degradative compounds are useful as they possess antioxidant and antimicrobial properties. The wastewater, after fungal treatment, can at least be used for crop irrigation to compensate for the scarcity of freshwater.

DECLARATION

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Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Morphometric Analysis of *Falco peregrinus peregrinus* Rescued in Pakistan

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ABSTRACT

Peregrine falcon *Falco peregrinus peregrinus* is recognized as the migratory species in the world. This falcon is one of the four subspecies of *Falco peregrinus.* These birds are poached in Pakistan during their migration. Fortunately, they were rescued by Pakistan Customs, Sindh Wildlife Department (SWD), and Raptor Center for Conservation and Rehabilitation, Pakistan (RCCRP) and were observed for this study. Several parameters were studied in the rescued birds for morphometric measurements including total length, wings pan, bill depth, bill width, bill length and tarsus in both male and female Peregrine falcon. Bill volume was also calculated with the given parameters which resulted in showing difference between the groups. Male and female individuals were also compared on the basis of body size and bill volume. The objective of this study was to determine the diversity among the individuals which will benefit while comparing adaptive radiations in avian fauna. This research can also be associated with diversification in birds for instance the structure of bill, wingspan, and total length which enables different functions in both sexes including feeding habits and flight patterns.

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RT planned and conducted experiments. KKY and JM funded the project and reviewed the manuscript. NS, JH, FA and NAR worte the manuscript and finalized it for publication.

Key words
Migratory birds, Indus flyway, Birds of prey, Avian diversity, Wingspan

INTRODUCTION

Peregrine falcons (*Falco peregrinus peregrinus*) is widely distributed throughout the world except Antarctica. It is one of the fastest birds in the world. While diving to catch the prey, their speed can reach up to 150 km/hr (Ponitz *et al*., 2014). Like other falcons, they also have slender bodies, a long narrow tail, and pointed wings. The upper parts are pale bluish. The under parts are flawlessly white, heavily marked with clean and tidy spots and bars. Due to variation in color and sizes, they are classified into 19 subspecies, out of which at least five are migratory (White *et al*., 2013). The study of these falcons required proper handling and favorable environment as suggested by Williams *et al.* (2020) that photography can be used as a tool for morphometric measurements which will further reduce the stress in birds.

Pakistan is a rich country in terms of avian biodiversity due to a variety of habitats from permanent snow zones to alluvial plains and sandy deserts, which extends to the seacoast in the south. Pakistan hosts four sub-species of Peregrine falcon; *F. p. peregrinus, F. p. babylonicus, F. p. calidus* and *F. p. peregrinator* (Dixon *et al*., 2017). *F. p. peregrinus* got our attention to study because it is a migratory and threatened species in Pakistan. During this study, uncontrolled illegal trade of falcons was observed (Ahmad and Khan, 2020). Peregrine falcons are mainly captured by a method called Khudhu, in local language of Pakistan. In this technique, live baits such as kestrils and laggar falcons. They are tied with the feathers of quail/ partridge and nylon mesh. As the migratory bird sees the opportunity to snatch the prey from laggar falcon (live bait), it entangles itself in nylon mesh (Ahmad and Khan, 2020; Shafiq and Idrees, 2006).

It is now accepted that the species evolution generally progress by a continuously transforming characters. These characters display noticeable hereditary variations along with the environmental factors (Falconer, 1996). Our study

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presents analysis of some morphometric measurements of wild Peregrine falcons (*Falco peregrinus peregrinus*) rescued by the Sindh Wildlife Department and the Pakistan Customs, which were captured illegally from Indus plains of Pakistan en route to their migration which include Hadero, Keenjhar, Haleji, and other Sindh lakes (Shafiq and Idrees, 2006; Ghalib *et al*., 2019; Khan *et al*., 2024). The birds were kept in a temporary rehabilitation center, provided by Raptor Center for Conservation and Rehabilitation, Pakistan during the span of this study.

Up till now, Peregrine falcons have not been the subject of morphometric study in Pakistan. This study projects on morphometric aspects related to the species ecology and biology (Debus and Rose, 2000) as the species can be used as a part of a broader scientific investigations for raptor communities (Aumann, 2001a, b, c; Falkenberg, 2011; Sutton, 2011).

MATERIALS AND METHODS

The specimen comprised of one subspecies of Peregrine falcon, *F. p. peregrinus,* (n=56; females= 27, males= 29). All the alive specimen was identified with the help of previously reported literature and indigenous knowledge, gathered from falconers (Grimmett *et al*, 2008). Measurements of total body length (from the highest point of skull, without bill, to the tip of the tail feathers), wingspan (from farthest primary feather (number 10) of one wing to another), bill length (from end of cere to the tip of bill), bill depth (from the highest point of maxilla to the shallowest part of mandible, without cere), bill width (highest and widest point, without cere) (Fig. 1) and tarsus length (tarsometatarsus) of each bird was measured by using Vernier caliper (for bill, minimum count 0.01mm), measuring tape (minimum count mm) and weighing balance (minimum count g). The bill volume (mm^3) was determined by using a formula, i.e.

$$\frac{\text{Bill length (L) x Bill depth (D) x Bill width (W)}}{3}$$

We made sure that the specimen had no broken feathers and the mandibles, which were completely closed. An individual bird was not handled more than ten minutes to minimize the stress which is a key factor in accuracy of the results. Measurements, including weighing of birds, were taken at the time of dawn when the birds were more relaxed and their crops were empty (Winker, 1998; Baldwin *et al*., 1931).

We assumed rescued falcons to be a portion of a greater population since they are migratory species. Descriptive statistics were calculated using Minitab 19. *t*-Test was performed for the analysis of variance to observe the significant differences between the mean values of the morphometric characters in male and female individuals. The morphometric data analyzed in this research allowed us to determine the variations in overall size of birds, which significantly indicate the diversity.

RESULTS

Table I shows the morphometric data of *F. p. peregrinus.* Females of Peregrine falcons were significantly larger than males in total length, body weight, wingspan, bill volume, and tarsus length. The mean total length was 422.63 ± 9.47 mm in females (range: 410-450 mm) and 381.86 ± 7.85 mm in males (range: 370-390 mm).

Table I. Descriptive statistics of morphometric characters of *Falco peregrinus peregrinus*(p-value 95%).

Morphometric characters	Sex	Total[1]	Mean ± SD[2] (Range)	DF[3]	Difference	T[4]	P[5]
Total length (mm)	Female	27	422.63 ± 9.47 (410-450)	50	40.77	17.47	0
	Male	29	381.86 ± 7.85 (370-390)				
Weight (g)	Female	27	914.99 ± 45.08 (794.14-979.65)	42	290.4	15.65	0
	Male	29	624.6 ± 88.3 (513.6-956.7)				
Wing-span (mm)	Female	27	997.89 ± 9.8 (988-1013)	30	77.4	7.64	0
	Male	29	920.45 ± 53.62 (830-972)				
Bill depth (mm)	Female	27	18.173 ± 1.574 (15.02-20.54)	53	2.394	5.37	0
	Male	29	15.779 ± 1.764 (13.23-18.65)				
Bill width (mm)	Female	27	12.827 ± 3.034 (9.08-18.01)	52	0.095	0.13	0.898
	Male	29	12.732 ± 2.714 (9.21-16.58)				
Bill length (mm)	Female	27	24.76 ± 0.91 (23.02-26.02)	46	3.764	11.54	0
	Male	29	20.995 ± 1.483 (19.26-23.03)				
Bill volume (mm^3)	Female	27	1931.1 ± 510.3 (1296-2798.1)	45	525	4.58	0
	Male	29	1432.9 ± 345.7 (935.3-1931.3)				
Tarsus (mm)	Female	27	58.3 ± 5.45 (50-67)	51	3.09	2.28	0.027
	Male	29	55.207 ± 4.609 (50-66)				

[1]N= Total, [2]Standard deviation, [3]Degree of freedom, [4]Test statistics, [5]Probability value

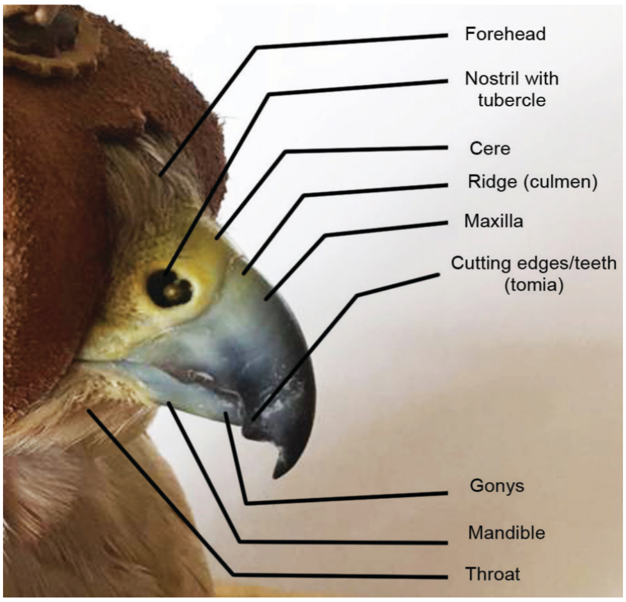

Fig. 1. Labelled mouth and bill parts of Peregrine falcon.

Similarly, wingspan ranged from 988 mm to 1013 mm in females and 830 mm to 972 mm in males, while tarsus length measured 58.3 ± 5.45 mm and 55.207 ± 4.60 mm in females and males, respectively. These differences confirm sexual dimorphism typical of birds of prey, where females tend to be larger. Although expected, quantifying such differences is important for ecological and taxonomic studies, aiding in accurate sex identification, understanding adaptive strategies, and monitoring population variation across regions or subspecies (Table II).

Table II. Allometry between different morphometric characters in different sexes of *F. p. peregrinus.*

Morphometric characters	Sex	N	y	r^2
Total length vs. Weight	Female	27	$29.123x^{0.57}$	0.0624
	Male	29	$23.262x^{0.5521}$	0.0086
Total length vs. Wing-span	Female	27	$198.97x^{0.2667}$	0.3608
	Male	29	$0.003x^{2.1237}$	0.5444
Total length vs. Bill volume	Female	27	$3E\text{-}15x^{6.7804}$	0.3953
	Male	29	$2E\text{-}22x^{9.6492}$	0.6125
Total length vs. Bill length	Female	27	$0.3919x^{0.6856}$	0.1657
	Male	29	$474956x^{-1.687}$	0.2449
Total length vs. Tarsus	Female	27	$4E\text{-}05x^{2.3362}$	0.3027
	Male	29	$0.1459x^{0.9979}$	0.0618
Bill length vs. Bill volume	Female	27	$5E\text{-}05x^{5.4115}$	0.612
	Male	29	$5E{+}06x^{-2.658}$	0.5051

DISCUSSION

Table II shows allometric relationship between different morphometric characters in the two sexes of *F. p. peregrinus*. The present study suggests that bill volume is highly significant in both male and female Peregrine falcons with respect to bill width, length, and depth. *F. p. peregrinus* possessed visibly diverse bill volume and the structure of the bill represents their feeding habits. Johansson *et al*. (1998) compared the bill size and the bill structure of Peregrines from the two different habitats and the result suggested no significant relationship. Similar findings are stated by Bierregaard (1978), which showed the relationship between bill depth and width among several groups of raptors, including *Falco*, as well as between bill depth and habitat.

Our study presents observation on bill volume. Bill volume showed greater growth rate than the body as whole. It was observed in both sexes. Similarly, bill length showed the highest growth rate relationship in females and lower growth rate relationship in males. A these are the first data on bill volume on raptors from Pakistan which is quite similar to the results of Beintema and Visser (1989) who used the bill length as universal age estimator and observed that the growth rate of body weight and bill length was constant throughout the developmental stages of chicks of Lapwings subfamily Vanellinae and other birds from order charadriiformes but the growth rate slowed down after 25 days.

This study also suggests that body weight has lower growth rate in both male (n=29) and female (n=27). Similar observations are reported in different birds such as, Teather and Weatherhead (1989) observed the nestlings of Great-tailed grackle or Mexican grackle (*Quiscalus mexicanus*) and noted that the larger sex had higher growth rate than the smaller one, Richter (1983) is of the view that smaller sex grows more quickly than the larger relative to their size in altricial birds (herons family Ardeidae, hawks family Accipitridae, owls family Strigidae and family Tytonidae, etc.), Nee *et al*. (1991) showed positive relationship in the abundance and body weight of several British birds, and Teather and Weatherhead (1994) denied any relationship between competing individuals that affect different patterns of growth and development in males and females.

The parameters observed during our study were also presented by a number of authors including research on African raptors kept by falconers and zoos, indicating male males of *Falco biarmicus* (Lanner falcon) had higher loads than females (Mendelsohn *et al*., 1989). Our result is in accordance with the above findings. Another study on bearded vulture *Gypaetus barbatus* highlights long and heavier wings in adults than in juveniles (Brown, 1988). We found that difference between adults and juveniles was non-significant.

In our study, wingspan of female Peregrine falcons were found to be significantly longer than males. This is due to "reversed sexual dimorphism or RSD" in raptors, where females are larger than males (Mueller and Meyer, 1985; Krüger, 2005; Sullivan *et al.*, 2019). Furthermore, smaller raptors capture prey more frequently than larger ones, this favors the male raptors for immediate chances of courtship (Safina, 1984). We have observed sexual dimorphism with significant difference between males and females in accordance with the above findings.

CONCLUSION

This is the first study on morphometric analysis of *Falco peregrinus peregrinus* from Pakistan. The study was designed to observe sexual dimorphism and any significant relationship between length, wingspan, bill volume, body weight, and tarsus length.

Our country lacks facilities/rehabilitation centers for birds of prey to deal with injured and sick with the goal of releasing them back to their natural habitat. Hence, it is highly recommended that work on establishment of rehabilitation centers on a smaller scale by concerned departments would change the current status of migratory species. The present study will be beneficial for the researchers or rescuing teams working on raptors in providing preliminary data on diversity among the individuals of same population, sexual dimorphism criteria, and growth parameters.

DECLARATIONS

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Ethical statement

We carried out this study under proper environmental conditions favorable for bird handling to reduce the stress in order to obtain accurate results. It is recommended that the use of photography as a tool for morphometric measurements can further reduce the stress in birds.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Plasmid Borne Antibiotic Resistance Factors in Indigenous *Shigella boydii* Isolated from Diarrheal Patients in Azad Jammu and Kashmir, Pakistan

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ABSTRACT

The antimicrobial susceptibility patterns for 110 *Shigella boydii* isolated from diarrhoeal patients admitted to hospitals in Azad Kashmir Pakistan were analyzed to determine their plasmid profile and changing trends in response to twenty antibiotics. Susceptibility of the isolated bacteria was determined against various antibiotics by antibiotic disc diffusion method. Plasmid DNA was isolated from the multiple antibiotic resistant strains of *S. boydii.* and was transferred to plasmid-less and sensitive to antibiotic strain of*Escherichia coli* HB101. The transformed cells were then examined for their sensitivity to ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) and sulfamethoxazole-trimethoprim (100 µg/ml). The isolates showed highest resistance against penicillin followed by carbenicillin, ampicillin, tetracycline, erythromycin, ceftizoxime, kanamycin, co-trimoxazole, piperacillin, amoxicillin, amikacin, streptomycin, nalidixic acid, gentamicin, chloramphenicol, cephalothin and ceftriaxone. All *S. boydii* isolates were sensitive to cefixime, ciprofloxacin and enoxacin. Multiple drug resistance (MDR) was observed ranging from three to ten drugs and was resistant to three or more antibiotics at level as high as 300µg/ml. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was PCaA (penicillin, carbenicillin, ampicillin). The plasmids were observed in (*28.0 %*) MDR strains of *S. boydii* which were found resistant to three or more antibiotics. The number of plasmids varied from one to seven. Analysis of plasmid DNA of *S. boydii* revealed that all the strains contained a heterogenous population of plasmids ranging between 23.1 kb to 2.0 kb. Based on molecular weight, the pattern of different plasmids was also very diverse. Depending on the number of plasmids, individual strains were grouped into nine different plasmid patterns. The plasmids (23.1 Kb and <23.1 Kb) could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *E. coli* HB101.

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SSA: Conceptualization, supervision, data curation
AAA: Sofware
MK: Investigation
AR: Writing, review, editing, supervision

Key words
***Shigella boydii*, Antibiotic resistance, R-plasmid, Shigellosis, Multiple drug resistance, Antimicrobial resistance determinants, Plasmid profile**

INTRODUCTION

Shigellosis is a public health problem in many parts of the world including Pakistan not only for morbidity but also for growth retardation and malabsorption. *Shigella* is a non-motile, nonspore-forming, facultative anaerobic Gram-negative bacterium. *Shigella* is a rod-shape and is lactose-fermenting bacterium causing dysentery (Yang *et al*., 2005). There are 4 species of *Shigella* classified on the basis of biochemical serological differences. Serogroup A: *S. dysenteriae* (12 serotypes), Serogroup B: *S. flexneri* (6 serotypes), Serogroup C: *S. boydii* (23 serotypes) and Sergoroup D: *S. sonnei* (1 serotype) (Niyogi, 2005). The *Shigella* genome includes a virulence plasmid that encodes conserved primary virulence determinants. The virulence plasmid encodes the ~30 kb Mxi-Spa type III secretion system (TTSS) and invasion plasmid antigens (Ipa proteins) required for invasion of the colonic and rectal epithelial cells (Sansonetti, 2001). In addition to

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the plasmid, many chromosomal genes, such as those encoded by the *Shigella* pathogenicity island (SHI)-1 and SHI-2, also contribute to virulence (Moss *et al.*, 1999). *Shigella boydii* are responsible for gastroenteritis that may progress to mucoid bloody diarrhoea. *S. boydii* (23 serotypes) is mainly endemic to the Indian subcontinent. In developing countries, shigellosis is widespread and causes extensive outbreaks. In industrialized countries, this disease has become rare, and it currently occurs as sporadic cases in migrant workers or those who travel to developing countries and is limited to epidemic episodes among children in daycare centers, individuals in custodial institutions, and homosexual men (Niyogi, 2005). Consequently, although shigellosis is a major public health concern, there is a great disparity between developing countries (over 163.2 million cases each year) and developed ones (1.5 million cases). Epidemics usually occur in areas with crowding and poor sanitary conditions, where transmission from person to person is common or when the organisms contaminate the food or water (Kotloff *et al.*, 1999). Dramatic outbreaks may also occur, particularly in the context of humanitarian disasters (wars, refugee camps). Shigellosis is not the most frequent cause of diarrhoeal disease, but its dysenteric form is the most severe: each year, it kills between 600,000 and one million people, mostly children in developing countries. Those infected with *Shigella* develop diarrhoea, fever, and stomach cramps starting a day or two after the exposure to the bacterium. In some persons, especially young children and the elderly, the diarrhoea can be so severe that the patient needs to be hospitalized.

Shigellosis is one of the acute enteric diseases for which antimicrobial therapy is generally required to manage infection and reduce fecal excretion of the bacterium to prevent further transmission. Antimicrobial resistance pattern, plasmid profile and serotype correlation of *Shigella* strains have been reported from several countries (Dutta *et al.*, 2002; Lin and Chang, 1992; Olukoya and Oni, 1990; Albert *et al*, 1990). Although *Shigella* spp. is intrinsically susceptible to all antibiotics that are active against Gram-negative bacilli, under antibiotic pressure, they have progressively acquired resistances to commonly recommended drugs (Hirose *et al.*, 2005; Toro *et al.*, 2005). The antibiotics commonly used for treatment are ampicillin, trimethoprim/ sulfamethoxazole, nalidixic acid, or ciprofloxacin. Appropriate treatment kills the *Shigella* bacteria and shortens the illness. Unfortunately, some *Shigella* bacteria have become resistant to antibiotics and using antibiotics to treat shigellosis can actually make the germs more resistant in the future (Nisa *et al.*, 2022). Indiscriminate use of the drugs and horizontal gene transfer has led to *Shigella* species becoming resistant to commonly used antibiotics (Noriega *et al.*, 1999). A high proportion of the resistant strains were found to be resistant to some of the most commonly used antibiotics such as tetracycline and streptomycin (Kotloff *et al.*, 2005). Most of the *Shigella* strains isolated from patients are resistant to ampicillin, sulfamethoxazole-trimethoprim, and nalidixic acid (Hossain *et al.*, 1998).

MDR in *Shegella* continues to pose a significant public health challenge particularly in developing countries. Whole genome sequencing and bioinformatic approaches unveiled more than 500 global plasmid entities and more than 1000 plamid mediated gene clusters from global databases. Asad *et al.* (2025) identified 28 antimicrobial resistance genes from nine antibiotic classes, with 75% orignating from plasmids.

The overuse of antibiotics is considered the main factor in the emergence and dissemination of antibiotic resistance. In order to ensure appropriate treatment, continual surveillance is required to determine which antibiotics are still active. The people in Azad Kashmir Pakistan face health hazards because of poor sanitation practices. The present study was aimed to investigate the virulence factors in locally isolated *S. boydii* and their possible role in infection. The object also was to suggest preventive measures. The strains of *S. boydii* resistant to commonly used antibiotics have been screened for plasmid DNA. The isolated plasmids have been characterized through biochemical and physical characteristics. The plasmid-less *E. coli* HB101 strains were transformed with plasmid DNA of multiple drug resistant (MDR) *S. boydii* isolates to identify the plasmid (s) involved in antibiotic resistance. Attempts have been made to determine any correlation between plasmid profile and serotype patterns of isolated shigella strains.

MATERIALS AND METHODS

This study was carried out in Azad Kashmir, which is a mountainous region and located 140 km. north-east of Islamabad (Pakistan). Approximately 4.3 million people live in the state of Azad Kashmir comprising rural and urban populations.

Bacterial strains

Shigella boydii strains were isolated from stools of patients suffering from diarrhoea admitted at different hospitals of Azad Kashmir (Pakistan), over a 5-year period. The samples were obtained from children (aged 0-5 years) and adults. The study subjects were both male and female. For the isolation of *S. boydii* a loop full of stool was mixed with 10 ml of sterile buffered peptone water and incubated at 37°C for 24 h. After incubation a loop full of culture was streaked on the SSA and MacConkey agar plates and were incubated at 37°C for 24 h. Non-lactose fermenting colonies (i.e., colorless) on MacConkey agar plates were inoculated on XLD agar and incubated at 37°C for 24 h. After incubation, red colonies with 2-4 mm diameter were marked and suspected colonies were subjected to subsequent Gram staining. All plates were incubated aerobically at 37 ºC for 24 h. From amongst the suspected *S. boydii* from both SSA and MacConkey agar, the non-lactose-fermenting (NLF) colonies were biochemically identified by Urea test, Triple Sugar Iron (TSI) test, Sulphide Indole Motility (SIM) test, and Simmons Citrate Agar – Microbial Utilization test. Serotyping was determined by Kligler's Iron Agar (KIA, DIFCO) test.

Chemicals and media

Chemicals and antibiotics used were obtained from Sigma Chemicals Co. and were of molecular biology grade. The culture media were purchased from DIFCO (USA). LB medium was used for the cultivation of bacteria and Muller Hinton agar DIFCO was used for susceptibility testing. Antibiotic susceptibility discs used were from OXOID, England. Antibiotics used in these studies were amikacin (Ak), amoxicillin (Am), ampicillin (A), carbenicillin (Ca), cefixime (Cfm), ceftizoxime (Cxm), ceftriaxone (Cz), cephalothin (Cl), chloramphenicol (C), ciprofloxacin (Cip), co-trimoxazole (Co), enoxacin (E), erythromycin (Er), gentamicin (G), kanamycin (K), nalidixic acid (Na), penicillin (P), streptomycin (S), sulfamethoxazole-trimethoprim (SxT) and tetracycline (T). All solutions were sterilized by Millipore (0.45mµ) filters and refrigerated.

Antimicrobial sensitivity testing

Antibiotic susceptibility tests of the collected strains of *S. boydii* were performed by antibiotic disc diffusion method (Bauer *et al.*, 1966) using filter paper discs. The minimum inhibitory concentrations of fifteen commonly used antibiotics were determined by agar dilution method using MICs: 25µg/ml, 50µg/ml, 100µg/ml, and 300µg/ml. MIC was defined as the lowest concentration on which the growth appeared. Reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested regularly as controls according to the National Committee for Clinical Laboratory Standards (1993)

Plasmid DNA isolation

Plasmid DNA was isolated from the multiple antibiotics resistant strains according to Birnboim and Doly (1976) to separate, identify and purify the plasmid DNA through agarose gel (Meyers *et al*, 1976). The plasmid DNA was purified by removal of RNA present in the solution with the help of RNase. To estimate the size of plasmid DNA, DNA Marker (Lambda DNA cut with *Hin*d-III) was used. After gel electrophoresis, plasmid DNA was stained with florescent, intercalating dye, ethidium bromide. DNA bands were visualized under UV transilluminator. Photographs of the gel were positioned over a short-wave UV light source that was taken with the help of gel documentation system GDS-5000 (UVP) and the images of DNA bands were obtained. Various plasmids DNA bands were individually cut out of the gel with a sharp razor, extracted, and purified by the usual molecular biological techniques (Weislander, 1979).

Transformation

All the isolates were tested for the ability to transfer their determinants. *E. coli* HB101 (plasmid less and sensitive to antibiotics) were transformed with different individually isolated plasmids. For this, 5 µl of plasmid DNA of multiple drug resistant (MDR) *S. boydii* was added to competent cells of *E. coli* HB101, prepared, incubated on ice for 30 min and then at 42 ºC for two min. One ml of pre-warmed LB broth was then added to this mixture and re-incubated at 37ºC at 60 rpm for 80 min. The whole mixture was then spread on two different Luria-Bertani agar plates containing ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) sulfamethoxazole-trimethoprim (100 µg/ml) and incubated at 37ºC overnight (Sambrook *et al*., 1983).

RESULTS

During the study period, out of 110 *S. boydii,* in 1994, 27 (12.0%) strains were recovered, whereas this number was 20 (11.9 %), 21 (11.0 %), 14 (11.5%) and 28 (13.6 %) in 1995, 1996, 1997 and 1998, respectively. *S. boydii* decreased from 12.0 % in 1994 to 11.9 % in 1995, 11.0 % in 1996 but again increased up to 11.5% in 1997 and the highest number (13.6 %) was recovered in 1998.

Over all the highest proportion of stool specimens infected with*S. boydii* were in the age group of >50-60 years (27.3%) followed in >60 years (23.1%), >30-40 years (20.6%), >10-20 years (17.6%), >20-30 years (16.7%), >40-50 years (14.3%) and >5-10 years (13.6%). The lowest infestation was observed in the age group >0-5 years (9.5%).

Antimicrobial sensitivity testing

Table I shows that the overall 65.4% *S. boydii* isolates were resistant to penicillin (P) followed by 51.8% to tetracycline (T), 49.1% to erythromycin (Er), 47.3% to ampicillin (A), 46.4% to ceftizoxime (CXM), 42.7%

Table I. Occurrence of antibiotics resistance of 110 *S. boydii* at four different concentrations, isolated from stools of patients with diarrhea in Azad Kashmir.

Antibiotics	No. of resistant isolates at			
	25 µg/ml	**50 µg/ml**	**100 µg/ml**	**300 µg/ml**
Amikacin (Ak)	36(32.7%)	34 (30.9%)	15 (13.6%)	3 (2.7%)
Ampicillin (A)	52(47.3%)	50 (45.4%)	30 (27.3%)	12 (10.9%)
Amoxicillin (Am)	35(31.8%)	33 (30.0%)	17 (15.4%)	2 (1.8%)
Carbenicillin (Ca)	47(42.7%)	46 (41.8%)	27 (24.5%)	13 (11.8%)
Cefixime (Cef)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Ceftizoxime (CXM)	51(46.4%)	49 (44.5%)	24(21.8%)	9 (8.1%)
Ceftriaxone (Cz)	21(19.1%)	17 (15.4%)	6 (5.4%)	1 (0.9%)
Cephalothin (Cl)	26(23.6%)	23 (20.9%)	7 (6.4%)	2 (1.8%)
Chloramphenicol (C)	29(26.4%)	25 (22.7%)	9 (8.2%)	3 (2.7%)
Ciprofloxacin (Cip)	00(0.0%)	00(0.0%)	00 (0.0%)	00 (0.0%)
Co-trimoxazole (Co)	39(35.4%)	37 (33.6%)	12 (10.9%)	4 (3.6%)
Enaxacin (E)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Erythromycin (Er) Gentamicin (G)	54(49.1%)	52 (47.3%)	16 (14.5%)	8 (7.2%)
Kanamycin (K)	30(27.3%)	27 (24.5%)	8 (7.2%)	5 (4.5%)
Nalidixic acid (Na)	43(39.1%)	41(37.3%)	14 (12.7%)	7 (6.4%)
Penicillin (P)	24(21.8%)	22(20.0%)	10 (9.1%)	3 (2.7%)
Sulfamethoxazole-Trimethoprim (SxT)	72(65.4%)	69 (62.7%)	45 (40.9%)	17(15.4%)
Streptomycin (S)	37(33.6%)	35 (31.8%)	11 (10.0%)	4 (3.6%)
Tetracycline (T)	34(30.9%)	29 (26.4%)	12 (10.9%)	3 (2.7%)
	57(51.8%)	54 (49.1%)	28 (25.4%)	12 (10.9%)

to carbenicillin, (Ca), 39.1% to kanamycin (K), 35.4% to co-trimoxazole (Co), 33.6% to sulfamethoxazole-trimethoprim (SxT), 32.7% to amikacin (Ak), 31.8 % to amoxicillin (Am), 30.9% to streptomycin (S), 27.3% to gentamicin (G), 26.4% to chloramphenicol (C), 23.6% to cephalothin (Cl), 21.8% to nalidixic acid (Na), and 19.1% to ceftriaxone (Cz). All *S. boydii* isolates were sensitive to cefixime (Cfm), ciprofloxacin (CIP) and enoxacin (E).

The MICs of twenty antibiotics against 110 strains of *S. boydii* are shown in a comparative account of the antibiotics resistance of isolates at four levels 25µg/ml, 50µg/ml, 100µg/ml and 300µg/ml in Table I. Generally, the isolates showed the highest frequency of resistance against penicillin (P) at all the four levels. The lowest frequency of resistance was against ceftriaxone (Cz) at all the four levels of antibiotics screened. At 100µg/ml level the isolates showed a considerable decrease in the resistance frequency of almost all the antibiotics tested. Multiple drug resistance was observed in this study ranging from three to ten drugs. Out of 584 isolates, screened for antibiotic resistance, 31% were resistant to three or more antibiotics at 25µg/ml, 26% were resistant to three or more antibiotics at 50µg/ml, 11% were resistant to three or more antibiotics at 100µg/ml and 4% were resistant to three or more antibiotics at 300µg/ml. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was PCaA at all the four levels shown in Table II.

Total 75 strains of *S. boydii* were processed for isolation of plasmids and 21 (28.0 %) of *S. boydii* carried plasmids. These were resistant against three or more antibiotics. The number of plasmids varied from one to seven. The plasmid pattern was determined by the presence or absence of a single plasmid within a group of strains.

In *S. boydii,* the analysis of plasmid DNA revealed that all the strains contained a heterogeneous population of plasmids ranging between 23.1 kb to 2.0 kb, (Fig. 1 Table III). The molecular size of all plasmids was determined by comparison with a bacteriophage lambda DNA digest with *Hind*-III. The most dominant plasmids were 2.3 Kb, 6.5 Kb, 2.0 Kb, >4.3 Kb, 4.3Kb, 23.1 Kb, <23.1 Kb and <6.5 Kb. The frequency with which they were encountered was 66.7%, 61.9 %, 61.9 %, 57.1%, 57.1 %, 47.6 %, 23.8 % and 23.8 %, respectively. Other plasmids were observed in lesser frequency. The frequency of 9.4 Kb plasmid was 14.3 %, for <4.3 Kb it was 9.5 % and for >2.3 Kb it was 4.8 %.

Based on molecular weight, the pattern of different plasmids was also very diverse. Depending on the number of plasmids, individual strains were grouped into nine different plasmid patterns, designated P1-P9, for 21 strains. Four strains (19.0 %) had pattern P1 (5 plasmids), three strains (14.3 %) had pattern P2 (2 plasmids), whereas three strains (14.3 %) had pattern P3 (6 plasmids), while another group of three strains (14.3 %) had pattern P4 (4 plasmids), two strains (9.5 %) had pattern P5 (5 plasmids), whereas two strains (9.5 %) had pattern P6 (4 plasmids), while another group of two strains (9.5 %) had pattern P7 (6 plasmids),whereas one strain (4.8 %) had P8 (3 plasmids) and the remaining one strain (4.8 %) had pattern P9 (2 plasmids).

Table II. Multiple antibiotic resistance patterns occurring in *S. boydii* isolated from stools of patients with diarrhea in Azad Kashmir.

Antibiotics resistance patterns	Percent of resistant isolates at			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
P, Ca, A	31	26	11	4
P, A, T	27	25	9	3
P, Ca, A, T	24	23	7	3
P, A, T, Er	22	21	7	3
P, Ca, A, Er	20	19	5	2
P, Ca, A, CXM	18	16	5	2
P, Ca, A, T, Er	15	14	4	1
P, C, A, T, CXM	13	10	3	1
P, Ca, T, CXM, K	10	7	2	1
P, Ca, A, T, K, Co	10	7	2	1
P, A, T, Er, K, Co	8	5	1	1
P, Ca, A, T, Er, Co, SxT, Am	7	4	1	-
P, A, C, Er, K, Co, Am, Ak, S, Na	2	2	-	-
P, Ca, A, T, Co, Am, Ak, S, Na, G	1	1	-	-
P, Ca, A, K, Am, Na, G, C, Cl, Cz	1	1	-	-

A, Ampicillin; AK, Amikacin; Am, Amoxicillin; Ca, Carbenicillin; Cef, Cefixime; CXM, Ceftizoxime; CZ, Ceftriaxone; Cl, Cephalothin; C, Chloramphenicol; Co, Co-trimoxazole; Er, Erythromycin; G, Gentamicin; K, Kanamycin; Na, Nalidixic acid; P, Penicillin; SxT, Sulfamethoxazole-Trimethoprim; S, Streptomycin; T, Tetracycline.

Transfer of antimicrobial resistance determinants and antimicrobial sensitivity testing

Of the 21 S. boydii strains, the plasmids of 18 strains were processed for transformation into *E. coli* HB101 separately for ampicillin (MIC-100 µg/ml), chloramphenicol (MIC-100 µg/ml) and sulfamethoxazole-trimethoprim (MIC-100 µg/ml), plasmids of 11 strains (61.1 %) for only ampicillin, 9 (50.0 %) for chloramphenicol, and 8 (44.4%) for sulfamethoxazole-trimethoprim resistance. Of the 18 transformations, 15 (83.3 %) were successfully accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim. Plasmids of three strains (no. BSb-721, BSb-785 and BSb-7004) were successfully transferred to *E. coli* Hb101 shown by the acquisition of resistance to ampicillin, and plasmids of another three strains (no. BSb-736, BSb-7003 and BSb-7008) with chloramphenicol resistance were also successfully introduced into *E. coli* HB101. Plasmids of 11 strains resistant to ampicillin, 9 strains resistant to chloramphenicol, and 8 strains resistant to sulfamethoxazole-trimethoprim were also successfully introduced into *E. coli* HB101.

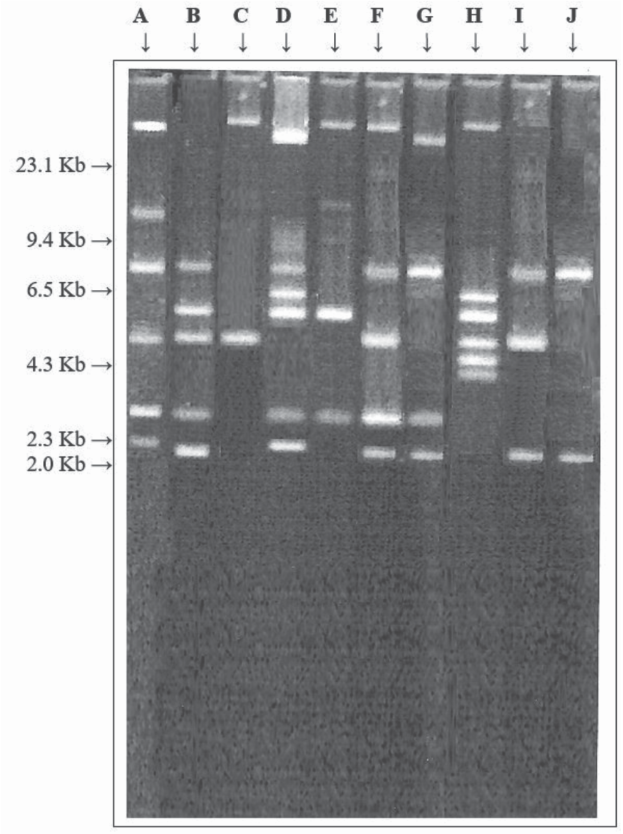

Fig. 1. Plasmid profile of representative *S. boydii* strains isolated from fecal samples of patients with gastroenteritis in Azad Kashmir. (Lane A, marker 9 DNA cut with*Hind* III; Lane B, BSb-721; Lane C, BSb-736; Lane D, BSb-741; Lane E, BSb-756; Lane F, BSb-785; Lane G, BSb-796; Lane H, BSb-7003; Lane I, BSb-7004; Lane J, BSb-7008). (B, author's name; Sb, *S. boydii*; digits, sample number).

In some multiple plasmid strains (no. BSb-741, BSb-756 and BSb-796), all the DNA bands of different molecular sizes were cut out of the gel, extracted, purified and then successfully transferred to *E. coli* HB101 individually. The plasmids (23.1 Kb and <23.1 Kb) could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *E. coli* HB101.

Table III. Transformation of plasmids of *S. boydii* into *E. coli* Hb101.

Sample no.	No. of plasmids	Molecular weight of plasmids which were individually transferred to *E. coli* HB101.	Transformed plasmids that conferred antibiotic resistance
741	6	<23.1Kb, 6.5Kb, <6.5Kb, >4.3Kb,2.3Kb,2.0Kb	<23.1Kb.
756	4	23.1Kb,9.4Kb,>4.3Kb,2.3Kb	23.1Kb.
796	4	<23.1Kb,6.5Kb,2.3Kb,2.0Kb	<23.1Kb

DISCUSSION

Shigella is a leading cause of shigellosis (bacillary dysentery) worldwide and a major cause of diarrhoeal disease in developed and developing countries (Soebel *et al.*, 1998). Antimicrobial resistance is now recognized as an increasingly global problem (Tenvor and Hughes, 1996). Resistance to commonly-prescribed antibiotics is an expanding global problem and has been observed in both developed and developing countries. Because of introduction of new antimicrobial agents is usually followed sooner or later by emergence of bacterial resistance to these drugs (Patwary, 1994). The problem of changing resistance patterns in *S. boydii* will remain an ongoing threat for both developed and developing countries. In the present study, it was noted that among the *Shigella* spp. isolated, (12.1%) isolates were *S. boydii* strains.

Shigellosis is primarily a childhood disease in both developed and developing countries, whereas epidemic shigellosis affects all age groups including Pakistan (Keusch and Bennish, 1991; Ahmed and Shakoori, 1996). However, the information about the etiology and drug sensitivity pattern of bacterial strains is lacking due to the lack of diagnostic facilities. In this study, *S. boydii* decreased from (12.0 %) in 1994 to (11.9 %) in 1995, (11.0 %) in 1996 but again increased up to (11.5 %) in 1997 and the highest number (13.6 %) was recovered in 1998. The highest proportion of stool specimens infected with *S. boydii* were in the age group of >50-60 years (27.3 %). The lowest infestation was observed in the age group >0-5 years (9.5 %). Almost similar results were reported by Ahmad *et al.* (2003) who recorded shigellosis in all age groups, but slightly higher in the age groups of >10-20 and 20-30 years. Khalil *et al.* (1998) reported highest infestation of *Shigella* in the age groups of 18-23 and 24-35 years. Similarly, Bhattacharya *et al.* (2005) reported that the majority (79%) of *Shigella* species were isolated from children aged less than five years in a recent study in Eastern Nepal.

In this study, *S. boydii* isolates were resistant to penicillin followed by tetracycline, erythromycin, ampicillin, ceftizoxime, carbenicillin, kanamycin, co-trimoxazole sulfamethoxazole-trimethoprim, amikacin, amoxicillin, streptomycin, gentamicin, chloramphenicol, cephalothin, nalidixic acid, and ceftriaxone. All *S. boydii* isolates were sensitive to cefixime, ciprofloxacin and enoxacin. However, the resistance to these commonly used antibiotics, especially tetracycline has already been documented from several parts of the world including Pakistan (Ahmed *et al*., 2003; Ahmed and Shakoori, 2001) and may reflect widespread overuse of the antibiotics especially, tetracycline. Analogous results were presented in a previous study by Dutta *et al.* (2002) who reported serovars of *S. dysenteriae* and *S. boydii* from Kolkata. Most of the *Shigella* isolates were multidrug resistance (MDR) i.e., resistant to antimicrobials like ampicillin, chloramphenicol, tetracycline, nalidixic acid and co-trimoxazole, amoxicillin. Ansaruzzaman *et al.* (2005) in a study in Bangladesh observed that the isolates were all susceptible to ampicillin, sulfamethoxazole-trimethoprim, nalidixic acid, ciprofloxacin and mecillinam but eight exhibited resistance to tetracycline.

The MICs of twenty antibiotics against one hundred and ten strains of*S. flexneri* are shown in a comparative account of the antibiotics resistance of isolates at four levels 25µg/ml, 50µg/ml, 100µg/ml and 300µg/ml in Table I. Generally, the isolates showed the highest frequency of resistance against penicillin at all the four levels. The lowest frequency of resistance was against ceftriaxone at all the four levels of antibiotics screened. At 100µg/ml level the isolates showed a considerable decrease in the resistance frequency of almost all the antibiotics tested. MDR was observed in this study ranging from three to ten drugs. Out of one hundred and ten isolates, screened for antibiotic resistance, 31% were resistant to three or more antibiotics at 25µg/ml, 26% were resistant to three or more antibiotics at 50µg/ml, 11% were resistant to three or more antibiotics at 100µg/ml and 4% were resistant to three or more antibiotics at 300µg/ml (Ahmed and Shakoori, 1996). Ahmad and Shakoori (1996) reported highest frequency of resistance against septran at 50 and 100µg/ml. Chloramphenicol resistance was 88.8%. In a recent study in Pakistan Ahmad and Shakoori (2001) documented

50% resistance of *Shigella* strains and Ahmad *et al*. (2003) reported 14.3% resistance of *Shigella* strains against chloramphenicol in Northern Areas of Pakistan. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was PCaA at all the four levels. Analogous results were reported by other investigators in many countries including Pakistan (Ahmed *et al*., 2003; Ahmed and Shakoori, 2001).

Bacteria have evolved numerous strategies for resisting the action of antibiotics and antibacterial agents. In many hospital units, exploitation of antibiotics is very intensive and this generates an enormous selective pressure for bacteria to acquire the means by which they may become antibiotic-resistant. Resistance to a particular agent may be accomplished by more than one resistance mechanism (Haribage *et al*., 2003). Multiple drug resistance in *Shigella* has complicated the situation in recent years (Gosh and Sehgal, 1998). The genes for resistance to ampicillin, chloramphenicol, spectinomycin, and tetracycline formed a linkage group located on the chromosome of the strains of all serotypes (Casalino *et al*., 1994). The drug resistance in bacterial population is may be due to a genetic and non-genetic mechanism. Regarding genetic mechanism most drug resistant microbes emerged as a result of genetic changes and subsequent processes by antimicrobial drugs. The drug resistance may be chromosomal DNA or plasmid DNA mediated. The plasmid mediated drug resistance is caused due to the presence of drug-resistant gene(s) harboring on the plasmid DNA. These gene(s) confer the drug resistance phenomenon in the host organism (Meyers *et al*., 1976).

In view of the overall high incidence of multiple drug resistance (MDR) among the *S. boydii*, the possibility of presence of R-plasmid was explored. This study revealed that (28.0 %) isolates of *S. boydii* carried plasmids. These were found resistant to three or more antibiotics used in this research work. The number of plasmids varied from one to seven. The number of plasmids varied from one to seven. *Shigella* species usually harbor a heterogeneous population of plasmids ranging in number from 2 to as many as 10 (Ansaruzzaman *et al*, 2005). In this study the analysis of plasmid DNA revealed that all the strains contained a heterogeneous population of plasmids ranging between 23.1 kb to 2.0 kb. Based on molecular weight, the pattern of different plasmids was also very diverse. Depending on the number of plasmids, individual strains were grouped into nine different plasmid patterns and were found among (MDR) *S. boydii* strains. These results are comparable with the results of a previous study by Ansaruzzaman *et al.* (2005) where they observed the multiple plasmids of different molecular sizes, of which the most common were 140, 3.4, 2.7 and 1.4 MDa. Two Bangladeshi isolates contained additional 1.2 and 1.6 MDa plasmids, and one of these isolates harbored a further plasmid of approximately 62 MDa. Antibiotic resistance did not correlate with the presence of any particular plasmid. The results of our study are also comparable with the results of Farshad *et al*. (2006) who observed that all the *Shigella* spp. Isolated from Iran harbored multiple plasmids, with an average of 9.5 plasmids (range, 5 to 14 plasmids) in each isolate of all strains and a mean of 10 plasmids in each isolate of *S. boydii*. The sizes of the plasmids from among all isolates ranged from 1 to 21 kb. Plasmids of 2 to 3 kb were the most frequently detected and were seen in about 96.34% of the isolates, while plasmids of 15 kb were detected in only 2.43% of all isolates. Similar results were reported in a previous study by Gebre-Yohannes and Drasar (1997) who showed that patterns of small plasmids of less than 15 kb, were similar within each of the individual *S. boydii* serotypes. Plasmids of about 3·3–3·7 kb were found in all strains of serotypes 2 and 4. Plasmids of about 4·3–4·6 kb were found in about 86% of strains. Serotypes 1, 2 and 3 were characterized by plasmids of about 5·6–5·7 kb. The 6·4·6–7 kb plasmid was found consistently in serotypes 1, 2, 3, 5, 8, 12 and 13 which were resistant to SSu or had an SSu resistance component in their phenotypes. Large plasmids (155–186 kb) were found in most S. boydii strains. Although pathogenicity tests are not used as criteria for the classification of members of the Enterobacteriaceae, the invasiveness of the isolates in the Sereny test, the presence of a 140 MDa plasmid (Sansonetti *et al*., 1982) and *Shigella* enterotoxin 2 (sen) gene and ipaH gene associated with invasiveness of the strains provide additional evidence that these isolates are representative of *Shigella.* The large plasmid was found to contain a gene conferring virulence, the large plasmid was very unstable and easily lost, only a small number of strains of *Shigella* species were found to have the large virulence plasmid (Vargas *et al*., 1999). In addition, Ahamed and Giri (2021) reported that 97% *Shigella* species harbored at least one plasmid. The number of plasmids varied from 1 to 9. The continuing emergence of drug resistant *Shigella* is narrowing considerably the efficacy of commonly used antibiotics in the treatment of shigellosis (Ahmad and Shakoori, 2001). Ahamed and Giri (2021) have shown that Shigella develops resistance through plasmid mediated quinolone resistance gene (*PMQR*) and quinolone resistance determining region (*QRDR*), efflux pumps gene (*gyrA, gyrB, ParC, ParE,* gyrase, topoisomerase IV) and mutations in drug binding regions.

The plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera (Sherley *et al*., 2004). In the present report, the plasmids of (MDR) S. boydii strains, were processed for transformation into *Escherichia coli* HB101 separately for ampicillin (MIC-100 µg/ml), chloramphenicol (MIC-100 µg/ml) and sulfamethoxazole-trimethoprim (MIC-100 µg/ml). The transformations of (83.3 %) were successfully accomplished as *Escherichia coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim. The plasmids (23.1 Kb and <23.1 Kb) could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *Escherichia coli* HB101. Similar results were observed where the 646–7 kb plasmid was found consistently in S. boydii which were resistant to SSu or had an SSu resistance component in their phenotypes (Ghosh and Sehgal, 1998). The conjugative drug resistance plasmids, most often coding for three or less drugs, were found in about 26% of drug resistant strains. R-factors, coding for AT resistance (in types 2 and 8), and ASSuT resistance (in type 4), were compatible with all reference plasmids tested. Plasmids belonging to incompatibility groups X and N were found in serotypes 5 and 10, respectively. To date there is no broadly available vaccine against *Shigella,* but several candidates are being evaluated in preclinical and clinical studies (Raso *et al*., 2023).

CONCLUSIONS AND RECOMMENDATIONS

Since the main route of transmission of shigellosis is through water, food and also person-to-person contact, the prevention and control strategies essentially include provision of safe water supply and adequate sanitation facilities, maintenance of good personal hygiene and food safety. Hand washing with plenty of water and soap is the most important single effective preventive strategy against shigellosis. It is emphasized that hands should be washed before eating, before feeding children, after defecation and after disposal of children's excreta. These measures are further reinforced in epidemic situations, the stringent control measures need to be instituted through simple but effective health education messages to the common masses.

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IRB approval and ethical statement

Ethical issues including plagiarism, informed consent, double publication and/or submission, redundancy etc have been completely observed by the authors.

The ethical approval for his study was taken from Institutional Review Board, University of the Punjab before sample collection. The informed consent forms were signed by the patient or close relative of the patient for current procedure.

Generative AI or AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Phylogeographic Insights and Genetic Divergence of *Trogoderma granarium*: A Comprehensive Analysis Using Consensus and Concatenated Gene Trees

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ABSTRACT

The presence of the globally devastating agricultural pest *Trogoderma granarium* (Khapra beetle) in Pakistan poses a serious threat to food security and stored grains, especially wheat. Wheat is a crucial economic and nutritional staple for the citizens of Pakistan. Effective management of this beetle relies on its accurate identification, as it closely resembles other Dermestidae species. Therefore, precise identification and understanding of the interspecific and intraspecific relatedness of the pest are essential for its management. This report focuses on the accurate detection and phylogenetic analysis of *T. granarium.* Molecular markers are the best tools for diagnosing and analyzing the phylogeny of the pest in any life phase and condition. For this purpose, partial sequences of two mitochondrial genes, 16S rDNA and cytochrome oxidase I, were used. Samples for this study were collected from various areas of Punjab, Pakistan. Phylogenetic trees, constructed from individual and concatenated gene sequences using neighbor-joining and maximum likelihood algorithms, indicate that this pest has a monophyletic origin.

Key words
***Trogoderma granarium*, Stored grain pest, Phylogenetic analysis, Concatenated, DNA, 16SrDNA, Cytochrome oxidase**

INTRODUCTION

Addressing global food security in the twenty-first century requires multi-pronged approaches. It is crucial to identify and enumerate the major contributors to annual yield losses. Among these factors, post-harvest losses caused by various infestations account for up to one-fifth of the total yield (Pimentel, 2019). The potential for such infestation is manifold when the pest in question is invasive. Invasive pests can survive in new habitats (Mack *et al*., 2000). The khapra beetle, *Trogoderma granarium* Everts, is one such invasive pest with a significant impact on stored grain losses. Its destructive activity is due to several inherent characteristics.

The khapra beetle has 142 known hosts (Athanassiou *et al*., 2019), an expansive temperature range (EPPO, 2013), a wide geographic range, the ability to outcome other species (Kavallieratos *et al*, 2017) and the abilities to undergo diapause (Banks, 1977). These abilities make pest management strategies challenging Pakistan is an agrarian economy where wheat is the main cereal staple for domestic use and export. The country, part of the Indian peninsula, is the origin of the khapra beetle (Stibick, 2007) and has a tropical climate conducive to its proliferation.

An outbreak of this beetle can cause significant losses to food security and exports. Pakistan ranks fourth in Khapra beetle interception frequency during shipment pretreatments by the US (Athanassiou *et al*., 2019), following India, Saudi Arabia, and Sudan, which is alarming. In addition to its destructive capabilities, the Khapra beetle is challenging to identify. Expertise is required to identify individuals at the adult or larval stages to the species level of the genus *Trogoderma*, sometimes necessitating dissection to inspect genitals (Castalanelli *et al*., 2011).

Available identification keys are often limited to specific habitats or developmental stages and do not cover

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all species of the genus *Trogoderma* (EPPO, 2013). The lack of accurate characterization can lead to the failure of pest management strategies (Mack *et al*., 2000). Therefore, developing new characterization strategies is more critical than developing new management strategies. Molecular techniques offer an alternative for identification with options including nuclear DNA or mitochondrial DNA. The *16S rDNA* and cytochrome oxidase subunit I (*CO1*) genes from the mitochondrial genome evolve at a higher rate than other mitochondrial genes (Hwang and Kim, 1999). This study has utilized the two molecular markers for characterization of Khapra beetle populations in Pakistan.

MATERIALS AND METHODS

Sampling and rearing of beetles

The Khapra beetle is prevalent across Punjab, Pakistan. Seventeen population samples were collected from various wheat warehouses in districts including Gujranwala, Okara, Sialkot, Lahore, Dera Ghazi Khan, Kasur, Shakargarh, Lodhran, Shaikhupura, KahnaNau, Sahiwala, Khushab, Gujrat, Multan, Jhang, Faisalabad and Layyah of Punjab province. A cluster sampling technique was used, visiting both government-run and privately owned warehouses. Beetle samples were collected in labeled plastic zipper bags with wheat grains and brought to the *Trogoderma* culture room at the Institute of Zoology, University of the Punjab, Lahore for further culturing.

The beetles were reared in the laboratory according to the pre-established protocol (Riaz *et al*., 2014). Fifth instar larvae of third generation were processed for isolation of DNA and amplification of 16S rRNA and cytochrome oxidase 1 (*CO1*) genes.

DNA extraction

The CTAB method of Doyle and Doyle (1990) was used with minor modifications for isolation of DNA. Larvae were homogenized in mixture of CTAB buffer (pH 7.8) and 2-mercaptoethanol (5:1) incubated at 65°C for thirty min followed by cooling at room temperature for ten min. A chloroform: iso-amyl alcohol mixture (24:1) was then added to the tubes, which were centrifuged at 11,000 rpm for 10 min. The supernatant from each tube was transferred to sterile eppendorfs to which chilled absolute ethanol was then added for precipitation of DNA. The tubes were then centrifuged at 14,000 rpm for 10 min. The supernatant was discarded, and 70% ethanol was added to each tube. After another centrifugation at 14,000 rpm for 3 min, the supernatant was discarded. The resulting pellet was dried at 60 °C for 30 min in an incubator. Finally, 10 µl of nuclease-free water was added to each tube, and the DNA pellet was dissolved with gentle tapping.

Amplification of 16S rRNA *and mitochondrial* CO1 *genes*

Once extracted, the DNA was amplified using species-specific mitochondrial 16S rDNA and cytochrome oxidase 1 (*CO1*) gene primers (Olson *et al*., 2014).

16S rDNA
Forward = 5'CTAAAATTGAAAATTTCTATACT3'
Reverse = 5'CTAGCCTGCTCCCTGATTGA3'.
CO1
Forward = 5'CAACATTTATTTTGATTTTTTGG3'
Reverse = 5'TCCAATGCACTAATCTGCCATATTA3'

Each DNA sample was subjected to PCR amplification in a 50 µl reaction mixture. Each PCR tube contained 5.0 µl of dNTPs (2.0 mM), 1.0 µl of 100 nM forward primer, 1.0 µl of 100 nM reverse primer, 4.0 µl of template DNA, 5.0 µl of 10X Taq buffer, 3.0 µl of 25 mM magnesium chloride, and 0.50 µl of Taq polymerase (Thermo Scientific cat# 00855243). The volume was adjusted to 50 µl with 30.50 µl of sterilized distilled water.

The PCR cycles (Bio Rad T100) included an initial denaturation at 95°C for fifteen min, followed by forty cycles of denaturation at 94°C for one minute, annealing at 42°C for one minute, and extension at 72°C for one minute, with a final extension at 72°C for five min. The amplified product was separated on a 2% agarose gel (Weal Tec MD-20) and sequenced by Macrogen, Korea.

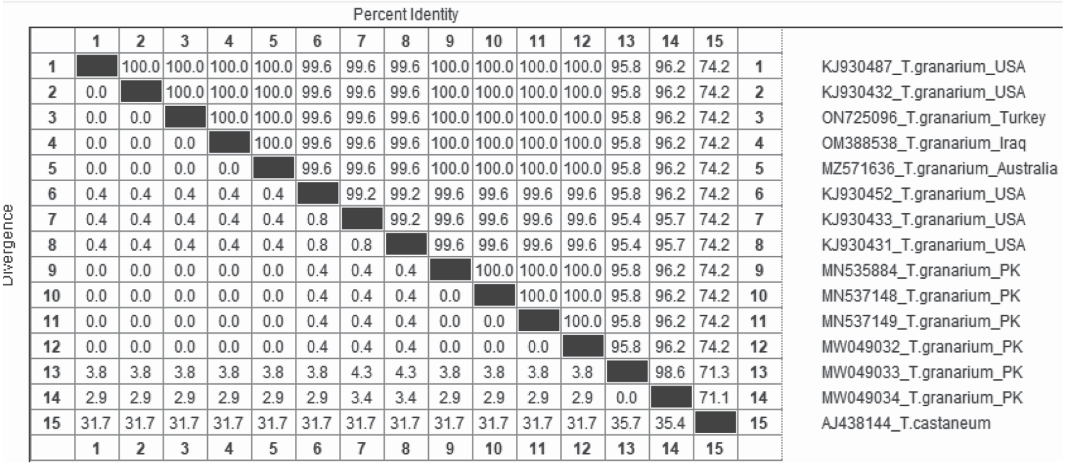
The 250bp PCR product amplified from 16S rDNA of Khapra beetle populations collected from Gujranwala (TR1), Okara (TR2), Sialkot (TR3), Lahore (TR4), Dera Ghazi Khan (TR5), and Layyah (TR6) were deposited in GenBank with accession number MN535884, MN537148, MN537149, MW049034, MW049033 and MW049032, respectively. Likewise the 800bp PCR product amplified from mitochondrial *CO1* gene of Khapra beetle populations of Gujranwala (IR-01), Okara (IR-02), Sialkot (IR-03), Lahore (IR-04), Dera Ghazi Khan (IR-05), Mughalpura (IR-06), Kasur (IR-07), Shakargarh (IR-08), Lodhran (IR-09), Khairpur (IR-10), Layyah (IR-11), Shaikhupura (IR-12), Manawa (IR-13), Wassan Pura (IR-14), Kahna Nau (IR-15), Khairpur (IR-16) and Layyah (IR-17) were deposited in GenBank with accession number OP341260, OP345940, OP346112, OP346574, OP349051, OP349099, OP349101, OP361281, OP352898, OP353625, OP354417, OP361318, OP354510, OP354511, OP354512, OP359420 and OP360011, respectively.

DNA sequence analysis

The sequences obtained from Macrogen were edited using DNASTAR Lasergene 7v7.1.0 software and aligned using the built-in version of CLUSTAL W (Thompson *et al*., 1994). Phylogenetic relationships were analyzed by constructing a phylogenetic tree using the neighbor-joining method (Tamura *et al*., 2013) on phylogeny.fr, funded by Reseau National des Genopoles (RNO). The percentage identity and divergence between the study group sequences and published sequences were calculated using genetic distance values from MagAlign, a DNASTAR software project (version 7v7.1.0).

RESULTS

Figures 1and 2 show the percentage identity of the consensus tree of *16S rDNA* and *MCOI* genes, respectively. Highlighted sequences in the gene tree represent the individuals of interest. The phylogenetic analysis of

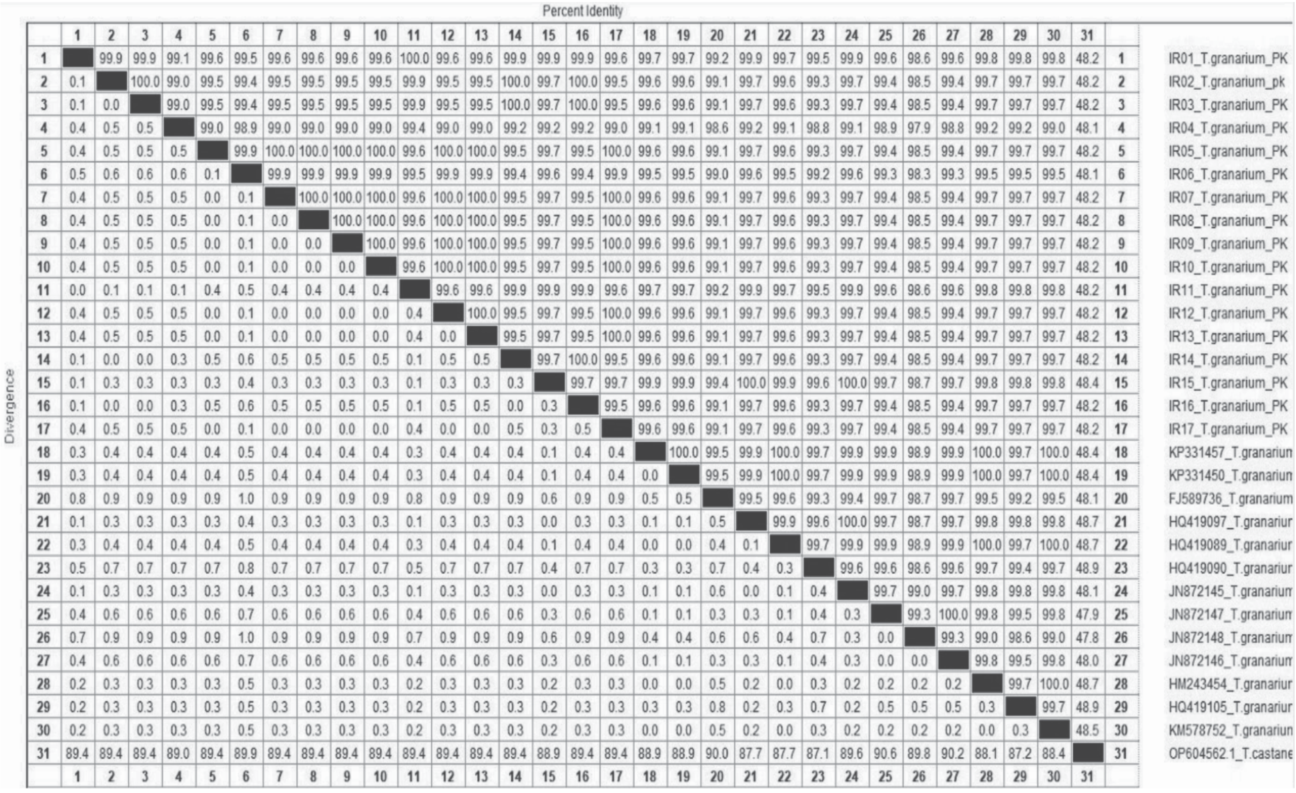


Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1	■	100.0	100.0	100.0	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	1	KJ930487_T.granarium_USA
2	0.0	■	100.0	100.0	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	2	KJ930432_T.granarium_USA
3	0.0	0.0	■	100.0	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	3	ON725096_T.granarium_Turkey
4	0.0	0.0	0.0	■	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	4	OM388538_T.granarium_Iraq
5	0.0	0.0	0.0	0.0	■	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	5	MZ571636_T.granarium_Australia
6	0.4	0.4	0.4	0.4	0.4	■	99.2	99.2	99.6	99.6	99.6	99.6	95.8	96.2	74.2	6	KJ930452_T.granarium_USA
7	0.4	0.4	0.4	0.4	0.4	0.8	■	99.2	99.6	99.6	99.6	99.6	95.4	95.7	74.2	7	KJ930433_T.granarium_USA
8	0.4	0.4	0.4	0.4	0.4	0.8	0.8	■	99.6	99.6	99.6	99.6	95.4	95.7	74.2	8	KJ930431_T.granarium_USA
9	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	■	100.0	100.0	100.0	95.8	96.2	74.2	9	MN535884_T.granarium_PK
10	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0	■	100.0	100.0	95.8	96.2	74.2	10	MN537148_T.granarium_PK
11	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0	0.0	■	100.0	95.8	96.2	74.2	11	MN537149_T.granarium_PK
12	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0	0.0	0.0	■	95.8	96.2	74.2	12	MW049032_T.granarium_PK
13	3.8	3.8	3.8	3.8	3.8	3.8	4.3	4.3	3.8	3.8	3.8	3.8	■	98.6	71.3	13	MW049033_T.granarium_PK
14	2.9	2.9	2.9	2.9	2.9	2.9	3.4	3.4	2.9	2.9	2.9	2.9	0.0	■	71.1	14	MW049034_T.granarium_PK
15	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	35.7	35.4	■	15	AJ438144_T.castaneum
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Fig. 1. Table showing the divergence and percent identity of *16S rDNA* genes.



Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31		
1	■	99.9	99.9	99.1	99.6	99.5	99.6	99.6	99.6	99.6	100.0	99.6	99.6	99.9	99.9	99.9	99.6	99.7	99.7	99.2	99.9	99.7	99.5	99.9	99.6	98.6	99.6	99.8	99.8	99.8	48.2	1	IR01_T.granarium_PK
2	0.1	■	100.0	99.0	99.5	99.4	99.5	99.5	99.5	99.5	99.9	99.5	99.5	100.0	99.7	100.0	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	2	IR02_T.granarium_pk
3	0.1	0.0	■	99.0	99.5	99.4	99.5	99.5	99.5	99.5	99.9	99.5	99.5	100.0	99.7	100.0	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	3	IR03_T.granarium_PK
4	0.4	0.5	0.5	■	99.0	98.9	99.0	99.0	99.0	99.0	99.4	99.0	99.0	99.2	99.2	99.2	99.0	99.1	99.1	98.6	99.2	99.1	98.8	99.1	98.9	97.9	98.8	99.2	99.2	99.0	48.1	4	IR04_T.granarium_PK
5	0.4	0.5	0.5	0.5	■	99.9	100.0	100.0	100.0	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	5	IR05_T.granarium_PK
6	0.5	0.6	0.6	0.6	0.1	■	99.9	99.9	99.9	99.9	99.5	99.9	99.9	99.4	99.6	99.4	99.9	99.5	99.5	99.0	99.6	99.5	99.2	99.6	99.3	98.3	99.3	99.5	99.5	99.5	48.1	6	IR06_T.granarium_PK
7	0.4	0.5	0.5	0.5	0.0	0.1	■	100.0	100.0	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	7	IR07_T.granarium_PK
8	0.4	0.5	0.5	0.5	0.0	0.1	0.0	■	100.0	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	8	IR08_T.granarium_PK
9	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	■	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	9	IR09_T.granarium_PK
10	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	■	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	10	IR10_T.granarium_PK
11	0.0	0.1	0.1	0.1	0.4	0.5	0.4	0.4	0.4	0.4	■	99.6	99.6	99.9	99.9	99.9	99.6	99.7	99.7	99.2	99.9	99.7	99.5	99.9	99.6	98.6	99.6	99.8	99.8	99.8	48.2	11	IR11_T.granarium_PK
12	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.4	■	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	12	IR12_T.granarium_PK
13	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.4	0.0	■	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	13	IR13_T.granarium_PK
14	0.1	0.0	0.0	0.3	0.5	0.6	0.5	0.5	0.5	0.5	0.1	0.5	0.5	■	99.7	100.0	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	14	IR14_T.granarium_PK
15	0.1	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.3	0.3	0.3	■	99.7	99.7	99.9	99.9	99.4	100.0	99.9	99.6	100.0	99.7	98.7	99.7	99.8	99.8	99.8	48.4	15	IR15_T.granarium_PK
16	0.1	0.0	0.0	0.3	0.5	0.6	0.5	0.5	0.5	0.5	0.1	0.5	0.5	0.0	0.3	■	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	16	IR16_T.granarium_PK
17	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.5	0.3	0.5	■	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	17	IR17_T.granarium_PK
18	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.1	0.4	0.4	■	100.0	99.5	99.9	100.0	99.7	99.9	99.9	98.9	99.9	100.0	99.7	100.0	48.4	18	KP331457_T.granariun
19	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.1	0.4	0.4	0.0	■	99.5	99.9	100.0	99.7	99.9	99.9	98.9	99.9	100.0	99.7	100.0	48.4	19	KP331450_T.granariun
20	0.8	0.9	0.9	0.9	0.9	1.0	0.9	0.9	0.9	0.9	0.8	0.9	0.9	0.9	0.6	0.9	0.9	0.5	0.5	■	99.5	99.6	99.3	99.4	99.7	98.7	99.7	99.5	99.2	99.5	48.1	20	FJ589736_T.granarium
21	0.1	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.3	0.3	0.3	0.0	0.3	0.3	0.1	0.1	0.5	■	99.9	99.6	100.0	99.7	98.7	99.7	99.8	99.8	99.8	48.7	21	HQ419097_T.granariur
22	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.1	0.4	0.4	0.0	0.0	0.4	0.1	■	99.7	99.9	99.9	98.9	99.9	100.0	99.7	100.0	48.7	22	HQ419089_T.granariur
23	0.5	0.7	0.7	0.7	0.7	0.8	0.7	0.7	0.7	0.7	0.5	0.7	0.7	0.7	0.4	0.7	0.7	0.3	0.3	0.7	0.4	0.3	■	99.6	99.6	98.6	99.6	99.7	99.4	99.7	48.9	23	HQ419090_T.granariur
24	0.1	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.3	0.3	0.3	0.0	0.3	0.3	0.1	0.1	0.6	0.0	0.1	0.4	■	99.7	99.0	99.7	99.8	99.8	99.8	48.1	24	JN872145_T.granariurr
25	0.4	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.4	0.6	0.6	0.6	0.3	0.6	0.6	0.1	0.1	0.3	0.3	0.1	0.4	0.3	■	99.3	100.0	99.8	99.5	99.8	47.9	25	JN872147_T.granariurr
26	0.7	0.9	0.9	0.9	0.9	1.0	0.9	0.9	0.9	0.9	0.7	0.9	0.9	0.9	0.6	0.9	0.9	0.4	0.4	0.6	0.6	0.4	0.7	0.3	0.0	■	99.3	99.0	98.6	99.0	47.8	26	JN872148_T.granariurr
27	0.4	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.4	0.6	0.6	0.6	0.3	0.6	0.6	0.1	0.1	0.3	0.3	0.1	0.4	0.3	0.0	0.0	■	99.8	99.5	99.8	48.0	27	JN872146_T.granariurr
28	0.2	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.0	0.0	0.5	0.2	0.0	0.3	0.2	0.2	0.2	0.2	■	99.7	100.0	48.7	28	HM243454_T.granariur
29	0.2	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.8	0.2	0.3	0.7	0.2	0.5	0.5	0.5	0.3	■	99.7	48.9	29	HQ419105_T.granariur
30	0.2	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.0	0.0	0.5	0.2	0.0	0.3	0.2	0.2	0.2	0.2	0.0	0.3	■	48.5	30	KM578752_T.granariun
31	89.4	89.4	89.4	89.0	89.4	89.9	89.4	89.4	89.4	89.4	89.4	89.4	89.4	89.4	88.9	89.4	89.4	88.9	88.9	90.0	87.7	87.7	87.1	89.6	90.6	89.8	90.2	88.1	87.2	88.4	■	31	OP604562.1_T.castane
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31		

Fig. 2. Table showing the divergence and percent identity of *MCOI* genes.

consensus and concatenated trees indicates that *T. granarium* individuals are monophyletic in origin (Figs. 3 4). However, the consensus trees (Figs. 3, 4) for *16S rDNA* sequences showed some differences. The NJ consensus tree revealed a separate clade for MW049033 and MW049034, while the ML consensus tree did not show this clade. Similarly, a separate clade for 16S sequences was observed in the NJ concatenated tree. The percent identity table (Fig. 2) showed no significant divergence among the reported and GenBank sequences for the *MCOI* gene. However, the percent identity table (Fig. 1) showed no prominent divergence among MN535884, MN537148, MN537149, and MW049032. In contrast, MW049033 and MW049034 showed divergences of 3.8% to 4.3% and 2.9% to 3.4%, respectively, for the *16S rDNA* gene. Therefore, the individuals in the current study are closely related to each other and to individuals reported worldwide, except for the 16S rDNA sequences of MW049033 and MW049034.

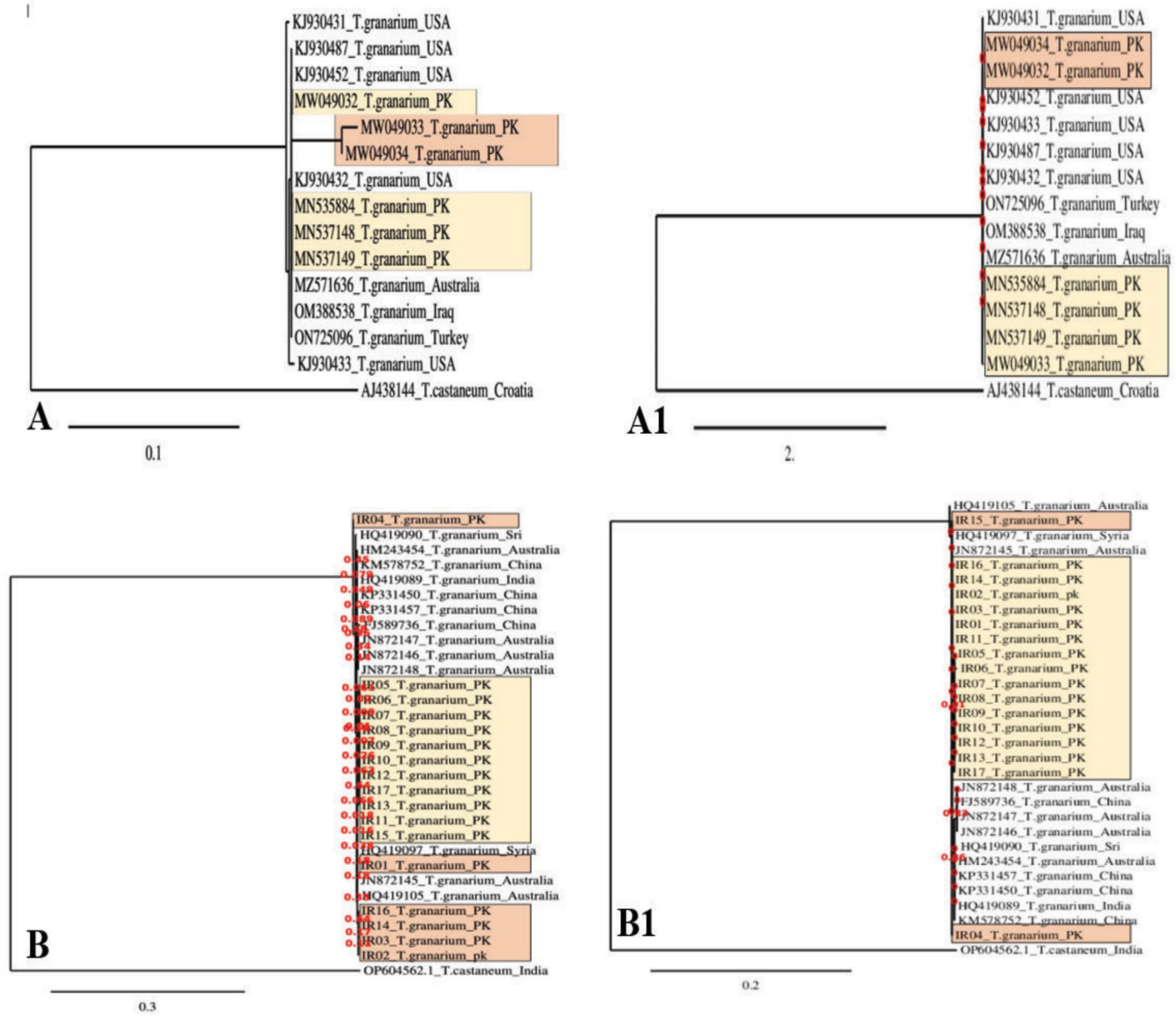


Fig. 3. The evolutionary history of *T. granarium* traced using neighbour joining and maximum likelihood methods based on 16S and *MCOI* sequences. Highlighted boxes represent the sequences reported from current study. Phylogenetic tree constructed through neighbour joining method (A) and maximum likelihood methods (A1), respectively 250 bp *16S rDNA* gene indicates the clustering of species at one node showing minimum divergence among the organisms (B, B1). Phylogenetic tree constructed through neighbour joining method (B) and maximum likelihood method (B1) of 800 bp MCOI indicates the clustering of species at one node showing minimum divergence among the individuals. OP604562.1 *T. castaneum* was used an outgroup.

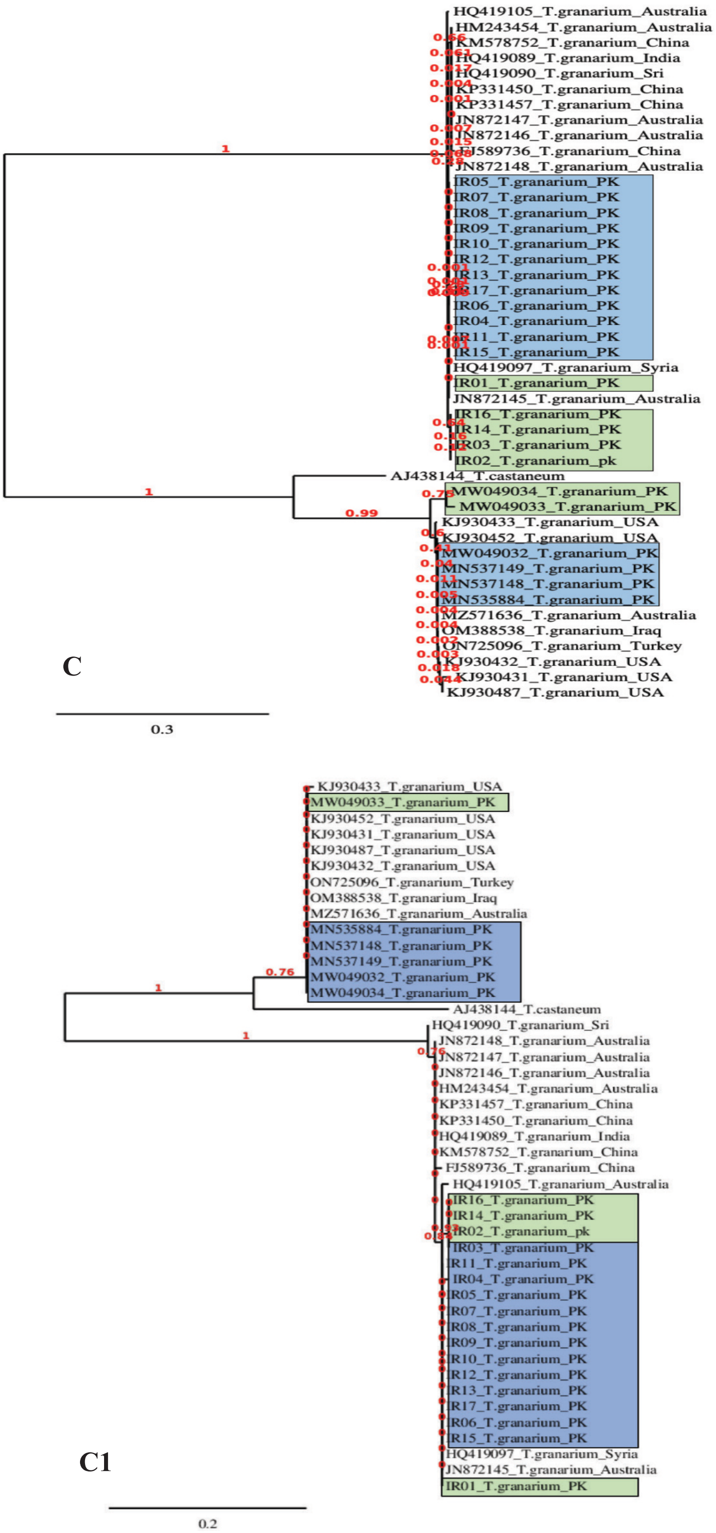


Fig. 4. Phylogenetic tree based on concatenation of *16S rDNA, MCOI* sequences of 23 isolates with 21 reference strains. The sequences were aligned using Neighbour Joining methods (C) and maximum (C1) likelihood methods. AJ438144 *T. castaneum* was used as an outgroup.

DISCUSSION

The utility of mitochondrial markers (16S and MCOI) for khapra beetle identification

Morphological keys are geographically restricted, of limited use without a verified voucher list, and challenging to identify without deep expertise because of the nuances in morphological landmarks, further complicating identification (Beal, 2003). However, studies on other invasive insect species has shown that molecular markers can provide useful information about population structure, gene flow, and dispersal pathways (Mikac and Clarke, 2006). This, in turn, helps establish the existence of cryptic species and provides insight into species dispersal (Loxdale and Lushai, 1998; Mikac and FitzSimmons, 2010; Mikac and Clarke, 2006). However, it is not possible to identify all animal species with universal molecular assays (Adam and Palmer, 2003; Damgraad, 2008; Dentinger *et al*., 2011). Olson *et al*. (2014) described that *CO1* and *16S rDNA* gene sequences from mitochondria can be diagnostic for *T. granarium. T. granarium* species associated with grain stores can be confirmed as khapra beetle by sequence data alone (Olson *et al*., 2014). Therefore, for this study, specific primers of *16S rDNA* and *MCO1* genes were used to analyze the collected species of the pest.

Phylogenetic diagnosis

Phylogenetic analysis of *16S rDNA* and *MCO1* genes provides a definitive means for diagnosing *T. granarium,* especially in cases of PCR products suspected of primer mis-annealing or contamination. Current study analysis shows that *T. granarium* collected from different locations in Punjab are monophyletic. In Pakistan, no work on the phylogenetic relationships of *T. granarium* has been reported yet. Thus, we take the opportunity to comment on the phylogeny of the insect pest and found that the individuals from Punjab are closely related and likely come from the same population.

CONCLUSION

This study provides a basis for both accurate molecular identification and divergence analysis of *T. granarium* based on partial DNA sequences of mitochondrial *16S rDNA* and *CO1* genes. Molecular data from other khapra populations across this part of the globe may help integrate a bigger picture.

DECLARATION

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Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Review Article

Understanding PRRS: Epidemiology, Pathogenesis, and Histopathology in the Context of Global Swine Health

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Key words
Porcine Reproductive and Respiratory Syndrome (PRRS), PRRS virus (PRRSV), Arterivirus, Swine health, Epidemiology of PRRS, High fever disease in pigs, Viral transmission mechanisms, PRRS vaccination challenges, Swine disease control

ABSTRACT

The disease that caused reproductive and respiratory problems in pigs was once known as "mystery swine disease" before being renamed "porcine reproductive and respiratory syndrome, (PRRS)" in the United States and Europe. In the early 1990s, the PRRS virus (PRRSV), an arterivirus, was discovered to be the disease's causative agent. Numerous studies have been carried out since then. Since spreading to Vietnam and Cambodia, "porcine high fever disease", PRRSV continues to cause severe illness in pigs in China as of 2010. This illness was first reported in 2006, when PRRSV was recognized as a critical virus linked to a high rate of mortality and morbidity (20%). The complete method of infection transmission is still unknown despite a great deal of research on epidemiology regarding the PRRS. This paper provides a brief historical summary of PRRS and the associated PRRSV. It lists the current objectives for eliminating or reducing PRRS as well as the areas whereby research is currently missing and preventing the creation of effective vaccines. It is hoped that this discussion will stimulate additional collaboration between researchers and swine veterinarians around the world to develop solutions that advance our knowledge of PRRS and PRRSV in an effort to eradicate this economically important disease.

INTRODUCTION

A well-known and economically important swine disease, porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in pregnant sows or respiratory tract distress in nursing pigs (Stadejek *et al*., 2002; Grebennikova *et al*., 2004). In the mid-1980s, the condition was first recognized as "mystery swine disease" or "blue ear disease" in the United States (Wensvoort, 1993). The PRRS virus (PRRSV), the causative agent, was first discovered in the US in 1987 (Wensvoort *et al*., 1991; Albina, 1997; Tian *et al*., 2007). Later, in the early 1990s, it was discovered throughout Asia and Europe (Murakami *et al*., 1994; Han *et al*., 2014). PRRS causes significant financial losses to the world's pig output, especially in enormous manufacturing systems (Young *et al*., 2021). In the USA alone, PRRS is thought to cost the pork sector roughly US$ 560 million in damages every year. A disease known as "porcine high fever syndrome (PHFS)" first appeared in the People's Republic of China in 2006 and quickly spread throughout the nation, inflicting extremely serious illness in pigs with severe fever (40–42 °C). The disease's characteristics included substantial death rates in suckling piglets, infants, and growers, as well as miscarriages in sows across all age groups. Pigs with PHFS were used to isolate PRRSVs in several Chinese facilities. The illness was linked to an unusually virulent strain of the PRRSVs, according to a later genomic and pathogenicity examination of those viruses. Highly pathogenic PRRS is the new term for the illness brought on by this novel variant strain (Neumann *et al*., 2005; Li *et al*., 2007; Tian *et al*., 2007; Zhou *et al*., 2008, 2009; King *et al*., 2017). Thus, in 2006, highly pathogenic PRRS (HP-PRRS) first appeared in China and has subsequently expanded to Southeast Asian nations. It has seriously harmed the region's pig productivity and put a strain on pig farmers (Zhou *et al*., 2008). To lower the financial losses brought on by this illness and stop it from spreading to other parts of the world, suitable control measures must be developed and put into place. This requires a thorough understanding of the disease's characteristics, the virus, and its epidemiology. In

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order to lay the groundwork for the creation of practical and effective disease management strategies, the goal of this study is to review the data currently available on HP-PRRS.

CHARACTERISTICS OF THE HP-PRRSV

Parkinson's disease is caused by an RNA virus that is a member of the genera Arterivirus, family Arteriviridae, order Nidovirales (Grebennikova *et al*., 2004) Genotype I, which represents the prototype Lelystad virus, which is the type of virus that is most common in Europe, and genotype II, represented by VR 2332, the prototype strain that was initially identified in North America, are two closely related but genetically and antigenically different strains. The genetic and antigenic makeup of these early isolates differed greatly from one another. Their genetic makeups are less than 70% similar. Disease control is constantly threatened by genetic and antigenic variability among isolates, even within a country. There are at least eight open-reading frames (ORFs) in the virus's 15–15.5 kb genome, which collectively encode over 20 complete proteins. The HP-PRRSV is an altered strain of the PRRSV linked to genotype II (North American genotype) of the virus, according to a genetic sequence study (Stadejek *et al*., 2002). Phylogenetic examination of the *ORF5* gene suggests that the HP-PRRSV may have descended from two of the PRRSVs that were previously discovered in China. Further analysis of a nonstructural protein (NSP2) revealed a trait unique to the HP-PRRSV: The discontinuous deletion of 30 protein amino acids (at locations 482 and 534-562) (Zhou *et al*., 2009). This loss is proposed as a genetic feature of the HP-PRRSV, even though it has been shown that the deletions are not the sources of the virus's increased virulence.

EPIDEMIOLOGY OF HP-PRRS VIRUS

Up to 70% of animals in Southeast Asia and China are still kept in traditional backyard and small-scale settings (Darith, 2017). This results in the proximity of production systems with varying sizes and levels of biosecurity, which are crucial factors that can influence the potential for disease spread and the impact that diseases may have on pig populations. Given that over 70% of pig farmers are smallholders, one of the main issues that persists is livestock producers' lack of awareness and comprehension of the advantages of disease control. Commercial pig farmers are aware of the advantages of disease control, but their understanding of the prerequisites for a cooperative national disease monitoring program is limited. The conventional farrow-to-finish methods are typically used by medium- and small-scale pig producers, frequently shows that the Figure 1 with very close age group mixing. direction of spread is more significant and more intense from sow farms to other farms.

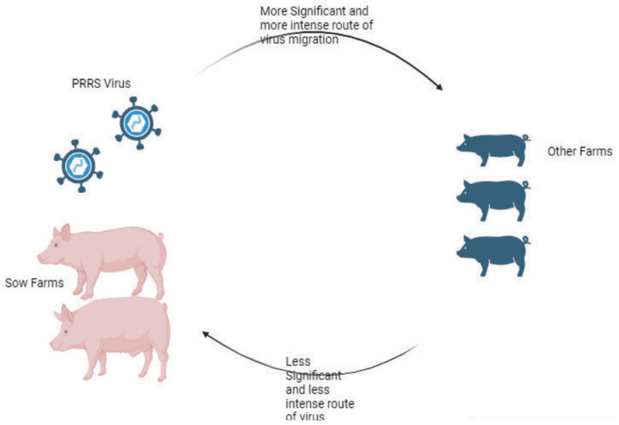

Fig. 1. Illustration of PRRSV transmission dynamics between sow farms and other pig farms. The figure highlights that virus migration from sow farms to other farms is a more significant and intense route compared to the reverse direction, which is less significant and less intense.

Replacement stock frequently originates from a range of places, is of unknown health, and is not properly quarantined before arrival. When it comes to interactions between farm workers and pigs outside of their farms, or between strangers and pigs housed on the farms, hygiene application is typically very inadequate or nonexistent. Vaccination is typically used to reduce disease in small-scale hog farming. Without proper supervision or veterinary advice, the use of large concentrations of antibiotic and antibacterial agent combinations is rather prevalent. Pork, which has been consumed at the highest rates about the entire amount of meat produced in various countries (Vietnam, China, Cambodia, as well as the Philippines), is one of the most important agricultural goods in the region. Commercial pig production rises in response to rising pork consumption. According to statistics, the main places where HP-PRRS has been spreading are those with larger populations of pigs. The Red River Delta in Vietnam is the primary region for heavy pig production in the country's north and is also a major hub for pig diseases, such as the rise of highly PRRS.

Live pigs, including piglets, fatteners, and finishers, are traded from this location to the southern region of Vietnam. The quick dissemination of PRRSV is likewise consistent with and probably explains pig migrations in these value chains. The illness may potentially be spreading between neighboring nations due to the transportation of HP-PRRS-infected pigs, including deceased pigs (Nilubol *et al*., 2012). The trans-boundary spread of HP-PRRSV from southern China to South-East Asia strongly implies the existence of bio-security lapses, such as the inability to regulate animal movements and border trade between neighboring nations. The spread of viruses within a nation also demonstrates the inadequacy of biosecurity measures, mostly due to unchecked human mobility in highly polluted regions, particularly at loading zones and slaughterhouses. Sharing such contaminated places with other cars could lead to a more community-wide spread of the virus.

Age of the pigs at the time of the infection

Klinge *et al*. (2009) showed that viraemias in 3-week-old pigs were noticeably longer than those in finishers or grownup pigs, regardless of the PRRSV isolate that was utilized as the inoculum. Similarly, 2-month-old pigs had much higher viral burdens within their lymph nodes the lungs, alongside tracheobronchial swabs over 6-month-old animals, regardless of the challenge strain's pathogenicity (Cho *et al*, 2006; Klinge *et al*, 2009). Additionally, Thanawongnuwech *et al*. (1998) showed that pulmonary macrophages from 4-week-old pigs generated higher viral titers than those from 4-months old pigs. All of the prior data provide indirect evidence that piglets are more pathogenic than finishers or older pigs, notwithstanding the lack of a precise evaluation. In farrow-to-finish farms, this can be important for controlling the illness, particularly in light of the potential for sows or newborn pigs from nurseries to become infected again.

HISTOPATHOLOGY OF PRRS

The porcine reproductive and respiratory syndrome virus is the root cause of PRRS. The indications of PRRS include interstitial pneumonia, thickened alveolar septa, and suppurative bronchopneumonia, with suppurative bronchopneumonia and proliferative and necrotizing pneumonia also observed, especially with virulent strains.

Lung lesions

Interlobular setae become thickened due to edema and proteinaceous exudates. Necrosis, degeneration can be seen in the lumen of bronchioles. Hyperplasia and hypertrophy can be seen. Interstitial pneumonia with vasculitis is's consistent finding (Wills *et al*., 1997). Figure 2 shows interstitial pneumonia and hyperplasia.

Lymphoid organs

Severe lymphoid depletion results in the formation of cavities in the germinal centers of lymphoid follicles. Figure 3 shows thrombus formation, congestion, and hemorrhages. There may also be fibrin deposition also (Hopper *et al*., 1992; Albina, 1997).

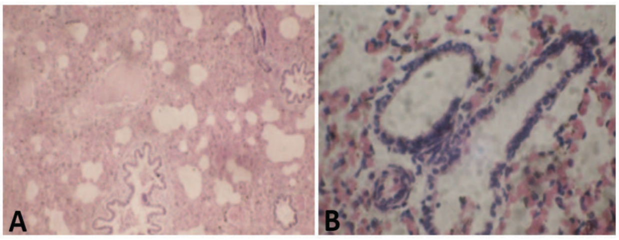

Fig. 2. A, Histopathological section of pig lung showing multifocal interstitial pneumonia with alveolar septal thickening and infiltration of inflammatory cells, characteristic of PRRSV infection. B, Higher magnification of affected lung tissue illustrating peribronchiolar lymphoid hyperplasia and bronchiolar epithelial damage, commonly observed in PRRSV-infected pigs. Stain: H and E stain. Magnification: A, 10x; B, 40x).

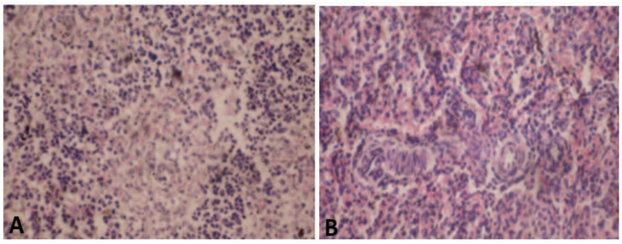

Fig. 3. A, Microscopic view of lymphoid tissue reveals prominent interfollicular vascular congestion. B, diffuse monocyte infiltration, indicating an active inflammatory response. Stain: H and E, Magnification: 40x.

Vascular lesions

Skin lesions consist of small round macules and papules and irregular patches; distributed to hindquarters, thorax, abdomen and margins of ears. Necrotizing and leukocytoclastic vasculitis of small blood vessels was shows microscopically, Figure 4 observed within the dermis. lymphocytic infiltration and lymphadenopathy can be seen (Hoff and Vandevelde, 1981; Kelly, 1995).

Reproductive lesions

Gross lesions in the umbilical cords varied, ranging from 1 to 2 cm segmental hemorrhagic regions to a full cord involvement that was enlarged and bled profusely. Gross cord lesions in virally infected fetuses were associated with necrotizing umbilical arteritis with per arterial bleeding, as demonstrated by a histopathologic study. In some experimentally infected sows, several foci of lymphoplasmacytic inflammation have been seen in the myometrium. Myometritis, endometritis, and placentitis of the mother placenta were noted in sows that were infected both naturally and in an experiment (Christianson *et al*., 1992). Figure 5A, B show moderate multifocal and perivascular lymphoplasmacytic inflammation along with interstitial edema (Stockhofe-Zurwieden *et al*., 1993).

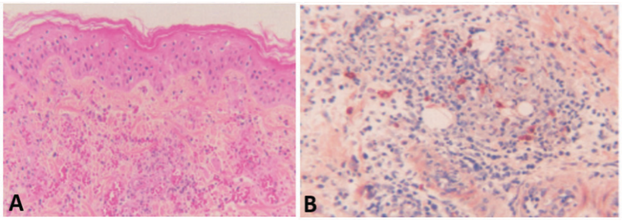

Fig. 4. A, Microscopic image reveals multifocal hemorrhages within the dermis along with perivascular. B, interstitial infiltration of lymphocytes, indicating an ongoing inflammatory process. Stain: H and E, Magnification: 40x.

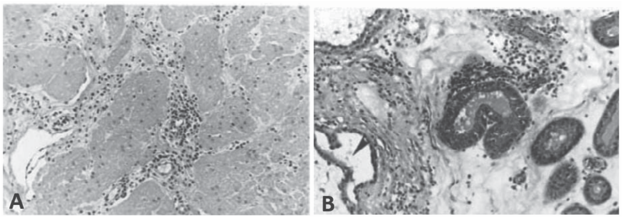

Fig. 5. A, Histopathological section showing moderate, multifocal inflammatory infiltrates scattered throughout the tissue. B, Perivascular accumulation of lymphocytes and plasma cells (lymphoplasmacytic inflammation), suggestive of a chronic immune response. Stain: Hematoxylin and Eosin. Magnification: .

Cellular infiltrates

A characteristic lesion of interstitial pneumonia is mononuclear cell infiltration of alveolar walls with normal airway epithelium, particularly in newborn pigs experiencing respiratory distress. Comparably, lymphoplasmacytic inflammation and edema development are evident (Guo *et al*., 2016).

Comparison between PRRS strains

In this investigation, three strains of porcine reproductive and respiratory syndrome virus-1 suffered segregation from pigs that tested negative for PRRS using porcine alveolar macrophages (PAM) cells. In 1993, strain 18794 was isolated from a pig in Denmark. In 2009 saw the isolation of strain ILI6 from Russian weaner pig lung tissue. Strain BOR59 was identified in 2009 from the lung tissue of a Belarusian swine that had died from respiratory disease symptoms.

In the current investigation, the virulence of two distinct strains of PRRSV-1 subtype 2 and an example of PRRSV-1 subtype 1 strain were evaluated. Four groups of eight-week-old pathogen-free pigs were set up and given fake inoculations, subtype 1 strain 18794, subtype 2 strain ILI6, or subtype 2 strain BOR59.

Overall, the outcomes revealed that the BOR59 strain is extremely pathogenic, although the severity of infection of the other subtype 2 strain, ILI6, was in the middle zone of that of the BOR59 and subtype 1 strains (Oleksiewicz *et al*., 1998; Stadejek *et al*., 2017).

Impact of immunopathology

The immunopathology of PRRS is complex and involves an overactive immune response, cytokine imbalance, and tissue damage, leading to the various disease manifestations (Albina *et al*., 1998). The immunopathology of PRRS significantly impacts both the pig industry and animal health. Some of the impacts are given below.

1. Reproductive failure: When pregnant sows are infected with the PRRS virus, it can cause abortion, stillbirth, and underweight piglets.
2. Respiratory illness: Pneumonia brought on by a PRRS virus infection in piglets and growing pigs can lower growth rates and raise mortality.
3. Immunological system suppression: The PRRS virus has the ability to lower immunological function, which increases pigs' susceptibility to recurrent infections.
4. Enhanced vulnerability to other illnesses: Porcine circovirus-associated disease (PCVAD) is more common in pigs infected with PRRS.

Histopathological findings in vaccinated animals

1. The testis and epididymis' adluminal compartments contain both mononuclear and multinuclear giant cells (Fig. 6A, B).
2. Damage and inflammation in the epididymis and testes (Fig. 6C, D).
3. Seminiferous epithelial cells are desquamated.
4. Apoptosis of bystander cells.
5. Lung alveolar edema and interstitial pneumonia.
6. Histiocytic proliferation and lymphoid depletion in the lymph nodes.
7. Multinucleated giant cells seen in lymph nodes and lungs.
8. Damage and inflammation in other organs, like the spleen and liver.
9. A histological investigation revealed luteinizing cysts formed in the ovaries instead of lesions of endometritis or myometritis. The vaccinated animals

had a decreased prevalence of these cysts. The development of an ovarian cyst impairs the ability to reproduce, leading to prolonged anestrus and complete infertility (Ziecik *et al*., 2023).

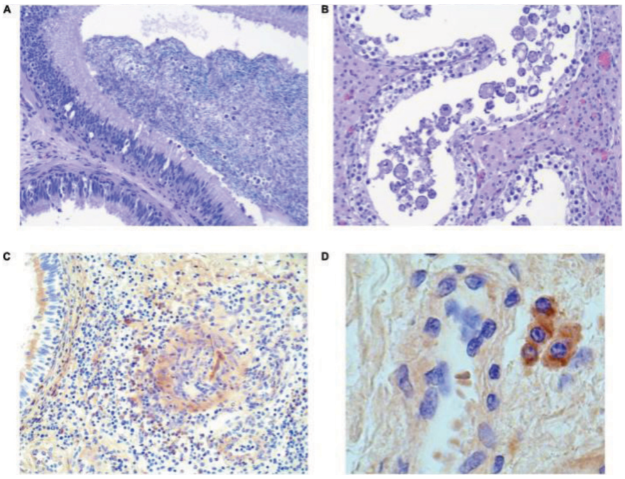

Fig. 6. A, Histopathological section showing the invasion of the epithelium by intraluminal mononuclear cells, indicating an inflammatory response. B, Enlarged and swollen cells present within the lumen, suggesting a pathological alteration in cellular morphology. C, Focally scattered macrophages within the testicular interstitium, reflecting localized immune activity. D, Macrophages dispersed in the interstitium of the epididymis, indicative of chronic inflammation. Stain: H and E. Magnification: 40x.

Diagnostic utility of histopathology in PRRS

1. Verification of infection: By detecting distinctive lesions and viral antigens in tissues, histopathology can verify the presence of PRRS virus infection.
2. Evaluation of disease severity: Histopathology can assess the degree of lesions linked to PRRS.
3. Distinguishing PRRS from other diseases: Histopathology can differentiate PRRS from other reproductive and respiratory conditions, allowing for a precise diagnosis.
4. Identification of secondary infections: Histopathology can detect secondary bacterial or viral infections, which often complicate PRRS cases.
5. Monitoring vaccine efficacy: Histopathology can evaluate the effectiveness of PRRS vaccines by examining lesions and viral antigens in vaccinated animals (Sarli *et al*., 2021).

Future directions in PRRS histopathology research

Immunohistochemically staining: Enhancing detection of PRRSV antigen and immune cell characterization in tissues.

Digital pathology: Implementing digital tools for image analysis and automated scoring of histopathological lesions.

Comparative pathology: Investigating PRRS lesions in different animal models and species.

Vaccine efficacy evaluation: Assessing the impact of various vaccines on PRRS lesions and immune responses (Renukaradhya *et al*., 2015).

Pathogenesis and transmission of PRRSV

The PRRS, commonly known as blue ear pig disease. It is a condition that can affect pigs and is caused by the virus betaarterivirus suid 1. This economically significant panzootic disease results in respiratory tract sickness in young pigs. Betaarterivirus sud 1 is an unclassified virus within the realm Riboviria, belonging to the kingdom Orthornavirae, phylum Pisuviricota, and class Pisoniviricetes. It is part of the order Nidovirales and falls under the family Arteriviridae. At the genus level, it is classified as Betaarterivirus, with the subgenus Eurpobartervirus. The full species name is Betaarterivirus sud 1 (Ma *et al*., 2018).

According to a 2021 study by Derald Holtkamp of Iowa State University, PRRS harms the US pork sector by $664 million annually. Based on data recently compiled by Pipestone Management Company, PRRS costs their system roughly $200 per sow year.

TRANSMISSION OF PRRSV

Zimmerman *et al.* (1997) reported that transmission of PRRSV occurred in pigs through direct or indirect contact. It can also be transmitted through inhalation.

The experiment was performed to detect PRRSV in muscle (longissimus dorsi), lymphoid tissue, and serum. 135 pigs (49 negative controls and 89 PRRSV-injected pigs). Thirteen out of eighty-nine (14.6%) muscle samples tested positive for qRT-PCR between 28 and 202 days following inoculation. Within these 13, PRRSV was recovered from three out of thirteen lymphoid tissue samples and four out of thirteen suited serum samples. Experiments were also performed to check the transmissibility of PRRSV through meat. No meat transmission was observed (Raymond *et al*., 2017).

Target cells and tissues

Many experiments were performed to detect the target cells and tissues of PRRSV, the virus was inoculated and detected at 3, 14, 21, 35 days of post-inoculation (DPI). On day 3 post inoculation, PRRSV replication was found in retropharyngeal lymph nodes, bronchial lymph nodes, tonsils, spleen, alveolar macrophages, and thoracic aortic lymph nodes. On day 14 post inoculation, the same tissues were PRRSV positive, except for the thoracic aortic and retropharyngeal lymph nodes. On 35 DPI, lung and alveolar macrophage PRRSV positivity was seen. The heart, bone marrow cells, and peripheral blood mononuclear cells did not contain PRRSV. During the acute stage of infection, PRRSV primarily replicates in lymphoid tissue and lung macrophages and remains in lung macrophages (Christianson *et al*., 1993).

Impact on reproduction and respiratory system

PRRSV can be transmitted horizontally or vertically. Horizontally, it is transmitted through body secretions such as semen. Abortion can occur at any stage of gestation (Lee *et al*., 2004). The virus has a direct effect on the conceptus and follows transplacental infection. Sometimes, gestation becomes prolonged and fetuses are infected transplacentally and often die in utero. Fetal circulation is also affected. Segmental hemorrhages in the umbilical cord can be seen (Lager and Halbur, 1996), along with cellular infiltration and cellular changes in the antibodies produced.

Lungs fail to collapse, and they exhibit distinct dark, mottled patches of pneumonia (Pallarés *et al*., 2002). Acute pneumonia is brought on by the acute cytocidal replication of PRRSV in alveolar lung macrophages. Expiration was more influential than inspiration. Lowering the compliance of the airways, decreased lung CO-transfer factor, and peripheral airway blockage were discovered while rebreathing test gases (He, CO) and using impulse oscillometry for non-invasive pulmonary function testing (Klein and Reinhold, 2001; Klein *et al*., 2004).

VIRAL REPLICATION

Basic research focuses on the process by which the virus enters its host cell, which is a critical first stage in the infection. Porcine alveolar macrophages (PAMs) are the primary target cells of PRRSV, which has a limited cell tropism. Target cell's restricted cell tropism is caused by the presence of particular entry mediators in the target cell. The virus's DNA is bound, internalized, and released by a number of cellular components, including heparin sulphate, CD163, porcine sialoadhesin (pSn), non-muscle myosin heavy chain 9 (MYH9), vitremin, CD151, and CD209 (DC-SIGN) (Van Breedam *et al*., 2010; Veit *et al*., 2014).

Mechanisms

PRRSV enters macrophages through receptor-mediated mechanisms, first binding to heparin sulfate glycosaminoglycans on the cell surface (Fig. 7). Then there is an increase in integration with pSn. Clathrin-mediated endocytosis identifies pSn as an internalization receptor. After that, the genome is released into the cytoplasm. CD163, a scavenger receptor cysteine rich (SRCR) for Hb clearance, is the most selective receptor for invasion and infection development. The viral glycoproteins GP2 and GP4 also bind to CD163. CD163 is essential for viral removal of coating and genetic release in conjunction with cellular proteases, such as trypsin-like serine proteases, cathepsin E, and aspartic proteases (Yu *et al*., 2019; An *et al*., 2020). The genome is used as a template that is translated to pp1 and pp1ab. Proteases hydrolyze polyproteins into mature non-structural proteins. The two essential components of nsps; nsp9 and nsp10 are assembled by the the transcription and replication complex of viruses. Making use of an interrupted transcription method, the RTC first produces both full-length and subgenome (sg)-length minus strands. The latter serve as templates for the production of plus-strand sg mRNAs, which are required for the expression of the structural protein genes found in the genome's 3´-proximal quarter (Gao *et al*., 2019).

Assembling and releasing the virion is the final step. Novel RNA genomes are bundled into nucleocapsids, which after budding from smooth internal membranes converted into enveloped virions by structural proteins. The new virions are then exocytosed from the cell (Snijder *et al*., 2013; Lunney *et al*., 2016).

Systemic effects

Most specifically, reproductive and respiratory systems are affected but other lymph organs and tissues also get disrupted. There are lesions present along with cellular infiltrates. PRRSV was also found in retropharyngeal lymph nodes, bronchial lymph nodes, tonsils, spleen, alveolar macrophages, and thoracic aortic lymph nodes.

Host factors influencing pathogenesis

Age

Nursery pigs are more susceptible to PRRSV than adults. Pulmonary intravascular macrophages in young pigs are more virulent to infection than those of adult pigs (Cho *et al*., 2006). Age has no bearing on the presence of antibody responses to PRRSV. Infection is restricted to cells of monocytic lineage, and fully developed pig alveolar macrophages are the primary target of spontaneous infection (Welch and Calvert, 2010).

Breed

Duroc, Hampshire pigs are susceptible to VR2385, a strain with high pathogenicity of PRRSV (Lee *et al*., 2004). The degree of resistance to PRRSV infection fluctuates

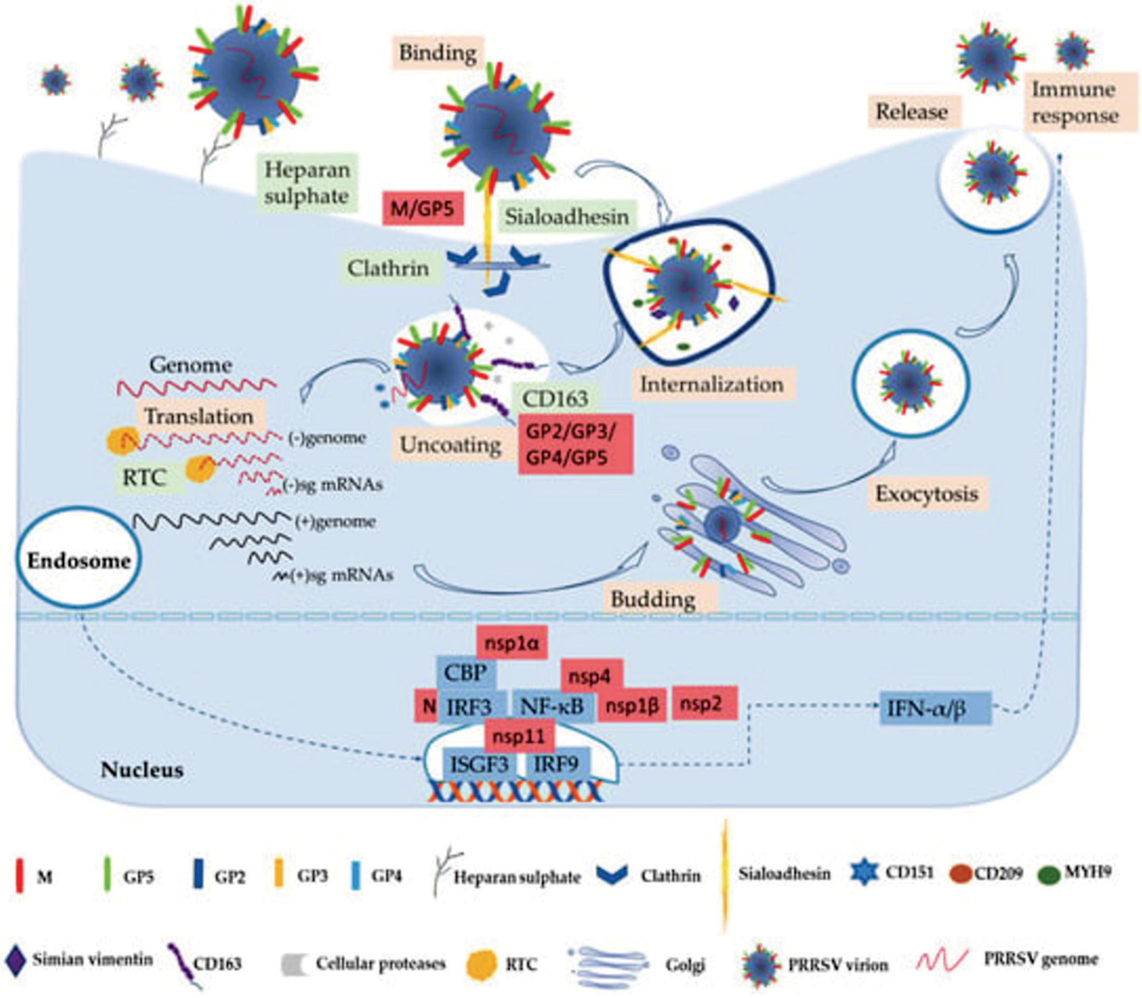

Fig. 7. Schematic representation of the viral entry pathway into the host cell. The diagram illustrates the various steps involved, including virus attachment to the host cell receptor, fusion with the host membrane, and subsequent entry via endocytosis or direct fusion. These processes are essential for viral infection and replication within the host cell (Nan *et al*., 2017).

among sow breeds and strains. Contrary to other breeds, including the Large White (LW), Meishan and Tongcheng (TC) are less prone to disease because of their enhanced ability to resist PRRSV (Vincent *et al*., 2005; You *et al*., 2020).

Other factors

The vulnerability of various pig breeds to PRRSV is influenced by environmental factors, pigs' nutritional and health conditions, the virulence of PRRSV, and other factors (Lunney and Chen, 2010).

Chronic infection and persistence

When PRRSV enters the thymus, it depletes T-cell precursors and modifies the TCR repertoire. Affected thymocytes are developed. The cytotoxic and helper K 2-T cells both exhibit repertoire diversification restriction. As a consequence, infection becomes prolonged and vital viral epitopes are sustained. Developed antibodies recognize virus but can't neutralize it.

Studies have shown that high levels of virus are detected in serum than the swab. Virus concentration decreases but is not eliminated from blood bloodstream and mucosal areas till three weeks have passed.

PRRSV diminishes K 2-T cell clones necessary for neutralizing epitope identification and impairs the thymus's developing K 2-T cell repertoire. As an outcome, the repertoire lacks certain T helpers needed for particular B cells to generate high-affinity antibodies capable of neutralizing PRRSV. It will lead the virus to persist longer (Fig. 8) (Duinhof *et al*., 2011; Robinson *et al*., 2018).

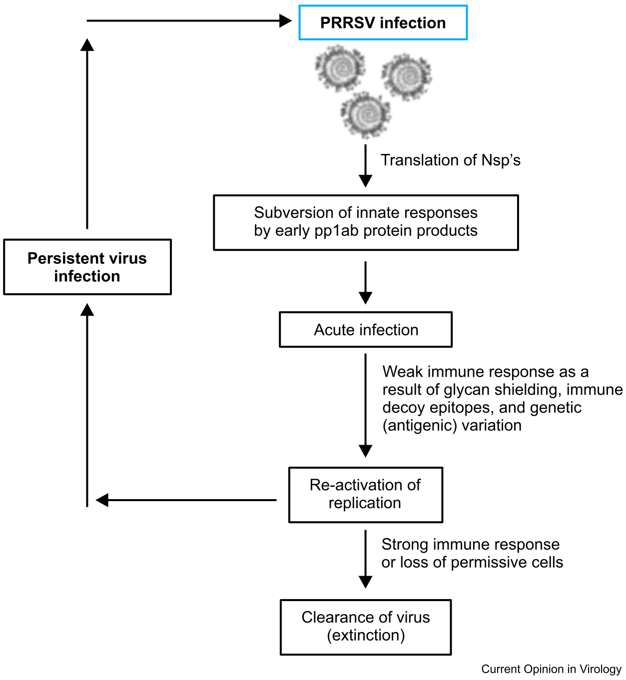

Fig. 8. Flow sheet diagram showing the persistence of virus in host cell (Ostrowski *et al*., 2002).

Overall pathogenesis

Primary replication takes place in lymphoid tissues when the virus has been transmitted to the tonsils or upper respiratory system. The next step is viremia, which might last for a few weeks. The spleen, thymus, tonsils, lymph nodes, and Peyer's patches are among the lymphoid tissues that the virus likes to infect. It spreads to pulmonary alveolar and intravascular macrophages and causes interstitial pneumonia. It causes abortion in late gestation by crossing the placenta.

CONTROL AND PREVENTION OF PRRS

These are the keys to managing PRRS (a) Create and rigorously implement biosecurity measures to stop PRRSV from entering or reentering, (b) Prevent the virus from spreading among or infecting sows in breeding herds. Currently, biosecurity, testing and removal, immunization, and management techniques like whole-herd depopulation/repopulation and herd closure are employed to combat PRRS.

Although there are vaccines available, their efficacy varies. This may be partly caused by the many viral strains that are out there, the viral load that infects the animal after vaccination, and vaccination-related processes or circumstances that influence immune response. As a result, the duration of immunity is unknown. Pig vaccination may be beneficial in herds with PRRS issues or herds at high risk of contracting PRRSV infection, even though it does not prevent PRRSV infection. Generally speaking, the goal of vaccination is to lower clinical losses. A vaccine technique is more affordable for pig farmers than other PRRS management methods, and it is workable for all sizes of pig producers (small, medium, and large). Commercially accessible PRRS vaccines come in two varieties: a live vaccination and a killed viral vaccine. The live vaccine is widely acknowledged for its ability to provide protection against genetically similar PRRS viruses. However, there are issues with its safety, cross-protective effectiveness, and immunogenicity. On the other hand, the PRRS killed vaccination is widely recognized for its safety, albeit its protection is restricted.

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Generative AI and AI-assisted technology statement

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Statement of conflict of interest

The authors have declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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