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ENDEMIC SEROPREVALENCE AND FARMER CONTROL MEASURES OF ANTHRAX AMONG RUMINATE ANIMALS IN PAKISTAN

N. Sardar[1,2], T. Yaqub[1*], A. A. Anjum[1], M. Javed[1], S. Ayub[3], N. Mukhtar[1], H. B. Aslam[1], Alan J. Wolfe[4], Daniel S. Schabacker[5], Sara Forrester[5], Mark Khemmani[4], Amin A. Aqel[6], M. F. Shahid[1,7], S. Yaqub[1], M. Ali[1], M. A. Shabbir[1], M. Z. Shabbir[1], M. Nawaz[1], K. Shafi and M. W. Aziz[1]

[1]Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[2] Department of Microbiology, University of Jhang, Pakistan; [3]Bacteriology Department, Institute of Public Health Lahore, Pakistan; [4]Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153, USA; [5]Argonne National Laboratory, Lemont, IL 60439, USA
[6]Faculty of Medicine, Mutah University, Al-Karak, Jordan; [7]Veterinary Research Institute, Lahore 53810, Pakistan

[*]Corresponding author's E-mail: tahiryaqub@uvas.edu.pk

ABSTRACT

Anthrax, caused by *Bacillus anthracis* (*B. anthracis*), is an important zoonotic disease of livestock that poses a threat to public safety. The present study aimed to determine the seroprevalence of *Bacillus anthracis* in sheep and cattle in endemic areas (Punjab and Balochistan provinces) of Pakistan by enzyme-linked immunosorbent assay (ELISA). Cross-sectional survey was also done to assess the control measures of the farmers. Out of 401 serum samples from sheep and cattle, a total of 68 (16.9%) samples were seropositive for anti-PA antibodies; seroprevalence of *B. anthracis* in sheep and cattle was 22.11% (48/217) and 10.86% (20/184), respectively. Seroprevalence was significantly higher ($p<0.05$) in Baluchistan province (33.3%, 44/132) compared to Punjab province (8.92%, 24/269). Most farmers (65.90%, 29/44) were not disposing carcasses as recommended. Around 77.28%, 17/22 of the veterinary officials reported the non-availability of vaccine against anthrax and 63.63% (14/22) stated the lack of cold chain for the vaccine storage and transportation. We concluded that seroprevalence of *B. anthracis* in unvaccinated sheep and cattle is evidence that the pathogen is circulating in Baluchistan and Punjab provinces. So must applied an active surveillance and, strengthened veterinary practices vaccination programs to control anthrax outbreaks in endemic areas.

Keywords: Seroprevalence, *Bacillus anthracis*, Endemic, Pakistan, Vaccination

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INTRODUCTION

Anthrax is a non-contagious zoonotic disease caused by Bacillus anthracis, a spore-forming gram-positive rod-shaped bacterium that was first identified in the late 19th century by Robert Koch (Koch, 1876). Anthrax is worldwide disease which endemic in South Asia and Sub-Saharan Africa (Dragon and Rennie, 1995). In Pakistan, anthrax is called "Phurki" in the local language of Punjab province (Rashid *et al*., 2020) and "Tack" in the local Balochi language of Baluchistan province (Davis *et al*., 1995). Anthrax is a severe disease of acute and per-acute form capable of infecting all warm-blooded animals including humans. In unfavorable conditions, *B. anthracis* forms heat- and chemical-resistant spores (Coffin *et al*., 2015). Sheep, goats, and cattle are affected by this disease after ingestion or inhalation of spores during grazing on contaminated pastures. Clinical manifestation of anthrax includes oozing of blood from natural orifices, absence of rigor mortis and, horse saw posture of the animal (Constable *et al*., 2017). Humans may get the infection if they come in contact with the infected animal carcass, their meat products or contaminated soil, or through occupational exposure (Organization, 2008, Sushma *et al*., 2021). Anthrax in humans occurs as cutaneous, pulmonary, or gastrointestinal forms based on the route of spore entry into the body (Kamal *et al*., 2011). Globally recommended measures include active surveillance, annual vaccination programs, and proper disposal of anthrax suspected carcasses to minimize the spread of infection for disease prevention (Ahsan *et al*., 2015).

The pathogenicity of *B. anthracis* depends upon two plasmids (pX01 & pX02). The pX01 encodes for edema factor (EF), lethal factor (LF) and protective antigen (PA), whereas pX02 encodes the capsule (UCHIDA *et al*., 1985). Together with and EF, PA plays an important role in disease progression. The PA is immunogenic and antibodies against it can be detected 11

days post-infection (Brenneman *et al*., 2011), making it an important biomarker for the laboratory-based diagnosis of anthrax. Detection of anti-PA antibodies through enzyme-linked immunosorbent assay (ELISA) is the most common diagnostic procedure to determine the seroprevalence of susceptible hosts (Sushma *et al*., 2021, Mukarati *et al*., 2021).

Annually, 10,000 to 100,000 anthrax cases in humans are estimated around the globe with a large number of cases occurring in the lower-middle-income countries of Sub-Saharan Africa and South Asia (Oncu *et al*., 2003). Animal movements between provinces and borders play a major role in disease dissemination and transmission (Wajid *et al*., 2020). The lack of a robust disease reporting and surveillance system in Pakistan makes it difficult to control infectious diseases like anthrax. Literature is scarce regarding outbreaks of anthrax in Pakistan. Some inconclusive evidence of anthrax and anthrax-like diseases is based merely on news and unpublished reports. A recent report on sporadic anthrax outbreaks suggests its presence in parts of Punjab, Khyber Pakhtunkhwa (KPK), and Baluchistan province (Rashid *et al*., 2020, Ashraf *et al*., 2014). Rashid and co-workers reported high seroprevalence against anthrax among the livestock (cattle, buffalo, sheep and goat) of central Punjab but this was estimated in response to anthrax vaccination practiced once a year (Rashid *et al*., 2020) no other study from Pakistan has yet been found.

Manifestation of clinical signs might not occur in some animals infected with *B. anthracis*; which could possibly transmit the infection to other animals (Turnbull, 2008). If gone unnoticed, this sub-clinical form of anthrax could pose a threat to public health. Since information on the dispersal of anthrax is lacking in Pakistan, a sero-surviellance was conducted in two different provinces (Punjab and Baluchistan) with the highest cattle and sheep population and where a varying number of anthrax outbreaks have been reported in the recent past (Arif *et al*., 2018). Poor husbandry practices and lack of awareness could present challenges for efficient control of diseases including anthrax. In Pakistan, literature is deficient about husbandry and vaccine practices of farmers and field veterinarians for anthrax which could provide an insight into reasons for repeated outbreaks of anthrax in Pakistan. Hence, this study aimed to estimate the seroprevalence of anthrax in sheep and cattle, and to identify the husbandry practices that contribute to anthrax outbreaks.

The results of this study will provide necessary information for the concerned authorities to develop novel interventions to control the disease.

MATERIALS AND METHODS:

This study was conducted from March, 2021 to May, 2021 in the two largest provinces of Pakistan **(**30.3753° N, 69.3451° E**),** Punjab and Baluchistan, they were selected based on the previous reports and observation of anthrax cases observed previously Districts Bahawalnagar, Bahawalpur, Multan, Muzaffargarh, Layyah from the Punjab and district Zhob, Quetta, Loralai from Baluchistan were selected for the current study (Figure 1).

Ethical Approval: The current study was conducted after approval from the office of research innovation and commercialization (ORIC), University of Veterinary and Animal Sciences, Lahore viz letter-number DR/1654 on 9-Feb-2021. Approved by Institutional Ethical Review Board, Institute of Public Health viz IERB/14/2019, all the data from human subjects was collected after informed verbal consent.

Collection of sheep and cattle serum samples: Convenient sampling was performed on the availability of herds and consent of farmer to participate in the study. A total number of 217 blood samples from sheep (Punjab, n = 126 & Baluchistan, n= 91) and 184 blood samples from cattle (Punjab, n=143 & Baluchistan, n= 41) were collected (Table 1). For blood collection at every farm/herd, 10% of the animal population was selected randomly for sampling. The blood was collected from the jugular vein of sheep and cattle using 5 ml and 10 ml disposable syringes, respectively. Approximately 3.5-4 ml blood from sheep and 6-7 ml blood from cattle was collected in gel vacutainers (ATLAS MEDO-O-VAC FARANSICO) to obtain serum.

Detection of Anti-PA-IgG: Enzyme linked Immunosorbent Assay (ELISA) was performed on serum samples using commercially available kits against cow (Cow Anthrax Protective Antigen IgG (Anti-PA-IgG) ELISA Kit; abx 055844) and sheep (Sheep Anthrax Protective Antigen IgG (Anti-PA-IgG) ELISA Kit; abx 055845). Briefly, the serum samples were diluted at 1:5 dilutions with the dilution buffer provided in the kit. One blank control, 2 positive and 2 negative controls (provided in the kit) were used without dilution buffer as per kit's instructions. The diluted samples and controls (50µl each) were added to the wells and incubated for 30 minutes at 37^0C, then washed 5 times with the washing buffer provided in the kit (1:20). 100µl of horseradish peroxidase enzyme conjugate was added and incubated for 30 minutes at 37^0C and the same washing procedure was performed as before. Next, 50µl each of substrates A and B were added to the wells and incubated for 15 minutes at 37^0C. Finally, 50µl stop solution was added and optical density (OD) was measured at 450nm. The cut-off value was detected by adding 0.15 (as

recommended in kit manual) to the negative control mean OD and sample OD values were then compared to the cut-off value . Samples with an OD value ≥ the kit value were considered positive.

Data collection: To understand the husbandry, health and vaccination practices of farmers and veterinarians regarding anthrax disease, a cross-sectional survey with close-ended questions (supplementary material) was also conducted. The participants of the study were owners/decision makers of the animal herds (n=44) from whom blood samples were collected and local veterinary officials (n=22) of the area involved in sampling. The participants enrolled for current study were based on the availability of herds and consent of farmers and veterinary officials.

Statistical analysis: The data was initially put into excel worksheet for cleaning and descriptive analysis was conducted. Statistical analysis was conducted using statistical package for social sciences 19. For categorical variables frequency, percentages and confidence intervals (CI) were given. The data on the seroprevalence was analyzed and mean ± standard deviation (SD) are given. Chi-square was used to determine the significant seroprevalence difference between provinces and animal species. The threshold for statistical significance was set at p-value ≤ 0.05 and calculated the Cochran–Mantel–Haenszel odds ratio between districts and sheep and cattle to adjusted Odds Ratio. Cochran–Mantel–Haenszel odds ratio were used to investigate the association between a binary predictor and a binary outcome variable. The data on the seroprevalence was analyzed and mean ± standard deviation (SD) are given.

RESULTS

The overall prevalence of anti-PA-IgG antibodies in all animals was 16.95% (n=68/401, 95%CI: 13.28 – 20.63). The seroprevalence of anti-PA-IgG antibodies in sheep [22.11% (n=48/217, 95%CI: 16.60 – 27.64)] was greater than in cattle [10.92% (n=20/184, 95%CI: 6.37 – 15.37)] with a p-value of 0.003 (Table 1). Seropositivity against *B. anthracis* was significantly higher in Baluchistan [33.33% (95%CI: 25.29 – 41.38)] than Punjab [8.92% (95%CI: 5.52 – 12.33)] province (p = 0.0001).

The cross-sectional survey data (Figure 2) revealed that the majority of the farms in the selected districts practiced free-range grazing (95.45%, 42/44) with considerably less stall feeding (4.54%, 2/44). The mean sheep flock sizes in Baluchistan and Punjab were 143±32 and 28±11, respectively. For cattle, the mean herd size was 5±2 in Baluchistan and 40±6 in Punjab province. Most farmers (77.27%, 34/44) reported anthrax manifestation in their respective areas as evidenced by sudden death (79.41%, 27/34) and oozing of terry red color from orifices of dead carcasses (44.11%, 15/34). Only 14.17 % (14/34) of the farmers who suspected anthrax reported the disease to the local veterinary/para-veterinary field staff. Out of surveyed participants, 68.18% (30/44) of the respondents reported the slaughtering of animals that were in grave condition and 56.81% (25/44) reported consuming meat of such animals domestically or selling it commercially. The survey results revealed that only 29.54 % (13/44) of the farmers were aware of vaccines and their protective benefits and only 11.36% (5/44) of the participants reported having their animals vaccinated against anthrax. Regarding carcass disposal practices, 65.90% (29/44) of the farmers disposed of dead animals in landfills.

The survey results revealed that 45.45% (10/22) of the field veterinary staff reported the occurrence of anthrax outbreaks in the past 12 months in their respective areas and 31.81% (7/22) of the participants informed about holding vaccination campaigns against major animal diseases. Most of the veterinary officials reported that vaccines against *B. anthracis* or necessary cold storage were not available (77.28%, 17/22; 63.63%, 14/22, respectively), and only 18.18 % (4/22) reported that they deliver vaccines to farmers living in remote areas by veterinarians themselves. No record keeping of the vaccination done or medicines applied was reported by any of the respondents. Of those respondents that administer vaccine, 54.54 % (12/22) were administered by veterinarians, and, whereas 45.45% (10/22) were administered by the farmers themselves. Occurrence of anthrax in vaccinated sheep flocks/ cattle herds was manifested by 54.54% (12/22) of the participants reported the occurrence of anthrax in the vaccinated sheep flocks/ cattle herds. Field veterinary staff also mentioned that 31.81 % (7/22) of the farmers were inclined towards vaccination of the healthy animals. The statistical analysis showed that results are significantly different between Punjab and Baluchistan provinces and, between sheep and cattle ($p<0.05$). The odds of animals getting sick in Punjab is low (0.96) as compared to the Baluchistan (3.33) with odds ratio of 2.32. The Cochran–Mantel–Haenszel odd ratio between Punjab and Baluchistan is 1.7631 which indicate that there is higher risk between them to adjusted odd ratio magnitude of confounding is 0.24.

Table 1: Details of animals sampled and seropositivity (%) of anthrax in Punjab and Baluchistan provinces of Pakistan

Study areas	Sheep			Cattle		
	Positive/Total	Positivity	95% CI	Positive/Total	Positivity	95% CI
Punjab	**11/126**	**8.7%**	**23.2 – 61.6**	**13/143**	**9%**	**4.3 – 13.8**
Bahawalnagar	6/40	15.0%	3.9 – 26.0	3/40	7.5%	0.6 – 15.6
Bahawalpur	2/21	9.5%	3.0 – 22.0	5/33	15%	2.9 – 27.3
Multan	0/19	-	-	2/27	7.4%	2.4 – 17.2
Muzaffargarh	0/22	-	-	0/21	-	-
Layyah	3/24	12.5%	0.7 – 25.7	3/22	13.6%	0.7 – 27.9
Baluchistan	**37/91**	**40.6%**	**30.5 – 50.7**	**7/41**	**17.1%**	**5.5 – 28.5**
Zhob	18/42	42.8%	27.9 – 57.8	4/19	21%	2.7 – 39.3
Quetta	15/28	53.5%	35.1 – 72.0	3/11	27%	0.9 – 53.5
Loralai	4/21	19%	2.2 – 35.8	0/11	0%	0

Table 2: Details of odds ratio and Cochran–Mantel–Haenszel odds ratio to adjusted odd ratio

Study Area	Odd Ratio	Odd ratio and Confidence interval	Cochran–Mantel–Haenszel odds ratio	Magnitude of Confounding	Chi-square test			
					Punjab vs Baluchistan		Punjab vs Baluchistan	
					χ^2	P	χ^2	P
Punjab	**0.96**	**C(0.41, 2.22)**						
Bahawalnagar	2.18	CI (0.58, 8.35)						
Bahawalpur	0.56	CI (0.11, 3.36)						
Multan	0.00	CI (0.00, 3.52)						
Muzaffargarh	-	0	**1.7631**	**0.24**	**31.2694**	**0.00001**	**8.9494**	**0.00277**
Layyah	0.90	CI (0.19, 4.25)						
Baluchistan	**3.33**	**CI (1.32, 8.39)**						
Zhob	2.18	CI (0.81, 8.70)						
Quetta	3.08	CI (0.63, 12.15)						
Loralai	-	CI (0.48, ∞)						

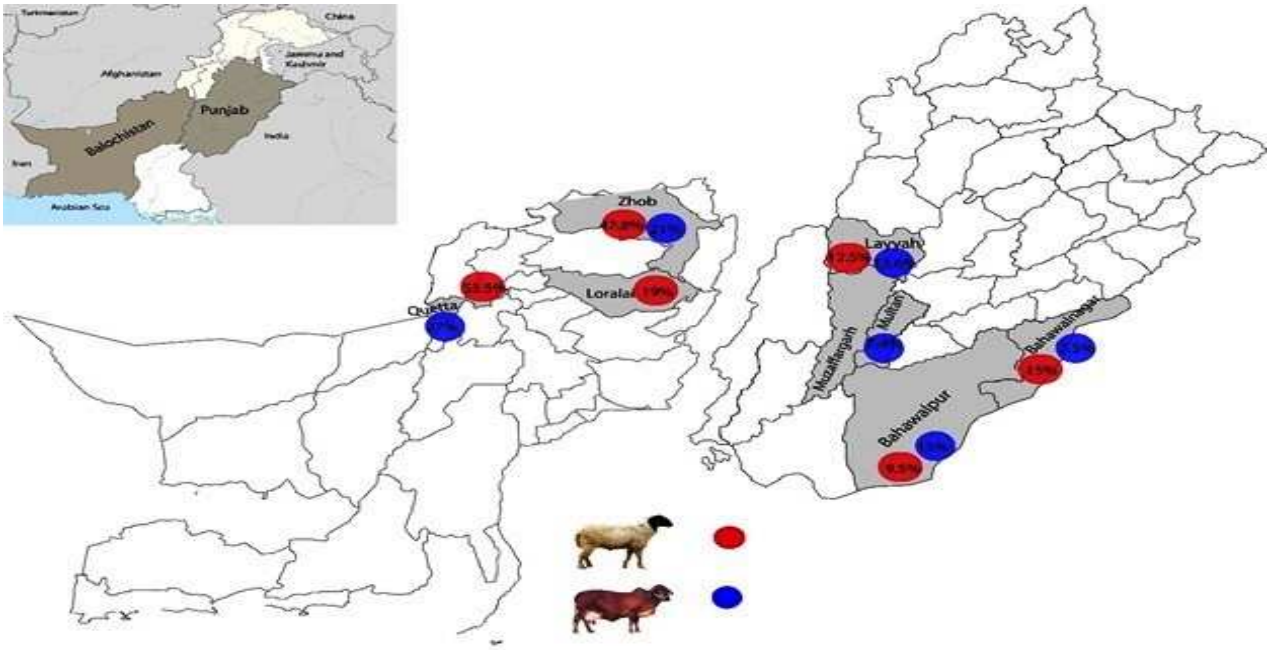

Figure 1: Mapping of seroprevalance of *B. anthracis* antibodies in different districts of Punjab and Baluchistan provinces

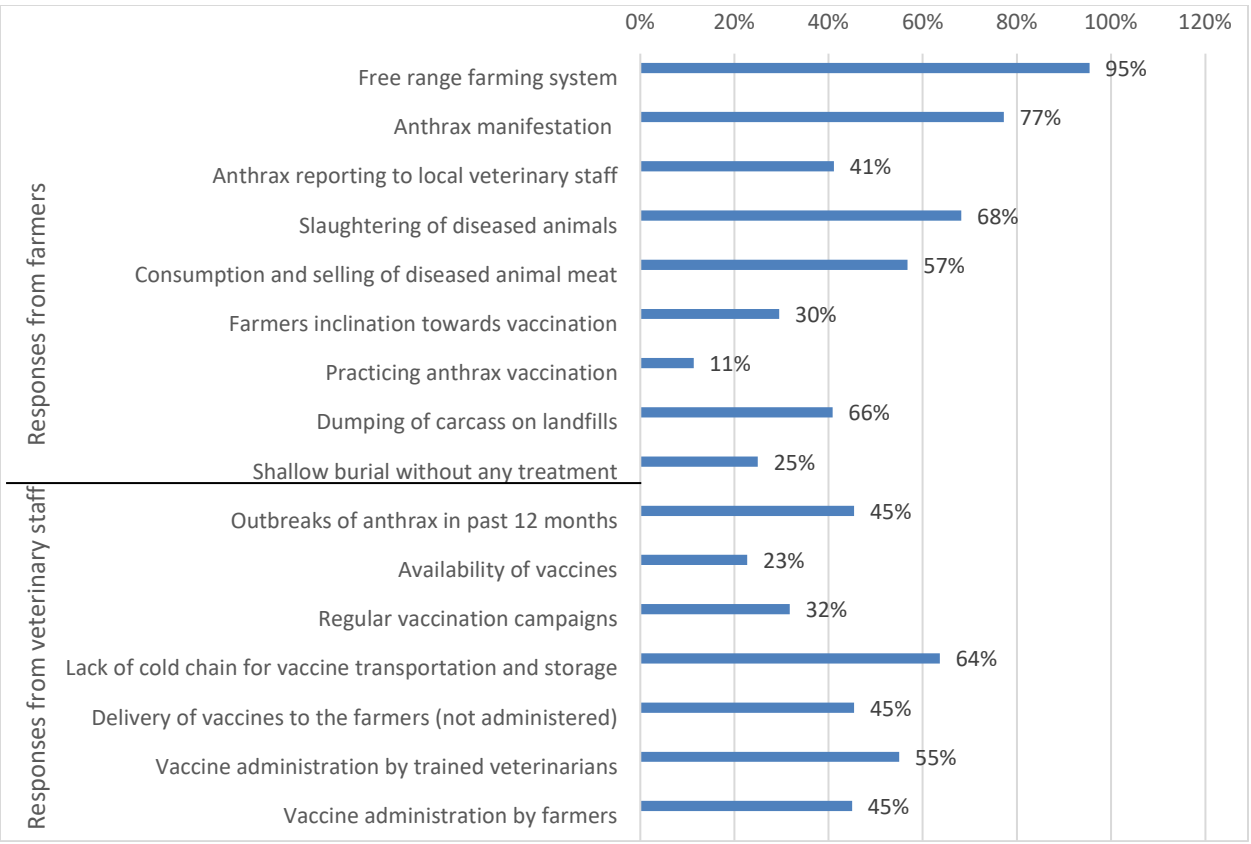

Figure 2. Production, husbandry and veterinary practices in anthrax endemic areas of Pakistan

DISCUSSION

The present study was designed to estimate the seroprevalence of *B. anthracis* in sheep flocks and cattle herds in anthrax endemic areas of Pakistan. Our findings indicate that higher seroprevalence was found in animals (sheep and cattle) sampled in Baluchistan compared to Punjab. Overall, a higher seroprevalence was found in sheep as compared to cattle in all study areas.

This study revealed a sero-positivity of *B. anthracis* in studied animals with no history of vaccination in the last six months as 16.95% (n=68/401, 95% CI: 13.28 – 20.63) and these results are comparable to that of the seroprevalence estimated in cattle in Zambia at 8% (n= 187) (Kingston *et al*., 2015) and Namibia at 8% conducted after 18 months of vaccination (Simbotwe *et al*., 2019). The sero-positivity found in present study was lower than the sero-positivity of cattle in Namibia (25%) estimated after 9-10 months of vaccination (Turnbull *et al*., 1992). The high seroprevalence in Namibia with vaccination history may be due to the reason that antibody titer decreases gradually after vaccination as described earlier (Simbotwe *et al*., 2019).

The higher seroprevalence found in Baluchistan might be due to the fact that Baluchistan shares borders with Afghanistan and Iran and un-controlled movements of animals could spread the infection across borders (Wajid *et al*., 2020). Moreover, existence of local nomadic farmers traveling with their animals in search of fresh pastures could disseminate infection along the way as suspected cases of anthrax in animals in these communities have been reported by local veterinarians and farmers (Arif *et al*., 2018). Furthermore, the slightly alkaline soil type of the hilly areas, rich in calcium and organic matter, could also support longer term survival of Bacillus anthracis spores in the environment (Mwakapeje *et al*., 2019) which could be the reason for repeated outbreaks of anthrax in Baluchistan.

The Seroprevalence of *B. anthracis* in sheep and cattle in the districts of South Punjab reported in this study (8.73% and 9.00%, respectively) was lower than a previous report by Rashid and co-authors (2020), in which the seroprevalence of sheep and cattle in central Punjab was 42.5% and 19.2%, respectively. The higher seroprevalence is likely due to the availability of vaccines and veterinary services in the districts of central Punjab. Moreover, intensive farming system is mostly practiced in central Punjab where stringent vaccination schedules are followed (https://vri.punjab.gov.pk/biologics-vaccine). In contrast, our findings of overall seroprevalence in south Punjab (8.92%) are comparable with those of Rashid *et al*., (2020), who calculated 5.5%

seroprevalence in the animal population of districts in south Punjab (Rashid *et al*., 2020).

Since the sheep and cattle included in this study were not vaccinated against *Bacillus anthrax* in the last six months, the sero-positivity of animals indicate a possible sub-clinical anthrax infection. However, this hypothesis requires further investigation in Pakistan. The previous study (Cizauskas *et al*., 2014) reported that antibody titer against anthrax decreases to significant levels in approximately six months in herbivores and boosters are recommended three months post-vaccination to ensure protective level of antibodies. Another study (Ndumnego *et al*., 2016) recommended that a booster three months post vaccination provides robust immunity against anthrax. Although it is recommended that an anthrax vaccine, developed by Veterinary Research Institute, Livestock & Dairy Development, Punjab be administered once a year, this recommendation may be re-visited.

One of the major factors underlying repeated anthrax outbreaks in Baluchistan province is the poor availability of vaccines (10%) for the animals, leaving 90% of the animal population unvaccinated and such findings have also been evidenced in a previous study conducted by Arif and colleagues (Arif *et al*., 2018). Poor anthrax vaccine coverage and less effective vaccination programs could result in repeated outbreaks of infectious diseases including anthrax (Chakraborty *et al*., 2012) in animals and these findings further corroborate the results of our study Moreover, due to the vastness of areas of Baluchistan province, farmers have less access to veterinary facilities and farmers have to manage their sick animals on their own, which maybe the reason for the repeated emergence of outbreaks like anthrax in study areas (Arif *et al*., 2018).

Present study reveals that farmers in Baluchistan province and two districts (Bahawalnagar and Bahawalpur) of Punjab province follow a nomadic lifestyle with a free-range pastoral grazing system. The two Punjab districts are mainly covered by desert and Baluchistan province is mainly covered with semi-arid hilly patches, experiencing dry weather. Due to scarce rainfall in Baluchistan province, pastoral grazing is practiced; 25% of the vast range land is used for grazing and animals are continuously moving to find new pastures and water sources (Arif *et al*., 2018). This movement of livestock for grazing and water has proven to increase the likelihood animals getting infected with new organisms and also disseminating pathogens to new places (Ekwem *et al*., 2021). In addition to the disease spread by movement of animals, the disease reporting was also very poor and the majority of farmers did not report the disease to veterinary officials. Our findings are in consistent with that of a study in Australia, where farmers were found reporting any sudden mortality suspected for anthrax have also been reported in other countries, such as Australia (Palmer *et al*., 2009).

It was found in this study that morbid animals were slaughtered for meat consumption domestically. Such practices of consuming meat of diseased animals were also reported in other lower-middle income countries like Ethiopia (Seid *et al*., 2020). Such practices could be of utmost importance for public health due to their zoonotic potential. Disposal practices if not practiced as per recommendations of the Office International des Epizooties (OIE) have the potential to contaminate the environment and could be the reason for repeated outbreaks in Pakistan. Moreover, scavengers can mechanically transmit the infection from improperly disposed carcasses to naïve environments (Bellan *et al*., 2013). A study in Uganda demonstrated that the butchering of animals suspected of anthrax is a potential risk factor for disease outbreak within 50 meters radius of slaughtering (Monje *et al*., 2020). The lack of vaccination records could result from farmers self-administering vaccines to their animals. Such malpractices also could be the reason for vaccination failure if injected improperly and/or transported without vaccine cold chain. The latter could interfere with the effectiveness of vaccines.

One of the limitation of this study includes the determination of anti-PA antibodies only in the serum samples by ELISA. These antibodies can be produced by both a natural infection and as a result of the live spore vaccine. In Pakistan, Sterne F-34 live attenuated spore vaccine is used for animals making differentiation antibody produced in disease or as a result of vaccine a difficult task. However, this possibility could be neglected through the fact that animals included in the study did not receive any vaccination against anthrax in last six months. Hence it can be assumed that PA antibodies result from natural exposure to Bacillus anthracis.

A convenient sampling method was adopted for this study due to the remote location of the areas sampled lack of any available data on the position of herds and flocks and, the pastoral farming system that keep animals on moving. Less number of sample were collected in case of cattle due to difficulty in restraining of cattle in the field conditions.

Conclusion: Socio-demographic and husbandry practices reveal that low vaccination rates are due to a lack of awareness about vaccination importance and poor coverage due to the vastness of the free-range area. Our findings warrant further active surveillance on a larger scale and across a wider geography to elucidate the potential link of sporadic outbreaks in endemic regions. We also recommend increased vaccine availability, vaccination coverage, and awareness among farmers

regarding anthrax and its potential veterinary and public health concerns.

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Author's contribution: NS, TY & AA: Designed the study, MWA, HBA, MFS & TY: Collected data and samples, NS, NM &MWA: Performed the experiments, TY, AAA, MJ & AJW: Overall supervision of the project, NS, MWA, HBA, MAS, NM, MZS: Writing original draft. TY, AJW, DSS, SF, MK & MN: Reviewing and editing the drafts.

Funding: The current study was supported by Defense Threat Reduction Agency (DTRA) under project no. HDTRA11710050.

Data availability statement: The data generated and analyzed during the study is available in table form.

Ethical Approval and participant consent: The current study was conducted after approval from the office of research innovation and commercialization (ORIC), University of Veterinary and Animal Sciences, Lahore viz letter-number DR/1654 on 9-Feb-2021. As the blood samples were taken from animals in the field so no ethical approval is required according to the Article 7.1 on the recommendation for animal welfare and Article 7.8 on use of animals in research and education of the World Organization for Animal Health Terrestrial Animal Health Code. The collection of blood samples from the animals in this study was done in consultation with the Livestock and Dairy Development Department (L&DD) and field veterinarians. The blood samples were collected during routine veterinary practice following the high standard of veterinary care, and after the permission of the dairy farms' owners. No clinical interventions were performed during the study.

Approved by Institutional Ethical Review Board, Institute of Public Health viz IERB/14/2019, all the data from human subjects was collected after informed verbal consent. The respondents were informed about the purpose of the study and the confidentiality of information was ensured and maintained as per the Helsinki Protocol.

The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication: Not applicable

Conflict of Interest: The authors declare no conflict of interest.

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