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Research Article

Culicoides: A neglected parasite of prime importance in selected areas of Pakistan

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ABSTRACT

Midges (Diptera: Ceratopogonidae) are the smallest two-winged blood-sucking flies having cosmopolitan distribution. Among them, biting midges have prime importance. By direct biting, they cause pain, severe irritation, and transmission of various pathogens, significantly impacting human and animal health. It was hypothesized that Culicoides' diversity prevails in different areas of Pakistan. Various factors as humidity, temperature, rainfall, and wind speed may affect the propagation of biting midges in Pakistan. The present study was designed for the description of Culicoides throughout the Summer, Winter, Autumn, and Spring seasons in Peshawar, Faisalabad, Rawalpindi, and Islamabad (Pakistan). Selected areas were targeted for sample collection biweekly. Light traps were placed in and around animal farms during the morning and evening. The samples trapped in the cup of light traps were transferred to the Eppendorf tubes containing 70% ethanol. After proper labeling, samples were transferred to the Molecular Parasitology Laboratory, University of Agriculture, Faisalabad, Pakistan for stereomicroscopic taxonomy. A total of 384 specimens were screened for identification of biting midges. Out of 384, 194 (50.52%) were *Culicoides imicola*, 124 (32.29%) were *Culicoides circumscriptas* and 66 (17.18%) were *Culicoides obsoletus*. The highest prevalence 50.52% was seen in Islamabad. The lowest prevalence, 0%, was observed in Faisalabad. The highest prevalence 54.68% was observed in Spring and the lowest prevalence (3.38%) was observed in winter. This study provides us the data on the distribution and associated determinants of biting midges in the selected districts of Pakistan. The factors that were associated with the distribution of biting midges were temperature, relative humidity, wind speed, and rainfall. In the future, this study will be helpful for broad spectrum identification of Midges at the molecular level.

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Introduction

Arthropods, consisting of invertebrates, have an outer covering on their body called a cuticle (Adler et al. 2004). Insecta, a class of arthropods, has most species of ecological societies of land and aquatic environments. One of the largest orders of Insecta is Diptera, which consists of more than one million species, including horse-flies, hover-flies, crane-flies, and many more; though, just 0.125 million species have been described (Picker et al. 2003). Biting midges (Insecta: Nematocera), small flies that often bite, have 39 species worldwide with an overall 1400 species and 29 subgenera (Atchley 1970). The word "midge" does not belong to any taxonomic group but has got species in many Nematoceran families. They are found on every land, excluding desert areas and frigid regions (Atchley and Wirth 1979).

Ceratopogonidae; commonly called punkies or biting midges, include harmful blood-sucking pests, which feed both on humans and animals. Females biting midges suck blood and other body fluids, while males feed on nectars of different plants (Yasin et al. 2023). Their bite is very painful, irritating the campers, fishers, hunters, hikers, gardeners, and other people who stay outdoors at the time of dawn and dusk. Sometimes during cloudy days, they become activated. The complete cycle can occur within 2-6 weeks, but it depends upon the species and atmospheric situation (Atchley 1967). Ceratopogonid midges are the main pollinator of "*Theobroma cacao*" because of their peculiar morphological and behavioral characteristics. Some of the Cecidomyiidae e.g. the Sorghum midge are well-known pests of plants. Many midges, except gall midges "Cecidomyiidae" are fresh-water inhabitants during their larval stages. The larvae of some Chironomidae consist of hemoglobin and are often called blood worms (Aussel and Linley 1994).

Biting midges are vectors of many pathogens of humans and animals. In South America, West Africa, Central Africa, Asia, and the Caribbean islands, *Culicoides* are the vectors for many filarial nematodes i.e. "*Mansonella"* (Ayllón et al. 2014). In the peak season of midges, many horses have severe hypersensitivity reactions and skin damage because of the bites of biting midges, which is called equine allergic dermatitis and affects the withers, many, and tail areas of the animals. Biting midges are the vectors of African horse sickness (Rozendaal, 1999), the Bluetongue (BT) virus, and the Schmallenberg virus (Aslam et al. 2023).

Biting midges drastically affect the livestock population and cause harmful diseases, such as BT and Epizootic hemorrhagic disease (Gerdes 2004). A special type of fauna is there in Pakistan, which is suitable for the development of different flies like mosquitoes, biting midges, black flies, and horseflies that can easily propagate and spread number of vector-borne viruses. Furthermore, excessive urbanization and land use lead to conducive spread of vector-borne diseases (Rozendaal 1999). Pakistan is indigenous to the biting midges they are among the vital factors which cause in decrease the economy of the country. There are only 24 species of biting midges that have been identified yet from six different locations in Pakistan. These locations are mainly the northern areas of Pakistan including Hunza, Gulmit, Mingora, Chilas, Kalam, and Dir (Battle and Turner 1971).

Keeping in view the dearth of knowledge about the *Culicoides* spp. in Pakistan and favorable environmental conditions. This research paper will highlight the diversity of *Culicoides* spp. prevailing in different areas of Pakistan and various factors like humidity, temperature, rainfall, and wind speed are responsible for the propagation of biting midges in the country.

Materials and Methods

Study area and sampling method

The study was conducted in the following selected areas of Pakistan i.e. Faisalabad, Islamabad, Rawalpindi, and Peshawar. These areas were targeted for the collection of biting midges because of different climatic conditions. A total number of 384 biting midges were collected from various livestock farms located in four districts of Pakistan irrespective of the type of animal and their age and sex. Samples were collected from the targeted districts with the help of biting midges collecting light traps and it was applied in the evening near the livestock vicinity in the dairy farm and placed properly. UV light in the light trap attracts the biting midges in the trap. The collected specimens were transported to the Department of Parasitology, University of Agriculture, Faisalabad. In the laboratory, the required specimens were isolated in tubes. For preservation, biting midges were placed in Eppendorf tubes containing 70% ethanol and then labeled properly. Identification of the adult midges was done by following taxonomic keys (Becker 1960; Bennett 1960; Mathieu et al. 2012).

Identification of specimens

The structural identification of the different species of *Culicoides* was done by examining the mouth parts (Labrum, Labium, Mandibles, Maxillae, and Hypopharynx), antennae structure (presence of Plumose and Pilose hairs), wing pigmentation pattern (presence of light and dark spots). A convenience examination was performed under, a stereomicroscope and for this purpose, each specimen was placed in an individual petri dish with the help of a fine needle and a fine camel hairbrush (used to expose different body parts of biting midges). Then each biting midge was

mounted separately on a glass slide. Specimens were treated with 100% ethanol with one dip then slide mounting was done by using DPX (Blackwell et al. 1992; Blackwell and Mordue 1994; Bishop et al. 2006).

Questionnaire-based surveillance

The details for each farm regarding age and sex of animals, housing system, floor pattern, watering system, animal keeping system and hygienic conditions were recorded in respect of number of biting midges collected from each district (Blackwell et al. 1995; Garros et al. 2011).

Statistical analysis

Data collected was arranged for each parameter. Prevalence and odds ratio were calculated by using SPSS software and were compared using multiple logistic regression.

Results

In total 100 dairy farms were located in the study districts (Faisalabad, Rawalpindi, Islamabad, and Peshawar) and no biting midge was seen on any dairy farm located in district Faisalabad. The species of collected biting midges were identified as *Culicoides imicola, Culicoides circumscriptus* and *Culicoides obsoletus*. Out of the collected biting midges, 194 were identified as *Culicoides imicola,* 124 were *Culicoides circumscriptus,* and 66 were *Culicoides obsolete*. The prevalence of biting midges regarding risk factors has been mentioned in Table 1. The $p<0.05$ shows that the prevalence of biting midges is significantly associated with study area, season, and species of midges but a p-value greater than 0.05 against risk factor age, gender, housing system, floor pattern, watering system, and farm hygienic conditions shows that there is no association of prevalence with these risk factors (Fig. 1).

Table 1: Prevalence of biting midges concerning risk factors

Variables	Level	Prevalence (% age)	Odd ratio	F-Calculated	P-value
Animal based Variables					
Age	Young	5.71	1.19	0.01	0.69
	Adult	3.76	2.97		
Gender	Male	4.08	0.08		
	Female	3.25	3.19		
Farm based Variables					
Housing System	Open	2.48	0.93	0.17	0.769
	Closed	1.23	0.76		
Floor Pattern	Uncemented	2.77	0.68	0.39	0.535
	Cemented	8.98	0.34		
	Partially cemented	1.36	0.97		
Watering System	Canal	6.25	0.12	0.19	0.664
	Tap	1.11	0.93		
	Pond	0.30	0.04		
Farm Hygienic condition	Poor	0.59	0.21	1.87	0.175
	Fair	2.10	0.85		
	Good	2.88	0.68		
Area based Variable					
Study districts	Faisalabad	0.00	0.00	31.73	0.000
	Peshawar	18.75	2.02		
	Rawalpindi	31.51	3.39		
	Islamabad	49.73	5.36		
Parasite based Variables					
Species of midges	*Culicoides imicola*	12.88	6.51	1161.90	0.000
	Culicoides circumscriptus	37.87	6.51		
	Culicoides obsoletus	20.16	6.51		
Environment based Variables					
Seasons	Spring	54.68	5.89	15.38	0.00
	Summer	31.25	3.36		
	Autumn	10.67	1.15		
	Winter	3.38	0.36		

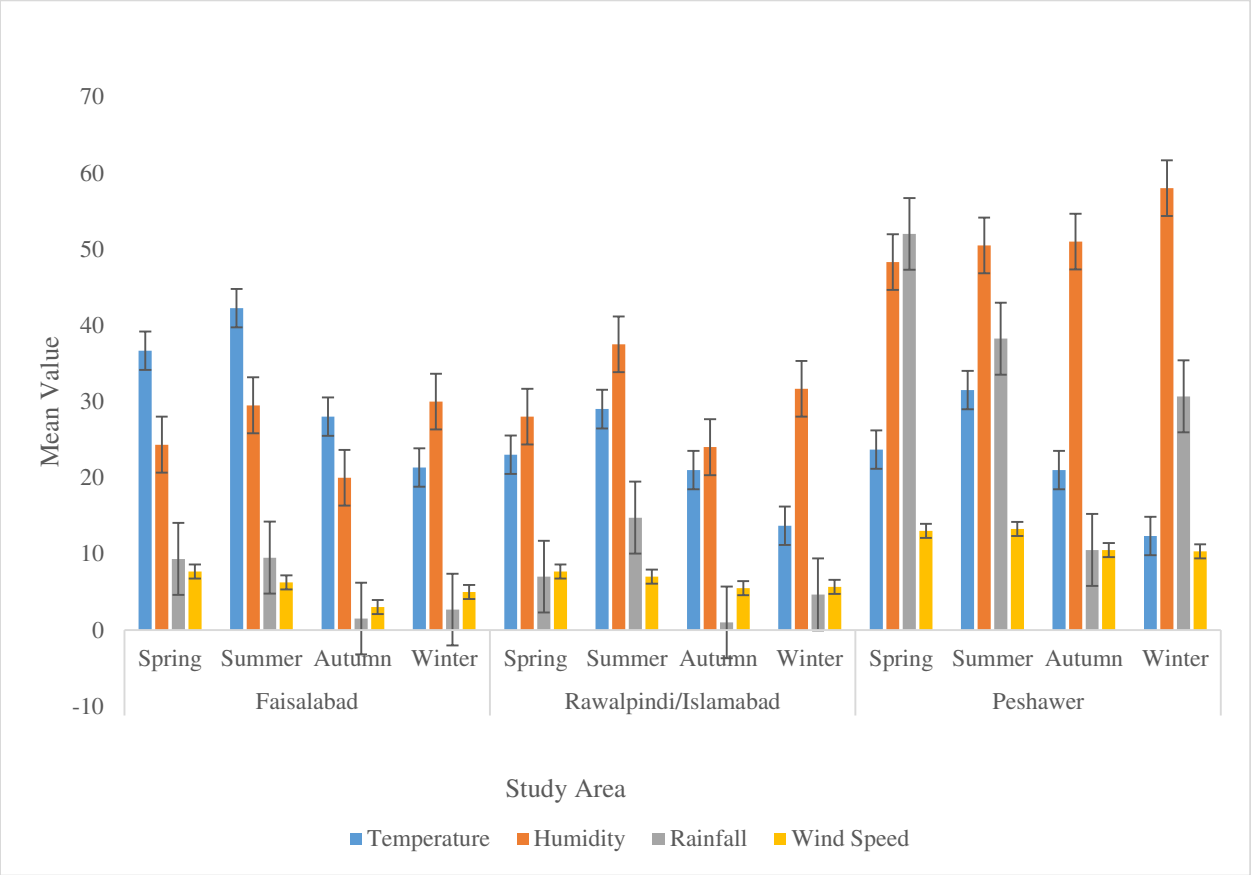

Fig. 1: Variation in environmental factors in study areas based on their mean values

Discussion

Parasitism is a major restriction in the growth and development of livestock population (Gerdes 2004; Kluiters et al. 2013) and Culicoides species complex play a main role in this context as this complex acts as potential vectors for various viral diseases like blue tongue and African horse sickness viruses (Laird 1946; Lamberson et al. 1992). Biting midge species are known to transmit 26 filarial nematodes, 66 types of viruses, and 15 types of protozoans to their host (Lardeux and Ottenwaelder 1997; Mathieu et al. 2012). Biting midges spread viruses of the family *Reoviridae, Rhabdoviridae,* and *Bunyaviridae* (Mullen 2009; Mathieu et al. 2012).

To find Culicoides specimens different identification keys were followed, and the results were matched with Rowley and Cornford (1972), as *Culicoides pulicaris* group, Drymodesmyia (Atchley and Wirth 1979) as *C. copiosus* group, Amossovia (Atchley 1967) as *C. guttipennis* group, Selfia (Atchley 1970) and Hoffmania (Ayllón et al. 2014). Species group keys composed of: *C. debilipalpis* group (Bartsch et al. 2009), *C. haematopotus* group (Atchley and Wirth 1979), *C. mohave* group (Aussel and Linley 1994), *C. palmerae* group (Atchley 1967), *C. piliferus* group (Becker 1960), *C. chaetophthalmus* group (Gerdes 2004), *C. stonei* group (Bartsch et al. 2009) and *C. pusillus* group (Rozendaal 1999).

Biting midges (*Culicoides)* were mostly observed during the Spring season in Pakistan. The results of our study were in line with Fasotte and Hubert (1989); Rozendaal (1999); Laird (1946); Garros et al. (2011); and Blackwell et al. (1992). They also reported that biting midges were found during seasons when the temperature remains 15°C to 25°C and relative humidity from 40 to 85 for their growth and reproduction. Our results are in line with the Fasotte and Hubert (1989); Rozendaal (1999); Laird (1946); Garros et al. (2011) and Blackwell et al. (1992) who reported a similar prevalence in the spring season. Gerdes (2004) described a higher abundance of *Culicoides imicola* sp. during Ssummer and in rainfall areas compared with other *Culicoides* sp. They also reported an increment in *Culicoides imicola* during the end period of Summer along with the increasing number of cases of African horse sickness and bluetongue. This showed a great contrast to our findings because of the area-based variations. Spring season favors the growth of

biting midges. The result of the present findings was in support of Bishop et al. (2006) who stated that various climatic conditions directly affect the distribution of biting midges (*Culicoides*) and some mosquitos. It is also evident that high temperature is usually associated with an increase and the abundance of transmissible diseases. The ambient temperature of district Faisalabad was recorded being 42°C. Relative humidity was 29.3% which is not suitable for biting midges' growth and survival. Therefore, not a single case of biting midges was in Faisalabad; as the biting midges cannot survive in high temperatures and moderate humidity, even their larvae or eggs die during such a high temperature. The average temperature recorded from Faisalabad, Rawalpindi, Islamabad and Peshawar was 25.29°C during Spring, Summer, Autumn, and Winter (Adler et al. 2004). According to Picker et al. (2003) and Atchley (1967), thermal limits are important for determining the distribution of *Culicoides*.

The average relative humidity, average wind speed, and average rainfall recorded in Faisalabad, Rawalpindi, and Islamabad throughout the year are in line with Bartsch et al. (2009) and Battle and Turner (1971); Becker (1960) who also reported that biting midges were in abundance while the temperature was 22°C, rainfall 18.5 mm and wind speed 7.1 mph. The environmental conditions of Rawalpindi, Islamabad, and Peshawar favor the growth and propagation of different species of biting midges, and the highest prevalence was observed in district Rawalpindi. While environmental conditions of Faisalabad were different, having high temperatures, low rainfall, and humidity, while wind speed was relatively slower than those of Rawalpindi, Islamabad and Peshawar that is why biting midges might not be found in Faisalabad.

Biting midges were not found in the Faisalabad district, whereas the biting midges were found and trapped in the districts of Rawalpindi, Islamabad, and Peshawar, respectively. The present results are in agreement with Blackwell et al. (1995); Garros et al. (2011) and Kluiters et al. (2013) who reported that biting midges are present on every continent except Antarctica and at altitudes above 1090 ft. Biting midges were found above an altitude of 1670 ft to 1090 ft that are in agreement with Mathieu et al. (2012) who also reported that biting midges were found at an altitude of 1200 to 2130 feet. During the present experiment, two peaks of abundance of biting midges were recorded. Similarly, Rozendaal (1999) reported a higher population of biting midges from August to September and a bit lower peak during November before the beginning of winter. Moreover, they reported a very low population of biting midges during Summer (May) which was the hottest month of the year in Maharashtra state of India. Our study is in line with the previous reports where higher abundance was reported in high-altitude areas like Rawalpindi, Islamabad, and Peshawar.

Our results are in agreement with McGregor et al. (2022) and Kar et al. (2022) those also reported that the biting midges (*Culicoides*) usually bite their host from dawn to dusk. The biting midges captured were all adult. Adult biting midges those were captured through light trapped were similar in size as reported by Songumpai et al. (2022), Fasotte and Hubert (1989) and Zhang et al. (2022) who also reported the size of biting midges being 1-4 mm in length. When biting midges were examined under a microscope, they exhibited certain features. Biting midges have compound eyes which are larger on the head and both eyes are closer to each other. Our findings are in agreement with Garros et al. (2011) who also reported that biting midges have large eyes and make the most of the head. It was recorded that the antennae of biting midges are similar to the study of Groschupp et al. (2023), Hadj-Henni et al. (2023), McGregor and Lewis (2023), Labuschagne et al. (2023), Yasin et al. (2023), Atif et al. (2021) and Lamberson et al. (1992) also reported that *Culicoides* have 15 segments of their antennae and have sensilla on their antennae. Our results are in agreement with the previous studies reported about the morphology of biting midges. The morphological characteristics of biting midges are related to *Chironomidae*, *Simuliidae*, and *Thaumaleidae* (Kar et al. 2022).

Our results showed that Frons splits antennae and is less noticeable in males than in females and Ocelli are missing. Our results are in line with Mehnaz et al. 2023; Fatima et al. 2019; Atif et al. 2022; Zaman et al. 2022.) Our results showed that the Ventral part of frons is antennae and clypeus that joins with mouthparts that make proboscis. Similar findings were also observed by Atchley (1967), Atchley (1970), and Picker et al. (2003).

The females of our study area were identified based on 3rd segment of palp as being swollen, which is a characteristic feature of females. A similar finding was also reported by Bartsch et al. (2009) and Ayllón et al. (2014). Palp was also seen having a small sensory pit in the 3rd segment. Similar, findings were also reported by Beckenbach and Borkent (2003) and Bishop et al. (2006). The irregularly patched palps having a small sensory pit on the 3rd segment was a confirmatory feature of females being identified.

Conclusion

Biting midges are prevalent in the districts of Rawalpindi, Islamabad, and Peshawar. High humidity favours the growth of biting midges. The highest abundance of biting midges was observed during late Spring and early Summer. Different husbandry practices like open housing systems, un-cemented floor patterns, tap watering systems,

chained animal keeping systems, and poor hygienic measures at farms have been found positively associated with a higher population of biting midges. The prevalence of these parasites on a broad scale will be helpful for future analysis of zoonotic diseases.

Ethical Statement
This study was conducted following the ethical guidelines mentioned by the university ethical committee.

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Competing Interest
The authors declare they have no conflicts of interests to disclose.

Consent for Publication
All the authors gave their consent for publication of the research paper.

Data Availability
The original data can be obtained from the corresponding author on demand.

Author Contribution
SM, MSS, MAA, and MNR designed the study ASA HS and AS conducted the research, and SM, MSS and MSC wrote the article and performed statistical analysis.

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