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Short Communication

SEROPREVALENCE OF *BACILLUS ANTHRACIS* PROTECTIVE-ANTIGEN IN NINE DISTRICTS OF CENTRAL PUNJAB, PAKISTAN

H. Rashid[1]*, K. Muhammad[1], M. Rabbani[1], M. S. Khan[1]. M. Z. Shabbir[1], A. Ahmad[1], M. A. Ali[1] and B. Jayarao[2]

[1]University of Veterinary and Animal Sciences, Lahore, Pakistan; [2]The Pennsylvania State University, University Park, PA, USA
Corresponding Author's Email: haroon.rashid@iub.edu.pk

ABSTRACT

Anthrax is a deadly and highly contagious, zoonotic disease caused by *Bacillus anthracis*. It is an acute, febrile disease of warm-blooded animals and humans. This study was conducted to investigate antibodies against anthrax (Protective-antigen) in four species of domestic animals (cattle, buffalo, sheep and goat) in nine districts of Punjab, Pakistan. The serum samples of the animals (n=680) were processed using indirect ELISA to determine antibodies against *Bacillus anthracis* protective-antigen (PA). The results showed that 23/120 (19.2%) cattle, 37/101 (36.6%) buffaloes, 124/285 (43.5%) goats and 74/174 (42.5%) sheep have protective level of anti-PA antibodies. Overall, seroprevalence of anti-PA antibodies was 37.9 % in 680 animals. Maximum seroprevalence was recorded in Lahore (82.4%) followed by Gujranwala (80.4%), Sahiwal (72.7%), Chakwal (63.6%), Sargodha (54.3%), Faisalabad (15.2%), Sheikhupura (14.1%), DG Khan (5.5%) and Attock (0%), respectively. It was concluded that eight out of nine districts of Punjab had animals positive for antibodies against PA of *B. anthracis* and all of the four species of domestic animals were seropositive. Continuous monitoring of animals for *B. anthracis* is highly recommended for keeping the threat under control.

Keywords: *Bacillus anthracis*, domestic animals, indirect ELISA, Punjab.

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INTRODUCTION

Anthrax is a contagious, acute, febrile and deadly zoonotic disease of warm-blooded animals and humans caused by *Bacillus anthracis,* which is an obligatory pathogen (Fasanella *et al.*, 2013; Hendricks *et al.*, 2014). The spores are formed when the vegetative bacterial cells are exposed to unfavorable conditions for its multiplication. The endospores thus formed are highly resistant to heat and chemical disinfectants (Coffin *et al.*, 2015). The affected carcass is not advised to be opened for necropsy and postmortem examination. The carcass is buried deep underground to prevent the spread of disease by scavengers and insects (Ebedes, 1977; Gunn, 2019). Anthrax spores from farm utilities may enter through open wounds and injured skin of animals and cause cutaneous form of anthrax (WHO, 2008). The spore may enter animal body through inhalation and result in deadly pneumonic form of anthrax (Greenfield *et al*., 2002). The spores may enter the animal body through drinking water or feed and induce gastrointestinal type of anthrax. The incidence of disease may rise as a result of malnutrition (OIE, 2004).

Generally, soil conditions for *B. anthracis* survival are reported as humus-rich, slightly acidic soils with pH 6.0 across the steppe and grassland soils (Hugh-Jones and Blackburn, 2009). Outbreaks are suggestive of strong geographical, local environment and climate relationship with communicable diseases which are influenced by various factors. The *B. anthracis* can infect animals directly from the soil or indirectly from fodder grown on infected soil (Radostits *et al.,* 2006).

Anthrax is diagnosed with striking absence of rigor mortis, gaseous decomposition and 'sawhorse' posture (Constable *et al.,* 2017). The natural orifices usually exude dark, tarry blood that does not clot. Contemporary confirmatory diagnosis is based upon microscopic examination of body fluids, culturing and inoculation of the organism on media and in lab animals (Radostits *et al.,* 2010).

In 1939, Max Sterne isolated a non-capsulated *B. anthracis* strain (34F2 strain) from which a live attenuated spore vaccine was developed. The strain used was avirulent for domestic animals, derived from subculture of an isolate from a case of bovine anthrax and is still used for routine immunization of livestock in many countries worldwide. A single inoculation provides protection for nine months, but a yearly boost is considered efficient. Effective immunity develops within a week but in horses this may take up to four weeks (Coetzer *et al.*, 1994). The live spore vaccine seems to retain some virulence in goats and llamas, but can also cause occasional losses in laboratory animals. The LD50 is as low as 10^3 spores in some inbred strains of mice (Welkos *et al.*, 1986).

In developing countries like Pakistan, anthrax may become a public health threat due to lack of extensive surveillance and research. Patchy and un-conclusive evidence suggests the circulation of bacteria in Cholistan desert, Thar Desert, parts of KPK and Balochistan. This evidence is based on some reports of Anthrax or Anthrax like diseases reported from Newspapers and some research papers (Ashraf *et al*., 2014; Durrani *et al*., 2017). Although aggressive antibiotic therapy can help in preventing disease but infected animals still die due to per acute nature of the disease and lethal toxins of the pathogen. In Pakistan, no field kits or laboratory tests are performed to diagnose anthrax. Very limited work has been carried out to detect Anthrax in soil based samples in Pakistan (Shabbir *et al.,* 2015). Scattered reports of disease occurrence have been suggestive of threat, but they usually go misdiagnosed. Transboundary transmission of disease might occur between neighboring countries e.g. India where diseases has been reported to be endemic (Patil *et al*., 2010), Afghanistan and Iran, where the disease is not yet reported effectively (Arya *et al*., 1982; Sadjadi *et al*., 1998). The districts of Pakistan neighboring these countries remain at risk of the disease. Hence, it is of prime importance to develop in-house system which could be used for laboratory detection and confirmation of anthrax in clinical samples. The main objective of current research was to find out seroprevalence of *B. anthracis* in central Punjab, Pakistan.

MATERIALS AND METHODS

A total of 680 animals (120 cattle, 101 buffalo, 285 goat and 174 sheep) were included in the study. The convenient sampling method was used to collect samples from the animals of consenting owners. The blood samples (n=680) were collected from jugular vein of domestic animals of nine districts of Punjab (Figure 1), using disposable syringes. Each blood sample (5mL of cattle/buffalo and 3 mL of sheep/goat) was processed for serum isolation (one mL of goat/sheep and 2 mL from cattle/buffalo) and properly labelled vials were stored at -80C till further use. The samples were screened for anti-protective antigen antibodies (PA) of *B. anthracis* using commercially available indirect ELISA kit (Serion-Virion) as per manufacturer's instruction.

In brief, each reagents and sera was brought to room temperature. Each of the test serum (5µL) was diluted as 1:40 using sample diluent (200µL) and was dispensed (100µL) together with calibrator and controls into the appropriate wells. For the blank reagent, 100µL sample diluent was dispensed in 1A well position. The plate was incubated for 30 minutes at the room temperature. The 300 µL of 1X buffer was used to wash the plates for three times. Paper towel was used to blot the plate. Enzyme conjugate (100µL) was added to each well and further incubated for 30 minutes in humidified chamber. All wells were cleared of fluid residues after incubation and washed three times with 300µL of 1X buffer. The pNPP substrate solution was dispensed 100µL and incubated at the room temperature for 30 minutes. Then, 100µL of stop solution was added and optical density (OD) was recorded at 450nm within 60 minutes against substrate blank at 650nm. The OD of each of the test, blank, negative and positive control samples were recorded.

Optical density values obtained were entered into Serion-Virion supplied data sheet to convert OD values to IU/mL of antibodies and processed for statistical analysis in SPSS version 19.0. The Chi-square test was used to determine the association of seroprevalence *B. anthracis (PA)* with district and species of animal. The district wise and species wise frequency distribution of anti-PA *B. anthracis* antibodies was determined.

Figure 1: Map of Punjab Highlighting Sampling Areas as black stars.

RESULTS

Out of 680 blood samples of domestic animals, species wise, maximum seroprevalence percentage of anti-PA antibodies was observed in sheep 74/174(42.5%) followed by goats 124/285(43.5%), buffaloes 37/101(36.6%) and cattle 23/120(19.2%), respectively. There was significant association between serum ELISA positivity and animal species (Chi square value=23.345 degree of freedom 3 and p= 0.000) (Table 1)

District wise, maximum seroprevalence percentage of anti-PA antibodies was recorded in Lahore (82.4, 42/51) followed by Gujranwala (80.4, 82/102), Sahiwal (72.7, 56/77), Chakwal (63.6, 7/11), Sargodha (54.3, 38/70), Faisalabad (15.2, 14/92), Sheikhupura (14.1, 13/92), DG Khan (5.5, 6/110) and Attock (0, 0/75), respectively (Table 2). There was significant association between serum ELISA positivity and district (Chi square value=308.872 degree of freedom 8 and $p < 0.000$) (Table 2).

DISCUSSION

Bacillus anthracis is a Gram positive, spore forming bacilli which causes a zoonotic and fatal disease in farm animals and humans called anthrax (Bhunia, 2018). The disease is caused by spores of *B. anthracis,* which infect animals and human beings through various routes (Schwartz, 2009). There are three clinical form of anthrax, which are based on route of infection i.e. cutaneous (skin), gastrointestinal (ingestion) and pulmonary (through inhalation of spores). Cutaneous anthrax infection mostly mild and remains limited to skin. However, in few cases, it can convert into systemic form when bacteremia occurs in the body. Hemorrhagic lesions can be developed on any part of body and can be fatal in bacteremic anthrax. Gastrointestinal (GI) anthrax occurs by eating the food contaminated with anthrax spores usually by eating contaminated meat. When anthrax spores enter the body through respiratory route into lungs, pulmonary or inhalational anthrax can occurs, which is the most severe form of anthrax. Pulmonary anthrax doesn't cause pneumonia, but causes hemorrhagic mediastinitis and pulmonary edema. The mortality was 92% previously but with the early detection and treatment it has been reduced significantly to 45% only in USA (Goel, 2015). The cattle act as sentinel for the disease. Sheep is the most affected animal (Hicks *et al*., 2012). The disease has modus operandi, which is mediated through a triangular toxin complex. Three non-toxic proteins namely PA, LF and EF of anthrax tripartite toxin co-assemble to produce a series of free or cell-bound toxic complexes. The first toxin is the Protective Antigen called (PA). PA 83 cleaves into two subunits PA 63 and PA 20. The PA 63 is the main protein which forms a heptamer and attaches 3 units of lethal toxin and Edema factor. This tripartites complex enters the cell through receptor mediated endocytosis, where the edema factor and lethal factor cause their main cellular toxicity. These three toxins are mainly produced by the bacteria and antibodies to these three are demonstrable at various levels of the disease (Bhatnagar, 2001). The anti-PA antibodies are formed a little later than anti-Edema factor antibodies and anti-lethal factor antibodies but the PA antibodies are more easily and clearly detectable. The anti-PA antibodies are evident in both disease form and sterne vaccinated animals but not demonstrable in the Pasteur strain vaccination. Both anti-LF and anti-EF antibodies are also observed in the sterne vaccinated animals. The classical Pasteur vaccine does not show antibody titers to any of the toxins in question. The sterne vaccine strain is a wild type attenuation, which has lost its ability to produce (Poly D glutmaic acid) proteinaceous capsule which is imperative for replication and survival of the germinal form of bacteria in the host where it causes disease. It is noteworthy that 90-95% of the cases presented of anthrax are of the cutaneous type which are non-lethal and recover after therapy within 60 days and show antibody titers against all antigens. ELISA detects all kinds of anti-anthrax antibodies in all the cases (Gosh *et al.,* 2015). The more severe form is the enteric form which shows the antibodies for all antigens of the anthrax. The more peracute form of anthrax is pulmonary or pneumonic form and locally called 'phurki or kala phephra', is highly fatal and death may result as early as within 24 hours and antibodies may not be demonstrable or no enough time for antibody production.

The anti-PA antibodies are formed as early as 11 days post infection (Brenneman *et al*., 2011). The presence and surge of antibodies against the various antigen of anthrax is also dependent on the strain and pathogenicity of the anthrax agent. Such as the vaccinal strains show slower antibody production while the pathogenic strains show higher titers as well as earlier antibody production indices (Hudson *et al*., 2008).

In the present study district wise, maximum seroprevalence percentage of anti-PA antibodies was recorded in Lahore (82.4, 42/51) followed by Gujranwala (80.4, 82/102), Sahiwal (72.7, 56/77), Chakwal (63.6, 7/11), Sargodha (54.3, 38/70), Faisalabad (15.2, 14/92), Sheikhupura (14.1, 13/92), DG Khan (5.5, 6/110) and Attock (0, 0/75), respectively. This may be due to the fact that the vaccine is being manufactured and marketed from VRI, Lahore and potential districts closer to the manufacturer are the ones in which more vaccination is being practiced. Another reason could be the awareness level of the farmers. One important factor hidden in the numbers is the fact that lower population of animals in the soil positive areas of Attock and Chakwal lead to lower number of animals tested for anti-PA antibodies, showing a lower percentage value. It could be due to

small sample size which might create sampling bias or may be due less density of human and animal population in the area.

In our study, species wise, maximum seroprevalence of anti-PA antibodies was observed in sheep 74/174 (42.5%) followed by goats 124/285 (43.5%), buffaloes 37/101 (36.6%) and cattle 23/120 (19.2%), respectively. It has been observed that cattle are the main host in which highest disease prevalence has been reported. Most of the animal keepers in Pakistan vaccinate cattle and buffaloes frequently, however they pay little attention to sheep and goat, however our findings indicate that a larger number of sheep and goats has been vaccinated against anthrax. It could be the result of carpet vaccination program conducted off and on by the veterinary and para veterinary staff of the Livestock and Dairy Department (L&DD) of Punjab.

In current study, Indirect ELISA against PA was used as a diagnostic test. In other serological studies, it has been observed that ELISA is more sensitive test to detect anti PA antibodies and capsular antigens compared to Anti-lethal factor antibodies (Harrison *et al*., 1989).

There was significant association between values of serum ELISA titer and district wise distribution of the disease (Chi square value=308.872 degree of freedom 8 and p<0.000) and these results are in agreement with Turnbull *et al.*, (2006). *B. anthracis* was not detected from soil of seven districts of Punjab (Rashid *et al.,* 2018) and the current sero-prevalence indices might be due to vaccinal titers of the domestic animals as the protective titers against Protective Antigen (PA) showing the presence of immunity.

Table 1. Animal Species-wise seroprevalence of Anti-PA *B. anthracis* Indirect ELISA antibodies of Punjab.

Sr. #	Animal Species	Serum sample tested for Anti-PA *B. anthracis* Indirect ELISA antibodies	
		Total	Positive (%)
1	Cattle	120	23(19.2%)
2	Buffalo	101	37(36.6%)
3	Goat	285	124(43.5%)
4	Sheep	174	74(42.5%)
Total		680	258(37.9%)

There was significant association between serum ELISA and animal type (Chi square value=23.345 degree of freedom 3 and p<0.000).

Table 2. District-wise prevalence of Anti-PA *B. anthracis* Indirect ELISA antibodies of Punjab.

Sr. #	District	Serum sample tested for Anti-PA *B. anthracis* Indirect ELISA antibodies	
		Total	Positive (%)
1	Attock	75	0(0%)
2	Faisalabad	92	14(15.2%)
3	Gujranwala	102	82(80.4%)
4	Sahiwal	77	56(72.7%)
5	Sheikhupura	92	13(14.1%)
6	DG Khan	110	6(5.5%)
7	Lahore	51	42(82.4%)
8	Sargodha	70	38(54.3%)
9	Chakwal	11	7(63.6%)
Total		680	258(37.9%)

There was significant association between values of serum ELISA titer and district wise distribution of disease (Chi square value=308.872 degree of freedom 8 and p < 0.000).

REFERENCES

Arya, L.S., A. Saidali, M.A. Qureshi, and M. Singh (1982). Anthrax in infants and children in Afghanistan. Indian. J. Pediatr. 49(4): 529-534.

Ashraf, S., H.R. Chaudhry, M. Chaudhry, Z. Iqbal, M. Ali, T. Jamil, and S.H.U. Rehman (2014). Prevalence of common diseases in camels of Cholistan desert, Pakistan. IUFS J. Biol. 2(4): 49-52.

Bhatnagar, R. and S. Batra (2001). Anthrax toxin. Crit. Rev. Microbiol. 27(3): 167-200.

Bhunia, A.K. (2018) *Bacillus cereus* and *Bacillus anthracis*. In: Foodborne Microbial Pathogens. Springer, New York, NY.

Brenneman, K.E., M. Doganay, A. Akmal, S. Goldman, D.R. Galloway, A.J. Mateczun, and L.W. Baillie (2011). The early humoral immune response to Bacillus anthracis toxins in patients infected with cutaneous anthrax. FEMS Immunol. Med. Microbiol. 62(2): 164-172.

Coetzer, J. A. W., G.R. Thomson, & R.C. Tustin, (1994). Infectious diseases of livestock with special reference to Southern Africa.

Coffin, J.L., F. Monje, G. Asiimwe-Karimu, H.J. Amuguni, and T. Odoch (2015). A One Health, participatory epidemiology assessment of anthrax (Bacillus anthracis) management in Western Uganda. Soc. Sci. Med. 129: 44-50.

Constable, P., K.W. Hinchcliff, S.H. Done, and W. Grünberg (2017). Systemic and multi-organ diseases. J. Vet. Med. 2002-2214.

Durrani, A.Z., Z. Bashir, and I. Rasheed (2017). Epidemiological study of common diseases and

their risk factors in camels in South Punjab, Pakistan. Microb. Pathog. 108: 6-12.

Ebedes, H. (1977). Anthrax epizo tics in Etosha National Park. Madoqua. (v10i2): 99-118.

Fasanella, A., G. Garofolo, M. Galella, P. Troiano, C. De Stefano, L. Pace, and R. Adone (2013). Suspect vector transmission of human cutaneous anthrax during an animal outbreak in Southern Italy. Vector Borne Zoonotic Dis. 13(10): 769-771.

Goel, A.K. (2015). Anthrax: A disease of biowarfare and public health importance. World. J. Clin. Cases. 3(1): 20–33. doi:10.12998/wjcc.v3.i1.20.

Ghosh, N., D. Gunti, H. Lukka, B.R. Reddy, J. Padmaja, and A.K. Goel (2015). Development & validation of a quantitative anti-protective antigen IgG enzyme linked immunosorbent assay for serodiagnosis of cutaneous anthrax. Indian. J. Med. Res. 142(2): 196–204. doi:10.4103/0971-5916.164258.

Greenfield, R.A., D.A. Drevets, L.J. Machado, G.W. Voskuhl, P. Cornea, and M.S. Bronze (2002). Bacterial pathogens as biological weapons and agents of bioterrorism. Am. J. Med. Sci. 323(6): 299-315.

Gunn, A. (2019). Essential forensic biology. John Wiley and Sons.

Harrison, L.H., J.W. Ezzell, T.G. Abshire, S. Kidd, and A.F. Kaufmann (1989). Evaluation of serologic tests for diagnosis of anthrax after an outbreak of cutaneous anthrax in Paraguay. Int. J. Infect. Dis. 160(4): 706-710.

Hudson, M.J., W. Beyer, R. Böhm, A. Fasanella, G. Garofolo, R. Golinski, and M. Mock (2008). Bacillus anthracis: balancing innocent research with dual-use potential. Intern. J. Med. Microbiol. 298(5-6): 345-364.

Hendricks, K.A., M.E. Wright, S.V. Shadomy, J.S. Bradley, M.G. Morrow, A.T. Pavia, and N. Pesik (2014). Centers for disease control and prevention expert panel meetings on prevention and treatment of anthrax in adults. Emerg. Infect. Dis. 20(2).

Hugh-Jones, M. and J. Blackburn (2009). The ecology of Bacillus anthracis. Molecular aspects of medicine. 30(6): 356-367.

Hicks, C.W., D.A. Sweeney, X. Cui, Y. Li, and P.Q. Eichacker (2012). An overview of anthrax infection including the recently identified form of disease in injection drug users. Intensive care Med. 38(7): 1092-1104.

OIE, 2004. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). Office International des Epizooties (OIE).

Organization W.H., Epizootics IOo. Anthrax in humans and animals. World Health Organization; 2008.

Patil, R.R. (2010). Anthrax: public health risk in India and socio-environmental determinants. Indian J. Community Medicine: Official Publication of Indian J. Community Med. 35(1): 189.

Radostits, O.M., C.C. Gay, K.W Hinchcliff, & P.D Constable, (Eds.). (2006). Veterinary Medicine E-Book: A textbook of the diseases of cattle, horses, sheep, pigs and goats. Elsevier Health Sciences.

Radostits, O.M., C.C. Gay, D.C. Blood, & K.W. Hinchcliff, (2010). A Textbook of Disease of Cattle, Sheep, Pigs, Goats and Horses. 10th Edn., Saunders publication co.

Rashid, H., K. Muhammad, M. Rabbani, M.S. Khan, T. Ghori, M. Chaudhry, and M.M. Sattar (2018). Prevalence and Distribution of Soil-Borne Bacillus anthracis in Different Districts of Punjab, Pakistan. Pakistan. J. Zool. 50(6): 2383-2386.

Sadjadi, J. (1998). Cutaneous anthrax associated with the kombucha mushroom in Iran. JAMA. 280(18): 1567-1568.

Schwartz, M. (2009). Dr. Jekyll and Mr. Hyde: A short history of anthrax. Mol. Asp. Med. 30: 347-55. 10.1016/j.mam.2009.06.004.

Shabbir, M.Z., T. Jamil, K. Muhammad, T. Yaqub, A. Bano, A.I. Mirza, and M.H. Chaudhary (2015). Prevalence and distribution of soil-borne zoonotic pathogens in Lahore district of Pakistan. Front. Microbiol. 6, 917. https://doi.org/10.3389/fmicb.2015.00917.

Turnbull, P.C., M.G. Broster, J.A. Carman, R.J. Manchee, and J.A.C.K. Melling (1986). Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect. Immun. 52(2): 356-363.

Welkos, S.L., T.J. Keener, and P.H. Gibbs (1986). Differences in susceptibility of inbred mice to Bacillus anthracis. Infect. Immun. 51(3): 795-800.