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Molecular Epidemiology of *Staphylococcus aureus* in Oral and Rectal Swabs from Bats in Pakistan

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Abstract: In Pakistan, bats are one of the dominant mammals that play an important role in the ecosystem in terms of pollination, seed dispersal, and control of pest insects. Bats have also played an important role in the emergence and transmission of zoonotic pathogens; however, most current studies focus on viral pathogens, not potential bacterial pathogens. This study was designed to estimate the prevalence and antibiotic profiling of *Staphylococcus (S.) aureus* in oral and rectal samples from bats captured in northern Pakistan and to determine the factors associated with infection. Two hundred individual bats of five species: *Pipistrellus javanicus* (n = 17), *Pipistrellus pipistrellus* (n = 10), *Rhinopoma microphyllum* (n = 48), *Rousettus leschenaultii* (n = 124), and *Scotophilus kuhlii* (n = 1) were captured for non-lethal collection of oral and rectal samples to isolate *S. aureus*. Bats were sampled from three sites: a natural cave, a man-made castle, and an animal shed, in Khyber Pakhtunkhwa and Punjab provinces. Oral (n = 200) and rectal (n = 200) swabs were collected from each individual bat using sterile cotton swabs specifically for use in bacteriological studies. Each isolate of bacteria was identified by using phenotypic tests and confirmed as *S. aureus* based on PCR assay. Out of a cumulative four hundred samples, 80 swabs were positive for *S. aureus* including 47 rectal and 33 oral swabs. Prevalence of *S. aureus* infection varied significantly among species, with *Rousettus leschenaultii* exhibiting the highest prevalence (n = 77; 37.90%). In addition to bat species, prevalence varied significantly among habitats but not between sex, age class, or reproductive status. This study confirmed the occurrence of *S. aureus* in oral and rectal microbiota of bats in Pakistan. Importantly, *S. aureus* isolates showed resistance to tetracycline, gentamicin, and erythromycin and carried resistant genes such as TetK, TetM, Erma, and aacA-D. In this regard, efforts should be taken to educate the local communities on how to minimize exposure to an antibiotic-resistant bacterial pathogen through contact with bats while simultaneously increasing the awareness of protecting bats as a vital component of our ecosystem.

Keywords: antibiotic resistance; Chiroptera; *Staphylococcus aureus*; microbiota; molecular identification; risk factors

1. Introduction

Pakistan has a high diversity of bat species with 50 recognized species belonging to 26 genera and 8 families [1,2]. There are numerous ecological and epidemiological traits of bats that make them suitable as reservoirs of pathogens [3,4]. Some bat species can be highly mobile which could facilitate the movement of pathogens between populations, such as Australian flying-fox individuals have been observed flying up to 50 km in a night [5,6]. Different bat species have varied social structures, some are solitary roosters and other species roost in colonies of hundreds to thousands of individuals and form mixed species roosts, a potential factor of transmitting pathogens to co-habituating bats. Furthermore, bats occupy a wide range of habitats from caves, woodlands, and forests to house openings, roof trusses, and crevices in urban areas. During the early migration season fruit bats such

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as *Eidolon helvum* often roost by the thousands in trees located in dense urban areas which increases human exposure to the bats' excreta [7–9].

Bats are reservoir hosts to zoonotic and non-zoonotic pathogens such as bacteria, fungi, and viruses [9,10]. However, most research has focused on viruses associated with bats but relatively little research has been conducted on bacterial associations [13]. Previous studies have shown that the nasopharynx and digestive system of fruit bats are probably colonized by the *S. aureus* complex which showed resistance against tetracycline and erythromycin [14]. Members of the *S. aureus* complex include *S. aureus*, *S. argenteus,* and *S. schweitzeri* [15]. In humans, *S. argenteus* can cause skin and soft tissue infections, as well as bacteremia [16,17]. *Staphylococcus schweitzeri*, on the other hand, mostly colonizes non-human primates and bats [14,18]. However, three cases of *S. schweitzeri* infection in humans have been documented, with a suspected zoonotic source [19]. Apart from *Staphylococcus spp.*, one study identified 34 bacterial species isolated from fecal swabs of bats kept in captivity. Most of these bacteria are not known to cause sickness in animals and humans except *Leptospira sp., Bartonella*, and *Barrelia*, of which some may be pathogenic to humans [20]. Some enteric bacteria such as *Shigella sp., Yersinia sp.,* and *Salmonella sp.,* which are causative agents of human and animal diseases that were also isolated from fecal and tissue samples (i.e., kidney, heart, spleen, liver, lung) of different species of bats [19–21].

Some bat species can be a source of multidrug resistance bacteria which pose a risk human and animal health [24–27]. Antimicrobial resistance genes of bacterial species have been isolated from bat guano samples of *Myotis blythii* and *M. myotis* [23], and antibiotic resistance genes were detected in fecal samples of wild and captive grey-headed flying foxes [28]. Antibiotic resistant genes have also been detected in other volant wildlife species [29]. For example, antibiotic resistant genes such as tet(M), erm(A), blaZ, erm(T), fexA, str, eta, and tst were detected in tracheal samples from white storks captured in residential areas [30]. Tetracycline is the drug of choice for the control of certain infections in dairy cattle and poultry, however, excessive use of such widely used antibiotics can result in the development of resistance. The prevalence of antimicrobial resistance (AMR) genes in wildlife species, including bats, could contribute to the epidemiology of what is a major threat to human populations all over the globe. In this circumstance, it is important to study AMR from a 'One Health' perspective that includes the diversity of free-ranging wildlife [29,30].

There are few studies on the occurrence of antibiotic resistant bacteria associated with oral and rectal microbiota in bats, however, such studies are lacking in Pakistan. The present study was conducted using molecular and bacteriological diagnostics to identify the presence and antibiotic resistance profile of bacteria in oral and rectal samples collected from bat species captured in KPK and Punjab regions of Pakistan. We also assess the environmental and demographic factors associated with *S. aureus* prevalence in sampled bat populations.

2. Materials and Methods

2.1. Study area

The present study was conducted at three sites in two different provinces of Pakistan specifically Khyber Pakhtunkhwa and Punjab (Figure 1). Bats were captured and sampled from Malakand caves and a nearby livestock shed in August 2018 and Sheikhupura fort in October 2019. Additional details on the three sampling sites is as follows:

Site I: A cave in district Malakand of Khyber Pakhtunkhwa province (34.4897° N, 71.7978° E), previously used for the mining of chromite but now has become a roosting site for bats after the government banned mining. The cave consisted of five passages which were interconnected mainly by a narrow passage internally. Forty-eight greater mouse-tailed bats (*Rhinopoma microphyllum*) and 42 fulvous fruit bats *(Rousettus leschenaultii*) were captured from this site.

Site II: Bats were captured from the cracks of a dilapidated brick shed traditionally used to house livestock. The shed was in Malakand district of Khyber Pakhtunkhwa province (34.07757 N° E71.83435° E), located near to Site I. We captured 10 common pipistrelles *(Pipistrellus pipistrellus*).

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Site III: Sheikhupura fort dates back to 1607 AD. Sheikhupura fort is a very old multi-story building, and due to its dilapidated condition bats occupied crevices in the brick buildings as their roosting site (31.70813° N, 73.99078° E). We captured 17 Java pipistrellus (*Pipistrellus javanicus)*, 82 fulvous fruit bats *(Rousettus leschenaultii*), and 1 Asiatic lesser yellow house bat *(Scotophilus kuhlii*).

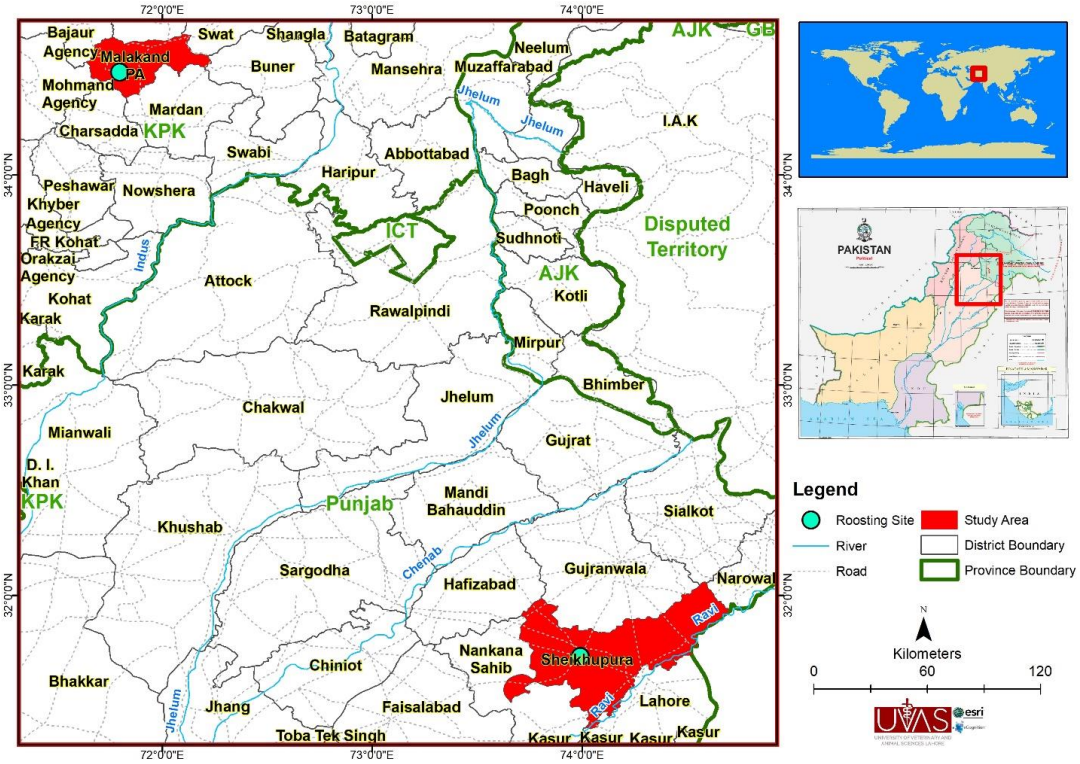

Figure 1. Study area map showing sampling sites in districts Malakand and Sheikhupura in Pakistan.

2.2. Collection of samples and data

Bats were captured (n = 200) by using different types of nets (hand and mist net) and were kept in hygienic cotton bags to prevent cross-contamination. Oral (n = 200) and rectal (n = 200) swabs were collected from each bat using a sterile cotton swab and transferred into an Eppendorf tube (1.5 mL) containing phosphate buffer solution and transported in a dry shipper to the laboratory for bacteriological studies. Bat species, age class, reproductive status, and sex were recorded in addition to the habitat type (natural vs. manmade). All bats were released after non-lethal sample collection.

2.3. Isolation and phenotypic identification of bacteria

Samples were enriched in nutrient broth and incubated at 37°C for 24 hours. After enrichment, samples were streaked on mannitol salt agar and kept at 37°C for 24 hours. Suspected colonies were subculture on tryptic soya agar plates for purification. After 24 hours of incubation, individual *S. aureus* colonies were identified colony morphology, shape, and color. Each isolate was identified using Gram staining, oxidase, catalase, indole, voges Proskauer, and urease tests according to standard procedure [33]. Finally, bacterial isolates were preserved in 50% glycerol till further analysis.

2.4. Molecular confirmation of bacteria

Nucleic acid was extracted from isolated bacteria using the FavorPerp TM DNA extraction mini kit (Favorgen Biotech Crop, Taiwan). Molecular confirmation of *S. aureus* using 16S rRNA gene of 484 base pairs was done [34]. For PCR initial denaturation was done at 94 ºC for 3 min, followed by 35 cycles of amplification with 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C

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for 30 sec. Five microliters of the amplified product were subjected to 1.5% agarose gel having ethidium bromide stain. Gel documentation system was used for the confirmation of PCR products (Gel Doc EZ imager). PCR products were sequenced in one direction only using primer 16S rRNA (1ST BASE, Malaysia) and sequences were checked using BioEdit (Version 7.2.5) and EMBOSS Seqret. New sequences were submitted to GenBank (accession numbers: OP001633- OP001637).

2.5. *Phenotypic and genotypic antibiotic resistance profiling*

After confirmation, *Staphylococcus aureus* isolates were further tested for antibiotic sensitivity against commonly used antimicrobials agents i.e. Tetracycline (30 µg), erythromycin (15 µg), and gentamicin (10 µg) by disc diffusion method [35]. Moreover, antibiotic-resistant genes of tetracycline such as Tetk and TetM were detected with modification as described previously [36]. PCR conditions for the detection of Tetk resistance gene were initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 30 sec, annealing 55°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 4 min for 30 cycles [36]. In the case of TetM gene PCR conditions were initial denaturation at 96°C for 3 min, denaturation at 96°C for 30 sec, annealing at 54°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 4 min for 30 cycles using a thermocycler (T100™ Thermal cycler, BioRad, Singapore). The detection of erythromycin resistance gene (ermA) was done as described by [37] with certain modifications; PCR conditions were initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, elongation 72°C for 30 sec, and final elongation at 72°C for 4 min for 30 cycles. Gentamicin resistance gene (aacA-D) was detected with modifications as described by[38]. PCR conditions were initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, elongation at 72°C for 30 sec, and final elongation 72°C for 4 min for 30 cycles.

2.6. Statistical analysis

The role of biological (bat species, age class, sex, and reproductive condition) and environmental (i.e., habitat type - natural, manmade) factors on the prevalence of *S. aureus* in oral and rectal swabs were analyzed using Chi-square tests. Post hoc pairwise Wilcox tests were performed if a factor had a significant influence on prevalence. A *p-value* less than 0.05 was considered significant. All statistical analyses were conducted using R ver. 4.2.2. with base functions [39]

3. Results

In our study, four hundred (200 oral and 200 rectal) swabs from 5 bat species were collected for isolation of *Staphylococcus aureus*. Overall, fifty-eight (14.5%) swabs were positive for *Staphylococcus aureus* and were found positive for voges proskauer, oxidase, and urease while negative for Gram stain, catalase, and indole test. We sequenced PCR products of the 16S rRNA gene for 5 samples, confirming these isolates were *S. aureus* (Genbank Accession numbers: OP001633- OP001637). All five sequences were identical across the 484 base pairs. The highest prevalence of *Staphylococcus aureus* was isolated from *Scotophilus kuhlii* (n = 1, 100%), followed by *Rousettus leschenaultii* (n = 47, 37.90%), and *Pipistrellus javanicus* (n = 4, 23.52%). Isolates of *Staphylococcus sp.* were recovered more frequently from rectal swabs (n=31/200, 15.50%) vs. oral swabs (n=19/200, 9.50%) of bats. Isolates were recovered from both rectal and oral swabs collected from 8 individual bats (4% of animals). For oral samples, the highest prevalence of *Staphylococcus aureus* was recorded in *Rousettus leschenaultii* (n = 16, 12.90%), followed by *Pipistrellus javanicus* (n = 2, 11.76%) and *Rhinopoma microphyllum* (n = 1, 2.08%), respectively. However, rectal samples positive for *Staphylococcus aureus* were highest in *Scotophilus kuhlii* (n = 1, 100%), followed by *Rousettus leschenaultii* (n = 25, 20.16%) and *Pipistrellus javanicus* (n = 2, 11.76%), respectively. Only two bat species had both swab types positive for *Staphylococcus aureus*, specifically *Rousettus leschenaultii* (n = 6, 4.84%) and *Rhinopoma microphyllum* (n = 2, 4.17%).

Table 1. Prevalence of *Staphylococcus aureus* isolated from oral and rectal swabs from bat species sampled at sites across northern Pakistan.

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Bat species	Swabs collected	*S. aureus* Prevalence (%)		
	Oral/Rectal (no. each)	Oral (95% CI)	Rectal (95% CI)	Total (95% CI)
Pipistrellus javanicus	17/17	11.76 (1.46 – 36.44)	11.76 (1.46 – 36.44)	23.52 (6.81 – 49.89)
Pipistrellus pipistrellus	10/10	0	0	0
Rhinopoma microphyllum	48/48	2.08 (1.31 – 17.20)	10.42 (3.47 – 22.58)	12.5 (4.73 – 25.25)
Rousettus leschenaultii	124/124	12.90 (11.47 – 25.62)	20.16 (17.66 – 33.57)	37.90 (29.35 – 47.05)
Scotophilus kuhlii	1/1	0	100 (2.50 – 100)	100 (2.50 – 100)
Total	200/200	13.50 (9.10 – 19.03)	19.50 (14.25 – 25.68)	29.00 (22.82 – 35.82)

The association between prevalence of *S. aureus* and factors such as sampling site, bat species, age, reproductive status, and sex is given in Table 2. The prevalence of *S. aureus* was significantly different ($p < 0.05$) amongst sampling sites and bat species. Sampling sites were also a contributing factor for the occurrence of *S. aureus* in oral and rectal samples which were collected from bats at roosting sites in caves (23.3%) and castle (37 %) while all bats collected from animal sheds were negative. We observed significant variation in prevalence by bat species: *Rousettus leschenaultii* (35.90 %), followed by *Pipistrellus javanicus* (23.52 %), *Pipistrellus pipistrellus* (0 %), and *Rhinopoma microphyllum* (12.5 %). While only one sample of *Scotophilus kuhlii* was captured and tested positive (Table 2). Male individuals (36.8 %) tended to be positive for *S. aureus* more often than female bats (24.1%), but the trend was not significant. There were no significant differences in infection status based on age class or reproductive status.

Table 2. Identification of biological and environmental factors associated with the prevalence of *Staphylococcus aureus.*

Factors	Variables	No. samples collected	Prevalence (%) (95% CI)	Chi-square χ^2	*p*-value
Site	Castle	100	37.00 (27.57 – 47.24)	8.60	**0.01**
	Cave	90	23.33 (15.06 – 33.43)		
	Animal Shed	10	0		
Species	*Pipistrellus javanicus*	17	23.52 (6.81 – 49.89)	17.90	**< 0.01**
	Pipistrellus pipistrellus	10	0		
	Rhinopoma microphyllum	48	12.5 (4.73 – 25.25)		
	Rousettus leschenaultii	124	37.90 (29.35 – 47.05)		
	Scotophilus kuhlii	1	100 (2.50 – 100)		
Age	Adult	149	29.53 (22.34 – 37.55)	0.01	0.91
	Juvenile	51	27.45 (15.89 – 41.74)		
	Lactating	2	50.00	5.11	0.28

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			(1.26 – 98.75)		
Reproductive Status	Non-breeding	90	22.22 (14.13 – 32.31)		
	Non-scrotal	1	0		
	Post-lactating	34	29.41 (15.10 – 47.79)		
	Scrotal	73	36.99 (25.97 – 49.09)		
Sex	Female	124	24.19 (16.95 – 32.70)	3.07	0.08
	Male	76	36.84 (26.06 – 48.69)		

Figure 1. PCR-based confirmation using primers for 16S rRNA of *Staphylococcus* isolated from oral and rectal swabs from five bat species. Sequences of these PCR products confirmed all samples were *S. aureus*. Lane M: (left to right) 100 bp ladder, Lane A to E: Field isolates of *S. aureus* from bat oral and rectal swab samples.

Based on the disc diffusion method, 58 isolates of *S. aureus* were subjected to antimicrobial susceptibility tests against three antibiotics: tetracycline, erythromycin, and gentamicin. *S. aureus* isolates were found to be highly resistant to tetracycline (43.10 %) and gentamicin (43.10 %). While 27.58 % isolates were also resistant to erythromycin (Table 3).

Table 3. Breakpoint values of each antimicrobial agent and phenotypic antimicrobial susceptibility profiles of 58 *Staphylococcus aureus* isolate detected in this study according to Clinical & Laboratory Standards Institute (CLSS).

Antimicrobial class	**Antimicrobial agents**	**Conc. (µg)**	***Staphylococcus aureus***		
			S	I	R
Tetracycline	Tetracycline	30 µg	15 (25.86)	18 (31.03)	25 (43.10)
Erythromycin	Macrolides	15 µg	20 (34.48)	22 (37.93)	16 (27.58)
Gentamicin	Aminoglycosides	10 µg	15 (25.86)	18 (31.03)	25 (43.10)

Further, all 58 isolates of *S. aureus* were analyzed for the presence of resistance genes using PCR assays. Among 58 isolates, 24 oral and 34 rectal isolates were subjected to tetracycline, erythromycin and gentamicin resistance genes detection. A comparatively higher number of oral *S. aureus* isolates were resistant against TetM (n = 18, 75 %), followed by TetK (n = 17, 70.08 %), aacA-D (n = 15, 62.5 %) and ermA (n = 8, 33.33 %) genes, respectively. Similarly in rectal isolates TetM and TetK genes were detected in 14 (41.17 %) and 12 (35.29 %) isolates while 13 (38.23 %) isolates of *S. aureus* carried aacA-

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D genes. In the case of erythromycin resistance gene; ermA was detected only in 6 (17.64 %) isolates (Table 4).

Table 4. PCR assays-based detection of antimicrobial resistance genes in *Staphylococcus aurues* isolated from oral and rectal samples from bat species in Pakistan.

Total isolates tested	Source of isolation	No. isolates	Antibiotic resistance genes in *Staphylococcus aureus*			
			TetK (%)	TetM (%)	ermA (%)	aacA-D (%)
58	Oral	24	17 (70.08)	18 (75.00)	8 (33.33)	15(62.5)
	Rectal	34	12 (35.29)	14 (41.17)	6 (17.64)	13 (38.23)
	Total	58	29 (50)	32 (55.17)	14 (24.13)	28 (48.27)

4. Discussion

Here we found that several bat species in Pakistan can harbor *S. aureus* in oral and fecal swabs, including strains with important antibiotic-resistant genes. *S. aureus* can cause serious infections both in animals and humans due to their zoonotic potential. In both developed and developing countries livestock and wildlife are the potential source for methicillin resistant *S. aureus* (MRSA) which can cause serious infections in humans and animals [40]. In this study we captured insectivorous and frugivorous bats and the highest prevalence of *S. aureus* (37.90% to 23.52%) was observed in fruit bats, although our sampling was not even and only included a few representative species from each category.

Previous studies have also identified *S. aureus* in diverse bat species globally. In Nigeria, a comparatively higher prevalence (78.6%) of *S. aureus* was found in fecal samples of straw-colored fruit bats (*Eidolon helvum*). Cross-species transmission of these *S. aureus* was inferred by multiple sequence typing (MLST) which revealed their close association with isolates of monkeys and other bats species in central and west Africa [41]. Another study conducted by Held *et al*. [14] in Gabon which sampled 55 *R. aegyptiacus* individuals found a much lower rate of *S. aureus* at 6.0 %. Held *et al*. [14] collected samples from bat species native to Gabon, i.e. *Hipposideros ruber, Rhinolophus sp., Miniopterus minor, Pipistrellus annulus*, and *Chaerephon pumila* and screened for *S. aureus* and *S. schweitzeri*. A low prevalence (4-6%) of *S. schweitzeri* and *S. aureus* was reported in *Micropteropus pusillus* and *Rousettus aegyptiacus. Staphylococcus* species were also prevalent in gut samples including feces, small intestine, and large intestine of *Myotis altarium* and *Rhinolophus sinicus* in China [42]. Additionally, some other studies have also reported low prevalence of *S. aureus* from African monkeys and bats [24,43]. In our analysis of 200 oral and 200 rectal swabs from four insectivorous and frugivorous bat species, we observed significant differences in prevalence of *S. aureus* among bat species. Variation has been seen in prevalence of bacteria with respect to host species of bats in Trinidad. *Salmonella spp*. was dominant bacteria isolated from two insectivorous (i.e. *Molossus major* and *M. ater*) and one fish-eating bat species (i.e. *Noctilio leporinus*), while *E. coli* was isolated from fruigivorous (i.e. *Artebius sp.* and *Carollia perspicillata*), sanguivorous (i.e. *Desmodus rotundus*, and *Diaemus youngi*), nectivorous (i.e. *Glossophaga sp*.), insectivorous (i.e. *Molossus major*, *Mormoops sp*., and *Pteronotus parnelli*), omnivorous (i.e. *Phyllostomus discolor*, *P. hastatus*, and *Pteronotus parnelli*) bats [22]. The interspecific factors that drive differences in *S. aureus* prevalence is not known, nor the mechanisms that lead to difference in prevelance even among co-roosting bat species. A study was conducted in Romania, in which bat guano, cave air and swabs of bat fur were collected from caves to determine of aerosolized microbes [44]. *Myotis capaccinii, M. daubentonii, M. myotis, Miniopterus schreibersii,* and *Rhinolophus euryale* were the main guano contributing bats species in the cave. Additional bacteria, including *E. coli*, *Klebsiella pneumoniae*, *Chryseomonas luteola*, *Salmonella spp*., *Micrococcus spp*., and *Streptococcus*; *Staphylococcus* were also detected in samples from Romania. Based on these findings it was concluded that guano of bats in caves had a higher concentration of airborne pathogens which could be a potential source of biohazards for other animals, including other bat species, as well as human beings [44].

We investigated the association of prevalence of *S. aureus* with respect to habitat type/location, bat species, age class, reproductive status, and sex. We only found significant differences between

locations and species in our study. We observed *S. aureus* in oral and rectal swabs collected from 5 bat species from natural caves but also man-made sites including an abandoned castle and livestock shed. Little is known about the potential for *S. aureus* cross-species transmission from bats to people or other species. Some previous studies have sugested cross-species transmission of *S. aureus* from bat, particuarlaly in areas of high human habitation. Five out of eight Egyptian fruit bats kept in Copenhagen Zoo with other animals were found positive for *S. aureus*. Moreover, it was also observed that some isolates prevalent in these zoo animals were comparable to those observed in the human population suggesting their zoonotic and reverse-zoonotic potential [48]. Similarly, bat guano samples collected from *Myotis blythii* and *M. myotis* roosts located inside a Church building in Slovakia were positive for *Staphylococcus sp*. It was suggested that the presence of bats and accumulation of bat guano inside human settlements may be a potential risk for human health [25]. Presence of *Staphylococcus* in bats living in diverse habitats, e.g. forests, university campus, and caves, have been reported from previous studies from Brazil, Nigeria, and Romania, respectively [24,44,45]. Sampling site specific prevalence of *Staphylococcus* species in the gut microbiome of lesser horseshoe bats were revealed in bats captured from caves in India using metagenomics and culture dependent methods [49]. Similar reports of isolation of *Staphylococcus* species in guano layers and caves have been reported in the United States, Serbia, Slovenia [47,50,51]. However, a study from Italy revealed that bats species such as *Tadarida teniotis, Miniopterus schreibersi, Myotis capaccinii, Myotis daubentoni, Pipistrellus kuhli* and *Myotis myotis* had a diverse microflora but no detection of Staphylococcus was noted. *Citrobacter freundii, Streptococcus faecalis, Pseudomonas putida, Proteus mirabilis, Kluyvera ascorbata, Klebsiella oxytoca, Escherichia coli, Escherichia blattae, Enterobacter cloacae* and *Hafnia alvei* were dominant bacterial species in enteric samples of these bats [52].

Species level differences in bacterial diversity and prevelance have been previously noted in other bat studies. Bats captured from the Atlantic forest of Brazil identified *Serratia marcescens, Pseudomonas* sp., *Klebsiella oxytoca* and *Escherichia coli* and found that baterial richness was greater in frugivorous bats as compared to nectarivores, insectivores, carnivores, and sanguivores. *Staphylococcus sp.* was also detected from oral and rectal samples [45]. These bats have the habit of seasonal migration, and it was also observed that they abandon their colonies during the rainy season. These factors might be responsible for seasonal variation in bacterial richness of bats [46]. In another interesting study, fecal samples of twelve bat species had varying levels of prevalence of *Staphylococcus*. These bats were captured from eight parts of Slovenia during their autumn migration across central Europe [47].

It is well documented that antimicrobial resistance is an emerging issue for veterinary, wildlife, and human health. We found that *S. aureus* isolates were resistant against tetracycline, gentamicin and erythromycin based on disc diffusion methods. These findings were supported by previous investigations outside of Pakistan, but with differeing results. Previous studies of guano samples of *Myotis blythii* and *M. myotis* from Slovakia using similar methods and found *S. aureus* isolates resistant to tetracycline and erythromycin while sensitive for gentamicin [25]. In contrast to our study, one study from forest areas of Gabon found that all isolates of *S. aureus* derived from bat pharyngeal swabs were susceptible to tetracycline [14]. In another study, the antibiotic resistance pattern was determined from *S. aureus* isolates from bat guano collected from a cave in Algeria. Seven isolates were sensitive to all antibiotics while remaining four isolates were shown resistant to fusidic acid, cefoxitin and penicillin G [27]. Characterization of *S. aureus* isolated from wild and zoo animals in Germany few isolates were resistant against tetracycline [26]. Samples collected from straw colored fruit bats (*Eidolon helvum*) in Nigeria found that all isolates were susceptible to tetracycline and gentamicin and only 7.4% isolates showed resistance against erythromycin [24]. This previously observed variation in antimicrobial resistance might be due to difference in bat species, roosting sites, and geographical regions, or perhaps due to variation in exposure to antibiotics, or resistance genes in the environment based on location, foraging patterns, and other factors.

In this study, we also investigated the prevalence and provided evidence of antibiotic resistance genes such as TetA in *S. aureus* isolated from oral and rectal samples of Pakistani bats. Such type of resistance genes detection pattern was also reported from other wild animals. In Spain tetracycline

resistance genes such as tet(K) and tet(L) were detected in *S. aureus* isolated from wild birds [29]. Another study from Spain reported detection of tet(M), erm(A), blaZ, erm(T), fexA, str, eta, tst, antibiotic resistant genes in tracheal samples of nestlings of white stork captured from human residential areas [30]. Apart from these, aacA-D genes associated with gentamicin resistance was reported from companion and wild animals in European countries which is verification of the presence of such genes in *S. aureus* isolates of bats [53,53]. Tetracycline is the drug of choice for the control of certain infections in dairy cattle and poultry birds. However, unregulated use of this antibiotic can be results in development of resistance, as observed in a study conducted by Liu *et al*. ([56] in China in *S. aureus* isolates of four different animals (cows, swine, chickens, ducks) and tetracycline resistance genes such tetK (22.38%) and tetM (52.45%) were detected.

5. Conclusions

In conclusion, the present study confirmed that multidrug resistance *S. aureus* is prevalent in the oral and rectal microbiota of bats in Pakistan. Sampling site (an environmental factor) and bat species (a biological factor) contributed significantly to differences in prevalence of *S. aureus*. We provide important baseline data and a first study for Pakistan and the locations and species we investigated. Together with other studies, our finding provide evidence that bats across the globe can harbor multidrug resistance bacteria which could pose human and animal health risks, and if associated with bat morbitity or mortality could be a threat to bat populations themselves. A deeper understanding of these bacterial associations and the animal and human interactions with bats are needed to understand and minimize the chance of cross-species transmission of such pathogens.

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